HERA

Human & Environmental Risk Assessment

on ingredients of household cleaning products

Subtilisins (Protease)

CAS No: 9014-01-1, 1395-21-7, 9073-77-2, 9001-92-7, 79986-26-8, 95979-76-3, 68909-17-1

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SUBTILISIN – HERA Report

1. EXECUTIVE SUMMARY

Subtilisins are proteolytic enzymes, mainly (>90 %) used in detergents and household cleaning products to remove proteinaceous deposits and stains. Subtilisins are of bacterial origin, and are produced by a fermentation process. The total amount of Subtilisin produced and used in the European Union in 2002 was about 1,000 tons of pure enzyme. The protease used in detergents is a globular protein with an average molecular weight of 27,000, consisting of 269 to 275 amino acids. The enzyme is characterised by amino acid sequence and three-dimensional structure as well by its biocatalytic activity in hydrolysing amino acid esters, amides and peptide bonds. The enzyme is active from pH 6 to 11, with a major activity in the pH range between 9 and 11. Subtilisins show good solubility but little stability in water.
Subtilisins are used in automatic dish wash detergents and in all types of powder and liquid household laundry detergents, and in laundry bleach additives. They are also used in industrial cleaning and laundering products. Minor quantities (<10%) are used in technical applications such as protein hydrolysate production, leather treatment, and in the textile and cosmetics industry. The Subtilisin concentration in household detergent and cleaning products is very low and depends on the type of product. The Subtilisin concentrations in products typically range between 0.007 % and 0.1 %.

Environmental Assessment

Subtilisins are proteins which are readily and ultimately biodegradable in the environment. Consequently, they are removed to a very high extent (> 99%) in sewage treatment plants as shown in simulation model tests and monitoring studies. An important aspect in the environmental exposure assessment is the fact that these enzymes are inactivated to a large extent under washing or cleaning conditions. Taking account of the existing facts and study results, an 80% reduction of protease activity in the washing process was conservatively assumed. Based on evidence that the inactivation of proteases is equivalent to the loss of their ecotoxic properties, the risk assessment has to take this fact into account.

The existing data on acute aquatic toxicity of Subtilisin cover a broad range of EC50 values (0.1 - 50 mg of active enzyme/l). The Predicted No Effect Concentration (PNEC) was conservatively derived from the lowest effect concentration in spite of seeming atypically low compared to the majority of the data. Therefore this PNEC-dertermining toxicity value is unsuitable to form the basis for the environmental classification of Subtilisins. Based on the HERA detergents exposure scenario, the environmental risk characterisation of Subtilisin revealed risk characterisation ratios below 1 in all environmental compartments. This allows the conclusion that the use of proteases in detergents does not provide a risk for the environment.

Human Health Assessment

The key health concern identified for Subtilisin is respiratory (Type 1) allergy. Consumers can be exposed to Subtilisins via the respiratory route during the task of dispensing detergent products in the washing machine (exposure up to 0.16 ng Subtilisin/m^3) or during handwash of laundry (0.01 ng/m^3), or by suddenly opening the dish washer during the cleaning step (<1.9 ng/m^3).

Since there is no well defined threshold for the induction of sensitisation a benchmark approach was used to assess the risk of consumers for respiratory allergy. An upper benchmark where allergic symptoms occur was established at 212 ng/m^3. Allergic symptoms can be excluded when exposure does not exceed a range of 1 ng/m^3 upon consumer use of laundry and cleaning products. There appears to be a complex relationship between frequency, magnitude and duration of

exposure and the generation of enzyme specific IgE antibody. Therefore a lower benchmark where risk of sensitisation is clearly absent cannot be given with sufficient accuracy. Since enzyme exposure associated with laundry products is calculated to be not more than 0.16 ng/m^3, adverse effects are not expected. Even under the worst-case situation (opening a dishwasher during the cleaning step) such effects are not to be expected as in reality the thresholds at which respiratory sensitisation and allergy occur are likely to be distinctly higher than mentioned above, thus making, the margin of safety proportionately greater.

Other than for respiratory allergy, there is also a hazard for skin and eye irritation by Subtilisins. Consumers may be exposed by skin contact during laundry hand wash (exposure up to a Subtilisin concentration of 0.0009%), by laundry pre-treatment using liquid detergent (0.09%), by hand dish wash (0.0001%) and by fabric wear with skin in contact with Subtilisin deposited during the wash (0.0035 μg/cm^2). As aqueous solutions of Subtilisin at concentrations up to 2% enzyme granulate (estimated 0.02 % aep) failed to show any irritation effects even on damaged human skin, and as the average NOEC value for humans is at 0.07 % (based on aep), it is concluded that skin contact with washing solutions containing Subtilisins is not a cause of concern. Skin contact with Subtilisin deposits on washed fabrics are not expected to cause skin irritation, as the levels of Subtilisin deposited on fabric are very small; even assuming all the material remains active and transfers to skin with 100% efficiency, the skin contact concentrations are several orders of magnitude below the No Observed Effect Concentration of 0.07 % mentioned above. Clinical studies which are in support of this conclusion have been published. In the course of laundry pre-treatment, skin contact with concentrated powder paste, or neat liquid detergent (maximum concentration 0.09%), may occur. If it does occur at all, the contact with skin is confined to a fraction of the hands (palms and/or fingers), and is of very short duration (typically a few minutes at most). The initially high Subtilisin concentration is usually diluted rapidly in the course of the pre-treatment task. Failing to rinse hands in water after contact with a laundry pre-treatment paste or liquid may result in (transient) skin irritation of the hands, which is expected to be mild in nature and can be easily avoided by prompt washing with water.

Exposure to the eye may occur through accidental splashes or spills during the handling of liquid detergent products. On basis of the experimental data and the concentrations employed in these studies as compared to the lower concentrations used in consumer products, accidental eye contact with Subtilisin from either neat liquid product or hand wash solutions is not expected to cause more than a mild transient irritation.

In conclusion it can be said, that use of Subtilisin in laundry and cleaning products represents no safety concerns for consumers.

Following the principles of the HERA Risk Assessment Guidance Document (A.I.S.E., 2005), this assessment is limited to the evaluation of risks to consumer health. An evaluation of occupational risks has been dealt with elsewhere.

2. CONTENTS

3. SUBSTANCE CHARACTERISATION

3.1. CAS no and grouping information

Substance group name:	Subtilisins
Synonyms and trade names:	Subtilisin Carlsberg, Subtilisin A, Subtilopeptidase A, Subtilisin BPN, Subtilisin B, Subtilopeptidase B, Subtilopeptidase C, Subtilisin E, Subtilisin 309, Subtilisin 147, Nagarse, Alcalase™, Savinase™, Maxatase™, Esperase™, Milezyme™, Opticlean™, Optimase™, BLAP, Durazyme™, Purafect™, Purafect OxP™, Kannase™, BLAP S, KAP™, Properase™.
CAS number:	9014-01-1 (The following numbers are also used, but less frequently: 1395-21-7, 9073-77-2, 9001-92-7, 79986-26-8, 95979-76-3, 68909-17-1).
Enzyme nomenclature number:	EC.3.4.21.62 (NC-IUBMB)

Subtilisins are defined by their catalytic mechanisms as serine proteases, with a distinct amino acid sequence and three-dimensional structure that differentiates them from other serine proteases. The catalytic triad consists of histidine, aspartic acid, and serine. The size of active Subtilisins varies from 18 kDa to 90 kDa, but all Subtilisins used in detergents have a size of approximately 27 kDa, consisting of 269 to 275 amino acids (Maurer, 2004). The enzyme is characterised by amino acid sequence and three-dimensional structure representing a typical globular protein of α-helical/ß-sheet structure. It is also characterised by its catalytic activity in hydrolysing amino acid esters, amides and peptide bonds.
They catalyze the hydrolysis of proteins with broad specificity for peptide bonds, and a preference for a large uncharged amino acid residue in the catalytic site pocket P1.
The enzymes are synthesized as a pre-pro-mature protein, transported over the cell membrane and processed to the mature enzyme. Sequence alignment shows that the Subtilisin enzymes used in the detergent industry belong to class I, family A (Siezen and Leunissen, 1997). The enzymes have an overall amino acid sequence homology of approximately 60-99%. The core of the various enzymes has a higher homology, but there are some regions with weaker homology. The latter regions are mostly found on the outer surface of the folded enzyme. These variations also account for the differences in specificity of immunological response to the various members of the Subtilisin family.

Subtilisins derived from different *Bacillus* species have different antigenic properties. In skin prick tests, no cross-reactivity between Subtilisins derived from *B. lentus*, *B. licheniformis* or *B. amyloliquefaciens* Subtilisin enzymes were found. Skin prick testing on new enzymes is now performed routinely in the enzyme industry prior to the introduction into manufacturing. This has also been confirmed in a recent clinical study (Sarlo et al., 2003). In this study, skin prick testing as well as RAST testing were conducted on an individual that was sensitized to *B. amyloliquefaciens* Subtilisin, but did not respond to *B. lentus* or *B. licheniformis* Subtilisin.

The preparations described in Chapter 5.2.1 are representative for all Subtilisin products on the market, and fall within the preparation categories mentioned above.

3.2. Chemical Structure and composition

Table 1: Physico-chemical properties of proteases

Parameter	Value	Remark
Macromolecular Description Physical state /Particle size	White crystals or powder (pure enzyme)	In detergents added as 1-5% preparation: stabilized liquid, slurry or coated granulate
Bulk density (kg/m^3)	**0.6 – 1.3** **1**	Granulates and Liquid Preparations Crystalline Enzyme
Melting point (°C)	Not relevant	Heating leads to destruction
Boiling point (°C)	*Not relevant*	Heating leads to destruction
Vapour pressure at 25 °C (Pa)	**1×10^{-6}**	Minimum value acc. to TGD
Water solubility at 25 °C (g/l)	**> 1 kg/l**	Henkel (2005)
Octanol-water partition coefficient: logPow	**- 3.1**	Henkel (2005)
Koc (l/kg)	**< 1.3**	Calculated acc. to TGD
Henry coefficient (unitless): log H	**-4**	Minimum value acc. to TGD
pH*	**7 – 10**	

* pH of substance in solution (range of isoelectric points)

Enzymes are primarily characterized by their biocatalytic or enzymatic activity. Protease activity is measured and described on the basis of different methods ranging from the determination of degradation products from natural or modified proteins (hemoglobin, casein, dimethylcasein) by Anson Units (AU), alkaline Delft Units (ADU), Novo Protease Units (KNPU), via the activity on chromogenic low molecular weight substrates, like peptide (AAPF) or amino acid derivatives (Glycine Units - GU) resulting in fluorescence and colour changes. There is no general activity determination method available for the activity determination and characterisation of all Subtilisin variants.
Enzyme preparations are characterised by their activity according to the specific methods of the producing company. In order to compare different enzyme preparations the amount of active substance is normally calculated from the activity via the specific activity of the enzyme, where the protein is determined by active site titration and/or quantitative and qualitative amino acid analysis. The resulting active enzyme protein (aep) content represents a value based on a theoretical pure and totally active enzyme.

3.3. Manufacturing Route and Production/Volume Statistics

3.3.1. Subtilisin Manufacturing Route

Enzymes are not simple synthetic chemicals but complex organic macromolecules produced by living organisms from which they are isolated. This affects the purity and the natural variation in molecular structure. In addition, nowadays most of the Subtilisin is produced using genetically engineered microorganisms (GMM), and increasingly also protein-engineered variants are produced. Therefore, a detailed description of the manufacturing route is necessary.

Subtilisin is produced by fermentation using microbial strains from the genus *Bacillus.* The species can be *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus*, or other Bacilli, each producing its own variant of the Subtilisin molecule. Although the primary structure of the Subtilisin enzyme protein is different in each variant, the overall tertiary structure, which determines the catalytic activity, is the same.

Nowadays usually genetically modified (engineered) microorganism (GMM) strains are used, to enhance productivity. Sometimes these strains are also producing protein-engineered Subtilisin variants to introduce a desired trait, such as enhanced oxidative stability. The changes made in the primary amino acid sequence of these variants are no different from the naturally occurring differences between Subtilisin derived from different strains. In order to maintain the overall 3-dimensional structure determining the specific Subtilisin catalytic activity, it is essential that certain regions of the amino acid sequence are conserved. This is the case for naturally occurring as well as protein-engineered variants. Almost super imposable 3-D structures may result from amino acid sequences showing only around 50% homology (e.g. Siezen and Leunissen, 1997). Generally, such enzymes are characterised by partial or full immunochemical identity.

The microorganisms used in the large-scale production of Subtilisin by fermentation methods, or their parents, have been used for decades and been shown by pathogenicity and toxicological testing to be safe to humans, animals and the environment (de Boer and Diderichsen, 1991; Priest et al., 1994; Pedersen et al., 2002). Use of these microorganisms takes place under containment in sealed installations. A system is employed in the manufacturing process which complies with Good Industrial Large Scale Practice (GILSP) containment level as outlined in the 1986 and 1992 reports issued by the Organisation for Economic Co-operation and Development (OECD) titled Recombinant DNA Safety Considerations and Safety Considerations for Biotechnology (OECD, 1986; OECD, 1992). For "Good Industrial Large Scale Practices fermentation experiments, the appropriate physical containment conditions need be no greater than those for the host organism unmodified by recombinant DNA techniques" (NIH, 1996). The use of GMMs under containment is regulated in Europe by the Contained Use EU-Directive (Commission Directive 90/219/EC, as amended). No production microorganism is left in the enzyme preparations.

For descriptive purposes, the manufacturing process can best be described as a three-part process: fermentation, recovery and enzyme formulation. These processes are described in more detail below.
The fermentation process used to grow the organism and produce the enzyme of interest (Subtilisin) is a submerged, aerobic and pure culture fermentation. The process is an integration of the laboratory propagation, the seed fermentation and the main fermentation. These processes, except for the laboratory propagation are carried out in sealed vessels carefully designed to prevent both the release of the production organism and/or the entry of other microorganisms. Usually the fermentation uses complex media to grow the microorganisms in, containing a carbon source which must be supplemented with special compounds, such as a nitrogen source, various nutrient salts, or certain trace elements. Soy meal, fish meal, cotton seed, low-quality protein materials such as casein or its hydrolysates, millet, stillage, and especially corn steep liquor can also be used as low-price nutrients. In addition, these chemically complicated mixtures contain trace elements and growth promoters.
Once the fermentation is completed, the fermentation broth is transferred to processing tanks.
The purpose of the recovery process is to separate the organism biomass from the enzyme, and then purify, concentrate, and stabilize the desired enzyme. The fermentation broth is stabilised with certain

process chemicals and flocculating agents are usually added to maximize biomass separation. This separation can be achieved by a series of filtration steps. A relatively solids-free stream (filtrate) is the purpose of this filtration. The filtrate is made free of production microorganisms, usually by an ultra-filtration step. All biomass is inactivated using (combinations of) heat, pH or other chemical inactivation steps to ensure the killing of all living production microorganisms.
The resulting filtrate is then concentrated by evaporation. The concentrated enzyme solution from the steps described above is standardised and stabilised. Stabilisation can be done in a variety of ways. Liquid formulations can be made with such diluents as salt, propylene glycol, sorbitol or other suitable substances and, if necessary, additional preservatives. Granular formulations are prepared by spraying layers of enzyme and stabilizing and colour coatings on a core of inorganic salts, or forming granulates by using mixers or extrusion processes. The granulated material is always coated with wax in order to prevent the release of enzyme containing dust from the preparation during further handling.

Such a preparation may contain minor amounts of other proteases and esterases (less than 1% on protein basis), as well as broth nutrients, metal salts and inert fillers, like salt, starch, sugar, polyethylene glycol. The products are handled in sealed containers, bags, kegs and big bags, tons for dry products and in Intermediate Bulk Containers (IBCs) for liquid products.

3.3.2. Production/Volume Statistics

In 2002, according to the enzyme producing companies a total of 1000 tons of Subtilisin was produced in the EU for the EU market. Less than 50 t/a were produced for the market of industrial and institutional cleaning and another amount of less than 50 t/a was targeted for other technical enzyme applications, like protein hydrolysate production, leather treatment, textile industry, etc. Thus according to the data of the suppliers the vast majority of the production (900 - 950 t/a) of Subtilisin was consumed in household detergents and cleansers in the EU.
The volume statistics when collected from the detergent producing companies resulted in 700 t/a representing 80% of the market. The total European market thus can be summarized to 875 t/a. Considering all uncertainties in such an evaluation, these data can be regarded as equal to the data estimated by the enzyme producers.
This amount of pure and active enzyme is contained in up to 30,000 t of granulated powder or liquid preparation used by the formulators. The enzyme protein concentration in these preparations ranges from 0.5 to 10%.

3.4. Use Applications Summary

Subtilisin preparations are used for removal of proteinaceous stains in powder and liquid laundry detergents and in automatic dish wash (and to a minor extent in hand dish wash) detergents. The enzyme content in detergent products has varied during the nearly forty years of use. The most recent information on the final concentration of enzyme protein in the HERA products is given in table 2. Subtilisin preparations are also used in industrial cleaning and laundering products and in other industrial applications, like protein hydrolysate production, leather treatment, textile industry. The use in HERA (household and cleaning) products clearly covers the vast majority (> 90%) of the enzyme production, and is the only use addressed in this assessment.

Table 2: Protease concentrations in HERA product range

PRODUCT CATEGORIES IN WHICH SUBSTANCE IS CONTAINED		SUBTILISIN CONCENTRATIONS IN COMMERCIAL PRODUCTS PERCENTAGE OF ACTIVE INGREDIENT IN THE COMMERCIAL PRODUCT		
		% weight		
		Minimum	Maximum	Typical
LAUNDRY REGULAR				
	Powder	0	0.059	0.009 - 0.023
	Liquid	0	0.040	0.010 - 0.04
LAUNDRY COMPACT				
	Powder	0	0.059	0.020 - 0.041
	Liquid/gel	0	0.090	0.016 - 0.080
	Tablet	0	0.065	0.020 - 0.065
	Gel	0	0.040	0.023 - 0.030
LAUNDRY ADDITIVES				
	Powder Bleach	0	0.04	0.005 - 0.023
	Liquid Bleach	0	0	0
	Tablet	0	0.06	0.060
HAND DISHWASHING				
	Liquid (Concentrate)	0	0.01	0
MACHINE DISHWASHING				
	Powder	0	0.099	0.007 - 0.075
	Tablet	0	0.099	0.050 - 0.098

4. ENVIRONMENTAL ASSESSMENT

4.1. Environmental exposure assessment

The following exposure assessment of detergent proteases (Subtilisin type) is based on the most conservatively estimated EU tonnage of 950 tons of active per year in HERA applications (household detergents and cleansers) which represent by far the most relevant application area of this protease. The higher estimate from the enzyme producers evaluation as compared to the estimate based on the detergent formulators evaluation has been chosen for the assessment representing the more conservative scenario.

4.1.1. Environmental fate

The general degradation pathway of proteins is a stepwise process starting with the splitting of peptide bonds in the protein polymer by proteolytic enzymes (proteases) forming lower-molecular oligopeptides which are subsequently degraded by peptidases to the monomeric amino acids (Stoner et al., 2004). Amino acids can be degraded further to carbon dioxide, ammonia and sulphide or they may be incorporated into biomass. Physical effects like heating, dilution, mixing of solutions with air, etc. lead to denaturation, (loss of activity and three dimensional structure) which is facilitating this proteolytical degradation process. Considering the common chemical features of enzymes and their general evaluation as substances easily accessible to biodegradation, it is not surprising that the existing biodegradability test data on Subtilisins underline the conclusion that these materials are rapidly and ultimately biodegraded in the environment.

4.1.1.1 Ready biodegradability

Table 3 summarises the existing data on the ultimate biodegradability of Subtilisines in OECD tests for ready biodegradability.

It turns out that the vast majority of the data measuring ultimate biodegradability by means of oxygen consumption (OECD 301 D) and carbon-removal (DOC), respectively, pass the OECD threshold degradation limit for ready biodegradability (60% BOD/COD and 70% DOC removal, resp.). It should be noted that there is no significant difference in the biodegradation rate and extent between wild type Subtilisins and protein engineered variants. This is in line with the general understanding of the common structure and properties of proteins irrespective of their specific activity pattern and their origin. Consequently, the exposure assessment of Subtilisins does not need to differentiate between wild type and protein engineered enzyme.
Subtilisins have been modified in their amino acid sequence by various protein engineering techniques in order to improve their cleaning or stability performance. Stability is relevant in terms of storage and in process stability, that is stability in the washing or cleaning process, e.g. vs. detrimental effects of bleach. A typical modification is the replacement of methionin 222 by amino acid residues that unlike methionine cannot be oxidised by hydrogen peroxide or other active oxygen compounds. The stabilisation effect of the modifications under storage or process conditions is not affecting the stability in the environment.

4.1.1.2 Completeness of ultimate biodegradabiliy

The conclusion of a complete biodegradability of detergent enzymes without formation of any recalcitrant intermediates was corroborated by the results from a number of additional investigations. Testing of Subtilisin in an inherent biodegradability test revealed very high degradation values of 95-100% DOC removal (28 d) in the Zahn-Wellens test (OECD 302 B) (Henkel 1990a). Similarly, a 99% DOC removal was determined in the Modified OECD Screening test (OECD 301E) when using the supernatant of the Zahn-Wellens test as inoculum (Henkel 1990a). Ultimately, Subtilisin was also tested in the metabolites screening test (Richterich & Gode 1988). Repeated additions of the enzyme concentrate to the modified OECD Screening test system over a 76-day period resulted in a 99 % DOC-removal (Henkel 1990a), hence, emphasising again that ultimate biodegradation of this detergent enzyme proceeds without formation of any recalcitrant products.

4.1.1.3 Biodegradation in sewage and river water

Swisher (1969) investigated the biodegradation of a Subtilisin specimen in die-away tests inoculated with river water, raw sewage and secondary effluent of a municipal sewage treatment plant, respectively. It was shown that the protease activity decreased within 1 day by 97% in river water, 96% in raw sewage and 100% in treated sewage. Hence, the conclusion from the ready biodegradability tests of Subtilisins is well confirmed, i.e. these enzymes lose their proteolytic properties very rapidly under environmentally relevant conditions. Based on the river water die-away test results, a rough estimate of the half-life of detergent proteases in surface waters could be calculated: $t_{1/2}$ = ca. 5 h (k = 0.146 h^{-1}). This figures contrasts considerably with the corresponding default value for readily biodegradable substances acc. to TGD which is used in the exposure assessment ($t_{1/2}$ = 30 d).

Table 3: Aerobic biodegradation in ready biodegradability tests

Test method	Enzyme preparation	Test result	Klimisch reliability code	Enzyme modification	Reference
OECD 301 D (Closed Bottle Test)	Subtilisin (BLAP F49)	56-68% BOD/COD (15 days)	2	protein-engineered	Henkel (1990a)
	Subtilisin (P300)	80-84% BOD/COD (15 days)	2	wild type	Henkel (1990a)
	Subtilisin (BLAP F49)	65-80% BOD/COD (28 days)	1	protein-engineered	Henkel (1995a)
	Subtilisin (PM 111)	79 % BOD/COD (28 days)	1	protein-engineered	Henkel (1992a)
	Subtilisin	62 – 73 % BOD/COD	4	n.i.	Schöberl & Huber (1988)
OECD 301 E (Modif. OECD Screening Test)	Savinase	85% DOC (22 days)	1	wild type	NICNAS (1993)
	Subtilisin	63 – 89 % DOC 56 – 69 % TOC	4	n.i.	Schöberl & Huber (1988)
	Subtilisin (Product C)	71 % DOC (28 days)	1	protein-engineered	Genencor (1996a)
	Subtilisin (Product H)	64 % DOC (28 days)	1	wild type	Genencor (1996a)
	Subtilisin (Product I)	55 % DOC (28 days)	1	protein-engineered	Genencor (1996a)
	Subtilisin (Product H)	79 % DOC (28 days)	1	wild type	Genencor (1996a)
	Alcalase®	93 % DOC (28 days)	1	wild type	Novo Nordisk (1992a), Ref. in: Bergman et al. (1997)
	Durazym™	88 % DOC (21 days)	1	protein-engineered	Novo Nordisk (1991a), Ref. in: Bergman et al. (1997)
	Esperase®	84 % (DOC, 28 days)	1	wild type	Novo Nordisk (1992b), Ref. in: Bergman et al. (1997)
	Savinase®	85 % DOC (28 days)	1	wild type	Novo Nordisk (1992c), Ref. in: Bergman et al. (1997)

n.i. = no information

4.1.1.4 Anaerobic degradation

There are no specific test data addressing the anaerobic biodegradation of detergent proteases. However, considering the excellent accessibility of proteins in general to anaerobic biodegradation which is made use of in the sewage sludge digestersit is highly likely that Subtilisins will be anaerobically decomposed like biomass in general. Hydrolytic processes transform the polymeric materials like proteins into their monomers, e.g. amino acids which are, ultimately, biodegraded yielding carbon dioxide and methane, unless they are used as building blocks for biomass formation.

4.1.1.5 Abiotic degradation

No specific information exists on the abiotic degradation of Subtilisins in the environment via hydrolysis or photolysis. Considering their excellent biodegradability it can be anticipated that possible abiotic degradation mechanisms may be of lower significance in the environment compared to biodegradation.

4.1.1.6 Bioconcentration

The bioconcentration potential of enzymes representing macromolecules subject to metabolism in any living organism, can generally be neglected. In particular, due to the high molecular weight of approximately 27000, the hydrophilic properties (high water solubility, logPow < 0) and their immediate accessibility to metabolic processes (biotransformation) it can be excluded that detergent-based proteases will bioconcentrate, i.e. will be present in aquatic organisms at concentrations higher than in the aqueous environment.

4.1.2 Removal

4.1.2.1 Inactivation in the washing process

Autoproteolytic reactions are the main reason for the inactivation of Subtilisin in the washing process and, hence, have a significant impact on the exposure assessment of these enzymes. Subtilisin is inactivated under the alkaline conditions of the washing or cleaning process enhanced by temperature, pH and the presence of surfactants and bleach. Studies into the decrease of the proteolytic activity in the washing cycle showed (Henkel 2003a) that proteases are completely inactivated at washing temperatures of 100 and 60 ^{o}C. At 40 ^{o}C the remaining activity ranged between 15 - 30% (determined 20 min after the washing step). Considering the fact that about 56 % of the washes are run at 40 ^{o}C, 33 % at 60 ^{o}C and 11 % at 90 ^{o}C in European households (Reynolds & Lindfors 1998), it can be concluded that detergent proteases contained in used washing liquors will enter the sewer system in an inactivated form to a very high extent. Based on the discussed distribution of washing temperatures and the findings on temperature-dependent enzyme inactivation, the HERA exposure assessment will conservatively assume an average 80% reduction of active proteases during the washing process. This figure does not take into account that Subtilisin used in automatic dishwashing detergents is completely deactivated as shown in tests conducted under the prevailing temperature conditions of 55 - 65 ^{o}C (Henkel 2003a).
It is important to note that the proteolytic activity of Subtilisin determines the ecotoxic effects of this protein towards aquatic organisms. This has been demonstrated in a study (Novo 1985) measuring both the proteolytic activity (expressed as 'active enzyme protein'/aep, see 4.2.1) of Alcalase® and its acute toxicity to fish (zebra fish, 96 h). The toxicity was depending on both the heat treatment prior to the test (room temperature vs. incubation at 60 °C and 90°C for 25 min) and the enzyme concentration (100 - 1000 mg/l). It could be shown that the enzyme was not toxic to fish after 90°C incubation (all concentrations) and after 60 °C incubation (concentrations $\leq$ 250 mg/l), respectively. This shows that the inactivation of proteases in the washing process is equivalent to the loss of their ecotoxic properties. The inactivation during the washing/cleaning process is irreversible. Reversible inactivation can only be seen under very specific conditions of slightly acid pH or presence of specific inhibitors like boric acid or boronic acid derivatives. Consequently, the extent of inactivation of proteases in the washing process must be taken into account for the exposure calculations within the risk assessment of Subtilisin.

4.1.2.2 Removal in sewage treatment plants

Based on the physico-chemical properties and the ready biodegradability of Subtilisin, the SimpleTreat model calculations in EUSES (acc.to EU TGD, Part II, Appendix II) for the removal in

waste water treatment plants predict an elimination rate of 87%. Expectedly, this is a very conservative assumption when comparing this figure with the results obtained in a laboratory model sewage treatment plant (OECD Confirmatory Test) at a 6-hour retention time. A removal of the proteolytic activity by 99.7 % was shown in this study conducted under GLP conditions (Henkel 1995b). Hence, a conservative removal rate of 99% in sewage treatment plants was used for the exposure assessment in the context of the HERA environmental risk assessment (see 4.1.3).

4.1.2.3 Monitoring studies in waste waters and treated effluents

While no data are available on the concentrations of detergent-based proteases in environmental media like surface waters, sediments and soils, several investigations exist into the protease concentrations in raw and treated waste waters. This allows a comparison of predicted and measured enzyme concentrations and of the removal rate in sewage treatment plants as well.
As it is known (Swisher 1969) that proteases are detectable in raw waste waters and in sewage sludges, two protease detection methods were used in the monitoring exercise. While the general protease activity measurement could not differentiate between the contributions of Subtilisin and sewage bacteria-derived proteases, an immunological (dot blot) protein detection method based on polyclonal antibodies allowed a specific detection and quantification of the major type of Subtilisin used in detergents.
A first study conducted by Henkel (Henkel 1993a) determined protease activities corresponding to 12-72 µg/l of Subtilisin in raw waste water and to less than 1.6 ug/l (detection limit) in the treated effluent. This would indicate a minimum protease removal in the range of 87-98%. However, the more specific immunological method with a detection limit in the ng/l-range could not detect any Subtilisin-specific protein, thus, confirming that the elimination of these detergent proteases in sewage treatment plants is virtually complete.
In a recent monitoring exercise (Henkel 2005), the protease activity and the presence of Subtilisin in the influents and effluents of two municipal waste water treatment plants was examined using again the described analytical methods for activity. The protease concentrations in the raw waste waters were found to be in the range of 45 - 600 ug/l, the effluent concentrations were detectable ranging from < 0.1 - 8 ug/l corresponding to an elimination rate of 85 - 99 %. With improved ELISA methodology Subtilisin-specific protein was detected in the raw waste waters in the range of 11 - 20 ug/l and close to or below detection level in the plant effluents.

4.1.3 PEC calculations

The exposure assessment of Subtilisin was conducted according to the TGD (2003) applying the EUSES exposure calculation tool. However, one important deviation from the standard calculation approach was made which is explained and justified in more detail. The PEC calculations by EUSES are based on consideration of a value for the fraction of the main source ($f_{main\ source}$ = 0.002) which applies for detergent ingredients (Industry Category 5) if the production volume is <1000 tons/a (cf. TGD, Part II, Appendix I). This value implies an increased local consumption tonnage by a factor 4 accounting for the local exposure variations in waste water (Hera, 2005). Despite the fact that Subtilisin has an EU consumption <1000 to/a (see 4.1), this enzyme represents an ingredient which is contained today in virtually all laundry detergents used in Europe. Therefore, a homogenous distribution of this substance in all European countries can be assumed, thus, allowing the neglect of the factor 4 and the application of $f_{main\ source}$ = 0.0005 as used for production volumes >1000 tons/a.

As discussed in chapter 4.1.2.2, a conservatively assessed removal rate of 99% in sewage treatment plants can be taken into account for the exposure assessment. As no data are available on the sorption of Subtilisin to sludge, in the present exposure assessment the fraction not going to surface water was assigned to "sludge" and "degraded" as predicted by Simple Treat. The concentration in dry sludge was then re-scaled, based on the new estimate for the fraction assigned to sludge.

It should be recalled that the EU consumption tonnage of Subtilisin (see 4.1) used as the starting point for the exposure calculations has been adjusted to the amount which is really entering the sewer, i.e. by taking account of the inactivation of the proteolytic activity by at least 80% in the washing process (see 4.1.2.1). Hence, the input tonnage for the exposure calculations is 950 x (1- 0.8) = 190 tons/year.

The following table summarises the output of the exposure calculations based on the described exposure assessment:

Subtilisin distribution in local compartments	**Environmental concentrations**
Concentration in STP influent (ug/l)	13.0
Concentration in STP effluent (ug/l)	0.13
Concentration in dry sewage sludge (ug/kg)	9.84
C_{local}	0.013
PEC Water (ug/l)	0.066
PEC Sediment (ug/kg)	0.056
PEC Agricultural Soil (ug/kg)	9.4×10^{-3}
Subtilisin distribution in regional compartments	
PEC Water (ug/l)	0.053
PEC Sediment (ug/kg)	0.039
PEC Agricultural Soil (ug/kg)	5.6×10^{-5}

The comparison of the predicted concentration of Subtilisin in STP influent with the results from corresponding monitoring data (see 4.1.2.3) shows an acceptable correspondence, i.e. the calculated values range in the same order of magnitude as the measured concentration (based on proteolytic activity). This can be considered as a good indication that the basic figures for the exposure assessment are realistic.
The terrestrial PEC values show that the exposure of the soil compartment to detergent proteases is very low in spite of the fact that the anaerobic biodegradability of proteins has not been considered in this exposure assessment. The comparison of $PEC_{regional}$ and C_{local} for surface water shows that the PEC_{water} is mainly driven by the PEC regional. This surprising result can be explained by the fact that the local Subtilisin concentration resulting from treated sewages is much lower (due to the high elimination in STP) than the background concentration. According to TGD (2003), the latter implies the assumption that 20% of sewage will enter surface waters without previous treatment and also assumes extremely conservative in-stream removal kinetics for readily biodegradable substances..

4.2 Environmental effects assessment

4.2.1 Ecotoxicity

The ecotoxicological data of a chemical are reported as an effect concentration usually expressed in the dimension 'mg/l'. However, in the case of Subtilisin the concentration of this substance in formulated products is expressed in enzymatic activity rather than weight. The activity is normally measured and described on the basis of different methods which do not allow a direct comparison of the individual concentration data. To be able to make use of all ecotoxicological data on Subtilisins available from individual companies, the existing effect data of the individual Subtilisin types were re-calculated to adjust them to a unified activity /protein relationship. Consequently, the tonnage data and the effect concentrations in this HERA report are uniformly based on weight (tons or mg, respectively)

expressed in 'active enzyme protein' (aep). It should be noted that the effect concentrations given in the original test reports on Subtilisins may therefore differ from the corresponding aep-based numbers used in this HERA effects assessment.

The existing data on the aquatic toxicity of Subtilisins are generally based on acute toxicity tests. No chronic toxicity studies could be taken into account for the effects assessment..

4.2.1.1 Acute aquatic toxicity

At first sight the toxic effects of Subtilisin on aquatic invertebrates and plants seem to be extremely variable (tables 5 and 6), not only between species, but also within species. One reason is that for some of the data published there is not a clear information available on the substance tested and the specific content of active enzyme protein. Another reason is probably that there is a lot of uncontrolled variation in the exposure side of the toxicity experiments, due to the time when these tests were done. Another reason can be found in the purity of the enzyme preparations and products. With increasing application of recombinant production strains the purity of the enzyme preparations increased. Another effect could theoretically be expected from differencies between protein-engineered and wild type strains. Yet there is no effect from GMO production strains or protein engineering of the enzymes is reflected in the data.

Fish:
The existing data on the acute toxicity of Subtilisin specimens to fish are summarised in Table 4. The LC50 values expressed in mg aep/l exibit a range between 1 - 72 mg aep/l. In a few cases of literature data it was not possible to normalise the reported toxicity values to aep so that these figures would be unsuitable for PNEC derivation. Although the data by Mann (1971) in general fit the overall picture, they are not based on standard test methods as used today (cf. 24-hour test duration) and, hence, are less suitable for PNEC derivation. Among the data based on standard test conditions and, hence, most suitable for PNEC derivation, the lowest LC50 value is 5 mg aep/l.
It should be noted that the fish toxicity data of wild type and protein engineered Subtilisin specimens do not show any significant difference underscoring that the genetic modifications of the protein molecule have no influence on its environmental behaviour.

Daphnia:
The acute daphnia toxicity data of Subtilisin specimens are shown in Table 5. The vast majority of data is based on standard test with *Daphnia magna* while an older study by Mann (1971) provided additional information on effects to the saltwater invertebrate Gammarus and to the sediment-dwelling species Tubifex. Apart from the effects data reported by Schöberl & Huber (1988) which do not allow a normalisation to aep, the available EC50 values range between 0.1 - 13 mg aep/l. Among the data based on standard test conditions and organisms and, hence, most suitable for PNEC derivation, the lowest EC50 value is 0.1 mg aep/l. It should be noted that this value does rather represent an extreme of the spectrum of daphnia toxicity test data while most of the values are in a range of around 1 mg aep/l. Hence, this rather atypical value may be used for a conservative environmental risk assessment of Subtilisin but is unsuitable to form the basis for the environmental classification of this enzyme group. Again, the daphnia toxicity data do not reveal any difference between the effect values of wild type and protein engineered Subtilisin specimens.

Algae:
The existing algal toxicity data on Subtilisin specimens are shown in Table 6. All data are based on tests with standard test species *Desmodesmus subspicatus* (syn. *Scenedesmus subspicatus*) or *Raphidocelis subcapitata* (syn. *Selenastrum capricornutum*) which exhibit, however, a broad range of effect concentrations ranging between 0.3 - 200 mg aep/l. A systematic difference between the effect values of wild type and protein engineered Subtilisin specimens was not recognisable. Among the data suitable for PNEC derivation, the lowest EC50 value is 0.3 mg aep/l.

4.2.1.2 Terrestrial toxicity tests

No data could be found on the terrestrial toxicity of Subtilisin. Hence, the PNEC calculation for this compartment are to be based on the aquatic toxicity test data.

4.2.1.3 Microorganisms

The existing data on the effects of Subtilisin towards sewage treatment plant-relevant bacteria are shown in Table 7. The tests were conducted with *Pseudomonas putida* which is representative of a bacterial species prevailing in activated sludge of municipal sewage treatment plants. As the data from Schöberl & Huber (1988) cannot be normalised to aep, the effect concentration to be used for PNEC derivation will be the one obtained in the respiration inhibition test, i.e. EC0 = 120 mg aep/l.

Table 4: Acute fish toxicity

Test species	Test specimen	Test guideline	Exposure time (h)	LC50 (mg aep/l)	Klimisch reliability code	enzyme modification	Remark/Reference
Danio rerio (syn. *Brachydanio rerio*) (Zebra barb)	Subtilisin BLAP (Li 128)	OECD 203	96	38	2	wild type	Henkel (1989a)
	Subtilisin BLAP (PM 111)	ISO 7346 / II	96	41	1	protein-engineered	Henkel (1992b)
	Subtilisin BLAP F49 (2.1)	EU 92/69/EWG	96	72	1	protein-engineered	Henkel (1995c)
	Savinase	OECD 203	96	200-400*> 1000* (heat inactivated enzyme)	2	wild type	NICNAS (1993)
	Alcalase	OECD 203	96	37	1	wild type	Novo Nordisk (1992d)
	Esperase	OECD 203	96	6	1	wild type	Novo Nordisk (1993a)
	Savinase	OECD 203	96	5	1	wild type	Novo Nordisk (1992e)
Oncorhynchus mykiss (syn. *Salmo gairdneri*) (Rainbow trout)	Subtilisin (Product C)	OECD 203; ASTM standard E729-88a	96	16	1	protein-engineered	Genencor (1996b)
	Subtilisin (Product H)	OECD 203; ASTM standard E729-88a	96	9.1	1	wild type	Genencor (1996c)
Poecilia reticulatus	Subtilisin Maxatase P	n.i.	24	25	4	wild type	Mann (1971)
Oncorhynchus mykiss	Maxatase P	n.i.	24	15 (young fish) 5 (eggs and larvae)	4	wild type	Mann (1971)
Rivulus cylindraceus	Maxatase P	n.i.	24	1	4	wild type	Mann (1971)
Anguilla anguilla (eel)	Maxatase P	n.i.	24	7.5 (sea water) 20 (brack water) 30 (fresh water)	4	wild type	Mann (1971)
Coregonus lavaretus wartmanni	Subtilisin	n.i.	96	350 *	4		Schöberl & Huber (1988)
Poecilia reticulata (Guppy)	Subtilisin	n.i.	24	25 *	4		Schöberl & Huber (1988)

*probably not based on aep

Table 5: Acute invertebrate toxicity

Test species	Test specimen	Test guideline	Exposure time (h)	EC50 (mg aep/l)	Klimisch reliability code	enzyme modification	Reference
Daphnia magna	Subtilisin BLAP (highly purified)	DIN 38412 (11)	24	2,6	2	wild type	Henkel (1989a)
	Subtilisin BLAP (PM 111)	DIN 38412 (11)	48	1,7	1	protein-engineered	Henkel (1993a)
	Subtilisin BLAP F49 (2.1)	EU 92/69/EWG	48	3,7	1	protein-engineered	Henkel (1995d)
	Subtilisin (Product C)	OECD 202	48	1.4	1	protein-engineered	Genencor (1996d)
	Subtilisin (Product G)	OECD 202	48	0.87	1	protein-engineered	Genencor (1997a)
	Subtilisin (Product H)	OECD 202	48	0.89	1	wild type	Genencor (1996e)
	Subtilisin	n.i.	48	160*	4	wild type	Schöberl & Huber (1988)
	Alcalase	OECD 202	48	13	1	wild type	Novo Nordisk (1992f)
	Esperase	OECD 202	48	0.1	1	wild type	Novo Nordisk (1993b)
	Savinase	OECD 202	48	0.6	1	wild type	Novo Nordisk (1992g)
	Durazym	OECD 202	48	3	1	protein-engineered	Novo Nordisk (1991b)
Gammarus salinus	Maxatase P	n.i.	24	200*	4		Mann (1971)
Tubifex sp.	Subtilisin (Maxatase P)	n.i.	24	50*	4		Mann (1971)

* data probably not based on active substance content

Table 6: Acute algae toxicity

Test species	Test specimen	Test guideline	Exposure time (d)	EC50 (mg aep/l)	Klimisch reliability code	enzyme modification	Reference
Desmodesmus subspicatus (syn. *Scenedesmus subspicatus*)	Subtilisin BLAP (highly purified)	38412 (9)	4	0.3	2	wild type	Henkel (1989a)
	Subtilisin BLAP (PM 111)	38412 (9)	4	3.0	1	protein-engineered	Henkel (1993b)
	Alcalase	OECD 201	3	50	1	wild type	Novo Nordisk (1992h)
	Esperase	OECD 201	3	4	1	wild type	Novo Nordisk (1993c)
	Savinase	OECD 201	3	200	1	wild type	Novo Nordisk (1992i)
	Durazym	OECD 201	3	> 150	1	protein-engineered	Novo Nordisk (1991c)
Raphidocelis subcapitata (syn. *Selenastrum capricornutum*)	Subtilisin (Product C)	OECD 201	3	1.5	1	protein-engineered	Genencor (1996f)
	Subtilisin (Product H)	OECD 201	3	0.39	1	wild type	Genencor (1996g)

Table 7: Bacterial toxicity

Test species	Test substance	Test guideline	Exposure time (h)	EC50 (mg aep/l)	Klimisch reliability code	enzyme modification	Remark/Reference
Pseudomonas putida	Subtilisin BLAP (highly purified)	DIN 384012 (27) (respiration inhibition)	0.5	> 120 (EC 0 = 120)	2	wild type	Henkel (1989a)
	Subtilisin	n.i.	n.i.	13,100 (EC10)*	4	n.i.	Schöberl & Huber (1988)

* EC10 is definitely not based on active enzyme protein

4.2.2 PNEC Calculations

The PNEC values of Subtilisin in the different environmental compartments are shown in the following table.

Environmental compartment	EC/LC50 (mg aep/l)	Assessment factor	PNEC
Aquatic organisms	0.1	1000	0.1 µg aep/l
Microorganisms	120 (EC 0)	100	1.2 mg aep/l
Sediment organisms	PNEC derived from aquatic effect data acc. to EUSES		0.085 µg aep/kg
Terrestrial organisms			0.017 µg aep/kg

The derivation of the PNEC for aquatic organisms is based on the most sensitive acute aquatic toxicity endpoint, i.e. the Daphnia EC50 = 0.1 mg aep/l. As data are available for fish, daphnia and algae, an assessment factor of 1000 is to be applied.
The PNEC for microorganisms in sewage treatment plants was conservatively derived from the *P. putida* respiration inhibition EC0 value (representing an EC50 surrogate) using an application factor of 100.
The PNEC values for sediment and terrestrial organisms were calculated by EUSES on the basis of the aquatic toxicity data.

4.3 Environmental risk characterisation

The results of the environmental risk characterisation of Subtilisin based on the modified EUSES standard exposure scenario (cf. 4.1.3) are summarised in the following table.

RCR Water	local regional	0.66 0.53
RCR Sediment	local regional	0.66 0.46
RCR Soil	local regional	0.54 3.2×10^{-3}
RCR STP*	local	1.1×10^{-4}

*sewage treatment plant (STP)

4.4 Discussion and conclusions

The outcome of the risk characterisation of Subtilisin in the present HERA environmental assessment does not indicate a concern for any of the environmental compartments. All risk characterisation ratios (RCR) are below 1 despite the fact that several conservative assumptions have been made regarding the exposure assessment (cf. 4.1.2.1 and 4.1.2.2). An additional conservative element is the derivation of the aquatic PNEC from an acute Daphnia toxicity data which is atypically low compared to the majority of the existing data. Therefore this Daphnia toxicity value is unsuitable to form the basis for the environmental classification of Subtilisins.
Finally, neither exposure nor effects assessment-relevant data provide any indication justifying a differentiation between Subtilisin produced by wild type strains and protein engineered material. Consequently, the environmental risk assessment applies to all representatives of this protease irrespective of their origin.

In sum, the present HERA environmental assessment shows that the use of proteases in detergents poses no concern in the environment.

5. HUMAN HEALTH ASSESSMENT

5.1 Consumer Exposure

5.1.1 Product types

Subtilisin proteases (Subtilisins) are the most commonly used enzymes in household laundry and cleaning products. Subtilisins are present in household laundry powders and liquids, laundry bleach additives, and in machine dishwashing powders and tablets. The Subtilisins concentration in products is very low and depends on the type of product. According to a 2003 A.I.S.E. survey, the Subtilisins concentration typically ranges between 0.007% and 0.098% in products (see table 2).

In addition to household detergents, Subtilisins are also used in some cosmetics and in a number of industrial applications including the leather, bakery and textile industries. In line with the scope of the HERA initiative, this assessment focuses on the use of Subtilisins in consumer laundry and cleaning products and does not consider other applications.

5.1.2 Consumer Contact Scenarios

Based on the product types, the following consumer contact scenarios were identified:

1. Direct skin contact with neat (laundry pre-treatment) or diluted consumer product (hand-washed laundry, hand dishwashing)

2. Indirect skin contact from fabrics containing deposited product

3. Inhalation of detergent dust generated when pouring the product into the machine or the hand washing receptacle.

4. Oral ingestion of residues deposited on dishes

5. Oral ingestion of residues in drinking water

6. Accidental or intentional overexposure

5.1.3 Consumer exposure estimates

5.1.3.1 Systemic exposure

Systemic exposure to Subtilisins associated to each of the consumer contact scenarios identified above is not quantitatively estimated in this assessment. This is contrary to the usual practice in HERA exposure assessments for most other chemicals. The reasons for not quantifying systemic exposure are as follows:

1. Subtilisins do not pose a hazard as a consequence of systemic exposure (see section 5.3 below). It is well known that the key hazard associated with Subtilisins is respiratory (Type 1) allergy. Other than allergy, eye, respiratory and skin irritation effects are the only hazards described for Subtilisins.

2. Subtilisins are present in very low levels in products (0.1% or less). Even assuming exaggerated, unrealistic conditions, levels of systemic exposure are not expected to exceed values of a few ng/kg bw/day. This conclusion can be supported by briefly considering each of the potential exposure routes:

I) Oral exposure to Subtilisins will lead to breakdown of the molecule into small peptides and amino acids as for any other ingested protein. In addition, the levels of Subtilisins deposited

on dishes and cutlery washed with products containing Subtilisin can be estimated not to exceed 50 picog per cm^2 and would lead to a theoretical maximum systemic dose of 4.5 ng/kg bw/day (this value was obtained as described in section 5.2.3.4.1 of the HERA TAED Risk Assessment and its derivation is described in detail there), if it were not broken down into peptides in the gastro-intestinal tract (which it is as mentioned before).

II) Inhalation: There is no significant systemic exposure by the inhalation route. The relevant endpoint related to inhalation is respiratory sensitisation which is addressed in section 5.2.1.3.2.

III) Dermal: absorption across intact skin is expected to be precluded by the large molecular size of the molecule. Assuming an exaggerated 1% weight fraction dermal absorption for the sake of argument, the systemic exposure to Subtilisin derived from direct dermal contact with neat liquid laundry compact detergent as a consequence of laundry pre-treatment would not exceed 7 ng/kg bw/day.

5.1.3.2 Inhalation exposure relevant for respiratory allergy

Estimation of exposure will be expressed in units of concentration of enzyme in air (e.g., ng/m^3). It will be referred to as $Exp_{resp\ all.}$

5.1.3.2.1 Inhalation of detergent during laundry washing tasks

Some studies (Van de Plassche et al.,1999) determined an average release of about 0.27 µg dust per cup of product used for machine laundering. Given the composition of powder laundry detergents (Table 2), up to 0.06% of the detergent dust can be expected to be Subtilisin, which translates into (0.27 µg x 0.0006) = 1.6 x 10-4 µg of Subtilisin in the dust. In the worst case assumption that all of the dust is inhaled during machine loading and considering a 1 m^3 volume (default for "direct individual's air space [TGD 2003]) instead of a realistic bigger room volume, the exposure to Subtilisin can be estimated as: $\mathbf{Exp_{resp\ all}}$ = 1.6 x 10-4 $\mu g/m^3$ = **0.16 ng/m^3**.

Levels of airborne Subtilisin concentrations to which consumers may be exposed to as a consequence of performing laundry tasks (dispensing of a powdered detergent into a sink and filling it with water) have actually been calculated and extrapolated from actual measurements of Subtilisin concentrations in air after a number of simulation experiments with prototype laundry detergents containing up to 0.06% Subtilisin. The description of these estimations and the experimental procedure for the measurements is detailed in Appendix 1: "Estimation of Exposure to Enzymes from Early Detergent Formulations". The level of airborne Subtilisin estimated for current types of detergents was 0.0057 ng/m^3. This value was obtained considering a detergent containing 0.034% Subtilisin. Assuming a linear relationship, the levels of airborne Subtilisin generated from use of a detergent containing 0.06% enzyme can be estimated as: $\mathbf{Exp_{resp\ all}}$ = **0.01 ng/m^3**.

5.1.3.2.2 Inhalation of detergent during dish washing tasks

Because of the nature and usage of the automatic dish washing products, inhalation exposure to Subtilisin by consumers may only take place if the dish washing machine is opened before the washing programme has ended. This is not an intended use scenario. However, it may be assumed that such event may occasionally take place. The potential exposure to enzyme could occur in theory if the vapour escaping from the opened dishwasher door contained enzyme. A worst case, non realistic exaggerated exposure can be conceived by considering the exposure derived from industrial dishwashing machines, when operators some time need to open the doors because of occasional interruption of the continuous operations. Measurement of enzyme (amylase) concentration under such conditions have been reported (A.I.S.E. Task Force "Enzyme exposure in industrial dishwashing",

1998) and are described in Appendix 2. The highest peak exposures detected were lower than **1.9 ng/m^3.** Considering the differences in product formulation, level of enzymes and set up of the machinery the household exposure is certainly below this value.

5.1.3.3 Dermal exposure relevant for irritation

5.1.3.3.1 Laundry hand wash

According to the HERA Table of habits and practices [*THPCPWE, Table of habits and practices for consumer products in Western Europe. Developed by A.I.S.E. (Association Internationale de la Savonnerie, de la Détergence et des Produits d' Entretien) within the HERA project in 2002*.], the maximum concentration of laundry detergent in the hand wash solution is 1 %. The highest level of Subtilisin in a laundry product is 0.09 % according to Table 2 above. Therefore, the concentration of Subtilisin to which consumers may be exposed can be expected to be **0.0009 % (w/v) or lower.**

5.1.3.3.2 Laundry pre-treatment

Pre-treatment of clothes with neat concentrated liquid laundry detergent may translate into contact of the hands with undiluted product. In such case, the maximum concentration of Subtilisin to which consumers may be exposed to is **0.09 % (w/v)**.

5.1.3.3.3 Fabric wear

Washing of fabrics with laundry detergents containing Subtilisin may result in deposition of Subtilisin on the fabric. Assuming that any Subtilisin deposited on fabric retains some proteolytic activity after the washing, drying and fabric adsorption process and that such proteolytic activity is available to the skin, one could argue that wearing of those fabrics may lead to skin irritation. The concentration of Subtilisin to which consumers may be exposed as a consequence of fabric wearing can be estimated as follows:

The levels of Subtilisin deposited on fabric were measured (ELISA) after real washing conditions with a number of commercial detergents, fabric compositions, and number of washing cycles. The highest levels detected (compact detergent, 15 wash cycles, synthetic fabrics) were 0.35 µg Subtilisin/g of fabric (Henkel, 2004 (1)). Using this highest deposition value and assuming a fabric density of 10 mg/cm^2 (P&G, unpublished data 1996), the amount of Subtilisin in contact with the skin can be estimated as: 0.35 µg/(g fabric) x 0.01 (g fabric)/cm^2 = **0.0035 µg/cm^2**.
Assuming a film thickness on the skin of 0.01 cm (Vermeire et al.., 1993), the concentration of Subtilisin in contact with the skin can be estimated as:
[0.0035 µg/cm^2] / [0.01 cm] = 0.35 µg/cm^3 = 0.35 µg/ml = 3.5 x 10^{-7} g/ml = 3.5 x 10^{-5} % (w/v) = **0.00003 % (w/v).**

5.1.3.3.4 Hand dish wash

According to the HERA Table of habits and practices [*THPCPWE, Table of habits and practices for consumer products in Western Europe. Developed by A.I.S.E. (Association Internationale de la Savonnerie, de la Détergence et des Produits d' Entretien) within the HERA project in 2002*.], the maximum concentration of dish washing detergent in the hand dish wash solution is 0.1%. The highest level of Subtilisin in a hand dish washing product is 0.01 % according to Table 2 above. Therefore, the concentration of Subtilisin to which consumers may be exposed can be expected to be **0.00001 % (w/v)** or lower.

5.1.3.4 Accidental or intentional overexposure

Accidental exposure of the eyes to Subtilisin may occur in consumers via splashes or spills with a formulated product. Therefore, the eye irritation potential has to be considered in the context of accidental exposure.

5.1.3.5 Indirect exposure from the environment

Indirect exposure to detergent Subtilisin from the environment is negligible (see data from ERA).

5.2 Hazard Assessment

The Subtilisins are protease enzymes of bacterial origin. Protease activity is defined as hydrolysis of proteins with broad specificity for peptide bonds. The enzymatic activity of Subtilisins is expressed at pH 6-11, with maximal activity at pH 10. At a pH of 4 or below, they are immediately inactivated.

Regardless of their specific substrate-pattern all enzymes are proteins, i.e. polymers of natural amino acids linked by peptide bonds. Depending on the specific sequence of the individual amino acids (primary structure) and the physical-chemical interactions between amino acid residues of the polymer and the surrounding (aqueous) medium, three-dimensional globular protein structures are formed which are decisive for their enzymatic activity and specificity.

The general characteristics of a protein are common to all types of enzymes irrespective of their specific activity pattern and their origin. Of course, this is also true for industrially produced Subtilisin enzymes mainly originating from genetically modified micro-organisms. Hence, based on the common principles of the enzyme/protein structures it can be concluded that there is a priori no difference between the metabolism of enzymes, i.e. their biosynthesis and catabolism as well, regardless of their origin from wild type or from genetically modified organisms. The same applies to the toxicological properties and the bioconcentration behaviour of proteinengineered Subtilisin variants, which essentially exhibit comparable properties in this respect, and are not different from natural occurring variants. See also: 3.3.1. Subtilisin Manufacturing Route.

Experimental toxicity tests have been carried out mainly with commercial enzyme preparations which typically contain between 0.5-10% enzyme protein, enzymatically inactive proteins, carbohydrate/ polysaccharide and inorganic salts. In the following sections the preparations tested are referred to by trade name where this was reported. The hazard assessment is based on published and unpublished data, as well as published studies presented in an Australian review (NICNAS 1993) and in the occupational risk assessment of Subtilisin protease by the UK Health and Safety Executive (HSE, 2003).

5.2.1 Summary of available toxicological data

5.2.1.1 Acute toxicity

5.2.1.1.1 Acute oral toxicity

To evaluate the acute oral toxicity Purafect™ in 33 % propylene glycol (Genencor 1995 (1)) was orally administered to two groups of ten fasted rats (five males and five females per group) at dose levels of 2000 and 5000 mg/kg. An additional group of ten rats was administered 33 % propylene glycol at dose level of 5000 mg/kg and served as a vehicle control. Observations were made at approximately 1 and 4 hours after dosing and daily for 14 days. Mortality was recorded daily. Body

weights were recorded prior to dosing, on day 7 and at termination or when found dead. All surviving animals were sacrificed on day 14 and a gross necropsy was performed. No clinical signs were observed in any animal in the vehicle control group during study. The only clinical sign observed at 2000 mg/kg was diarrhoea in three animals. Clinical signs observed at 5000 mg/kg included decreased activity, abnormal gait, abnormal stance, dyspnoea, diarrhoea, prostration and chromodacryorrhea. All surviving animals were normal by day 2 at 2000 mg/kg and day 3 at 5000 mg/kg. There was an increase in mean body weight of all surviving animals on day 7 and 14. None of the animals died in the vehicle control group or at 2000 mg/kg. Five of ten animals (one male and four females) died at 5000 mg/kg. Necropsy of the animals that died on study revealed discoloured, distended and fluid-filled stomach and intestines. No visible lesions were observed in any animal at terminal necropsy. Based upon the results, the estimated acute oral LD_{50} for Purafect™ in 33 % propylene glycol was determined to be greater than 2000 mg/kg for female rats and greater than 5000 mg/kg for male rats, or 5000 mg/kg for combined sexes.

In acute oral toxicity tests with Alcalase™concentrate (Novo Nordisk 1981 (1), 1981 (2), 1985 (1), and inactivated Alcalase™ (Novo Nordisk 1985 (2)) the LD_{50} values were 1.5 g aep/kg in mice and 0.83 g aep/kg in rats. When Alcalase™ is inactivated by treatment with hydrochloric acid, the toxicity is significantly reduced. Thus, the proteolytic activity contributes essentially to the toxic effect. The following clinical signs were reported at doses near to or exceeding the LD_{50} values: Piloerection within the first 30 minutes after administration, decreased motor activity, increased respiration, ptosis and diarrhoea (within 2-4 hours after administration). Also high gait and mydriasis have been observed. Nearly all deaths occurred within 24 hours from dosing. In surviving animals, faeces were normal 24 hours after dosing. In animals which died due to administration of Alcalase™, autopsy revealed dilated intestines with dark-brown mucous or haemorrhagic contents, sometimes described as a thin, dark, or watery content; further, the mucous membrane of the intestine showed loose and indistinct structures. The cause of death is ascribed to gastrointestinal disturbance/bleeding. In animals which survived until they were sacrificed, no organ changes related to the treatment were observed. From these studies it is concluded that the main clinical signs and the causes of death are ascribed to the gastrointestinal disturbances.

Savinase™ was tested in another acute oral toxicity study (Novo Nordisk 1991 (1)) with 20 rats divided into four groups. The test solution was Savinase™ dissolved in tap water to a 15 % concentration. Application was once by oral gavage at a dose volume of 10 ml/kg (corresponding to 1500mg/kg = 0.37 g aep/kg). Clinical signs were recorded daily, whilst body weight was recorded on day 1, 8 and 15, day 1 being the day of treatment. There were no premature deaths. All animals suffered from diarrhoea and skin irritation around the anus for the first three days of dosing. There were no notable effects on body weight and body weight gain or any other signs of toxic effects related to treatment. The conclusion of this study is that the LD_{50} for Savinase™ was >1.5 g/kg (0.37 g aep/kg) and the "no effect level"<1.5 g/kg body weight (0.37 g aep/kg body weight).

In an acute study on rats (Novo Nordisk 1987 (1)) with two batches of inactivated Savinase™ no adverse effects were seen in any animal when dosed with 5 or 10 g/kg of the two batches, respectively.

A further study in rats was performed with Esperase™ (Novo Nordisk 1970 (1)). A sample of Esperase™, activity 7.4 KNPU/g, was prepared as a 33.3% w/v suspension in tap water and administered orally, by gavage, in a single dose to male rats and the animals were observed for 2 weeks. The dose levels were 5.15, 10.16 and 15.52 g/kg. Mortality was seen within 48 hours after dosing. The estimated LD_{50} values were between 5.15-10.16 g/kg, (0.15-0.29 g aep/kg).

In another study the acute oral toxicity of Esperase™ (Novo Nordisk 1982 (1)), activity 60.5 KNPU/g, was investigated in mice. Groups of mice were administered the test substance by gavage in doses of 3.0, 4.5, 6.0 and 7.5 g/kg using tap water as solvent and dosed at a constant volume of 40 ml/kg. The surviving mice from all treated groups developed diarrhoea within 24 hours after dosing, and mortality occurred until 5 days after dosing. The LD_{50} values were calculated according to Finney, and were 7.3 g/kg for males and 6.8 g/kg for females, which corresponds to 1.7 and 1.6 g aep/kg, respectively.

The same result could be observed in another acute oral toxicity study in mice dosed with Esperase™ (Novo Nordisk 1982 (2)), activity 54.4 KNPU/g. Groups of mice were administered the test substance by gavage in doses of 3.0, 4.5, 6.0 and 7.5 g/kg using tap water as solvent and dosed at a constant volume of 40 ml/kg. The surviving mice from all treated groups developed diarrhoea within 24 hours after dosing, and mortality occurred until 5 days after dosing. The LD_{50} values were calculated according to Finney, and were 10.3 g/kg for males and 6.4 g/kg for females, which correspond to 2.1 and 1.3 g aep/kg, respectively.

Gavage studies have been conducted in rats with two Subtilisin preparations, Savinase ™ and Opticlean-M (NICNAS, 1993). Groups of 10 male and female rats were given 0, 1.48, 1.6, 2.0, 2.65, 3.65, 4.0 or 4.44 g/kg Savinase™ powder in aqueous suspension and were observed for 14 days. An LD_{50} value of around 3 g/kg was determined. All treatment-related deaths occurred within 24 hours of dosing. No treatment-related histopathological changes were observed. In a similar study design, groups of 5 male and female rats were given 5 g/kg Opticlean-M granules, finely ground and suspended in 1% aqueous methylcellulose and observed for 14 days (NICNAS, 1993). No deaths occurred. All rats showed piloerection shortly after dosing which resolved by day 2. Macroscopic examinations did not reveal any adverse effects.

In another study, the acute oral toxicity of two enzyme preparations, a Subtilisin Carlsberg preparation, containing 5-15% enzyme, 8% other proteins and 60% inorganic salts, and a Subtilisin BPN preparation, containing 5-15% enzyme, 25-30% other proteins and 8-25% inorganic salts, were assayed in the rat (Griffith et al., 1969). Enzyme preparations were diluted to 20% aqueous solutions and dosed to groups of ten rats, which were then observed for 14 days. The LD_{50} values of the 20% solutions of the enzyme preparations were reported as 3700 mg/kg for Subtilisin Carlsberg and 9000 to above 10,000 mg/kg for Subtilisin BPN.

These published data are shown in relation to the in-house data of several enzyme producing companies in table 1 “acute oral toxicity” (?). In contrast to the data for confectionated enzyme products (liquid and granulated enzyme products), the majority of the data shown in table 1 resulted from the test of enzyme concentrates.

Summary of acute oral toxicity

All data were based on the test of enzyme containing concentrates and preparations, containing the enzyme protein in a range of 1 to approx. 50%. These Subtilisin preparations are of low oral toxicity after single exposures in rats with LD50 values ranging from 3 to more than 10 g/kg. When calculated on the basis of theoretical pure active enzyme substance the LD50 values are in the range of 1100 mg aep/kg, with the lowest figure at 100 mg aep/kg.
When the enzymes were inactivated, the toxicological potential is decreased by orders of magnitude. Thus, the proteolytic activity contributes essentially to the toxic effect, especially under the specific conditions of the gavage administration.

5.2.1.1.2 Acute inhalation toxicity

Three groups of five male and five female albino rats each were exposed for 4 hours, using nose-only exposure methods, to aerosol concentrations of Purafect™ FN 2 (Genencor 1991 (1)). The exposure concentrations were 2.1, 2.8 and 1.4 mg/L for Groups IV, V and VI, respectively. The aerosol was characterised by a mass median aerodynamic diameter from 3.4 to 3.6 microns. Mortalities were observed at all three exposure levels, 3 of 10 at 2.1 mg/L, 3 of 10 at 2.8 mg/L and 2 of 10 at 1.4 mg/L. Significant pharmacotoxic signs observed over the course of the study included laboured breathing, lethargy and ataxia. The ataxia noted immediately post-exposure was probably more related to confinement in the restraint tube rather than test material related, since the highest incidence was noted at the lowest exposure level. All groups lost body weight on day 1. Group IV males and females and Group V females exhibited weight loss on day 3, while Group VI animals and Group V males exhibited depressed weight gain. All groups exhibited weight gain on days 7 and 14. Animals which

died on study exhibited pulmonary congestion, while animals that survived to the 14-day necropsy exhibited no significant macroscopic abnormalities. Based on the results of this study, the 4 hour LC_{50} was found to be greater than 2.8 mg/L.

In another Savinase™ study (Novo Nordisk 1978 (1)) with four groups, each containing seven male and seven female rats, were exposed continuously for four hours to different levels of the dust of Savinase™. The levels within the chambers ranged from 0.058 mg/L to 0.157 mg/L. All rats were observed at frequent intervals throughout each four hour exposure period and checks on appearance and behaviour were made subsequently during the 14 day post-exposure observation period. All animals, dying as a result of exposure or killed at the end of the 14 day post-exposure observation period, were subjected to gross pathology. The respiratory tract was macroscopically examined in detail, the lungs dissected out and weighed in order to calculate the lung to body weight ratio for each animal. Increased urination and defaecation were noted during exposure for all animals in all groups. Deaths occurring during exposure were confined to the group with the highest exposure level. Five animals died between two hours and fifty minutes and the end of the four hour exposure period. The macroscopic pathology revealed changes considered related to the effect of the dust of Savinase™ in the lungs of all animals that died as a result of exposure. These changes were typified by massive haemorrhage, congestion and oedema. Nothing abnormal was detected in the lungs of animals in the control group.
Brown areas on the lungs of surviving test animals killed at the end of the 14 day post exposure observation period were considered to be the result of accumulation of haemosiderin pigment within lung alveolar macrophages and the accumulation of these macrophages and pigment near the surface.
The LC_{50} (four hours) for the particulate aerosol generated for Savinase™ was estimated from the mortality curve to be 0.13 mg/L of chamber air. For this study can be concluded that the LC_{50} (four hours) for Savinase™ was 0.13 ± a standard error of 0.017 mg/L of chamber air (0.13 mg/L = 0.0177 mg aep/L). A major factor in the cause of death of these rats that died as a result of exposure was an acute pneumonia, typified by massive pulmonary oedema together with lung congestion and haemorrhage. The changes seen during this study are considered to be typical of the action and result of inhalation of a highly concentrated proteolytic enzyme powder.

Studies concerning Alcalase™ (Novo Nordisk 1993 (1)) were carried out during the period 1980-1985. The methods used were essentially meeting the recommendations in the OECD TG 403 (1981) and EEC-guidelines (1984). All studies were carried out as nose only studies, with an exposure time of 4 hours. All air concentrations are given as actual concentrations. Treatment related clinical observations were: Struggling, increased urination and defaecation, blood-stained snouts or nasal bleeding and respiratory distress. Further, the animals appeared subdued and hunched. The amount of food, consumed by animals surviving the exposure, was below normal in the first few days of the observation period. Corresponding to the reduced intake of food, the rate of weight gain and the body weight was reduced in this period. Autopsy of dead animals revealed abnormalities of the lungs in form of oedema and haemorrhage. These findings are considered to have caused the deaths. Animals surviving to sacrifice showed either no dose-related organ changes or some reminiscences of lung changes, such as brown areas on the lung surface. Lung to body weight ratios in animals which died following the exposure were very high due to the oedema and haemorrhage, whereas the ratios in surviving animals were only slightly higher than those of the controls. It can be concluded that the LC_{50} (4 hours) for Alcalase® is in the range of 0.47 to 1.05 mg/L (~ 6.12 to 21.5 AU/m^3 ~ 0.1 to 0.4 mg aep/L). A major factor in the cause of death was massive pulmonary oedema together with lung congestion and haemorrhage. The lung changes were reversible, if survived. The reactions observed during the studies are considered to be typical reactions after inhalation of proteolytic enzyme powders.

Single exposure studies have been conducted in the rat with two Subtilisin preparations, Savinase™ and Opticlean P™ Conc. (NICNAS, 1993). Groups of 7 male and female rats were exposed nose-only to 0, 0.058, 0.070, 0.132 or 0.157 mg/L Savinase™ powder for 4 hours. Around 70% of particles were in the respirable range (below 5.5 µm in diameter). In a similar study, groups of 5 male and female rats were exposed nose-only to 0, 0.108, 0.196, or 0.298 mg/L Opticlean P™ Conc. powder. Around 75% of particles were in the respirable range (below 5.5 µm in diameter). No information on the enzymatic

activity of these preparations was given. At the end of the 14-day observation period macroscopic examinations of the abdominal, thoracic and cranial cavities were performed, the lungs were weighed and lung to bodyweight ratios determined. No microscopic examinations or lung function tests were performed.
LC_{50} values of 0.130 mg/L (Savinase™) or 0.229 mg/L (Opticlean P™ Conc) were determined with deaths occurring within 24 hours at exposure greater than 0.070 mg/L for Savinase™ and greater than 0.196 mg/L for Opticlean P™ Conc. Signs of toxicity were similar for both enzyme preparations and were consistent with a destruction of lung tissues due to the proteolytic activity of these enzymes. During exposure animals from all treated groups showed respiratory impairments, which resolved by day 7. Other clinical signs included blood/brown staining around the snout and also around the jaws and urogenital areas of rats. All signs were cleared by day 10. In addition, all treated animals showed an initial decrease in bodyweight and food consumption but this returned to normal by day 3. Macroscopic examinations of the lungs of dead animals revealed increased lung weights, haemorrhage (particularly for Savinase™ exposed rats), congestion and oedema. "Slightly" increased lung weights were found in rats surviving to the end of the study. Brown areas attributable to haemosiderin accumulation or grey areas were also visible on the lungs of these rats.

The effects of single inhalation exposure to Subtilisin were studied in groups of 18 male albino rats, 9 male albino rabbits and 9 male Hartley guinea pigs, exposed whole body for 6 hours to 0.001 to 0.0368 mg/L of a 12% preparation of the Subtilisin Carlsberg enzyme Alcalase™ in a matrix of inactive proteins, non-proteinaceous organic material and inorganic salts (Richards et al., 1975). The preparation was in the form of a dry powder, particle size unknown. Based on the enzymatic activity of dust sampled from the exposure chamber, the authors estimated that animals had been exposed to 0.1 to 4.4 µg/L enzyme. Animals were killed at 1, 4 and 16 days post-exposure, and necropsy performed with limited histopathology (lungs, liver, kidneys, spleen, tracheobronchial lymph nodes, adrenal glands). There were no treatment-related deaths. No clinical signs were apparent in rats or rabbits. However in guinea pigs, hyperactivity followed by sneezing, excessive salivation and laboured breathing were noted at concentrations of 4.2 µg/L enzyme preparation (0.5 µg/L enzyme) and above. Pathological changes were only evident in the lungs. No changes were observed in the lungs of rats or rabbits exposed to 1 µg/L enzyme preparation (0.1 µg/L enzyme). The authors did not clearly report findings from guinea pigs at this concentration. In rats and rabbits exposed to concentrations of 4.2 µg/L enzyme preparation (0.5 µg/L enzyme) and more, small patchy areas of haemorrhage were observed. More extensive damage consisting of haemorrhage, pulmonary oedema and congestion was visible in guinea pigs at this concentration. In all three species pathological changes had resolved by day 16.

Two other studies are available in which guinea pigs were exposed to purified Subtilisin (Markham and Wilkie, 1979) or 3% aqueous concentrations of Alcalase™ or Maxatase™ (Goldring et al., 1970). However the exposure conditions are not well described, with no information provided on airborne concentrations and particle sizes of the aerosols. On this basis it is only possible to draw qualitative conclusions from these studies. The toxicities seen were lung haemorrhage and oedema, which is consistent with observations in all other studies.

Summary of acute inhalation toxicity

There are no human data on the effects of single exposures to Subtilisin. Single exposure inhalation studies in animals indicate that Subtilisin, depending on its concentration, may cause direct effects on the lungs, such as haemorrhage, congestion and oedema, probably reflecting the proteolytic activity of these enzymes. No other tissues appear to be affected. Four-hour LC_{50} values between 0.13 and 1.05 mg/L were obtained in the rat. Enzyme preparations described above showed an enzyme content of 14% to 36%, respectively, of theoretical pure active enzyme protein. When calculated on the basis of this aep the average LC_{50} range from 0.1 to 0.2 mg aep/L, with the exception of a single value found at 17.7 µg aep/L.

5.2.1.1.3 Acute Dermal Toxicity

Due to the lack of experimental data it was not possible to determine an LD_{50} value. But according to their relatively large molecular weight Subtilisin enzymes are not expected to be able to penetrate through the intact skin and, therefore, it can be expected that these enzymes do not exert any acute systemic toxicity upon dermal exposure.

Summary of Acute Dermal Toxicity

The effects of single dermal exposures have not been studied but given the predicted lack of dermal absorption, systemic toxicity would not be anticipated by this route.

5.2.1.2 Corrosiveness/Irritation

5.2.1.2.1 Skin Irritation

Studies in animals

The skin irritation of enzyme protein on animal skin is measured and scored as Primary Irritation Index (PII) with the maximum attainable score of 8.0.

One group of six rabbits was dosed dermally with undiluted Purafect™ FN2 (Genencor 1991 (2)). Purafect™ FN2 (≈ 0.05 gaep/dose) was applied to the back of each rabbit to one intact and one abraded site under one-inch square gauze patches secured with Dermiform® tape. The test sites were then wrapped with gauze bandaging, occluded with plastic film and secured with Dermiform® tape. A collar was also applied to each rabbit. After the end of the 4-hour exposure period the test sites were washed with tepid tap water and dried with disposable towels. The test sites were evaluated for dermal irritation using the Draize method approximately 1, 24, 48 and 72 hours after patch removal and on day 7. Very slight to well-defined erythema was observed at both sites on all rabbits at 1 hour. The erythema generally remained at these levels through 48 hours. By 72 hours, however, the erythema diminished significantly or cleared altogether. Very slight to slight degrees of oedema were additionally observed for all rabbits at on or both test sites at 1 hour, continuing to hour 48 in three rabbits. Test sites on three rabbits cleared by hour 24, all sites were clear of oedema by 72 hours. The test sites on all rabbits cleared of dermal irritation by day 7. The abraded test sites exhibited higher group average dermal irritation scores for erythema than the intact test sites at the 24, 48 and 72 hour evaluations with the 24 hour irritation score exhibiting the greatest difference between the intact and the abraded test sites. There were no remarkable differences observed in oedema between the intact and abraded sites. Desquamation was additionally observed at one or both sites of four rabbits on day 7. Based on the dermal irritation properties observed Purafect™ FN2 would be considered mildly irritating. The Primary Irritation Index (PII) was 1.3 (≈ 0.05 g aep/dose).

In another primary dermal irritation test using the same study design (Genencor 1991 (3)) two groups of three rabbits each were dosed dermally with Purafect™ FN2 concentrate (≈ 0.05 gaep/dose) and FNA concentrate (≈ 0.05 gaep/dose), and two groups of six rabbits were dosed dermally with Multifect™ P-3000 UF concentrate (≈ 0.05 gaep/dose). All materials were applied undiluted as received to the back of each rabbit, on one side the skin was intact and the other side abraded, under one-inch square gauze patches secured with Dermiform® tape. The test sites were wrapped with gauze bandaging, occluded with plastic wrap and overwrapped with Dermiform® tape. A collar was also applied to each rabbit. The test and control articles remained in contact with the test side for approximately 4 hours. Following the exposure period, the bandaging materials and collar were removed and test sites washed with tepid tap water and dried with disposable towels. The test sites were evaluated for dermal irritation using the Draize method 30-60 minutes after patch removal and at 24, 48 and 72 hours after patch removal. One rabbit dosed with FNA and one rabbit dosed with Purafect™ FN2 concentrate exhibited very slight erythema and very slight oedema at the abraded site

at 24 hours. These findings cleared by 48 hours in the FNA treated rabbit as did the oedema in the Purafect™ FN2 treated rabbit, but the erythema in the Purafect™ FN2 treated rabbit persisted to 48 hours. The Purafect™ FN2 treated rabbit was clear of all findings by 72 hours. No dermal irritation was observed at either test side of rabbits treated with Multifect™ P-3000 UF concentrate. Based on the dermal irritation properties observed Purafect™ FN2 and FNA are both considered mildly irritating, and Multifect™ P-3000 UF concentrate is considered non-irritating.

The potential irritant and/or corrosive effects of Purafect™ FN2 (PR119) (≈ 0.05 gaep/dose) (Genencor 1994 (3)) were evaluated on the skin of New Zealand White rabbits. Each of six rabbits received a 0.5 ml dose of the test article as a single dermal application on intact skin. The dose was held in contact with the skin under a semi-occlusive binder for an exposure period of four hours. Following the exposure period, the binder was removed and the remaining test article was wiped from the skin using gauze moistened with distilled water. Test sites were subsequently examined and scored for dermal irritation for up to 7 days following patch removal. Exposure to the test article produced very slight erythema to mild blanching on 6/6 test sites and moderate to severe oedema on 5/6 test sites at the 1 hour scoring interval. The dermal irritation progressed to eschar on 5/6 test sites by the 24 hour scoring interval and generally persisted throughout the remainder of the test period. The dermal irritation resolved completely in 1/6 animals by study termination (day 7). Additional dermal findings noted during the period included eschar exfoliation, which was noted on 5/6 test sites. Under the conditions of this test, Purafect™ FN2 is considered to be a moderate irritant to the shin of the rabbit. The calculated Primary Irritation Index for this sample of Purafect™ FN2 was 4.75.

Using the same test design as above the potential irritant and/or corrosive effects of Purafect™ FN3 (PR330) (≈ 0.05 gaep/dose) (Genencor 1994 (1)) were evaluated. Test sites were subsequently examined and scored for dermal irritation for up to 14 days following patch removal. Exposure to the test article produced moderate to severe erythema, focal/pinpoint to severe blanching and slight to moderate oedema on 6/6 test sites at the 1 hour scoring interval. The dermal irritation progressed to eschar on all test sites by the 24 hour scoring interval. The dermal irritation generally persisted through study day 7. Following the day 7 scoring interval, the dermal irritation generally diminished but did not resolve completely in any animals by study termination (day 14). Additional dermal findings noted during the period included eschar exfoliation and desquamation, which were noted on 6/6 and 1/6 test sites, respectively. Under the conditions of this test, Purafect™ FN3 is considered to be a severe irritant to the skin of the rabbit. The calculated Primary Irritation Index for the test article was 6.13.

In another test of the same design the potential irritant of Purafect™ FN3 (PR330) (≈ 0.05 gaep/dose) (Genencor 1994 (2)) were evaluated. Test sites were subsequently examined and scored for dermal irritation for up to 7 days following patch removal. Exposure to the test article produced mild to moderate blanching and moderate to severe oedema on 6/6 test sites at the 1 hour scoring interval. The dermal irritation progressed to eschar on 6/6 test sites by the 24 hour scoring interval and generally persisted throughout the remainder of the test period. The dermal irritation did not resolve in any animals by study termination (day 7). Additional dermal findings noted during the period included eschar exfoliation, which was noted on 6/6 test sites. Purafect™ FN3 is therefore considered to be a severe irritant to the skin of rabbit. The calculated Primary Irritation Index for the test article was 6.58.

Again Purafect™ FN3 (PR330) (≈ 0.05 gaep/dose) (Genencor 1994 (5)) was used in another test of the same design to examine the potential irritant and/or corrosive effects on the skin of New Zealand White rabbits. Test sites were subsequently examined and scored for dermal irritation for up to 72 hours following patch removal. Exposure to the test article produced blanching and moderate to severe oedema on 6/6 test sites at the 1 hour scoring interval. The dermal irritation progressed to eschar on 6/6 test sites by the 24 hour scoring interval and persisted in all animals throughout the remainder of the test period (72 hour scoring interval). Additional dermal findings noted during the period included clear fluid exuding from the test area, which was noted on 6/6 test sites. Also noted during the study were clinical observations of abrasion like lesions on lateral abdominal and shoulder areas of 2/6 test animals. These clinical observations may have been attributed to leakage of the test article from the

exposure site. Under the conditions of this test, Purafect™ FN3 is considered to be a severe irritant to the skin of the rabbit. The calculated Primary Irritation Index for the test article was 7.42.

In another study of the same test design Purafect™ OxP (≈ 0.05 gaep/dose) (Genencor 1994 (4)) was evaluated on the skin of rabbits. The test sites were subsequently examined and scored for dermal irritation for up to 14 days following patch removal. Exposure to the test article produced erythema (very slight to well-defined) to mild blanching on 6/6 test sites and very slight to moderate oedema on 5/6 test sites at the 1 hour scoring interval. The dermal irritation progressed to focal/pinpoint to severe eschar on 4/6 test sites by the 48 hour scoring interval. The dermal irritation generally diminished during the remainder of the test period and resolved completely in all animals by study day 14. Additional dermal findings noted during the period included eschar exfoliation, which was noted on 3/6 test sites. Under the conditions of this test, Purafect™ OxP is considered to be a moderate irritant to the skin of the rabbit. The calculated Primary Irritation Index for the test article was 3.38.

A further study of this type was performed with Purafect™ OxP (≈ 0.015 gaep/dose) (Genencor 1994 (6)). Test sites were subsequently examined and scored for dermal irritation for up to 72 hours following patch removal. Exposure to the test article produced blanching and slight to moderate oedema on 5/6 test sites and well-defined erythema with focal and/or pinpoint areas of blanching and moderate oedema on 1/6 test sites at the 1 hour scoring interval. The dermal irritation progressed to eschar in 6/6 test sites by the 24 hour scoring interval and persisted throughout the remainder of the test period in all animals (72 hour scoring interval). Additional dermal findings noted during the period included clear fluid exuding from the test site, which was noted on 5/6 test sites. Under the conditions of this test, Purafect™ OxP is considered to be a severe irritant to the skin of the rabbit. The calculated Primary Irritation Index for the test article was 5.67.

In another study (Novo Nordisk 1977 (2)) a primary skin irritation test was performed on Savinase™ using two groups each containing six albino rabbits. On the intact and abraded skin of the clipped back of each of the animals, a 0.5 ml sample of Savinase™ was applied under an occlusive patch of gauze. Readings were made 24 and 72 hours after the initial application of the patches. The test substance solutions were: 25% w/v Savinase™ in an aqueous solution (0.017 g aep/0.5 ml sample) and 25% w/v Savinase™ in Sørensen's borate buffer (0.017 g aep/0.5 ml sample). Under the test conditions employed, both buffered and non buffered solution were indistinguishable by effect; each provoked a mild to moderate erythematous response in the entire group at 24 hours, with a single animal displaying a barely perceptible oedema. At 72 hours the reactions had subsided and only the last-mentioned animal displayed a barely perceptible erythema. In conclusion Savinase™ can be classified as a "mild irritant" (Primary Irritation Index < 2) to rabbit skin.

The primary skin irritation potential was also evaluated for Esperase™ (Novo Nordisk 1970 (1)) with the activity of 7.4 KNPU/g in six albino rabbits. Prior to administration, the hair was clipped from the back of the animals where after 0.5 g (0.014 g aep/0.5 g) of the test material was applied to one abraded and one intact area. The treated areas were covered with a gauze patch for 24 hours. The readings were performed 24 and 72 hours after the initial application of the patches, and scored according to Draize. The average scores of the two readings were used to determine the primary irritation score. The results showed that all rabbits were negative, thus the primary irritation score was 0. In conclusion, Esperase™ is neither irritating to the abraded nor to the intact skin of rabbits in a concentration of 0.014 g aep/0.5 g.

The skin irritancy of Savinase™ and Opticlean-M has been studied in rabbits (NICNAS, 1993). In the Savinase™ study, groups of 6 rabbits received topical applications (0.5 ml) of 5% aqueous solutions of the enzyme preparation at either pH 7.0 or pH 9.1, to both intact and abraded skin. Test sites were covered with an occlusive dressing for 24 hours and scored at the time the dressing was removed (24 hours) and 72 hours after application. Reactions described as mild to moderate erythema were observed in all rabbits at 24 hours irrespective of treatment, accompanied in two animals (treatment not specified) by very slight oedema. This had diminished to very slight erythema, seen in only five animals, by 72 hours. In the Opticlean-M study, 500 mg of granulated enzyme preparation, moistened with distilled water, was applied to the skin of 3 rabbits and test sites covered with a semi-occlusive

dressing for 4 hours. Test sites were then washed and animals observed for 4 days. The only sign of skin irritation was slight erythema, observed in all rabbits on day 1 only.

In an earlier study, 0.5 ml of 1% aqueous solutions of detergent with and without Subtilisin enzyme were applied under an occlusive dressing to the skin of albino rabbits for 24 hours (Griffith et al., 1969). From information in the paper, it can be calculated that the final enzyme protein concentrations would have been around 0.0001-0.00045%. Scores were read at patch removal and at 72 hours, and primary irritation indices of 1.3 without enzyme and 3.7 or 4.8 with enzyme calculated according to the method of Draize. These scores suggest mild irritancy. Given that the irritation produced in the preceding studies using much higher concentrations of enzyme in the absence of a detergent matrix was fairly mild, it is not clear if the apparent increased irritancy of the detergent with added enzyme was entirely due to the very low concentration of enzyme or to another factor. Overall this study is of limited use in determining the skin irritant properties of Subtilisin.

Griffith et al. (1969) also investigated the effects to rabbits of daily dermal exposure to a detergent formulation containing 0.25% enzyme protein for 13 weeks. Very few details were reported. It appears that this formulation produced a greater degree of irritation than the detergent without the enzyme. These results are consistent with the single exposure results obtained by Griffith et al. (1969) but are not sufficient to assess the skin irritant properties of Subtilisin.

Further studies performed with liquid enzyme products ((Novo Nordisk 1983 (1), 1981 (9), 1981 (10), 1981 (12), 1981 (13)) are only mentioned in the appending tables. The results of these studies correspond to the studies on Subtilisin concentrates.

Summary of skin irritation (animals)

Depending on the type of enzyme preparation (especially the concentration of the enzyme) and the conditions of enzyme application, subtilisin preparations were found to show a range of effects from non-irritant to severe irritant.

Studies in human volunteers

Alcalase™ concentrate was tested in a human skin irritation study (Novo Nordisk 1978 (3)) for the determination of the ID_{50} (dose required to produce irritation in 50% of the subjects). The activity was 14.3 AU/g (~ 25.1% active enzyme protein (aep)). The ID_{50} determination was performed on 10 adults in a twenty-four hour patch test (0.05 ml on 1 cm² patch) with range of concentrations (0.05%, 0.1, 0.25, 0.5, 0.75% w/v). As a result the ID_{50} was determined at ~ 0.25 % w/v (~ 0.06 % aep ~ 31 µg aep/cm²) and the NOEL was assessed at ~ 0.05 % w/v (~ 0.013 % aep, ~ 6.3 µg aep/cm²).

Another test (Novo Nordisk 1980 (1)) was carried out with Alcalase™ activity 6.73 AU/g. Groups of ten subjects were exposed via a Duhring Chamber to Alcalase™ in water on respectively normal and scarified skin of the volar forearm. New solutions (100 µl) were applied daily for three consecutive days, i.e. a continuous exaggerated exposure for 3 days. Each subject was exposed to 4 different concentrations of Alcalase™ in water, 0.66 %, 0.33 %, 0.13 %, and 0.07 %, respectively.

The results showed that Alcalase™ in water can incite a concentration dependent irritation on normal and scarified human skin. The NOEL was assessed at ~ 0.07 % w/v (~ 0.008 % aep, ~ 8 µg aep/cm²).

Esperase™ with the activity of 7.4 KNPU/g (~ 2.81% active enzyme protein (aep)), was tested (Novo Nordisk 1970 (1)) for the determination of the ID_{50} skin irritation in 10 human volunteers according to the method described by Kligman et al. 1967. The exposure was under an occlusive patch left on the skin for 24 hours to concentrations ranging from 0.25 to 5 %w/v. The ID_{50} was determined at ~ 1.2 % w/v corresponding to 0.03 % aep ~ 8 µg aep/cm².

In another study (Novo Nordisk 1970 (1)) Esperase™, activity 7.4 KNPU/g, was tested for skin irritation in human volunteers. Aqueous solutions of Esperase™ 5 and 10 %, ($1.4x10^{-3}$ and $2.8x10^{-3}$), a paste, 90% (0.026 g aep/g), were tested for 24 hours under occlusive dressing. Also a 0.2 % aqueous solution ($5.7x10^{-5}$ g aep/ml), was tested and left for 72 hours with readings made every 24 hours. The results showed no reaction to the test material. To conclude, Esperase™ induced no irritating effects to human skin applied in high concentrations (0.026 g aep/g) in this study.

In order to improve the knowledge of the irritating properties, an additional test (Novo Nordisk 1978 (4)) with Savinase™ was performed on human volunteers. In accordance with the method described by Kligman and Wooding, an exaggerated occluded human patch test was performed to evaluate the irritation potential using 10 human volunteers. The test substance was Savinase™ with the activity of 53.9 KNPU/g (~ 13.6 % active enzyme protein (aep)) diluted in water to a concentration of 1% was applied once daily for 10 days at a dose of 0.25 ml per pad (0.0003 g aep/0.25 ml). Under the test conditions employed, Savinase™ did not cause any instances of primary skin irritation, so in conclusion, the NOEL for this Savinase was above 0.1 % w/v ~ 0.14 % aep, ~ 68 µg aep/cm².

For the following studies published in the scientific literature there is no precise information available on the exact active enzyme protein content or enzymatic activity. Therefore it is not possible to compare the results on the basis of active substance or activity. Due to the years when the studies were made it can be estimated that the active substance content of the products tested was in the range of 1% aep.

A series of studies, including both single and repeated exposures to intact skin and skin pre-treated with dilute acid or alkali or tape stripped, has been conducted to investigate the irritancy of the Subtilisin preparation Maxatase™ (Valer, 1975a). Groups of between 20 to 100 panelists were used for each investigation. No details of the volunteers were provided. In studies of normal skin, the enzyme preparation in aqueous solution at pH 5 or 8.5 was applied once to the forearm under occlusive patches at concentrations of 0.25-20% and test sites observed 48 hours after patch removal. Additional studies were carried out in which 5 x 24 hour applications were made of 0.25-5% aqueous solutions at pH 8.5. A period of 24 hours elapsed between each application and test sites were scored 24 hours after removal of the last patch. Distilled water buffered to the appropriate pH was used as a control. No effects could be attributed to the Maxatase™.

Further studies were performed in which 3 x 24 hour applications of 0.25-5% aqueous solutions of Maxatase™ at pH 8.5 were made to skin artificially irritated by a prior 24 hour application of either dilute sodium hydroxide or dilute carbolic acid. Buffered distilled water was used as a control. No irritation was apparent. In contrast, when a single 72 hour application was made to intact skin of 0.25-5% concentrations in a 70% DMSO solution or Maxatase™ was applied once for 24 hours as 0.25-5% aqueous solutions to tape stripped skin immediately after stripping, concentrations of 2% or more produced irritant reactions in a few individuals (between 1-4 out of 40 tested). The numbers of individuals affected and severity of the reactions increased with increasing concentration. One control subject given 70% DMSO showed grade 1 skin irritation as did one control subject with buffered distilled water applied to tape stripped skin. Overall, this study shows that dilute aqueous solutions of Maxatase™ are not irritant to intact skin but that Maxatase™ may be irritant to damaged skin.

Studies have been conducted with volunteers to examine the irritancy of detergents containing Subtilisin variants BPN' and Carlsberg (Griffith et al., 1969). These studies were aimed at assessing consumer exposure and included patch testing, arm washing, hand immersion and home use studies. It is not clear if any of the volunteers used for this study had previously been exposed to the enzyme preparations or detergents that were tested. At least 6-10 individuals, possibly many more, received each treatment but the actual numbers tested are unclear from the report. No erythema was observed in patch tests of a 0.2% solution of the enzyme preparation itself (actual enzyme concentration 0.01-0.03%) applied once to back skin. When detergent formulations were tested, no or faint to moderate erythema was apparent in individuals given single or triple applications every other day of detergent formulations (actual enzyme concentrations ranging from 0.00009-0.0072%). However, the detergent containing the least enzyme appeared to produce the strongest skin reactions. Hence, the role of

Subtilisin enzymes in the irritation observed in this study is uncertain. Given the low concentrations of enzymes present in the detergent solutions in these studies, the lack of skin reactions is of questionable relevance to occupational or consumer conditions. Since there is no information available on the composition of the different detergents used, this study does not allow to reach conclusions of the protease irritation potential.

In a three-phased randomised, double-blind, cross-over experiment with 26 adult patients with atopic dermatitis the reaction of atopic persons towards enzyme containing detergents was tested (Andersen et al. 1998). In the first phase patients continued to use their normal laundry detergent. In phase II patients used trial detergent with or without added enzymes and during phase III patients were given the opposite trial detergent. A total of 25 patients finished the study. The primary efficiency parameters were inter-period changes in corticosteroid usage and changes in the SCORAD (SCORing Atopic Dermatitis) score. The secondary efficacy parameters were altered subjective symptoms scored during the final two weeks of each interval. Analysis of all data revealed no statistical differences in any of the primary or secondary parameters comparing treatment and placebo periods. The data seem to exclude that atopic dermatitis may be exacerbated during one month exposure to enzyme-enriched detergent. Since no significant irritant capacity was detected in atopic dermatitis patients, it seems unlikely that consumers with "normal skin" will experience any skin discomfort when enzyme enriched detergents are used.

Studies performed with crystalline and crude Alcalase™ are only mentioned in the appending tables. The results of these studies show a NOEL of 5.8 µg aep/cm^2 (crude) and 33.4 µg aep/cm^2 (crystalline).

Epidemiology Data / Human experience

There have been a few reports of skin lesions described as "primary irritant reactions" in workers who handle powdered concentrated enzyme preparations directly (Flindt, 1969; Newhouse et al., 1970; Gothe et al., 1972). Workers had itching and burning skin, sometimes with visible lesions, mainly in the areas of the fingertips, the wrists, under the collar and on the forehead. Newhouse et al. (1970) reported outbreaks of skin rash in detergent manufacturing workers shortly after th introduction of a powdered Subtilisin preparation (Alcalase™) into a detergent manufacturing process.

Subsequent improvements in work practices and the use of personal protective equipment abolished the problem. The rapid onset of these rashes supports the conclusion that these are irritation rather than allergic responses. A temporal link between exposure to a Subtilisin A™ preparation and skin problems was also noted by 12 out of 64 detergent manufacturing workers studied by Gothe et al. (1972). Unfortunately, no tests were performed in either of these studies to determine if these lesions were due solely to Subtilisin or if other components of these Subtilisin preparations were involved. When Watt et al. (1973) looked at the reporting of skin rash in a health surveillance scheme over a 2-year period, it appeared that greater numbers of workers reported skin rash in the March assessments (11-14 %) compared with the September/October assessments (3-7 %). This seasonal pattern suggests non work-related factors may be of importance for this particular group of workers although information from additional years would be helpful to confirm this pattern of reporting. Overall, the role of Subtilisin in the skin problems reported in these studies has not been adequately explored. However, the possibility of a link between skin problems and direct skin contact with concentrated Subtilisin preparations under poorly controlled occupational conditions cannot be excluded.

There have been a limited number of published papers on the public health aspects of enzymatic laundry detergents. Two large studies indicated that the addition of enzymes to laundry powders did not increase the incidence of primary irritation among users (Griffith et al., 1969; Mason Bolam et al., 1971). Two small studies indicated that early domestic use of these products was associated with dermatitis (Jensen, 1970; Ducksbury and Dave, 1970). As dermatitis may also occur in some individuals when using non-enzymatic detergents (Adams, 1983), it is difficult to identify which factor(s) causes the dermatitis. Exposure of consumers, including infants, to fabrics washed in the

enzyme containing detergents or pre-soak agents did not increase the risk of primary skin irritation (Griffith et al., 1969; White et al., 1985).

In vitro studies using excised skin

A study is available in which skin biopsy specimens from the soles of human feet were incubated in Savinase™ solutions (Imai, 1991). The results suggested that the external surface of the skin was unaffected by the enzyme, but as skin from the soles of the feet is likely to be highly keratinised it is of doubtful relevance for predicting effects on skin from the hands or forearms. Hence, no useful conclusions can be drawn from this study, which will not be discussed further.

Summary of Skin Irritation (humans)

In human volunteer studies aqueous solutions containing up to 20% of a typical Subtilisin preparation (aep estimated at approx. 0.2%) were not irritant to intact skin but were irritant in concentrations of 2 % (aep estimated at approx. 0.02%) or more to damaged skin.

At higher concentrations, most proteases behave as primary irritants and the effect is considered due to the proteolytic activity of the enzyme. In studies with volunteers where the active enzyme protein content was determined, ID_{50} was in the range from 0.008 % aep ~ 8 µg aep/cm² to more than 0.1 % aep ~ 68 µg aep/cm², and NOEL was in the range of 0.013 % aep ~ 6 µg aep/cm² to more than 0.1 % aep ~ 68 µg aep/cm². Significant differences in the results were observed, depending especially on the type of application (occlusive, semi-occlusive dressing, repeated dosing over more than 24 hrs., scarified skin). In addition, the irritation effect is strongly dependent on the physico-chemical water activity, the pH and of course the activity and concentration of the protease. Further, the studies performed with human volunteers are based on rather few subjects and when effects are seen, the dose-response curve for proteases is steep. This which might add to the variability seen in these studies that were performed over a 10 yr period but by the same investigator, Dr. Albert M. Kligman.

Workers handling concentrated enzyme preparations without personal protective equipment reported skin problems mainly on the fingertips and on the wrist and neck where perspiration and chafing could exacerbate any irritation. The contributory role of Subtilisin in the skin problems reported in these studies has not been adequately explored.
Studies with volunteers with enzyme containing detergents did not result in irritating effects in patch tests, wash tests and wear tests where patients with atopic dermatitis were tested for the effect of detergents (+/- enzymes) in a double blind study.

5.2.1.2.2 Eye Irritation

***In vitro* studies**

Corrosion of bovine cornea was examined with BLAP S (F 49) granulate in a bovine cornea test (Henkel 1995 (1)). Four bovine eyes from freshly slaughtered animals were exposed to 2.15 % (w/w) equivalent 5000 HPE/ml and 8.60 (% w/w) equivalent 20000 HPE /ml of the enzyme solution applied on intact cornea. The test solutions were prepared one hour prior use. The eyes were stored in 0.9 % sodium chloride at 37°C. The corneas of all eyes were proved to be intact by applying a Fluorescein SE Thio-solution onto the bovine eyes and investigating the corneas by UV-light. The test substances were applied on the eyes for 30 seconds and the test solution was washed off by 0.9 % sodium chloride solution. The application of Fluorescein SE Thio was repeated and the corneas of all eyes were investigated for damages. After macroscopic evaluation a sample of each cornea was fixed in 10 % formalin and histologically examined. In the macroscopic and histological evaluation no corrosive effect could be observed up to 8.6 % (w/w).

The same test design was performed with BLAP S (BLAP S 200) granulate (Henkel 1995 (2)) in a bovine cornea test. In this study four bovine eyes from freshly slaughtered animals were exposed to 2.63 % (w/w) equivalent 5000 HPE/ml and 10.52 (% w/w) equivalent 20000 HPE /ml of the enzyme solution. The macroscopic and histological evaluation did not show any corrosive effect up to 10.52 % (w/w).

***In vivo* studies**

Eye irritation studies (Novo Nordisk 1984 (1), 1984 (2)) were carried out on two specimen of Alcalase™ 2.0T-Granulate, activity 2.59 AU/g and 2.41 AU/g in a modified Draize test with 6 rabbits. In each test, three animals were rinsed after four seconds and the other three animals were treated without rinse. The readings were done 24, 48, 74 hours, and 7 days after application of 3 mg granulate. The test substance was ground to a dusty powder before application. The instillation caused reactions up to diffuse redness with slight swelling of the conjunctiva. The reaction was at peak level at the reading 24 hours after the treatment. No reactions were observed after 7 days. The reaction of the rinsed eyes was significantly less severe, and only one animal in one of the tests showed a slight reaction. The scores according to Draize for sample (1) were with rinse: 0.66 and without rinse 3.33. The scores concerning erythema and oedema were on cornea: 0/4, on iris: 0/2, for redness: 0.89/3 and swelling: 0.1/4. For sample (2) the following scores were stated: with rinse: 0 and without rinse 2.67. The scores concerning erythema and oedema were on cornea: 0/4, on iris: 0/2, for redness: 0.33/3 and swelling: 0/4.

In another study (Novo Nordisk 1970 (1)) the eye irritation potential of Esperase™, activity 7.4 KNPU/g was evaluated in six albino rabbits. 0.1 g, ($2.8x10^{-3}$ g aep/0.1 g), of the enzyme powder was placed in one eye, whilst the other untreated eye served as a control. The eyes were not rinsed following application. The reaction to the test material was read according to the scoring system of Draize for damage to the cornea, iris and the conjunctivae at 24, 48 and 72 hours after application. The results showed a score of 1 for redness, chemosis and discharge in the conjunctivae for 5/6 rabbits. The readings were negative at the 48 hours reading. To conclude Esperase™ in powder form is mildly irritating to the rabbit eye and the duration of the irritation is relatively short.

Another test (Novo Nordisk 1978 (5)) for ocular irritancy was performed with Esperase™ concentrate activity 59.0 KNPU/g, in a 5 % w/v concentration in water. Eight albino rabbits were used and 0.1 ml of the 5 % w/v concentration, (activity $1x10^{-3}$ g aep/0.1 ml), was instilled into the conjunctival sac. The treated eyes of five of the rabbits were washed with tap water 5 minutes after treatment, and the remaining 3 rabbits were washed with tap water 24 hours after treatment. The results showed that the 3 rabbits which were washed after 24 hours, exhibited redness, score 1, up to 24 hours after instillation and one of these rabbits also showed a slight opacity of the cornea at the 24 hour's reading. Only 1/5 of the remaining rabbits showed a score 1 for conjunctival redness at 24 hours. To conclude, the Esperase™ concentrate in a 5 % w/v concentration in water, dosed as 0.1 ml in the eye (0.001 g aep/0.1 ml), was classified as negative as to potential for injury in the eye in this study.

A study has been conducted to determine the irritancy to the eye of a 5% aqueous solution of the powdered enzyme preparation Savinase™ (NICNAS, 1993). Eight rabbits received 0.1 ml of the solution into the left eye, the right eye served as a control. Although the amount of test substance used in this study is in line with current OECD guidelines, the test substance itself is a dilute solution of the enzyme preparation. The treated eyes of 5 rabbits were washed 5 minutes after application and the treated eyes of the remaining 3 rabbits were washed 24 hours after application. Eyes were scored at 1, 24, 48, 72 hours and 7 days and also examined with fluorescein dye. Of the eyes washed after 5 minutes, 4 showed slight conjunctival chemosis and redness at one hour of which 2 also showed corneal opacity. These reactions did not persist. Only one rabbit showed slight conjunctival redness at the 24 hour evaluation and all changes had cleared by 48 hours. Of the eyes washed after 24 hours, slight redness was apparent in one eye after 1 hour but no effects were apparent at the end of the 24 hour exposure period. Another eye showed signs of corneal opacity and conjunctival redness up to 48 hours and the third eye showed signs of corneal opacity up to 24 hours, conjunctival oedema up to 72 hours and conjunctival redness up to 7 days post application.

Powdered enzyme preparations containing 5-15% of either Subtilisin Carlsberg or Subtilisin BPN™ were instilled into one eye of each of 3 rabbits per enzyme (3 mg per eye) (Griffith et al., 1969). This amount of test substance is small in comparison with the 100 mg required by current OECD guidelines. Actual scores were not reported, but this treatment produced "moderate conjunctival irritation and transient corneal haziness, which cleared in 2-4 days", results being similar with the two enzyme preparations. One and 10% aqueous solutions were of comparable irritancy to the powdered preparations.
These results are similar to those obtained in the previous study and show that Subtilisin can cause eye irritation. Given that the test material in the first study was in a dilute solution and given the small amount of test substance used in the second study, on the basis of these results it can be reliably concluded that Subtilisin is an eye irritant.

One study performed with an Esperase liquid product (Novo Nordisk 1981 (11)) is only mentioned in the appending tables. Concentrated liquid product Esperase was regarded as strongly irritant, the 5% dilution was irritant to eye.

Epidemiology data/ human experience

In an investigation of 355 factory workers exposed to Subtilisin, 3 complained of eye irritation and 3 were diagnosed as having mild conjunctivitis (Witmeur et al., 1973). It is unclear whether these are the same three people. From the limited information available, it is not possible to conclude that Subtilisin were the cause of these eye effects.

Summary of Eye Irritation

Depending on its concentration in preparations, Subtilisin can be irritating to the eye. A concentrated product was strongly irritant to rabbit eyes, whilst 5-15% dilutions were at most moderately irritant, with no irreversible eye damage observed. A 5% aqueous solution was non-irritating to the eyes of rabbits in a modern guideline study. In the studies where the active enzyme protein content is known, 0.03 mg aep in 0.1 ml was negative, and between 0.05 mg and 3.5 mg aep/0.1 ml were slightly irritating.

5.2.1.2.3 Respiratory Irritation

There were no studies of sensory irritation in the respiratory tract in animals available. Respiratory changes were observed during the exposure phase of the single inhalation exposure studies quoted in section 5.2.1.1.2. In the rat studies cited by NICNAS (1993), respiratory impairments persisted for several days beyond the exposure period and were accompanied by blood or brown staining around the snout. These respiratory changes are considered to reflect pathological damage to the respiratory tract and do not necessarily indicate sensory irritant potential.

Summary of Respiratory Irritation

No studies have specifically examined the ability of Subtilisin to cause sensory irritation in the respiratory tract. Findings from inhalation studies in animals suggest that Subtilisin can cause inflammatory damage to the respiratory tract epithelium rather than elicit sensory irritation effects.

5.2.1.3 Sensitisation

5.2.1.3.1 Skin sensitisation

Studies in animals

Skin sensitisation studies (Henkel 1990 (1)) were conducted according to the Buehler method and to OECD TG 406 with BLAP 70. A group of 10 guinea pigs were induced and threefold challenged for six hours by epicutanous occlusive application of 0.1 ml (0.00012 g aep/0.1 ml) of enzyme preparation. The same concentration was used as well for the induction and for the challenge phase. The results showed no signs of sensitisation in all tested animals.

Another experiment (Henkel 1995 (3)) was performed according to the Buehler method with BLAP S. In this test a group of 20 guinea pigs were induced and twofold challenged for six hours by epicutanous occlusive application of 0.5 ml of 0.42 % Subtilisin (charge 29). The re-challenge was carried out with 0.05 ml of 0.21, 0.42, 0.63 % enzyme preparation. These results showed no contact hypersensitivity and no sensitisation in all tested animals. Skin reactions could be observed only in three animals in the first challenge phase.

An evaluation (Novo Nordisk 1970 (1)) of the skin sensitisation potential of Esperase™, activity 7.4 KNPU/g, was made according to the Landsteiner guinea pig sensitivity test. Ten animals received intra-dermal injections of a 0.01 % solution ($2.8x10^{-6}$ g aep/ml) of the test material every other day to a total of ten injections (0.5-1.0 ml/animal). Two weeks after the last injection the animals were challenged with a single intra-dermal injection. The scoring of the areas was made 24 hours after each of the injections, an average was calculated for the first 10 injections, and a comparison was made to the reaction after challenge. The results showed no differences between the average reactions of the ten first injections compared to the last injection. To conclude, the 0.01% solution of Esperase™ showed no evidence of skin sensitising activity.

Skin sensitisation studies conducted according to the Buehler method have been conducted with two Subtilisin preparations, Savinase™ and Opticlean-M (NICNAS, 1993). In the Opticlean-M study, a group of 10 guinea pigs received topical applications of a 10% aqueous solution of this Subtilisin preparation for 6 hours per day, 3 days per week for 3 weeks. No information was provided concerning skin responses during the induction phase, but it was stated that this concentration was "slightly" irritant in a preliminary study. A negative control group was exposed to water. Furthermore, the evidence from skin irritation studies presented above confirms that a concentration of 10% would be irritating. Two weeks after the final induction, both test and control animals were challenged at different skin sites to those used for induction, with a 5% aqueous Opticlean-M solution.
All test animals showed well-defined erythema up to 72 hours post-challenge with most animals also showing oedema. In contrast, only slight localised erythema was apparent on the skin of 3/10 control animals challenged with Opticlean-M. The results from the control animals suggest that the concentration used at challenge (5 %, one application only) was somewhat irritant, and this does cast some doubt about the nature of the skin reactions (allergic or irritant) observed in the test animals. This doubt is compounded by the fact that a 10 % concentration was applied on 9 occasions during induction in the test animals, suggesting that quite pronounced skin reactions would have occurred, possibly leading to hyper-irritability. This is a condition whereby the skin becomes more susceptible to irritants in a widespread fashion, not localised to the original site of exposure (Kligman and Basketter, 1995). Awareness of this condition, which is known to occur in humans ("angry-back" syndrome) has arisen relatively recently, and would not have been known about at the time of this study. Overall therefore, although the results of this study are apparently positive, there are uncertainties as to whether they represent a true skin sensitisation response.

Studies in humans

Clinical Data

A human repeat insult patch test (Novo Nordisk 1982 (5)) based on the Kligman Human Maximisation method Alcalase™ 2.5L, actual activity 2.82 AU/g, was performed to study the potential for human skin sensitisation. The concentrations in the main study were 0.25 % in the induction phase and 0.25, 0.10, 0.025, and 0.010 % w/v in water in the challenge phase. The pilot, as well as the main study were made without sodium lauryl sulphate (SLS) pre-treatment, since the test material itself is sufficiently irritant above the concentration used for induction (i.e. 0.25%). 26 human volunteers participated in the main investigation. Patches with 0.5 ml/patch were applied 5 times to the upper left arms and held in place for 48 hours. During induction, the skin was examined either one or two days after removal of each patch. Two weeks after application of the final "insult patch", challenge patches with 4 different test concentrations were applied to both arms of each subject. At challenge, skin reactions were scored 48 hours and 96 hours after patch application, i.e. 1 and 48 hours after removal of the patches. All members of the panel experienced mild to moderate skin irritation by the induction application of 0.25 % w/v Alcalase™ 2.5L. At challenge, there were several cases of moderate skin irritation due to the exposure to the highest concentration (0.5 ml, 0.25 % w/v), and several cases of mild irritation due to application of 0.10 % w/v. When the individual reactions observed after the challenge were compared with the reactions observed during the induction phase, two subjects were questionable as to an allergenic nature of the reaction. These two subjects were re-challenged after approximately one month, and it was considered that the reactions were of an irritant and not an allergenic nature. Two subjects failed to complete the induction series of patches and were dropped from the study, and two subjects only had 4 induction patches applied, but were, nevertheless, included in the challenge phase of the study. From these findings it can be concluded that Alcalase™ 2.5L may cause a moderate degree of skin irritation at concentrations of 0.25 % w/v in water, whereas the solution did not lead to skin sensitisation in the 32 subjects who completed the study.

An earlier sensitisation test (Novo Nordisk 1978 (6)) was performed as a human patch maximisation test to determine the contact-sensitising potential of Savinase™, more thoroughly, using 25 volunteers, by the method described by Kligman. As a pre-test found Savinase™ to be irritating to skin, pre-irritation by sodium lauryl sulphate solution was omitted. The study was divided into two phases; an induction phase and a challenge phase. The test substance solution was: Savinase™ in 10 % petrolatum (Pharmacopoea Nordica, editio danica volumen II, 1963, p. 629), the dose being 0.3 g (0.0410 g aep/dose). All results proved negative in this maximisation test.

The ability of Esperase™, activity 7.4 KNPU/g, to induce contact sensitisation was evaluated in 25 volunteers by the Kligman Maximisation Method (Novo Nordisk 1970 (1)). The study involved five continuous applications of 48 hours duration. The 2.5 % aqueous solution (7.1×10^{-4} g aep/ml) of the test material was applied under an occlusive patch to skin which was inflamed by prior application of sodium lauryl sulphate. The volunteers were challenged two weeks later at a new site by a 48 hour occlusive patch, using 0.01 % aqueous solution (2.8×10^{-6} g aep/ml) of the enzyme. The results showed marked inflammatory reactions on the arm during the induction period. At challenge there was no evidence of a dermatitis having the characteristics of an allergic reaction. The study concluded that the potential of Esperase™ to induce contact allergy under the test conditions employed was very low.

In a delayed contact sensitivity study (Novo Nordisk 1970 (1)) as a human volunteer trial a 2.0 %, (5.7×10^{-4} g aep/ml) solution of Esperase™, activity 7.4 KNPU/g, was applied under an occlusive bandage to the arm of volunteers, and the potential of inducing contact sensitisation was evaluated. The bandage was removed 24 hours later and the area was observed for reactions. This procedure was repeated three times a week for three weeks. The volunteers were challenged two weeks after the last application with another occlusive patch, which was removed after 24 hours. The reactions were graded according to the same scale. A total of 47 volunteers completed the test. The results showed no evidence of reaction to the test material in the induction phase or in the challenge phase.

A series of studies involving repeat insult patch tests in human volunteers has been conducted with the Subtilisin preparation Maxatase™ (Valer, 1975 B). In one of these studies groups of 100 volunteers were administered 15 occlusive applications, 5 days per week for 3 weeks, of Maxatase™ in aqueous solution at concentrations of 0.25-5 %, buffered to pH 8.5. The applications were made to intact skin of the forearm and kept in place for 24 hours. No volunteer had previous contact with proteolytic enzymes or enzyme containing detergents. Volunteers were challenged with 0.1-1 % aqueous solutions of the respective preparations, buffered to pH 8.5. Challenge patches were placed at the induction site and onto previously unexposed skin and held in contact for 48-hours. Negative results were obtained.

Three additional groups of volunteers were subjected to the same test protocol as above. These groups comprised the volunteers from a previous skin irritation study (see Valer, 1975 A, section 5.2.1.2.1); a group of 100 volunteers with occupational contact dermatitis attributed to enzyme containing detergents; and a group of 100 volunteers with occupational contact dermatitis due to agents other than biological detergents with no previous contact with proteolytic enzymes or enzyme containing detergents. The skin applications administered to the volunteers from the irritation study were regarded as induction applications for the purposes of this study. The induction regime for volunteers in the remaining two groups consisted of 5 x 24 hour occlusive applications of Maxatase™ in aqueous solution at concentrations of 0.25-5 %, buffered to pH 8.5. No skin reactions were reported in any group at challenge.

A final series of experiments was performed using a group of 380 housewives who had used enzyme containing detergents regularly at least 3 months. Participants were induced with 0.1-1% aqueous solutions of Maxatase™ plus a 0.25, 0.75 or 1% solution of a biological detergent. It is not clear if 3 x 24 hour induction applications were made or a single 48 hour application. Participants were challenged with the same treatment they had received at induction. No skin reactions were observed.
Overall, none of the several hundred volunteers in this series of investigations showed evidence of skin sensitisation to Maxatase™. Measures had been taken in some volunteers during induction, e.g. tape stripping to remove the stratum corneum, to maximise the potential for dermal penetration.

Griffith et al. (1969) conducted repeat insult patch tests in 1478 volunteers. Nine induction applications of 0.25-1 % aqueous solutions of detergents containing Subtilisin were made, each for 24 hours over 3 weeks. The Subtilisin concentrations of these test solutions would have been 2.5 x 10-4 to 7 x 10-3 %. Control formulations without enzyme were included. Challenge was carried out 10 to 14 days after the induction phase at the induction sites and previously unexposed skin. There were no signs of skin sensitisation at challenge. However, given the very low concentrations of enzyme tested no conclusions can be drawn about the skin sensitisation potential of the more concentrated enzyme.

Occupational studies

Patch testing was carried out in a study conducted in two Danish factories employing more than 400 workers in the production of the Subtilisin preparation Alcalase™ (Zachariae et al., 1973). When large scale production began, around 43% reported skin problems, falling
to 21% two years later following the introduction of measures to reduce exposure (mainly personal protective equipment). In this study, 79 production workers were identified with skin problems described as 'itching' or 'burning' of the skin affecting mainly the hands, forearms, face and perspiring areas of the body. Occasionally erosions or pustules were observed on the fingertips. Twelve workers with no known contact with detergent enzymes were selected to act as controls. No further details of the exposed workers or controls were available.

Initially 18 workers and the 12 controls were patch tested with 0.5, 0.1 and 0.01% aqueous Alcalase™ solutions. Patches remained in contact with the skin for 48 hours. Four controls and two workers gave erythematous reactions, stated to be "typical of a mild reaction to primary irritants", to the 0.5% solution and one worker reacted to the 0.1% solution. In addition to the initial 18 workers, a further 61 workers were patch tested with the 0.01% solution. No positive reactions were observed in either

workers or controls. Overall, from the limited information available, it is not possible to draw any firm conclusions about the causes of dermatitis in these workers, but the results do not suggest that skin sensitisation had occurred.

A brief case report describes a baker who developed dermatitis of the palms 5 months after working concentrated *B. subtilis*-derived enzyme tablets, used to improve bread texture (Smith et al., 1989). The report stated he was not handling any other irritants.
He patch-tested negative to 0.1%, 1% and 10% Subtilisin in petrolatum, and to other flour additives. Hence, there was no evidence for skin sensitisation due to Subtilisin in this worker.

Consumer studies

Jensen (1970) investigated 13 people with severe hand dermatitis following the use of a Subtilisin-containing detergent. An unusual feature was that a secondary light-induced eczema developed on distant parts of the body in 10 cases. Occlusive patch tests were positive in 4 of the 12 patients tested with a 0.5% solution, but were negative at 0.25%.
Detergent without enzyme gave negative results. Ten controls, not further described, failed to react to either concentration. The author noted that sufficient time had not elapsed between this test and healing of the dermatitis to rule out the possibility of a false positive result. Overall therefore it is not possible to draw any conclusions about the skin sensitisation potential of Subtilisin from this study.

Another report discusses 12 "home helps" who developed intense irritation, blistering and oedema of the hands after using detergents containing Subtilisin enzymes (Ducksbury and Dave, 1970). Six of these 12 had reacted on first use of the detergent. All 12 gave negative reactions on patch testing with a 0.1% aqueous solution of enzyme-containing detergent. The fact that reactions occurred after a single or very few exposures strongly indicates that the dermatitis was irritant in origin, and the contribution made by the detergent itself as opposed to Subtilisin is uncertain.

In a later study, 80 consumers with dermatoses (mainly atopic eczema) on the trunk and limbs after wearing clothes washed in enzyme-containing detergents were patch tested with 5% Alcalase™ in petrolatum (20-minute exposure) (White et al., 1985). A further 60 consumers were patch tested with 1% aqueous Subtilisin (48 hour exposure). Negative results were obtained in each case. This report provides no evidence for skin sensitisation potential for Subtilisin.

Further studies performed with liquid enzyme products (Novo Nordisk 1982 (6), 1981 (15), 1981 (14), and slurries (Novo Nordisk 1979 (1)) are only mentioned in the appending tables. The results of these studies correspond to the studies on Subtilisin concentrates.

Summary of skin sensitisation

Extensive patch testing in large-scale human volunteer studies has shown no evidence for the ability of Subtilisin to induce skin sensitisation. Negative results have also been obtained in patch tests in Subtilisin-exposed workers. Furthermore, no confirmed cases of skin sensitisation caused by Subtilisin have been identified in workers engaged in the manufacture/use of these enzymes. Very little testing has been conducted in animals; although an apparently positive result was obtained in a single study in guinea pigs. There were, however, doubts about whether the skin reactions observed were irritant or allergic in nature. This "positive" finding has not been confirmed in other animal studies, and there are no further meaningful animal data. When considering the balance of evidence, it seems pertinent to note that the large molecular weight of Subtilisin suggests that it would not be able to penetrate intact skin and initiate the cellular responses associated with delayed hypersensitivity. Overall, the weight of human evidence indicates that Subtilisins should not be regarded as skin sensitisers.

5.2.1.3.2 Respiratory sensitisation and allergy

Studies in animals

Although there is not yet a generally accepted animal model in which to test the ability of a substance to induce asthma, a number of studies have been performed which offer some insight into the ability of Subtilisin to produce an allergic response following administration into the respiratory tract.

Groups of 8 guinea pigs were induced by inhalation exposure to aqueous aerosols containing 0.0083, 0.041, 0.15, 0.39. 1.9 or 15 µg/L Subtilisin A™ for 15 min/day for 5 days (Thorne et al., 1986). A control group was exposed to air only. Five days post-induction animals were placed in whole body plethysmographs and challenged with a 20-minute exposure to 1.9 µg/L aqueous enzyme. An additional group of guinea pigs induced with 15 µg/L was challenged on day 17 to investigate the effect of a delayed challenge. A 36% increase in respiratory rate within the first hour post-challenge was regarded as an immediate positive reaction, and a similar increase after this time was regarded as a positive late reaction. Reactions at induction were not reported. On challenge, no immediate responses were seen at the two lowest doses. Late reactions were not looked for in these animals. Both immediate and a few late onset reactions were observed in animals induced with 0.15 µg/L or more. Given the lack of information on respiratory effects seen during induction, it is not possible to determine if the reactions are allergic or due to irritation. As part of this study, 12 guinea pigs were exposed for 20 minutes once only to 1.9 µg/L, with challenge at this concentration 7 days later. The mean increase in respiratory rate observed following the first exposure was 14.5% (range 0 - 33%). At challenge, 2 guinea pigs showed immediate and one a late positive reaction. Additionally, 25 guinea pigs were exposed for 6 hours per day, 5 days per week to 0.68 ng/L for 11 weeks followed by 1.5 ng/L for 6 weeks. A further 10 negative control animals exposed to air and 5 positive control animals "hyper-immunised" by inhalation, intra-peritoneal and intra-dermal exposures were included. All animals were challenged 17 to 22 days later with 1.9 µg/L for 20 minutes. None of the guinea pigs given the prolonged induction regime, and no negative control animal responded, compared with 4 of the 5 "hyper-immunized" animals. These results suggest the existence of a threshold for the induction of a state of increased responsiveness.

A subsequent publication detailed results from serological tests in the animals used in this study (Hillebrand et al., 1987). A dose-related increase in Subtilisin-specific antibodies, primarily IgM but also IgG was observed in sera from guinea pigs exposed to 0.0083 - 1.9 µg/L.
Ritz et al. (1993) compared antibody responses to Alcalase™ using the guinea pig intratracheal test (GPIT) with an inhalation exposure regime, and showed that both exposure routes produced similar responses.

Kawabata et al., (1996) administered weekly intratracheal doses of Subtilisin Carlsberg (Alcalase™) in a detergent matrix for up to 8 weeks to groups of 5 mice. Alcalase™-specific IgE and IgG1 antibodies measured 5 days after the last dose showed a good correlation with the number and magnitude of doses given. Further experiments showed that the addition of the detergent matrix markedly enhanced the antibody response (both IgE and IgG1) compared to Alcalase™ alone.

The same group of workers also developed a mouse intranasal model (MINT) (Robinson et al., 1996). Groups of 4 - 5 female mice were exposed to Alcalase™ in saline on days 1, 3 and 10 by placing 5 µl of enzyme solution outside each nostril and allowing the mouse to inhale. Blood was collected 5 days after the last dose to measure the enzyme specific IgG1 titre as a surrogate for IgE. A clear dose-related increase in IgG1 titre was obtained. Additional studies were performed using a 0.5 µg Alcalase™ dose to determine the IgE response and the effects of an extended dosing regime. The results showed a negligible IgE response. When dosing was extended to up to 9 weekly doses, the IgG1 response levelled off after 5 weekly doses. In contrast, a small IgE response was only apparent after 8 weekly doses or more. This small IgE response is notably different to the IgE response obtained on intratracheal dosing and may result from different patterns of distribution of the enzyme with the two dosing procedures.

The antibody response to Alcalase™, Savinase™ and Subtilisin B (containing 35, 21 and 5% protein respectively) has been investigated in the GPIT and MINT (Sarlo et al., 1997; Robinson et al., 1998). Enzymes were administered on an equivalent protein weight basis in these studies and the range of doses for each enzyme was selected to enable a dose response curve of antibody titre versus dose to be constructed. In both test systems the relative potency of Subtilisin B was about one third to one half of that of the other two preparations.

Additional studies reporting increased antibody responses to Subtilisin administered to the respiratory tract have been performed in guinea pigs and rabbits (Richards et al., 1975; Markham and Wilkie, 1976; Markham et al., 1979; Cernelc and Urbanc, 1982). These studies do not add new information to that already presented and will therefore not be discussed further.

Summary of respiratory sensitisation and allergy (animals)

Subtilisin can induce an antibody response in guinea pig and mouse models. However, a relationship between the strength of antibody response and any corresponding respiratory changes in these animals has not been established. Although animal models might predict enzyme allergenicity/antigenicity in humans, the degree of correlation between relevant allergy epitopes in humans and experimental animals is not yet fully understood (A.I.S.E. 2002).

Studies in humans / Human experience

Occupational studies (Occupational asthma/allergic rhinitis)

Studies with bronchial and/or nasal challenges (Franz et al., 1971) included open bronchial challenges with various concentrations of a commercial Subtilisin preparation in buffered saline in 10 detergent manufacturing workers with work-related symptoms of asthma. Five control subjects, not further described, were also tested. Immediate reactions (15-45% falls in peak expiratory flow rate, PEFR) were seen in 9/10 workers and in 0/5 controls. In seven workers the reduction in PEFR was accompanied by wheezing. Five of these 9 subjects were followed for an additional 10 hours and all had a late reaction. All five subjects noted the similarity of the work and challenge-related symptoms. In 4 of the 10 workers, single breath carbon monoxide diffusing capacity was also measured before and serially after provocation. In 3/4, a decrease by around one third was seen. The reduction persisted for 3 to 5 months in two people and more than 5 months in the third. The reason for the prolonged reduction in carbon monoxide diffusing capacity is unclear. As part of this study, 25 workers from this factory reporting work-related asthma (including the 10 described above) and 11 workers reporting work-related rhinitis, underwent skin prick tests and passive transfer tests (used to determine the presence of specific antibodies). The same commercial enzyme preparation was used. Skin prick tests were positive in 22/25 with symptoms of asthma compared to 3/11 with symptoms of rhinitis. Five individuals from a control group of 227 subjects also responded. No details of these control subjects were provided. Passive transfer tests with 5 sera (those responding most strongly in the skin prick test) using two volunteer subjects were all positive. Overall, the results show that Subtilisin enzymes can cause occupational asthma mediated by an immunological mechanism.

Bernstein (1972) reported results from bronchial and nasal challenge studies and skin prick tests with two Subtilisin preparations (Alcalase™ and an amylase-protease mix**,** containing around 5-10% enzyme protein) in 14 domestic and occupational (including laundry and cleaning workers) users of enzyme-containing detergents. Twelve of these subjects gave a positive response to one or both of these preparations in skin prick tests. In the bronchial challenge studies, subjects inhaled first saline and then, at 10 minute intervals, increasing concentrations of enzyme until a positive response was obtained. In the nasal challenge studies, subjects inhaled saline and then a single dose of enzyme preparation.
Of the 7 subjects who underwent bronchial challenge, 6 had an immediate response (5 to Alcalase™ and 1 to Amylase Protease) measured as reductions in Forced Expiratory Volume in one second (FEV1) 10-40% compared with baseline values. All 7 subjects reported a marked late response

between 4-8 hours after challenge and in one subject this was measured as a 75% drop in Peak Expiratory Flow (PEF). Late measurements were not performed for the other subjects. Five normal and 5 asthmatic controls (asthma not due to enzymes) were also challenged; none reacted. The subject who had not given a clearly positive response was re-challenged two months later. On this occasion challenge produced a gradual decline in FEV1 to around 80% of baseline at 24 hours with concomitant reductions in forced expiratory flow. Respiratory parameters had not returned to baseline after 5 days. At this time the carbon monoxide diffusing capacity was also reduced to around 75% of baseline.
The remaining 7 subjects underwent nasal challenge. All had marked immediate reactions to Alcalase™ (2) or Amylase Protease (5), characterised by increased nasal resistance, increased nasal secretions and subjective feelings of difficult nasal breathing. The reactions corresponded to the enzyme product used by the subject. Three normal controls and 5 with allergic rhinitis not due to enzymes were also challenged and none responded. The results of this study suggest that Subtilisin enzymes are capable of causing asthma and rhinitis.

Dijkman et al. (1973) reported results from non-blinded bronchial challenge studies in six detergent manufacturing workers with work-related symptoms of wheeze (3/6), breathlessness (5/6) and 4/6 nasal irritation which developed between 1-5 months after first contact with Maxatase™. Symptoms typically occurred in the evening or at night, and lasted several days or weeks after "heavy" exposure. Five workers showed non-specific bronchial hyper-responsiveness to histamine. All six were challenged with saline and nebulised Maxatase™ solution; four had an early response to the enzyme and all six gave late responses (decreases in FEV1 and vital capacity (VC) of up to 50%). The late phase reactions were reportedly associated with malaise, headaches, muscle pains and slight fever. In two of these workers, late phase reactions were prolonged, taking 10 hours and 8 days respectively before FEV1 and VC returned to their pre-challenge levels. Oxygen saturation and carbon dioxide tension of arterial capillary blood, and carbon monoxide diffusing capacity were unaffected. An asthmatic with no history of exposure to these enzymes did not react to challenge with the enzyme. Five of the 6 gave a positive skin prick test, the negative result being obtained in the worker with an isolated late reaction at bronchial challenge.

Bronchial challenge tests with an aerosol of dilute Alcalase™ have been carried out in 29 Australian workers with previous occupational exposure to this enzyme; the results were presented in more than one publication (Gandevia and Mitchell, 1970; Mitchell and Gandevia, 1971 A). The group included both symptomatic and asymptomatic workers and some who did and did not respond in a skin prick test. No further details of these workers were available. Non-blinded challenges were performed after a 24-48 hour monitoring period to establish baseline FEV values. A negative response at first challenge was followed by a second challenge 24 hours later using a 10-fold greater concentration of Alcalase™. A control solution was not used. A 10% or greater decrease in FEV1 on challenge was regarded as a positive reaction. The results were very briefly reported. Twenty workers showed an immediate, delayed, dual or nocturnal asthmatic reaction and it appears that these workers also reacted to skin prick tests with Alcalase™. Workers who did not have an asthmatic reaction following bronchial challenge did not tend to react to skin prick tests. No comparison was made between responses to challenge and the reporting of respiratory symptoms at work. Eleven of the 29 workers also developed fine bubbling rales on inspiration and expiration, including 7 who gave a positive response on challenge and 4 who gave negative responses. There was no clear relationship between the development of rales and the reporting of work-related respiratory symptoms and the cause of these rales could not identified. Overall, this study is limited by brief reporting and by the fact that the bronchial challenges were not carried out under blinded conditions. However, the results are consistent with the view that Alcalase™ is a potential cause of occupational asthma.

Radermecker and Booz (1970) report case histories of three individuals who developed asthma following exposure to the Subtilisin enzyme preparation Maxatase™. Two of these were workers employed in the manufacture of detergents. Both workers developed symptoms of asthma after around three months exposure to enzymes. At the time of diagnosis, both gave positive reactions in skin prick tests. "Normal" controls, not further described, did not react to similar skin prick tests. The third case, a housewife with a history of hay fever, had symptoms of asthma while using enzyme-containing detergents. The symptoms disappeared once she stopped using biological detergents. At the time of

diagnosis she gave positive skin prick reactions to Maxatase™ and to grass pollen. One of the workers underwent blinded bronchial challenge testing with an aerosol of 1% Maxatase™ in a physiological solution and to the vehicle alone with a 10-day interval between each challenge. He showed a dual response to Maxatase™ with a maximal fall in FEV1 of 70%, but no reaction to the vehicle. The housewife also underwent a bronchial challenge to Maxatase™ and was reported to have showed a "substantial" immediate reaction. No further details were provided. These findings suggest that the Subtilisin preparation Maxatase™ is a potential cause of asthma.

Paggiaro et al. (1984) investigated 6 detergent factory workers with work-related asthma rhinitis and/or conjunctivitis for between 3 months and 11 years prior to the study. It was reported that hygiene control was "poor" and workers received "considerable exposure" to Subtilisin enzymes. Four workers had hyper-responsive airways as assessed by non-specific challenge with "Betanecolo" and in three of these, baseline spirometry revealed moderate bronchoconstriction. Two types of specific bronchial challenge were performed, both likely to have been under non-blinded conditions; inhaling an aerosol of "crude" proteolytic enzyme solution and tipping a detergent powder containing encapsulated (low dust) Alcalase™ from one tray to another. A 15% or greater decrease in FEV1 was regarded as a positive result. Five normal and five asthmatic controls, not further described, were also tested. All 6 workers reacted to the crude enzyme aerosol (5 immediate and 1 dual responses). They were calculated to have received doses ranging from 46 to 924 µg. Two reacted to the detergent plus encapsulated Alcalase™ (1 immediate, 1 dual). No control subject reacted to either challenge procedure. Five of the 6 were skin prick and RAST positive to crude enzyme and/or Alcalase™ (no controls reacted). The worker with the negative skin prick test result gave a dual reaction on challenge to both the crude enzyme preparation (263 µg) and the encapsulated Alcalase™. The results of these investigations suggest that Alcalase™ may have been the cause of the occupational asthma in these workers.

Nasal challenge tests have been conducted in detergent workers exposed to Maxatase™ and Esperase™ (Vanhanen et al., 2000). At this factory, enzymes had been used in an encapsulated form since the mid-1970s to reduce exposures. A questionnaire was used to gather information on work-related symptoms. Skin prick tests and RASTs were used to assess immunological status. Workers with work-related symptoms and positive skin prick responses to Subtilisin were given single blind nasal challenge tests. Total dust and protease exposure levels were measured using both area and personal sampling.
In all, 76 workers took part, corresponding to a participation rate of 95%. Of these, 40 were engaged in manufacturing work and 36 were office-based managerial and sales staff. Symptoms at work, mainly stuffy nose or rhinorrhoea, were reported by 19 (47%) of the manufacturing workers compared with 4 (11%) of the office staff. Five manufacturing workers also reported cough and one occasional dyspnoea at work, two reported skin problems and two eye irritation.
One of the 4 office staff with nasal symptoms also reported work-related cough. Skin prick tests and RASTs revealed 8/40 manufacturing workers and 0/36 office staff to have specific IgE to both Subtilisin preparations. One of these workers had been diagnosed with occupational asthma and rhinitis due to protease three years earlier. The remaining 7 skin-prick positive workers underwent nasal challenge. Five gave a positive response to both preparations. Their length of employment ranged from 7 to 25 years. Results for one worker were inconclusive, and one could not be challenged due to nasal polyposis. The positive results for the 5 workers seem to be reliable evidence for allergic rhinitis due to Subtilisin, given that they were conducted under single blind conditions, and as the criteria for a positive response (nasal swelling and volume of mucous production) seem unlikely to be influenced by psychological factors. However, work-related nasal symptoms were also reported in a further 14 workers in whom no challenge tests were performed.
Dust measurements were gathered for the production of laundry detergents and dish-washing detergents. Total personal dust levels for the laundry detergent line ranged from 0.07-1.3 µg/L (4-hour TWA). The protease content in most of these samples was below the limit of detection of 50 ng/m^3 (samples were collected over periods ranging from 2-5 hours). The personal sampling data were only slightly higher than those for area sampling. Measurements in the dish-washing detergent line indicated very similar total dust levels but higher levels of protease. Historical exposure data were not provided but past exposures were likely to have been higher as the plant was modernised in the 1980s.

The method of quantifying protein content and activity used in this study is seen as inappropriate. The same holds true for the recovery of the enzyme from the filters. Thus the values found are considered to be not correct. Due to this we cannot correlate the exposure levels to sensitization levels and health effects.

Pepys et al. (1969) reported dual reactions (21 - 59% falls in FEV1) in three detergent manufacturing workers on open bronchial challenge with Alcalase™. A healthy control, not further characterised, did not react to the enzyme solution. Skin prick tests were also performed with Alcalase™ and Maxatase™ and all three workers responded positively. Two of these workers were also tested with a purified Subtilisin enzyme (Koch-Light) and reacted strongly.

Double-blind and open challenge tests were conducted in 12 housewives with asthma or rhinitis and in whom positive reactions in RAST and skin prick tests to Subtilisin enzyme were obtained (Zetterstrom 1977). The tests involved the subjects measuring out and tipping detergent with or without Alcalase™ in conditions designed to simulate normal exposures during machine washing. Equivocal results were obtained under the double blind conditions. On open challenge, 8 of the 12 subjects experienced symptoms mainly rhinitis which persisted or were followed by a late reaction in four subjects. In 3 cases, peak expiratory flow rate (PEFR) was measured; in one subject PEFR was unaffected and falls of only 10% and 15% (negative and borderline responses respectively) were recorded for the other two. Unfortunately, the report did not state what if any reactions occurred to the detergent without enzyme, hence it is difficult to draw any firm conclusions from this study.

A recent case report has been published by Lemiere et al. (1996) purporting to show a late asthmatic reaction to Subtilisin. However, the subject was challenged by breathing the vapour from an aqueous solution of the enzyme cleaner used by the subject at work. Given the low volatility of Subtilisin, it doubtful if the subject actually inhaled the enzyme during the challenge procedure. Therefore, no conclusions can be drawn from this study.

Additional challenge studies have been reported by Wuthrich and Schwarz-Speck, (1970), Reinheimer and Utz (1971), Rosemeyer and Wuthrich (1974) and Gonzalez-Zepeda et al. (1975). In each study, positive immediate responses were obtained on bronchial or nasal challenge with Subtilisin enzyme preparations. However, the conditions under which these challenge studies were performed were poorly reported such that a clear interpretation of the results is not possible. Hence, these studies will not be discussed further.

UK SWORD statistics

SWORD (Surveillance of Work related and Occupational Respiratory Disease, UK) statistics dating back to 1989 show that between 1 and 15 cases of occupational asthma due to detergent enzymes occur each year. The specific enzymes involved have not been reported. From around 1990, enzymes other than Subtilisin began to be used in detergent formulations, hence it is not possible to determine how many of the cases reported to SWORD are specifically due to Subtilisin.

Studies with supportive evidence for occupational asthma

Juniper and Roberts (1984) described 55 cases of asthma attributed to Alcalase™ on the basis of positive skin prick tests, symptoms of breathlessness, sweating and wheezing and lung function data. These cases were identified between 1968 and 1975 during routine health surveillance of 1642 workers exposed to Alcalase™. This paper covers the initial assessment and follow-up examinations in 1982. The onset of respiratory symptoms following occupational exposure to Alcalase™ was either immediate, late, dual and/or nocturnal, and the average drop in FEV1 during an episode was 37% (range 13 - 45%); FVC also dropped 25% (range 6 - 35.5%). Physical examination revealed dyspnoea and bronchospasm and in several cases coarse scattered rales were evident. No specific tests were undertaken to confirm that Subtilisin was the cause of these effects. In each case once the subject was removed from occupational exposure to enzymes, FEV1 and FVC returned to normal over periods ranging from 1 day to 22 months. Annual chest x-rays revealed no abnormalities. The pattern of

results in these 55 workers is consistent with the development of occupational asthma, and although bronchial challenge tests were not conducted to confirm the cause, at the time when these cases arose, Subtilisin was the only enzyme used in detergent manufacture, pointing to a causal role of Subtilisin in these cases.

A further 8 workers with work-related symptoms of asthma (8) and rhinitis (3) from a total of 110 workers at a detergents manufacturing plant were studied by Perdu et al. (1992). Five of these 8 were employed in the washing powder conditioning section and 3 were senior managers with only occasional exposure to enzymes. The onset of symptoms in these workers coincided with the introduction of a new Subtilisin preparation Biozym P300S. Previously the factory had been using Savinase™ and Maxatase™. Microscopic examination of the three preparations revealed the shape of the Biozym granules (cylindrical) to be different to the shape of Savinase™ and Maxatase™ granules (spherical) and the capsule coating the grains was abnormal or damaged in 85% of the Biozym grains compared to only 2-5% of grains of the other preparations. It was also noted that poor hygiene practices were in operation. Airborne exposures were not measured. Health investigations were performed on these 8 workers following a period of absence from work for an unspecified time. All 8 gave positive responses to P300S, Savinase™ and Maxatase™ in skin prick tests. A group of 10 "healthy, non-asthmatic" subjects were similarly tested with the three preparations and none reacted. FEV1 and FVC values for the 8 workers were generally within predicted values. However, the FEV25/75 was 10 - 30% below predicted values for 6/8, and 5/8 had hyper-responsive airways as assessed by histamine challenge. Although no formal follow-up investigations were reported it was noted that these individuals returned to work and remained symptom-free once the use of P300S was discontinued. Overall, the pattern of onset and recovery from asthmatic symptoms points to exposure to the Subtilisin preparation P300S as the cause.

Health evaluation/surveillance studies

Following a report by Flindt (1969) suggesting that enzymes may be a cause of work-related asthma, it has been standard procedure at large detergent manufacturing plants worldwide to carry out routine health surveillance of all employees. This includes a pre-employment medical examination, involving lung function tests, chest x-rays, skin prick tests and/or RASTs. These investigations are repeated annually. Periodically, results from such health surveillance schemes have been published and these are presented below. Results from health evaluation studies have been presented alongside the health surveillance data since the same range of tests was carried out.

Hence, the following reports provide only supportive background information:

Juniper et al. (1977), Flood et al. (1985) and Cathcart et al. (1997) presented health surveillance results from five UK detergent manufacturing plants, spanning a period of 20 years. At four of these plants, the inclusion of Subtilisin enzymes into detergent products had begun in the late 1960s, the fifth only started to manufacture enzyme detergents in 1983 and the manufacture of enzyme detergents had been suspended at two factories between 1975 and 1983. At each site, static sampling to monitor airborne enzyme levels was carried out and industry guidance ensured that sampling was consistent between each site. Chest x-rays and immunological tests ceased to be conducted in the 1980's. In the analyses presented below, only lung function data from males over the age of 25 and with at least 18 months employment (4 years in Cathcart et al., 1997) were considered. Women and younger males were excluded. Data from workers who transferred between exposure categories were not included in the analyses presented below in order to assess the relationship between health effects and exposure group. During the time covered by these papers, exposure levels (reported as combined annual means for all 5 factories) fell from around 100 ng/m^3 pure crystalline Subtilisin in 1969 to 6 ng/m^3 in 1971, 2 - 4.5 ng/m^3 in 1972 - 1975 and 0.7 - 1.8 ng/m^3 in 1976 to 1993 (data obtained from continuous static sampling over several hours). This does not give any indication of the day to day or location to location variations in airborne enzyme levels nor the personal exposures that would have been received by individual workers. Over the 20 year period, 166 cases of occupational asthma thought to be due to enzymes were recorded at these five factories, 7-39 per year between 1968 and 1974 (total 140), 0-5 cases per year between 1975 and 1980 (total 17) and 0-4 per year after 1980 (total 9). This

trend is confirmed by actual figures (presented by K. Sarlo and A. Newman-Taylor at the ACGIH conference Cincinnatti 2004, that also included the data from the the publications by Vanhanen et al. (2000) and Cullinan et al. (2000).
Diagnosis was on the basis of positive skin prick reactions, symptoms and lung function tests. There was no indication of the exposure conditions for these workers and it was not possible to identify what proportion of the total workforce these workers represented. Fifty five of these workers are discussed above in more detail (Juniper and Roberts, 1984).
Juniper et al. (1977) reported a relationship between exposure group and the percentage of employees giving positive skin prick reactions to the Subtilisin preparations Alcalase™ and Maxatase™. Numbers were 233/619 (38%) from the high exposure group, 17/180 (9.4%) from the medium exposure group, 10/353 (3%) of the low exposure group and 28/490 (6%) from the intermittent high exposure group. Most workers with positive reactions did so for the first time within the first 6-24 months of employment. In some workers, who had given weak reactions there was evidence of reversion from skin prick positive to skin prick negative status despite their continuing exposure to enzymes. Skin prick data were not available for subsequent analyses.
Overall, these results indicate that a proportion of workers in the detergents industry develop work-related asthma-like symptoms which appear to be related to the use of Subtilisin. Also there appears to be a relationship between positive responses to Alcalase™/Maxatase™ in skin prick tests and exposure category.

Newhouse et al., (1970) conducted a health evaluation in 271 (98% of total) production, shipping and warehousing employees at a detergent manufacturing plant with follow-up 6 months later. When Subtilisin was first introduced into the manufacturing process workers initially reported skin rashes, and 8-10 weeks later, rhinorrhoea and respiratory conditions including bronchitis and asthma. The report suggests that sneezing and nasal irritation due to detergent powders were common before enzymes were introduced. The initial health assessment took place five months after Alcalase™ was introduced on a commercial scale, one month after a range of measures had been introduced to reduce exposures to Subtilisin containing dusts.
Concurrent with this survey, monthly area sampling was performed. In the first set of samples the proteolytic activity of airborne dust ranged from 11 - 103 x 10-6 Anson units.m^3. The final set of samples revealed that the proteolytic activity of airborne dust was between 0.3 - 6 x 10-6 Anson units.m^3. The corresponding mass of Subtilisin per m^3 air was not reported. These data suggest that over the period of this study, there was a marked improvement in airborne dust levels.
The only results from the initial survey which could be related to exposure status (based on job title) were from skin prick tests; 57 workers tested positive with the greatest frequency of positive tests in workers with the greatest exposure (e.g. 45% in those handling concentrated Alcalase™ compared to 0% in shipping). Across the whole workforce, 117 workers reported symptoms of "acute chest disease", not further characterised. It is not clear if this was linked to the introduction of enzymes. A further 10 reported symptoms of nasal irritation which were thought to be related to non-enzyme components of the detergent powder. Eleven of the 271 workers had obstructive lung disease but whether this was occupationally related is unknown. No lung abnormalities were identified in any of the 271 workers from chest x-rays. Pre- and post-shift FEV1 data were available for 46 workers, no job-title information was provided. A mean drop of 231 ml was recorded for 15 workers with chest symptoms and positive skin prick tests and also in 9 workers with symptoms but negative skin prick tests; a mean drop of 100 ml was recorded in 9 asymptomatic workers with positive skin prick tests; and a mean drop of 20 ml was recorded in 13 asymptomatic, skin prick negative workers. Exposure data for these workers were not given. These findings tend to suggest greater drops in FEV1 across a workshift in skin prick positive workers.
In the follow-up investigation 6 months later, 32 of the 62 workers (from the group of 103 studied in the follow-up investigation) with respiratory symptoms at the time of the initial survey said that these symptoms had not recurred. Of the 41 who had earlier been free of symptoms, 4 developed them for the first time (three of these had been skin prick positive 6 months before). Skin prick tests in the follow-up study were performed with Alcalase™, Maxatase™ and purified Subtilisin and most workers who reacted did so to all three agents. Fifty two of the 56 previously skin prick positive workers still reacted. Nine of 47 previously negative workers were now skin prick positive. Overall, the follow-up results suggest that in a period when the proteolytic activity of dust in the factory was

falling, there was a slight increase in the overall prevalence of workers with skin prick positive results, but that the overall prevalence of workers with respiratory symptoms fell slightly.

Health surveillance results spanning 3 years were published by Greenberg et al. (1970), Watt et al. (1973) and Pepys et al. (1973). The results covered 121 workers, thought to represent nearly all employees at a detergent manufacturing factory. The initial survey was conducted 23 months after the introduction of Subtilisin enzymes into the manufacturing process. No exposure data were presented. At the initial investigation, 17 workers reported cough, 26 dyspnoea, 15 chest pain, and 77 eye/nose symptoms. Spirometry revealed 31 to have a reduced ventilatory capacity as judged by an FEV1:FVC ratio of 70% of the predicted value. A greater proportion of these 31 workers reported respiratory symptoms compared with the rest of the workforce. Skin prick tests were performed using Alcalase™ (42 positive), Maxatase™ (36 positive) and purified Subtilisin (42 positive); a total of 48 (40%) reacted to at least one agent. Pepys et al. (1973) noted that only 2 positive reactions were obtained from prick tests with purified Subtilisin in 2500 patients attending the author's allergy clinic. It was noted that the mean FEV1 for skin prick positive workers (2.64 l) was less than that of skin prick negative workers (3.04 l) but no further detail concerning the distribution of FEV1 data was provided. Overall, this study provides no clear evidence for the induction of asthma by Subtilisin, although the high prevalence of skin prick positive tests and respiratory symptoms in this workforce raise concerns for asthmagenic potential.

Sarlo et al. (1997) presented skin prick test results from a plant producing granulated and liquid detergents, processes involving 250 and 150 workers respectively. Savinase™ and Alcalase™ were present in the granule detergent, and Alcalase™ and Subtilisin B™ in the liquid. Three cases of rhinitis were reported in the granulated enzyme facility during the study, the cause was not identified. All employees in the liquid detergent facility remained asymptomatic. Between 1986 to 1991 3.3% of granule workers were skin prick positive to Alcalase™ and 5.2% to Savinase™; 11.6% of the liquid workers were skin prick positive to Alcalase™ and 6.7% to Subtilisin B™. Alcalase™ is antigenically distinct to the other two Subtilisin enzymes which are derived from a different Bacillus species.

A health evaluation has been conducted in workers exposed for 2 years to an encapsulated Subtilisin preparation, Esperase™ (enzyme content 8-10%) in the dry bleach industry (Liss et al., 1984). This study is limited chiefly by the small numbers of workers (13) exposed to the enzyme. Although 6/13 exposed workers reported respiratory symptoms, the reporting of respiratory symptoms in a control group of 9 unexposed workers was similar (4/9). Baseline spirometry showed no differences between the exposed and non-exposed workers. Although there was a statistically significant mean decline in FEV1 of 114 ml over a workshift in exposed workers, no conclusions can be drawn from this observation because pre- and post-shift measurements were not made in the control group. RASTs for enzyme specific IgE and ELISAs for enzyme specific IgG gave positive scores in 3 and 4 exposed workers respectively. No evidence for occupational asthma can be derived from this study.

Weill et al. (1971, 1973 and 1974) presented three consecutive sets of health surveillance data for 110 of 611 workers from two enzyme detergent manufacturing factories. At one plant, for 3 years prior to the initial investigation, concentrated Subtilisin preparations had been diluted to make the "enzyme complex" for addition to detergents. Airborne Subtilisin levels ranged from <1 to 30 ng/L with peaks of up to 1000 ng/L.
The second factory opened 6 months prior to this study, and "enzyme complex" rather than the concentrated preparation was used; airborne Subtilisin levels of <1 to 20 ng/L with peaks of up to 60 ng/L were recorded. All measurements were obtained from static sampling for one hour. Thirteen of 60 workers from the "processing" factory reported asthma-like symptoms but there was no association with level of exposure (based on job title). None of 50 workers from the second factory reported respiratory symptoms.
Results from skin prick tests with the Subtilisin preparation showed that for processing plant workers, 52% were positive in the high exposure group, 35% in the medium and 16% in the low. In the second plant, 45 and 53% workers in the high and intermediate exposure groups respectively were positive. No positive reactions were obtained from the low exposure group. However, given that there were no personal sampling data the reliability of the exposure categorisation is uncertain. The follow-up

reports suggested poor lung function in workers at the second assessment with apparent improvement by the third assessment. However, whether this was due to workers with poor respiratory health leaving these first plant it is unclear which of these were skin prick positive, hence there is no firm evidence for Subtilisin-induced occupational asthma from this study.

The relationship between work-related cough and exposure to Subtilisin A™ was investigated in 64 workers from two detergents manufacturing plants (Gothe et al. (1972)). Results from an interview with a physician showed that work-related cough was present in 18/33 (55%) with direct enzyme exposure; 7/17 (41%) with indirect enzyme exposure and 0/14 with no enzyme exposure. However, when workers were asked about cough in relation to use of Subtilisin A™, only 4/33 (12%) with direct exposure noticed a clear relationship compared with 3/17 (18%) with indirect exposure. There were no differences in FVC and FEF25-75 and chest x-ray findings between the three groups. Other respiratory symptoms were not commented upon. No conclusions can be drawn from this study.

Results from a health evaluation of 98 detergent workers out of a total workforce of 175 were reported by Mitchell and Gandevia (1971 B). Reasons why 77 workers did not participate were not reported. The investigation was conducted 18 months after Alcalase™ had been introduced into the manufacturing process. Sixty two workers reported immediate onset enzyme-related rhinitis. "Less severe" nasal symptoms also occurred with exposure to other detergent components. The number of affected workers was not reported. Forty nine workers reported enzyme-related asthma-like symptoms with immediate, delayed and/or nocturnal onset. It was stated that some workers who did not react to skin prick tests experienced these symptoms immediately after "exceptionally heavy" exposures, suggesting an irritant response. More commonly, symptoms only developed after several months occupational exposure. The relationship between reporting of symptoms and exposure was not explored.
It was noted that positive skin prick test reactions were obtained in a greater percentage of those with upper respiratory tract symptoms (77%) than in those with no such symptoms (48%). No such relationship was observed for the reporting of lower respiratory tract symptoms. There were no deficits in pulmonary function parameters or chest x-ray findings that could be related to enzyme exposure (judged according to job category) or symptoms. In the absence of appropriate challenge tests, the cause of the asthma-like symptoms and rhinitis in this workforce cannot be clearly identified, although the apparent association between upper respiratory tract symptoms and skin prick positive tests is suggestive of the possibility of allergic rhinitis due to Subtilisin.

Witmeur et al. (1973) analysed five consecutive sets of health surveillance data specifically from 355 workers at two Danish biotechnology plants engaged in the manufacture of Alcalase™. No Alcalase™ workers reported symptoms of work-related asthma. This included a 36 workers employed for more than 10 years in enzyme production. There was no evidence of any deficits in FEV1 relating to enzyme exposure intensity or length of employment, and all chest x-rays appeared normal. Positive RAST reactions to Alcalase™ were obtained for only 9 workers, around 3% of those tested.

In a subsequent paper, health surveillance results from workers from the above two Danish plants engaged in the manufacture of another Subtilisin preparation, Esperase™, were briefly presented (Zachariae et al., 1981). A total of 667 workers were identified who had been exposed to Esperase™ over the 10 year period covered by this paper, but health data were only presented for the 31 who had given positive RAST reactions. Of these 31, 16 reported shortness of breath and chest tightness, 6 nasal and throat irritation or rhinitis, 2 frequent coughing with no other symptoms, and 9 did not report respiratory tract symptoms. Lung function and chest x-ray data were not reported. These findings are suggestive of the possibility of occupational asthma and allergic rhinitis caused by Esperase™, but as no challenge tests were performed, and as no comparative symptom data were reported for the RAST negative workers it is not possible to draw any conclusions from this report.

Several other publications are available describing Subtilisin-exposed workers or domestic users of enzyme detergents, with reports of exposure-related symptoms and/or skin prick test or RAST data (Wuthrich and Ott, 1969; Belin et al., 1970; Mc Murrain, 1970; How and Cambridge, 1971; Shapiro and Eisenberg 1971; Dolovich and Little 1972; Nava et al., 1973; Little and Dolovich 1973;

Zetterstrom and Wide, 1974; Belin and Norman 1977; Cernelc et al., 1977 and 1981; Pepys et al., 1985; Colomer et al., 1990; Biagini et al., 1996; Johnsen et al., 1997; Cullinan et al., 2000). These studies are more limited in the scope of their investigations than those already cited, and as they add no further insights into the evidence for Subtilisin-induced occupational asthma and allergic rhinitis, they will not be discussed further.

Summary of occupational asthma/allergic rhinitis

Evidence from bronchial and nasal challenge studies in detergent workers shows that Subtilisin can cause occupational asthma and allergic rhinitis. When Subtilisin was first introduced into the detergents manufacturing process there were a large number of cases of occupational asthma attributed to enzymes each year (7-39 before 1975).

It is likely that all of these cases were due to Subtilisin as that was the only enzyme used in detergent manufacture over that time period. Since then, enzymes are granulated and hygiene conditions have increasingly improved, and there has been a corresponding drop in the number of cases of enzyme-related asthma per year. However, no personal exposure data are available and there is no information concerning the exposure-response relationships for Subtilisin-induced asthma/allergic rhinitis. There is some evidence to suggest that the prevalence of workers with positive skin prick test results increases with increasing background levels of exposure to Subtilisin, but there is not sufficient information from which to identify a threshold below which positive reactions in skin prick tests would not be elicited.

Consumer studies

In the late 1960's, dusty, un-encapsulated, powdered enzyme preparations were handled in detergent manufacturing plants. Poorly controlled exposure to these enzyme preparations led to the production of allergen-specific immunoglobulin E (IgE) antibodies in workers, and, in some cases induced respiratory (type I) allergy among workers (Flindt, 1969; Pepys et al., 1969). As a consequence of the respiratory occupational detergent enzyme allergies the question of the consumer situation was investigated in the late 1960s and the early 1970s. The most comprehensive study was published in November 1971 by the *ad hoc* Committee on Enzyme Detergents of the U.S. National Academy of Sciences (PB 204 118, 1971). The Committee found only six unequivocal cases of detergent enzyme caused sensitisations. This conclusion was based upon information from American consumers and physician letters. It was estimated that in the U.S. 50 million households used powdered enzyme containing laundry detergents in July 1969, and that many of these households had used them since December 1966.

It is important here to emphasize that sensitisation does not predict the likelihood with which respiratory symptoms will occur (A.I.S.E., 2002). Sensitisation is not a clinical outcome or disease, but is a marker of exposure (SDA, 2005).

Other authors have also shown that the hazard of sensitisation occurs for consumers in situations where enzyme is released as dust or aerosol, in particular when enzyme is incorporated in the detergent as a dusty powdered material (Belin et al., 1970; Schmitt GJ, 1974; Zetterstrom and Wide, 1974; Bernstein, 1972; Rosemeyer and Wuthrich, 1974; Pepys et al., 1973). However the number of sensitised consumers was extremely low, and only a few sporadic cases of allergies were observed in consumers, including some women handling clothing of industrial workers containing dusty enzymes brought home for laundering. (Zetterstrom and Wide, 1974; Belin et al., 1970; Bernstein, 1972).

Since the early 1970s detergent enzymes are always incorporated as practically non-dusting granulated and coated enzyme preparations. Recent studies have demonstrated the negligible risk of consumers to become sensitised (Sarlo et al., 2003).

Enzymatic detergents do not appear to increase the potential for skin sensitisation or respiratory sensitisation in consumers, including atopic individuals (Griffith et al., 1969; Higman, 1985, Pepys et al., 1985).

5.2.1.4 Repeated dose toxicity

By ingestion, enzymes are expected not to produce chronic toxicity. This is due to the fact that enzymes are proteins, which are susceptible to destruction by the digestive enzymes. Therefore, it is expected that enzymes will not accumulate in the body and that no significant chronic adverse effects would be produced.
By dermal application, enzymes are not expected to be absorbed to any relevant extent due to their large molecular weight. The only effects expected would be localised skin reaction(s) at the application site for some enzyme preparations. Therefore, it is expected that enzymes will not accumulate in the body and that no significant systemic adverse effects would be produced.

5.2.1.4.1 Oral administration

5.2.1.4.1.1. Subacute toxicity

In a 28-day oral toxicity study (Genencor 1995 (2)) in rats, Purafect™ PR329 in 33 % propylene glycol was examined. Purafect™ PR329 in 33 % propylene glycol was incorporated into Certified Purina® Rodent Lab Chow at concentrations of 3500 (low), 7500 (mid) or 15000 (high) ppm. Ten males and ten females in each dose group were fed the treated diet *ad libitum* for 28 consecutive days. A vehicle control group, also consisting of ten males and ten females was fed basal (untreated) diet containing 33 % propylene glycol. Throughout the treatment phase, animals were observed daily for the presence of any clinical signs. Body weight and food consumption measurements were performed weekly on each animal. Prior to terminal sacrifice, ophthalmology was performed on surviving animals. At the end of the treatment period, blood samples were obtained for clinical, biochemical and haematological analyses. Overnight urine collections were performed prior to the scheduled terminal sacrifice. Following 28 days of treatment, surviving animals were euthanized and a gross pathological examination was performed. Tissues collected at the time of necropsy for histopathological examination were retained in 10 % neutral buffered formalin. No statistically significant or biologically significant histopathological, ophthalmological or urinalytical findings that could be associated with the presence of the test article were observed in this study. However, reductions in food consumption compared to the controls were observed in the first week of the study, but only in mid dose males and in high dose animals. This finding is attributed to the poor palatability of the diet mixture. Consequently, these animals exhibited reductions in daily weight gain values, when compared to appropriate controls during this same period. However, food consumption and daily body weight gains for all treated animals were comparable to those of control animals for the remainder of the study. Although statistically significant differences were found in mean values for some organ weights, isolated clinical biochemical and haematological parameters, when compared to pertinent controls in this study, these differences are not considered to be biologically nor physiologically significant. Based upon the results of this study, a No-Observed-Adverse-Effect-Level (NOAEL) for the food additive Purafect™ PR329 in 33 % propylene glycol in rats is greater than 15000 ppm.

Another study (Novo Nordisk 1980 (4)) with Alcalase™ was carried out similar to OECD TG 407 (1981) and EEC-guideline No. B. 7 (1984). Two different substances were tested: A: Alcalase™, batch Nos. MIF 415-424 and B: Ash-mix, which is the ash components qualitatively and quantitatively in the same composition as present in A, Alcalase™, batch Nos. MIF 415-424. The substances were suspended in tap water and administered by gavage as stated below: A: 5 g enzyme + 15 ml water and B: 3.36 g ash mix B + 15 ml water. 100 SPF Wistar rats from Zentralinstitut für Versuchstiere, Hannover, in the weight range of 69-112 g at the start of dosing, were used. 10 rats/dose/sex were used. Haematological examinations were carried out on day 0 and day 30 of treatment. A full histopathological examination was only made on 3 males and 3 females from the high dose level and the control group.

Doses:

Group No.	Dosed with	Content of daily dose			
		AU/kg bw	mg aep/kg bw	ASH mg/kg bw	Ml/kg bw
1A	Susp. A	16.82	295	1682	9.0
2A	Susp. A	6.73	118	673	3.6
3A	Susp. A	2.02	35	202	1.1
B	Susp. B	0	0	1682	9.0
C	Tap water	0	0	0	9.0

In general, the rats disliked the taste and/or smell of the test compounds, especially the active enzyme. Consequently, they tried to avoid the gavage sometimes resulting in fatal aspiration of the suspension. After 10 days, group 1A was diminished by 40% owing to enzyme aspiration. Therefore, the rest of the group was sacrificed and a completely new group 1A was started. Some of the rats developed transient diarrhoea. This symptom seemed to be dose-related. Only one animal from groups B and C developed diarrhoea. No dose-related changes were detected among the biochemical and haematological parameters. The relative organ weights of the adrenals were dose-related and slightly elevated. The histological picture of this and the other organs showed no abnormalities which could be related to the treatment.

The object of another study (Novo Nordisk 1981 (3)) with Alcalase™ was to determine the oral maximum tolerated dose of Alcalase™ in dogs. The investigation was made in two parts. The test substance was Alcalase™ with an activity of 20.6 AU/g ~ 0.36 g aep/g. The test substance was administered by gavage with a constant dose volume of 10 ml/kg bw/day.
In **part A**, two Beagle dogs were dosed daily for 4 weeks, at weekly increasing dose levels of 100, 250, 500, and 1000 mg Alcalase™/kg bw/day ~ 36, 90, 180, and 360 mg aep/kg bw/day. The animals were observed clinically every day, and weights were recorded 3 times every week. Laboratory investigations were made pre-trial and at the end of the treatment for each dose level. The laboratory examinations met the OECD and EEC-recommendations for subchronic oral toxicity-non-rodent: 90-day study. In addition, a test for occult blood in the faeces was carried out. At the end of the 4-week dosing period, both animals were sacrificed and subjected to a macroscopic examination. Furthermore, the weights of the liver, kidneys and spleen were recorded. In **part B**, two other Beagle dogs, one of each sex, were dosed daily for 14 days at a dose level of 500 mg/kg bw/day ~ 180 mg aep/kg bw/day. The method was similar to the method used in part A. Laboratory investigations were expanded with urinalysis for glucose, protein, urobilinogen, ketones, blood, and pH.
Part A: At 250 mg/kg bw/day ~ 90 mg aep/kg bw/day and above, extremely loose faeces were a regular observation. It most frequently occurred about 3 hours after dosing. Faecal production tended to be normal the following morning. Frequent occurrence of faecal blood in loose post-doses faeces were noted throughout the study, and at dose levels of 500 mg/kg bw/day ~ 180 mg aep/kg bw/day and above, the male showed instances of emesis. Finally, the male lost body weight and showed inappentence at 1000 mg/kg bw/day ~ 360 mg aep/kg bw/day. The male glutamic oxaloacetic transaminase values were at the lower limits of normality following dosing at 5000 mg/kg bw/day ~ 180 mg aep/kg bw/day. The male also showed a reduction in glutamic pyruvic transaminase, possibly dose-related over the study period. Autopsy revealed localised areas of intestinal reddening in both animals.
Part B: In addition to the clinical signs described for part A, instances of emesis were observed occasionally resulting in blood-stained vomitus. There were no effects on body weights or food consumption. Clinical chemistry revealed a reduction in alkaline phosphatase over the study period. Finally, blood pigments were observed in a pre-terminal urine sample from the male, and both preterminal faecal samples were positive for occult blood. Autopsy revealed intestinal abnormalities in both animals, the colon of the female being severely reddened.

In order to estimate the influence of the inactivated enzyme a study (Novo Nordisk 1982 (4), amended in 1985) with **inactivated** Alcalase™ was performed. The protocol of this study was in every respect

equivalent to the study described above. The test substance was inactivated Alcalase™ with an activity of approx. 19 AU/g before inactivation.
In **part A**, the doses were 0.3, 1.0, 3.0 and 9.0 g/kg bw/day ~ 100, 300, 1000, and 3000 mg inactivated enzyme/kg bw/day. In **part B**, the dose was 5 g/kg bw/day ~ 1700 mg inactivated enzyme/kg bw/day. **Part A**: At 9.0 g/kg bw/day ~ 3000 mg inactivated enzyme/kg bw/day, loose or liquid faeces were observed approximately 4.5-5.5 hours after dosing. At 3.0 and 9.0 g/kg bw/day ~ 1000 and 3000 mg inactivated enzyme/ kg bw/day, haemoglobin and read blood cell values for both dogs were elevated in comparison with pre-trial data, although within normal limits. Na^+-values at the lower limits of normality were obtained from both dogs after dosing with 1.0 g/kg bw/day ~ 330 mg inactivated enzyme/kg bw/day. Autopsy revealed no abnormalities. **Part B:** No dose-related clinical symptoms were observed, and no abnormalities were found in the laboratory investigations. Autopsy revealed no abnormalities.

In another study (Novo Nordisk 1982 (3) amended in 1985) with Savinase™, activity 53.9 KNPU/g, 0.1365 g aep/g) groups of ten male and ten female rats were given Savinase™, suspended in tap water, one dose daily by oral gavage. The test substance solutions were Savinase™ 1.21 (0.17 g aep/kg), 0.49 (0.07 g aep/kg) and 0.17 (0.02 g aep/kg) g/kg body weight, administered at a dose volume of 10 ml/kg body weight. The control animals received tap water. The animals were observed for 30 days after which they were sacrificed and autopsies were performed. Gross pathology as well as organ weight and histological examinations were performed. Haematology was performed on blood samples taken on day -1 and day 29, day 1 being the first day of treatment. During one of the ultimate days of treatment urine specimens were collected within a 24 hour period. One male and one female from the highest dose level group and two males and one female from the intermediate dose level group died as a result of dosing errors (acute aspiration pneumonia). If death occurred within the first days of treatment the dead rat was replaced. One female rat of the intermediate dose level group was sacrificed due to dehydration and diarrhoea at day 4. The signs of intoxication observed at 0.07 g aep and 0.17 g aep of Savinase™ per kg body weight were decreased weight gain, food utilization and blood haemoglobin concentration. In conclusion 0.17 g (0.02 g aep/kg) Savinase™ per kg body weight, were well tolerated and regarded as the "no-observed-adverse-effect level".

In another study (Novo Nordisk 1970 (1)) carried out with Esperase™, activity 7.4 KNPU/g (0.028 g aep/g), the enzyme was administered in the diet at two dose levels for 4 weeks. Groups of five males and females were fed either basal laboratory diet or basal laboratory diet including 0.5% Esperase™ ($1.4x10^{-4}$ g aep/g diet) or 2% ($5.6x10^{-4}$ g aep/g diet). During the dosing period the animals were observed for general appearance, body weight and food consumption (weekly), unusual faecal consistency, mortality and after sacrifice, for gross post mortem changes in organs. The results showed mortality (3/5 males and 1/5 females) which occurred within the first two weeks in the highest dosed group. The gross pathology of these animals revealed an empty gastrointestinal tract and general emaciation. The bodyweight gain was considered equal for the control and the lowest dosed group, and it was clearly decreased in the highest dosed group. Also the average weekly food consumption was lower for the high dose group. In animals sacrificed after 4 weeks of treatment there were no abnormalities that could be attributed to treatment. To conclude, mortality was seen in the group fed 2% Esperase™ in the diet, ($5.6x10^{-4}$ g aep/g diet), where 4 out of 10 animals died during the study. It was concluded that the empty gastrointestinal tract indicated that the cause of death was starvation due to an unpalatable diet.

A further study (Novo Nordisk 1991 (2)) was performed to evaluate the toxic potential of Esperase™, activity 5.42 KNPU/g, and to find suitable dose levels for subsequent prolonged studies in rats. Five groups of five male rats were dosed orally by gavage, for 14 days at a constant volume of 5 ml/kg body weight. The dose levels were based on an activity of 5.45 KNPU/g to 0, 10, 5, 2.5 and 1.25 KNPU/kg, which corresponds to 0, 1.83, 0.92, 0.46 and 0.24 g/kg. (The actual measured activities based on the g weighed material were 0, 9, 4.7, 2.4 and 1.2.). The animals were observed daily, weighed on days 1, 5, 8, 12 and 14, and subjected to gross pathology at termination. The results showed no indication of toxicity attributed to treatment with Esperase™. To conclude, Esperase™ batch PPA 3366, dosed orally to rats by gavage for 14 days at dose levels up to 9 KNPU/kg, (1.83 g/kg), with a corresponding activity of 0.035 g aep/kg bodyweight, induced no signs of toxicity.

Savinase™ and Opticlean P™ have each been tested in a 28-day gavage study in rats in which high doses of around 1000 mg/kg/day were used (NICNAS, 1993). One animal given Savinase™ (dose group not reported) was sacrificed on day 4 due to dehydration and diarrhoea. Treatment-related effects in both studies included reductions in body weight gains (magnitude not specified), reduced food consumption and minor alterations in a few haematological and biochemical parameters noted in some animals from all dose groups. There were no clear treatment- related effects on organ weight or histopathology in either group.
From the brief details provided no clear conclusions can be drawn, but given that these enzymes are proteins it is unlikely that they would survive the digestive processes intact, hence, any effects from high dose oral exposure are likely to be localised to the digestive tract epithelium.

5.2.1.4.1.2. Subchronic toxicity

A subchronic 90 days toxicity study (Genencor 1994 (7)) with Multifect P-3000 was performed in 160 rats. Dietary administration of 5,000, 15,000 and 50,000 ppm of protease concentrate for 90 days resulted in a dose related enlargement (hypertrophy of the serous acinar cells) of the submandibular salivary glands of the male and female rats. No treatment-related changes were present in the other tissues evaluated from the male and females rats receiving 50,000 ppm of the protease concentrate. A few incidental findings occurred in both the control rats and the rats receiving 50,000 ppm of protease concentrate at essentially comparable incidences and were of the usual type and incidence commonly seen in Sprague Dawley rats. The presence of the incidental lesions did not interfere in the evaluation of the test substances as used in this study. Since the primary change in the enlarged salivary glands was hypertrophy and no degenerative or inflammatory changes were present in the affected salivary glands these changes appear to be not a toxic effect on this organ. There were no degenerative changes present or other evidence that the acinar hypertrophy was an adverse or toxic effect. It represents a reversible, compensatory, physiologic response to the increase level of proteolytic enzyme in the diet. There is no reason to believe the test material would pose a safety concern in humans, particularly at the comparatively very low levels of human exposure. Based on these results, the dose level of 50,000 ppm of the protease concentrate can be considered to be a no adverse effect level.

In a 13 weeks oral toxicity study (Novo Nordisk 1981 (4)) according to OECD-guideline No. 408 Subtilisin Carlsberg was administered to groups of male and female CD rats in doses of 0, 160, 400 and 1000 mg/kg/day by gavage at a constant dose volume of 10 ml/kg. The major clinical observation was increased difficulty of dosing animals receiving 1000 mg/kg/day (360 mg aep/kg bw/day). This difficulty, resulting from struggling during dosing, caused several of the 16 premature deaths due to either aspiration of the compound or direct mechanical damage. For males a dose related reduction in body weight gain was observed. There was only a slight dose-related reduction in food consumption in both sexes. Examinations revealed a significant increase of absolute and relative thyroid weights in all treated males. This finding is, however, not judged to be of great significance as the thyroid gland is known to be very difficult to dissect precisely for weighing also shown in the present study by the coefficient of variability above 20% in all groups. Further, the histopathology did not reveal any changes and historical data showed that the thyroid weight data in the control animals for both males and females in the present study were unusually low. Today, weighing of the thyroid gland is no longer required according to the OECD test guideline 408. Relative lung weights were increased in male rats receiving 400 or 1000 mg/kg/day. Taking these effects into account the NOEL was considered to be 160 mg/kg/day in this study which means 58 mg aep/kg/day.

Gastrointestinal disturbance and bleeding was also the main adverse effect in a 13 weeks study (Novo Nordisk 1981 (5)) with Beagle dogs performed in accordance with OECD TG 409. Subtilisin Carlsberg was given by gavage with a constant dose volume of 10 ml/kg in doses of 3, 30 and 300 mg/kg/day. Per dose and per sex 3 dogs were used. The only symptom after treatment with 30 mg/kg/day was infrequent loss of faecal consistency. Animals receiving 300 mg/kg/day tended to pass loose faeces within 1-5 hours after dosing throughout the study period. The faeces were sporadically blood-stained. Instances of vomitus were rare. Animals treated with 300 mg/kg/day also had reduced values for haemoglobin concentration, red blood cell count, and packed cell volume during weeks 6 and

12. The same animals had marginally low values for total protein and albumin. However, none of these changes were considered to be of major toxicological significance. No adverse findings related to the treatment were disclosed by gross pathology, histopathology, or analysis of organ weights. The NOEL in this study was considered to be 30 mg/kg/day. Since the active enzyme protein content of the preparation used was determined at almost exactly 50%, the NOEL based on aep can be given at 15 mg/kg/day.

This result is in contrast to the occasional emesis and liquid faeces found with inactivated enzyme at a dose of 1600 mg/kg/day (Novo Nordisk 1983 (2)). The method applied in this test was equivalent to the method applied above (Novo Nordisk 1981 (5)) but the test was carried out with inactivated Alcalase. (actual activity before inactivation: 18.5 AU/g ~ 0.32 g aep/g). The test substance was administered by gavage with a constant dose volume of 20 ml/kg bw and with a 20 ml washout of the gavage tube. Doses were 0.3, 1.2, and 5 g/kg bw/day ~ 97, 390, and 1600 mg inactivated enzyme/kg bw/day. The only clinical signs were occasional emesis and passing of soft or liquid faeces from animals treated with 1.2 and 5 g/kg bw/day ≈ 390 and 1600 mg inactivated enzyme/kg bw/ day. No adverse findings related to treatment were disclosed by gross pathology, histopathology, or analysis of organ weights. In dogs treated with inactivated Alcalase, NOEL was 0.3 g/kg bw/ day (0.097 g inactivated enzyme/kg bw/day). The only symptom when treated with 5 g/kg bw/day (1.6 g inactivated enzyme/kg bw/day) was occasional emesis and passing of soft or liquid faeces. There were no signs of systemic toxicity.

Esperase™ was administered (Novo Nordisk 1991 (7)) orally to groups of male and female rats in doses of 1, 3, and 5 g/kg/day by gavage at a constant dose volume of 5 ml/kg over 13 weeks according to OECD TG 408. The results showed a slight reduction in bodyweight gain for males in the intermediate and high dose group, but without any concomitant change in food efficiency ratios. A dose related increase in water consumption was seen in all groups. At histopathology focal hyperplasia of the keratinized epithelium of the stomach was seen in 2/20 males and 2/20 females in the high dose group. However, the low incidence provides insufficient evidence of a treatment related effect. The NOEL in this study was 1 g/kg/day.

In another study no treatment-related changes were observed in rats fed diets containing 0.0015% or 0.0025% of a Subtilisin preparation in detergent for 28 days (Griffith et al., 1969).

5.2.1.4.2 Inhalation

In a study in which Cynomolgus monkeys were exposed to a 2:1 mixture of the Subtilisin preparations Alcalase™ and Milezym 8X™ (Coate et al., 1978), groups of 9 monkeys (E) were exposed (whole-body) for 6 hours/day, 5 days per week for 6 months to 0 or 1.18 µg/L Subtilisin preparation (MMAD 2.8 µm). Additional groups of 9 monkeys were similarly exposed to this mixture together with 100 µg/L detergent (DE), or to 100 µg/L of detergent alone (D). Five animals per group were sacrificed at the end of the 6 months; the remaining animals were allowed a 4-week recovery period before sacrifice. Only combined necropsy findings were given. Observations were made throughout the study, including clinical signs, body weight, lung function and tests for specific IgE. At the end of the study, skin prick tests were performed. At necropsy a wide range of tissues was examined microscopically, including detailed examinations of the upper respiratory tract and lungs.
During the study, there were no treatment-related deaths in the E group animals, whereas 2 died in the D group and 3 in the DE group, prompting a reduction of the exposure period to 17 weeks for the DE group. Decedents showed severe respiratory difficulties prior to death. Laboured breathing was more prominent in E monkeys than in monkeys from other groups. There were no changes in pulmonary function (flow resistance during inspiration and expiration, changes in carbon monoxide diffusing capacity [DLCO], breathing rate, tidal volume) in E group monkeys. However, pulmonary function test data from DE and D group animals showed evidence of small airways constriction, the effects were most marked in DE group animals.
Body weight was not affected in E or D group animals, but was reduced in the DE group. At necropsy, one E group animal showed pulmonary inflammatory changes including bronchiolar epithelial hyperplasia and hypertrophy, bronchiolar wall fibrosis, diffuse alveolitis and pneumocyte hyperplasia. Alveolar and perivascular pigmentation were also seen. The severity of these changes was not clearly

described. The remaining animals in this group had similar lung histology to the controls. Chronic inflammatory changes and fibrosis were seen in the lungs of all DE and D animals, and were more severe in DE than D animals. There was no evidence that E or DE monkeys developed Alcalase™- or Milezym™-specific IgE as measured by RAST and by passive cutaneous anaphylaxis, nor could Alcalase™- or Milezyme™-specific IgG, IgA, IgM or IgE be demonstrated in pulmonary tissue by immunofluorescence. However, IgM to Alcalase™ and/or Milezyme™ was found in sera from E and DE monkeys taken 4 months into the study (Cashner et al., 1980). This apparent discrepancy might reflect the reduced sensitivity of the immunofluorescence technique. No Subtilisin-specific antibodies were detected in D monkeys. Given the fact that pathological changes were observed in only one E group monkey, it is difficult to determine if this was treatment-related or a spontaneous finding.

Overall therefore, no conclusions can be drawn from this study in relation to the effects of long-term repeated inhalational exposure to Subtilisin.

An inhalation study over 8 weeks was performed with Subtilisin Carlsberg and Esperase™ in guinea pigs. The test was carried out using 56 animals in the highest concentration and 30 animals in the other groups and control. The enzyme concentrations of the two enzymes were identical and were 100, 10, 1 and 0.1 µg/L. The test groups were exposed for a period of one hour, once every 7 days, in whole body chambers. Particle size profiles were determined using a microscope and histograms of the size were produced. These analyses showed that the dust particles for both enzymes were readily respirable. In general the test animals were observed throughout the exposure periods and all animals were examined daily between exposure periods. The results showed treatment related mortality for Esperase™ at 100 µg/L (corresponding to $1.5x10^{-3}$ mg aep/L) in 3/56 animals immediately after the 6th exposure. The histopathology of these animals showed signs of an acute pulmonary hypersensitivity reaction which was considered the cause of death. Irritation of the skin in form of localised erosions were seen in solitary animals for both enzymes. Other clinical signs such as some nose bleeding and lachrymation was observed in solitary animals, being most prevalent in the high dose groups but also seen in single animals with no obvious relation to dose. Respiratory patterns of animals were altered during the study but there was no obvious difference between the enzymes. A dose-related sensitisation determined by histamin release from the perfused lung of treated animals was detected and confirmed by passive cutaneous anaphylaxis and Schultz-Dale tests. Even at the lowest exposure concentration slight sensitization occurred in some animals. Based on this extensive study, the enzymes were considered to have similar allergenic potential to guinea pigs when exposed to the dust by inhalation (Novo Nordisk 1972 (1)).

Guinea pigs were exposed to 17 µg/L atmosphere of Savinase dust for a period of one hour, once every day for a total of 8 weeks (1 week equals 7 days) in whole body chambers. Particle size profiles were determined using a Quantimet automatic image analyser. The guinea pigs were observed during the exposure periods and all animals were examined daily between the exposure periods. There were no mortalities during the experiment. Irritation of the skin in the form of localised erosions was seen in 6 out of 32 animals. Lesions were found generally on the back. During exposure general irritation indicated by sneezing and lachrymation was observed. Respiratory patterns of animals during the first two exposures were changed, so that the respiratory rates were increased. This was probably due to the inhalation of particulate material rather than to an allergenic response. During exposures two to five inclusive, more marked changes in respiratory pattern were observed in some animals. Typically these showed various signs of respiratory distress, some breath holding, arrhythmias and prolongation of expiration (Novo Nordisk 1974 (1)).

5.2.1.4.3 Dermal Administration

A repeated dose dermal 28-day toxicity study (Novo Nordisk 1978 (2)) was performed with Savinase™ similar to the method described in OECD TG 410, using 32 albino rabbits, divided into four groups. The study was performed with application of Savinase™ on abraded as well as on intact skin.

The test substance solutions were:
Group 1: water (control) at a dose volume of 2 ml/kg
Group 2: 0.1% w/v aqueous sodium tripolyphosphate (control) at a dose volume of 2 ml/kg
Group 3: Savinase™ in 0.5 % w/v concentration in an aqueous solution at a dose volume of 2 ml/kg (0.0014 g aep/kg/day)
Group 4: Savinase™ in 0.5 % w/v concentration in 0.1 % w/v aqueous sodium tripolyphosphate at a dose volume of 2 ml/kg (0.0014 g aep/kg/day).
The doses were applied for 28 consecutive days. On each application day, the animals were washed with lukewarm water four hours after the application to prevent a build-up of excessive residues.
Animals were observed daily for ill-health and for signs of reaction to treatment. For haematology studies blood samples were taken by venopuncture from all rabbits before commencement and after four weeks of treatment, haemograms and blood chemistry was performed. All surviving rabbits were sacrificed after 28 days of treatment and all rabbits were subjected to detailed macro- and microscopic pathology. Overt signs of reaction to treatment were absent. No systemic effects were observed and the behaviour and general appearance of all animals were normal throughout the study. Skin reactions were confined to sporadic, minimal grade (barely perceptible) erythematous responses. The observed responses were considered to be related to the daily mechanical handling and material application and not to the applied materials per se. Histopathological examination of the treated skin revealed slight acanthosis, occasional patchy parakeratosis and a variable, slight infiltration of the underlying dermis by mononuclear and eosinophil leucocytes. There were minor changes in the lungs of most animals from all groups and sporadically occurring parasitic lesions in various organs. None of those were related to treatment. In conclusion, under the conditions of this test, repeated daily applications of buffered or non-buffered Savinase™ were without effect and thus the test substance may be considered harmless. Reactions were confined to microscopically detected minor changes at the site of application.

Summary of subchronic toxicity

After oral administration Subtilisin did not show evidence for significant adverse systemic effects. Local effects at the site of contact can be attributed to the proteolytic action on the gastro-intestinal epithelium. This assumption is supported by histopathological findings. The dose levels at
which localised (non-systemic) effects were seen in various repeated dose studies were consistent with the exposure doses generating similar responses in the acute studies. No clear evidence for an adverse effect was found in Cynomolgus monkeys exposed for 6 months to 1.18 µg/L. No adverse effects were found in a 28-day dermal repeated dose study on rabbits performed similar to OECD TG 410.

5.2.1.5 Genetic Toxicity

Introduction
Genetic toxicity is not expected for enzyme preparations in general in the light of the following:

- Availability of extensive negative mutagenicity data on enzyme preparations
- Documented and recognised lack of genotoxic effects with enzyme preparations in both bacterial and mammalian systems.
- Substantial amounts of genotoxicity data are available from regulatory agencies in support of enzymes used in food and feed.

The following data confirm this expectation.

5.2.1.5.1 *In vitro*

Ames tests in two independent test series (Henkel 1995 (4)) according to OECD TG 471 (1981) and EU guideline were carried out with the Subtilisin preparation BLAP S granulate in *S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537, TA 1538 with and without metabolic activation. The incubation time was 48 hours. In the first test aqueous concentrations of 8, 40, 200, 1000, 5000

µg/plate and in the second test aqueous concentrations of 50, 100, 200, 400, 800 µg/plate were used. No mutagenic activity was found.

In another experiment Multifect™ P-3000 (Genencor 1995 (3)) was tested for mutagenic activity in the *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a confirmatory assay according to procedures similar to OECD TG 471 and 472. The tester strains used in this mutagenicity assay were *S. typhimurium* TA98, TA100, TA1535, TA1537, and *E. coli* WP2uvrA. The mutagenicity assay was conducted in both the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™ induced rat liver (S9 mix) with five doses of test article along with concurrent vehicle, negative, and positive controls, using three plates per dose. The results of the initial assay were confirmed in an independent experiment. In the initial dose range finding experiment, the test article Multifect™ P-3000 caused a dose-responsive enhancement or overgrowth of the bacterial background lawn with *S. typhimurium* TA100 and *E. coli* WP2uvrA, only in the presence of S9 mix. This enhancement indicated that the test article was interfering with the selective conditions of the assay system. No enhancement of the bacterial lawn was observed with either tester strain in the absence of S9 mix. In addition, no enhancement of the bacterial lawn was observed with the formulation ingredients only (MP-STAB) with either tester strain in either the presence or absence of S9 mix. Due to the interference with the test system observed with Multifect™ P-3000 with tester strains TA100 and WP2uvrA in the presence of S9 mix, a second dose range finding experiment was conducted in which the "treat and plate" method of exposure was used rather than the standard "plate incorporation" method. The treat and plate exposure method allows the test article to be separated from the tester strain following a defined exposure period. This experiment was performed with tester strains TA100 and WP2uvrA, and ten doses ranging from 10.3 to 7,690 µg per ml, both in the presence and absence of S9 mix using three plates per dose. For the treat and plate exposure method, the doses were expressed as µg of test article per ml of treat and plate reaction mixture (0.5 ml of S9 mix or phosphate buffer, 0.1 ml of tester strain, and 0.05 ml of test article dose). The dose range covered was equivalent to 6.67 to 5,000 µg per plate using the plate incorporation exposure. In the dose range finding study with Multifect™ P-3000 and with MP-STAB using the treat and plate exposure method, no interference with the selective conditions of the test system were observed and no cytotoxicity was observed up to the maximum dose tested 7,690 µg per ml. For this reason, the treat and plate exposure method was used in both the initial and confirmatory mutagenicity assays. The doses of Multifect™ P-3000 tested in the mutagenicity assays were 154 up to 7,690 µg per ml (equivalent to 100 up to 5000 µg per plate). The results of the positive and negative controls indicated a valid experiment. The results of the *Salmonella-E. coli*/Mammalian-Microsome Reverse Mutation Assay with a confirmatory assay indicate that under the conditions of this study, in both the initial and confirmatory assay, Multifect™ P-3000 did not cause a positive increase in the number of revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

In the same experimental setup Purafect™ FN3 (PR329) in 33 % propylene glycol was tested (Genencor 1995 (4)) for mutagenic activity in the *Salmonella-E. coli*/Mammalian-Microsome Reverse Mutation Assay with a confirmatory assay. Also in this case the initial dose range finding experiment, showed an enhancement or overgrowth of the bacterial background lawn for both *S. typhimurium* tester strain TA100 and *E. coli* tester strain WP2uvrA, indicating that the test article was interfering with the selective conditions of the assay system by supplying additional histidine and tryptophan. A second dose range finding experiment was conducted using the treat and plate method of exposure like for Multifect™ P-3000 above. Using the treat and plate exposure method, no interference with the selective conditions of the test system was found and no cytotoxicity was observed up to the maximum tested dose, 7,690 µg per ml. For this reason, the treat and plate exposure method was used in both the initial and confirmatory mutagenicity assays. The doses tested in the mutagenicity assays were 154 up to 7,690 µg per ml (equivalent to 100 up to 5000 µg per plate). The results of the *Salmonella-E. coli*/Mammalian-Microsome Reverse Mutation Assay with a confirmatory Assay indicate that under the conditions of this study, in both an initial and confirmatory assay Purafect™ FN3 (PR329) in 33 % propylene glycol, did not cause an increase in the number of revertants per plate of any of the tester

strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

Another Ames test (Novo Nordisk 1991 (8)) was carried out with Savinase™ in a study similar to OECD TG 471 (1983). The test was performed on *S. typhimurium* strains TA 1537, TA 1535, TA 100 and TA 98 in a liquid culture assay with and without the presence of a metabolic activation system (rat liver S-9 mix). Bacteria were exposed to 6 dose levels of the test material (half-log intervals) in the first test and to 6 more narrowly spread dose levels in the second test with 10.0 mg per ml incubation mixture as highest dose level. The cultures were incubated in a phosphate buffered nutrient broth for 3 hours. After incubation the test substance was removed by centrifugation prior to plating. The viabilities of all cultures were determined, and the sensitivity of the individual test organisms was confirmed using standard mutagens. As Savinase™ is a proteolytic enzyme, the enzymatic activity inactivated the S-9 mix. Therefore in all tests with application of S-9 mix heat-inactivated test substance was used. All results were confirmed by conducting two independent experiments. No dose-related and reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to Savinase™ either in the presence or absence of the S-9 mix. It can be concluded that no mutagenic effect of the Savinase™ was detected in the four strains of *S. typhimurium* used in the presence or absence of metabolic activation (S-9 mix).

Another bacterial reversion assay (Novo Nordisk 1981 (6)) was performed with Alcalase™. The study was performed in accordance with the current OECD TG 471 (1997) and the EU guidelines on 5 strains of *S. typhimurium* and *E. coli* WP2 uvrA (pKM101). The test substance used was Alcalase™ actual activity 20.6 AU/g ~ 0.36 g aep/g. The tests were carried out as treat-and-plate tests, i.e. the bacteria were incubated (treated) for 3 hours with the test substances in a nutrient broth, isolated by centrifugation, re-suspended in buffer, and poured on to minimal medium plates. As positive controls, 2-aminoantracene, sodium azide, 2-nitro-fluorene, 9-aminoacridine and methylmethanesulphonate were used. As negative control the vehicle (water) was used. The negative control values were within the normal range. The positive control groups all produced satisfactory responses in their relevant strains. No mutagenicity was observed in the *S. typhimurium* strains, the test substance being toxic to the bacteria at the highest concentration tested (9.1 mg/ml incubation mixture). In the case of the *E. coli* strain, a small dose-related increase in the number of colonies per plate was observed in the absence of S-9 mix. This result, however, was not reproduced in 2 subsequent re-tests, and was therefore considered to be an incidental finding. It can be concluded that Alcalase was not mutagenic in the 5 strains of *S. typhimurium* and the *E. coli* strain used.

In addition mutagenic activity of Esperase (Novo Nordisk 1991 (3)) was tested with *S. typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100 in a liquid culture assay. The mutagenic potential of Esperase, activity 5.42 KNPU/g, was investigated using four strains of *S. typhimurium* with or without the metabolic activation system S9. The principles of the study were based on OECD TG 471(1983). The test assay used was a liquid culture, where bacteria were exposed to 6 doses of the test solution for 3 hours before plating the bacteria onto a minimal medium. After a suitable period of incubation, the revertant colonies were counted and compared to the number of spontaneous revertants in untreated and positive control cultures. In all tests using metabolic activation (S9 mix), the test substance was heat inactivated. This was performed due to the proteolytic nature of Esperase which otherwise would inactivate the metabolic activation system. All results were confirmed by conducting two independent experiments. The results showed no dose related and reproducible increase in revertants with any of the bacterial strains exposed to Esperase, either in the presence or absence of the S9 mix. To conclude there was no indication of mutagenic activity of Esperase in dose levels up to 6.1 mg per ml ($1.3x10^{-4}$ g aep/ml) incubation mixture in the presence or absence of metabolic activation under the test conditions employed.

Ames tests have been conducted with the Subtilisin preparations Savinase™ (tested in 1977) and Opticlean P™ (tested in 1987) in *S. typhimurium* strains TA 1535, TA 100, TA 1537, TA 1538 and TA 98 (NICNAS, 1993). Few details were reported. In the Savinase™ study, aqueous concentrations of 33 - 10 000 µg/plate were used and tests were conducted with and without S9-mix. In the Opticlean

P™ study, aqueous concentrations of 15 - 1500 µg/plate were used, again tests were conducted with and without S9-mix. No information on cytotoxicity was provided. Negative results were reported for both preparations.

An *in vitro* mammalian cytogenetic test (Henkel 1993 (1)) was performed in V 79 cells of the Chinese hamster with the Subtilisin preparation BLAP PM 111 granulate according to OECD TG 473 and EPA regulations. A single amino acid exchange of Subtilisin PM111 was assessed for its potential to induce structural chromosomal aberrations. Cells were treated for 4 hours in the presence of metabolic activation and for 18 and 28 hours without metabolic activation. The test concentrations were for experiment 1 without S9 mix 3.0, 30.0, 50.0 µg/ml (18 hours) and 50.0 µg/ml (28 hours) and with S9 mix 0.3, 1.0, 2.0 µg/ml (18 hours) and 2.0 µg/ml (28 hours). For the second experiment the tested concentrations were without S9 mix 10.0, 30.0, 50.0 µg/ml (18 hours) and 50.0 µg/ml (28 hours) and with S9 mix 0.3, 1.0, 1.8 µg/ml (18 hours) and 1.8 µg/ml (28 hours). It was concluded that BLAP PM 111 under the conditions of the test system, produced no evidence of damage to chromosomal structure in a Chinese hamster cell line.

BLAP S was also tested (Henkel 1995 (5)) for its potential to induce structural chromosomal aberration in the *in vitro* mammalian cytogenetic test with V 79 cells of the Chinese hamster. The experiments were performed in the absence and presence of metabolic activation by a rat liver microsomal fraction (S9-mix) according to OECD TG 473. Cultured V 79 Chinese hamster cells were treated with concentrations up to 3 µg/ml test substance without metabolic activation and up to 5 µg/ml test substance with metabolic activation. The test substance was applied as emulsion in culture medium. Treatment was performed for 4 hours. It was concluded that BLAP S did not induce chromosomal aberrations in Chinese hamster cell line under the experimental conditions reported.

The ability of Multifect™ P-3000 (Genencor 1995 (5)) to induce chromosomal aberrations in cultured whole blood human lymphocytes with and without metabolic activation was tested in an *in vitro* assay similar to OECD 473. The maximum concentration of 20.0 µl/ml was used in the activation assay and 4.00 µl/ml for the non activation assay. Dosing was achieved using a 2 % (20 µl/ml) dosing volume for the activation assay and using a 1 % (10 µl/ml) dosing volume for the non activation assay. Cultures were harvested 22.1 hours after initiation of treatment. The highest dose level tested in the activation assay was achieved using the neat test article. The diluent for preparing the dilutions of the test article for the subsequent dose levels in the assay with metabolic activation and all dose levels in the assay without metabolic activation was phosphate buffered saline (PBS). The solvent control cultures were treated with 20.0 µl/ml of MP-STAB (formulation ingredients alone) in the activation assay and 10.0 µl/ml of MP-STAB for the non activation assay. No significant increase in cells with chromosomal aberrations or in polyploidy was observed at all concentrations. In the confirmatory assay, replicate cultures were incubated with up to 4.00 µl/ml of Multifect™ P-3000 without metabolic activation and with up to 20.0 µl/ml with metabolic activation in 21.9 and 45.8 hour aberrations assays. No significant increase in cells with chromosomal aberrations or in polyploidy was observed at all concentrations. Multifect™ P-3000 was considered negative for inducing chromosomal aberrations in cultured whole blood human lymphocytes both in the presence and absence of an exogenous activation system. These results were confirmed in independently conducted confirmatory trials with two harvest times.

Purafect™ FN3 (PR329) UF Concentrate in 33 % Propylene Glycol was tested in the same experimental setup (Genencor 1996 (1)) as used for Multifect™ P-3000 described above to evaluate its ability to induce chromosomal aberrations in cultured whole blood human lymphocytes with and without metabolic activation. Osmolality of the test article solution and the solvent control (33 % polypropylene glycol) provided were evaluated and 54.0 mg/ml was evaluated as highest possible concentration for testing in this assay. The solvent control was used for dosing the solvent control cultures. All dilutions were prepared using phosphate buffered saline. In the preliminary test for mitotic suppression and dose determination, concentrations up to 54.0 mg/ml were tested. All dosing was achieved using a dosing volume of 5 % (50.0 µl/ml). Based on the observed reductions in the mitotic index as compared with the solvent control culture, the initial trial of the chromosomal

aberrations assay was conducted testing concentrations from 3.38 up to 432 µg/ml without metabolic activation and from 17.0 up to 1620 µg/ml with metabolic activation in 22 hour assays. All dosing was achieved using a 1 % (10 µl/ml) dosing volume of the test solutions and the solvent control article. Cultures dosed with 3.38 up to 54.0 µg/ml without metabolic activation and with 33.8 up to 270 µg/ml in the activation assay were evaluated for chromosomal aberrations. No significant increase in cells with chromosomal aberrations, polyploidy or endoreduplication was observed at the concentrations analysed. In the confirmatory assay, replicate cultures were incubated with up to 108 µg/ml in a non activation assay for 22.2 and 45.8 hours. An additional assay without metabolic activation was conducted with a 3-hour treatment testing dose levels of up to 270 µg/ml and a 22.2 hour harvest. Replicate cultures were incubated with up to 270 µg/ml with metabolic activation in 22.2 and 45.8 hour assays. Cultures dosed with 6.75, 13.5, 27.0, and 54.0 µg/ml from the 22.2 hour non activation assay and with 27.0, 54.0, 81.0, and 108 µg/ml from the 45.8 hour non activation assay were evaluated for chromosomal aberrations. Cultures dosed with 108, 162, 216, and 270 µg/ml from the 22.2 and 45.8 hour activation assays and the 3 hour exposure/22.2 hour harvest non activation assay were evaluated for chromosomal aberrations. No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed at the concentrations analysed. Due to the lack of sufficient toxicity at 108 µg/ml in the 45.8 hour nonactivation assay (confirmatory trial), this assay was repeated testing dose levels of 54.0 up to 5400 µg/ml with a 46 hour harvest. Cultures dosed with 108, 162, and 216 µg/ml were evaluated for chromosomal aberrations. No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed at the concentrations analyzed. The test article, Purafect^{TM} FN3 (PR329) UF Concentrate in 33% Propylene Glycol, was considered negative for inducing chromosomal aberrations, polyploidy, or endoreduplication in cultured whole blood human lymphocytes with and without metabolic activation. These results were verified in independently conducted confirmatory trials.

The chromosome damaging potential in human lymphocytes of Esperase^{TM}, activity 5.42 KNPU/g, was investigated in two independent experiments (Novo Nordisk 1991 (4)) according to OECD TG 473. The highest dose level was 5000 µg/ml, and the experiments were both performed in the absence or presence of metabolic activation by S-9 mix. Due to the proteolytic activity of Esperase, the Esperase preparation used was inactivated by acid treatment for using with the S-9 mix. Negative (solvent) control cultures and positive controls were used as references. In experiment 1, treatment in the absence of S-9 was continuous for 20 hours and treatment in the presence of S-9 was for 3 hours followed by a 17 hours recovery period prior to harvest. In experiment 2, a delayed sampling time was included. Treatment in the absence of S-9 was continuous for 20 or 44 hours and treatment in the presence of S-9 was for 3 hours followed by a 17 or 41 hours recovery period prior to harvest. The results showed that treatment of the cell cultures with Esperase in the absence of S-9 mix had the same range of aberrant cells at all sampling times in both experiments and that these results did not significantly differ from the negative control. To conclude, Esperase did not induce chromosome aberrations in human lymphocytes when tested up to 5000 µg/ml, ($1x10^{-4}$ g aep/ml), in the absence and presence of S-9.

The gene mutation potential for Esperase (Novo Nordisk 1991 (5)), activity 5.42 KNPU/g, was assayed in the HPRT locus (6-thioguanine resistance) in mouse lymphoma cells in two independent experiments. The highest dose level was 5000 µg/ml, and the experiments were both performed in the absence and presence of metabolic activation by S-9 mix. Due to the proteolytic activity of Esperase, the Esperase preparation used was inactivated by acid treatment for using with the S-9 mix. Negative (solvent) control cultures and positive controls were used as references. In the first experiment, treatment was performed with a wide range of doses in the absence and presence of S-9, followed by plating 7 days after treatment to investigate the viability and the 6-thioguanine resistance. In the second experiment, a narrower dose range was used to maximise the chance of detecting any dose related effect. The results showed that treatment of the cell culture with Esperase up to 5000 µg/ml, in the absence and presence of S-9, did not induce any statistically significant increase in mutation frequency at the HPRT locus of the L5178Y mouse lymphoma cells. To conclude, Esperase had no mutagenic activity in this test system when tested up to 5000 µg/ml, ($1x10^{-4}$ g aep/ml), in the in the absence and presence of S-9.

Summary of Genetic Toxicity (*in vitro*)

Subtilisin was uniformly negative in a variety of good quality *in vitro* tests, including bacterial reverse mutation assays (Ames tests), a gene mutation test in mammalian cells (HPRT test in mouse lymphoma cells) and several chromosome aberration studies in Chinese hamster cells and human lymphocytes.

5.2.1.5.2 *in vivo*

An *in vivo* bone marrow cytogenetic test (Novo Nordisk 1981 (7)) with male Chinese hamsters was in principle carried out according to the lines of the current OECD TG 475. The test substance used was Alcalase™, reported activity 20.6 AU/g ~ 0.36 g aep/g. Following a toxicity study with Alcalase®, the cytogenetic study was carried out by daily administration for 5 consecutive days, as follows: A vehicle control group of 10 animals receiving distilled water orally, a low dose group of 10 animals receiving Alcalase™ 200 mg/kg bw (~ 72 mg aep/kg bw) orally, and a high dose group of 12 animals receiving Alcalase™ 2000 mg/kg bw (~ 720 mg aep/kg bw) orally for 2 days, but after several deaths, only 1000 mg/kg bw (~ 360 g aep/kg bw) for the remaining 3 days. Further, a positive control group of 10 animals received ethyl methane sulphonate (EMS) 200 mg/kg bw by intraperitoneal injection daily for 5 days. Four hours after the last dose, each animal was intraperitoneally injected with 6 mg/kg colchicine, and two hours later they were sacrificed. While EMS induced large numbers of aberrant cells, Alcalase™ did not significantly increase the frequency of such cells, even when administered in toxic doses.

NICNAS (1993) cites a bone marrow chromosomal aberration test (Novo Nordisk 1977 (3)) in which Savinase™ in aqueous solution was administered to 5 Chinese hamsters by gavage for 5 days at doses of 0, 60, 300 and 1500 mg/kg. Three hours after the last dose cell division was arrested and bone marrow cells collected. A positive control group given cyclophosphamide was also included. Treatment related deaths occurred in all Savinase™ dose groups and it was necessary to dose additional animals to ensure that 5 animals per dose group were available for analysis. Overall, one animal per group from the low and intermediate dose groups died and 6 animals given 1500 mg/kg died. The cause of death was not reported. No effects on body weight gain and no clinical signs of toxicity were observed in animals completing the 5 day treatment. Negative mutagenic results were obtained for Savinase™.

A dominant lethal assay (Novo Nordisk 1977 (4)) was performed on groups of 10 male mice given 5 daily doses of 0, 100 or 1000 mg/kg/day aqueous Savinase™. A positive control group given trimethyl phosphate was also included. Each male was allowed to mate with 3 females per week for 8 consecutive weeks. At the end of this period, males were sacrificed and reproductive organs were examined histopathologically. Pregnant females from each mating were sacrificed on day 4, 9 or 18 of gestation where possible. Presumed non-pregnant animals were sacrificed 18 days after pairing and the reproductive organs were examined. No treatment-related deaths occurred and no clinical signs of systemic toxicity were observed. Mating performance was comparable between groups. Treatment with Savinase™ did not affect the conception rate, rate of zygote development, pre-implantation or post-implantation loss, number of viable foetuses nor produce an increase in the number of foetal abnormalities. In contrast, treatment with trimethyl phosphate (positive control) produced abnormal zygote development and no viable foetuses in females from the first week of mating and a significant increase in post-implantation losses in females from the second week of mating. Overall, there was no evidence that Savinase™ induced a dominant lethal effect.

Summary of Genetic Toxicity (*in vivo*)

The results of the *in vivo* test systems confirmed the results from the *in vitro* assays. The *in vivo* tests in somatic cells with Alcalase™ and Savinase™ and in germ cells with Savinase™ were negative. The results support the conclusion that Subtilisin is not genotoxic.

5.2.1.6 Carcinogenicity

Carcinogenicity is not expected for enzyme preparations in general since:

- there is no indication in the published literature that detergent enzymes possess carcinogenic properties
- It has been demonstrated that the systemic bioavailability for enzymes is expected to be extremely low and toxicologically insignificant.
- As proteins, enzymes are readily biodegraded in the gastrointestinal tract resulting in negligible bioavailability.

There were no experimental studies on the carcinogenic potential of Subtilisin available. However, a carcinogenic potential is not to be expected. Because there are no indications for a carcinogenic effect of Subtilisin (see above), the performance of such studies is not warranted on animal welfare grounds.

5.2.1.7 Reproductive and Developmental Toxicity

In order for a chemical to affect the reproductive system, the chemical must be absorbed into the systemic circulation and be delivered to the respective target organs or must be able to affect the endocrine system. A potential of Subtilisin for reproductive and/or developmental toxicity is not expected since:

- As proteins, enzymes are readily biodegraded in the gastrointestinal tract resulting in negligible bioavailability.
- Due to their large molecular weight, enzymes do not readily penetrate the skin or mucous membranes to attain consequential concentration in the systemic circulation.
- Enzymes are not structurally related to any known endocrine disrupter.

There were no animal studies available on the reproductive toxicity of Subtilisin. Because adverse effects on the reproduction are not to be expected (see above), the performance of such studies is not warranted for animal welfare grounds.

For the same reason there are only rather old studies available for the developmental toxicity of Subtilisin. All of these were tested on rats in several studies according to OECD TG 414.

Pregnant CD rats were treated daily with Alcalase^{TM}, activity 6.73 AU/g ~ 0.12 g aep/g (Novo Nordisk 1976 (1)). The method used essentially meets with the recommendations of OECD TG 414. Alcalase^{TM} was administered by gavage with a constant dose volume of 10 ml/kg bw/day and doses of 0, 300, 1000 and 2000 mg/kg bw/day. Neither the body weight gains of the dams nor the weights of the foetuses were reduced at any of the dose levels. There was no evidence of developmental toxicity in any of the treated groups.

Pregnant CD rats were treated daily with Alcalase^{TM} activity 20.6 AU/g ~ 0.36 g aep/g (Novo Nordisk 1981 (8)). The study was performed according to OECD TG 414. Alcalase^{TM} was administered by gavage with a constant dose volume of 10 ml/kg bw/day and doses of 0, 150, 475 and 1500 mg/kg bw/day. Toxicity to the dams was demonstrated by dose-related clinical signs of stress, the death of one animal at the highest dose level, and by dose-related reduction in body weight gain and food consumption. A slight degree of generalised toxicity was demonstrated in the foetuses by dose-related increase in the incidence of retardation of skull ossification. This was statistically significant at the highest dose level. There was no evidence of teratogenic effects, even at maternally toxic dose levels.

Pregnant rats were treated daily with Savinase^{TM} with 50, 150 and 500 mg/kg bw/day (0.0068, 0.0205 and 0.0683 g aep/kg bw/day) on gestation days 6 - 18 per gavage with a constant volume of 5 ml/kg bw/day (Novo Nordisk 1977 (5)). No adverse effects were observed for the dams or developing foetuses. At the highest dose there was a marginal increase in the number of undersized pups, but no other effects on the pregnant females or developing foetuses were found.

Pregnant rats were treated daily with Esperase™ , activity 5.42 KNPU/g (Novo Nordisk 1991 (6)). The method used was in accordance with OECD TG 414. Esperase™ was administered by gavage with a constant dose volume of 10 ml/kg bw/day and doses of 0, 1000, 3000 and 5000 mg/kg bw/day (0.02, 0.06 and 0.1 g aep/kg bw/day). Clinical observations, body weight, food and water consumption and macroscopic pathology, which included counting of the corpora lutea in each ovary, and the implantation sites were recorded. The foetuses were examined for visceral and skeletal abnormalities, including the state of skeletal ossification. No adverse effects were seen on the foetuses at dose levels up to 5000 mg/kg bw/day.

Summary of Reproductive and Developmental Toxicity

The data show that Subtilisin does not produce developmental effects in animal experiments. The NOAEL in the studies conducted was 475 mg/kg bw/day (171 mg aep/kg bw/day) for maternal toxicity and > 5000 mg/kg bw/day (> 0.1 g aep/kg bw/day) for developmental toxicity. The observed results are in agreement with the expectations since reproductive effects were not anticipated as systemic distribution to the reproductive organs is not likely to occur via potential routes of exposure.

5.2.2 Identification of critical endpoints

The data presented in Chapter 5.2.1 show that the key hazard associated with Subtilisin is respiratory (Type 1) allergy. Other than respiratory allergy, eye and skin irritation effects are the only hazards described for Subtilisins. From repeated dose toxicity studies there is no indication that Subtilisins produce a systemic effect. All effects seen in repeated dose toxicity studies relate to local effects and were due to the proteolytic activity of the enzyme at the site-of-contact. These effects were generally seen at concentrations that were equal or higher than those that induced irritation and/or allergy and are therefore not considered as key hazards within the scope of this assessment.

Subtilisin is of low acute oral toxicity, but has shown toxicity in animals after inhalational exposure, due to the proteolytic activity on the respiratory epithelium. Again, these effects were associated with concentrations that were generally higher than those which induced irritation and/or allergy and are therefore not considered as key hazards in this assessment. Subtilisin was not genotoxic in a wide variety of good quality in vitro and in vivo tests, and there is no indication for reproductive or developmental toxicity, or a carcinogenic potential.

5.3 Risk Assessment

For type 1 respiratory sensitisation and allergy towards enzymes a traditional margin of safety calculation cannot be done as there is no well defined “No Observed Effect level” (NOEL). Indeed, there are only limited data available on dose responses, so as a consequence, a benchmark approach is used to assess risk. A clear benefit of this strategy is that it can be based entirely on human data. In this situation, the need is to identify exposure levels associated both with the causation of respiratory allergy and levels where such adverse effects are not generated.

As detailed in the SDA consumer risk assessment document (SDA, 2005), values of estimated or measured exposures are compared to the highest exposure level previously shown not to induce the generation of allergen-specific antibody (the “No Observed Effect Concentration”, or NOEC), or to the lowest exposure level previously shown to induce the generation of allergen-specific antibody (the ”Lowest Observed Effect Concentration,” or LOEC). The threshold for inducing the generation of IgE antibody presumably lies between these two levels. Such comparisons require a consideration of the uncertainty in estimated exposures, as well as uncertainty in the NOEC or LOEC. At a point somewhere between these two levels, there will exist a threshold. The existence of a threshold for allergen-specific antibody production to enzymes must be considered a reasonable assumption, as similar thresholds are generally assumed for most biological effects (Cohrssen and Covello, 1989).

It is important to emphasize that sensitisation does not predict the likelihood of respiratory symptoms occurring (AISE, 2002). Sensitisation is not a clinical outcome or disease, it is only an indication or a marker of exposure (SDA, 2005).

From occupational data, a decrease in exposure to enzymes led to a sharp decline in the incidence of allergic symptoms among workers until the symptoms were eliminated. In addition, the rate at which workers developed IgE antibody to enzymes also declined with a decline in exposure (for a review see Schweigert, 2000; Sarlo and Kirchner, 2002). These studies demonstrated a dose-response relationship for antibody production and elicitation of symptoms and support the existence of thresholds for both events. It is reasonable to assume that such thresholds and dose-response relationships exist for consumer exposures.

5.3.1 Respiratory Sensitisation and Allergy Benchmarks

A detailed discussion of benchmark data for consumer exposure to enzymes is presented elsewhere (SDA, 2005). What follows represents the key points.

There are a few documented cases of consumers who used dusty laundry products in USA and Sweden in the late 1960s and early 1970s and became allergic to the enzymes in the product (PB 204 118. US National Academy of Sciences, 1971; Belin et al., 1970; Zetterstrom, 1974). An analysis of 1,645 individual Swedish serum samples showed that 15 individuals had enzyme specific IgE antibody (0.91%). These 15 were also skin prick test (SPT) positive to the enzyme. Exposure data (sink fill for hand laundry) have been generated retrospectively to simulate the exposure to these materials.. This was done by reconstructing a similar type of product many years later, and measuring exposure under simulated use conditions. The estimated average peak levels were 212 ng/m^3 for this use This example demonstrates the effects resulting from high exposure over a short duration that occurred on a regular basis.

Some of these 15 individuals reported symptoms of allergy when they used the dusty enzyme-containing laundry powder. A provocative test of some of these consumers showed that 8 of 12 patients who had IgE antibody to enzyme had symptoms after challenge with enzyme-containing product (laundry powder mixed with enzyme). None of the 12 patients had symptoms from exposure to garments and bed linen laundered with enzyme containing granule laundry product (Zetterstrom, 1977). This is an example of a benchmark where the generation of enzyme specific IgE antibody and the elicitation of symptoms were associated with an exposure to enzyme-containing product.

The ACGIH proposed a occupational limit of 60 ng/m^3, which has been applied with great success for some decades (Sarlo, 2003). In consumer use, airborne levels of enzymes contained in household laundry would generally be undetectable, but can be calculated to be 0.01ng/m^3 (see 5.1.3.2 above). Thus the levels are several orders of magnitude below the factory limit as well as those seen in the Swedish studies (Belin, 1970). A highly unlikely worst case scenario for consumer enzyme exposure could be associated with automatic machine dishwashing (see section 5.1.3.2.2) where the airborne enzyme level might reach levels in the ng/m^3 range. The benchmark here is the abrupt opening of institutional dish washers using higher enzyme concentrations, where levels of approximately 2 ng/m^3 have been determined. However, this is still 30x lower than the ACGIH limit which was related to daily workplace exposure, not an ad hoc exposure associated with a “misuse” situation.

A retrospective evaluation of nearly 2,500 patients that attended an allergy clinic in the early 1970’s showed that at least 80% used coated enzyme laundry detergents for almost 2 years and none developed IgE antibodies to enzymes (Pepys et al., 1973). Continued skin testing of consumers of granulated and encapsulated laundry products over the years confirmed these original findings that exposure to enzymes via laundry use does not result in IgE production (Pepys et al., 1985). In addition baseline skin prick testing of prospective employees in the detergent industries has shown no reaction

to detergent enzymes among this population. This observation supports Pepys's work that exposure to enzymes via laundry use will not lead to allergen-specific antibody production among consumers.

Additional support also can be derived from studies carried out in non-European situations. Laundry pre-treaters containing proteolytic enzymes have been produced and sold at high volumes in the U.S. since the mid-1990's. Although there have been no indications of allergic symptoms among consumers, previous work had indicated the potential to produce significant concentrations of enzyme in air using trigger sprayers. For example, a study has been conducted to characterize aerosols to which a consumer could be exposed from a trigger spray containing a prototype enzyme laundry product (SDA, 2005). For the purpose of this study, a prototype, non-commercial water-based formulation containing 0.5% protease enzyme was used, and it produced an average range of 67 - 121 ng/m^3 of protease in the air (depending on sampling method) over a 10.5 minute period of simulated product use. A controlled prospective clinical study of ninety-six atopic users of a laundry prespotter containing protease was carried out in 2001 (Weeks et al., 2001 A). After exaggerated usage of the prespotter product daily for six months, no subject became skin prick test positive to the protease. This result is consistent with the safety record for this class of prespotter product used by tens of millions of consumers. The estimated exposure in the study just mentioned were 12-17 ng/m^3 for a period of 10 minutes daily (Weeks et al., 2001 B).
A two year prospective study among 581 atopic women in the Philippines showed no IgE production to enzymes after use of enzyme-containing granule detergent for hand laundry supplemented with an enzyme-containing synthetic laundry bar (exposures from bar use for hand laundry ranged from 0.004 to 0.026 ng/m^3). These women also used the bar for personal cleansing with measured exposures less than 0.01 ng/m^3 (Cormier, 2004). Another study (conducted in Egypt) reported that exposures up to 0.5 ng/m^3 over a one year period did not give rise to sensitisation (SDA, 2005).

In summary, an upper benchmark where adverse effects occur is 212 ng/m^3 and adverse effects (allergic symptoms) are absent when exposure is in the range of 1 ng/m^3 or less (Peters et al., 2001).
These estimates, of course are highly dependent upon a number of parameters, such as: Particle size distribution, exposure duration and frequency, atopic status and smoking habits.
Since enzyme exposure associated with laundry products is calculated to be no more than 0.01 ng/m^3, adverse effects are not expected. In reality, the thresholds at which respiratory sensitisation and allergy occur are likely to be distinctly higher than mentioned above, thus making, the margin of safety proportionately greater.

5.3.2. Skin/Eye Irritation

Concentrated Subtilisin is an irritant to skin and eyes. The irritation potential of aqueous solutions of Subtilisin depends on the concentration. As reported in the irritation hazard section (5.2.1.2) of this assessment, aqueous solutions of Subtilisin at concentrations up to 2% enzyme granulate (estimated 0.02 % aep) failed to show any irritation effects even on damaged human skin. The average NOEC value for humans is at 0.07 % (based on aep). It is noted that the irritation effect is strongly dependent also on the water activity, on pH and on the activity of the protease.

As Subtilisin concentrations in washing solutions are well below 0.02 %, the contact of skin with such solutions does not pose a relevant risk for irritation.

Skin contact with Subtilisin deposits on washed fabrics will also not cause skin irritation. The levels of Subtilisin deposited on fabric are very small; even assuming all the material remains active and transfers to skin with 100% efficiency, the skin contact concentrations (see section 5.1.3.3.3) are several orders of magnitude below the 0.07 % figure mentioned above. Clinical studies which demonstrate the validity of this conclusion have been published (Bannan et al., 1992: Rodriguez et al., 1994).

In the course of laundry pre-treatment, skin contact with concentrated powder paste, or neat liquid detergent may occur (maximum Subtilisin concentration 0.09%). If it does occur at all, the contact

with skin is confined to a fraction of the hands (palms and/or fingers), and is of very short duration (typically a few minutes at most). The initially high Subtilisin concentration is usually diluted rapidly in the course of the pre-treatment task. The contact with liquid detergent products is not comparable with the contact of aqueous solutions of the same enzyme concentration, due to low water activity and the reversible inhibition of the protease to achieve the storage stability required for a consumer product.

Failing to rinse hands in water after contact with a laundry pre-treatment paste or liquid may result in (transient) skin irritation of the hands, which is expected to be mild in nature and can be easily avoided by prompt washing with water.
On the basis of the experimental data reported in 5.2.1.2 and the Subtilisin concentrations employed therein and compairing these concentrations to the lower levels used in consumer products, accidental eye contact with Subtilisin from either neat liquid product or hand wash solutions is not expected to cause more than a mild transient irritation.

5.4 Discussion and Conclusions

The very low levels of Subtilisin in household formulations (< 0.1%), together with its high molecular weight, and its fast denaturation and hydrolysis in the body, will not lead to a relevant systemic exposure to the active substance; estimates of systemic concentrations are in the order of a few ng/kg bw/day only, even under unrealistic worst-case conditions. From repeated dose toxicity studies there is no indication of systemic effects. All effects seen in repeated dose toxicity studies relate to local effects and were due to the proteolytic activity of the enzyme at the site-of-contact. These effects were generally seen at concentrations that were equal or higher than those that induced irritation and/or allergy and are therefore not considered as key hazards within the scope of this assessment.

Respiratory (Type 1) allergy is the critical endpoint for detergent enzymes such as Subtilisin. This became evident in 1969 when in occupational medical surveillance, Subtilisin was identified as the agent responsible for respiratory health effects in workers (Flindt, 1969). At that time, Subtilisin was added to detergents as a dry powder prone to cause enzyme containing dust when handled. In contrast to the situation then, today´s detergent enzymes, including Subtilisins, are solely used in the form of non-dusting, coated granulates or non-volatile liquids.
Consumers can be exposed via the respiratory route to subtilisin during the task of dispensing powder products in the washing machine (0.16 ng/m^3) or in the sink for hand wash (0.01 ng/m^3), or by suddenly opening the dish washer during the cleaning step (< 1.9 ng/m^3). Since there is no well defined threshold for the induction of sensitisation a benchmark approach was used to assess the risk of consumers. An upper benchmark where adverse effects occur is 212 ng/m^3. A lower benchmark where Allergic symptoms under occupational conditions do not occur is in the range of 1 ng/m^3 (Peters et al., 2001). There appears to be a complex relationship between frequency, magnitude and duration of exposure and the generation of enzyme specific IgE antibody. These estimates, of course are highly dependant upon a number of parameters, such as: Particle size distribution, exposure duration and frequency, atopic status and smoking habits. Since enzyme exposure associated with laundry products is calculated to be no more than 0.01ng/m^3, adverse effects are not expected. In reality, the thresholds at which respectively respiratory sensitisation and allergy occur are likely to be distinctly higher than mentioned above (1ng/m^3), thus making, the margin of safety proportionately greater.
This is supported by the observation that the risk of allergy in consumers was extremely low following the widespread use of products containing detergent enzymes in the 60'and 70's when the materials were un-coated.

Other than for respiratory allergy, there is also a hazard for skin and eye irritation by subtilisins. Consumers may be exposed by skin contact during laundry hand wash (0.0009%), by laundry pre-treatment using liquid detergent (0.09%), by hand dish wash (0.0001%) and by fabric wear with skin in contact with Subtilisin deposited during the wash (0.0035 µg/cm^2). As aqueous solutions of Subtilisin at concentrations up to 2% enzyme granulate (estimated 0.02 % aep) failed to show any

irritation effects even on damaged human skin, and as the average NOEC value for humans is at 0.07 % (based on aep), it is concluded that skin contact with washing solutions containing Subtilisins is not a cause of concern.
Skin contact with Subtilisin deposits on washed fabrics are not expected to cause skin irritation, as the levels of Subtilisin deposited on fabric are very small; even assuming all the material remains active and transfers to skin with 100% efficiency, the skin contact concentrations are several orders of magnitude below the 0.07 % figure mentioned above. This is confirmed by clinical studies (Bannan et al., 1992: Rodriguez et al., 1994). In the course of laundry pre-treatment, skin contact with concentrated powder paste, or neat liquid detergent (maximum concentration 0.09%), may occur. If it does occur at all, the contact with skin is confined to a fraction of the hands (palms and/or fingers), and is of very short duration (typically a few minutes at most). The initially high Subtilisin concentration is usually diluted rapidly in the course of the pre-treatment task. Failing to rinse hands in water after contact with a laundry pre-treatment paste or liquid may result in (transient) skin irritation of the hands, which is expected to be mild in nature and can be easily avoided by prompt washing with water.

Exposure of the eyes may occur through accidental splashes or spills during the handling of liquid detergent products. On basis of the experimental data and the concentrations employed in these studies as compared to the lower concentrations used in consumer products, accidental eye contact with Subtilisin from either neat liquid product or hand wash solutions is not expected to cause more than a mild transient irritation.

In conclusion it can be said, that use of Subtilisin in laundry and cleaning products represents no safety concerns for consumers.

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7. CONTRIBUTORS TO THE REPORT

AMFEP (Association of Manufacturers and Formulators of Enzyme Products; Leading Trade Association), Genencor, Henkel, McBride, Novozymes, Procter & Gamble, Unilever

8. ABBREVIATIONS

AAPF	succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-Nitro-anilide (protease substrate)
ACGIH	American Conference of Governmental Industrial Hygienists
aep	active enzyme protein
AISE	Association Internationale de la Savonnerie, de la Détergence et des Produits d'Entretien
AMFEP	Association of Manufacturers and Formulators of Enzyme Products (EU)
AU	Anson Units (measure for proteolytic activity using hemoglobin as substrate)
BOC	Biological Oxygen Demand
COD	Chemical Oxygen Demand
DOC	Dissolved Organic Carbon
EUSES	European Union System for Evaluation of Substances
FEV	Forced Expiration Volume
GMO	Genetically Modified Organism
GPIT	Guinea Pig Intratracheal Test
GU	Glycine Units (measure for proteolytic activity using a glycine-ester substrate)
HERA	Human and Environmental Risk Assessment
HPRT	Hypoxanthine Phosphoribosyl Transferase Test
HSE	Health and Safety Executive (UK)
KNPU	Kilo Novo Protease Unit
LOEL/LOEC	Lowest Observed Effect Level/Lowest Observed Effect Concentration
LOAEL/LOAEC	Lowest Observed Adverse Effect Level/ Lowest Observed Adverse Effect Concentration
NC-IUBMB	International Union of Biochemists and Molecular Biologists Nomenclature Committee
NOEL/NOEC	No Observed Effect Level/No Observed Effect Concentration
NOAEL/NOAEC	No Observed Adverse Effect Level/No Observed Adverse Effect Concentration
n.i.	no information
NICNAS	Australia's National Industrial Chemicals Notification and Assessment Scheme
OECD	Organisation for Economic Cooperation and Development
OECD TG	OECD Testing Guideline
OEL	Occupational Exposure Limit
PEC	Prediced Effective Concentration
PEFR	Peak Expiratory Flow Rate
PNEC	Predicted No Effect Concentration
RAST	Radio-Allergo-Sorbent-Test
RCR	Risk Characterisation Ratios
SDA	Soap and Detergents Association (USA)
SDIA	Soap and Detergent Industry Association (UK)
STEL	Short-term Exposure Limit
STP	Sewage Treatment Plant
SWORD	Surveillance of Work related and Occupational Respiratory Disease, UK
TGD	EU Technical Guidance Document
TWA	Time Weighted Average

9. APPENDICES

9.1 Appendix 1

Estimation of Exposure to Enzymes from Early Detergent Formulations (from SDA 2005)

Summary

Detergent products made today are orders of magnitude less dusty than products used by Swedish consumers in the late 1960's and described in the study conducted by Belin et al. (1970). Peak airborne enzyme exposures to Swedish consumers described in the Belin study are estimated at 212 ng/m^3. This conclusion is based on the descriptions from the Belin paper and analyses done by the Procter & Gamble Company to measure potential exposure using a product formulated to the same consistency as was used during in the late 1960s by Swedish consumers. The later estimate of exposure, together with the allergy data from the Belin paper, were used to create an "effect" benchmark. In addition, exposure to a less dusty detergent formulation was reported by Hendricks (1970) to not be associated with an allergic antibody response. The data from Hendrick, together with an estimate of exposure, were used to create a "no effect" benchmark.

Background

In the 1970s several papers (Belin et al., 1970; Hendricks, 1970; Zetterstrom, 1974) were published describing allergy in several Swedish consumers to enzyme-containing granular detergent products and subsequent reaction upon re-exposure to these products. Belin's report is significant as it documents a case where consumer use of a detergent resulted in the development of IgE antibody to enzyme and allergic symptoms upon exposure to dusty laundry product. Unfortunately, while formulation concentration of enzyme was described, the actual airborne enzyme dust concentration was not determined. There is, therefore, no airborne enzyme level to associate with producing the allergy during use of the product. These papers are of significance to the enzyme risk assessment process because the allergy caused by normal use constitute an "effect" that can be used to determine risk to modern enzyme-containing products. However, to make them useful, an estimate of potential exposure is necessary.

During that same time period, Hendricks published a paper (1970) describing the lack of allergic antibody among consumers that used granular detergents with an improved enzyme coating that reduced dustiness and, hence, exposure, to enzyme protein. These detergents were made from enzyme stocks that had undergone new granulation processes and reduced enzyme protein dust generation during handling of these products. The significance of this work is that with the improved forms of enzymes the exposures to enzyme proteins were reduced and no IgE antibody was made. If potential exposure were available then this report could be used as the basis for a "no effect" benchmark.

The purpose of this Appendix is to describe methods used by the Procter & Gamble Company to estimate exposures from the reports of Belin, and Hendricks. With these estimates of exposure, the data contained in these reports could be used as "effect" and "no effect" benchmarks.

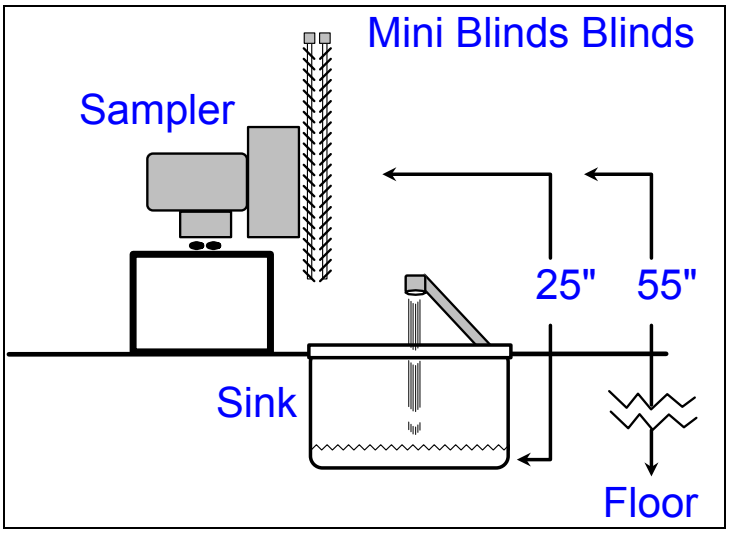
Methods Used to Estimate Exposures of Swedish Consumers to Enzymes in Early 1960's Detergents

Preparation of dusty laundry product. Granular laundry detergent products were formulated to simulate products used by Swedish consumers in the late 1960's. The formulations contained anionic and nonionic surfactants, silicate builders, and perborate bleach. A coated enzyme preparation of subtilisin protease was added to produce a final concentration of 667 µg enzyme protein/g of detergent product.

Product micronization.
Granules are typically in the order of 200-500 micron, while spray dried powder is typically 100-25 micron, depending on the kind of enzyme/strain. In this exposure estimation study a micronized product with a uniform particle size of 1 micron was used. Real powdered enzyme product is larger than the 1 micron used for the study. Normally smaller particle size means more dust and more exposure, so the study using 1 micron actually is a worst case estimation of the 60's exposure.

Approximately 100 g of detergent product was micronized (finely ground) to a powdered material with a uniform, 1 micron particle size using an Ultra Centrifugal Mill Type ZM1 (Brinkman Instruments, Inc., Westbury, NY, USA) with 1.0 mm stainless steel sieve. This micronized sample was then used in the detergent dispensing studies.

Product dispensing, sink filling, and air collection while using dusty enzyme product.
Five samples of 50 g each were weighed from the micronized finished detergent product into 100 mL disposable beakers. Using the setup shown in the figure below, the faucet was turned on and water allowed to reach to 45°C without blocking the sink drain. One second before dispensing the product, a General Metal Works type HV2000P air sampler (General Metal Works, Inc., Cleveland, OH, USA) fitted with a 10 cm diameter GF/C glass fiber filter and calibrated to a flow rate of 0.33 m^3/min was turned on. The sink drain was then blocked by use of a stopper and immediately one of the fifty g samples of micronized detergent was dispensed into the sink at a height of 12 inches above the sink bottom and a time equal zero minutes. The sink was allowed to fill to a volume of 8 L at which point the air sampler was turned off and the time recorded. The faucet flow rate was about 8 L/min. The air sampler was positioned at breathing zone height, perpendicular to the front of the person performing the dissolution task, and facing the water dispensing area of the sink. The sampling height was 55 inches from the floor to center of the sampler and 25 inches from the bottom of the sink. Two sets of adjustable miniblinds were positioned immediately in front of the air sampler. One set of blinds was opened at a 315° angle relative to the air sampler intake. This set of blinds touched the air sampler. The other set was positioned next to the first set and touching but with the blinds angled in an opposite direction 45° relative to the sampler intake. In this manner, the blinds would act as a deflector for splashing or splattering of diluted product as washing and rinsing occurred but without interrupting air flow. Four more trials with 50 g samples were conducted. In between each trial, the sink was cleaned and the room cleared of airborne enzyme by room exhaust and use of high-powered fans. In addition to room checks after clean out, one additional test was run to demonstrate the splatter prevention effectiveness of the mini-blind set up by dispensing product following the same procedure but without turning the sampler on. This test was meant to show if any enzyme solution was splattering onto the open-faced pads due to sink filling and potentially biasing results to high values.



Air sample extraction for analysis. Upon collection, each pad was removed, placed in a 50 mL conical tube and immersed in 25 mL of a enzyme extraction buffer consisting of 500 mM NaCl, 20 mM Tris, 0.1% BSA, 20 mM thiosulfate, 1 mM calcium chloride, and 0.1% Tween 20, pH 8.2. Each tube was incubated for a minimum of 18 hours at 10°C prior to analysis. At the time of analysis, the pads were removed and discarded. The extracted solution in the tube was analyzed for enzyme protein concentration. Previous work had shown that maximum extraction was obtained within one hour of incubation in this solution or twenty minutes of incubation if the sample was rotated along the longitudinal axis of the tube at about 25 rpm.

Enzyme measurement. Solutions prepared from air collections were analyzed by an Enzyme Linked Immunosorbant Assay (ELISA) to quantitate enzyme protein present. The ELISA method used was a modification of the method described by Miller, et al. (1994) and was used to measure subtilisin protease in these solutions. As conducted in these studies, this assay system in combination with the above air collection procedure had an effective measurement range of 6 to 597 $\pm$ 6 ng/m^3 protein in airborne dust for the five minute sampling period.

Protein assignment. Protein was assessed by the Kjeldahl Total Nitrogen Method (Bradstrut 1965). This method has proven to be the most practical protein method for application across a wide variety of enzyme classes used in detergents. All enzyme measurements are based on standards calibrated by this protein method.

Results and Discussion

Exposure during granular detergent dispensing into a sink and filling with water. Results of air collections conducted following procedures outlined above for the product dispensing tasks are shown below.

Test	Airborne Subtilisin Protease Concentration (ng/m^3)	
Pre-trial area check	none detected	
Product Dispense Trial 1	118	
Clean up Check	none detected	
Product Dispense Trial 2	218	
Clean up Check	none detected	**Average:**
Product Dispense Trial 3	165	
Clean up Check	none detected	212 ng/m^3
Product Dispense Trial 4	387	$\pm$ 104
Clean up Check	none detected	Standard deviation
Product Dispense Trial 5	170	
Clean up Check	none detected	
Dispensing without sampler on	none detected	

The airborne subtilisin protease concentration measured during product dispensing and sink filling ranged from 165 to 387 ng/m^3 with an average of 212 ng/m^3. No airborne enzyme was detected after cleanup indicating that there was no carryover from test to test. The splatter test also indicated that the mini-blinds were effective in preventing enzyme contamination from splatter as the sink filled with water.

The product used by Swedish consumers described in the Belin paper (Belin et al. 1970) was said to be "powdered" and positions it as different from the less dusty detergent containing "granular enzyme". One can conclude from this information that the enzyme stock put into the detergents that Swedish consumers were using was the dustier, powdered form of the enzyme used by the industry in the 60's and early 70's. Zetterstrom (1977) who later carried out follow-up work in an attempt to help evaluate the clinical history of the occurrences reported by Belin and others provides more details on the wash conditions that were used. In his studies, Zetterstrom used 50 g doses of detergent in 14-L capacity laundry wash basins containing 8 to 10 L of wash solution.

Based on this information, measurement of airborne enzyme during detergent dispensing into a sink was set up using dustier enzyme, 50 g detergent dispenses, and sink fill volumes of 8 L. The dustier enzyme came from a micronization process typically used for laboratory preparation of granular detergents for analysis. During the micronization process, the product is ground to a fine powder reducing particle diameters to about 1.0 micron. Micronized enzyme could, therefore, be used to simulate the powdered enzyme-containing detergent formulations of the 1960's and early 1970's.

The air flow rate used in the dispensing study is also a point that requires some perspective. The pumps used are air samplers that sample at about 330 to 400 L/min. For use with a 10 cm diameter filter, the cross sectional flow rate through the filter is 330 to 400 L/min/total filter area, or 3.8 to 5.1 L/min/cm^2. Compared to the breathing rate and flow rate through the nose per area and assuming a 1 cm diameter for the nostril, a 10 to 16 L/min breathing rate per area would equal 10 to 16 / (1 cm/2) ^{2} x 3.14) x 2 nostrils) or (6.4 to 10.2 L/min)/cm^2. The 10 to 16 L/min breathing rate is the rate specified by Hendricks for housewives doing light work during the laundering process. Clearly, the cross sectional flow rate is slower for the air sampler at 3.8 to 5.1 L/min/cm^2 than air flow through the nose at 6.4 to 10.2 L/min/cm^2. Further, the room in which these studies were conducted had a total volume of 304,483 L. Only 0.11 to 0.13% of total room air was sampled per minute using this sampling set up. Slower cross sectional flow rate than the nose and sampling a tenth of a percent of the total room volume supports that using these pumps would not bias collected enzyme levels higher than what the person actually captures during breathing.

On the basis of the dispensing measurements then, it is reasonable to assume that the Swedish consumers were exposed to enzyme concentrations at about the 212 ng/m^3.

Estimates of Exposure from Coated Enzymes in Detergents

Results and Discussion

There is significant modification that must be made when evaluating the data presented in the Hendricks paper. This change deals with the assumption applied in the paper that respirable enzyme dust of 20 micron size and lower is the only material to be concerned with in regards to the development of allergic antibody. However, it is now well established that larger particles trapped in mucosal areas such as the nasal passage can also contribute to the generation of IgE antibody to enzyme. Based on this occurrence, the Hendricks (1970) exposure number for consumer exposure to enzymes must be expanded to include all particles that are collected at the breathing zone by the air sampler. Since there are no further data available on what percentage of total enzyme dust was collected at larger particle sizes, a best estimate is needed. This can be done by assuming that a proportional formula ratio of the enzyme protein is maintained in the total dust collected. Hendricks' paper indicates that the level of 20 micron and lower size enzyme dust particles collected during pouring is 1/8th of the level expected for the amount of detergent collected. Using this value, the total enzyme ingredient dust that would be present in the detergent dust collected is 8 x 0.5 ng or 4 ng enzyme ingredient @ 1.5 AU/g (comment: enzyme activity is expressed here in the Anson Unit, AU). This translates to enzyme protein for a two minute pour as (4 ng enzyme ingredient) x (1.5 AU/g ingredient) / (30 AU/g enzyme protein), or 0.2 ng airborne enzyme protein per two minute pour. (Note that in calculating this number, the 30 AU/g enzyme protein value comes from experimentally

measured total protein determination of the ingredient as 5% and, thus, (1.5 AU/g ingredient) / (0.05 g protein / g ingredient) = 30 AU/g protein).

Converting this amount into a more meaningful concentration per cubic meter is difficult. Unfortunately, the Hendricks publication never directly indicated the volume of air corresponding to the detergent dust collections. Looking closely at some of the descriptions of the Bendix air sampler and taking into account Hendricks' emphasis of the importance of breathing rates throughout his publication, it is likely that the pump was chosen and set up to get closer to a breathing air sampling rate. The description of the preliminary work does indicate flow rates of 3 to 4 CFM (convert to m^3/min) for measuring collection efficiency and comparisons to bellows-derived flows simulating breathing versus constant flow. Hendricks' experimental outcome suggested that there was no difference in the airborne concentrations determined by either sampling system and that constant flow would suffice. This work was also done with Bendix air samplers. Therefore, it is likely that the air flow rates used to collect detergent dust were rates of 3 to 4 CFM (convert to m^3/min). An average of 0.0991 m^3/min was used in this assessment. While this flow rate is still faster than the breathing rate emphasized in the paper (0.0991 m^3/min vs. 0.016 m^3/min), the information in the paper does not indicate that the pump ever sampled at a lower rate. For a pouring time of two minutes, a total of 0.198 m^3 was sampled. Given this information, the airborne enzyme concentration during a two minute product scooping and pouring of a simulated detergent product was calculated to be 0.2 ng enzyme protein / 0.198 m^3or 1.01 ng/m^3.

Conclusions

Several papers have been evaluated in order to discern a best estimate of exposure to enzyme aerosol during product use. In particular, the Belin paper reports that consumers' in the 1960's became allergic to enzymes in the detergents they were using for laundering. As this is a recorded event, an exposure level would be very useful in carrying out risk assessments. The work reported here simulated those use conditions with a product containing dusty enzyme, as was used at the time of Belin's Swedish consumer's experiences and determined a value of 212 ng/m^3 as the best estimate of exposure. The Hendricks paper also provides and opportunity to assess exposure and relative product dustiness at the time that enzyme encapsulation processes were applied to reduce enzyme dustiness. While several assumptions had to be made in order to derive an exposure in ng/m^3 terms, a value of 1.01 ng/m^3 was determined.

A comparison of calculated exposures is shown below:

			Protein	Exposure
Time Period	**Enzyme Form**	**Detergent Dose (g)**	**Dose / use (µg)**	**during use (ng/m^3)**
mid 1960's to early 1970's	dusty, powder	50	33,500	212
1970	Coated granular product	78.1	46,860	1.01

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9.2 Appendix 2

Enzyme exposure in industrial dihwashing (A.I.S.E. Task Force, 1998)

A.I.S.E. Task Force „Enzyme exposure in industrial dishwashing“

Final report

May 7, 1998

Task Force Members:
Peter Angevaare[1], Christiaan De Vos[2], Mark Fieldsend[3], Günter Hellmann[4], Karl-Heinz Maurer[5], Annette Prentoe[6], Margaret Richold[7], Freek Schepers[1], Merete Simonsen[6], Matthias Strutz[8], Dorte Ulrik[6]

[1] DiverseyLever, P.O.Box 10, NL-3600 Maarssen;
[2]Genencor International N.V., Komvest 43, B-8000 Brugge;
[3]Unilever, Port Sunlight Laboratory, Bebington, Wirral L633JW, UK,
[4]Henkel-Ecolab GmbH & Co., D-40554 Düsseldorf;
[5]Henkel KGaA, D-40191 Düsseldorf;
[6]Novo Nordisk A/S, DK-2880 Bagsvaerd;
[7]Unilever, SEAC, Sharnbrook, Bedford MK44 1LQ, UK;
[8]Dr.Weigert Chemische Fabrik, Mühlenhagen 85, D-20539 Hamburg

ENZYMES IN MACHINE WARE WASH APPLICATIONS

1. Introduction

1.1 History of Enzyme Exposure and Occupational Exposure Limits in the Detergent Industry

Detergent enzymes are recognised respiratory allergens (Flindt, 1969). When first used in Europe in the 1960's the form of enzyme used was a dry dusty powder, and shortly after their introduction respiratory problems among the work force were encountered (Juniper & Roberts 1984). This was controlled by the introduction of encapsulation together with better air monitoring and medical surveillance and outbreaks of enzyme induced occupational asthma in the detergent industry are now rare. Because of this knowledge any new application is viewed very carefully for safety. An Occupational Exposure Limit for subtilisin proteases of 60 ng/m^3 (STEL\ceiling) has been recommended by the ACGIH (American Conference of Governmental Industrial Hygienists) and has been ratified by a number of regulatory agencies. Within the detergent industry this has been voluntarily reduced to 15-20 ng/m^3 for proteases so as to ensure the official OEL is never exceeded and in recognition of a potential adjuvant effect that the presence of surfactant plus enzyme may present.

Although all detergent enzymes are potential respiratory allergens, some classes of enzyme, e.g. bacterial amylases, are more potent than others, based on animal studies (Sarlo et al, 1997 and Unilever 1997 personal communication). This finding has resulted in a voluntary reduction within the detergent industry of the OEL to 5-7 ng/m^3 when such amylases are handled.

Enzymes such as protease and amylase have been added to retail machine dish wash formulations for many years without incident either during their production or when used by the consumer. More recently, the addition of amylase to formulations used in the Industrial Machine Ware Wash Sector (MWW) has been introduced. Before this practice becomes widely accepted it was considered desirable by the Trade Associations representing the interests of the detergent industry (Association Internationale de la Savonnerie et des Produits d'Entretiens - A.I.S.E.) and the enzyme producers (Association of Manufacturers of Fermentation Enzyme Products - AMFEP) that the level of airborne enzyme resulting from this practice should be established. There were two reasons for this, one being the likelihood that airborne enzyme could be released in significant amounts together with aerosol and strong detergent in close proximity to workers needing access to the machine and secondly recognising that this work force may also be considered "consumers" i.e. that they would fall outside the jurisdiction of normal care and surveillance that might be afforded to workers in the detergent and enzyme producing industries. An investigative programme was therefore initiated.

1.2 Terms of Reference

The task force on Enzyme Exposure in I&I dishwashing was instituted as a follow up of the A.I.S.E. Internal Workshop on Safety of Enzymes in Industrial Machine Dishwashing, that took place in Brussels on March 6, 1997. The task force recruited their members from A.I.S.E. and AMFEP member companies.

The terms of reference were defined as follows:
1. To determine the most appropriate analytical methodology, including sampling procedure and method of analysis.
2. Agree best approaches to defining habits and practices with key parameters for evaluation being frequency of door opening, enzymes peaks, frequency of peaks, duration of peaks.
3. Use this approach to generate the data.
4. Report outcome of the above to A.I.S.E./AMFEP Enzymes safety Working Group so that an OEG for amylase in industrial machine dishwashing can be proposed.

1.3 The Industrial Dishwashing Machine

Industrial dishwashers come in different sizes and can use different modes of operation. The single-tank dishwasher typically uses a batch-wise process, whereas the bigger multi-tank dishwashers operate in a continuous mode.

In single-tank machines, the rack containing the dishes, stays in a fixed position during the entire wash cycle. A circulation pump is connected with the wash tank and flushes the detergent solution through rotating spray arms over the dishware. This circulation typically takes about 1 minute. At the end of this period, rinse aid containing fresh hot water is sprayed over the dishware. Single-tank machines are usually equipped with a hood, which can usually be opened at any time during the wash cycle. An automatic valve then switches off the water circulation and protects the operator from being sprayed by hot and usually alkaline solutions. Since there are no enzyme containing products in the market for this type of dishwasher and the use of household automatic dishwasher detergents results in much lower enzyme concentrations in the tank, the AISE task force did not take these machines into account in the monitoring of airborne enzymes.

Figure 1 shows a typical multi-tank machine, containing different zones for washing, rinsing and drying the dishware. Hot, rinse aid containing fresh water is introduced into the rinse zone and cascades into one or more wash zone before it enters the drain. A conveyer belt transports the dishware in counter-current direction from (pre-)wash to drying zone.

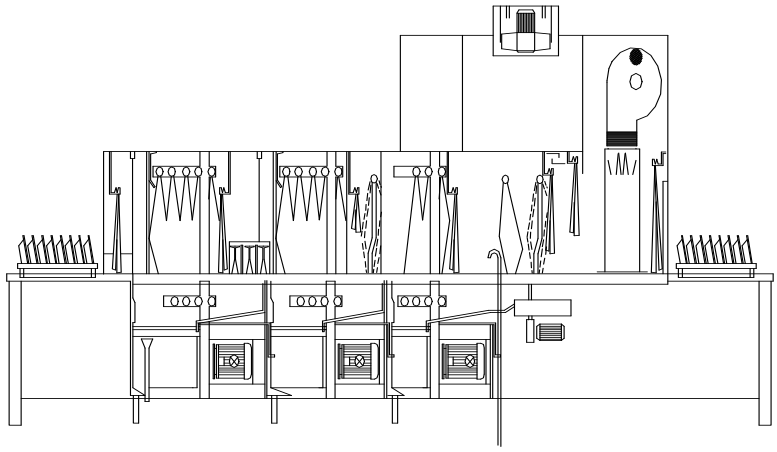

Figure 1: A typical multi-tank dishwasher (dishes enter the machine at the left)

The mechanical action in this type of dishwasher is usually very high. Flushes in the order of 1000 litres wash solution per hour over the dishware are not exceptional. This high mechanical action forms aerosols inside the machine. Since both the entrance and exit of the multi-tank machines are open during operation, aerosols may be released and inhaled by the kitchen personnel. Moreover, each tank can be accessed through a door at the side of the machine. For some machines, opening the door causes the machine to switch off immediately. For others, the machine needs to be switched off manually at a control panel. Upon opening a side-door, the machine operator can potentially be exposed to high levels of aerosol. The task force therefore decided to monitor enzyme exposure under normal use conditions as well as using a scenario involving opening one of the side-doors.

Multi-tank dishwashers exist with either internal air circulation or ventilation of air from inside the machine to outside the building. Internal air circulation improves the heat economy of the dishwash process. However, the effect of air circulation on the potential exposure to airborne enzymes was not studied by the task force. Only air was monitored around dishwashers with air ventilation.

1.4 Enzymes in Institutional Dishwashing

In the institutional dishwashing very high alkaline detergents are used for efficient cleaning. Even with the use of such detergents, starch layers can build up in a continuous process on the plates. These layers in the past had to be removed in a separate batch process at different time intervals.
By continuous dosage of amylase to the last tank, containing the fresh detergent solution, the removal of starch can be integrated into the process. With the dosage of amylases, a significant reduction of the alkalinity in the detergent is possible, without loss of cleaning performance.
The high alkaline detergent formulations are highly irritating in accidental skin contact. This corrosive effect could be reduced significantly by the introduction of less irritant enzymatic formulations.

2. Habits and Practices

Exposure during normal use of the enzymatic detergent is potentially possible by aerosols escaping the tunnel system at the entrance and the exit, which is closed by textile strip curtains.
Besides this potential exposure during normal use, staff can be exposed to aerosols coming from the inside of the multi-tank dishwashing machine by the following habits and practices, which are common in the institutional use of dishwashers.
The data are based on information from technical salesmen of DiverseyLever and Dr. Weigert (Table 1).

1. In regular use, the side door of a multi-tank dishwashing machine is opened one to four times a day to empty and clean the tanks. The cleaning time varies from one minute to one hour. Switch off the machine, wait for 10 - 90 seconds before opening the side door and 10 to 300 seconds before cleaning of the tanks begins.

2. In case of unexpected events like poor loading, oversized articles or conveyor problems, which under exceptional conditions may happen up to ten times a day, the side door is opened for approx. 5 to 180 seconds after waiting for approx. 0 to 60 seconds before opening the door respect. 5 to 60 seconds before handling begins.

Table 1: Habits and Practices with respect to door opening

reason for opening side door	**frequency (times a day)**	**duration (seconds)**	**time switch off - opening (seconds)**	**time switch off - handling (seconds)**
regular	1 - 4	60 - 3600	10 - 90	10 - 300
unexpected event	0 -10	5 - 180	0 - 60	5 - 60

3. Measurement Protocol Enzyme Aerosol Conditions for Institutional Dishwashing Machines

The task was to collect a data set of concentrations of airborne amylase found around institutional multi-tank machines. The protocol for measuring exposure was agreed by the task force and included normal use conditions and a few 'worst case' conditions (door opening, increased enzyme concentrations). Similar test were carried out at the Henkel-Ecolab site in Düsseldorf, Germany, and at the DiverseyLever site in Maarssen, the Netherlands. The analysis of enzyme on the filter was done by Novo Nordisk using an ELISA method.

3.1 Objectives

1. Confirm level of airborne enzyme encountered in normal use
2. Measure levels of airborne enzyme when side door is opened (misuse or worst case) reflecting the results from habits and practices. A number of 10 openings for a minute each with no delay was seen as the worst case.
3. Measure level of airborne enzyme when side door is opened after a delay (potential solution)

3.2 Safety Issues

For safety reasons the following points were observed in all sampling procedures: Protective facemasks (P3) and protective gloves were used. Personnel was subject to enzyme sensitisation monitoring program as directed by local company regulations. No unauthorised personnel was allowed to the room during experiments. After last experiment on last day the room was cleaned (not by contract personnel). Lab coats were washed after experiments.

<u>Check List before Sampling</u>

- Precheck that Teflon-filters do not become too wet during sampling.
- Check on product concentration in all tanks before start (alkalinity titration)
- Check on enzyme concentration in all tanks before start sampling (must be same)
- Check on enzyme concentration in all tanks after sampling complete
- Check enzyme consumptions (pumping rate of weight loss from container). No exact measurements, just control.

- Check temperature wash and rinse baths
- Check pressure of pumps before and after experiments
- Check on dosage rinse aid (o.g. time of pump + weight difference must be equivalent to 0.5 g/l)
  Temperature and humidity in room where experiments are done
- Establish the temperature and flow scheme in machine (must be typical for normal operation)
- Check air-flow at entrance, open door as measured by anemometer

3.3 Sampling Procedure

Apparatus: Air sampler Gravicon VC 25 was used in all experiments. Positioning on a tripod.

Sampling time: 30 min at 300 l/min (60 min at 0 and 10 mg/l Termamyl 300L)

Filters: Teflon filter Millipore FLAP 14250, 1 μm

Storing and dispatching of filters:
All handling of filters before and after sampling should be done by tweezers.
After sampling, filters are to be carefully folded and put into a plastic beaker with lid, volume approximately 200 ml.
During storage and shipment filters must be kept cold (4°C).
Filters were send by courier to: Novo Nordisk A/S, Bagsvaerd, Denmark
Novo Nordisk was informed prior to sending the filters.

Exact location of sampler
The sampler was located as close as possible to the position of the head of a potential worker handling the machine.

At the high concentration: measurement with the sampler positioned at 1. Exit and 2. Entrance
Exit: height of the sampler: 1.5 m, at the level of the exit, 40 cm from the machine.

Open door: in Düsseldorf (4 tank machine): 3rd door, 10 cm below door upper closure, shield air exhaust to prevent turbulences by sampler into the machine
in Maarssen (3 tank machine): last door

Conditions / Loading of the machine
- Enzyme dosed in tank 3 (or 4 in a four tank machine)
- Detergent dosed in tank 3 (or 4)
- Racks (6 plates / rack) with dishes to be continuously run through machine
- Speed: 1 m/min (approx.)

3.4 Experimental set-up:

Preparations:
Installation of equipment and testing with running dishwasher to determine maximum sampling rate in practice (nominal 300 l/m).
Check the wetness of Teflon-filters under practical sampling conditions. In case of filters become wet, GF/C glass microfiber could be used as an alternative.

Table 2: Experiments

No	Enzyme Concentration	Proposed Time	Remarks	Sampling time
1	0 mg/l Termamyl 300L	09:00-10:00	Normal use measurement	60 min
2	10 mg/l Termamyl 300L	10:30-11:30	Normal use measurement	60 min
3	50 mg/l Termamyl 300L	12:00-12:30	Normal use measurement	30 min
4	50 mg/l Termamyl 300L	12:30-13:30	Peak exposure 10 times 1min, 5 min rest	30 min
6	50 mg/l Termamyl 300L	13:30-14:30	as 4,(30 sec delay before opening door)	30 min
7	100 mg/l Termamyl 300L	15:00-15:30	Normal use measurement	30 min
8	200 mg/l Termamyl 300L	16:00-16:30	Normal use measurement at exit	30 min
9	200 mg/l Termamyl 300L	17:00-17:30	Normal use measurement at entrance	30 min

Experiment no. 5 was ommitted for not being practicable.

3.5 Filter Treatment

Storage and shipment
The filters did not become wet during sampling for up to 60 min. Filters were kept cold just after exposure and were send by courier within 1-2 days after air sampling.

Termamyl stability on filters
On one occasion the shipment took longer than expected (5 days) and a stability study therefore was set up to investigate if this delay was deleterious.

Termamyl was dissolved in the wash tank solution at different concentrations and spiked onto filters. Filters were obtained using a low volume air sampler operating at 25 l/min and suction was performed for 30 minutes.

The results indicated that stability of the Termamyl wash tank solution on the filters correlated to the amount of enzyme spiked on the filters. The immunochemically activity on the filters declined after 4 days and it therefore was recommended to have exposed filters analyzed within 3 days after air sampling. In addition it turned out that the ELISA signal on the filter was much more stable than in detergent solution. (Novo Nordisk Report: "Stability of Termamyl in wash solution and on spiked filters").

Elution
Exposed filters from the air sampler Gravicon VC 25 were eluted in 25 ml PBS 0.15M, DMF 10 V/V% for one hour.

3.6 ELISA Measurements

Reagents
The ELISA was carried out in accordance to a Novo Nordisk Standard Operation Procedure with the following antibody reagents and conjugate:

Catching antibody: Monospecific rabbit anti Termamyl, Novo Nordisk A/S
Antigen standard: Termamyl 19-1197 (122.5 KNU/g)
Detecting antibody: Guinea pig anti Termamyl, Novo Nordisk A/S
Conjugate: (HRP) Rabbit anti Guinea pig P0141, DAKO

Detection limit
The detection limit in the ELISA was 0.06 ng enzyme protein/ml. The recovery from the filters was >95% for amounts of > 25 ng/filter (Novo Nordisk report „Recovery of Termamyl from spiked filters“)

Interaction of detergent and rinse aid
Prior to analyzing exposed filters, interaction of liquid detergent or rinse aid was evaluated.
Termamyl standard was added to a solution containing either 0.5 g/l of rinse aid (Clinmat perfect, Henkel-Ecolab) or 5 g/l of detergent solution, containing 0.01% NaOH and 1% NTA.
It was shown that at dilution rates of more than 1:8 interaction of Clinmat Perfect and detergent in the assay could be neglected. (Novo Nordisk Report “Interaction of liquid detergent in Termamyl ELISA”).

4. Results

4.1 Exposure under Normal Use Conditions

According to the companies producing enzymatic detergents, enzyme dosages used in practice do not exceed 100 mg/l Termamyl 300 L. Under these conditions the exposure levels did not exceed a level of 0.1 ng/m^3 under normal use conditions.
Whereas there were detectable levels of enzyme on most of the filters from Düsseldorf, exposure levels did not exceed the detection limit of the ELISA method in the filters from Maarssen, unless a concentration of 200 mg/l Termamyl 300L was used (Table 3).
The differences in exposure levels can partly be explained by the time lag between sampling and measurement. Whereas the filters from Düsseldorf were measured the day following sampling, the filters from Maarssen, due to transport problems, were analysed four to seven days after sampling. Considering the stability and a standard deviation increasing with decreasing protein levels, the difference in detectable enzyme levels can be explained. For this reason the data above detection limit from the Maarssen filters were corrected for a 30% loss during a maximum storage time of 7 days (in Table 3).

4.2 Exposure under Worst Case Simulation Conditions

Under the conditions of worst case simulation with 50 mg/l Termamyl 300L all levels found were below 2 ng/m^3. A 30 sec waiting period between stop of the machine and door opening had only limited effect, reducing the level to values of 1.2 ng/m^3. The effect might be more significant at higher enzyme levels.
Under conditions of a 4fold increased concentration of 200 mg/l average levels of 0.6 to 2.8 ng/m^3 were found without door opening at exit and entrance respective.
Additional measurements done in Maarssen determined the enzyme amount by enzyme activity at a concentration of 100 mg/l Termamyl 300L. When the data based on activity were compared to the ELISA data the activity derived data seemed to be significantly higher. This observation was contradictionary to the expectations, as normally ELISA levels are expected to be higher than activity levels.
All results are compiled in Table 3.

4.3 Comparison of Protein Determination based on Activity with Protein Determination based on ELISA

The determination of exposure levels by ELISA is dependent on the integrity of the relevant protein epitopes (antibody recognition site), in the same way that activity measurement strongly depends on the integrity of the enzyme active site. The application of the enzyme in institutional dishwashing occurs under conditions of relatively high alkalinity and elevated temperatures. To exclude the

possibility of different stability of the relevant epitopes under the conditions of application another set of experiments was started in a special subgroup of the task force. Participants of this group were Margaret Richold, Dennis Leadbeater, Rene Crevel (Unilever), Dorte Ulrik, Annette Prentoe (Novo Nordisk) and Karl Maurer (Henkel).

Table 3: Results of the exposure measurement

Sampling Location/Day D - Düsseldorf M - Maarssen	**Sampling time (min)**	**theoretical conc. of Termamyl L 300 DX (mg/l)**	**Application method**	**concentration measured by loss of weight (mg/l)**	**concentration measured by activity analysis start conditions (mg/l)**	**Termamyl ng/filter***	**ng/m³ air**	**∅ Termamyl exposure in ng/m³ air**
D 1,2 M 1,3	60	0	normal use measurement, exit	- -	0.0 / 1.8 0.0 / 0.5	- / - <1 / <2	0.000 / 0.000 < 0.04 / < 0.08	**0.0** **< 0.06**
D 1,2 M 1,3	60	10	normal use measurement, exit	10.2 / 10.2 9 - 11	9 / 17 5 / 3	2 / - <1 / <2	0.089 / 0.000 < 0.08 / < 0.16	**0.05** **< 0.1**
D 1,2 M 2,2	30	50	normal use measurement, exit	49 / 49 47 - 53	46 / 50 36 / 38	2 / - < 1 / < 2	0.178 / 0.000 < 0.08 / < 0.16	**0.09** **< 0.1**
D 1,2 M 2,2	30	50	peak exposure 10 x 1 min/2 min. pause	49 / 52 47 - 53	22 / 14	6 / 8 < 1/ <2	1.600 /2.133 < 0.3 / < 0.5	**1.87** **< 0.4**
D 1,2 M 2,2	30	50	peak exposure 10 x 1 min/1.5 min. pause 30 sec delay before opening the door	49 / 52 47 -53	18 / 20	3 / 6 <1 / <2	0.800 / 1.600 < 0.3 / < 0.5	**1.20** **< 0.4**
D 1,2 M 1,3	30	100	normal use measurement, exit	95 / 95 95 - 105	63 / 43 45 / 53	- / 2 < 1 / <2	0.000 / 0.178 < 0.08 / < 0.16	**0.09** **< 0.1**
D 1,2 M 1,3	30	200	normal use measurement, exit	207 /193 190 - 210	167 / 168 71 / 74	3 / 9 8 / 5	0.267 / 0.800 < 0.6 / < 0.5	**0.53** **0.6**
D 1,2 M 1,3	30	200	normal use measurement, entrance	207 / 193 190 - 210	70 / 62	5 / 5 53 / 12	0.444 / 0.444 4.7 / 1.0	**0.44** **2.8**

* Maarssen values corrected for 30 % loss during transport/storage

In a first experiment activity and ELISA were both determined on identical filters in different laboratories the next day after sampling. Filters were sampled in Düsseldorf during 30 min sampling time, at the entrance of the machine and with a controlled concentration of 200 mg/l Termamyl 300L. These filters were transfered overnight to the laboratories of Unilever and Novo Nordisk and tested for activity and ELISA signals (Henkel-Ecolab Report „Validation of analytical methods“).
The antibodies and the ELISA protocols of Novo Nordisk and Unilever gave almost identical results, although the protein content in one case was based on Kjeldahl nitrogen determination and in the other case was calculated using the specific activity of the pure enzyme. In the same test it could be shown that when identical corrections for protein content were applied similar values were obtained on identical filters, when protein was determined by activity or by ELISA. The protein levels based on activity turned out to be somewhat lower than by ELISA, which was according to expectations. Standard deviation was generally high for all values, due to the low signals since the enzyme content was at the limit of detection. Standard deviation generally was higher for the activity determination. Under test conditions (200 mg/l Termamyl, normal use) the values given in Table 4 were received.

Table 4: Airborne Enzyme Concentrations as calculated on the basis of ELISA and Activity Determination

Protein Determination	**Airborne Enzyme Protein (ng/m^3)***
ELISA	0.9 - 2.2
Activity	0.6 - 1.5

* Unilever Data

These data are in good agreement with the data from the first exposure measurement.

4.4 Stability of ELISA Signal and Activity in Detergent Solution

At the same time the stability of ELISA signal and Activity were tested in detergent solution under application conditions at 60 °C. Both signals decreased with approximately the same velocity when followed over a 60 min. period (Novo Nordisk Report „Stability of Termamyl in Detergent heated to 60°C“). Experiments at DiverseyLever and Henkel gave the same results.
From these data it could be concluded that the stability of the antibody recognition site is not different from the stability of the amylase activity under application conditions. The low stability of the enzyme under application conditions explains the reduced actual enzyme concentrations in table 3, when measured by activity. Low stability could also give an explanation for the significant differences between measured enzyme concentrations in aerosols and the calculated enzyme concentrations based on determination of fluorescent dye in air samples, collected in preliminary experiments prior to the work done by the task force.

5. Recommendations

Based on habits and practices and the exposure data collected the following recommendations were established by the task force:
This survey has established that under normal use and mis-use situations, the emission of airborne enzyme is below targets set and agreed within the detergent industry. For detergent amylase (*Bacillus licheniformis* amylase) this target OEL is 5-7 ng/m^3. It is recommended that a similar standard is adopted for this application when using enzymes in MWW systems, and that it is never exceeded even under worst case operating conditions, taking into consideration the allergenic characteristics of the enzyme.

It is recognised that the exposures measured and reported in this review have been established for a multi tank MWW system with external air ventilation. No tests have been made on systems with internal air circulation. It is strongly recommended that whenever enzymatic formulations are considered for other Industrial Cleaning Applications, the airborne enzyme emissions are also established under conditions of normal use and under conditions of foreseeable nonintended use or misuse.

ELISA (Enzyme linked immunosorbent assay) is the recommended method of measuring airborne enzyme. The ELISA should be tested for accuracy and reliability, same as in the example given the ELISA was tested for specificity, detection limit, yield from filter, and the stability of the epitope.

The Soap and Detergent Industry associations in Europe and USA have published guidance documents (SDIA 1991 and SDA 1995) for enzyme users covering control of airborne emissions, monitoring systems and medical surveillance programs. It is recommended that similar information and guidance is provided to users of enzyme containing formulations in MWW systems.

6. References of Appendix 2

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9.3 Appenix 3

HERA Risk Assessment / Subtilisin toxicity data / - Tables -

1.0. Acute toxicity
1.1 Acute oral toxicity (5.2.1.1.1.)

1.1. Acute oral toxicity						
Product name	**Test species / Route**	**Guide-line**	**Value LD 50**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Purafect FN3 (prot.eng. var. 3) concentrate	rat / gavage	OECD 423	> 5000 mg/kg (> 0.5 g aep/kg bw)	2	Combined sexes	Genencor 1995 (1) 06.03.1995 / PH 402-GNC-001-94
Alcalase PPA 1180 concentrate	10 mice / gavage	OECD EEC	3.9 g/kg bw (79.5 AU/kg bw) 4.1 g/kg bw (84.0 AU/kg bw) LD50: 1.5 g aep/kg bw	1	slightly toxic	Novo Nordisk 1981 (1) Novozymes / Alcalase GL/111382a Study No. 0281, 1981-04-28
Inactivated Alcalase PPA 1180 concentrate	10 mice / gavage	OECD EEC	Activity after inactivation: 0.003 AU/g 13.5 g/kg bw ≈ 0.04 AU/kg bw LD50 inactivated: > 4.9 g aep/kg bw	1	slightly toxic	Novo Nordisk 1981 (2) Novozymes / Alcalase GL/111382a Study No. 3081, 1981-10-07
Alcalase PPA 1619 concentrate	5 rats / gavage	OECD EEC	1.8 g/kg bw ≈ 28.8 AU/kg bw LD50: 0.51 g aep/kg bw	1	moderately toxic	Novo Nordisk 1985 (1) Novozymes / Alcalase GL/111382a Study No. 4984a, Ph-850638, 1985-01-31

1.1. Acute oral toxicity						
Product name	**Test species / Route**	**Guideline**	**Value LD 50**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Inactivated Alcalase PPA 1619 concentrate	5 rats / gavage	OECD EEC	Activity after inactivation: 0.0037 AU/g LD 50 inactivated:: > 6.0 g/kg bw ≈ > 1.3 g aep/kg bw	1	slightly toxic	Novo Nordisk 1985 (2) Novozymes / Alcalase GL/111382a Study No. 6185b, Ph-853473, 1985-11-04
Alcalase PPA 1180 concentrate	10 rats / gavage	OECD EEC	2.3 g/kg bw ≈ 47.4 AU/kg bw LD50: 0.83 g aep/kg bw	1	moderately toxic	Novo Nordisk 1981 (2) Novozymes / Alcalase GL/111382a Study No. 0481, 1981-10-07
Savinase PPA 3352 concentrate	20 rats / gavage	OECD 401	> 1.5 g/kg bw ≈ 0.37 g aep/kg bw no effect level: < 1.5 g/kg bw ≈ 0.37 g aep/kg bw	1	Non toxic to rats	Novo Nordisk 1991 (1) Novozymes / Savinase / MTM / PNi / F-9201974 Study No. 91539, 1991-11-26 NiB/PNi, F-915067
Inactivated Savinase PPA 2153 PPA 2154 concentrate	rats / gavage	OECD 401	PPA 2153: 41.2 KNPU/g PPA 2154: 41.8 KNPU/g Dose: 5 respective 10 g/kg	1	No adverse effects	Novo Nordisk 1987 (1) Novozymes / Savinase / MTM / PNi / F-9201974/ Study No. 7687, 1987-10-14 ASB/PNi, F-871832
Esperase SP-72 AB 13 concentrate	rat / gavage		LD 50 values between 5.15 - 10.16 g/kg bw (0.15 - 0.29 g aep/kg bw)	2 Non GLP	Practically non toxic	Novo Nordisk 1970 (1) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / WARF Institute Inc., 1970-01-16, WARF NO. 9120651

1.1. Acute oral toxicity

Product name	Test species / Route	Guideline	Value LD 50	Klimisch reliability code	Remarks	Reference / Report Date / Report No
Esperase PEIK 133 concentrate	mice / gavage		LD 50 values of males: 7.3 g/kg bw (1.7 g aep/kg bw) LD 50 values of females: 6.8 g/kg bw (1.6 g aep/kg bw)	1 GLP	Practically non toxic	Novo Nordisk 1982 (1) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / Study No. 6481, NN, 1982-02-12, HaGA/PNi, Ph-820837
Esperase PEIK 137 concentrate	mice / gavage		LD 50 values of males: 10.3 g/kg bw (2.1 g aep/kg bw) LD 50 values of females: 6.4 g/kg bw (1.3 g aep/kg bw)	1 GLP	Practically non toxic	Novo Nordisk 1982 (2) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / Study No. 6381, NN, 1982-02-12, HaGA/PNi, Ph-820838
Savinase	Groups of 10 male and female rats / gavage		0, 1.48, 1.6, 2.0, 2.65, 3.65, 4.0, 4.44 g/kg Savinase powder in aqueous suspension LD50: 3 g/kg bw	4	No treatment related histo-pathological changes were observed.	NICNAS, 1993
Opticlean-M	Groups of 5 male and female rats / gavage		5 g/kg Opticlean-M granules, ground and suspended in 1 % aqueous methylcellulose	4	No deaths occurred	NICNAS, 1993
Subtilisin Carlsberg Subtilisin BPN	Groups of 10 male and female rats / gavage /		Enzyme content: each 5-15 %, diluted to 20 % aqueous solutions LD50: Subtilisin Carlsberg: 3,7 g/kg Subtilisin BPN: 9 - 10 g/kg	4		Griffith et al, 1969

1.2. Acute inhalation toxicity (5.2.1.1.2.)

1.2. Acute inhalation toxicity						
Product name	**Test species / Exposure duration**	**Guideline**	**Value LC 50**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Purafect FN2 (prot.eng. var. 2) concentrate	3 x 10 rats / 4 hours	OECD 403	> 2.8 mg/L ≈ > 0.28 mg aep/L	2	mortality: 3/10 at 2.8 mg/L, 3/10 at 2.1 mg/L, and 2/10 at 1.4 mg/L	Genencor 1991 (1) 19.07.1991 / 540-043
Savinase SP 88 FPF 312-320 concentrate	56 rats / 4 hours	Standard but not compliance to OECD No. 403	0.0177 mg aep/L	1	Death of rats as result of exposure was an acute pneumonia, typified by massive pulmonary ooedema together with lung congestion and haemorrhage, typical of the action and result of inhalation of a proteolytic enzyme powder.	Novo Nordisk 1978 (1) Novozymes / Savinase / MTM / PNi / F-9201974/ HRC No. NVO64/7818, 1978-01-24
Alcalase PPA 1618 concentrate	5 rat / 4 hours	OECD 403	LC50: 0.47 to 1.05 mg/L ≈ 6.12 to 21.5 AU/ m^3 ≈ 0.1 to 0.4 mg aep/L	1	Massive pulmonary ooedema together with congestion of the lungs and haemorrhage, typical of the action resulting from inhalation of a proteolytic enzyme concentrate.	Novo Nordisk 1993 (1) Novozymes / Alcalase GL/111382a
Savinase	Groups of 7 male and female rats / nose-only / 4 hours		0, 0.058, 0.070, 0.132, 0.157 mg/L Savinase powder (70 % of particles in the respirable range) LC50: 0.130 mg/L	2	Destruction of lung tissues (haemorrhage, congestion, ooedema) due to proteolytic activity. Death occurring within 24 hours at exposure greater than 0.070 mg/L.	NICNAS, 1993
Opticlean P concentrate	Groups of 7 male and female rats / nose-only / 4 hours		0, 0.108, 0.196, 0.298 mg/L Opticlean P powder (75 % of particles in the respirable range) LC50: 0.229 mg/L	2	Destruction of lung tissues (haemorrhage, congestion, ooedema) due to proteolytic activity. Death occurring within 24 hours at exposure greater than 0.196 µg/L.	NICNAS, 1993

1.2. Acute inhalation toxicity						
Product name	**Test species / Exposure duration**	**Guideline**	**Value LC 50**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase	Groups of 18 male rats, 9 male rabbits, 9 male Hartley guinea pigs / whole body exposure / 6 hours		No LC 50 Test conditions: 0.001 to 0.036 mg/L of a 12 % preparation of dry powder (0.1 to 4.4 µg/L enzyme)	4	No treatment-related deaths. No clinical signs in rats and rabbits. Pathological changes were only evident in lungs of guinea pigs (4.2 µg/L enzyme prep. (0.5 µg/L enzyme)), no changes in the lungs of rats and rabbits exposed to (1µg/L enzyme prep. (0.1 µg/L enzyme)). In all three species pathological changes had resolved by day 16.	Richards et al, 1975

2.0. Skin irritation (5.2.1.2.1.)

2.1. Skin irritation (animals)

2.1. Skin irritation (animals)						
Product name	Test species / Exposure duration / Conditions	Guideline	Concentrations / Scores	Klimisch reliability code	Remarks	Reference / Report Date / Report No
Purafect FN2 PR119, prot. eng. var. 2 concentrate	6 rabbits / 4 hours / occluded patch (intact & abraded) / with wash	OECD 404	Prim. Irritation index: 1.3 (≈ 0.05 g aep/dose)	2	mild irritation (intact skin)	Genencor 1991 (2) 18.01.1991 / 540-044
Purafect FN2 PR 119, prot. eng. var. 2 concentrate	6 rabbits / 4 hours / occluded patch (intact & abraded) / with wash	OECD 404	Prim. Irritation index: 0 (≈ 0.05 g aep/dose)	2	one animal very slight at 48 h (cleared by 72 h) at abraded, no irritation at intact skin	Genencor 1991 (3) 29.07.1991 / 540-053
Multifect P-3000 concentrate UF	12 rabbits / 4 hours / occluded patch (intact & abraded) / with wash	OECD 404	Prim. Irritation index: 0 (≈ 0.05 g aep/dose)	2	No irritation	Genencor 1991 (3) 29.07.1991 / 540-053

2.1. Skin irritation (animals)						
Product name	**Test species / Exposure duration / Conditions**	**Guideline**	**Concentrations / Scores**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date Report No**
Purafect FNA	6 rabbits / 4 hours / occluded patch (intact & abraded) / with wash	OECD 404	Prim. Irritation index: 0 (≈ 0.05 g aep/dose)	2	one animal very slight 24 h (cleared by 48 h) at abraded, no irritation at intact skin	Genencor 1991 (3) 29.07.1991 / 540-053
Purafect FN2 (PR119, prot. eng. var. 2) concentrate	rabbit / 4 hours	OECD 404	Prim. Irritation index: 4.75 (≈ 0.05 g aep/dose)	2	moderate irritation	Genencor 1994 (3) 11.10.1994 / 3342.5
Purafect FN3 (PR330, prot. eng. var. 3) concentrate	6 rabbits / 4 hours / semi-occlusive / with wash	OECD 404	Prim. Irritation index: 6.13 (≈ 0.05 g aep/dose)	2	severe irritation	Genencor 1994 (1) 11.10.1994 / 3342.3
Purafect FN3 (PR330, prot. eng. var. 3) concentrate	6 rabbits / 4 hours / semi-occlusive / with wash	OECD 404	Prim. Irritation index: 6.58 (≈ 0.05 g aep/dose)	2	severe irritation	Genencor 1994 (2) 11.10.1994 / 3342.4
Purafect FN3 (PR330, prot. eng. var. 3)	6 rabbits / 4 hours / semi-occlusive / with wash	OECD 404	Prim. Irritation index: 7.42 (≈ 0.05 g aep/dose)	2	severe irritation	Genencor 1994 (5) 21.10.1994 / 3342.8
Purafect OxP concentrate	6 rabbits / 4 hours / semi-occlusive / with wash	OECD 404	Prim. Irritation index: 3.38 (≈ 0.05 g aep/dose)	2	moderate irritation	Genencor 1994 (4) 11.10.1994 / 3342.6
Purafect OxP	6 rabbits / 4 hours / semi-occlusive / with wash	OECD 404	Prim. Irritation index: 5.67 (0.015 g aep/dose)	2	severe irritation	Genencor 1994 (6) 21.10.1994 / 3342.9
Savinase SP 88 FPF 312-320 concentrate	12 rabbits / intact and abraded skin / 0.5 ml applied under patch of gauze / occlusive / 24 hours		25 % w/v Savinase in water: 0.017 g aep/0.5 ml 25 % w/v Savinase in Sørensen´s borate buffer: 0.017 g aep/0.5 ml Prim. Irritation index: < 2	1	mild irritant	Novo Nordisk 1977 (2) Novozymes / Savinase / MTM / PNi / F-9201974/ LRS report No. 77/NTL25/178, 1977-05-27

2.1. Skin irritation (animals)						
Product name	**Test species / Exposure duration / Conditions**	**Guideline**	**Concentrations / Scores**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date Report No**
Esperase SP-72 AB13 concentrate	6 rabbits / patch test / abraded and intact skin / 24 hours		applied dose: 0.5 g (0.014 g aep/0.5 g) Prim. Irritation index Draize: 0	2 Non GLP	no irritation	Novo Nordisk 1970 (1) Novozymes / Esperase / TiH/PNi / F-9203233a / Edition 2 / WARF Institute Inc., 1970-01-16 WARF No. 9120651
Alcalase 2.5L liquid product	rabbits / occlusive / 4 hours / with wash:	Code of Federal Regulations, title 16 § 1500.41	Erythema / ooedema: 1.4/4 and 0.1/4 Prim. Irritation index Draize: 1.3	1	Alcalase 2.5L is slightly irritant to skin.	Novo Nordisk 1983 (1) Novozymes / Alcalase GL/111382a / Study No. 8082, Ph-830628, 1983-01-06
Savinase	Groups of 6 rabbits / intact and abraded skin / / occlusive / 24 hours		0.5 ml of 5 % aqueous solutions at pH 7.0 und 9.1	2	Mild to moderate erythema at 24 hours irrespective of treatment, 2 animals very slight ooedema	NICNAS, 1993
Opticlean-M	3 rabbits / semi-occlusive / 4 hours / test sides washed / observation for 4 days		500 mg granulated enzyme preparation, moistened with water	2	Slight erythema in all rabbits on day 1 only	NICNAS, 1993
Subtilisin	Rabbits / occlusive / 24 hours		0.5 ml of 1 % aqueous solutions detergent with and without Subtilisin (0.0001-0.00045 % ep) Prim. Irritation score without enzyme: 1.3 Prim. Irritation score with enzyme: 3.7 or 4.8	2	mild irritancy	Griffith et al, 1969

2.1. Skin irritation (animals)						
Product name	**Test species / Exposure duration / Conditions**	**Guideline**	**Concentrations / Scores**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Savinase 8.0L PPA 1186 Liquid product	6 rabbits / abraded and intact skin	Code of Federal Regulations	Undiluted Savinase 8.0L / application dose: 0.5 ml (0.0114 g aep/dose) Prim. Irritation index Draize: 1.1	1	mild irritant	Novo Nordisk 1981 (12) Novozymes / Savinase / MTM / PNi / F-9201974/ Study No. 1681, 1981-11-20, HaGA/PNi
Savinase 8.0L PPA 1186 Liquid product	12 rabbits / abraded and intact skin / readings 24 and 72 hours after application	Code of Federal Regulations	1 % (w/w) Savinase 8.0L diluted in liquid base detergent application dose: 0.5 ml (0.0001 g aep/dose) undiluted liquid base dose: 0.5 ml Prim. Irritation index Draize of Savinase in liquid base detergent: 2.6 Prim. Irritation index Draize of undiluted liquid base detergent: 2.9	1	moderate irritant	Novo Nordisk 1981 (13) Novozymes / Savinase / MTM / PNi / F-9201974/ Study No. 1681, 1981-11-20 HaGA/PNi
Esperase 8.0L PPA 1185 Liquid product	6 rabbits / intact and abraded skin / 24 hours	Code of Fed. Regulations 1979	0.5 ml (0.017 g aep/0.5 ml) Prim. Irritation score Draize: 1.7 Score 2 for erythema on both abraded and intact sides in all animals Score 2-3 for oedema in 2/6 animals	1 Non GLP	mild irritant	Novo Nordisk 1981 (9) Novozymes / Esperase / TiH/PNi / F-9203233a / Edition 2 / NN, 1981-07-06, HaGA/PNi Study No. 1681
Esperase 8.0L PPA 1185 in detergent and pure detergent	12 rabbits / intact and abraded skin / 24 hours	Code of Federal Regulations 1979	1 % w/w Esperase dissolved in detergent: 0.5 ml (1.8×10^{-4} g aep/0.5 ml) Prim. Irritation score: 2.6 Concentrated detergent without Esperase: Prim. Irritation score: 2.9	1 Non GLP	both moderate irritants Addition of 1 % Esperase 8.0L to the detergent did not influence the irritation potential significantly.	Novo Nordisk 1981 (10) Novozymes / Esperase / TiH/PNi / F-9203233a / Edition 2 / NN, 1981-11-20, HaGA/PNi Study No. 1681

2.2. Skin irritation (humans)

2.2. Skin irritation (humans)						
Product name	**Test species / Conditions / Exposure duration**	**Guide-line**	**Concentrations / Scores**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase MIF 415-424 concentrate	10 human volunteers / normal skin and criss-cross scarified skin / occlusive Duhring chamber / 100 µl for 3 consecutive days		Alcalase concentrations in water: - 0.07 % (0.004 AU/mL) - 0.13 % - 0.33 % (0.02 AU/mL) - 0.66 % (0.04 AU/mL)	1	Irritation (24 h) with concentrations higher than 0.07 %, sharp dermatitis (24 h) with 0.66 % Reaction is more intense on scarified skin; appl. of 0.33 % (0.02 AU/ml) can evoke a very sharp dermatitis in 24 hours.	Novo Nordisk 1980 (1) Novozymes / Alcalase GL/111382a / IVY Research protocol: 4335/02. 1980-04-25
Alcalase concentrate	ID50 determination of 4 enzymes / 10 adults / patch test / 24 hours		0.05 ml on 1 cm² patch with range of concentrations (0.05, 0.1, 0.25, 0.5, 0.75% w/v) ID50: ~ 0.25 % w/v (~ 0.06 % aep) NOEL: ~ 0.05 % w/v (~ 0.013% aep, ~ 6.3 µg aep/cm^2)	2		Novo Nordisk 1978 (3) Memo of 2004-09-09, 2004-35622-02, NiB/DSc IVY Research Lab. /A. M. Kligman 1978
Esperase SP-72 AB13 concentrate	Kligman: human volunteers / occlusive patch test / 24 - 48 hours		1.2 % solution ($3.4x10^{-4}$ g aep/ml)	2	Primary irritant due to the proteolytic activity. 1.2 % solution caused primary irritancy to 50 % of the individuals tested. Corresponding value for crystalline Alcalase was 3 %.	Novo Nordisk 1970 (1) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / WARF Institute Inc. , 1970-01-16 WARF No. 9120651

2.2. Skin irritation (humans)						
Product name	**Test species / Conditions / Exposure duration**	**Guide-line**	**Concentrations / Scores**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Esperase SP-72 AB13 concentrate	human volunteers / occlusive patch test / 24 and 72 hours		Aqueous solutions (24 hours) : 5 % ($1.4x10^{-3}$ g aep/ml) 10 % ($2.8x10^{-3}$ g aep/ml) Paste (24 hours): 90 % (0.026 g aep/g) Aqueous solutions (72 hours): 0.2 % ($5.7x10^{-5}$ g aep/ml)	2	No irritating effects to human skin in high concentrations (0.026 g aep/g)	Novo Nordisk 1970 (1) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 /
Esperase	ID50 determination on 10 adults / patch test / 24 hours		range of concentrations: 0.25, 0.5, 1.0, 2.0, 4.0, 5.0 % w/v ID50: ~ 1.2 % w/v (~ 0.03 % aep) NOEL: 0.19 % w/v (~ 0.01 % aep, ~ 2.7 µg aep/cm^2)	2		Novo Nordisk 1970 (2) Memo of 2004-09-09, 2004-35622-02, NiB/DSc Hospital of the University of Pennsylvania/A. M. Kligman 1970
Savinase SP 88 FPF 312-320 concentrate	Kligman and Wooding: 10 human volunteers / patch test		1 % in water / 0.25ml/pad (0.0003 g aep/0.25 ml) = 0.12% aep	1	No danger of primary skin irritation in normal intended use in humans	Novo Nordisk 1978 (4) Novozymes / Savinase / MTM / PNi / F-9201974/ IVY No. 3708, 1978-03-20 Kligman
Maxatase	Groups of between 20 to 100 panelists / normal skin / occlusive 1. application once, observation after 48 hours 2. 24 hours between each appl., scored 24 hours after last patch removal		1. Enzyme prep. at pH 5 or 8.5 0.25 – 20 % applied once 2. 5 x 24 hours application of 0.25-5 % aqueous solutions at pH 8.5	3	No effects could be attributed to Maxatase.	Valer, 1975 A

2.2. Skin irritation (humans)						
Product name	**Test species / Conditions / Exposure duration**	**Guide-line**	**Concentrations / Scores**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Maxatase	human volunteers / intact and pre-treated skin / occlusive 1. 3 x 24 hours application, skin artificially irritated by a prior 24 hour appl. of diluted NaOH or dilute carbolic acid 2. single 72 hour appl. to intact skin (A) and 24 hours to tape stripped skin (B)		1. 0.25-5 % aqueous solutions at pH 8.5 2. 0.25-5 % in a 70 % DMSO solution of Maxatase (A) and 0.25-5 % aqueous solutions (B)	3	1. no irritation was apparent 2. dilute aqueous solutions of Maxatase are not irritant to intact skin but may be irritant to damaged skin. (Concentrations of 2 % or more produced irritant reactions in 1-4/40 individuals.)	Valer, 1975 A
Subtilisin BPN Subtilisin Carlsberg	Consumer exposure studies: patch testing, arm washing, hand immersion, home use studies a) single application to back skin b) single or triple application every other day		a) 0.2 % solution of enzyme preparation (0.01- 0.03 % ep) b) detergent formulations (0.00009 to 0072 % ep)	3	a) No erythema observed b) no or faint to moderate erythema The detergent containing the least enzyme appeared to produce the strongest skin reactions.	Griffith et al, 1969
Alcalase crystalline	ID50 determination on 10 adults / patch test / 24 hours		ID50: ~ 1.2 % w/v (~ 0.42 % aep) NOEL: 0.19 % w/v (~ 0.07 % aep, ~ 33.4 µg aep/cm^2)	2		Novo Nordisk 1970 (2) Memo of 2004-09-09, 2004-35622-02, NiB/DSc Hospital of the University of Pennsylvania/A. M. Kligman 1970
Alcalase crude	ID50 determination on 10 adults / patch test / 24 hours Activity: 1.5 AU/g (2.6 % aep)		ID50: ~ 3.0 % w/v (~ 0.08 % aep) NOEL: 0.45 % w/v (~ 0.01 % aep, ~ 5.8 µg aep/cm^2)	2		Novo Nordisk 1970 (2) Memo of 2004-09-09, 2004-35622-02, NiB/DSc Hospital of the University of Pennsylvania/A. M. Kligman 1970
Savinase 8.0L	Consumer exposure test / 29 human volunteers / 10 min / hand wash on 4 consecutive days		Savinase 0.5 % w/v in liquid detergent	1	No evidence of an overall deterioration in skin condition was observed.	Novo Nordisk 1980 (3) Memo of 2004-09-09, 2004-35622-02, NiB/DSc Inveresk, 1980

2.2. Skin irritation (humans)						
Product name	**Test species / Conditions / Exposure duration**	**Guide-line**	**Concentrations / Scores**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Savinase concentrate	10 adults / occlusive / once daily for 10 days to volar forearm		1 % w/v Savinase ID50 resp. NOEL: > 1% w/v (~ 0.14 % aep, ~ 68 µg aep/cm^2)	2	No irritation observed.	Novo Nordisk 1978 (3) Memo of 2004-09-09, 2004-35622-02, NiB/DSc IVY Research Lab. /A. M. Kligman 1978
Laundry detergent with and without enzyme	25 adult patients with atopic dermatitis / three phased randomised double-blind cross-over experiment		1. phase: normal laundry detergent 2. phase: trial detergent with or witout added enzyme 3. phase: opposite trial detergent		No significant irritant capacity detected in atopic dermatitis patients.	Andersen et al, 1998

3.0. Eye irritation
3.1. Cornea corrosion
3.2. Eye irritation (5.2.1.2.2.)

3.1. Cornea Corrosion						
Product name	**Method**	**Test species / Exposure duration / Route**	**Guideline**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
BLAP S (F 49) SAT 950215 granulate	Bovine cornea test	4 bovine eyes from freshly slaughtered animals / 30 sec 2.15 (% w/w) 5000 HPE/ml 8.60 (% w/w) 20000 HPE /ml application on intact cornea		2	No corrosive effect up to 8.6 % (w/w).	Henkel 1995 (1) R0500069 Scantox Germany 23.06.1995 / Lab. Nr. 0110

3.1. Cornea Corrosion						
Product name	**Method**	**Test species / Exposure duration / Route**	**Guideline**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
BLAP S (BLAP S 200) SAT 940899 granulate	Bovine cornea test	4 bovine eyes from freshly slaughtered animals / 30 sec 2.63 (% w/w) 5000 HPE/ml 10.52 (% w/w) 20000 HPE /ml application on intact cornea		2	No corrosive effect up to 10.52 % (w/w).	Henkel 1995 (2) R00500068 Scantox Germany 23.06.1995 / Lab. Nr. 0111

3.2. Eye irritation (5.2.1.2.2.)

3.2. Eye irritation						
Product name	**Method / Test species / Exposure**	**Guide-line**	**Concentration / Score**	**Klimisch reliability code**	*Remarks*	**Reference / Report Date / Report No**
Alcalase PPA 1631 granulate	Modified Draize / 6 rabbits: 3 animals rinse after 4 sec, 3 animals without rinse / reading after 24, 48, and 72 hours and 7 days		Dose: 3 mg granulate cornea: 0/4 iris: 0/2 redness: 0.89/3 swelling: 0.1/4	1	3 mg Alcalase 2.0T-Granulate Scores acc. to Draize: - rinse: 3.33, + rinse: 0.66 Reactions up to diffuse redness with slight swelling of the conjunctiva. Peak level at reading 24 hours after the treatment. No reactions after 7 days. Reaction of the rinsed eye significantly less severe, one animal in one of the tests showed a slight reaction.	Novo Nordisk 1984 (1) Novozymes / Alcalase GL/111382a / Study No. 5084a, Ph-843685, 1984-11-26
Alcalase PPA 1630 granulate	Modified Draize / 6 rabbits: 3 animals rinse after 4 sec, 3 animals without rinse / reading after 24, 48, and 72 hours and 7 days		Dose: 3 mg granulate cornea: 0/4 iris: 0/2 redness: 0.33/3 swelling: 0/4	2	3 mg Alcalase 2.0T-Granulate Scores acc. to Draize: - rinse: 2.67, + rinse: 0	Novo Nordisk 1984 (2) Novozymes / Alcalase GL/111382a / Study No. 5084a, Ph-843686, 1984-11-26

3.2. Eye irritation						
Product name	**Method / Test species / Exposure**	**Guideline**	**Concentration / Score**	**Klimisch reliability code**	***Remarks***	**Reference / Report Date / Report No**
Esperase SP-72 AB13 concentrate	6 rabbits / no rinse		Enzyme powder: 0.1 g ($2.8x10^{-3}$ g aep/0.1 g) redness: 1 chemosis and discharge in the conjunctivae for 5/6 rabbits	2 Non GLP	Mildly irritating and relatively short duration of the irritation (negative after 48 hours)	Novo Nordisk 1970 (1) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / WARF Institute Inc. , 1970-01-16 WARF No. 9120651
Esperase EK 23 concentrate	8 rabbits / instillation into the conjunctival sac 5 animals washed after 5 min / 3 animals washed after 24 hours	US Federal Register test 1972	0.1 ml of 5 % w/v in water (0.001 g aep/0.1 ml) conjunctival redness: 1/5, score: 1	2 Non GLP	Negative with 0.001 g aep/0.1 ml	Novo Nordisk 1978 (5) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / NN, 1978-12-13, RKH/PNi.
Savinase	8 rabbit eyes: 5 rabbits washed 5 min after application / 3 rabbits washed 24 hours after application	OECD	5 % aqueous solutions of powdered enzyme preparation application: 0.1 ml/eye	2	4 of 5 eyes washed <u>after 5 min</u>: showed slight conjunctival chemosis and redness and 2 also showed corneal opacity. Reactions not persist. Eyes washed <u>after 24 hours</u>: 1. eye: slight redness after 1 h, no effect after 24 h 2. eye: signs of corneal opacity and conjunctival redness up to 48 h 3. eye: signs of corneal opacity up to 24 h, conjunctival ooedema up to 72 h and conjunctival redness up to 7 days post application	NICNAS, 1993

3.2. Eye irritation						
Product name	**Method / Test species / Exposure**	**Guideline**	**Concentration / Score**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Subtilisin Carlsberg Subtilisin BPN	3 rabbits		powdered enzyme preparation (containing 5-15 % ep) application: 3 mg/eye	2	Moderate conjunctival irritation and transient corneal haziness, which cleared in 2-4 days, results being similar with both enzyme preparations. 1 and 10% aqueous solutions were of comparable irritancy to the powdered prep.. These results show that Subtilisin can cause eye irritation.	Griffith et al , 1969
Esperase 8.0L PA 1185 Liquid product	Screening test: 3 rabbits / 1 hour Main test: 6 rabbits	Code of Federal Regulations 1979	Screening: 0.1 ml (0.0035 g aep/0.1 ml) Main test: 5 % w/w Esperase 8.0L (0.18 mg aep/0.1 ml)	2 Non GLP	Concentrated Esperase 8.0L was regarded strongly irritant in a screening test. Esperase 8.0L 5% w/w in water was regarded irritant to eye.	Novo Nordisk 1981 (11) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / NN, 1981-07-07, HaGA/PNi Study No. 1681

4.0. Sensitisation (5.2.1.3.)
4.1. Skin sensitisation (5.2.1.3.1.)
4.1.1. Skin sensitisation (animals)

4.1.1. Skin sensitisation (animals)

Product name	Method / Test species / Exposure duration	Substance concentrations / Route	Guideline	Klimisch reliability code	Remarks	Reference / Report Date / Report No
BLAP 70 LI 128 granulate	Modified method acc. Buehler: Epicutanous occlusive application: Induction and threefold challenge phase / 10 guinea pigs / 6 hours	topical application of 0.1 ml of 0.00012 g aep/0.1 ml for induction and challenge phase	OECD No. 406	1	No sensitisation 0 / 10	Henkel 1990 (1) 13.03.1990 / TBD891007
BLAP S granutate	Modified method acc. Buehler: Epicutanous occlusive application: Induction and twofold challenge phase / 20 guinea pigs / 6 hours	topical application of 0.5 ml of 0.42 % Subtilisin Charge 29 rechallenge with 0.05 ml of 0.21 %, 0.42 % and 0.63 %	OECD No. 406	1	0 / 20: No contact hypersensitivity No sensitisation 3 / 20: skin reaction only in the first challenge	Henkel 1995 (3) 28.07.1995 / R9500979
Esperase SP-72 AB13 concentrate	Landsteiner guinea pig sensitivity test 10 guinea pigs / intradermal injections	0.01 % solution ($2.8x10^{-6}$ g aep/ml) every other day to a total of ten injections (0.5-1.0 ml/animal) and one single injection after 14 days		2 Non GLP	0.01 % showed no evidence of skin sensitising activity	Novo Nordisk 1970 (1) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / WARF Institute Inc., 1970-01-16 WARF No. 9120651

4.1.1. Skin sensitisation (animals)						
Product name	**Method / Test species / Exposure duration**	**Substance concentrations / Route**	**Guideline**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Opticlean-M	Buehler method / 10 guinea pigs / induction: topical applications for 6 hours/day, 3 days/week for 3 weeks	Induction phase: 10 % aqueous solution of sublilisin preparation Challenged phase (2 weeks later): 5 % aqueous solution at different skin sides as induction		2	Induction phase: irritant in 10 % concentration. Challenged phase: 10/10 showed well-defined erythema up to 72 hours most animals also showed ooedema. Only slight localised erythema was apparent on skin of 3/10 control animals challenged with Opticlean-M. Question whether skin reaction is allergic or irritant!	NICNAS, 1993

4.1.2. Skin sensitisation (humans)

4.1.2. Skin sensitisation (humans)						
Product name	**Method / Test species / Exposure duration**	**Substance concentrations / Route**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase 2.5L SP 255 PPA 1318-1	Human repeat insult patch test based on the Kligman human maximization method / 26 human volunteers / 48 hours	induction phase: 0.25 % in water challenge phase: 0.25, 0.10, 0.025, and 0.010 % w/v in water - 0.5 ml/patch - 5 times		1	Moderate degree of skin irritation with 0.25 % w/v in water but do not lead to skin sensitisation.	Novo Nordisk 1982 (5) Novozymes / Alcalase GL/111382a ISC Project Nos. 0033 and 0034, Report No. 0034, Ph-823772, November 1982

4.1.2. Skin sensitisation (humans)						
Product name	**Method / Test species / Exposure duration**	**Substance concentrations / Route**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Savinase SP 88 FPF 312-320 concentrate	Human patch test based on the Kligman human maximization method / 2 phases / 25 human volunteers	Savinase in 10 % petrolatum / dose: 0.3 g (0.0410 g aep/dose)		1	No danger of contact sensitisation in normal intended use in humans.	Novo Nordisk 1978 (6) Novozymes / Savinase / MTM / PNi / F-9201974 IVY No: 3708, 1978-06-06 Kligman
Esperase SP-72 AB13 concentrate	Delayed sensitisation Human repeat occlusive patch test according Kligman Maximisation method / 48 hours / 25 human volunteers	2.5 % aqueous solution ($7.1x10^{-4}$ g aep/ml) in five continuous applications of 48 hours duration two week later new side: 0.01 % aqueous solution ($2.8x10^{-6}$ g aep/ml)		2	Very low potential to induce contact allergy.	Novo Nordisk 1970 (1) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / WARF Institute Inc., 16.01.1970, WARF No. 9120651
Esperase SP-72 AB13 concentrate	Delayed contact sensitisation Human volunteers / occlusive bandage / 3 times a week for 3 weeks / 24 hours 47 human volunteers	2 % aqueous solution ($5.7x10^{-4}$ g aep/ml)		2	No evidence of SP 72 inducing contact dermatitis.	Novo Nordisk 1970 (1) Novozymes / Esperase / TiH/PNi / F-9203233° / Edition 2 WARF Institute Inc. , 27.08.1970, WARF No. 9120651
Alcalase 2.5L liquid	Kligman human maximization method / 30 adults / 5 induction patches of 48 hours at a concentration of 0.25 % w/v	challenge two weeks later with four concentrations: 0.010, 0.025, 0.10, 0.25 %w/v		2	Can cause moderate human skin irritation at concentration of 0.25 % w/v (~ 0.012 % aep = 15 µg aep/cm^2) upon repeated contact but does not lead to skin sensitisation.	Novo Nordisk 1982 (6) Memo of 2004-09-09, 2004-35622-02, NiB/DSc Ian Smith Consultancy, 1982

4.1.2. Skin sensitisation (humans)						
Product name	**Method / Test species / Exposure duration**	**Substance concentrations / Route**	**Guideline**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Savinase 8.0L (SP 240) PPA 1186 liquid product and concentrate PBCT 005	Human patch test based on the Kligman human maximization method / 3 phases / 22 human volunteers	Savinase 8.0L in water (challenge phase): 0.1 % (0.000023 g aep/g) 0.2 % (0.00005 g aep/g) 0.3 % (0.00007 g aep/g) 0.5 % (0.0001 g aep/g) Savinase concentrate PBCT 005 in water: 0.0036 % v/v (0.0069 g aep/g) Every applied dose: 0.25 ml		1	Did not elicit skin sensitisation though it is an irritant to skin.	Novo Nordisk 1981 (15) Novozymes / Savinase / MTM / PNi / F-9201974/ IRI Project No. 117791, report no. 2185, December 1981
Esperase SP 241 PPA 1185 liquid	Human repeat insult patch test according Kligman Maximisation method / 2 phases / 24 human volunteers	Esperase Liquid SP 241 PPA 1185: 0.5, 1.0, 1.5, and 2 % v/v (3.5×10^{-4} g aep/0.5 ml) gave scores of severe irritation reduced concentrations: 0.1, 0.2, 0.3, and 0.5 % v/v (8.8×10^{-5} g aep/0.5 ml)		1	Esperase Liquid did not elicit any skin sensitisation	Novo Nordisk 1981 (14) Novozymes / Esperase / TiH/PNi / F-9203233A/ Edition 2 IRI project No. 117388, report No. 2184, December 1981, Ph-822980

4.1.2. Skin sensitisation (humans)						
Product name	**Method / Test species / Exposure duration**	**Substance concentrations / Route**	**Guideline**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Esperase Batch PPA 1978-10-11 slurry and Esperase slurry / liquid detergent mixture	Human repeat insult patch test according Kligman Maximisation method / 2 phases / 25 human volunteers with slurry 23 human volunteers with mixture	Esperase slurry: Applied doses induction phase: 0.25 ml of 0.1 % w/v ($8.5x10^{-6}$ g aep/0.25 ml) Applied doses challenge phase: 0.1, 0.04, 0.02 and 0.01 % w/v Esperase slurry (0.5 % w/v) / liquid detergent mixture: Applied doses induction phase: 0.25 ml of 0.4 % v/v ($1.7x10^{-7}$ g aep/0.25 ml) Applied doses challenge phase: 0.4, 0.16, 0.08 and 0.04 % v/v		1	Esperase liquid and the Esperase/detergent mixture, although skin irritants, did not elicit skin sensitisation.	Novo Nordisk 1979 (1) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 / IRI project No. 112243, report No. 1298, April 1979
Maxatase	repeat insult patch tests / groups of 100 human volunteers / 15 occlusive applications / 5 days per week for 3 weeks / intact skin	1. Maxatase aqueous solution 0.25 - 5 % (pH 8.5), 24 hours 2. Challenge with 0.1 - 1% aqueous solutions (pH 8.5) Challenge patches were placed at the induction site and onto previously unexposed skin and held in contact for 48-hours.		2	Negative results	Valer, 1975 B

4.1.2. Skin sensitisation (humans)						
Product name	**Method / Test species / Exposure duration**	**Substance concentrations / Route**	**Guideline**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Maxatase	groups of 100 human volunteers with occupational contact dermatitis: a) attributed to enzyme containing detergents b) with occupational contact dermatitis due to agents other than biological detergents with no contact with enzymes 5 x 24 hours / occlusive applications (same protocol as above)	Maxatase aqueous solution 0.25 - 5 % (pH 8.5) as an induction of reference group		2	No skin reactions were reported in any group at challenge.	Valer, 1975 B
Maxatase	Group of 380 housewives used enzyme containing detergents regularly at least 3 month (3 x 24 hour induction applications or a single 48 hour application)	0.1 - 1% Maxatase aqueous solution plus 0.25, 0.75 or 1 % solution of a biological detergent for induction and challenge		2	No skin reactions were observed.	Valer, 1975 B
Detergents containing Subtilisin	repeat insult patch tests / 1478 human volunteers / 15 occlusive applications / 5 days per week for 3 weeks / intact skin / 24 hours	9 induction application of 0.25-1% aqueous solutions each for 24 hours over 3 weeks, test solutions: 0.00025 to 0.007 % ep Challenge was carried out 10 to 14 days after the induction phase at the induction sites and previously unexposed skin.		2	No signs of skin sensitisation at challenge. Due to the very low concentr. of enzyme tested no conclusions can be drawn about the skin sensitisation potential of the more concentrated enzyme.	Griffith et al, 1969

4.1.2. Skin sensitisation (humans)						
Product name	**Method / Test species / Exposure duration**	**Substance concentrations / Route**	**Guideline**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase	a) 18 workers in Subtilisin production / patch test / 48 hours b) 61 workers / patch test	a) 0.5, 0.1 and 0.01 % aqueous solution b) 0.01 % aqueous solution			a) 4 controls and 2 workers showed erythema (typical of a mild reaction to primary irritants) to the 0.5% solution and one worker reacted to the 0.1% solution. b) No positive reactions in either workers or controls.	Zachariae et al, 1973
Subtilisin containing detergent	13 people with severe hand dermatitis / occlusive patch test	0.25 and 0,5 % solution			4/12 positive with 0.5 % solution but negative at 0.25 %, detergent without enzyme: negative. No sufficient time had elapsed between this test and healing of the dermatitis to rule out the possibility of a false positive result. No conclusions for sensitisation from this study.	Jensen 1970
Subtilisin	12 "home helps" with intense irritation, blistering and ooedema of the hands after using detergents containing Subtilisin enzymes	0.1% aqueous solution of enzyme-containing detergent			6/12 reacted on first use of the detergent, 12/12 negative reactions on patch testing with a 0.1% aqueous solution Reactions occurred after a single or very few exposures, strongly indication that the dermatitis was irritant in origin, and the contribution made by the detergent itself as opposed to Subtilisin is uncertain.	Ducksbury and Dave, 1970

4.1.2. Skin sensitisation (humans)						
Product name	**Method / Test species / Exposure duration**	**Substance concentrations / Route**	**Guideline**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase	a) 80 consumers with dermatoses (clothes washed in enzyme-containing detergents) / patch test / 48 hour b) 60 consumers / 48 hours	a) 5 % Alcalase in petrolatum b) 1 % Alcalase aqueous solution			negative results in each case No evidence for skin sensitisation potential for Subtilisin.	White et al, 1985

4.2. Inhalation sensitisation (5.2.1.3.2.)

4.2.1. Inhalation sensitisation (animals)

4.2.2. Inhalation sensitisation (humans)

4.2.1. Inhalation sensitisation (animals)						
Product name	**Test species / route / exposure duration**	**Substance concentrations**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Subtilo-pepti-dase A	Groups of 8 guinea pigs / inhalation exposure	induction phase (15 min/day for 5 days): aqueous aerosols: 0.0083, 0.041, 0.15, 0.39, 1.9, or 15 µg/L challenged phase (20 min): 1.9 µg/L delayed challenge with group of induced (15 µg/L) guinea pigs challenged on day 17		3	36% increase in respiratory rate within the first hour post-challenge as an immediate positive reaction, similar increase after this time as a positive late reaction. No reactions at induction. On challenge, no immediate responses at the two lowest doses. Immediate and a few late onset reactions observed in animals induced with 0.15 µg/L or more. Given the lack of information on respiratory effects seen during induction, it is not possible to determine if the reactions are allergic or due to irritation.	Thorne et al , 1986
Subtilo-pepti-dase A (same study as above)	a) 12 guinea pigs / inhalation exposure b) 25 guinea pigs / inhalation exposure / 6 hours/day for 5 days/week for 11 weeks	a) induction (20 min once) with 1.9 µg/L, challenged 7 days later with 1.9 µg/L b) 0.68 ng/L for 11 weeks followed by 1.5 ng/L for 6 weeks, 5 positive control animals "hyperimmunized" by inhalation, intra-peritoneal and intradermal exposures were included All animals were challenged 17 to 22 days later with 1.9 µg/L for 20 minutes.		3	a) The mean increase in respiratory rate observed following the first exposure was 14.5% (range 0 - 33%). At challenge, 2 guinea pigs showed immediate and one a late positive reaction. b) None of the guinea pigs given the prolonged induction regime, and no negative control animal responded, compared with 4 of the 5 "hyperimmunized" animals. These results suggest the existence of a threshold for the induction of a state of increased responsiveness.	Thorne et al , 1986
Subtilisin	Serological test / guinea pigs	0.0083 - 1.9 µg/L		3	Dose-related increase in Subtilisin-specific antibodies, primarily IgM but also IgG observed in sera. IgG is the major antibody involved in immediate antibody mediated respiratory reactions in the guinea pig.	Hillebrand et al, 1987
Alcalase	guinea pig intra-tracheal test (GPIT) with an inhalation exposure regime			2	Both exposure routes produced similar responses. As this was a method-development study it is not discussed further.	Ritz et al, 1993

4.2.1. Inhalation sensitisation (animals)						
Product name	**Test species / route / exposure duration**	**Substance concentrations**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase	groups of 5 mice / weekly / intratracheal doses for up to 8 weeks	Alcalase in a detergent matrix			Alcalase-specific IgE and IgG1 antibodies measured 5 days after the last dose showed a good correlation with the number and magnitude of doses given. Further experiments showed that the addition of the detergent matrix markedly enhanced the antibody response (both IgE and IgG1) compared to Alcalase™ alone.	Kawabata et al, 1996
Alcalase	a) groups of 4 - 5 female mice / mouse intra-nasal model (MINT) / exposure on days 1, 3 and 10 b) additional studies up to 9 weekly doses	a) Inhalation of Alcalase in saline, 5 µl of enzyme solution, blood collection 5 days after the last dose b) Studies with 0.5 µg Alcalase dose, determination of IgE response and effects of an extended dosing regime.			a) A clear dose-related increase in IgG1 titre was obtained. b) negligible IgE response. When dosing was extended to up to 9 weekly doses, the IgG1 response levelled off after 5 weekly doses. In contrast, a small IgE response was only apparent after 8 weekly doses or more.	Robinson et al, 1996
Alcalase Savinase Subtilisin B	GPIT and MINT	Alcalase containing 35% ep, Savinase 21% ep, Subtilisin B 5% ep			In both test systems the relative potency of Subtilisin B was about one third to one half of that of the other two preparations.	Sarlo et al, 1997 and Robinson et al, 1998

4.2.2. Inhalation sensitisation (humans)

4.2.2. Inhalation sensitisation (humans)						
Product name	**Test species / route / exposure duration**	**Substance concentrations / procedure**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Subtilisin	a) 10 detergent workers with work-related asthma bronchial an/or nasal challenges / open bronchial challenges b) 25 workers with work-related asthma / 11 workers with work-related rhinitis / skin prick tests and passive transfer tests	a) various concentrations of Subtilisin in buffered saline b) as above			a) Immediate reactions in 9/10 workers b) Skin prick tests positive in 22/25 with symptoms of asthma compared to 3/11 with symptoms of rhinitis. Subtilisin enzymes can cause occupational asthma mediated by an immunological mechanism.	Franz et al, 1971
Alcalase and Amylase/ Protease*	14 domestic and occupational users of enzyme-containing detergents a) skin prick tests b) bronchial and c) nasal challenge studies	Alcalase and Amylase/ Protease preparations containing around 5-10% ep			a) skin prick tests: 12/14 positive response to one or both of these preparations b) bronchial challenge: 6/7 immediate response, reductions in FEV1 10-40% compared with baseline values. b) nasal challenge: 7/7 immediate reactions, characterised by increased nasal resistance, increased nasal secretions and subjective feelings of difficult nasal breathing Subtilisin enzymes are capable of causing asthma and rhinitis.	Bernstein, 1972 * Amylase/Protease is a mixture of amylase and protease (Subtilo-peptidase B from B. subtilis)
Maxatase	non-blinded bronchial challenge / 6 detergent workers with symptoms developed between 1-5 months after first contact with Maxatase	saline and nebulised Maxatase solution			4/6 early response, 6/6 late responses, in 2/6 late phase reactions prolonged, taking 10 hours and 8 days respectively before FEV1 and VC returned to their pre-challenge levels, 5/6 positive skin prick test.	Dijkman et al, 1973

4.2.2. Inhalation sensitisation (humans)						
Product name	**Test species / route / exposure duration**	**Substance concentrations / procedure**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase	29 symptomatic and asymptomatic workers with previous occupational enzyme exposure/ non-blinded challenges after a 24-48 hour monitoring period	aerosol of dilute Alcalase (Negative response at first challenge followed by a second challenge 24 hours later using a 10-fold greater concentr.			20/29 immediate, delayed, dual or nocturnal asthmatic reaction. 11/29 workers developed fine bubbling rales on inspiration and expiration, including 7 who gave a positive response on challenge and 4 who gave negative responses. Results are consistent with the view that Alcalase is a potential cause of occupational asthma.	Gandevia and Mitchell, 1970; Mitchell and Gandevia, 1971a
Maxatase	asthmatic workers from detergent factory (asthma after 3 months exposure) / housewife (history of hay fever, asthma while using enzyme-containing detergents) / bronchial challenges	aerosol of 1% Maxatase in a physiological solution and vehicle alone with a 10-day interval between each challenge			Worker showed a dual response to Maxatase with a maximal fall in FEV1 of 70%, but no reaction to the vehicle. The housewife showed a "substantial" immediate reaction. Subtilisin preparation Maxatase is a potential cause of asthma.	Radermecker and Booz, 1970
Alcalase	6 detergent factory workers with work-related asthma rhinitis and/or conjunctivitis for between 3 months and 11 years prior to the study	inhalation of an aerosol of "crude" proteolytic enzyme solution, tipping a detergent powder containing encapsulated (low dust) Alcalase from one tray to another			6/6 workers reacted to the crude enzyme aerosol, doses ranging from 46 to 924 µg. Two reacted to the detergent plus encapsulated Alcalase. 5/6 were skin prick and RAST positive to crude enzyme and/or Alcalase. The worker with the negative skin prick test result gave a dual reaction on challenge to both the crude enzyme preparation (263 µg) and the encapsulated Alcalase. Alcalase may have been the cause of the occupational asthma in these workers.	Paggiaro et al, 1984
Maxatase and Esperase	Nasal challenge tests / 76 detergent workers / skin prick tests and RASTs / single blind nasal challenge tests	Total personal dust levels for laundry detergent line from 0.07-1.3 µg/L (4-hour TWA). Protease content below the limit of detection of 50 ng/m^3 (collected over 2-5 hours)			8/40 manufacturing workers and 0/36 office staff showed specific IgE to both Subtilisin preparations. Nasal challenge with 7 skin-prick positive workers: 5 positive responses to both preparations. Work-related nasal symptoms were also reported in a further 14 workers in whom no challenge tests were performed. Plant was modernised in the 1980s.	Vanhanen et al, 2000

4.2.2. Inhalation sensitisation (humans)						
Product name	**Test species / route / exposure duration**	**Substance concentrations / procedure**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase Maxatase	3 detergent manufacturing workers / open bronchial challenge (Alcalase) / skin prick tests (Alcalase and Maxatase)				3/3 workers: dual reactions (21-59 % falls in FEV1), skin prick tests positive. 2/3 reacted strongly positive with a purified Subtilisin enzyme (Koch-Light).	Pepys et al, 1969
Alcalase	12 housewives (asthma or rhinitis, positive reactions in RAST and skin prick tests) / double-blind and open challenge tests	Simulation of normal exposures during machine washing by measuring out and tipping detergent with or without Alcalase			Equivocal results were obtained under the double blind conditions. On open challenge, 8/12 experienced symptoms mainly rhinitis which persisted or were followed by a late reaction in 4/8. In 3/12 peak expiratory flow rate (PEFR) was measured; in 1/12 PEFR was unaffected and falls of only 10% and 15% were recorded for the other two.	Zetterstrom, 1977
Alcalase	55 cases of asthma / skin prick tests / lung function data / cases during health surveillance of 1642 workers between 1968 and 1975				Pattern of results in 55 workers is consistent with the development of occupational asthma. Although bronchial challenge tests were not conducted to confirm the cause, at the time when these cases arose, Subtilisins were the only enzyme used in detergent manufacture, pointing to a causal role of Subtilisin in these cases.	Juniper and Roberts, 1984
Biozym P300S, Savinase and Maxatase	8 workers with work-related symptoms of asthma (8) and rhinitis (3) / health investiga-tions following a period of absence from work for an unspecified time	Differences in shape: Biozym granules (cylindrical), Savinase and Maxatase granules (spherical), capsule coating damaged in 85% of the P300S grains and only 2-5% of grains of the other preparations.			Onset of symptoms in workers coincided with the introduction of a new Subtilisin Biozym P300S (previously Savinase and Maxatase). 8/8 positive responses to P300S, Savinase and Maxatase in skin prick tests. A group of 10 "healthy, non-asthmatic" subjects were similarly tested with the 3 preparations and none reacted. Workers remained symptom-free once the use of P300S was discontinued. Overall, the pattern of onset and recovery from asthmatic symptoms points to exposure to the Subtilisin preparation P300S as the cause.	Perdu et al, 1992

4.2.2. Inhalation sensitisation (humans)						
Product name	**Test species / route / exposure duration**	**Substance concentrations / procedure**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Subtilisin Alcalase and Maxatase	20 years of routine health surveillance of employees / medical examination: lung function tests, chest x-rays, skin prick tests and/or RASTs	Exposure level fell from 100 ng/m^3 pure crystalline Subtilisin in 1969 to 6 ng/m^3 in 1971, 2-4.5 ng/m^3 in 1972-1975 and 0.7-1.8 ng/m^3 in 1976 to 1993			166 cases of occupational asthma due to enzymes recorded at five factories. Subtilisins were the only enzymes, no specific challenges were performed to confirm Subtilisin impact. Relationship between exposure group and number of employees with positive skin prick to Alcalase and Maxatase: 233/619 high exposure group, 17/180 medium exposure group, 10/353 low exposure group, 28/490 intermittent high exposure group. Proportion of workers in the detergents industry develop work-related asthma-like symptoms which appear to be related to the use of Subtilisins. Relationship between positive responses to Alcalase/Maxatase in skin prick tests and exposure category observed.	Flindt, 1969 Juniper et al, 1977 Flood et al, 1985 Cathcart et al, 1997 Juniper and Roberts, 1984
Subtilisin Alcalase	health evaluation in 271 workers in production, shipping and warehousing with follow-up 6 months later / initial health assessment: 5 months after Alcalase introduction and one month after exposure reduction / monthly area sampling	first set of samples: pro-teolytic activity (pa) of airborne dust from 11-103x10^6 Anson units/m^3 final set of samples: pa between 0.3-6 x10^6 Anson units/m^3 , marked improvement in airborne dust levels			Subtilisin introduction causes skin rashes, and 8-10 weeks later, rhinorrhoea and respiratory conditions including bronchitis and asthma. Skin prick tests from initial survey: 57 positive (exposure dependant), 117 workers: symptoms of "acute chest disease". No lung abnormalities were identified from chest x-rays.	Newhouse et al, 1970
Alcalase Maxatase and Purified Subtilisin	Follow-up of study above / 103 workers / skin prick tests				32 of 62 workers (from group of 103) with respiratory symptoms at the initial survey: symptoms had not recurred. 4/41 earlier free of symptoms developed them for the first time (3/41 were skin prick positive 6 months before). Most workers reacted to all 3 agents. 52/56 previously skin prick positive workers still reacted. 9/47 previously negative workers now skin prick positive. Overall, the follow-up results suggest that in a period when the proteolytic activity of dust in the factory was falling, there was a slight increase in the overall prevalence of workers with skin prick positive results, but that the overall prevalence of workers with respiratory symptoms fell slightly.	Newhouse et al, 1970 (same as above)

4.2.2. Inhalation sensitisation (humans)						
Product name	**Test species / route / exposure duration**	**Substance concentrations / procedure**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Subtilisin Alcalase Maxatase	121 workers / health surveillance over 3 years / initial survey 23 months after the introduction of Subtilisin enzymes	No exposure data were presented.			Initial investigation: symptoms of cough (17), dyspnoea (26), chest pain (15), eye/nose (77), reduced ventilatory capacity (31). Skin prick tests results: Alcalase (42 positive), Maxatase (36 positive), purified Subtilisin (42 positive); a total of 48 (40%) reacted to at least one agent. Study provides no clear evidence for the induction of asthma by Subtilisins, although the high prevalence of skin prick positive tests and respiratory symptoms in this workforce raise concerns for asthmagenic potential.	Greenberg et al, 1970 Watt et al, 1973 Pepys et al, 1973
Savinase Alcalase Subtilisin B	skin prick test/ 250 and 150 workers from a plant producing granulated and liquid detergents	Savinase and Alcalase in granule detergent; Alcalase and Subtilisin B in the liquid			All employees in the liquid detergent facility asymptomatic, between 1986 to 1991, 3.3% of granule workers skin prick positive to Alcalase and 5.2% to Savinase; 11.6% of the liquid workers skin prick positive to Alcalase and 6.7% to Subtilisin B. Alcalase is antigenically distinct to the other two Subtilisin enzymes which are derived from a different Bacillus species.	Sarlo et al, 1997
Esperase	health evaluation / 13 workers of dry bleach industry / exposed for 2 years	Esperase: encapsulated Subtilisin preparation (content 8-10% ep)			6/13 exposed workers reported respiratory symptoms (unexposed workers: 4/9). RASTs for enzyme specific IgE and ELISAs for enzyme specific IgG gave positive scores in 3 and 4 exposed workers respectively. No evidence for occupational asthma can be derived from this study.	Liss et al, 1984
Subtilisin	3 consecutive sets of health surveillance / 110 of 611 workers / 2 enzyme detergent manufacturing factories / static sampling for one hour	First factory: airborne Subtilisin levels: <1 - 30 ng/L with peaks of up to 1000 µg/ m^3 Second factory: airborne Subtilisin levels: <1 - 20 ng/L with peaks of up to 60 ng/L			13/60 (first factory) asthma-like symptoms, 0/50 workers (second factory) respiratory symptoms. Skin prick tests from first factory: 52% positive in high, 35% in medium, 16% in low exposure group. Second plant: 45 and 53% workers positive in the high and inter-mediate, negative in the low exposure group. Overall, although there were workers with asthma-like symptoms in the first plant it is unclear which of these were skin prick positive, hence there is no firm evidence for Subtilisin-induced occupational asthma from this study.	Weill et al, 1971, 1973 and 1974
Subtilisin A	64 workers / 2 enzyme detergent manufacturing factories				Work-related cough in 18/33 with direct enzyme exposure; 7/17 with indirect enzyme exposure; 0/14 with no enzyme exposure. Workers asked about cough in relation to use of Subtilisin A, 4/33 with direct exposure noticed a clear relationship compared with 3/17 with indirect exposure. No conclusions can be drawn from this study.	Gothe et al, 1972

4.2.2. Inhalation sensitisation (humans)						
Product name	**Test species / route / exposure duration**	**Substance concentrations / procedure**	**Guide-line**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase	health evaluation of 98 workers / 18 months after Alcalase introduction				62/98 immediate onset of enzyme-related rhinitis, 49/98 enzyme-related asthma-like symptoms with immediate, delayed and/or nocturnal onset. Irritant response after exceptionally heavy exposures. More commonly, symptoms only developed after several months occupational exposure. In the absence of appropriate challenge tests, the cause of the asthma-like symptoms and rhinitis in this workforce cannot be clearly identified.	Mitchell and Gandevia, 1971b
Alcalase	5 consecutive sets of health surveillance / 355 workers / 2 biotechnology plants				No symptoms of work-related asthma in Alcalase workers (36 workers employed > 10 years in enzyme production). There was no evidence of any deficits in FEV1 relating to enzyme exposure intensity or length of employment, and all chest x-rays appeared normal. Positive RAST reactions to Alcalase were obtained for only 9 workers, around 3 % of those tested.	Witmeur et al, 1973
Esperase	health surveillance / 667 workers from the above 2 biotechnology plants / exposition over 10 year				Health data for 31 workers with positive RAST reactions. 16/31 shortness of breath and chest tightness, 6/31 nasal and throat irritation or rhinitis, 2/31 frequent coughing, 9/31 no respiratory tract symptoms. Possibility of occupational asthma and allergic rhinitis caused by Esperase. No challenge tests were performed, and no comparative symptom data were reported for the RAST negative workers. It is not possible to draw any conclusions from this report.	Zachariae et al, 1981

5.0. Repeated dose toxicity (5.2.1.4.)
5.1. Oral administration (5.2.1.4.1.)
5.1.1. Oral subacute toxicity (5.2.1.4.1.1.)
5.1.2. Oral subchronic toxicity (5.2.1.4.1.2.)

5.1.1. Oral subacute toxicity

Product name	Test species / Exposure / Route	Guideline	Dose / Value NO(A)EL	Klimisch reliability code	Remarks	Reference / Report Date / Report No
Purafect FN3 (PR329, prot. eng. var. 3) concentrate	60 rats / 28 days / diet	OECD 407	3500, 7500 15000 ppm NOAEL: > 15000 ppm ≈ 0.075 g aep/kg bw/day	2	None. No adverse effects at all three dosages tested.	Genencor 1995 (2) 03.04.1995 / PH 436R-GNC-001-94
Alcalase MIF 415-424 concentrate	100 rats / 30 days / gavage	OECD 407	Administered dose: 2.02 - 16.82 AU/kg bw/day ≈ 0.035 - 0.300 g aep/kg bw/day	1	Dose related diarrhoea and struggling during treatment No dose related changes detected among the biochemical and haematological parameters.	Novo Nordisk 1980 (4) Novozymes / Alcalase GL/111382a Study 1980-02-15
Alcalase PPA 1180 concentrate	2 x 2 dogs / 30 days / gavage	OECD 408	0.1 g/kg bw/day ≈ 2 AU/kg bw/day ≈ 0.04 g aep/kg bw/day	1	Increasing signs of gastro-intestinal disturbances with: 0.25 - 1.00 g/kg bw/day ≈ 5.0 - 20.6 AU/kg bw/day ≈ 0.09 - 0.36 g aep/kg bw/day	Novo Nordisk 1981 (3) Novozymes / Alcalase GL/111382a IRI Project No. 416788, Study No. 1038, May 1981
Inactivated Alcalase PPA 1365 concentrate	2 x 2 dogs / 30 days / gavage	OECD 408	Activity **before** inactivation: 19 AU/g 5 g/kg bw/day ≈ 1.7 g inactivated enzyme/kg bw/day	1	Induction of liquid faeces with: 9.0 g/kg bw/day ≈ 3.0 g inactivated enzyme/kg bw/day	Novo Nordisk 1982 (4) Novozymes / Alcalase GL/111382a IRI Project No. 630235, Report No. 2426, November 1982

5.1.1. Oral subacute toxicity						
Product name	**Test species / Exposure / Route**	**Guideline**	**Dose / Value NO(A)EL**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Savinase SP 88 concentrate	20 rats / 30 days / oral gavage		0.17 g/kg bw/day ≈ 0.02 g aep/kg bw/day	1	0.17 g (≈ 0.02 g aep/kg bw/day) were well tolerated and regarded the "no-observed-adverse-effect level"	Novo Nordisk 1982 (3) Novozymes / Savinase / MTM / PNi / F-9201974 Study No. 1576, 1982-01-29 LM-H/VBH/PNi, corrected 1985-08-23, Ph-852567
Esperase SP-72 AB 13 concentrate	10 rats / diet at two dose levels for 30 days		Feeding doses: 0.5 % SP-72 (1.4×10^{-4} g aep/g day diet) 2 % SP-72 (5.6×10^{-4} g aep/g day diet)	2 Non GLP	Mortality was seen in the group fed 2% SP-72 in the diet, where 4/10 animals died during study by starvation due to an unpalatable diet.	Novo Nordisk 1970 (1) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 WARF Institute Inc. 1970-01-16, WARF No. 9120651
Esperase PPA 3366 concentrate	25 rats / gavage / 14 days at a constant volume of 5 ml/kg bw		14 days dose level: up to 9 KNPU/kg ≈ 1.83 g/kg day ≈ 0.035 g aep/kg bw day	1 GLP	no signs of toxicity	Novo Nordisk 1991 (2) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 Study No. 90130, NN, 1991-05-23, NiB/PNi, F-910132
Savinase Opticlean P	28-day gavage study in rats		High doses around 1000 mg/kg/day		Treatment-related effects in both studies included reductions in body weight gains, reduced food consumption and minor alterations in a few haematological and biochemical parameters noted in some animals from all dose groups. There were no clear treatment-related effects on organ weight or histopathology in either group.	NICNAS, 1993

5.1.2. Oral subchronic toxicity (5.2.1.4.1.2.)

5.1.2. Oral subchronic toxicity						
Product name	**Test species / Exposure / Route**	**Guideline**	**Dose / Value NO(A)EL**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Multifect P-3000 concentrate	160 rats / 90 days / diet	OECD 408	5000, 15000, 50000 ppm NOAEL: > 50000 ppm/day of a ca. 10% aep concentrate ≈ 250 mg aep/kg bw/day	2	hypertrophy of submandibular gland	Genencor 1994 (7) 09.11.1994 / PH 470-GNC-001-94
Alcalase PPA 1180 concentrate	156 rats / 90 days / gavage	OECD 408	Dose levels: 10 ml/kg of 0, 160, 400, 1000 mg/kg/day NOEL: 0.4 g/kg bw/day (8.2 AU/kg bw/day) (0.14 g aep/kg bw/day)	1	The major clinical oberservation was increased difficulty of dosing animals receiving 1000 mg/kg/day. Reduced body weights in males with: 1 g/kg bw/day (20.6 AU/kg bw/day (0.36 g aep/kg bw/day	Novo Nordisk 1981 (4) Novozymes / Alcalase GL/111382a IRI Project No. 416809, Report No. 2126, September 1981
Alcalase PPA 1180 concentrate	18 dogs / 90 days / gavage	OECD 409	Dose levels: 10 ml/kg of 3, 30, 300 mg/kg/day NOEL: 3 mg/kg bw/day (0.06 AU/kg bw/day) (0.001 g aep/kg bw/day)	1	Gastro-intestinal disturbance/ bleeding Infrequent loss of faecal consistency after treatment with: 30 mg/kg bw/day ≈ 0.6 AU/kg bw/day ≈ 0.01 g aep/kg bw/day	Novo Nordisk 1981 (5) Novozymes / Alcalase GL/111382a IRI Project No. 416793, Report No. 2123, September 1981
Inactivated Alcalase PPA 1389 concentrate	18 dogs / 90 days / gavage	OECD 409	Activity **before** inactivation: 18.5 AU/g ≈ 0.32 g aep/g 0.3 g/kg bw/day ≈ 0.097 g inactivated enzyme/kg bw/day	1	No signs of systematic toxicity, occasional emesis and liquid faeces with : 5.0 g/kg bw/day ≈ 1.6 g inactivated enzyme/kg bw/day	Novo Nordisk 1983 (2) Novozymes / Alcalase GL/111382a IRI Project No. 63 Register 0298, Report No. 2622, June 1983

5.1.2. Oral subchronic toxicity						
Product name	**Test species / Exposure / Route**	**Guideline**	**Dose / Value NO(A)EL**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Esperase PPA 3366 concentrate	20 rats / gavage / 90 days	OECD 408	dose levels: 5 ml/kg of 1.0, 3.0, 5.0 g/kg bw/day NOEL: 1 g/kg bw/day	1 GLP	Rats dosed for 90 days resulted in changes at 5.0 g/kg (0.10 g aep/kg) and 3.0 g/kg (0.06 g aep/kg) for both sexes and at 1.0 g/kg (0.02 g aep/kg) for males. There were no abnormal findings for females dosed 1.0 g/kg.	Novo Nordisk 1991 (7) Novozymes / Esperase / TiH/PNi / F-9203233A / Edition 2 IRI project No. 450347, report No. 7680, 1991-04-25, F-914756

5.2. Inhalation administration (5.2.1.4.2.)

5.2.1. Inhalation subchronic toxicity

5.2.1. Inhalation subchronic toxicity						
Product name	**Test species / Exposure / Route**	**Guide-line**	**Dose / Value NO(A)EL**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase and Milezym 8X	Groups of 9 monkeys / inhalation / whole-body / 6 hours per day / 5 days per week / 6 months / combined necropsy / observation of clinical signs, body weight, lung function, tests for specific IgE / skin prick test at the end of the study / 5 animals/group killed after 6 months; remaining animals allowed to recover 4 weeks before being killed		a) 2:1 mixture of the Subtilisin preparations 0 or 1.18 µg/L Subtilisin preparation (MMAD 2.8 µm) **(E)** b) Mixture a) together with 100 µg/L detergent **(DE)**, or to 100 µg/L of detergent alone **(D)**		No treatment-related deaths in E group, whereas 2 died in D and 3 in DE. Laboured breathing in E but no changes in pulmonary function. Small airways constriction in DE and D group, most marked in DE. Body weight not affected in E or D, reduced in DE. One E group animal showed pulmonary inflammatory changes. Chronic inflammatory changes and fibrosis were seen in the lungs of all DE and D animals, and were more severe in DE than D animals. No evidence that E or DE monkeys developed Alcalase or Milezym specific IgE as measured by RAST and by passive cutaneous anaphylaxis, nor could Alcalase- or Milezyme-specific IgG, IgA, IgM or IgE be demonstrated in pulmonary tissue by immuno-fluorescence.	Coate et al, 1978

5.2.1. Inhalation subchronic toxicity						
Product name	**Test species / Exposure / Route**	**Guide-line**	**Dose / Value NO(A)EL**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase and Milezym 8X	monkeys (small group sizes) / 4 months / inhalation				IgM to Alcalase and/or Milezyme was found in sera from E and DE monkeys. No Subtilisin-specific antibodies were detected in D monkeys. No conclusions can be drawn from this study in relation to the effects of long-term repeated exposure to Subtilisin.	Cashner et al, 1980
Subtilisin Carlsberg and Esperase	longterm inhalation study / 86 guinea pigs: 56 highest concentr. and 30 other groups and control / 8 weeks / 1 hour / once every 7 days / whole body chambers		enzyme concentrations: 100, 10, 1 and 0.1 µg/L		Mortality at 100 µg/L in 3/56 animals immediately after the 6th exposure. Irritation of the skin in solitary animals for both enzymes. Other clinical signs: nose bleeding, lacrymation. Dose-related sensitisation of treated animals was confirmed in assays and the enzymes were considered to have similar allergenic potential.	Novo Nordisk 1972 (1) Novozymes/ Esperase / TiH / PNi/ F-9203233A / Edition 2 HRC 5612/72/1008, 1972-12-19
Savinase	Guinea pigs / inhalation / 1 hour / once every day / 8 weeks / whole body chambers / respiratory patterns during the first 2 exposures changed (respira-tory rates increased)		atmosphere of Savinase dust (17 µg/L)		No mortalities during the experiment. Skin irritation in 6/32 animals, general irritation. This was probably due to the inhalation of particulate material rather than to an allergenic response. Various signs of respiratory distress, some breath holding, arrhythmias and prolongation of expiration.	Novo Nordisk 1974 (1) Novozymes/ Savinase / MTM / PNi/ F-9201994 / Edition 2 HRC NVO41/74102, 1974-05-06

5.3. Dermal administration (5.2.1.4.3.)

5.3.1. Dermal subacute toxicity

5.3.1. Dermal subacute toxicity						
Product name	**Test species / Exposure / Route**	**Guideline**	**Dose / Value NO(A)EL**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Savinase SP 88 FPF 312-320 concentrate	Dermal toxicity / 32 rabbits / 28 days / intact and abraded skin / 4 hours then wash	Standards but not in compliance with OECD No. 410	Savinase in 0.5 % w/v in aqueous solution: 2 ml/kg (0.0014 g aep/kg/day) Savinase in 0.5 % w/v in 0.1 % w/v aqueous sodium tripolyphosphate: 2 ml/kg (0.0014 g aep/kg/day)	1	Repeated daily applications of buffered or non-buffered Savinase were without effect and thus the test substance may be considered virtually harmless. Reactions were confined to microscopically detected minor changes of the site of application.	Novo Nordisk 1978 (2) Novozymes / Savinase / MTM / PNi / F-9201974/ LSR report No. 78/NTL27/085, 1978-03-06

6.0. Genetic toxicity (5.2.1.5.)

6.1. Genetic toxicity *in vitro*

6.1. Genetic toxicity - in vitro						
Product name	**Test species / Conditions / Exposure duration**	**Test / Guideline**	**Test substance concentr.**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
BLAP S (F 49) SAT 950177 granulate	*Salmonella typhimurium* strains: TA 98, TA 100, TA 1535, TA 1537, TA 1538 with and without metabolic activation / 48 h incubation	Ames Test: two independent tests OECD 471, 92/69/EEC, L383A, Annex V b 14	1st test : 8, 40, 200, 1000, 5000 µg/plate 2nd test: 50, 100, 200, 400, 800 µg/plate	1	no mutagenic activity	Henkel 1995 (4) 19.07.1995 / R9500804

6.1. Genetic toxicity - in vitro						
Product name	**Test species / Conditions / Exposure duration**	**Test / Guideline**	**Test substance concentrations**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Multifect P-3000	*S. typhimurium* & *E. coli* / single / treat & plate	Ames test OECD 471 and 472	> 7690 µg/ml equivalent to > 5000 µg/plate of a 4% aep product	2	negative	Genencor 1995 (3) 19.06.1995 / 16834-0-409R
Purafect FN3 (PR329, prot. eng. var. 3) concentrate	*S. typhimurium* & *E. coli* / treat & plate	Ames test OECD 471 and 472	> 7690 µg/ml equivalent to > 5000 µg/plate of a ca. 10% aep product	2	negative	Genencor 1995 (4) 16.02.1995 / 16592-0-409R
Savinase PPA 3352 concentrate	*S. typhimurium* strains: TA 98, TA 100, TA 1535, TA 1537 / with and without metabolic activation / 3 hours incubation	Ames Test: two independent tests Standards but not in compliance with OECD 471	6 doses in first test, 6 more narrow doses in second test with 10 mg/ml incubation mixture as highest dose level	1	no mutagenic effect	Novo Nordisk 1991 (8) Novozymes / Savinase / MTM / PNi / F-9201974 Study No. 91535, 1991-09-11, PBjP, F-913716
Alcalase PPA 1180 concentrate	5 strains of *S. typhimurium*: TA 1535, TA 100, TA 1537, TA 1538 and TA 98 & *E. coli* WP2 uvrA (pKM101) / 3 hours incubation	Ames test OECD 471 and 472	up to 20.6 AU/g ≈ 0.36 g aep/g	1	Up to 20.6 AU/g ≈ 0.36 g aep/g no mutagenic effects in the five tested *Salmonella* strains and *E. coli*	Novo Nordisk 1981 (6) Novozymes / Alcalase GL/111382a / IRI Project No. 704170, Report No. 2031, April 1981
Esperase PPA 3366 concentrate	*S. typhimurium* strains: TA 98, TA 100, TA 1535, TA 1537 / with and without metabolic activation / 6 doses / 3 hours incubation	OECD 471	up to 6.1 mg/ml ($1.3x10^{-4}$ g aep/ml)	1 GLP	No indication of mutagenic activity in dose levels up to 6.1 mg/ml ($1.3x10^{-4}$ g aep/ml) incubation mixture in presence and absence of metabolic activation.	Novo Nordisk 1991 (3) Novozymes / Esperase / TiH/PNi / F-9203233A/Edition 2 Study No. 91514, NN, 1991-04-25, PBjP, F-911207

6.1. Genetic toxicity - in vitro						
Product name	**Test species / Conditions / Exposure duration**	**Test / Guideline**	**Test substance concentrations**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Savinase (tested in 1977) Opticlean P (tested in 1987)	*S. typhimurium* strains TA 1535, TA 100, TA 1537, TA 1538 and TA 98	Ames tests	Savinase: aqueous concentr. of 33-10 000 µg/plate with and without S9 Opticlean P: aqueous concentr. of 15 - 1500 µg/plate with and without S9		No information on cytotoxicity was provided. Negative results were reported for both preparations.	NICNAS, 1993
BLAP PM 111 granulate	V 79 Chinese hamster cell line treatment interval 4 hours with metabolic activation treatment intervals 18 and 28 hours without metabolic activation	Chromosomal aberration: Single amino acid exchange: two independent tests OECD 473, EPA regulations	Exp. 1: without S9 mix: 18 h: 3.0, 30.0, 50.0 µg/ml 28 h: 50.0 µg/ml with S9 mix: 18 h: 0.3, 1.0, 2.0 µg/ml 28 h: 2.0 µg/ml Exp. 2: without S9 mix: 18 h: 10.0, 30.0, 50.0 µg/ml 28 h: 50.0 µg/ml with S9 mix: 18 h: 0.3, 1.0, 1.8 µg/ml 28 h: 1.8 µg/ml	1 GLP	No damage of chromosomal structure.	Henkel 1993 (1) R0500067 CCR Project 315808 February 17, 1993
BLAP S (F 49) Granulate Charge 29	V79 Chinese hamster cell line with and without metabolic activation 4 hours	Chromosomal aberration: two independent tests OECD 473, 84/449/EEC, Annex V of 67/548/EEC part B	up to 3 µg/ml without metabolic activation up to 5µg/ml with metabolic activation	1	No induction of chromosomal aberrations.	Henkel 1995 (5) R9500906 / 25.07.1995
Multifect P-3000	Human lymphocytes (whole blood) / multiple	Chromosomal aberrations (OECD 473)	max. dose 20 µl/ml of a 4% aep product	2	negative	Genencor 1995 (5) 22.06.1995 / 16834-0-449CO

6.1. Genetic toxicity - in vitro						
Product name	**Test species / Conditions / Exposure duration**	**Test / Guideline**	**Test substance concentrations**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Purafect FN3 (PR329, prot. eng. var. 3) concentrate	Human lymphocytes / 3 H , 19.3 H and 43.2 H	Chromosomal aberrations (OECD 473)	max. dose 216 µg/ml of a ca. 10% aep product	2	Confirmatory assay (45.8 hour) inactivation assay redone as no toxicity was detected at the highest concentration used (108 µg/ml). Repeat assay: toxicity sufficient. No chromosomal aberrations, polyploidy or endoreduplications.	Genencor 1996 (1) 30.05.1996 / 17373-0-449CO
Esperase PPA 3366 concentrate	Chromosomal aberration (cultured human lymphocytes) / with and without metabolic activation	OECD 473 EEC Annex V, B 10	Highest dose level: 5000 µg/ml ($1x10^{-4}$ g aep/ml)	1 GLP	No induction of chromosome aberrations in human lympho-cytes when tested up to 5000 µg/ml ($1x10^{-4}$ g aep/ml) in absence and presence of metabolic activation.	Novo Nordisk 1991 (4) Novozymes / Esperase / TiH/PNi / F-9203233A/Edition 2 Hazelton Microtest Report No. 2HLRENOD.023 F-913199, 1991-07-01
Esperase PPA 3366 concentrate	Gene mutation test / mutations to 6-thioguanine resistance in mouse lymphoma cells using a fluctuation assay with and without metabolic activation	OECD 476	Highest dose level: 5000 µg/ml ($1x10^{-4}$ g aep/ml)	1 GLP	No mutagenic activity when tested up to 5000 µg/ml ($1x10^{-4}$ g aep/ml) in absence and presence of metabolic activation.	Novo Nordisk 1991 (5) Novozymes / Esperase / TiH/PNi / F-9203233A/Edition 2 Hazelton Microtest Report No. 2MLRENOD.023 F-911235, 1991-04-05

6.2. Genetic toxicity - *in vivo*

6.2. Genetic toxicity - in vivo						
Product name	**Test / test species / Exposure duration**	**Guideline**	**Test substance concentr.**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase PPA 1180 concentrate	Bone marrow cytogenetic test (Chinese hamster) 6 hours	OECD 475 EEC B 11	low dose group of 10 animals: 10 x 72 mg aep/kg bw ≈ 200 mg/kg bw high dose group of 12 animals: 12 x 720 mg aep/kg bw ≈ 2 g/kg bw 10 positive controls (EMS, 200 mg/kg)	1	No evidence of damage to chromosomal structure in male Chinese hamster bone marrow cells.	Novo Nordisk 1981 (7) Novozymes / Alcalase GL/111382a IRI Project No. 704186, Report No. 2043, May 1981
Savinase SP 88 FPF 312-320 concentrate	Bone marrow cytogenetic test (Chinese hamster) 15 animals	OECD 475	1500 mg/kg bw (0.2047 g aep/kg) 300 mg/kg bw (0.0409 g aep/kg) 60 mg/kg bw (0.0082 g aep/kg)	1	No evidence of damage to chromosomal structure in male Chinese hamster bone marrow cells.	Novo Nordisk 1977 (3) Novozymes / Savinase / MTM / PNi / F-9201974 LRS Report No. 77/NTL24/344, 1977-10-18 NICNAS 1993
Savinase SP 88 FPF 312-320 concentrate	Rodent dominant lethal test 40 male mice and sufficient female mice	Standards but not in compliance with OECD 478	0.1 g/kg bw/day (0.0137 g aep/kg) 1.0 g/kg bw/day (0.1365 g aep/kg)	1	No dominant lethal mutations in sperm cells of male mice.	Novo Nordisk 1977 (4) Novozymes / Savinase / MTM / PNi / F-9201974 LRS Report No. 77/NTL23/407, 1977-12-31

7.0. Developmental toxicity

7.0. Developmental toxicity						
Product name	**test species / route**	**Guideline**	**Test substance concentration**	**Klimisch reliability code**	**Remarks**	**Reference / Report Date / Report No**
Alcalase MIF 415-424 concentrate	pregnant rats / gavage	OECD 414	Supply on day 2 of gestation: 10 ml/kg bw 300 mg/kg bw/day (36 mg aep/kg/day) 1000 mg/kg bw/day (120 mg aep/kg/day) 2000 mg/kg bw/day (240 mg aep/kg/day)	1	No teratogenic effect up to 2 g/kg bw/day (0.25 g aep/kg/day)	Novo Nordisk 1976 (1) Novozymes / Alcalase GL/111382a IRI project no. 406392, report no. 524, June 1976
Alcalase PPA 1180 concentrate	pregnant rats / gavage	OECD 414	Supply on day 1 of gestation: 10 ml/kg bw 150 mg/kg bw/day (54 mg aep/kg/day) 475 mg/kg bw/day (171 mg aep/kg/day) 1500 mg/kg bw/day (540 mg aep/kg/day)	1	No teratogenic effect up to 1.5 g/kg bw/day (0.54 g aep/kg/day)	Novo Nordisk 1981 (8) Novozymes / Alcalase GL/111382a IRI project no. 704317, report no. 2102, August 1981
Savinase SP 88 FPF 312-320 concentrate	85 pregnant rabbits / gavage	Standards but not in compliance with OECD 414	From day 6 to day 18 inclusive of gestation volume-dosage: 5 ml/kg bw 50 mg/kg bw/day (0.0068 g aep/kg/day) 150 mg/kg bw/day (0.0205 g aep/kg/day) 500 mg/kg bw/day (0.0683 g aep/kg/day)	1	50 (0.0068 g aep/kg/day) and 150 (0.0205 g aep/kg/day) mg/kg bw/day had no adverse effect upon the dam or developing foetus. At 500 mg/kg bw/day (0.0683 g aep/kg/day): marginal increase in the number of undersized pups, but no other adverse effects upon the pregnant female or developing foetus.	Novo Nordisk 1977 (5) Novozymes / Savinase / MTM / PNi / F-9201974 LRS Report No. 77/NTL22/412, 1977-12-22
Esperase PPA 3366 concentrate	pregnant rats / gavage	OECD 414	days 6 to 16 after mating volume-dosage: 10 ml/kg bw 1000 mg/kg bw/day (0.02 g aep/kg/day) 3000 mg/kg bw/day (0.06 g aep/kg/day) 5000 mg/kg bw/day (0.10 g aep/kg/day)	1 GLP	5000 mg/kg bw/day (0.1 g aep/kg/day is non teratogenic)	Novo Nordisk 1991 (6) Novozymes / Esperse / TiH/PNi / F-9203233A / Edition 2 IRI project no. 490149, report no. 7698, 25 July 1991, study no. 91703, 1991-12-06, NiB/PNi, F-915076

Klimisch reliability code: The Klimisch reliability code is a systematic approach in order to harmonize the data evaluation process worldwide.

In this context reliability is differentiated into four categories:
1 = reliable without restrictions
2 = reliable with restrictions
3 = not reliable
4 = not assignable
This classification scheme is helpful for subsequent assessments and should increase the clarity of evaluation.

Remark: Animals are counted without control animals as far as possible to differentiate.