



Human & Environmental Risk Assessment on ingredients of household cleaning products

α -AMYLASES, CELLULASES AND LIPASES

CAS No:

α -Amylase:	9000-90-2
Cellulase (β -(1,4)-Glucanase):	9012-54-8
Lipase:	9001-62-1

This report is based on the rationale described in the HERA Risk Assessment on Subtilisins (Protease) <http://www.heraproject.com> and builds on the data presented there for protease enzymes to assess the use of other enzymes used in household detergents.

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α -AMYLASE, CELLULASE and LIPASE - HERA Report (Bridging Document)

1. EXECUTIVE SUMMARY

This report is based on the rationale described in the HERA Risk Assessment on Subtilisins (Protease) <http://www.heraproject.com> and builds on the data presented there for protease enzymes to assess the use of other enzymes used in household detergents.

1. Substance Characterisation and Usage

Amylases, cellulases and lipases used in detergents are hydrolytic enzymes, used in detergents and other technical applications like textile or pulp and paper industry to remove deposits and stains. Amylase is acting versus starch containing stains, lipase against natural fats and oils. Cellulase is used to exhibit effects on cotton fibers like anti pilling, colour brightening, and antigreying. The enzymes are of bacterial or fungal origin, and are produced by a fermentation process. Each of the enzymes is characterised by its amino acid sequence and three-dimensional structure as well as by its biocatalytic activity in hydrolysing glycosidic bonds (amylase and cellulase) or ester bonds (lipase).

The total amount of these enzymes used in detergents in the European Union in 2002 is in a range of 150 tons for amylases, 15 tons for cellulases and 8 tons for lipases (pure active enzyme protein). The concentration of amylases, cellulases or lipases in household detergent and cleaning products is very low and depends on the type of product. According to a 2003 A.I.S.E. survey, the concentrations in products typically range between 0.002 and 0.09%.

2.. Environmental Assessment

Amylases, cellulases and lipases are proteins which are readily and ultimately biodegradable in the environment. An important aspect in the environmental exposure assessment is the fact that these enzymes are inactivated (loss of enzymatic activity) to a large extent under washing or cleaning conditions. These enzymes do not show relevant ecotoxic properties.

This allows to conclude that the use of amylases, cellulases and lipases in detergents does not pose a risk for the environment.

3. Human Health Assessment

The key health concern identified for all enzymes is respiratory (Type 1) allergy. Consumers can be exposed to enzymes via the respiratory route during the task of dispensing powder products in the washing machine and during hand wash of laundry, or by suddenly opening the dish washer during the cleaning step. The exposure has been determined for the detergent protease Subtilisin which is considered the most critical enzyme type. This is seen due to the higher frequency of use, the higher concentration in the products, and the intrinsic irritation hazard (HERA Subtilisin Risk Assessment). Subtilisin has irritation properties due to its catalytic activity (breakdown of proteins) while amylases, cellulases and lipases are not irritant. Since amylases, cellulases and lipases are used in lower concentrations the exposure can be expected to be at equal or lower levels compared to Subtilisin.

According to human experience data the allergic potency of amylases, cellulases and lipases is considered to be in the same range as Subtilisin.

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. For Subtilisin an upper benchmark where allergic symptoms occur was established at 212 ng/m³. Allergic symptoms can be excluded when exposure does not exceed a range of 1 ng/m³. There appears to be a complex relationship among frequency, magnitude and duration of exposure to the generation of enzyme specific IgE antibody. Therefore a lower benchmark where occurrence 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/m³, 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.

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

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3. SUBSTANCE CHARACTERISATION

3.1. CAS No and Grouping Information

Substance name: α -amylase, cellulase, hemicellulase, lipase

Synonyms and Trade Names:

α -Amylases: Duramyl™, Purastar™, PurastarOx™, Purastar HPT™, Purastar ST™, Termamyl™, Natalase™, BAN®, Stainzyme™

Cellulases: β -(1,4)-Glucanase, Carezyme™, Celluzyme™, Endolase™, Puradax™, Renozyme™, Biotouch NCD, Clazinase®

Lipases: Lipolase™, Lipex™, LipoPrime™, Lipolase ultra

Function and CAS numbers:

α -Amylases catalyses the endo-hydrolysis of 1,4- α -D-glycosidic linkages in polysaccharides like starches, glycogen, and oligosaccharides containing three or more 1,4- α -linked glucose units. They can be found under a number of CAS numbers. The industry is normally referring to the CAS number 9000-90-2.

Cellulases cleave β -1,4-glycosidic bonds in cellulose, a major and complex component of plant cells and an important raw material of the pulp & paper and the textile industries. A mixture of different enzymes is needed to hydrolyse the bonds between different oligosaccharides. They are the intermediates in the breakdown process of cellulose. Cellulases from fungi are naturally produced as a complex mixture of enzymes, with over 13 EC numbers in the family 3.2.1.x., that act as a consortium to degrade cellulose to glucose. This consortium consists of endoglucanases (EC 3.2.1.4, CAS 9012-54-8), cellobiohydrolases (EC 3.2.1.91, CAS 37329-65-0) and beta-glucosidases (EC 3.2.1.21, CAS 9001-22-3). Since the active washing principle is based upon the endoglucanase activity only, the natural mixtures of these enzymes, are usually sold as "cellulase" under EC 3.2.1.4. Since 1995 an increasing number of bacterial or fungal monocomponent cellulases, containing single endoglucanase enzyme species (E.C. 3.2.1.4, CAS 9012-54-8), have been introduced in the detergent enzyme market.

Lipases hydrolyse triacylglycerol substrates, as they are present in fats and oil. The lipase normally used is triacylglycerol lipase (EC 3.1.1.3), which is described by CAS 9001-62-1.

According to their structure and the specificity of the reaction catalysed, the NC-IUBMB has classified the enzymes using the E.C. nomenclature, see above.

3.2. Chemical Structure and Composition

The physico-chemical properties as listed in the HERA context are similar or identical for all detergent enzymes. The information given here is the same as for the Subtilisin protease.

Table 1: Physico-chemical properties of detergent amylases, cellulases and lipases

Parameter	Value	Remark
Macromolecular Description Physical state / Particle size	White crystals or powder (pure enzyme)	In Detergents added as 0.2 - 3% 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)	<i>Not relevant</i>	Heating leads to destruction
Vapour pressure at 25°C (Pa)	1 x 10 ⁻⁶	Minimum value acc. to TGD
Water solubility at 25 °C (g/L)	< 0.8 kg/L	(at pH 7, 21° C) Henkel 2005
Octanol-water partition coefficient: log K _{ow}	- 2.95	(at 21° C) 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	

Enzymes are catalytic proteins or polypeptides: they consist of amino acid residues coupled via peptide bonds in an essentially global 3D-structure. They are primarily characterised by their biocatalytic or enzymatic activity. 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.

Typical detergent amylases are *Bacillus* amylases like the *Bacillus licheniformis* amylase, which has a neutral isoelectric point and a broad pH optimum between pH 5 and 9. The enzyme is characterised by a molecular weight of 58 kD (Aehle, 1997).

There is a broad range of detergent cellulases used both from fungal as well as from bacterial origin. All of them are characterised by β -1,4-endoglucanase activity. The pH optimum ranges from neutral to moderately alkaline conditions. The molecular weight ranges from 20 kD to 80 kD. There are glycosylated as well as unglycosylated molecules, depending on the origin and the production strain. Also there is not a uniform picture with respect to the existence of cellulose binding domains (CBD) (Aehle, 2004).

Typical detergent lipases are derived from *Thermomyces lanuginosus* (formerly known as *Humicola insolens*) and are produced using *Aspergillus oryzae* as a host. A number of variants of the wild type molecule are used (Aehle, 2004)

3.3. Manufacturing Route and Production/Volume statistics

3.3.1. Enzyme 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 detergent enzymes are produced using genetically engineered microorganisms (GMM), and increasingly also protein-engineered variants are produced.

α -Amylase is produced using microbial strains from the genus *Bacillus*, in a process similar to that described for Subtilisin (HERA Subtilisin risk assessment, 2005). Cellulases and lipases can originate from bacterial origin as well as from fungal origin.

As for Subtilisin, usually genetically modified (engineered) microorganism (GMM) strains are used, to enhance productivity. The hosts for recombinant production are *Bacillus* species for bacterial enzymes like the α -amylase and *Trichoderma* and *Aspergillus* species for fungal enzymes. Sometimes these strains are producing protein-engineered 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 than the naturally occurring differences between enzymes derived from different strains.

The fermentation, down stream and confectioning process for these enzymes is in principal not different from the information given in the HERA Subtilisin risk assessment. The final enzyme products used as raw materials for the detergent production are also similar in their structure and composition. As these enzymes are produced from similar microbial strains as proteases, in many enzyme preparations traces of protease are present. This was particularly true for the preparations used in the older toxicological tests. In more recent years most production amylase, cellulase or lipase strains have been modified by genetic engineering to be deficient in protease activity. The presence of traces of protease is the most likely cause of any residual irritancy of amylase, cellulase or lipase preparations. As the production strains are optimised these traces will be diminished.

3.3.2. Production/Volume Statistics

In 2002 according to the enzyme producing companies 150 tons of amylase, 15 tons of cellulase and 8 tons of lipase was produced in the EU for the EU detergent market. In all cases this is presumed to represent less than 50% of the production of these enzyme types, since there are more enzyme applications e.g. in textile and pulp & paper industry and more enzyme producing companies supplying enzyme preparations for such fields. The volume statistics when collected from the detergent producing companies resulted in the data shown in table 2 representing 80% of the market. The total enzyme consumption in the European market thus can be extrapolated to the data shown in the same table. Considering all uncertainties in such an evaluation, these data can be regarded as well comparable to the data estimated by the enzyme producers. The tonnage data covering the total formulator market have been used for the environmental exposure analysis in the present HERA risk assessment.

This amount of pure and active enzyme is contained in up to 7,000 t of granulated powder or liquid preparation used by the formulators. The enzyme protein concentration in these preparations is ranging from 0.2 to 3%.

Table 2: Volume statistics on amylase, cellulase and lipase usage in HERA products (Europe)

	Enzyme Producers (tons/a)	HERA Formulators (80%) (tons/a)	Total Formulator Market (tons/a)
Amylase	150	124	155
Cellulase	15	8.3	10.4
Lipase	8	7.6	9.5
Protease (Subtilisin)	900-950	700	875

3.4. Use Applications Summary

α -Amylase, cellulase and lipase preparations are used for removal of stains in powder and liquid laundry detergents. α -Amylase is also used in automatic 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 3. The use in HERA (household and cleaning) products is considered to cover in the order or less than 50% of the enzyme production, and is the only use addressed in this assessment. No release of active enzyme into the environment can be found in other technical processes, like in textile treatment and in the pulp & paper industry, due to the recycling of process solutions and in-process loss of enzyme activity. Thus the release of these industrial applied enzymes into the environment is not further considered. Only HERA products where enzyme usage has been reported are listed in table 3 to table 5.

Table 3: α -Amylase concentrations in HERA product range

PRODUCT CATEGORIES IN WHICH SUBSTANCE IS CONTAINED		RANGE OF USE LEVELS OF SUBSTANCE AS 100% OF ACTIVE INGREDIENT		
		% weight		
		Minimum	Maximum	Typical
LAUNDRY REGULAR		–	–	–
	Powder	0	0.018	0.002 - 0.013
	Liquid	0	0.025	0 - 0.009
LAUNDRY COMPACT				
	Powder	0	0.04	0.003 - 0.03
	Liquid/gel	0	0.025	0.002 - 0.015
	Tablet	0	0.042	0.002- 0.035
	Gel	0	0.02	0.004 - 0.02
LAUNDRY ADDITIVES				
	All categories	0	0.005	0.002 - 0.013
MACHINE DISHWASHING				
	Powder/Gel	0	0.09	0.002 - 0.06
	Liquid	0	0	0
	Tablet	0.005	0.09	0.007 - 0.04

Table 4: Cellulase concentrations in HERA product range

PRODUCT CATEGORIES IN WHICH SUBSTANCE IS CONTAINED		RANGE OF USE LEVELS OF SUBSTANCE AS 100% OF ACTIVE INGREDIENT		
		% weight		
		Minimum	Maximum	Typical
LAUNDRY REGULAR		–	–	–
	Powder	0	0.03	0.0003 - 0.03
	Liquid	0	0.003	0 - 0.002
LAUNDRY COMPACT				
	Powder	0	0.007	0.0005 - 0.007
	Liquid/gel	0	0.003	0.0001 - 0.003
	Tablet	0	0.005	0.0002 - 0.005

Table 5: Lipase concentrations in HERA product range

PRODUCT CATEGORIES IN WHICH SUBSTANCE IS CONTAINED		RANGE OF USE LEVELS OF SUBSTANCE AS 100% OF ACTIVE INGREDIENT		
		% weight		
		Minimum	Maximum	Typical
LAUNDRY REGULAR		–	–	–
	All categories	0	0.006	0 - 0.001
LAUNDRY COMPACT				
	Powder/Tablet	0	0.01	0 - 0.002
	Liquid/gel	0	0	0

4. Environmental Assessment

This report is based on the rationale described in the HERA Risk Assessment on Subtilisins (Protease) <http://www.heraproject.com> and builds on the data presented there for protease enzymes to assess the use of other enzymes used in household detergents.

4.1. Environmental exposure assessment

The following exposure assessment of detergent amylase is based on the most conservatively estimated EU tonnage of 155 tons of active amylase protein per year in HERA applications (household detergents and cleansers), the evaluation of cellulase and lipase is based on the extrapolated tonnages of the total formulator market, i.e. 10.4 and 9.5 tons per year, respectively.

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. Physical effects like

heating, dilution, mixing of solutions with air, etc. lead to denaturation, i.e. loss of activity and changes of the three-dimensional structure, which facilitates 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 amylase, cellulase and lipase underline the conclusion that these materials are rapidly and ultimately biodegraded in the environment.

4.1.1.1.1. Ready biodegradability

It turns out that all available data measuring ultimate biodegradability by means of oxygen consumption (OECD 301 C) and carbon-removal (OECD 301 E), respectively, amply surpass the OECD threshold degradation limits for ready biodegradability (60% BOD/COD and 70% DOC removal, resp.).

It should be noted that in accordance with the findings on detergent proteases (HERA, 2004), there is no significant difference in the biodegradation rate and extent between wild type enzymes 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 all detergent enzymes does not need to differentiate between wild type and protein-engineered enzyme species.

Table 6: Ultimate biodegradation of detergent enzymes in OECD ready biodegradability tests

Enzyme	Product	Genetic origin	Guideline	Degradation	Reliability (Klimisch score)	Literature
Lipase	Lipolase™	modified	OECD 301 E	99% DOC removal	1	(Bergman 1997)
Cellulase	Carezyme	modified	OECD 301 E	84% DOC removal	1	(Bergman 1997)
	Celluzyme	wild type	OECD 301 C	78% BOD/COD	1	(Greenough 1991)
	Clazinase®	wild type	OECD 301 E	92% DOC removal	1	(Genencor 1995)
Amylase	Termamyl®	wild type	OECD 301 E	99% DOC removal	1	(Bergman 1997)
	Duramyl™	modified	OECD 301 E	99% DOC removal	1	(Bergman 1997)

4.1.1.2. Biodegradation in sewage and river water

No specific data on the biodegradation of amylase, cellulase and lipase in sewage and in river water are available. However, the pertinent information on proteases from literature and from enzyme producing companies may be largely applicable to these enzymes, too, given their common chemical composition as explained in 3.2. and 4.1.1..

Swisher (1969) investigated the biodegradation of a detergent protease 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 enzymes is well confirmed, i.e. these enzymes lose their catalytic 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: $t_{1/2} = \text{ca. } 5 \text{ h}$ ($k = 0.146 \text{ h}^{-1}$).

This figure contrasts considerably with the corresponding default value for readily biodegradable substances acc. to TGD, which is being used in the present HERA exposure assessment ($t_{1/2} = 30$ d). Although there are no concrete experimental data, it can be postulated that the loss of the enzymatic activity of amylases, cellulases and lipases follows similar kinetics as determined for proteases.

4.1.1.3. Anaerobic degradation

There are no specific test data addressing the anaerobic biodegradation of detergent enzymes. However, considering the excellent accessibility of proteins in general to anaerobic biodegradation which is made use of in the sewage sludge digesters it is highly likely that amylases, cellulases and lipases 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.

4.1.1.4. Abiotic degradation

No specific information exists on the abiotic degradation of amylases, cellulases and lipases in the environment via hydrolysis or photolysis. However, considering their excellent biodegradability it can be anticipated that possible abiotic degradation mechanisms will be of lower relevance for their environmental fate than biodegradation.

4.1.1.5. 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 20 kD - 80 kD, the hydrophilic properties (high water solubility, $\log K_{ow} < 0$) and their immediate accessibility to metabolic processes (biotransformation) it can be excluded that detergent-based enzymes 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

Proteolytic reactions are the main reason for the inactivation of detergent enzymes in cleaning processes and, hence, have a significant impact on the exposure assessment of these enzymes. The activity of amylases, cellulases, and lipases used in detergents is strongly affected by the alkaline conditions of the washing or cleaning process enhanced by temperature, pH and the presence of proteases, surfactants and bleach. Studies into the decrease of the enzyme activity in the washing cycle showed (Henkel 2003a) that the amylases, cellulases and lipases used in detergents are completely inactivated at washing temperatures of 100 and 60 °C. At 40 °C the remaining activity of amylases ranged between 40 - 60% (determined 20 min after the washing step), cellulases, and lipases were found to have a residual activity in the range of 15 - 25%. Considering the fact that about 56% of the washes are run at 40 °C, 33% at 60 °C and 11% at 90 °C in European households (Reynolds & Lindfors 1998), it can be concluded that detergent enzymes 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 60% reduction of active amylases, and a 80% reduction each of cellulases and lipases during the washing process.

4.1.2.2. Removal in sewage treatment plants

Based on the physico-chemical properties and the ready biodegradability of amylases, cellulases and lipases, the SimpleTreat model calculations (acc. to Appendix II of TGD, Part II) for removal in waste water treatment plants predict an elimination extent of 87.3%. This is a very conservative assumption considering the 99.7% primary biodegradation determined for a protease (removal of the proteolytic activity) in a laboratory model sewage treatment plant (OECD Confirmatory Test) (Henkel 1995b). However, as neither concrete experimental data on the enzymatic activity removal of amylases, cellulases, and lipases under sewage treatment plant conditions nor corresponding monitoring data from real waste water treatment plants is available, the conservative default value of the TGD was used in the HERA environmental risk assessment.

4.1.2.3. PEC calculations

Detergent enzymes represent an ingredient group, which is contained today in most laundry detergents used in Europe. Therefore, it is justified to assume a homogenous distribution of these substances in all European countries so that the predicted environmental concentrations will mainly depend on the individual per capita detergent consumption. The exposure scenario applied in this HERA risk assessment follows the current HERA methodology (HERA 2005) and corresponds to the TGD process (2003). As no data are available on the sorption of amylases, cellulases and lipases to sludge, in the HERA exposure assessment the fraction not going to surface water was assigned to "sludge" and "degraded" as predicted by SimpleTreat applying the same physico-chemical default data for proteins as previously used for Subtilisin (HERA, 2005): Molecular weight: 2000 g/mol (EUSES max. default), Vapour pressure: 1×10^{-6} Pa (EUSES min. default), $\log K_{ow}$: -1, water solubility 100 g/L.

It should be recalled that the EU consumption tonnages of amylases, cellulases and lipases (see 4.1) used as the starting point for the exposure calculations have been adjusted to the amount which is really entering the sewer, i.e. by taking account of the inactivation of the enzymes by at least 60 and 80%, respectively in the washing process (see 4.1.2.1). Table 7 shows the input tonnage for the exposure calculations of the individual enzymes and summarises the output of these calculations:

Table 7: Basic input data and summary of output data of the environmental exposure assessment of detergent-based amylase, cellulase and lipase

	Amylase	Cellulase	Lipase
Input data: Tonnage entering STP (to/a)	62	2.1	1.9
Output data: Distribution in local compartments:			
Concentration in STP influent ($\mu\text{g aep/L}$)	16.8	0.57	0.515
Concentration in STP effluent ($\mu\text{g aep /L}$)	2.13	0.07	0.065
Concentration in dry sewage sludge ($\mu\text{g aep /kg}$)	0.0127	4.3×10^{-4}	3.9×10^{-4}
C_{local} ($\mu\text{g aep /L}$)	0.213	7.2×10^{-3}	6.5×10^{-3}
PEC Water ($\mu\text{g aep /L}$)	0.234	7.9×10^{-3}	7.2×10^{-3}

PEC Sediment ($\mu\text{g aep /kg}$)	0.199	6.75×10^{-3}	6.1×10^{-3}
PEC Agricultural Soil ($\mu\text{g aep /kg}$)	0.012	4.1×10^{-4}	3.7×10^{-4}

	Amylase	Cellulase	Lipase
Distribution in regional compartments:			
PEC Water ($\mu\text{g aep /L}$)	0.021	7.15×10^{-4}	6.5×10^{-4}
PEC Sediment ($\mu\text{g aep /kg}$)	0.021	5.2×10^{-4}	4.7×10^{-4}
PEC Agricultural Soil ($\mu\text{g aep /kg}$)	1.4×10^{-5}	4.8×10^{-7}	4.3×10^{-7}

4.2. Environmental Effects Assessment

4.2.1. Acute aquatic toxicity

The following tables summarise the existing information about the acute aquatic toxicity of amylases, cellulases, and lipases. Test material is normalised to active enzyme protein content in the preparation. The preparation may vary in purity and enzyme concentration over a broad range. No chronic data are available for these enzymes.

Table 8 (a, b, c): Acute aquatic toxicity data of detergent-based amylases, cellulases and lipases

Amylases	LC/EC₅₀ (mg aep/L)		
	Fish	Daphnia	Algae
Termamyl (Novo Nordisk, 1992 b,c,d)	>320	450	112

Cellulases	LC/EC₅₀ (mg aep/L)		
	Fish	Daphnia	Algae
Celluzyme (Greenough et al. 1991)	>1000	1000	
Puradax HA 400E (Genencor International 1996 a,b,c)	>300	>1000	>1000

	LC/EC₅₀ (mg aep/L)

Lipases	Fish	Daphnia	Algae
Lipolase (Greenough et al. 1996)	>1000	>1000	99

Amylases

Acute toxicity to fish (test species: *Oncorhynchus mykiss* (Rainbow trout)) was tested by Novo Nordisk (Novo Nordisk, 1992 b) with α -amylase (Termamyl) according to test guideline OECD 203. After an exposure time of 96 hours the LC50 was found to be greater than 320 (mg aep/L). For this study the Klimisch reliability code stated was 1.

Another study (Novo Nordisk, 1992 c) on acute toxicity was performed with invertebrates (test species: *Daphnia magna*) according to guideline OECD 202. Based on the results of this study the 48 hour EC50 was determined at 450 mg aep/L (Klimisch reliability code: 1)

Furthermore an acute algae toxicity study (Novo Nordisk, 1992 d) with α -amylase (Termamyl) was carried out according to guideline OECD 201 (test species: *Scenedesmus subspicatus*). The EC50 value was 112 (mg aep/L) after an exposure time of 72 h (Klimisch reliability code: 1).

The algal toxicity formed the most sensitive endpoint of the aquatic toxicity of amylases. Therefore, EC50 = 112 mg aep/L was used for the PNEC derivation of amylase.

Cellulases

A 96 h semi-static test for acute toxicity in zebra fish (Method ISO/DIS/7346) was also carried out at 0, 0.1, 1.0 and 10 g aep/L. The 10 g aep/L group died of oxygen depletion, attributable to biodegradation of SP 227. All other fish survived (Greenough et al, 1991).

Greenough et al. (1991) described acute toxicity studies into an alkaline cellulase (SP 227, Celluzyme) on *Daphnia magna* and Zebra fish (*Brachydanio rerio*). With daphniae (IC(I)₅₀ values for 24 and 48 hours were > 1 g aep/L and about 1 g aep/L respectively. At 0.6 g/L no immobilized daphniae were observed.

An alkaline cellulase was also tested for acute toxicity for zebra-fish and daphnia. A 96 hour semi-static (the test solutions were renewed daily) test for acute toxicity in zebra fish was determined according to the appendix of the EU-guideline 92/69/EEC from the 31st July 1992. The product was tested over a concentration range of 125 to 1000 mg aep/L. The observed LC50 was 330 mg aep/L for zebra-fish (Genencor International, 1996 a).

Daphnia was tested in a 48 hour static test (without renewing the test solutions) according to the same guideline over a concentration range of 62.5 to 1000 mg aep/L. The observed EC50 was >1000 mg aep/L (Genencor International, 1996 c).

The same alkaline cellulase was also tested for chronic algae (*Scenedesmus subspicatus*) toxicity according to the same guideline. A 72 hour static test with a concentration range of 31.25 to 1000 mg aep/L was performed and produced a EC50 of >1000 mg aep/L (Genencor International (1996 b).

Regarding fish toxicity of cellulases, two findings about EC50 >100 mg aep/L are available. As a conservative approach, EC50 = 100 mg aep/L was assumed for the PNEC derivation of cellulase.

Lipases

Lipolase was tested for acute toxicity on daphniae (*Daphnia magna*) over a 24 hours period under static conditions (Greenough, 1996). With 1 g aep/L no immobilization was observed after 24 hours. Analysis of samples revealed a significant loss of enzyme activity during the 24 h period. The mean value for enzyme activity was 82% after 2 h and 76% after 24 h.

A 96 h semistatic test for acute toxicity was carried out with carp (*Cyprinus carpio*) at 1 g aep/L. Solutions were renewed every 24 h. No effects were observed after 96 hours of exposure (Greenough, 1996).

Algal cultures were exposed to five concentrations (10 - 160 mg aep/L) of Lipase (Greenough, 1996). The EC50 for inhibition of growth after 72 h was 97 mg aep/L and the EC50 for inhibition of maximum growth rates (24 - 72 h) was 99 mg aep/L. The no observed effect level was 40 mg aep/L.

Again, algae (EC50 = 99 mg aep/L) proved to be the most sensitive endpoint of the aquatic toxicity of lipases. Therefore, this value was used for the PNEC derivation of lipase.

4.2.2. Terrestrial toxicity tests

No data could be found on the terrestrial toxicity of amylases, cellulases or lipases. Hence, the PNEC calculations for this compartment are being based on the aquatic toxicity test data.

4.2.3. Microorganisms

No published data are available on the effects of amylase, cellulase and lipase towards sewage treatment plant-relevant microorganisms. As these enzymes are produced by bacteria as well as by fungi in nature no adverse effects on other microorganisms are expected at environmentally relevant concentrations. For a conservative derivation of the PNEC_{microorganisms} the effect concentration used in the HERA risk assessment of Subtilisin (EC50 = 3.6 mg aep/L in the cell growth inhibition test) is being used. This is an over-estimation because the inhibitory effect of Subtilisin is attributable to its proteolytic activity, which is absent or greatly reduced in other enzyme preparations.

4.2.4. PNEC calculations

The PNEC values of amylase, cellulase and lipase derived for the different environmental compartments are shown in the following tables.

Table 9a: PNEC values of amylase

Environmental compartment	EC/LC50 (mg aep/L)	Assessment factor	PNEC
Aquatic organisms	112	1000	112 µg aep/L
Microorganisms	3.6	10	360 µg aep/L

Sediment organisms	PNEC derived from aquatic effect data acc. to EUSES	95 µg aep/kg
Terrestrial organisms		19 µg aep/kg

Table 9b: PNEC values of cellulase

Environmental compartment	EC/LC50 (mg aep/L)	Assessment factor	PNEC
Aquatic organisms	100	1000	100 µg aep/L
Microorganisms	3.6	10	360 µg aep/L
Sediment organisms	PNEC derived from aquatic effect data acc. to EUSES		85 µg aep/kg
Terrestrial organisms			17 µg aep/kg

Table 9c: PNEC values of lipase

Environmental compartment	EC/LC50 (mg aep/L)	Assessment factor	PNEC
Aquatic organisms	99	1000	99 µg aep/L
Microorganisms	3.6	10	360 µg aep/L
Sediment organisms	PNEC derived from aquatic effect data acc. to EUSES		84 µg aep/kg
Terrestrial organisms			17 µg aep/kg

4.3. Environmental risk characterisation

The results of the environmental risk characterisation of amylase, cellulase and lipase based on the exposure (Table 7) and effects assessment (Table 9) are summarised in the following table.

Table 10: Environmental risk characterisation of amylase, cellulase and lipase

		Amylase	Cellulase	Lipase
RCR Water	local	0.002	7.9×10^{-5}	7.2×10^{-5}
	regional	1.8×10^{-4}	7.2×10^{-6}	6.6×10^{-6}
RCR Sediment	local	0.002	7.9×10^{-5}	7.3×10^{-5}
	regional	2.2×10^{-4}	6.1×10^{-6}	5.6×10^{-6}
RCR Soil	local	6.3×10^{-4}	2.4×10^{-5}	2.2×10^{-5}
	regional	7.4×10^{-7}	2.8×10^{-8}	2.5×10^{-8}
RCR STP	local	0.006	1.9×10^{-4}	1.8×10^{-4}

4.4. Discussion and conclusions

The outcome of the risk characterisation of amylase, cellulase and lipase in the HERA environmental assessment does not indicate a concern for any of the environmental compartments. All risk characterisation ratios (RCR) are far below 1 despite the fact that conservative assumptions have been applied in the exposure and effects assessment.

In summary, the present HERA environmental assessment shows that the use of amylases, cellulases and lipases in detergents is not of concern for the environment.

5. HUMAN HEALTH ASSESSMENT

This report is based on the rationale described in the HERA Risk Assessment on Subtilisins (Protease) <http://www.heraproject.com> and builds on the data presented there for protease enzymes to assess the use of other enzymes used in household detergents.

5.1. Consumer Exposure

5.1.1. Product Types

Amylases, cellulases and lipases are used in household laundry and cleaning products. These enzymes may be present in household laundry powders and liquids, and in machine dishwashing powders and tablets. The enzyme concentration in product is very low and depends on the type of product. According to a 2003 A.I.S.E. survey, the enzyme concentration typically ranges between 0.007% and 0.05% (see table 3, 4 and 5) in product.

In addition to household detergents, amylases, cellulases and lipases are also used in a number of industrial applications including the pulp & paper, starch, bioethanol and textile industries. The amylases used in baking are fungal amylases and substantially different from the bacterial amylases used in industrial settings. In line with the scope of the HERA initiative, this assessment focuses on the use of amylases, cellulases and lipases 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)
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. Accidental or intentional overexposure

5.1.3. Consumer Exposure Estimates

5.1.3.1. Systemic Exposure

Systemic exposure to amylases, cellulases and lipases 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. These enzymes 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 detergent enzymes is respiratory (Type 1) allergy. Respiratory allergy is the only hazard described for detergent enzymes other than Subtilisin protease. Systemic exposure is not relevant for any of those hazard endpoints.

2. Amylases, cellulases and lipases are present in very low levels in products (0.045% 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 detergent enzymes will lead to breakdown of the molecule into small peptides and amino acids as for any other ingested protein. In addition, the levels of amylases deposited on dishes and cutlery washed with products containing amylases can be estimated not to exceed 50 picograms per cm² 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 assessment], if it were not broken down into peptides in the GI (which it is as mentioned before).

II) Inhalation: there is no significant systemic exposure by the inhalation route. The relevant endpoint related to inhalation exposure is respiratory sensitisation, which is addressed in section 5.2.1.3.

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/m³). It will be referred to as $Exp_{\text{resp all}}$.

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 3), up to 0.04% of the detergent dust can be expected to be amylase, which translates into (0.27 µg x

0.0004) = 1.1×10^{-4} μg of amylase in the dust. The same calculation for cellulase and lipase results in 0.8×10^{-4} μg cellulase and 0.16×10^{-4} μg lipase. In the worst case assumptions 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 enzyme can be estimated as:

$$\begin{array}{llll} \text{Exp}_{\text{resp all}} & \text{amylase} & = & 1.1 \times 10^{-4} \mu\text{g}/\text{m}^3 = & \mathbf{0.11 \text{ ng}/\text{m}^3 \text{ amylase}} \\ & \text{cellulase} & = & 0.8 \times 10^{-4} \mu\text{g}/\text{m}^3 = & \mathbf{0.08 \text{ ng}/\text{m}^3 \text{ cellulase}} \\ & \text{lipase} & = & 0.16 \times 10^{-4} \mu\text{g}/\text{m}^3 = & \mathbf{0.016 \text{ ng}/\text{m}^3 \text{ lipase}} \end{array}$$

Levels of airborne enzyme 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 of the HERA Subtilisin Risk Assessment: “Estimation of Exposure to Enzymes from Early Detergent Formulations”. The levels of airborne Subtilisin estimated for current types of detergents was $0.0057 \text{ ng}/\text{m}^3$. This value was obtained considering a detergent containing 0.034% Subtilisin. Assuming a linear relationship, the levels of airborne enzyme generated from use of a detergent containing the enzyme concentrations as described in tables 3 - 5 can be estimated as:

$$\text{Exp}_{\text{resp all}} = \mathbf{0.006 \text{ ng}/\text{m}^3 \text{ amylase, } 0.005 \text{ ng}/\text{m}^3 \text{ cellulase and } 0.001 \text{ ng}/\text{m}^3 \text{ lipase.}$$

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 enzymes by consumers may only take place if the dish washing machine is opened before the washing program 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 vapor 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 has been reported [AISE Task Force “Enzyme exposure in industrial dishwashing”, 1998]. The highest peak exposures detected were lower than $1.9 \text{ ng}/\text{m}^3$.

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 amylase in a laundry product is 0.04% according to Table 3 above. Therefore, the concentration of amylase to which consumers may be exposed can be expected to be **0.0004% (w/v) or lower**. The levels for cellulase and lipase will be 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 amylase to which consumers may be exposed to is **0.04% (w/v)**.

5.1.3.3.3. Fabric Wear

Washing of fabrics with laundry detergents containing amylase, cellulase and lipase may result in deposition of enzymes on the fabric. Even assuming that any amylase, cellulase or lipase deposited on fabric retains some activity after the washing, drying and fabric adsorption process and that such enzymatic activity is available to the skin, this will not lead to any skin effects, when the enzyme does not show irritating activities.

Amylase

Amylases in the past have been associated with protease activity, and therefore irritating effects were seen in the past with amylase preparations as well. Due to amylase production with recombinant DNA technology strains such activity is practically absent in recent products. Independent of the presence of such side activities in amylases the risk assessment of these is covered by the Subtilisin risk assessment. Nevertheless the amylase activity is potentially available to the skin. The concentration of amylase to which consumers may be exposed as a consequence of fabric wearing can be estimated as follows:

The levels of amylase 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, of cotton, cotton/synthetic mix and synthetic fabrics) were 0.018 µg amylase/g of fabric (Henkel, 2004 (1)). Using this highest deposition value and assuming a fabric density of 10 mg/cm² (P&G, unpublished data 1996), the amount of Subtilisin in contact with the skin can be estimated as: 0.018 µg/(g fabric) x 0.01 (g fabric)/cm² = **0.00018 ug/cm²**. 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:
[0.00018 µg/cm²] / [0.01 cm] = 0.018 µg/cm³ = 0.018 µg/ml = 0.18 x 10⁻⁷ g/ml = 0.18 x 10⁻⁵ % (w/v) = **0.000018 % (w/v)**.

Cellulase

Cellulases are normally associated with minimal proteolytic side activities. The concentration of cellulases to which consumers may be exposed as a consequence of fabric wearing can be estimated as follows:

The levels of cellulase 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, especially cotton fabrics) were 0.18 µg cellulase/g of fabric (Henkel, 2004 (1)). Using this highest deposition value and assuming a fabric density of 10 mg/cm² (P&G, unpublished data 1996), the amount of cellulase in contact with the skin can be estimated as: 0.18 µg/(g fabric) x 0.01 (g fabric)/cm² = **0.0018 ug/cm²**.

Assuming a film thickness on the skin of 0.01 cm (Vermeire et al., 1993), the concentration of cellulase in contact with the skin can be estimated as:

$$[0.0018 \mu\text{g}/\text{cm}^2] / [0.01 \text{ cm}] = 0.18 \mu\text{g}/\text{cm}^3 = 0.18 \mu\text{g}/\text{ml} = 1.8 \times 10^{-7} \text{ g}/\text{ml} = 1.8 \times 10^{-5} \% \text{ (w/v)} = \mathbf{0.000018 \% \text{ (w/v)}}.$$

Lipase

Lipases are normally not associated with only reduced proteolytic side activities, since they were from the very beginning produced by recombinant production strains. The concentration of lipases to which consumers may be exposed as a consequence of fabric wearing can be estimated as follows:

The levels of lipase 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 0.034 μg lipase/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 lipase in contact with the skin can be estimated as: $0.034 \mu\text{g}/(\text{g fabric}) \times 0.01 (\text{g fabric})/\text{cm}^2 = \mathbf{0.00034 \mu\text{g}/\text{cm}^2}$.

Assuming a film thickness on the skin of 0.01 cm (Vermeire et al., 1993), the concentration of lipase in contact with the skin can be estimated as:

$$[0.00034 \mu\text{g}/\text{cm}^2] / [0.01 \text{ cm}] = 0.034 \mu\text{g}/\text{cm}^3 = 0.034 \mu\text{g}/\text{ml} = 0.34 \times 10^{-7} \text{ g}/\text{ml} = 0.34 \times 10^{-5} \% \text{ (w/v)} = \mathbf{0.000003 \% \text{ (w/v)}}.$$

5.1.3.4. Accidental or Intentional Overexposure

Eye irritation

Accidental exposure of the eye to amylase, cellulase and lipase will occur in consumers only via splashes or spills with a formulated product. Since all these enzymes have no eye irritation potential, this potential is not considered.

5.2. HAZARD ASSESSMENT

5.2.1. Summary of available toxicological data

The hazard assessment of *Bacillus* α -amylase can be evaluated from the occupational risk assessment by the UK Health and Safety Executive (HSE, 2003). The assessment of the lipase used in detergents has been published (Greenough et al., 1996) as well as a safety evaluation of an alkaline cellulase (Greenough et al., 1991). Only the most essential studies are described in this section.

5.2.1.1. Acute toxicity

Amylase

Inhalation - Studies in animals

Two brief reports are available of single inhalation exposure studies with BAA (*B. subtilis*), conducted according to OECD guidelines. In the first report, rats (5 per sex per group) were exposed to either 1.6 mg/L of a production BAA batch ADTA 202-204, a mixture of two batches prepared by the standard production process (45.9% of particles < 4.7 µm) or 1.08 mg/L of a “salt-free” BAA batch PPY 1316, prepared from production batch ADTA 202-204 by removal of NaCl (33.3% of particles < 4.7 µm), for 4 hours (IRI, unpublished¹). An air-exposed control group was also included. The actual amount of enzyme protein in the test aerosols was 0.114 mg aep/L (production batch) and 0.258 mg aep/L (salt-free batch). No deaths occurred. Slight respiratory depression and red nasal staining were observed in all BAA-exposed rats during exposure only. No other treatment-related findings were noted. These batches were known to contain small amounts of residual protease activity. The extent to which this protease may have contributed to the effects observed in BAA exposed rats is unclear. Andersen *et al* (1987) also reported a study in which rats were exposed to 1.6 mg/L BAA preparation (highest concentration attainable) derived from a genetically modified strain of *B. subtilis* for 4 hours. Total organic solids comprised 83.3% of the test substance. This would include both active and inactive enzyme as well as other organic material and is representative of the organic composition of the commercial product. No adverse effects were observed.

Oral exposure - Studies in animals

Two brief reports are available of the effects of oral exposures to BAAs. Both studies were conducted according to OECD guidelines. In the first study, groups of 10 male and 10 female rats were given an aqueous suspension of 0, 4 or 10 g/kg of BAA (“salt free” batch PPY 1316, enzyme derived from *B. subtilis*) by gavage (Novo Nordisk, unpublished¹). The actual enzyme content of this batch was 239 mg aep/g. In the second study, groups of 5 male and 5 female rats were given an aqueous suspension of 0 or 5 g/kg BAA preparation, derived from *B. licheniformis*, the actual enzyme content of the preparation was 60.13 mg aep/g (Novo Nordisk, unpublished²). At the end of the 14-day observation period rats were sacrificed and a macroscopic post mortem examination was performed. No treatment-related effects were found in neither of the studies. The findings from these studies suggest that BAAs are of low acute oral toxicity.

Cellulase

Inhalation - Studies in animals

In an acute inhalation study (Greenough and McDonald, 1984) a group of five male and five female Sprague-Dawley rats weighing 176-195 g were confined in a nose-only inhalation chamber and exposed for 4 hours to an atmospheric concentration of 3.48 ± 0.6 mg alkaline cellulase/L, this being the highest concentration that could be maintained over the 4-hour period. Batch PPC 1317 was used as supplied, and the test atmosphere was generated by means of a Wright dust feeder. Particle size distribution studies indicated that the mass mean diameter was 7.8 µm with a geometric standard deviation of 2.1 µm.

The inhalation LC₅₀ was not demonstrated in this limit test other than to indicate that the value exceeded 3.48 mg/L.

Oral exposure - Studies in animals

For an acute oral toxicity study in rats and mice (Agger, 1982 a, b) batch PPC 1317 was suspended in tap-water and was given as a single oral dose by gavage to groups of five male and five female animals

after overnight fasting. Mice of the NMRI strain weighing 18 - 23 g were treated with 5, 7.5, 10 or 12.5 g/kg body weight and Wistar rats weighing 65-84 g were given 2.5, 5, 7.5 or 10 g/kg. The animals were observed daily for 14 days after dosing and were then killed and autopsied.

Dose-related decreased motor activity and diarrhoea was observed in the mice. The LD₅₀ in mice was calculated to be 8 g/kg body weight (95% confidence limits, 7.5 - 8.6). The rats showed no clinical signs and all of them survived treatment and the 14-day observation period; therefore the LD₅₀ was demonstrated to exceed 10 g/kg.

Lipase

Inhalation - Studies in animals

In an acute inhalation toxicity study in rats (McDonald, 1988) a group of five male and five female Sprague-Dawley rats weighing 115-142 g were confined in a nose-only inhalation chamber and exposed for 4 hours to an atmospheric concentration of 0.74 ± 0.1 mg/L, this being the highest concentration that could be maintained over the 4-hour period. Test batch A was used as supplied and the test atmosphere was generated by means of an Aerostyle (Aerosyte Co. Ltd. London, UK) dust generator. Particle size distribution studies indicated that the mass mean diameter was 8.2 μ m with a geometric standard derivation of 3.0 μ m.

The inhalation median lethal concentration (LC₅₀) was not demonstrated in this limit test other than an indication that the value exceeded 0.74 mg/L, this being the highest concentration that could be maintained over the exposure period.

Oral exposure – Studies in animals

The acute oral toxicity was tested in rats (Stavnsbjerg, 1988a). Test batch B was suspended in tap water and given once orally by gavage to groups of five male and five female animals after overnight fasting. Wistar rats weighing 70-76 g were given 5 g/kg. The animals were observed daily for 14 days after dosing and were then killed and subjected to autopsy.

The rats showed no clinical signs, and all survived treatment and the 14-day observation period. The median lethal dose (LD₅₀) was therefore demonstrated to exceed 5 g/kg.

5.2.1.2. Corrosiveness/Irritation

Amylase

Skin irritation - Studies in animals

Three studies have been conducted to investigate the skin irritation potential of BAAs derived from *B. subtilis*. In the first two studies, intact and abraded skin sites on groups of 3 rabbits were exposed to 20 or 50% aqueous suspensions of a “salt-free” BAA batch PPY 1316 or a 50% aqueous suspension of a production BAA batch ADTA 202-204, the batches are also described in the beginning of section 5.2.1.1. (Novo Nordisk, unpublished³). Occlusive dressings were used and the duration of exposure was 24 hours. The reactions were evaluated 30-60 min. after patch removal and after further 48 hrs. Primary irritation scores (the total erythema and edema scores are added in both readings and the averages of the scores for intact and abraded skin are combined; this combined average is referred to as the primary irritation score or index) were for the “salt-free” batch 0.6 (20%) and 1.0 (50%). The 50% suspension of the production batch gave a primary irritation score of 0.2. Erythema was stated to be the most pronounced reaction to treatment. These preparations contained proteases (known to have irritating

properties) as an impurity, hence it is not clear if these mild skin reactions were a response to BAA or were due to the “residual” proteolytic activity of the preparation. In the third study, a primary irritation score of 0.21 was obtained following a 4-hour exposure to a BAA derived from a genetically modified strain of *B. subtilis* (total organic solids comprised 83.3% of the test substance) (Andersen et al, 1987). The test method of these skin irritation studies was in compliance with the Code of Federal Regulations 1979, title 16, § 1500.41. Overall, given the low scores that were obtained for BAAs even with “residual” proteolytic activity it is considered that BAAs have a low potential to cause skin irritation.

Eye irritation - Studies in animals

Very mild redness of conjunctiva was seen in only one of six rabbits at 24 hrs. after instillation of a BAA derived from a genetically modified strain of *B. subtilis* in an eye irritation study conducted according to the method described in Code of Federal Regulations 1979, title 16, § 1500.42 (Andersen et al, 1987). No other effects were seen in this study.

Eye irritation - Studies in humans

Signs of skin irritation, not further described, were observed at all concentrations in a repeat insult patch test in which human volunteers received nine topical applications of 1, 2.5, 5 or 10% BAA derived from *B. subtilis*, vehicle was distilled water (ISC, unpublished). The magnitude of responses increased with increasing concentration such that the use of the 10% concentration was discontinued and was replaced for the rest of the study by a 0.5 % BAA. At challenge, using the same concentrations of BAA, there were no significant reactions indicative of skin sensitisation. The reaction scores during induction period suggest, however, that although skin irritation does not appear to occur following a single exposure, repeated dermal exposure to higher concentrations of BAA may result in irritation. The irritation is most probable due to residual protease activity present in the amylase preparation.

Cellulase

Skin irritation - Studies in animals

Two primary skin irritation tests (Stavnsbjerg, 1983 and 1984 a) were carried out (one on each of Batch nos PPC 1247 and PPC 1317) following the method of the Code of Federal Regulations 1979, Title 16, No. 1500.41, adapted to OECD Guidelines for Testing of Chemicals. The backs of six albino rabbits were clipped free of hair and 0.5 g of SP 227 was introduced under gauze (1 in. x 1 in.). Test sites were divided into abraded and intact skin. Patches were secured for the 4-hr exposure period and covered by impervious material. After 4 hr, patches and the remaining SP 227 were removed with water and any skin reactions were evaluated about 1, 24, 48 and 72 hr after removal. A primary irritation index (PII) was calculated and was used to classify SP 227 as follows: PII = 0, non-irritant; $PII > 0 \leq 2$, mild irritant; $PII > 2 < 5$, moderate irritant; $PII > 5$, severe irritant. SP 227, Batch 1247 had a PII of 0.06 and was thus classified as a mild irritant to skin; SP 227, Batch 1317 had a PII of 0 and was classified as non-irritant to skin. The mild irritant category for Batch 1247 resulted from three rabbits having very slight oedema at the dosing site about 1 hr after patch removal. The irritation potential is furthermore depending on the amount of protease contamination in the enzyme test preparation.

Eye irritation - Studies in animals

Two eye irritation tests (Agger, 1982 d; Stavnsbjerg, 1984 b) were carried out (one on Batch PPC 1247 and one on Batch PPC 1317) following the OECD Guidelines for Testing of Chemicals with evaluations made following the Code of Federal Regulations, Title 16, No. 1500.42. Six albino rabbits were used per test: The volume of 0.1 ml SP 227 was instilled into the conjunctival sac to the left eye and the grade of ocular reaction was recorded at 1, 24, 48 and 72 hr after instillation. None of the batches of SP 227 produced any corneal or iris reactions at any time point; with Batch PPC 1317 there was also no conjunctival reaction and this batch was regarded as negative in its potential for eye injury. However, Batch PPC 1247 produced conjunctival reddening in four out of six rabbits after 1 hr, and the reddening was still present in one of the rabbits after an extended observation period of 7 days, but only as a score 1 reading, i.e. the mildest score different from the normal state. Anyway, as a result of the prolonged but mild reaction, this batch was regarded as positive in its potential for eye injury. The proteolytic activity of protease contamination in the enzyme preparation has a significant influence on the test result.

Lipase

Skin irritation - Studies in animals

Three primary skin irritation tests (Berg, 1988 b, d and 1990) in rabbits were carried out (two with test batch C and one with test batch A mixed with equal volumes of propyleneglycol) following the method of the Code of Federal Regulations, Title 16, No. 1500.41 (1979), adapted to OECD Guidelines for Testing of Chemicals (1981 a). The backs of 12 albino rabbits were clipped free of hair and 0.5 g of test material was introduced under gauze (2.5 x 2.5 cm). Test sites for two of the tests were divided into abraded and intact skin. Patches were secured for the 4-hr exposure period and covered with impervious material. Patches and the remaining test material were removed with water after 4 hr and any skin reactions were evaluated at approximately 1, 24, 48 and 72 hr after removal. A primary irritation index (PII) was calculated and used to classify the test material: 0, non-irritant; $0 \leq 2$, mild irritant; $2 < 5$, moderate irritant; and > 5 , severe irritant.

Test batch C had a PII of 0 and 0.27 and was thus classified as a mild irritant to skin; test batch A had a PII of 0 and was classified as non-irritant to skin. The mild irritant category for batch C resulted from four rabbits having very slight erythema and oedema at the dosing site 0.5 – 72 hr after patch removal. The mild irritation is most probable due to residual protease activity present in the lipase preparation.

Eye irritation - Studies in animals

An eye irritation test (Berg, 1988a) in rabbits was carried out with test batch C following the OECD (1981 b) with evaluations made following the Code of Federal Regulations (1980). Three albino rabbits were used, with the volume of approximately 0.1 ml of test material being instilled into the conjunctival sac of the left eye and the grade of ocular reactions being recorded at 1, 24, 48 and 72 hr after instillation. The test material produced no corneal or iris reactions at any time point and produced conjunctival reddening in one rabbit after 1 hr only. No effects were seen in any of the other animals at any reading. As a result, this batch was regarded as negative in its potential for eye injury.

5.2.1.3. Sensitisation

Amylase

Skin sensitisation

- Studies in animals

The potential for BAAs derived from *B. subtilis* to induce skin sensitisation following topical dermal application has been investigated in two studies. In the first study (IRI, unpublished²), a group of 20 guinea pigs was induced with nine, 6-hour, topical applications of a 50% BAA solution (in distilled water). The batch was a production batch, ADTA 202-204, which is also described in the beginning of section 5.2.1.1. Two weeks after the final induction patch was removed, animals were challenged with a further application of the 50% solution and sites were scored at 24 and 48 hours. A positive control group (20 animals) was induced with 0.2% DNCB in a similar manner and further groups of 20 animal were included as irritancy (presumably vehicle) controls for both the BAA and DNCB treatments. These animals remained untreated during the induction phase of the study. No positive reactions were observed from any BAA test or control animal to challenge at a concentration of 50% BAA solution in distilled water. DNCB produced the appropriate responses in controls. The second BAA study was performed with a BAA derived from a genetically modified strain of *B. subtilis* using the procedure as described above. The test material was applied undiluted during induction and challenge, while the positive control animals were induced with 0.1% DNCB in 70% v/v aqueous ethanol. No dermal responses to challenge with the undiluted test material were seen in negative or test group animals, but all positive control animals responded to DNCB (Andersen et al, 1987). Overall therefore under the conditions of this assay, BAA was not identified as a potential skin sensitiser. Given the large molecular size of BAA enzymes and consequent lack of dermal absorption potential, these results are predictable.

- Studies in humans

A human repeat insult patch test (HRIPT) has been conducted in human volunteers using a BAA derived from *B. subtilis* (ISC, unpublished). In the main test, concentrations of 1, 2.5, 5 or 10% BAA (vehicle distilled water) were applied to the skin of 89 volunteers. These concentrations were selected on the basis of skin irritation findings from a preliminary study including 12 subjects where concentrations of 20, 30, 40 and 50% BAA were applied. From the responses it was clear that none of these levels would provide acceptable irritation levels for the main study. Therefore concentrations of 1, 2.5, 5 or 10% BAA in distilled water were chosen. During the 3-week induction period, patches were applied 3 times per week (on Monday, Wednesday and Friday) and left in place for 24 hours. Fourteen days after the final application, challenge patches (same concentrations as during the induction) were applied to both arms of each subject. The responses were graded after 48 and 96 hours. During induction, skin irritation was observed at each of the four concentrations. The magnitude of responses increased with increasing concentration such that the use of the 10% concentration was discontinued and replaced with a 0.5% concentration for the rest of the study. At challenge, using the same concentrations of BAA, i.e. 0.5, 1, 2.5 or 5% BAA, there were no significant reactions indicative of skin sensitisation. As challenge was carried out on healthy intact skin, it is likely that the enzyme was unable to cross the skin, hence a negative result was to be expected.

Respiratory sensitisation

- Studies in animals

The ability of a BAA derived from *B. licheniformis* to induce an antibody response has been investigated in three different animal models. Sarlo et al (1997a) and Robinson et al (1998) used the guinea pig intratracheal test (GPIT). In this test, groups of 10 guinea pigs received intratracheal doses of BAA once a week for 12 weeks and blood samples were collected 24 hours before dosing on weeks 3, 4, 5 and 12 to determine the antibody response. Under the conditions of this test, guinea pigs mounted an IgG₁ response to BAA (IgG₁ is an antibody linked with allergic symptoms in the guinea pig). Sarlo et al (1997a) stated that the appearance of immediate onset respiratory symptoms (respiratory distress such as periodic diaphragmatic spasms or retractions) corresponded to the appearance of IgG₁ antibodies in the sera. The publication does not state whether or not the severity of respiratory changes mirrored the strength of the antibody response. These researchers compared the IgG₁ responses obtained for BAA to the IgG₁ responses obtained from concurrent studies with a proteolytic Subtilisin enzyme (used as a reference substance for this test). A ten times smaller dose of BAA in terms of protein dose was required to give an equivalent IgG₁ response to that for the reference substance. In a separate experiment it was demonstrated using the GPIT that the IgG₁ response to BAA was enhanced when BAA and a proteolytic Subtilisin enzyme were dosed together (Sarlo et al, 1997b).

The antibody response to a BAA derived from *B. licheniformis* has also been investigated using the mouse intranasal test (MINT) (Robinson et al, 1998). Groups of 5 anaesthetised mice were exposed to different concentrations of BAA in saline (typical range between 0.003 - 10 µg enzyme protein/animal) on days 1, 3, 10, 17 and/or 24 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. The range of doses for each enzyme was selected to enable a dose response curve of antibody titre vs dose to be constructed. The authors showed that mice exposed to BAA mounted a measurable IgG₁ response to this enzyme and that increasing the number of doses increased the magnitude of the antibody response to BAA.

The mouse IgE test has also been used to investigate the antibody response to a BAA derived from *B. subtilis* (Hilton et al, 1994). Groups of 10 female BALB/C mice were given two intraperitoneal injections of BAA, each consisting of 250 µL of 1% test material protein in PBS (i.e. each animal received 2.5 mg enzyme protein, which is approx. 1000 x higher dose per animal than used in the above method). Mice given the saline vehicle alone acted as controls. The injections were given a week apart and 1 week after the second dose blood levels of BAA-specific IgG, IgE and total serum IgE were measured. A strong IgG response was observed and BAA-specific IgE was also found. However, BAA did not induce a detectable increase in total serum IgE levels, which is the normal criterion for a positive response in this assay. The publication stated that higher doses of enzyme were not given because it was thought that the enzymatic activity might adversely affect the health of the animals. The authors considered that on the basis of these findings with BAA, and for three other proteins, the mouse IgE test was not a suitable model for investigating respiratory sensitisation caused by proteins.

The studies show that BAAs 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. Overall, the ability of animal models to predict enzyme-induced asthma in humans has not yet been demonstrated.

- Studies in humans (occupational asthma)

In some workplaces skin prick tests are performed as a screen for identifying those who may be at risk of developing occupational asthma. These tests involve intracutaneous administration of the test substance; a wheal and flare reaction within 15-20 minutes indicates the presence of substance specific IgE. It is the IgE antibody which is largely associated with the immunological mechanism of asthma in relation to proteins, and this underlies the rationale for performing the skin prick test.

UK SWORD statistics

Three cases of occupational asthma due to BAAs have been reported to SWORD in 1999. These cases all occurred among detergent workers and HSE understands that they derived from a particular factory where there was inadequate control over a period of time. Prior to 1999, no cases of occupational asthma have been reported to SWORD that can be specifically linked with BAAs, although because of the nature of the scheme it is not always possible to identify the precise causal agent for individual cases.

Cellulase

Skin sensitisation - Studies in animals

This study (Skin sensitisation: delayed contact hypersensitivity in guinea-pigs (Cuthbert and D'Arcy-Burt, 1984)) was performed after the method described by Buehler (1965). SP 227 (Batch no. 1317) and dinitrochlorobenzene (DNCB, an established sensitiser), were tested for induction and challenge procedures on groups of 20 guinea-pigs each. A closed patch with 0.5 ml test solution was applied to the clipped back of the guinea-pigs for 6 hr on each of three occasions, test solutions for SP 227 being prepared as 50% (w/w) in distilled water and for DNCB as 0.2% (w/w) in propylene glycol. 2 week after the third application the animals were challenged with identically prepared patches. Two further groups, each of 20 previously untreated guinea-pigs were also exposed simultaneously with the challenge using identical patches, in order to check for irritancy. The incidence and severity of the reactions were examined 24 and 48 hr after final patch removal; health status was monitored throughout the study.

In both the SP 227 challenge and SP 227 irritancy groups, negative results were recorded. In the DNCB irritancy group there were negative results, and in the DNCB challenge group 15/20 guinea-pigs showed evidence of sensitisation. It was concluded that sensitisation had not been shown with SP 227.

Lipase

Skin sensitisation - Studies in animals

Skin sensitisation: delayed contact hypersensitivity in guinea pigs (Berg, 1988c): A modified Draize test was performed after the method described by Johnson and Goodwin (1985). Test batch C was tested for induction and challenge procedures on groups of 20 guinea pigs each.

The test consisted of an induction phase, followed by a resting period and a subsequent challenge phase to prove whether sensitisation occurred. The induction was performed by four intradermal injections of 0.1 ml of test material at a concentration of 500 LU/g, at sites overlying the axillary and inguinal lymph nodes in 20 animals. 10 animals were injected similarly with 0.9% sterile saline (negative control). All animals were challenged epicutaneously using a 6-hr occluded patch 13 days later with test material (30,000 LU/g). One week later all animals were challenged intradermally in the flank with 0.1 ml test material (200 LU/g). All animals were rechallenged epicutaneously 1 week later to confirm the first epicutaneous challenge.

At the first epicutaneous challenge positive responses were seen in three of 20 test animals, whereas negative responses were noted in all the control animals. The intradermal challenge did not reveal any significant difference between test and control animals and the second epicutaneous challenge produced positive responses in one test animal and four control animals. Negative results were recorded in both the rechallenge and irritancy groups. It was therefore concluded that sensitisation had not been shown with the lipase.

5.2.1.4. Repeated Dose Toxicity

Amylase

Studies in animals

No studies have been conducted to examine the systemic effects of repeated inhalation or dermal exposures to BAAs. Studies of up to 90 days duration have been conducted by the oral route in both rats and dogs. None of these studies were reported in any detail and the actual amount of enzyme in the formulations tested in these studies is unclear. No findings of toxicological significance were observed in either species exposed to any of the BAA formulations tested other than “slight” reductions in food consumption at high dietary doses (5% of the diet or more) and irrigative changes in the stomach of rats gavage with 3000 mg/kg/day or more (Novo Nordisk, 1990; LSR, unpublished; IRI, unpublished³; Andersen et al, 1987; Novo Nordisk, unpublished⁴; Mackenzie et al, 1989).

Studies in humans

No information is available on the effects, other than allergen city, of exposure to BAAs in humans.

Cellulase

Studies in animals

In a 4 week study (Husband and Wood, 1983), groups of five male and five female Sprague-Dawley rats were dosed once a day by gavage at dose levels of 0 (control), 0.3, 1 or 3 g/kg body weight. SP 227 (Batch PPC 1317) was suspended in distilled water and administered at a dose volume of 10 ml/kg. In a subsequent 13 week study (Perry et al, 1990) groups of 20 male and 20 female Sprague-Dawley rats were fed diets formulated with SP 227 (Batch no. PPC 2809) to give dose levels of 0, 0.12, 0.6 and 3 g/kg body weight.

Routine clinical observations, and determinations of body weights and food consumption were undertaken throughout the study periods. Haematological and clinical chemical investigations were carried out at the end of the treatment periods and all rats were subjected to gross pathological examination and organ-weight analyses. Microscopic examination was carried out on a comprehensive list of tissues taken from animals in the control and high-dose groups in both studies.

No treatment-related clinical signs were seen in either study. There was a slight dose-related increase in body-weight gain (approx. 7%) in the males from the 4 week study and a reduction in body-weight gain in both sexes at the high-dose level in the 13 week study (10% in males and 20% in females). A slight reduction in food consumption was also noted for the high-dose rats in the 13 week study. Water consumption was not affected by treatment in either study.

Haematological and clinical chemical investigations did not reveal any changes after 4 week of treatment. In the 13 week study, there was an increase in alkaline phosphatase of 26% in intermediate-dose females and 68% in high-dose females. Gross and microscopic pathology and organ-weight analyses gave no indication of adverse responses to treatment in either study.

Lipase

Studies in animals - Subacute oral toxicity

In a two week study (McDonald and Parkinson, 1988) groups of 10 male and 10 female Sprague-Dawley rats were dosed once per day by gavage at dose levels of 0 (control), 0.2, 2 or 10 g/kg. Test batch A was suspended in distilled water vehicle and administered at a dose volume of 10 ml/kg.

In a subsequent 13 week study (Perry et al, 1989) groups of 20 male and 20 female Sprague-Dawley rats were administered test batch A dissolved in distilled water at a dose volume of 10 ml/kg to give dose levels of 0, 0.2, 1 and 5 g/kg.

Routine clinical observations, body weights and food consumption measurements were undertaken throughout the study periods. Terminal haematology and clinical chemistry investigations were performed and on completion of the respective treatment periods all animals were subjected to gross pathological examination and organ weight analyses. Microscopic examination of a comprehensive list of tissues was undertaken for control and high-dose animals in both studies.

No treatment-related clinical signs were seen in either study. Body weight gain, and food and water consumption were not affected by treatment. Haematology and clinical chemistry investigations did not reveal any changes. Gross and microscopic pathology and organ weight analysis gave no indication of adverse responses to treatment.

5.2.1.5 Genetic Toxicity

Amylase

Studies in bacterial systems

The mutagenic activity of a BAA derived from *B. licheniformis* has been examined in *S. typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 (Novo Nor disk, 1989). Owing to the presence of histidine in crude enzyme preparations, a treat-and-plate protocol was followed. Bacterial cultures were exposed for three hours to a range of six concentrations of the BAA preparation up to 10 mg dry material/ml, in phosphate-buffered nutrient broth both with and without rat liver S-9. The actual enzyme content of this test material was 63.9 mg aep/g dry material. All tests included appropriate controls. After incubation, the exposed cells were separated from the test material by centrifugation prior to plating. Negative results were obtained for this enzyme, both with and without rat liver S-9 mix. Positive and negative control cultures responded as expected. The results were verified by a second independent test.

Similarly negative results were obtained for a BAA derived from *B. subtilis* when tested as described above at concentrations of up to 10 mg test material/ml (Andersen et al, 1987).

Studies in mammalian systems

The ability of a BAA derived from *B. licheniformis* to induce chromosome aberrations has been assessed in human lymphocytes, obtained from one donor only (Microtest, unpublished). Duplicate cultures were used. Chromosome aberrations were scored from cultures exposed to 2113, 3250 or 5000 µg/ml BAA preparation, actual enzyme content was 60.13 mg aep/g (5000 µg/ml was apparently a concentration which induced mitotic inhibition both with and without S9, approximately 57% and 52% respectively). Appropriate vehicle and positive control cultures were included. At 5000 µg/ml a non-reproducible increase in total structural and numerical aberrations was observed in one of the two replicates and small increases in polyploid cells (over concurrent controls) were observed in some treated cultures both in the absence and presence of S-9. There were no effects at lower concentrations. Given that the aberrations were seen at the top dose only and were not reproducible, it is considered that the results of this test should be regarded as negative.

Studies in vivo

Andersen et al, (1987) found no evidence to indicate that BAA was clastogenic in an *in vivo* bone marrow chromosomal aberrations study in rats. Dose levels of up to 5000 mg/kg were administered, by the oral route. A clear positive result was obtained for the positive control substance chlorambucil.

Cellulase

Studies in bacterial systems

In a gene mutation test (Pedersen, 1984) SP 227 (Batch no. PPC 1317) was examined for mutagenic activity using *Salmonella typhimurium* strains TA 1535, TA 100, TA 1537 and TA98.

Since the test material was a crude enzyme preparation containing free amino acids such as histidine, a liquid culture assay was used. Bacteria were exposed to five doses of SP 227 (from 0.1 to 10 mg/ml incubation mixture at half-log intervals) in a phosphate buffered nutrient broth for 3 hr. After incubation the test substance was removed by centrifugation before plating. The numbers of revertants to prototrophy and of viable cells were estimated.

The test was conducted in the presence and absence of metabolic activation - a liver preparation from male rats pretreated with Aroclor 1254, and the co-factors required for mixed-function oxidase activity (S-9 mix). The sensitivity of the individual bacterial strains was confirmed by significant increases in the number of revertant colonies induced in similar liquid conditions by diagnostic mutagens.

No dose-related increases in revertants to prototrophy were obtained in any of the tests performed. All results were confirmed in an independent experiment. It was concluded that there were no indications of mutagenic activity of SP 227 in the presence or absence of metabolic activation.

Studies in mammalian systems - Chromosome aberrations

For testing chromosome aberrations (Asquith, 1983) groups of five male and five female Sprague-Dawley rats were dosed orally by gavage for five consecutive days with 0, 0.3, 1.0 or 3.0 g SP 227 (Batch no. PPC 1317) kg body weight/day and killed 6 hr after the final dose. A further group of five male and five female rats was given a single ip dose of 100 mg methyl methanesulphonate/kg as a positive control and these rats were killed after 24 hr. One hour before the rats were killed they were treated with democolcine to arrest cells in metaphase. Slides of bone marrow cells were prepared and examined microscopically for chromosomal damage and mitotic indices were derived.

The positive control rats showed clear chromosome damage. There were no significant increases in chromosome aberrations, excluding gaps, in males or females at any dose level of SP 227. Female rats treated with SP 227 showed increases in aberrations including gaps at all doses but with no clear dose relationship and only statistically significant (chi squared = 5.02; P = 0.025) at 3.0 g/kg/day. Treatment with SP 227 had no effect on the mitotic capacity of bone marrow cells and it was concluded that SP 227 was not a chromosome mutagen for the rat *in vivo*.

Lipase

Studies in bacterial systems - gene mutation

In a gene mutation test (Pedersen, 1988) test batch A was examined for mutagenic activity using *Salmonella typhimurium* strains TA1535, TA100, TA1537 and TA98 and *Escherichia coli* WP2uvrA (pKM101).

Lipase activity was removed from the test material by ultrafiltration before testing because of its destructive action on the metabolic activation system (S-9) and the bacterial cell wall. A liquid culture assay was used. Bacteria were exposed to five doses of test material (from 0.1 to 10 mg/ml incubation mixture at half-log intervals) in a phosphate buffered nutrient broth for 3 hr. After incubation the test substance was removed by centrifugation before plating. The numbers of revertants to prototrophy and viable cells were estimated. The test was conducted in the presence and absence of metabolic activation, namely, a liver preparation from male rats pre-treated with Aroclor 1254 and cofactors required for mixed function oxidase activity (S-9 mix). The sensitivity of the individual bacterial strains was

confirmed by notable increases in the number of revertant colonies induced in similar liquid conditions by diagnostic mutagens.

No dose-related increases in revertants to prototrophy were obtained in any of the tests performed; all results were confirmed in an independent experiment and it was concluded that there were no indications of mutagenic activity in the presence or absence of metabolic activation.

Studies in mammalian systems - Chromosome aberrations

Chromosome aberrations (Marshall, 1988) were examined with test batch A in an *in vitro* cytogenetics assay using human lymphocyte cultures from a male and a female donor. Treatments were performed in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction (S-9) from Aroclor-1254 induced animals. The test compound dose levels for analysis were selected by determining mitotic indices from a broad range of doses up to 5000 µg/ml. There was no clear evidence of any treatment-related mitotic inhibition at any of the dose levels analysed.

Appropriate negative (solvent) control cultures were included in the test system and contained low incidences of chromosomal aberrations within historical solvent control ranges. Methyl methanesulfonate and cyclophosphamide (CPA) were used as positive control chemicals in the absence and presence of liver S-9, respectively. Both compounds induced statistically significant increases in the incidence of chromosomal aberrations. It was considered important that the presence of lipase did not affect the activity of the S-9 liver preparation. This was achieved by showing that S-9-mediated clastogenicity of CPA was not reduced in the presence of test material up to 5000 µg/ml.

Treatment of cells with test material in both the absence and presence of S-9 resulted in numbers of aberrations which were similar to those observed in concurrent negative controls. A small but statistically significant increase in “total aberrations including gaps” which pushed the category total in the female donor outside the normal range, was observed at the intermediate dose level in the absence of S-9 but was not considered to be of biological significance. There were no significant differences between treated cells and controls at any other dose level in either the absence or presence of S-9 and aberration frequencies fell within historical control ranges.

It is concluded that the test material was unable to induce chromosome aberrations in human lymphocytes when tested up to 5000 µg/ml in either the absence or presence of S-9.

5.2.1.6. Carcinogenicity

Amylase: Carcinogenicity studies have not been conducted for any BAA. However, no carcinogenic potential would be predicted for this class of substance

Cellulase: no data available

Lipase: no data available

Carcinogenicity is not expected for enzyme preparations in general since:

- there is no indication in the public 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 amylase, cellulase, lipase as well as for Subtilisin available. However, a carcinogenic potential is not to be expected. Because there are no indications for a carcinogenic effect of these enzymes (see above), the performance of such studies is not warranted for animal welfare grounds.

5.2.1.7. Developmental Toxicity

Amylase

Studies in animals - Fertility

Two BAAs, one derived from *B. stearothermophilus* and one derived from a genetically modified strain of *B. subtilis* have been evaluated for effects on fertility in one-generation studies in rats (Mackenzie et al, 1989). BAA derived from *B. stearothermophilus* was fed for 13 weeks to groups of 12 male and 24 female rats in diets containing 0, 36 or 72 units of BAA/g food. The same concentrations of BAA from genetically modified *B. subtilis* were fed to groups of 26 male and 26 female rats for 4 weeks. After these initial dosing periods, parental rats were mated and continued on their respective diets during mating and through to weaning. Litters were examined on the day of delivery and after four days pups were culled to give litters of 10 or 8 pups respectively, each litter contained equal numbers of male of female pups where possible. At weaning, F₁ pups were randomised into groups of 20 males and 20 females and these pups continued on the diet received by their parents for 13 weeks. The remaining pups were killed at weaning. Extensive biochemical and haematological examinations and urinalysis were conducted on parental animals before the start of the study, on F₁ animals given BAA derived from *B. stearothermophilus* seven weeks into the post-weaning phase and on F₁ animals given either BAAs at the end of the post-weaning phase. Full necropsies were performed on the parental animals. Culled pups were examined externally when killed and microscopic examinations of tissues were performed on a range of tissues from pups allowed to continue with the test diet after weaning. No treatment-related effects on fertility or other findings of toxicological significance were observed for either enzyme.

5.2.2. Identification of critical endpoints

The data presented in Chapter 5.2.1 show that the key hazard associated with amylase, cellulase and lipase used in detergents is respiratory (Type 1) allergy. This observation is in full agreement with the results with the detergent protease Subtilisin.

Other than respiratory allergy, eye and skin irritation effects are the only hazards described for amylase and cellulase. For lipase a mild skin irritation potential was observed. The problem with the identification of critical endpoints for allergy and irritation is that the test material always contains certain amounts of protease which has an intrinsic higher potential for allergy and irritation than amylase, cellulase and lipase have.

Since none of these enzymes is used in detergents without proteases, the irritation potential of protease and protease contamination is covered by the risk assessment of Subtilisin.

5.3. RISK ASSESSMENT

For type 1 respiratory allergy to enzymes a traditional margin of exposure calculation cannot be done, as there is no well defined no effect level. 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 induction of IgE and levels where such effects are not generated. Sensitisation is used as a biomarker but is not representing an adverse health effect.

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 JJ and Covello VT, 1989). 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).

There are no documented cases of consumers sensitised against amylases, cellulases and lipases. This is consistent with the fact, that they have been used in detergents only with high quality granulated or liquid enzyme products.

The ACGIH proposed an occupational limit of 60 ng/m³ for Subtilisin, 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/m³ (see 5.1.3.2 above). Thus the levels are several orders of magnitude below the factory limit as well as those seen in Swedish studies documenting cases of allergy to Subtilisin in consumers (Belin, 1970). An average peak exposure of 212 ng/m³ was estimated for those cases.

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/m³ range. The benchmark here is the abrupt opening of institutional dishwashers using higher enzyme concentrations, where levels of approximately 2 ng/m³ Amylase 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 prospective skin prick testing of 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. 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/m³ 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/m³ 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/m³). These women also used the bar for personal cleansing with measured exposures less than 0.01 ng/m³ (Cormier, 2004). Another study (conducted in Egypt) reported that exposures up to 0.5 ng/m³ 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/m³; and adverse effects (allergic symptoms) are absent when exposure is in the range of 1 ng/m³ 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/m³, 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 and Eye Irritation Benchmarks

In animal studies concentrated amylase, cellulases and lipases are mildly irritant to skin and eyes. The irritation potential of aqueous solutions of these enzymes depends on the concentration. As reported in the irritation hazard section (5.2.1.2) of this assessment, aqueous solutions of these enzymes at concentrations up to 10% aep amylase in a single exposure failed to show any irritation effects.

As amylase concentrations in washing solutions are well below 10%, the contact of skin with such solutions does not pose a relevant risk for irritation.

Skin contact with amylase, cellulase and lipase deposits on washed fabrics will also not cause skin irritation. The levels of irritation caused by the possible contaminating substance 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 10% figure mentioned above.

In the course of laundry pre-treatment, skin contact with concentrated powder paste, or neat liquid detergent may occur (maximum amylase 0.04%, cellulase 0.03% and lipase concentration 0.01%). 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 enzyme 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 enzyme 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 enzyme concentrations employed therein and comparing these concentrations to the lower levels used in consumer products, accidental eye contact with enzymes 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

Respiratory (Type 1) allergy is the critical endpoint for all detergent enzymes. This became evident in occupational medical surveillance, when in 1969 the protease 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, amylases, cellulases and lipases are solely used in the form of non-dusting, coated granulates or non-volatile liquids.

Consumers can be exposed via the respiratory route to amylases, cellulases and lipases during the task of dispensing powder products in the washing machine (amylases: 0.11 ng/m³, cellulases: 0.08 ng/m³, and lipases: 0.016 ng/m³) or in the sink for hand wash (amylases: 0.006 ng/m³, cellulases: 0.005 ng/m³ and lipases: 0.001 ng/m³) or by suddenly opening the dish washer during the cleaning step (amylase < 1.9 ng/m³). 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/m³. Allergic symptoms under occupational conditions can be excluded when exposure is in the range of 1 ng/m³ (Peters et al., 2001). There appears to be a complex relationship among frequency, magnitude and duration of exposure to 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 0.01 ng/m³, 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, thus making, the margin of safety proportionately greater.

It has to be noted that in view of the widespread use of products containing detergent enzymes, the risk of allergy in consumers was extremely low even in the 60's and 70's when the materials were uncoated.

Other than for respiratory allergy, there exists a hazard for skin and eye irritation by preparations of amylases, cellulases and lipases due to contaminating protease. Consumers may be exposed by skin contact during laundry hand wash (amylases: 0.0004%), by laundry pre-treatment using liquid detergent (amylases: 0.04%), and by fabric wear with skin in contact with these enzymes deposited during the wash at levels that are by a factor of one to ten below the levels of Subtilisin. Since the irritation effects of these enzymes were also seen at a tenfold level of that of Subtilisin and contact with Subtilisins is not a cause of concern, it can be concluded that there is no risk seen in such a skin contact with amylases, cellulases and lipases. The level of contaminating protease is in most modern enzyme preparations derived from genetically engineered production strains much reduced relative to the older preparations (see 3.3.1).

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

6. REFERENCES

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7. CONTRIBUTORS TO THE REPORT

AMFEP (Leading Trade Association), Genencor, Henkel, McBride, Novozymes, Procter&Gamble, Unilever

8. ABBREVIATIONS

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)
BAA	Bacillus amyloliquefaciens α -Amylase
BOC	Biological Oxygen Demand
bw	body weight
CBD	Cellulose Binding Domains
COD	Chemical Oxygen Demand
DNCB	Dinitrochlorbenzene
DOC	Dissolved Organic Carbon
ERA	Environmental Risk Assessment
EUSES	European Union System for Evaluation of Substances
GI	Gastrointestinum
GMM	Genetically Modified Microorganisms
GPIT	Guinea Pig Intratracheal Test
HERA	Human and Environmental Risk Assessment
HSE	Health and Safety Executive (UK)
IUCLID	International Uniform Chemical Information Database
LAS	Linear Alkylbenzene Sulphonate
LOEL/LOEC	Lowest Observed Effect Level/Lowest Observed Effect Concentration
LOAEL/LOAEC	Lowest Observed Adverse Effect Level/ Lowest Observed AdverseEffectConcentration
MINT	Mouse Intranasal Test
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
NICNAS	Australia's National Industrial Chemicals Notification and Assessment Scheme
OECD	Organisation for Economic Co-operation and Development
OECD TG	OECD Testing Guideline
PNEC	Predicted No Effect Concentration
RAST	Radio-Allergo-Sorbent-Test
RCR	Risk Characterisation Ratio
STP	Sewage Treatment Plant
SDA	Soap and Detergents Association (USA)
SDIA	Soap and Detergent Industry Association (UK)
TAED	Tetraacetylenethylenediamine
TGD	EU Technical Guidance Document
TOS	total organic carbon solids, defined as $100\%-(A+W+D)\%$, where A = ash content, W = water content, D = content of diluents TOS in some studies is used to define the amount of enzyme protein