

REVIEW ARTICLE

The toxicology and immunology of detergent enzymes

David Basketter¹, Ninna Berg², Francis H. Kruszewski³, Katherine Sarlo⁴, and Beth Concoby⁵

¹DABMEB Consultancy Ltd, Sharnbrook, Bedfordshire MK44 1PR, UK, ²Novozymes A/S, Bagsvaerd, Denmark,

³American Cleaning Institute, Washington DC, USA, ⁴Procter & Gamble, Cincinnati, OH, USA, and

⁵Genencor, A Danisco Division, Palo Alto, CA, USA

Abstract

Detergent enzymes have a very good safety profile, with almost no capacity to generate adverse acute or chronic responses in humans. The exceptions are the limited ability of some proteases to produce irritating effects at high concentrations, and the intrinsic potential of these bacterial and fungal proteins to act as respiratory sensitizers, demonstrated in humans during the early phase of the industrial use of enzymes during the 1960s and 1970s. How enzymes generate these responses are beginning to become a little clearer, with a developing appreciation of the cell surface mechanism(s) by which the enzymatic activity promotes the T-helper (T_H)-2 cell responses, leading to the generation of IgE. It is a reasonable assumption that the majority of enzyme proteins possess this intrinsic hazard. However, toxicological methods for characterizing further the respiratory sensitization hazard of individual enzymes remains a problematic area, with the consequence that the information feeding into risk assessment/management, although sufficient, is limited. Most of this information was in the past generated in animal models and *in vitro* immunoassays that assess immunological cross-reactivity. Ultimately, by understanding more fully the mechanisms which drive the IgE response to enzymes, it will be possible to develop better methods for hazard characterization and consequently for risk assessment and management.

Keywords: IgE allergy, enzymes, detergents, toxicology

Introduction

It has long been recognized that enzymes, such as those produced industrially by bacterial and fungal fermentation that are used in detergent products, have the potential to give rise to respiratory allergy in suitably exposed individuals (Flindt, 1969; Pepys et al., 1969; Zachariae et al., 1981; Cullinan et al., 2000). Indeed, the historical experience of industrial use of these enzymes several decades ago demonstrated that this was very much the case in practice (reviewed in Sarlo, 2003). Furthermore, there is evidence that indicates that they are among the more potent respiratory allergens that are known (Baur et al., 1998; Heederik et al., 2002). However, when looking at the total number of patients reported by occupational or chest physicians, less cases of asthma are ascribed to industrial enzymes compared to other respiratory allergens like isocyanates, laboratory animals, and wood dust (McDonald et al., 2000).

Major progress has been made to reduce the health risks associated with occupational exposure to industrial enzymes and respiratory allergy to enzymes of consumers using enzyme-containing detergent products is not reported. In many countries of the world the current situation seems to be one of an increasing degree of atopicity in the general population (Holgate, 2004; Eder et al., 2006) along with occasioned heightened levels of concern surrounding materials that possess the intrinsic ability to induce sensitization of the respiratory tract. The bacterial and fungal enzymes, including those used in modern detergents (Johnsen et al., 1997; Olsen and Faholt, 1998), fall under this description well, as their main intrinsic hazard is the ability to cause sensitization of the respiratory tract. Consequently, it was very timely that the American Conference of Governmental and Industrial Hygienists re-addressed the general topic of the safety of such enzymes, with particular focus on the

Address for Correspondence: Dr David A. Basketter, DABMEB Consultancy Ltd, Sharnbrook, Bedfordshire MK44 1PR, UK.
Tel: 44(0)1234782944. Fax: 44(0)1234782944. E-mail: dabmebconsultancyld@me.com

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end-point of respiratory sensitization for occupational health (ACGIH, 2004).

Other papers in this volume consider important occupational hygiene aspects associated with the use of enzymes (Johnson et al., 2012). In this paper, we review the toxicology and immunology of enzymes, examining the aspect of the sensitization potential of these materials. The most important questions relate to how the potential intrinsic hazards can be identified and characterized so as to provide an adequate basis for safety assessment. However, consideration is also given to the developing understanding of why certain proteins, and especially enzymes, seem particularly able to stimulate the formation of IgE antibodies.

Why are enzymes allergens?

It is entirely natural that, when exposed to foreign proteins, the human immune system mounts a response to ensure the non-self material is efficiently removed. Such processes have of course evolved very much in the context of the maintenance of self in the face of invasion by foreign organisms and the proteins they produce. Hence, in common with other mammals, humans possess a highly evolved defensive system, with both natural and adaptive immune mechanisms (Mak and Saunders, 2008; Playfair and Chain, 2009). However, of the vast array of foreign proteins to which the human body is exposed, only a very modest number have commonly been associated with IgE-mediated allergy. These proteins currently comprise a select group of ≈ 1000 members; i.e., one database (AllFam; <http://www.meduniwien.ac.at/allergens/allfam>) in mid-2008 contained a total of 954 protein allergens. What renders these proteins, and notably enzymes, allergenic?

The innate immune response evolved principally as a rapid, frontline system for dealing with invading organisms (e.g., viruses, bacteria, parasites), and acts in concert with, and as a modifier of, the adaptive immune system. The adaptive immune response is characterized by memory, sensitivity, and specificity. Lymphocytes, divided into T- and B- types (Cooper et al., 1965), and subsequently functional sub-populations of T-lymphocytes, are critical to adaptive immune responses (Mosmann et al., 1986). The T-helper (T_H)-2 sub-population is stimulated by certain chemical allergens (Dearman et al., 1996, 2009) and proteins (Colavita et al., 2000), and these T_H -2 cells are responsible for promoting the differentiation of antibody responses towards the production of IgE by B-lymphocytes. The current understanding of this process has been well reviewed recently and does not need to be repeated here (Nakayama and Yamashita, 2008), but it is interesting to note that further developments in T-helper cell biology continue and may contribute significantly to this story in the future (Bettelli et al., 2008).

Since all foreign proteins have the potential to generate an immune reaction, the main question to address is why certain proteins generate the quality and quantity

of response that makes them able to act as powerful respiratory allergens. The majority of these proteins fit into a fairly restricted number of families (Radauer et al., 2008) and likely contain one or more immunogenic/allergenic epitopes capable of stimulating T_H -2 driven immune responses. Unfortunately, these authors conclude 'The small number of protein families that contain allergens and the narrow functional distribution of most allergens confirm the existence of yet unknown factors that render proteins allergenic' (p 847). Additional signals are required for both the initiation and the evolution of the immune/allergenic response. Encountering a protein in the presence of 'danger signals' (Matzinger, 1994, 2002) divert the immune response from the normal tolerance/elimination pathway to a T_H -2 response with the development of IgE. Importantly, enzyme proteins also possess other active properties, i.e., their enzymatic function, which may also impact the characteristics of the immune response.

Evidence has been accumulating which suggests that proteolytic activity of enzymes can act as T_H -2 adjuvants (reviewed in Chapman et al., 2007) providing additional signals to push the immune response to allergy. Whilst much of this work has not been carried out directly on the types of proteolytic enzymes used in detergents products, it is reasonable to suppose that the results may be applied to them, not least since the types of catalytic activity involved are similar. Furthermore, the pathways that may be involved in this adjuvant activity are being elucidated. Cysteine/serine proteases have been shown to cleave the low affinity IgE receptor (CD23) from B-lymphocytes which was proposed as a key part of the mechanism of feedback inhibition of IgE production, and so increasing the levels of IgE (Shakib et al., 1998). Other proteolytic actions also promote the production of 'danger signals', by damaging directly the respiratory epithelium (Adam et al., 2006; Reed, 2007) and/or its repair (Hughes and Page, 2007), stimulate the migration of pro-inflammatory cells (Shin et al., 2006) and the recruitment of dendritic cells (Pichavant et al., 2005).

Proteases also interact with the transmembrane proteins referred to as protease-activated receptors (PARs), whose activation on epithelial cells leads directly or indirectly to a plethora of pro-inflammatory consequences (reviewed in Reed and Kita, 2004; Chapman et al., 2007). These actions appear to be due to the proteolytic activity, as inactivated materials lack the property (Kheradmand et al., 2002). This does of course present a difficulty for non-proteolytic enzymes, which still possess a similar degree of respiratory allergenic potential, a paradox discussed, but not resolved, in a review by Chapman et al. (2007). It is possible to conjecture that just as proteases may directly attack susceptible sites on cell membrane proteins such as PARs, so other types of enzymes such as lipases may modify lipid membrane structure and indirectly affect cell membrane protein function, and amylases and cellulases may have some other indirect, but equally effective, action. However, at present it is not

possible to confirm or refute whether specific types of enzymes action are always directly linked to allergenicity.

The toxicology of enzymes

The toxicology of enzymes is unremarkable, with the exception of potential irritating effects usually associated with some proteases at high concentrations, and the allergenic effects of enzymes. Like anything one is exposed to, the dose makes the poison; thus, with certain enzymes, a sufficient excess in exposure can lead to irritation. Enzymes are like the majority of food proteins so that oral exposure to the gut is entirely without effect, except perhaps at very high, unrealistic, exposure levels (Griffith et al., 1969; Pariza and Foster, 1983; Ashby et al., 1987; Greenough et al., 1991, 1996; Hjortkjaer et al., 1993; Pariza and Johnson, 2001; van Dijck et al., 2003). Any direct dermal toxic effect is limited to skin irritation when dealing with proteases at substantial exposure. No doubt, sufficient exposure by inhalation would produce acute irritating effects, certainly of a nuisance dust type (although enzymes are now supplied as non-dusty encapsulates), but this is limited because of the controls put in place concerning the allergenic effects of enzymes (see below).

Enzymes have been evaluated for genotoxic effects (although it should be kept in mind that the assays were designed for the evaluation of chemicals, not proteins). Nevertheless, enzymes are not mutagenic in the standard screening assays used in genetic toxicology, specifically the Ames test, and are not clastogenic in chromosome aberration tests. This is expected based of their intrinsic properties and given appropriate methods of production (Griffith et al., 1969; Pariza and Foster, 1983; Ashby et al., 1987; Greenough et al., 1991, 1996; Hjortkjaer et al., 1993; Pariza and Johnson, 2001; van Dijck et al., 2003). Importantly, these tests also ensure that any residual materials from the production fermentation are also without effect.

Repeated exposure studies on a number of enzymes have been completed. Most often this has been done in relation to enzymes to be used in foodstuffs and so exposure has been carried out via the oral route. Without exception, enzymes have shown no significant effects (Griffith et al., 1969; Pariza and Foster, 1983; Ashby et al., 1987; Greenough et al., 1991, 1996; Hjortkjaer et al., 1993; Pariza and Johnson, 2001; van Dijck et al., 2003). This is an entirely predictable outcome as the materials being evaluated appear to the test system simply as another source of food proteins. Acute eye and skin irritation tests show that the enzymes are not irritating, with the exception of the proteases where these are classified as mildly irritating. Occlusive patch tests on humans showed that the proteases had no effect levels greater than 5 µg active enzyme protein/cm². We are not aware of any *in vivo* repeat dermal exposure studies, but, since there is no toxicity by the oral route and percutaneous penetration of proteins is extremely low (Smith Pease et al., 2002), this

is of no toxicological relevance, a conclusion confirmed in a human 10-day daily occluded patch test study (Berg, unpublished data).

It has been recognized for many years that, where there is excessive occupational exposure, then skin irritation may result (Griffith et al., 1969; Zachariae et al., 1973). However, either in the occupational setting or in all normal uses and reasonably foreseeable misuses of detergent products containing proteolytic enzymes, skin irritation does not occur (reviewed in Basketter et al., 2008). In contrast, the amylases, lipases, and cellulases used in detergents lack any skin (or eye) irritation hazard and, thus, cannot give rise to any occupational or consumer risk (Griffith et al., 1969; Zachariae et al., 1973; Pariza and Foster, 1983; Ashby et al., 1987; Greenough et al., 1991, 1996; Hjortkjaer et al., 1993; Pariza and Johnson, 2001; Smith Pease et al., 2002; van Dijck et al., 2003; Basketter et al. 2008).

The other skin end-point of interest is contact sensitization. The clinical experience with enzymes used in detergent products clearly shows a lack of contact skin sensitization potential. This is based on data from controlled clinical studies along with careful observations made from occupationally exposed populations. Patch test studies in humans show the lack of skin sensitization of protease enzymes. In contrast, testing enzymes in predictive tests long established and validated for the identification of potential skin sensitizers among chemicals can lead to positive responses to enzymes. These tests include the guinea pig maximization test (Magnusson and Kligman, 1970; Wahlberg and Boman, 1985; Cronin and Basketter, 1994), the occluded patch test of Buehler (Buehler, 1965; Basketter and Gerberick, 1996), and the murine local lymph node assay (LLNA) (Dean et al., 2001; Gerberick et al., 2005). These models report the results of several hundred tests on chemicals, and so demonstrate that the methods are valid, but were not in fact designed to cope with proteins (ICCVAM, 2008). Unsurprisingly, when guinea pigs and mice are suitably exposed to the bacterial and fungal enzymes used in detergents, positive results can be obtained, exactly as they should be for any foreign protein introduced into such a test system (Coenen et al., 1995; Bergman and Broadmeadow, 1997; Festersen et al., 2008). Consistent with the guidance from new EU legislation (Commission of the European Union (EU), 2006), the model data do not match the experimental or diagnostic clinical experience (White et al., 1985; Hjortkjaer et al., 1986; Andersen et al., 1998; Belsito et al., 2002; Brinch and Pedersen, 2002). We can, therefore, conclude that detergent enzymes do not present any skin sensitization hazard.

However, it is quite clear that enzymes can give rise to significant immunopathology (reviewed in Bernstein and Sarlo, 2006). Enzymes are respiratory sensitizers, and sufficient exposure can lead to symptoms of allergy including asthma. The first introduction of bacterial proteases into detergent products was associated with a serious outbreak of occupational asthma (Flindt, 1969; Pepys et al., 1969;

Zachariae et al., 1981; Cullinan et al., 2000; Sarlo, 2003). Industry guidance and practice has since then ensured that this health risk is firmly controlled (The International Association for Soaps, Detergents and Maintenance Products, 2002; US SDA, 2005). However, the experience served to highlight that enzymes possess a significant respiratory sensitization hazard and that predictive test methods were lacking. In response, over a period of many years, effort was directed towards the development of test methods for the predictive identification of enzyme protein allergens. The most well-described system employed intra-tracheal instillation of enzymes in groups of guinea pigs with subsequent measurement of the anaphylactic antibody response (Sarlo et al., 1991; Ritz et al., 1993). An alternate method using intra-dermal injection in the guinea pig was also described (Blaikie et al., 1995). Both methods showed detergent enzymes to be potent respiratory allergens and that the protease enzymes were also adjuvants for allergic antibody responses. Both methods also delivered similar results for a limited panel of commonly used detergent enzymes (proteases, amylases, and lipases), and to a degree assisted in characterizing the relative potency of enzyme allergenic hazard. This information then contributed to the establishment of occupational exposure limits (Basketter et al., 2012). Both guinea pig tests were animal- and time-intensive, and limited data were generated on dose response.

Attempts were also made to develop a simpler procedure using a murine model, the mouse intra-nasal test (MINT; Robinson et al., 1996, 1998; Sarlo et al., 2000). The method was based on the co-regulation in the mouse by interleukin-4 of the production of IgG₁ (readily measured by enzyme linked immunosorbent assay) and IgE. The mouse model is less time intensive as compared to the guinea pig models and lends itself to evaluation of a broad range of doses. This approach similarly identified enzyme proteins as potential respiratory allergens, and provided some evidence of differential capacities in this respect. Others reported some difficulties with the approach however (Blaikie and Basketter, 1999a, b), and the mouse strain used is critical to how the enzymes are judged as allergens. The reality today is still that there are no standard sensitization assays of any kind for enzymes (or other proteins). However, the commonly used detergent enzymes of various classes have been tested in both the guinea pig and mouse models, and these models showed that the enzymes fall within a certain range of 'potency'. The existing occupational guidelines are sufficient to control sensitization and subsequent development of asthma/allergy among detergent workers. Therefore, as 'new to the world' enzyme backbones are introduced, the animal tests provide an experimental option to assess how similar they are to the existing enzymes and, hence, judge the risk to workers.

Test methods—current perspectives

Toxicological safety assessment is based on the identification of intrinsic hazards, their characterization in

terms of potency and, very importantly, the estimation of exposure. It is reasonable to assume that novel enzymes to the detergent industry are likely to be potential respiratory allergens. Some novel enzymes are in fact just variants of protein structures that are already well known and well characterized. Thus, where the production organism, the enzyme backbone, allergenic epitopes, and patterns of immunological cross-reactivity with antibodies to existing enzymes all point to very close identity, the read across would be appropriate. That is, the same control measures applied to existing enzymes would be appropriate for the variants. Where there are more fundamental differences in structure and/or amino acid sequence, or the functionality of the enzyme has been substantially changed (which therefore might indicate that the immune system would perceive it differently), then additional information must be sought. The *in vivo* methods mentioned can be used to assess whether the new enzyme behaves in a similar manner to the benchmark enzymes (Sarlo et al., 1991, 1997, 2000; Ritz et al., 1993; Blaikie et al., 1995; Robinson et al., 1996, 1998; Blaikie and Basketter, 1999a, b; The International Association for Soaps, Detergents and Maintenance Products, 2002; Larsen et al., 2007). Similar outcomes to the benchmark enzymes indicate that exposure control guidelines used for the benchmarks should be sufficient for the new enzyme. Significantly different outcomes vs the benchmarks could prompt a review of whether the existing guidelines are appropriate for the new enzyme.

As with any *in vivo* test in toxicology, the key question is whether the outcomes (in this case relative allergenicity vs benchmarks) derived from the animal models is relevant for man, particularly in respect of the types of exposure experienced in factory and consumer situations. In the field of human respiratory sensitization to enzymes, as well as to other proteins, exposure response relationships for the induction of sensitization and elicitation of clinical responses are not always well understood (Larsen et al., 2007), but probably the best information that exists is in fact for the enzymes used in detergents and as such this provides highly valuable benchmark data (Hjortkjaer et al., 1986; Sarlo et al., 1991, 1997; Ritz et al., 1993; Robinson et al., 1998; Brinch and Pedersen, 2002; The International Association for Soaps, Detergents and Maintenance Products, 2002; US SDA, 2005; Bernstein and Sarlo, 2006). Decades of practical experience has shown that control of exposure to enzymes based on the guidance on allergenicity derived from *in vivo* tests in the guinea pig and the mouse has permitted the avoidance of IgE-mediated occupational diseases (The International Association for Soaps, Detergents and Maintenance Products, 2002; Sarlo, 2003; US SDA, 2005). The legitimacy of this conclusion has been reinforced by situations where exposure control has lapsed and led to an outbreak of occupational asthma (Cullinan et al., 2000).

A similar concern regarding the relative paucity of benchmark human data and dose response information applies to the development of *in vitro* assays in this area (Roggen

et al., 2006). At present, an extensive research program is underway in the European Union EU, called Sens-it-iv (2008). This EU-funded work has focused on an examination of the utility of an airway epithelial cell line (A549) as a potential *in vitro* tool for the investigation of respiratory allergy. Much work in this area remains to be done, as noted in a review by Verstraelen et al. (2008). Even when established, *in vitro* assay would most probably judge new enzymes against existing benchmarks and so lead to the establishment of exposure limits similar to those already in place and which have been shown for a number of years to be highly protective (The International Association for Soaps, Detergents and Maintenance Products, 2002; Sarlo, 2003; US SDA, 2005; Basketter et al., 2012).

Lastly, it is important in this section to note that whereas no respiratory allergy to enzymes has been reported in consumers using enzyme-containing detergent products, a minority of those exposed occupationally do develop IgE despite control, leading to airborne concentrations generally in the very low ng/m³ range. These individuals are at greater risk of having clinical disease if exposed to higher levels of the enzyme, but the simple presence of IgE is not, of itself, a disease state. It does though reinforce the need for constant vigil in terms of occupational hygiene, as well as the need for regular monitoring of the workforce, measures that would ensure any inaccuracy in the setting of exposure limits for a novel enzyme would be identified before clinical problems occurred.

Conclusions

The enzymes of bacterial and fungal origin that are widely used in industry and particularly in fabric washing detergents are generally of little interest from a toxicological point of view. Both their acute and chronic effects are generally unremarkable. Their sole toxic activity of any significance, the capacity to cause allergy in the respiratory tract, is, however, one which must be the subject of stringent control. All enzymes of non-human origin should be assumed to possess this intrinsic hazard. The tools and techniques that have been applied by the detergent industry have shown that even these rather potent respiratory allergens can be handled safely in the factory and, where a proper risk assessment has been applied, that they can be used without any significant risk to the consumer.

Declaration of interest

DB was paid for the preparation of this manuscript; the other authors are employees of the detergent enzyme industry or of its trade association in the US.

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