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Experiences from Occupational Exposure Limits Set on Aerosols Containing Allergenic Proteins

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Occupational exposure limits (OELs) together with determined airborne exposures are used in risk assessment based managements of occupational exposures to prevent occupational diseases. In most countries, OELs have only been set for few protein-containing aerosols causing IgE-mediated allergies. They comprise aerosols of flour dust, grain dust, wood dust, natural rubber latex, and the subtilisins, which are proteolytic enzymes. These aerosols show dose-dependent effects and levels have been established, where nearly all workers may be exposed without adverse health effects, which are required for setting OELs. Our aim is to analyse prerequisites for setting OELs for the allergenic protein-containing aerosols. Opposite to the key effect of toxicological reactions, two thresholds, one for the sensitization phase and one for elicitation of IgE-mediated symptoms in sensitized individuals, are used in the OEL settings. For example, this was the case for flour dust, where OELs were based on dust levels due to linearity between flour dust and its allergen levels. The critical effects for flour and grain dust OELs were different, which indicates that conclusion by analogy (read-across) must be scientifically well founded. Except for subtilisins, no OEL have been set for other industrial enzymes, where many of which are high volume chemicals. For several of these, OELs have been proposed in the scientific literature during the last two decades. It is apparent that the scientific methodology is available for setting OELs for proteins and protein-containing aerosols where the critical effect is IgE sensitization and IgE-mediated airway diseases.

Keywords: airway allergy; prevention; risk assessment; standard setting

INTRODUCTION

Prevention of occupational diseases were originally hazard based (Luxon, 1984), but is still used, for example, for prevention of diseases due to airborne infectious agents (Trajman and Menzies, 2010) and nanomaterials (Yokel and MacPhail, 2011). However, the many hundreds of occupational exposure limits (OELs) set after World War II (Nielsen and Øvrebø, 2008) allow together with determined airborne exposures (Harper, 2004; Centers for Disease Control and Prevention, 2011) risk-based prevention approaches. Thus, setting of OELs is the first step in a risk-based management of occupational hazards (Ding *et al*., 2011), although it has to be realized that the OEL setting methodology is still under development (Schenk and Johanson, 2011). However, only few of the OELs are set due to IgE-mediated airway allergy to proteins and protein-containing aerosols, although many are respiratory allergens (Deutsche Forschungsgemeinschaft; DFG, 2011) and important industrial products. We therefore review the basis for the setting of such OELs with the purpose to identify methodological principles, which may be useful for setting OELs for other proteins and

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protein-containing aerosols where IgE-mediated airway allergy is the critical effect; the focus of our study is the OEL setting methodology and the exposure–response relationships based on representative examples.

GENERAL PRINCIPLES FOR SETTING OCCUPATIONAL EXPOSURE LIMITS AND COMPARISONS WITH EFFECTS OF PROTEIN CONTAINING AEROSOLS

Airborne concentrations below the OELs are considered to protect nearly all occupationally exposed individuals against adverse effects (ACGIH, 2011; DFG, 2011). A prerequisite for establishing a health-based OEL is that adverse reactions are exposure-dependent and that there is an exposure level where adverse effects no longer appears, i.e. it is possible to establish a no-observed-(adverse)-effect level (NO(A)EL) for the offending effects (Nielsen and Øvrebø, 2008).

Allergens are agents, which can provoke undesirable and specific immune responses, including allergic asthma and allergic rhinitis (HCN, 2008). For toxicological reactions, in general, one NO(A) EL is considered for the key effect. However, for allergic reactions two phases have to be considered (Heederik *et al*., 2002). First, exposure to an allergen may induce 'sensitization' that implies production of specific antibodies or activated immune cells. Sensitization is not *per se* a disease (HCN, 2008). Second, 'elicitation' of symptoms occurs with further exposures to the allergen at a sufficient exposure level (Nielsen *et al*., 2002; HCN, 2008; Basketter *et al*., 2010). There may be differences between levels that induce sensitization and those that induce elicitation of symptoms (Baur *et al*., 1998; Baur, 2003). Thus, two limits may be set, one where no sensitization is observed and another one that prevents the elicitation of allergic reactions in already sensitized individuals (Reiter, 2002; Basketter *et al*., 2010). Sensitization is recommended as the key effect, i.e. a critical effect used in standard settings, by the Health Council of The Netherlands (HCN, 2008). For aeroallergen exposures, risk assessment may be evaluated by means of the airborne concentration as with OELs in general.

It may be complicated establishing thresholds for allergen exposures due to inter-individual variations in susceptibility to both sensitization and elicitation. Those differences may be caused by genetic variations (e.g. atopy versus non-atopy; atopics are subjects especially prone to develop IgE-mediated allergy), age-dependent effects and lifestyle factors (Baur, 2003; HCN, 2008). Smoking may, for example, promote sensitization due to an adjuvant effect (Nielsen *et al*., 2005). Also, co-exposure to endotoxins may play a role in development of sensitization and asthma (Jones, 2008). However, 'practical' NOAELs have been proposed for several environmental and occupational allergen exposures (Baur *et al*., 1998; Baur, 2003); we use the word 'practical' to indicate an apparent threshold, for example, from epidemiological studies, where no significant excess prevalence is observed or that nearly all workers do not have adverse reactions.

The prerequisite for setting an OEL is the existence of a clear exposure–response relationship. This has been demonstrated for several occupational airway allergens (Baur *et al*. 1998; Heederik *et al*. 1999; Baur 2003; Cullinan *et al*., 2003a; HCN, 2008; Brant *et al*. 2009). This has also been shown for indoor and outdoor allergen exposures to house dust mites, cockroaches, pets, pollen, and moulds. The exposure–response relationship often shows a monotonic increase in sensitization and development of allergy with increasing allergen exposure (Nielsen *et al*. 2002; Brant *et al*. 2009). However, it may sometimes show a bell-shaped relationship (Heederik *et al*., 2002) as is the case with cat allergens where high exposure levels may induce tolerance (Erwin *et al*., 2005). A similar relationship has been observed in laboratory animal workers exposed to rat (Jones, 2008) and mouse allergens (Peng *et al*., 2011), which may be due to a 'modified T helper cell type 2 (Th2) response' where specific IgG4 antibodies are thought to play a protective role (Erwin *et al*., 2005; Jones, 2008). Also, regulatory T cells can play an important role in development of tolerance (Fujita *et al*., 2012). However, in cross-sectional studies a bell-shaped relationship may also be due to a healthy worker effect (Heederik *et al*., 2002). It is neither reliable nor ethically defendable to attempt to use other parts of a potential bell-shaped curve than the left increasing part, which expresses a classical exposure–response relationship. Also, the general trend that IgE sensitization and IgE-mediated allergies increase with exposure levels has been observed, for example, at exposures to enzymes used in the detergent industry (Flindt, 1969; Pepys, 1992; Brant *et al*., 2009).

As for toxicological reactions of chemicals, the exposure–response relationships for allergens have three important features: the steepness of the relationship, the position of the exposure–response curve, and the existence of a threshold (Heederik *et al*., 2002; Cullinan *et al*., 2003b). Thus, different proteins have different sensitization potencies

(Basketter and Kimber, 2011). For example, sensitization to rat urinary allergens occurs in the pg m^{-3} range, sensitization to fungal α-amylase in the ng m^{-3} range, whereas sensitization to wheat, pig, and cow proteins occurs in the μg m^{-3} range (Heederik *et al*., 1999). That different allergens have different potencies are also deduced from environmental allergen exposures as only a limited number of allergens are of major importance in the general population (Nielsen *et al*., 2002). In a recent comprehensive review of enzymes used in the production of food and animal feed, it was found that only 17 of 71 enzymes were linked to respiratory allergies (Martel *et al*., 2010). In another comprehensive study, it was found that a small number of protein families contained the allergenic proteins; the allergens were frequently found among proteins able to cause hydrolysis of proteins, polysaccharides and lipids, proteins binding metal ions or lipids, transport proteins, storage proteins, and proteins from the cytoskeleton (Radauer *et al*., 2008). Thus, the number of allergy cases in a population (burden-of-disease) depends both on the potency of the allergens, the exposure levels, the number of exposed individuals, the presence of adjuvants, and the particle size. In the German population in 1999, the number of occupational asthma cases caused by various exposures was in the order flour > latex > food and feed (Baur, 2003).

Prerequisites for establishing exposure–response relationships are appropriate analytical methods as discussed by Nieuwenhuijsen *et al*. (2006). Enzyme-linked immunosorbent assays (ELISAs) are one of the traditional methods for quantification of proteins. The ELISAs are typically of the 'sandwich' type with capture antibodies (Abs), capturing the specific protein. This is followed by a reaction with a detecting Ab, which is coupled with an enzyme system for the quantification. Both types of Abs can be polyclonal or monoclonal (Freymuth *et al*., 1986; Nerurkar *et al*., 1987; Erali *et al*., 1996; Evans *et al*., 1998; Heederik *et al*., 1999; Kumar *et al*., 2008). ELISAs with mono- and polyclonal Abs may show similar results (Aldeen *et al*., 1998; Jensen and Ankley, 2006) or different results (Aldeen *et al*., 1998; Kazim *et al*., 1998) as different Ab-binding epitopes may be used in assays (Kwak and Yoon, 1996; Kazim *et al*., 1998). A critical evaluation of performance of an ELISA is always needed (Heederik *et al*., 1999; Jensen and Ankley, 2006) else absolute results and cross-comparison between studies may be hampered.

Airborne allergens are often collected on filters by means of a pump. Sampling may be in the breathing zone by a person-carried filter cassette and a pump or it may be by a high volume static sampler. The filter content of allergens is eluted and then analyzed by immunochemical methods (Houba *et al*., 1997; Heederik *et al*., 1999; Baur, 2002; Renström, 2002; Korpi *et al*., 2004) or by chromatography and advanced mass spectrometry (Stevenson *et al*., 2010). Both immunochemical methods with introduction of multiplex-analyses (Earle *et al*., 2007) and physico-chemically based methods are undergoing a strong technological development that will allow a more sensitive and precise exposure assessment. Also, a near real-time analytical system has been developed for quantification of airborne subtilisin (serine protease) dust. Dust was captured in a continuously washed cyclone followed by determination of enzyme activity in a bioreactor and automatic determination of the amount of released fluorophor every 5–6 min; the limit of detection was ~5 ng m^{-3} (Rowell *et al*., 2007). However, exposure-assessment from enzyme activity may not be allergen-specific (Heederik *et al*., 2002).

Overall, OELs may be set for proteins and their bioaerosols as they may have practical NOAELs and show dose-dependent adverse effects, and appropriate analytical methods can be established.

ALLERGENIC PROTEINS AND BIOAEROSOLS FROM LISTS OF OCCUPATIONAL EXPOSURE LIMITS

We retrieved OELs for allergenic proteins and bioaerosols set due to IgE-mediated reactions from the USA (ACGIH, 2011), German (DFG, 2011), UK (Health and Safety Executive; HSE, 2007) and the Japanese (Omae, 2007) lists, and from OEL documentations by the Health Council of The Netherlands (www.healthcouncil.nl) and the EU Scientific Committee on Occupational Exposure Limits (SCOEL, 2003; 2008). Flour dust is considered an airway sensitizer (DECOS, 2004; HSE, 2007; SCOEL, 2008; ACGIH, 2011). Grain dust is also considered sensitizing (HSE, 2007). The US list (ACGIH, 2011) considers western red cedar wood dust (from *Thuja plicata*) a sensitizer and the UK list (HSE, 2007) considers softwood and hardwood dust sensitizing. The natural rubber latex proteins were only on the US list (ACGIH, 2011). The subtilisins (proteolytic enzymes) were also on several lists as airway sensitizers (HSE, 2007; ACGIH, 2011).

Due to difficulties in identifying NOAELs, no OEL is set in the German list for the bioaerosols (DFG, 2011). However, a hazard warning ('danger of sensitization of the airways') was set for several bioaerosols ('animal hair, epithelial and other

material derived from animals', 'cereal flour dust', 'natural rubber latex', 'ricinus protein', 'soy bean constituents' and some types of woods, *Terminalia superba, Thuja plicata,* and *Triplochiton scleroxylon*) and enzymes (α-amylase, bromelain, cellulase, papain, pepsin, phytase, subtilisins, and xylanases).

SELECTION OF BIOAEROSOLS FOR EVALUATION

Both the complex aerosols and enzyme-containing aerosols are comprised by the term 'bioaerosols' (Douwes *et al*., 2003). We selected flour dust and grain dust as representatives of complex aerosols. Wood dust was excluded as it has several independent critical effects. The effects include decrease in lung function, development of sinonasal cancer and asthma (HCN, 2000; SCOEL, 2003; ACGIH, 2010). Western red cedar dust is the prominent example of a wood dust type that can cause asthma (ACGIH, 2010). Subtilisin was selected as an example of an enzyme-containing dust being methodologically relevant for evaluation of other industrial enzymes.

The evaluations are based on clinical and epidemiological studies where practical NOAEL may be obtained (Sarlo *et al*., 2010); we focus on studies where exposure concentrations and immunological effects have been reported. No validated animal model exists for prediction of sensitization and airway allergy in humans (Boverhof *et al*., 2008; Basketter and Kimber, 2011), but animal models have been used for ranking of allergenicity of enzymes (Schweigert *et al*., 2000; Sarlo and Kirchner, 2002). No *in vitro* method allows assessment of sensitization potencies (Basketter and Kimber, 2011). The specific literature searches are in Appendix 1.

COMPLEX BIOAEROSOLS

Flour dust

Flour dust is the finely milled and processed grains of mainly wheat, rye, millet, barley, oats, corn cereals (ACGIH, 2001), or a combination of these. The protein content of wheat flour can exceed 10% (ACGIH, 2001; Del Moral *et al*., 2007; Tatham and Shewry, 2008). Mature grain contains over 1000 different proteins (Tatham and Shewry, 2008) and at least 40 different allergens with a high degree of cross-reactivity between the different cereal allergens (ACGIH, 2001; Tatham and Shewry, 2008). Several studies have shown a linear relationship between airborne allergen levels and flour dust concentrations (ACGIH, 2001; Jacobs *et al*., 2008), allowing flour dust concentrations to be used as a proxy for allergen exposures. Other compounds such as enzymes may be added to flour dust (ACGIH, 2001). The risk assessment strategies have used different endpoints such as specific sensitization (ACGIH, 2001; DECOS, 2004) or symptoms (SCOEL, 2008).

The American Conference of Governmental Industrial Hygienists (ACGIH, 2001) has summarized the epidemiological studies on flour dust. In general, these studies showed increased prevalence of upper and lower airway symptoms and decreased lung function at flour dust exposures exceeding 1 mg m^{-3} and reaching levels above 50 mg m^{-3}. Sensitization occurred at dust levels as low as 0.5 mg m^{-3}, with a significant risk at ≥1 mg m^{-3}. A part of the prevalence of respiratory symptoms was due directly to irritation and not associated with sensitization. Sensitization was considered the critical effect and if prevented, it was considered also to prevent the irritant-induced effects. A threshold limit value (the OEL) was set at 0.5 mg m^{-3} with the purpose to minimize sensitization. The value applies for all cereals and is measured as inhalable dust.

In The Netherlands, comprehensive data collection and evaluation of the allergic effects of wheat, rye, barley, and oats flour dust were undertaken. Air monitoring data for inhalable dust was compared with symptoms from the respiratory tract and the eyes, including rhinitis, asthma, and conjunctivitis. The main part of the work-related asthma and rhinitis was due to IgE-mediated allergy against flour dust proteins. The allergic effects were considered the critical effects, although non-specific irritation of dust was also encountered. To prevent symptoms, the risk evaluation was based on prevention of sensitization to flour dust allergens, although sensitization *per se* is not an allergic illness. It was accepted that sensitization often precedes the onset of allergic symptoms, thus preventing sensitization prevents the onset of symptoms. Furthermore, sensitization was exposure-dependent, but no threshold could be identified. In consequence, the additional risk to specific sensitization was estimated from a linear non-threshold extrapolation. The 0.1%, 1%, and 10% additional risk of sensitization corresponded to 0.012, 0.12, and 1.2 mg m^{-3}, respectively, of inhalable flour dust where the exposure was for 8 h per day, 5 days per week during a life-long employment. Also, the epidemiological studies indicated that symptoms, including those due to irritation, were apparent at about the level of 10% additional risk of sensitization (DECOS, 2004). If based on symptoms, this level may be considered a lowest-observed-(adverse)-effect level (LO(A)EL) or close to a NOAEL in occupational settings.

The SCOEL based its evaluation on the documentation by the Dutch Expert Committee on Occupational Standards (DECOS, 2004) and used symptoms as the endpoint in the evaluation. It was acknowledged that no trustworthy threshold could be identified. However, the risk of nasal symptoms appears to increase at concentrations ≥1 mg m^{-3} and the risk of asthma above 3 mg m^{-3}. Both asthma and sensitization are rare in the range of 0.5–1.0 mg m^{-3} inhalable dust. From a pragmatic point of view, the SCOEL concluded that an OEL of 1 mg m^{-3} of inhalable dust would protect the majority of exposed from onset of disease and that the envisaged symptoms would be mild. However, exposures below 1 mg m^{-3} may trigger symptoms in already sensitized workers (SCOEL, 2008). Thus, the SCOEL did not set an OEL due to the lack of a well defined threshold but gave advice about the level where an OEL may be set by authorities.

The exposure–response relationships used in the ACGIH, the DECOS, and the SCOEL documentations can be compared with recent studies. Thus, in a US bakery study (Page *et al*., 2010), the higher-exposed group (geometric mean dust level: 3 mg m^{-3}, range: >0–65 mg m^{-3}) was compared with the lower exposure group (0.24 mg m^{-3}, range: ~0–1.4 mg m^{-3}); mean tenure was 13 and 16 years, respectively. The respective exposures to α-amylase were 2.1 ng m^{-3} (range: 0.1–11 000 ng m^{-3}) and 0.12 ng m^{-3} (range: 0.02–1.2 ng m^{-3}). Wheeze (15% versus 1%), runny nose (16% versus 4%), stuffy nose (18% versus 6%), and frequent sneezing (21% versus 8%) were significantly more prevalent symptoms in the higher-exposed compared with the lower exposed. The sensitization prevalence to α-amylase was 6% and 0%, respectively, and sensitization to wheat 27% and 6%, respectively, with the cut-off for IgE at ≥0.35 kU L^{-1}. The prevalences in the lower exposed group were close to the prevalences in US blood donors. The exposure levels in this study embrace the OELs in the ACGIH (2001) and the SCOEL (2008) documentations.

A study was conducted in British bakeries, where investigated exposures were wheat flour dust and added enzymes, namely, fungal and bacterial amylase, glucose oxidase, amyloglucosidase, and *Aspergillus niger*-derived cellulase, hemicellulase, and xylanase. The median flour dust concentrations were from 2.1 to 5.2 mg m^{-3}. The mean time working in the baking industry was 13.1 years. The common work-related symptoms were nasal irritation (28.9%), eye irritation (13.3%), cough and chest tightness (10.2%), and wheeze and phlegm (7.6%); ocular and nasal symptoms were considered to be due partly to a direct mucous membrane irritation. Sensitization to workplace allergens was 14%. Nasal and ocular irritation was more prevalent among the sensitized and among the atopics. No association was observed between work-related nasal and eye symptoms, and the lung function parameters. Work-related chest tightness and decreased lung function were more frequent among sensitized workers. Atopy and current smoking in atopics were important risk factors for sensitization. The prevalence of sensitization to wheat flour allergens was higher than the prevalence of sensitization to the added enzymes. This suggests that a good control of flour dust exposures not only controls sensitization to the wheat allergens but also to the added enzymes (Harris-Roberts *et al*., 2009). Overall, this study indicates that adverse effects occur at flour dust exposures above 2 mg m^{-3}.

Among Korean bakery employees with a mean period of 3.9 years in the bakery, 5.9% were skin prick test positive to wheat flour, 2.3% to rye flour, 3.9% to yeast, and 0.5% to fungal α-amylase. Work-related respiratory symptoms were reported by 17%, where 13.5% had lower respiratory symptoms. The employees were grouped according to their mean wheat dust levels. The minimal, intermediate, and high exposure group had a mean exposure level of 0.01 (range: 0.00–0.35), 1.16 (range: 0.02–5.97), and 3.04 (range: 0.07–11.27) mg m^{-3}, respectively. The prevalence of lower respiratory symptoms was 10% in the minimal group and 15% in the combined intermediate and high exposure groups that was statistically significant. However, no exposure-dependent effect was observed with being skin prick test positive to wheat allergens. Employees with work-related lower respiratory symptoms had a significantly higher sensitization rate to wheat than those without work-related respiratory symptoms (Hur *et al*., 2008).

Overall, the exposure–response relationships used in three documentations are in agreement with recent studies. The OEL documentations used different endpoints, sensitization, and respiratory symptoms. Sensitization prevalence to enzymes added to flour dust was lower than sensitization to the flour dust allergens. Thus, an appropriate control of flour dust exposures not only seems to control sensitization to the wheat allergens but also to the added enzymes. The other cereal allergens are considered to have similar potencies as the wheat allergens. Additionally, airborne dust levels may be used as proxy for airborne flour dust allergen levels.

Grain dust

Grain dust, although related to flour dust, is a more complex bioaerosol as it, in addition to seed

components, also contains other constituents, for example, from plants, animals (including mites and insects), and microorganisms, where especially endotoxin is considered to play an important role (Swan *et al*., 2007; HCN, 2011). Occupational exposure limits have been set in The Netherlands (1.5 mg m^{-3} as inhalable dust; HCN, 2011), in the Japan (1 mg m^{-3} as respirable dust and 4 mg m^{-3} as 'total' dust; Omae, 2007), the USA (4 mg m^{-3} as total dust; ACGIH, 2001), and the UK (10 mg m^{-3}; HSE, 2007). The critical effect used in The Netherlands was the decrease in lung function, in the USA the effects were upper respiratory tract, eyes and skin irritation, bronchitis symptoms, and decrease in pulmonary function, whereas in the UK it was respiratory sensitization, which apparently occurred at higher exposure levels (Swan *et al*., 2007).

Recent studies corroborate the endpoints used in the OEL settings. Two cross-sectional studies in Canadian grain workers were conducted 30 years apart. In the first, the mean total grain dust level was 6 mg m^{-3} and in the second, 2 mg m^{-3} by area sampling. The first cohort had significantly more lower respiratory symptoms (chronic cough, chronic phlegm, shortness of breath, and occasionally wheeze), but there was no significant difference in current asthma. Additionally, the forced vital capacity (FVC) and the forced expiratory volume in 1 s (FEV_1) were significantly lower in the first cohort (Dimich-Ward *et al*., 2011). Additionally, a longitudinal study was initiated in 1990–1991 with a follow-up until 2003–2004 in male grain farmers and male non-farming controls in Canada. Grain farmers had a significant excess annual decline in forced vital capacity in comparison to the control group. Also, the prevalence of wheeze, dyspnoea, and phlegm was greater in 1990–1991 in grain farmers; this was not significantly increased later in the follow-up period (Senthilselvan *et al*., 2010).

Overall, the used critical effects (allergic airway diseases for flour dust and decreased lung function for grain dust) suggest that non-allergenic effects may occur at exposures, which are below levels causing significantly increased sensitization. Also, the comparison between the critical effects at flour dust and grain dust indicates that conclusion by analogy (read-across) for setting of OELs should be done with caution and has to be well founded scientifically.

ENZYMES

Industrially used enzymes are obtained from plants and mammalian tissue or produced in culturable microorganisms (Baur, 2005; Olempska-Beer *et al*., 2006; Green and Beezhold, 2011). Recently, the recombinant DNA technology has made it possible to tailor enzymes with specific properties and produce them in microorganisms (Baur, 2005; Olempska-Beer *et al*., 2006). The genetically engineered enzymes constitute a considerable part of the industrially used enzymes (Baur, 2005). In general, enzymes are potent sensitizers and potent inducers of airway allergies (Baur, 2005; Green and Beezhold, 2011).

Several reviews have recommended setting of OELs for protein allergens (Baur *et al*., 1998; Baur, 2003; Brant *et al*., 2009; Basketter *et al*., 2010). For enzymes, OELs have been set only for the subtilisins and only by the ACGIH (2011) and the HSE (2007). In Germany, subtilisins and several other enzymes are classified as airway sensitizers without setting OELs (DFG, 2011). Overall, setting of OELs for enzymes is hardly started, although the enzyme industry is rapidly growing.

General experiences from industrial exposures to industrial enzymes

Experiences with enzymes in the detergent industry unequivocally demonstrated exposure-dependent effects of sensitization and development of allergies, mainly rhinitis, conjunctivitis, and asthma. The alkaline and heat-stable proteolytic enzymes introduced into detergent products in the 1960s led to high exposures (up to the μg m^{-3} range) and sensitization of 50–70% of the workers, with nearly 20% of these individuals suffering from occupational allergies, including asthma (Schweigert *et al*., 2000; Sarlo and Kirchner, 2002). In addition to proteases, other enzymes such as amylases, lipases, and cellulases are commonly used by the industry (Schweigert *et al*., 2000).

Strict exposure control programs with in-house OELs reduced the exposures to low levels (≤15 ng m^{-3} range), which reduced sensitization to low levels and prevented the onset of allergic symptoms (Schweigert *et al*., 2000; Sarlo and Kirchner, 2002). In the establishing of this low exposure level, it had been taken into account that the detergent matrix behaves as an immunological adjuvant (Schweigert *et al*., 2000). An important finding was that induction of sensitization was observed at exposure concentrations that were lower than concentrations needed to elicit enzyme-induced allergic symptoms (Schweigert *et al*., 2000; Sarlo and Kirchner, 2002).

Long-term studies did not show a consistent trend in accelerated decrease in lung function due to enzyme exposure (Juniper *et al*., 1977; Flood *et al*., 1985; Cathcart *et al*., 1997). In the detergent industry,

enzymes are not known to induce hypersensitivity pneumonitis or emphysema (Schweigert *et al*., 2000) or contact allergy with delayed type hypersensitivity (Schweigert *et al*., 2000; Sarlo *et al*., 2010). This suggests that IgE-mediated allergies, especially asthma, are key effects of enzyme exposures in the detergent industry.

SUBTILISINS

Biochemistry

Fungal proteases include serine, cysteine, aspartic, and metalloproteinases. Proteases can degrade epithelial barriers and induce inflammation by inducing proinflammatory cytokines through protease-activated receptors, by activation of the kinin system, the coagulation and the fibrinolytic cascades, and the activation of endogenous proteases, by degradation of protease inhibitors and by interference with the complement system. Additionally, many proteases are prominent inducers of IgE-mediated allergies as asthma and rhinitis (Yike, 2011). Protease activity of enzymes may provide strong immunological (danger) signal promoting development of IgE-mediated airway allergies (Porter *et al*., 2011). Expressing the HLA-DQ8 polymorphism may be associated with genetically increased susceptibility to develop sensitization and airway allergies to subtilisins (Xue *et al*., 2005).

The subtilisins constitute a serine protease family where the catalytic site contains the Asp-His-Ser triade (Krem and Di Cera, 2001; Gupta *et al*., 2002; Tripathi and Sowdhamini, 2008). Subtilisins are widely distributed among prokaryotic organisms (Tripathi and Sowdhamini, 2008). Commercially, subtilisins are produced in Bacillus species (Gupta *et al*., 2002; Maurer, 2004).

In the 1960s, subtilisins were introduced in detergents (Maurer, 2004; Saeki *et al*., 2007). Subtilisin BPN' was from *B. amyloliquefaciens*, subtilisin E from *B. subtilis*, subtilisin Carlsberg from *B. licheniformis*, and subtilisin NAT from *B. subtilis (natto)* (Gupta *et al*., 2002; Saeki *et al*., 2007). These subtilisins have a high amino acid homology (Gupta *et al*., 2002) and constitute one clan of the subtilase family A enzymes (Saeki *et al*., 2007). In the 1980s, the high-alkaline subtilisin proteases were introduced (Maurer, 2004; Saeki *et al*., 2007), including M-protease from *B. clausii* (Maurer, 2004; Saeki *et al*., 2007), Savinase from *B. clausii* (Maurer, 2004; Saeki *et al*., 2007; Fujinami and Fujisawa, 2010), and NKS-21 (Saeki *et al*., 2007; Fujinami and Fujisawa, 2010). A lower amino acid similarity (~60%) was found between the M-protease and the subtilisins BPN' and Carlsberg, but the catalytical triad was similar (Saeki *et al*., 2007). These second-generation proteases, including M-protease, belong to another clan of the subtilase family A enzymes (Saeki *et al*., 2007; Fujinami and Fujisawa, 2010). In the 1990s, protein-engineered enzymes appeared (Maurer, 2004) with the purpose to improve temperature, high pH, storage and oxidation stability (Gupta *et al*., 2002), and the ability to function in cold water (Fujinami and Fujisawa, 2010). For example, the alkaline protease, KP-43, which was resistant to chemical oxidants, was introduced in laundry detergents. This enzyme has the Asp-His-Ser catalytical triad, but the amino acid homology between the M-protease and the two subtilisins, BPN' and Carlsberg, was low (~25%). KP-43 belongs to a third clan of the subtilase family A enzymes (Saeki *et al*., 2007). Protein engineering is mainly in the species *B. amyloliquefaciens, B. subtilis*, and *B. lentus* (Bryan, 2000).

Occupational exposure limits

Soon after the introduction of a subtilisin in detergent products, severe IgE-mediated asthma reactions appeared among workers in detergent factories (Flindt, 1969; Pepys *et al*., 1969). These and other studies prompted the ACGIH to establish an OEL for subtilisins in the early 1970s; the ACGIH OELs are termed Threshold Limit Values (TLVs®). The TLV® was derived from experiences in the surfactant industry. ACGIH (2001) set a ceiling level of 60 ng m^{-3} of the 100% active pure enzyme; a ceiling level is a concentration that should not be exceeded during any part of the working exposure (ACGIH, 2011). This requires a well-controlled working environment with exposures considerably lower than the ceiling level (Hewett, 1997). The endpoints considered had the purpose to minimize the potential for symptoms such as sore throat, nasal congestion, cough, wheezing, headache, and skin irritation, and more severe effects as airway obstruction, pulmonary oedema, and allergic respiratory sensitization (ACGIH, 2001). The value is one of the lowest OELs ever set by the ACGIH and it is still recommended. However, the TLV® for subtilisins has been criticized for not being protective (Heederik *et al*., 2002; Cullinan *et al*., 2003b; Douwes *et al*., 2003), which advocates for a re-evaluation. The OEL is 40 ng m^{-3} in the UK (HSE, 2007). No OEL has been set for other industrial enzymes by ACGIH (2011) in the UK (HSE, 2007), in Japan (Omae, 2007), by the Health Council of The Netherlands or by the SCOEL.

Clinical and epidemiological studies

From the period up to 2000, 15 published studies of subtilisins were reviewed by van Kampen and Merget (2002). Afterward, reports on sensitization (van Rooy *et al*., 2009) and development of airway allergies to medical instrument cleaning detergent with subtilisins (Adisesh *et al*., 2011) still appeared. For setting OELs, quantitative exposure–response relationships are mandatory, but from both periods such relationships were limited.

A major prospective study, included 1642 workers, was conducted over a 7-year period (1968–1975) in a factory producing enzyme-containing washing powder. The airborne dust enzyme activity (glycine units m^{-3}) decreased markedly in the first few years of the survey. After introduction of a subtilisin, 34 workers (2.1%) with no previous chest symptoms developed breathlessness, sweating, and wheezing at exposures. The percentage of SPT positive workers increased with exposures and more atopics developed a SPT positive reaction compared with non-atopics. For example, in the highly exposed group, 75% of the atopics became SPT positive compared with 40% of the non-atopic workers (Juniper *et al*., 1977).

Sensitization was studied in a plant producing dry bleach; encapsulated subtilisin was used. The airborne enzyme level was from <0.05% and up to ~40% of the TLV® (60 ng m^{-3}) by area sampling. The mean aerodynamic particle size was ~5 µm. The average duration of employment among exposed workers since introduction of the enzymes was ~17 months with a range of 1 month to 2 years. Upper and lower airway symptoms were similar in exposed and non-exposed workers. Among 12 enzyme-exposed workers, three exposed workers developed specific IgE antibodies to the protease. None of the 11 non-exposed workers developed specific IgE antibodies. One of the sensitized workers had eye and chest symptoms that appeared to be due to occupational exposure. In another sensitized worker, symptoms were considered equivocal. The last positive worker had no symptoms. The study concluded that sensitization to subtilisin may occur below the TLV® (Liss *et al*., 1984).

In a cross-sectional study in a detergent factory, 40 exposed workers were compared with 36 non-exposed people. In general, the subtilisin protease exposures were ≤16 ng m^{-3} by area sampling, although exposure up to 1500 ng m^{-3} had been measured. Eight workers were sensitized to proteases, one had asthma and seven had rhinitis. None in the control group was sensitized. The authors concluded that it is possible that the TLV® for subtilisins (60 ng m^{-3}) may allow sensitization or at least symptoms in sensitized workers exposed to the used protease (Vanhanen *et al*., 2000).

Outbreaks of sensitization, upper airway symptoms, and asthma still occurred in the detergent industry where strict exposure controls were not followed. In such a case, the geometric mean concentration was 4.3 ng m^{-3} and the highest value 57 ng m^{-3} in 1997 to an unspecified protease; the type of sampling was not reported. Of 74 employees who started working in or after 1997, five had a positive response to protease. However, several enzymes were used, where amylase was found to be a more potent sensitizer than the protease (Cullinan *et al*., 2000).

In four Chinese detergent manufacturing plants (A1, A2, B1, and B2), the geometric mean total dust concentration was from 0.2 to 3.1 mg m^{-3} (range: 0.02–13 mg m^{-3}) and the geometric mean enzyme concentration from 0.5 to 2.2 ng m^{-3} (range: 0.01–10 ng m^{-3}) by area sampling. The proteases were savinase and alkalase. Nasal irritation, sneezing, throat irritation, and cough were significantly increased compared with non-exposed controls. No increase was observed in symptoms at a total dust concentration of 0.2 mg m^{-3}, and the benchmark dose was estimated to be 1.4 mg m^{-3} for sneezing and higher for the other symptoms. No severe allergic response and asthma case was reported. The non-specific dust effects may therefore have played an important role in causing the symptoms. In the A1 plant, the mean enzyme level was from 0.5 to 1 ng m^{-3} and the prevalence of sensitization to savinase was 3.2%; alkalase was not studied. In plant B1, the median enzyme concentration was from 0.5 to 1 ng m^{-3}, and the sensitization prevalence to both savinase and alkalase was 3.7%. In plant A2, the geometric mean enzyme concentration was ~2.1 ng m^{-3}, and the prevalence of sensitization to savinase 15% and to alkalase 7.5%. In plant B2, the geometric mean enzyme concentration was 1.6 ng m^{-3} and 8.7% were sensitized to savinase and 31% to alkalase. This study suggests that 2.2 ng m^{-3} can be suggested as the NOAEL for allergic symptoms and 1 ng m^{-3} as the LOAEL for sensitization (Zhang *et al*., 2004).

In a case-reference analysis of a cohort of employees in a European detergent factory, lung diseases were not increased significantly at 4 ng m^{-3} of an unspecified protease but significantly increased at 8 ng m^{-3}. Eye and nose symptoms were increased at 2 ng m^{-3}. The authors mention that only the protease level was measured, although amylases and cellulase were also used, and that irritant dust and non-occupational reactions may have contributed to the findings above these levels (Brant *et al*., 2009). In this study, the NOAEL for chest diseases was 4 ng m^{-3}.

Overall, these findings support that practical NOAELs for a number of industrially relevant proteolytic enzymes may be in the low ng m^{-3} range. Ideally, NOAELs may be set for each specific enzyme and OELs established in a case-by-case manner. However, such an approach is not possible from the available date in the peer-reviewed literature. A strategy could be to set a common OEL for the subtilisins and the related proteases. In this case, the OEL has to protect against all members of the families. Two studies (Cullinan *et al*., 2000; Brant *et al*., 2009) suggest an OEL in the low ng m^{-3} range. From the Brant *et al*. (2009) study, the lower airway symptoms were not increased at 4 ng m^{-3} that is supported by the Zhang *et al*. (2004) study. Eye and nose symptoms were increased at 2 ng m^{-3} (Brant *et al*., 2009) but may have been due to effects of non-allergic irritants (Zhang *et al*., 2004). The OELs set by the ACGIH and in the UK are bypassed by the much lower exposure levels presently achieved in the detergent industry. The lower in house OELs in industry take into account that other constituents of detergents apart from enzymes may have adjuvant effects (Schweigert *et al*., 2000; Sarlo and Kirchner, 2002; Sarlo, 2003; Basketter *et al*., 2010).

CONCLUSION

Few OELs have been set for protein-containing bioaerosols, where the critical effect is IgE-mediated sensitization and allergy. Where such OELs have been set, analytical methods for determination of airborne allergen levels have been established. This is a prerequisite for establishing quantitative exposure–response relationships and thus for the setting of the OELs. Where linearity exists between the allergen content and dust levels, an OEL may be based on the dust level as a proxy for allergen exposure.

No appropriate animal model exists for predicting sensitization and airway allergy. Thus, OELs have to be based on clinical and epidemiological findings. In this case, it may not be possible to establish a 'true' NOAEL, which may be reduced by one or more assessment factors to set an OEL. Nevertheless, epidemiological studies may establish exposure levels where no excess response due to occupational exposure is observed and thus fulfilling the requirement that 'nearly all workers may be exposed, day-after-day, over a working lifetime, without adverse health effect' (ACGIH, 2011). Thus, the use of clinical or epidemiological studies to derive OELs for allergens do not deviate from OEL settings for other endpoints, but for transparency, uncertainties always have to be highlighted and discussed in OEL documentations.

Two endpoints, IgE-mediated sensitization and respiratory symptoms, can both be used for setting OELs as is the case with flour dust; both endpoints should always be discussed and evaluated. For simplicity, it is tempting to use read-across to set OELs for related types of exposures. If used, it must be scientifically founded. For example, read-across is not possible between flour dust and grain dust due to the more complex bioaerosol of grain dust.

OELs have been set for the proteolytic enzyme subtilisin, but surprisingly no other OEL is set for industrial enzymes, although many are high-volume compounds. The values for subtilisins seem neither in agreement with recent studies nor with industrial experiences. This suggests re-evaluation of the values. Also, an attempt to set OELs for other enzymes has repeatedly been mentioned in the scientific literature during the last two decades. As we have highlighted here, the scientific methodologies are available.

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APPENDIX 1: LITERATURE SEARCH

At the first search level, we retrieved OELs for allergenic bioaerosols set due to IgE-mediated reactions from the USA (ACGIH, 2011), German (DFG, 2011), UK (HSE, 2007) and the Japanese (Omae, 2007) lists, and from OEL documentations by the Health Council of the Netherlands (www.healthcouncil.nl) and the EU SCOEL. The searches also identified relevant OEL documentations and governmental reports, which were consulted and relevant cross-references retrieved.

At the second search level, we performed specific searches in PubMed for retrieval of informative studies in English and German. The hits at each search were screened from the title or if not clear additionally from the abstract. To select recent informative studies on OEL setting to supplement the studies in Nielsen and Øvrebo (2008), we used the search terms: 'industrial hygiene AND risk management AND review'(1679 hits; 01.11.2011), 'occupational exposure limit* AND review'(hits 138; 04.11.2011), 'occupational hygiene AND measurement* AND review' (non-informative) and 'elisa assay AND monoclonal antibody* AND polyclonal antibody* AND comparison' (56 hits; 04.03.2012). Together with the cross-references, the references from Nielsen and Øvrebø (2008), the retrieved studies, and studies from our own files, these studies were the basis for the Introduction section and the section 'General principles for setting occupational exposure limits and comparison with effects of protein-containing aerosols'. Also, these references were used together with the retrieved references from the search 'Western red cedar AND asthma AND mechanism* AND review' (5 hits; 02.01.2012) for the two sections 'Allergenic proteins and bioaerosols from lists of occupational exposure limits' and 'Selection of bioaerosols for evaluation', and the searches 'wheat AND flour AND protein AND content' (198 hits; 13.04.2010), 'flour dust AND lung AND sensitization' (7 hits; 30.11.2011), 'bakery AND sensitization'(48 hits; 30.04.2011), 'grain dust AND lung AND effect*' (149 hits; 30.11.2011) for the section 'Complex bioaerosols'.

The additional searches: 'industrial enzyme AND airway AND allergy' (22 hits; 06.04.2011), 'enzyme* AND occupational exposure limit*' (13 hits; 05.12.2011), 'Olempska-Beer Z' (7 hits; 06.03.2012), 'subtilisin AND asthma' (4 hits; 04.05.2010), 'subtilisin AND allergy' (28 hits; 13.07.2011), the enzyme number '3.4.21.62' (361 hits; 13.07.2011). A search was performed in Google Scolar: 'asthma AND subtilisin*' (first 1000 of 1930 hits) were used for the sections 'Enzymes' and 'Subtilisins'. For Subtilisins, all studies with exposure–response relationships from occupational settings were included.

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