



Categorisation of protein respiratory allergens: The case of Subtilisin

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ABSTRACT

Characterisation of the relative sensitizing potency of protein and chemical allergens remains challenging, particularly for materials causing allergic sensitization of the respiratory tract. There nevertheless remains an appetite, for priority setting and risk management, to develop paradigms that distinguish between individual respiratory allergens according to perceptions of the hazards and risks posed to human health. One manifestation thereof is recent listing of certain respiratory allergens as Substances of Very High Concern (SVHC) under the provisions of REACH (Registration, Evaluation, Authorisation and restriction of Chemicals). Although priority setting is a laudable ambition, it is important the process is predicated on evidence-based criteria that are transparent, understood and owned. The danger is that in the absence of rigorous criteria unwanted precedents can be created, and confidence in the process is compromised. A default categorisation of sensitizers as SVHC requiring assessment under the authorisation process is not desirable. We therefore consider here the value and limitations of selective assignment of certain respiratory allergens as being SVHC. The difficulties of sustaining such designations in a sound and equitable way is discussed in the context of the challenges that exist with respect to assessment of potency, and information available regarding the effectiveness of exposure-based risk management.

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1. Introduction

For effective toxicological evaluations it is necessary to identify the intrinsic hazards associated with substances, and to assess likely risks to human health. If such evaluations indicate a potential for substantial or severe human health risks then it is of course legitimate to focus particular attention on those materials that are deemed to be of particular concern.

Under the provisions of Article 57(f) of REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) such materials may be designated as Substances of Very High Concern (SVHC) if they possess the intrinsic potential to cause severe, even life-threatening, effects in humans at relatively low levels of exposure (European Chemicals Agency, 2007; Ter Burg and Jongeneel, 2011). Both skin and respiratory sensitising chemicals are being considered under REACH for categorisation as SVHC. Three chemical respiratory sensitizers have been given first priority in the evaluation process (diazene-1,2-dicarboxamide [ADCA], hexahydro-2-benzofuran-1,3-dione [HHPA], and hexahydromethylphthalic anhydride [MHHPA]) and have already been listed on the REACH SVHC candidate list (<http://echa.europa.eu/candidate-list-table>).

In 2011, the National Institute for Public Health and the Environment (RIVM) in the Netherlands stated that a proper risk management option for respiratory sensitizers could be to qualify them as SVHC in accordance with Article 57(f) of REACH (European Chemicals Agency, 2007). The provisions under article 57(f) concern “substances – such as those having endocrine disrupting properties, or those having persistent, bioaccumulative and toxic properties or very persistent and very bioaccumulative properties, which do not fulfil the criteria of points (d) or (e) – for which there is scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) and which are identified on a case-by-case basis in accordance with the procedure set out in Article 59”.

In the RIVM report (Ter Burg and Jongeneel, 2011), Subtilisin (a proteolytic enzyme of bacterial origin widely used in detergent products) was listed as a possible SVHC candidate. In this respect, it is of note that bacterial and fungal enzymes have for many years been widely used industrially and are well recognized as being associated with occupational respiratory allergy. The ability of proteins to cause allergic sensitization of the respiratory tract is, however, not limited exclusively to enzymes. Although detergent enzymes, and enzymes generally have been implicated as respiratory allergens, there are a variety of other proteins from diverse sources that are known to be associated with sensitisation of the respiratory tract and occupational asthma (including, for example,

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egg and other food proteins, bakery proteins, and protein dander from experimental animals) (Basketter et al., 2012a,b; Martel et al., 2010; Baur, 2013; Holsapple et al., 2006; Basketter and Kimber, 2011).

There are approximately 400 enzymes listed in the European Inventory of Existing Commercial Chemical Substances (EINECS) (EINECS, 2012). Of those, 17 enzymes (13 enzymes on EINECS, 1 enzyme on ELINCS [European List of Notified Chemical Substances], and 3 enzyme groups) are listed in Annex VI of the EU's CLP (Classification, Labelling and Packaging) regulation (United Nations Nations, 2011). The common harmonized classification of these 17 enzymes is that of respiratory sensitizer. In general, enzymes, regardless of their catalytic activities, are potential respiratory sensitizers, whereas the weight of evidence from humans indicates that enzymes are not able to cause skin sensitization (Basketter et al., 2008, 2010, 2012, 2012a; Human and Environmental Risk Assessment on ingredients of household cleaning products: protease (Subtilisin), 2007; Human and Risk Assessment on ingredients of household cleaning products: α -AMYLASES, 2005). Except where there is compelling evidence to the contrary, all enzymes should be regarded as having the inherent potential to cause sensitization of the respiratory tract and should be classified "H334: Hazard Category 1: May cause allergy or asthma symptoms or breathing difficulties if inhaled" in accordance with the CLP Regulation (United Nations Nations, 2011). However there have been successful innovations, such as encapsulation and other formulation changes to craft non-inhalable forms of the products that reduce consumer risk. In addition, it has been possible to determine safe exposure levels of occupational and consumer exposures (Basketter et al., 2010; Sarlo et al., 2010).

The above listing of respiratory sensitizers on the REACH SVHC candidate list has potentially important implications. These are reflected by a letter issued by CEFIC (European Chemical Industry Council) dated March 12th 2013 and entitled "Industry's viewpoint on the Roadmap on Substances of Very High Concern" (CEFIC, 2013). In that letter it is stated that there may be significant repercussions in designation of a material as a SVHC, and that there has emerged a "black list" effect. As a consequence the designation should be used only sparingly and in circumstances where there is a clear need. In the present paper, the argument is made that an appropriate analysis of available risk management options should be explored in advance of any substance being proposed to be placed on the SVHC candidate list. The point is that the designation as SVHC may not be appropriate or necessary if there are available relevant approaches and procedures to mitigate the potential risks to human health. Thus, SVHC designation should be applied with due discretion.

In this commentary we will consider the case for the proposal that Subtilisin is a candidate for designation as a SVHC. In this context we will specifically address two issues: (a) whether there exists a legitimate evidence-based argument for the differential categorisation of protein respiratory allergens according to their intrinsic potency, and (b) whether, in the case of Subtilisin in particular, there is reason to believe that there exists a substantial risk to human health. These are important issues because they inform our appreciation of the extent to which it is possible currently to evaluate accurately the relative sensitizing properties of allergen, and the principle of risk-based decision making.

2. The relative sensitizing potency of respiratory allergens

At least in theory there are several ways in which the potency of allergens could be defined. However, for the purposes of hazard characterization and risk assessment the most important and most valuable metric is based on an understanding of the level of

exposure that will trigger the acquisition of sensitization. There is available a recent detailed critique of the uncertainties associated with evaluation of the potency of respiratory allergens (Basketter and Kimber, 2011), and therefore a similarly detailed account is not required here.

Although the difficulties in determining the relative potency of protein respiratory allergens are not as great as those associated with chemical respiratory allergens, there are nevertheless important challenges (Basketter and Kimber, 2011).

Those challenges can be best illustrated by reference to the characterization of contact allergens. Here, the local lymph node assay (LLNA) can be used to compare and contrast the ability of contact allergens to cause the proliferation and clonal expansion of responsive T lymphocytes in lymph nodes draining the site of exposure. It is well established that this proliferative response by draining lymph node cells provoked by encounter with a contact allergen is causally and quantitatively correlated with the acquisition of sensitization. At a practical level, therefore, the relative skin sensitizing potency of a contact allergen is measured by consideration of dose response profiles in the LLNA, and identification of the concentration of allergen required to induce a certain level of response (specifically, a 3-fold increase in proliferation compared with concurrent vehicle-treated controls). This value is defined as EC₃; the Effective Concentration of a contact allergen required to stimulate a 3-fold increase in proliferation (Kimber et al., 2001).

On this basis it is clear that for adoption of a similar approach for assessment of the relative potency of respiratory allergens it will be necessary to identify biomarkers or responses that correlate quantitatively with sensitization of the respiratory tract. There is a continuing debate about the immunological processes through which chemicals cause sensitization of the respiratory tract. The picture is somewhat clearer in the case of protein allergens, and there is a general consensus that IgE antibodies plays a pivotal role.

In brief the development of allergy induced by exposure to proteins can be summarized as follows. Encounter with sufficient protein allergen, by an appropriate route, provokes an adaptive immune response that is associated with the elaboration of allergen-specific IgE antibodies. These antibodies distribute systemically and associate with mast cells that are found in virtually all vascularized tissues. At this point sensitization has been achieved. If the now sensitized subject encounters the same protein (and for the purpose of this article by inhalation into the respiratory tract), then that protein will bind with, and cross-link, IgE antibodies displayed on the mast cell surface. This in turn causes mast cell activation and the release by degranulation of both preformed and newly synthesized mediators (such as vasoactive amines and leukotrienes) that act in concert to drive the inflammatory response that is recognized clinically as respiratory allergy. Symptoms may range from mild itching, congestion and rhinitis, to severe asthma.

Against this background there are two main strategies that have been pursued for the identification and characterization of protein respiratory allergens, although it is important to acknowledge that neither approach has been formally validated for this purpose. The first is to attempt to measure directly the ability of test proteins to induce and elicit respiratory hypersensitivity reactions in experimental animals. The second is to consider instead the capacity of proteins to provoke IgE responses. In many instances the two approaches have been used in tandem (Karol et al., 1989; Kawabata et al., 1986; Ritz et al., 1993; Robinson et al., 1996, 1998; Sarlo et al., 1991; Sarlo and Karol, 1994). The former approach lacks the resolving power to provide a useful and reliable approach for the assessment of relative sensitising potency, and therefore measurement of IgE responses is the more attractive approach.

However, although IgE antibody is clearly identified as a critical effect molecule, and is the most obvious candidate as a correlate of sensitization, there are both technical challenges and mechanistic

uncertainties that need to be acknowledged and negotiated if the elaboration of IgE is to be considered as a potential measure of relative potency.

At a technical level the primary issue is one of the accuracy and sensitivity of detection. Compared with other classes of antibody, IgE is produced in only very small amounts. The enzyme-linked immunosorbent assay (ELISA) is commonly the method of choice for measuring antibody levels, but this is not necessarily the most appropriate option for assessment of IgE responses. Thus, ELISA assays for the measurement of IgE antibody that are both sensitive and selective can be difficult to engineer, and may vary substantially in quality and reliability. An important factor is that the stimulation of IgE antibody responses will almost always be accompanied by IgG antibody, with the latter being produced in much larger amounts, and possibly by a factor of between 100- and 1000-fold (Basketter and Kimber, 2011). Competition between IgE and IgG antibodies for antigenic determinants on the target protein can confound the interpretation of ELISA assays designed to detect the former. The other alternative is to employ a method known as passive cutaneous anaphylaxis (PCA). Although, if configured appropriately, this method is selective for IgE antibody, and relatively sensitive, it is expensive and requires a substantial number of animals. Moreover, PCA is technically demanding and unsuitable for routine use.

It is clear, therefore, that there are important technical challenges associated with the measurement in both humans and experimental animals of IgE antibody responses. However, even if those challenges can be addressed successfully there remains the question of the degree to which IgE antibody levels correlate with the extent of sensitization. That is, although there is no doubt that IgE antibody plays a pivotal role in the acquisition of sensitization to protein allergens, it does not necessarily follow that there is a clearly defined relationship between the two. For instance, it is known that the level of specific IgE antibody found within the blood of subjects sensitized to protein allergens does not necessarily correlate closely with either the elicitation of allergic symptoms following challenge, or with the severity of allergic reactions. An illustrative example is provided by food allergy where there is often a mismatch between sensitization (measured as a function of IgE antibody) and clinical reactivity to the relevant protein (Buck et al., 2010).

Despite the reservations summarized above, IgE antibody does provide probably the most relevant biomarker of sensitization, and is legitimate to consider it as a readout for assessment of the relative potency of protein allergens. However, even if reliable and reproducible assays could be designed to measure accurately induced IgE antibody responses in experimental animals, the assessment of relative potency based upon such measurements would not be facile. Thus, for instance, it would be necessary to define what level of IgE antibody production would be indicative of a sensitization hazard, and the approach would then be to compare proteins based upon what level of exposure would be required to provoke that concentration of antibody. A theoretical difficulty with that approach is that in reality the elicitation of any level of IgE antibody could be regarded as representing a hazard, but it would not be technically realistic to expect to measure reliably very low levels of IgE antibody. Leaving aside that concern, and assuming that a relevant target concentration of IgE antibody could be identified, defining the amount of protein necessary to provoke that level of response would not be an easy undertaking. At the very least, fairly extensive dose–response analyses would be necessary. In addition, it would be necessary to integrate an understanding of threshold levels of protein allergen with considerations of the route, frequency and duration of exposure.

Finally, and importantly, it is necessary to keep in mind that, as yet, there exists no compilation of protein (or chemical) respiratory

allergens categorised according to their relative sensitizing potency in humans and against which predictions from experimental systems could be tested (Basketter and Kimber, 2011).

Taken together, the view currently is that although in theory it might be possible to compare the sensitizing potential of protein allergens as a function of the level of exposure required to elicit a certain plasma concentration of specific IgE antibody, this has not yet been accomplished, let alone validated, in any systematic way.

The conclusion drawn, therefore, is that there is currently no tried and tested experimental method for characterizing the relative sensitizing potential of protein respiratory allergens. In the absence of such a method there is no certain experimental basis for ranking protein allergens as a function of sensitizing potency. Given that position, there is presently no prospect of providing an evidential basis for making recommendations for the selective designation of certain protein allergens as being Substances of Very High Concern.

3. The availability of effective risk management strategies and SVHC designation

Subtilisin represents an intriguing choice in the context of candidature for SVHC designation since this enzyme is perhaps the best described, and most thoroughly understood, of all protein respiratory sensitizers.

The purpose here is to address the question of whether there are available effective strategies to manage the human health risks associated with exposure to this enzyme.

The original introduction of Subtilisin into detergent products in the late 1960s was associated with IgE-mediated occupational asthma (Flindt, 1969; Pepys et al., 1969; Pepys, 1992). Those observations, and others from elsewhere, drew attention from the American Conference of Governmental Industrial Hygienists (ACGIH), and in the early 1970s ACGIH established an occupational exposure limit (OEL) of 60 ng/m³ American Conference of Governmental Industrial Hygienists, September 2004. [In the UK a somewhat lower OEL of 40 ng/m³ has been adopted by the Health and Safety Executive for protease enzymes (UK Health and Safety Executive, 2004)]. Although there have been suggestions that it is not sufficiently protective (Heederick et al., 2002; Cullinan et al., 2003), the prevailing view is that the ACGIH OEL of 60 ng/m³ has in practice worked well and that its adoption has to a very large extent resulted in adequate control of worker exposure (Basketter et al., 2010, 2012, 2012b; Baur, 2013; Sarlo et al., 2010; Cathcart et al., 1997; Sarlo and Kirchner, 2002; Sarlo, 2003). The general success of the application of this OEL is illustrated further by the fact that in the absence of adequate control there have been reports of occupational asthma (Cullinan et al., 2000; Vanhanen et al., 2000; Van Rooy et al., 2009).

It has to be acknowledged, however, that even when the OEL is adhered to, and appropriate controls appear to be in place, there are very occasional cases of enzyme-induced respiratory allergy, mostly upper respiratory allergy manifested as rhinitis (Basketter et al., 2010; Vanhanen et al., 2000; Brant et al., 2009). This is not unexpected. Even in a well-controlled workplace environment there may be accidents, peak levels or short exposure excursions beyond the OEL that may be sufficient to support the acquisition and expression of sensitization.

Nevertheless, it is well established that the acquisition of sensitization (to proteins and chemicals) is strictly dose-dependent, and that there are threshold exposure levels below which sensitization will fail to develop. Detergent enzymes are no exception to this rule (Basketter et al., 2012b; Sarlo et al., 2010; Flindt, 1969; Pepys, 1992; Brant et al., 2009), and therefore there is no reason why adherence to a relevant OEL should not prevent the development

of sensitization in the workplace. That theoretical expectation is borne out by practice and the evidence is that the ACGIH OEL has served the needs of risk management with considerable effectiveness. Even if it were to be argued that the risk of occupational allergy (in those, for instance, who display unusual levels of susceptibility) remains too high, even under conditions where the guideline OEL is adhered to, this would not be an indictment of the risk management strategy *per se*. Rather, such an argument would make the case that the threshold levels for the development of sensitization to detergent enzymes in some subjects were such that a reduction in the recommended OEL should be considered in order to protect more effectively a susceptible subpopulation. However, it must be emphasized that the available evidence indicates that the ACGIH OEL does in fact provide for an effective control of exposure, and that occupational health problems are associated with circumstances where those effective risk management measures have not been maintained (for example, Cullinan et al., 2000). That being the case, then reducing the OEL would have no impact on sensitization, not least since for sensitized subjects (paradoxically) the level of exposure required for elicitation of an allergic reaction is the same as, or higher than, that required for the acquisition of sensitization (Basketter et al., 2012b). For consumers, there have been no reports of respiratory allergy since the early 1970s and the prevalence of enzyme-specific IgE antibody among the general population is very rare (Sarlo et al., 2010).

The conclusion drawn is that there is available for prevention of allergic sensitization to Subtilisin an effective, and tried and tested, risk management strategy based around control of occupational exposure levels. The ACGIH OEL of 60 ng/m³ has generally worked very well, as evidenced in a recent overview of 5 years of health surveillance and air monitoring in the detergent industry (Basketter and Kruszewski, 2013).

4. The candidacy of Subtilisin as SVHC

From the information summarized above two important inferences can be made. The first is that it is not possible currently to determine the relative sensitizing potency of protein respiratory allergens. The second is that the application of the ACGIH OEL provides the basis for an effective risk management strategy to reduce or prevent the acquisition of sensitization to Subtilisin and the potential development of clinical symptoms, the worst case scenario being occupational asthma.

On the basis of the available information it is, therefore unreasonable, to propose that Subtilisin be designated SVHC. The arguments are that:

- There is no basis for identifying Subtilisin as being more of a risk to human health compared with the many other protein respiratory allergens that have industrial application. If it is not possible currently to distinguish between protein respiratory allergens with respect to their relative sensitizing potency then there are no scientific grounds for categorizing differently from other enzyme allergens or for that matter other protein respiratory allergens.
- There is a tried and tested strategy, based on control of workplace exposure to Subtilisin, that has been proven to be very effective in managing human health risks.
- The rare occurrence of clinical symptoms to subtilisin are only seen occupationally, and the early stages of elicitation (e.g. rhinitis) can be reversed by cessation of exposure, without effect on future quality of life. Further, subtilisins do not cross-react immunochemically with other allergens or enzymes (Bindslev-Jensen et al., 2006), thus other allergens will not induce further effects.

Taken together those arguments make a case that categorisation of Subtilisin as SVHC is both inappropriate and unnecessary.

5. Concluding comments

A case is made here against the classification of Subtilisin as SVHC. A legitimate question is whether it matters if Subtilisin is designated in this way. The authors are of the view that the decision is important and relevant for two main reasons:

1. It is simply good practice that toxicology, and decisions reached on the basis of toxicological information, should be evidence-based. There is simply no evidential basis for the differential categorisation of Subtilisin from other proteins, whether they are enzymes or not. Indeed, the same principle applies to chemical as well as protein respiratory allergens, that sub-categorisation on the basis of intrinsic potency is premature.
2. Designation of SVHC should be reserved for materials where there is a real risk to human health, where use has to be specifically authorised, and where the expectation is that use will be phased out. Although there is no doubt that Subtilisin is a human health hazard due to its potential to cause respiratory allergy, there is available a proven and very effective risk management strategy to protect human health. The designation of SVHC should be reserved solely for instances where there is a tangible health risk, and inclusion in this category of Subtilisin, where the health risk can be managed effectively, will only serve to devalue the currency of this categorisation and reduce its usefulness.

Conflicts of interest

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