

## ORIGINAL ARTICLE

## Sensitising effects of genetically modified enzymes used in flavour, fragrance, detergent and pharmaceutical production: cross-sectional study

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**ABSTRACT**

**Objectives** The use of genetically engineered enzymes in the synthesis of flavourings, fragrances and other applications has increased tremendously. There is, however, a paucity of data on sensitisation and/or allergy to the finished products. We aimed to review the use of genetically modified enzymes and the enormous challenges in human biomonitoring studies with suitable assays of specific IgE to a variety of modified enzyme proteins in occupational settings and measure specific IgE to modified enzymes in exposed workers.

**Methods** Specific IgE antibodies against workplace-specific individual enzymes were measured by the specific fluorescence enzyme-labelled immunoassay in 813 exposed workers seen in cross-sectional surveys.

**Results** Twenty-three per cent of all exposed workers showed type I sensitisation with IgE antibodies directed against respective workplace-specific enzymes. The highest sensitisation frequencies observed were for workers exposed enzymes derived from  $\alpha$ -amylase (44%), followed by stainzyme (41%), pancreatinin (35%), savinase (31%), papain (31%), ovozyme (28%), phytase (16%), trypsin (15%) and lipase (4%). The highest individual antibody levels (up to 110 kU/L) were detected in workers exposed to phytase, xylanase and glucanase. In a subgroup comprising 134 workers, detailed clinical diagnostics confirmed work-related symptoms. There was a strong correlation ( $r=0.75$ ,  $p<0.0001$ ) between the symptoms and antibody levels. Workers with work-related respiratory symptoms showed a higher prevalence for the presence of specific IgE antibodies against workplace-specific enzymes than asymptomatic exposed workers (likelihood ratio 2.32, sensitivity 0.92, specificity 0.6).

**Conclusions** Our data confirm the previous findings showing that genetically engineered enzymes are potent allergens eliciting immediate-type sensitisation. Owing to lack of commercial diagnostic tests, few of those exposed receive regular surveillance including biomonitoring with relevant specific IgE assays.

**INTRODUCTION**

The use of enzymes in industry and everyday products has increased tremendously in recent years, but remains largely unrecognised by the public and medical professionals. Food, beverage, detergent, perfume, pharmaceutical, textile and chemical industries are increasingly using enzymes in biotechnological processes for the synthesis of volatile and non-volatile chemical compounds contributing,

**What this paper adds**

- New developments of industrial processes in a variety of industries, fuelled by consumer pressure for low-fat foods and natural flavours, have resulted in an explosion in the production of flavours, fragrances and other industrial applications using enzyme technology. Engineering the enzyme protein may change its allergic properties, posing new potential health risk.
- The individual very high specific IgE antibody levels in our cross-sectional pilot study show that the industrial enzymes are potent sensitisers, eliciting immediate type I allergic reactions. A correlation between the workplace exposure, the level of sensitisation and respiratory symptoms was observed.
- Our findings stress that the commercially available ELISA or ImmunoCAP reagents so far are directed against the native not modified enzymes. However, specific IgE analysis should be directed against workplace-specific (modified) proteins for use in human biomonitoring.

among others, to the fragrance, taste and flavour of products.<sup>1,2</sup> The desire of consumers for natural flavours in products and aroma in low-fat foods has driven the considerable growth in this sector to about US\$10 billion from the beginning of new millennium.<sup>3</sup> Though the conventional routes of chemical flavour synthesis are still used, the biotechnological generation of enzymes, especially of aroma compounds, is growing. New flavours have been produced by chemical transformation of natural substances, direct extraction from plants or by enzymatic/microbial/fungal biosynthesis (biotechnology).<sup>4</sup> Whereas the first process does not legally allow the product to be labelled as 'natural', the synthesis of aromatic compounds in microbial/fungal<sup>5</sup> systems allows the product to be classified as 'natural' by the European and US legislations.<sup>6</sup> A large variety of such enzymes are now on the market (table 1), mostly genetically modified with the help of recombinant DNA technology and expressed in different species increasing the numbers of proteins with potential sensitising properties into thousands. However, there is still little current information on the possible sensitising

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**Table 1** Examples of known industrial enzyme applications

Application	Enzymes used
Food processing, food aroma	Amylases, proteases, aspartases, cellulases
Baby foods	Trypsin
Brewing industry	Barley enzymes, amylases, glucanases, proteases $\beta$ -glucanases, arabino xylanases, amylo glucosidase, pullulanases, aceto lactate decarboxylase
Fruit juices	Cellulases, pectinases
Dairy industry	Rennin, lipases, lactases, microbial-produced enzymes
Meat tenderisers	Papain
Starch industry	Amylases, amyl glucosidase, glucoamylases, glucose isomerase
Paper industry	Amylases, xylanases, cellulases, ligninases
Biofuel industry	Cellulases, ligninases
Contact lens cleaners	Proteases
Rubber industry	Catalases
Photographic industry	Protease (ficin)
Molecular biology	Restriction enzymes, DNA ligase, polymerases
Fragrance and flavours industry	Lipolytic enzymes, proteases, alcohol dehydrogenase, amine oxidase, glutaminases, hydroxylases, feruloyl esterase, phenolic acid esterase, pectinase, eugenol- <i>O</i> -methyltransferase, acetyl-CoA-benzyl alcohol, acetyltransferase
Pharmaceutical industry	Papain, bromelain, trypsin, pancreatinin, lipase, amylases
Detergent and laundry and dish wash Industry	Phosphatase, urase Primarily proteases, amylases, lipases, cellulases, (ie, stainzyme, savinases termamyl), functional in washing/cleaning and fragrance generation

effects in enzyme manufacturing or consumer exposure which may be oral, inhaled or cutaneous. The allergenic potency of airborne dust or liquid aerosol containing enzymes has been reported in the 1960s–1990s.<sup>7–10</sup> Since then, the number of enzymes produced and used by the industry has increased tremendously with numerous workers being exposed and many developing respiratory symptoms.<sup>11–16</sup> Previous studies from Vanhanen *et al.*,<sup>17</sup> Brisman *et al.*<sup>12</sup> and Baur<sup>11</sup> have shown up to 50% of workers studied developed conjunctivitis, rhinoconjunctivitis, asthma or hypersensitivity pneumonitis due to sensitisation. Most of the evidence comes from the detergent<sup>16</sup> and food industries,<sup>18</sup> but there are also indications of corresponding problems in the pharmaceutical industry, laboratories and also in consumers of enzyme-containing products, including cosmetics, fragrance products,<sup>19</sup> detergents<sup>20</sup> or soft-lens fluids. More recently, Brant *et al.*<sup>21</sup> and van Rooy *et al.*<sup>22</sup> have found strong evidence of an association between detergent enzyme exposure, the development of respiratory diseases and an increased risk of sensitisation and respiratory allergy in detergent workers.

New applications of enzymes bearing different physico-chemical properties, including different molecular weights, etc, include new flavours and aroma in low-fat food. Many fungi and yeasts have been found to produce *de novo* such odorous compounds. These include enzymes involved in the cheese flavour biosynthesis such as lipophilic lipases delivering food flavour triggering ketones from glycerides cleaved to fatty acids or mono-oxidases yielding lactone-triggered flavour.<sup>23</sup> Fresh fish aromas can be produced by lipoxygenases and hydroxyperoxidases which oxidise fatty acids for the production of leaf aldehydes and leaf alcohols and liberate alcohols and carbonyls from polyunsaturated fatty acids. Lipozyme-mediated transesterification yields methionylesters with flavour attributes.<sup>24</sup> New starter industrial cultures of *Lactococcus lactis*, *Lactobacillus*, *Streptococcus* and *Propionibacterium* provide enzymes degrading casein and convert methionine through new pathways into flavour formatting aromatic products used for cheese ripening.<sup>25</sup> Also, glutamate dehydrogenase is applied to enhance flavour

formation in some cheeses.<sup>26</sup> Allergenic enzymes such as  $\alpha$ -amylase, proteases such as papain, and other flour additives are increasingly used in bakeries to accelerate the baking process and to improve the product properties, adding these enzymes to a long list of possible causes of baker's asthma.<sup>11</sup>

Enzymes are potent sensitisers eliciting immediate-type allergic reactions with the formation of specific IgE antibodies. Unfortunately, owing to the variety and diversity of antigenic structures of these new enzymes, commercial diagnostic tests are rarely available. Current guidelines recommend the measurement of specific IgE for surveillance in workers exposed to high-molecular-weight sensitising agents.<sup>27–28</sup> Hidden allergens can also be a problem in food safety for general consumer and pose a challenge in mandatory labelling practice.<sup>29</sup> As enzymes are known to be allergenic, it is likely that the introduction of new enzymes or enzyme mixtures will increase the risk of allergy in the absence of preventive measures.

The aim of our study was to identify any sensitisation to the 'new' enzymes in workers involved in their production and application. We aimed to stress the enormous challenges in developing suitable assays of specific IgE to a variety of enzymes and hence performing human biomonitoring studies.

## METHODS

### Data collection

We received blood for antibody testing from 813 workers, all occupationally exposed to enzymes and comprising the whole workforce that use mostly genetically modified enzymes in the food, chemical, detergent and pharmaceutical industries. In 2 industries using 6–10 enzymes, we also received clinical data, including case histories by questionnaire, physical examination and lung function testing from all 134 exposed workers (see online supplementary appendix 1). Commercial restrictions limited our access to clinical data for the remainder to allow detailed correlations between the clinical symptoms and the level of sensitisation for the whole group. However, we were informed by the occupational physicians that individual workers complained of nasal symptoms, conjunctivitis, wheezing or

dyspnoea in the workplace in the group for which we lacked access to clinical data.

We screened individual workers for the presence of specific IgE antibodies against enzymes used in their workplace between 2007 and 2013 in pilot analyses across respective industries. Each sample was tested for IgE antibodies to the enzymes to which they were exposed: phytase, xylanase, glucanase, cellulase, savinase and/or  $\alpha$ -amylase. We are not allowed to provide any information on the modifications performed on the enzymes used at the individual workplaces, nor any information on the type of the industrial use. Data from workers exposed to specific experimental enzyme modifications (n=10) were excluded due to low number of exposed workers.

### Immunoassays

Workplace-specific genetically engineered enzymes, which may differ in the primary, secondary or tertiary structure from the host enzyme, were coupled to solid phases and used for IgE antibody analyses in the immunoassay. This method was developed in our laboratory for human biomonitoring surveillance studies and/or independent expert investigation of the symptomatic workers. The method has been published elsewhere<sup>30, 31</sup> (see also online supplementary appendix 2). The creation of non-commercial hydrophilic polymer encased in a capsule (CAPs) follows our established in-laboratory standard protocol: 3 mL allergen solution (workplace-specific enzyme proteins) using biotin-XX-NHS in the ratio of 5:1 (5 mole activated biotin to 1 mol protein) biotinylated for 2 hours. The purification of the mixture takes place over Sephadex G25 column (prepacked column, PD 10 from GE Health Care), prewashed with 1% bovine serum albumine (BSA) solution and 1× PBS solution; the column is eluted with PBS, and nine fractions are collected. The protein concentrations from each fraction are measured using Bradford assay method (BioRad). The first three protein collecting samples are merged together. If the protein concentration exceeds the concentration of 1.4 mg/mL, the sample has to be diluted and repeatedly measured.

The determination of the biotinylation grade is carried out by a photometric measurement of the  $\Delta$ OD500/HABA/avidin/sample over a HABA/avidin kit from Sigma. The biotinylated sample should have a degree of biotinylation of 1, confirming that a biotin molecule has bound to a protein molecule. The CAP was coupled to Phadia streptavidin-CAP (o212, Streptavidin CAPs, provided from Phadia) sponges on microtiter plates. The Cap-Washer Device (Phadia/Thermo-Fisher) washed streptavidin-CAPs on the microtiter plates with the biotinylated protein solution (biotinylation, ~1; amount of protein per CAP Sponge, 20–30  $\mu$ g; volume per CAP Sponge, 50  $\mu$ L); this was then incubated in the protein solution for 30 min. Specific IgE (allergen concentration in kU/L) against the specific enzyme used at the given workplaces was measured following the addition of 40  $\mu$ L patient serum (antigen).

Specific IgE antibodies measured by the fluorescence enzyme-labelled immunoassay (the specific IgE expressed in kilo units per litre (kU/L)) were corrected with the WHO reference of human serum IgE; 1 kU=2.4 ng/L. Recombinant enzymes used in the workplace of tested workers were biotinylated and coupled with CAP cellulose sponges; the specific IgE antibodies were detected in patients' sera using an ImmunoCAP-specific IgE fluorescence detector and seven-point calibration curves (ImmunoCAP is a registered trade name of Phadia/Thermo-Fisher, Freiburg, Germany). For ImmunoCAP-specific IgE, the limit of detection (LOD) is 0.02 kU/L, the limit of quantitation (LOQ) is 0.2 kU/L for IgE and the upper limit of

calibration is 100 kU/L. The cut-off points used in the study were: 0.35 kU/L.

Commercial ImmunoCAP conjugates (Phadia, see below) used in routine clinical laboratories were applied in parallel with similar analytical procedures (for the calibration curves and control sera). If possible, commercial native enzymes were used as a control. These included commercially available enzymes (Phadia): savinase (*Bacillus amyloliquefacies*), cellulase (*Aspergillus niger*), amylase (*A. oryzae*) and xylanase (*A. niger*). The commercial native enzymes tested in a parallel experiment showed little or no cross-reactivity with the respective native enzymes, except for savinase (determined in separate preliminary experiments, data not shown). For the controls, the commercial ImmunoCAP conjugates (sIgE,  $\alpha$ -amylase, k87, Phadia/Thermo Fischer Scientific, Freiburg, Germany) were used.

To test individual conjugates and to validate the assay, a pool serum from positive enzyme patients was used. Positive patients are determined in a separate preliminary experiment, pooled and used as a positive control.

All immunological methods were validated routinely with control serum samples and additional standard set points (two analytic standards, one with low concentration and the other with high concentration, were used as set points). For validation of the assays, the following controls were included: pooled positive and negative patients (for each enzyme)/control sera (pooled sera from patients sensitive to other allergens like isocyanates and additionally second pooled sera from reference patients with no exposure), analytical standards (also used as set points for quality control), human serum albumine (HSA) solution and biotin control samples.

The measured day-to-day precision was <12% RSD (per cent of relative SD). The assay validation was performed according to the good laboratory practice rules. Separate control studies with HSA solution showed that IgE values above 0.02 kU/L can be considered as specific being more than 2 RSD or 10% analytical variation. The variability between the in-laboratory method and the commercial assay method was 0.5–20% (for lower and upper edges of analytical failure) for the IgE values for  $\alpha$ -amylase and savinase. The other enzymes tested show <10% cross-reactivity with their native forms, for instance Termamyl showed no cross-reactivity with the native, genetically non-modified amylase form.<sup>31</sup>

### Clinical diagnosis of patients with enzyme exposure history

Clinical diagnoses were based on the outcomes of the medical and occupational history and clinical findings. An adaptation and translation into German<sup>32</sup> of the ATS and David Bernstein, University of Cincinnati Medical Center, surveillance questionnaires identified work-related allergy symptoms such as rhinorrhoea, sneezing, conjunctival irritation, wheezing, cough, shortness of breath and fever, and the questionnaire included workplace exposures including potential risk factors such as hygiene aspects. The type of job, age, sex and smoking habits were also included. Furthermore, diagnosis was based on the results of specific IgE tests with enzymes, skin test reactions to common allergens and enzymes, measurements of forced expiratory volume in 1 s (FEV1) and forced vital capacity (FVC). The individual asthma diagnosis for each patient followed previously published guidelines.<sup>27, 28</sup>

### Data analysis

The patients were grouped according to the enzymes used at individual workplaces based on the native enzyme origin, that is, Termamyl (a heat-stable  $\alpha$ -amylase produced by a genetically

modified strain of *Bacillus licheniformis*) was grouped as amylase (except for stainzyme, which is not only a liquid amylase, but also itself comprises a group of several divergent genetically modified enzymes, including Blaze, Evity, stainzyme plus, etc); savinase is also given a separate group, since this alkaline protease, with various applications in detergent, food (ie, in production of infant milk) or lances production/cleaning, is not genetically modified itself, but is produced by submerged fermentation of genetically modified microorganisms.

Immunological data were expressed as means (min–max). Each analysis was repeated with independent preparations. The Wilcoxon signed rank test was used to compare differences between individual IgE measurements to enzymes and ANOVA with Barlett's post-test used for correlations between groups. Pearson's correlation test was used for correlations between diagnosis probability estimates and the specific immunoglobulin binding. The relative prevalences for the presence of specific IgE antibodies in symptomatic workers were calculated from the contingency tables using a logistic model. The data analyses were performed with GraphPAD Prism V6.0 Software (GraphPad Software, San Diego, California, USA).

## RESULTS

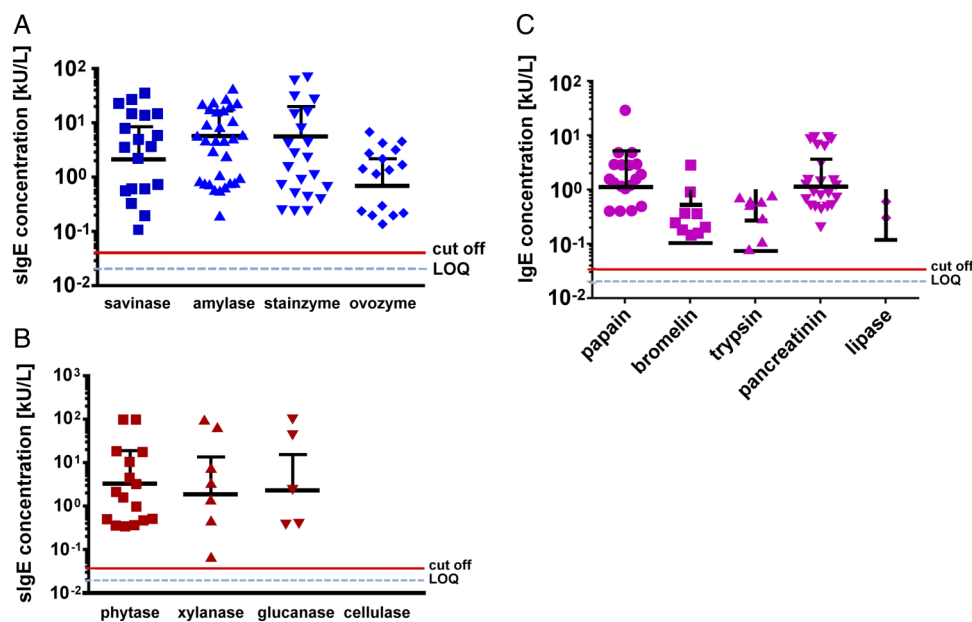
Complete exposed workforces in individual workplaces using genetically modified enzymes in food, beverage, chemical, detergent and pharmaceutical industries provided samples for specific IgE testing. Each sample was tested for IgE antibodies to the enzymes to which they were exposed, phytase, xylanase, glucanase, cellulose, savinase and/or  $\alpha$ -amylase. Samples were received from 813 workers. Men comprised 66%, aged 20–60 years and women 34%, aged 20–50 years.

Specific IgE antibody levels ranged from 0 to 100 kU/L for all analysed enzyme species groups (figure 1). Twenty-three per cent of exposed workers showed the presence of specific IgE antibodies (n=187) against their workplace enzymes; the highest frequencies were observed for  $\alpha$ -amylase with

44% sensitisation, followed by stainzyme (41%), pancreatinin (35%), savinase (31%), papain (31%), ovozyme (28%), phytase (16%), trypsin (15%) and lipase (4%). The median antibody levels for all enzymes tested were relatively low (below the clinical cut-off points of <0.35 (0.02–100) kU/L) for all enzymes tested.

Results for the enzymes separated into groups, based on common exposure groups (application), are shown in figure 1A–C. The workers were exposed for 0.3–10 years to 2–4 enzymes in their individual workplaces. Note that the first group (figure 1A) was the most heterologous group comprising workers from various industries from food processing to wash, clean and home care product industries (including flavour and fragrances). Group A has the highest number of sensitised patients (10.7%, n=87). The group consists of workers using mainly various  $\alpha$ -amylase forms in food starch and detergent industries (ie, Termamyl) using amylases with various protein engineering changing the performance profile of the native amylase enzyme. Since the amylase stainzyme is produced in a different way (by fermentation of genetically modified enzymes), it has been analysed separately (shown in a separate lane). Since some of the workers exposed to amylases were also exposed to proteases, this group includes also proteases, like savinase. Notably, only 3.4% workers in the grouped showed specific antibodies to all four enzymes at the time of analyses. However, since the commercial secrecy limited our access to the industrial hygiene data on previous exposure to individual enzymes, no further detailed evaluation was possible.

Figure 1A shows the groups of savinase,  $\alpha$ -amylase, stainzyme and ovozyme, where much higher mean values were observed. With respect to the individual enzymes the mean value was 4.27 kU/L (n=86), for the  $\alpha$ -amylases; 7.75 kU/L (n=71) for the stainzyme (n=67), it was slightly higher with the value of 11.95 kU/L. For ovozyme (n=67), the mean value was 2.66 kU/L. In total, 137 workers within the group had values below the LOQ.



**Figure 1** The presence of specific IgE antibodies against industrial enzymes. Across respective industries (detergent, food, chemical and pharmaceutical industries), workers were screened (n = 813 analyses) for the presence of specific IgE (sIgE) antibodies against individual workers-specific, modified enzymes. The data show the individual levels of specific IgE by enzyme groups (A–C) as mean ( $\pm$ SD) values. Additionally, the lower LOQ (blue dotted line) and the cut-off values (red line, CAP Class 0) are shown. LOQ, limit of quantification.

The second group (figure 1B) and the third group (figure 1C) include workers exposed to enzymes used in various industrial applications, including food, beverage, dietary and chemical or pharmaceutical products. As mentioned above, the assembling of the individual group is based on indicated multiple exposures. When analysing the group phytase, xylanase, glucanase and cellulose (figure 1B), the following mean values were detected; for phytase, 3.33 kU/L (n=80); for xylanase, 1.86 kU/L (n=98); for glucanase, 2.24 kU/L (n=67); and for cellulase, <0.35 kU/L (n=16). Figure 1C comprises the last group bromelain, papain, trypsin, pancreatin and lipase showing mean values of 1.1 kU/L for papain (n=55), <0.35 (0.02–3) kU/L for bromelain (n=52), <0.35 kU/L for trypsin (n=50), 1.2 kU/L for pancreatin (n=59) and <0.35 kU/L for lipase (n=45). Despite multiple exposures, no cross sensitisation could be monitored. In total, 221 workers showed values below the LOQ.

When comparing further individual antibody levels, we have analysed the 75% percentile of sIgE value levels for all individual enzymes tested. Those were not very high with 0.6 (0.02–68) kU/L for savinase, 4.9 (0.02–68) kU/L for  $\alpha$ -amylase, 2.3 kU/L (0.02–110) for stainzyme (n=67), <0.35 (0.02–43) kU/L for ovozyme, <0.35 (0.02–100) kU/L for phytase, <0.35 (0.02–100) kU/L for xylanase, <0.35 (0.02–100) kU/L for glucanase, <0.35 (0.02–0.6) kU/L for cellulase, <0.35 (0.02–30) kU/L for papain, <0.35 (0.02–3) kU/L for bromelain, <0.35 (0.02–1) kU/L for trypsin, <0.35 (0.02–10) kU/L for pancreatin and <0.35 (0.02–10) kU/L for lipase. However, some individual sIgE antibody levels were as high as 110 kU/L in all enzyme groups.

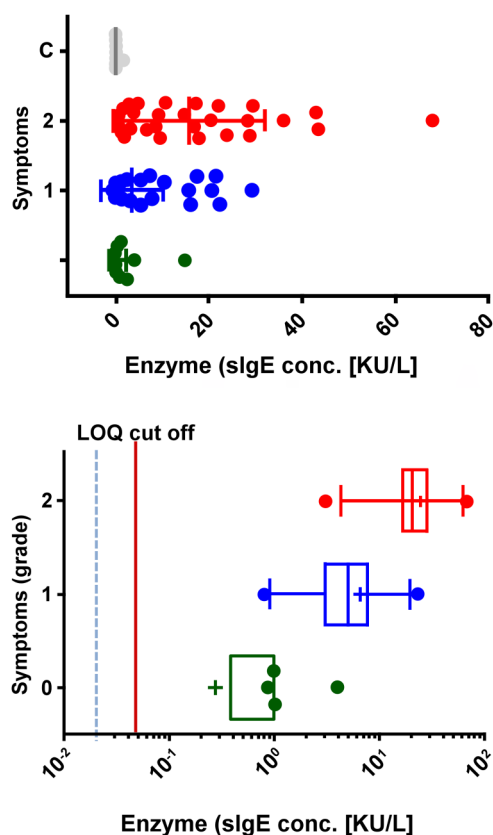
In a representative group of 134 workers, questionnaire data were available to correlate specific IgE with symptoms (figure 2). Of them, 64% were asymptomatic, 19% had work-related rhinitis and/or conjunctivitis, and 17% had work-related wheezing and/or asthmatic dyspnoea. Pearson's correlation analysis showed a significant correlation between symptoms and specific IgE ( $r=0.75$ , 95% CI 0.61 to 0.84,  $p<0.0001$ ). Within the small group analysed, no correlation to atopy status was recorded.

As a control (C), the workers were tested for sIgE antibodies against amylase (commercial test, non-modified protein).

Workers with work-related symptoms, including rhinitis/conjunctivitis or other respiratory symptoms, such as wheezing and asthmatic dyspnoea, showed significantly higher prevalences for the presence of specific IgE antibodies against workplace-specific enzymes as compared with asymptomatic group ( $p<0.0001$  with Fisher's exact test with a likelihood ratio of 2.32, a sensitivity of 0.92 and a specificity of 0.60).

## DISCUSSION

We report a pilot cross-sectional study through various industries where workers were exposed to modified enzymes to detect possible sensitisation to the enzymes used. We found sensitisation to workplace-specific enzyme proteins in 23% of 813 workers tested. In a small subgroup (n=134) where full clinical data were available, 36% reported work-related symptoms of asthma or rhinitis. Symptomatic workers had significantly higher levels of specific IgE than asymptomatic exposed workers ( $r=0.75$ , 95% CI 0.61 to 0.84,  $p<0.0001$ ). The prevalence of sensitisation was particularly high in workers exposed to enzymes derived from  $\alpha$ -amylase, stainzyme or pancreatin with 35–44% sensitised. These enzymes were mainly found in the wash, clean and home care product industries. Detecting sensitisation required the development of assays to the altered enzymes rather than native enzymes. We have recently described a case of allergy to the recombinant enzyme *Termamyl*<sup>®</sup> in a



**Figure 2** Work-related respiratory symptoms after exposure to enzymes. Occurrence of specific IgE antibodies in exposed workers based on the presence (1, 2) or absence (0) of respiratory symptoms. For Y axis, 0=no work-related symptoms, 1=exposure-related rhinitis and/or conjunctivitis and 2=exposure-related respiratory symptoms including wheezing or asthma symptoms. Note that the groups were for the worst symptoms (no overlapping). The X axis shows specific IgE concentrations for the enzymes tested within the group. As a control (C), the serum was screened (in a parallel assay) for the sIgE levels of native amylase for possible cross-reactivity (using commercial assay CAP-System, see the Methods section for more details). The lines indicate the respective mean ( $\pm$ SD) values for the respective sIgE antibodies directed against a workplace-specific enzyme. The lower graph shows the same data as box and whiskers plot with 10–90 percentile (mean is shown as a '+' value). Additionally shown are the LOQ (blue dotted line) and the cut-off values (red line, CAP Class 0). LOQ, limit of quantification.

modern 'clean' factory<sup>31</sup> showing sensitisation to this genetically engineered bacterial  $\alpha$ -amylase, but no cross-reactivity with fungal  $\alpha$ -amylase. Similar absence of immunological cross-reactivity has been shown in tests with polyclonal antibodies.<sup>33</sup>

The industrial control of exposures to enzymes can be achieved with air monitoring (ambient monitoring) or worker sensitisation (biomonitoring). Both of the methods rely on the structure and antigenic properties of the protein used in the assay. While the dust samples are mainly collected on filters and measured with the help of ELISA using the antibodies directed against the enzyme protein,<sup>33</sup> the IgE tests, published in the literature use either ELISA, Radio-Allergo-Sorbent-Test (RAST)<sup>34</sup> or fluorescence labelled immunoabsorbent assays. The National Institute of Health, NIH, food guidelines issued in December 2010<sup>35</sup> identified the fluorescence enzyme-labelled (ie, ImmunoCAP) methods as a 'gold standard' for in vitro IgE testing. However, there are not many workplace-specific IgE tests available. The RAST method has not been available

commercially for more than 10 years. In 2010, the US National Institute of Allergy and Infectious Diseases also recommended that the RAST measurements of specific immunoglobulin E for the diagnosis of allergy be abandoned in favour of testing with more sensitive fluorescence enzyme-labelled methods. Despite the use of the altered enzymes used in this study, circulating IgE antibodies may remain undetectable despite a convincing clinical history because antibodies may be directed towards submolecular antigenic determinants that are revealed or altered during industrial processing of the enzyme.<sup>31</sup>

Industry has developed several *in vitro*, *in silico* and rodent screening models to predict protein sensitising potential, especially in the food industry for 'novel' food proteins.<sup>36</sup> Regardless of the assay applied, the host enzyme protein used in the system appears to be the major limiting step in the test system.<sup>5</sup> The commercially available ELISA or ImmunoCAP systems are directed against the native enzymes not the modified enzymes, if available at all. However, as mentioned above, changing the primary, secondary or tertiary protein structure may change its sensitising properties and allergenic potency. The workplace-specific enzyme should be used for diagnosis. If the workplace-specific enzyme protein is available, the most suitable test system should use such enzyme in the test method applied for human biomonitoring.

Previous studies<sup>11 13 17</sup> have shown sensitising reactions with conjunctivitis, rhinitis, asthma or hypersensitivity pneumonitis. Most of the data have come from the detergent and food industries. Early exposure to enzymes was reported where enzyme powders leading to high levels to airborne dust caused respiratory allergy in the workplace and in housewives using these products. Following public complaints, the industry changed the production process encapsulating enzymes. Despite enzyme encapsulation decreasing dust level and improving occupational hygiene, respiratory disorders continue to occur. Enzyme sensitisation was found in 22% of the workers in a modern detergent factory with 47% of the workers who developing respiratory symptoms.<sup>17</sup> Cullinan *et al*<sup>16</sup> reported major problems in the packing-refilling area of a detergent factory where 77% had a positive enzyme skin-prick test reaction, 69% had upper respiratory symptoms and 55% had work-related asthma. The levels were only a little lower in the production area where 33% had positive enzyme skin-prick test results, 25% had upper respiratory symptoms and 19% had work-related asthma. The cross-sectional and case-referent analysis by Brant *et al*<sup>21</sup> and van Rooy *et al*<sup>22</sup> showed differences between individual workplaces. In the engineering section, 30% had a positive enzyme skin-prick test result, 26% upper respiratory symptoms and 25% work-related asthma. A total of 108 workers with the highest exposures to proteases,  $\alpha$ -amylase, lipase and cellulose and sensitisation to at least 1 enzyme, reported more work-related symptoms with a prevalence ratio (PR) of 4.2 compared with the least exposed group.<sup>22</sup> Johnsen *et al*<sup>37</sup> showed sensitisation to all types of enzymes handled in an enzyme-producing plant, most often in the production areas and laboratories with 8.8% of workers developing clinical enzyme allergy during the first 3 years of employment.

Occupational asthma cases due to phytase and savinase have been reported in agricultural and dishwashing industries.<sup>34 38</sup> Caballero *et al*<sup>39</sup> described sensitisation to fungal enzymes in the animal feed industry and Elberling *et al*<sup>19</sup> mucosal symptoms elicited by fragrances in a general population-based data. Hannu *et al*<sup>20</sup> documented reactive airways dysfunction syndrome from inhalation of dishwasher powder.

In 2002, the European Commission<sup>6</sup> together with Austrian Environmental Protection Agency published a report summarising current knowledge and legislation on enzyme applications and regulations within the European countries pointing out that "Though the scientific literature investigated indicate that enzymes have the potential for sensitization of the respiratory tract, at present, no validated test methods exist to determine and to predict sensitization via inhalation". No recommendations could be given regarding test methods that could be routinely used for the evaluation of respiratory sensitisation. They proposed that the application of the precautionary principle and generally label enzymes with the R42 warning "may cause sensitisation via inhalation". Based on literature review, there are few indications that enzymes are skin sensitisers. However, all enzymes may be potential skin irritants and cause urticaria.

### Limitations and conclusions

Limitation of our study is that commercial secrecy has limited our access to data and authority to identify specific bioengineered enzyme formulae. Nevertheless, we have been allowed access to exposure histories and serum from exposed workers, with additional clinical data in a subgroup. This subgroup with clinical data was not randomly selected from all workers tested, thus possible selection bias could not be excluded. This stresses the necessity of independent future studies. Published industry-funded research points to a decrease in the incidence of respiratory sensitisation and allergy to enzymes,<sup>40</sup> unfortunately often lacking detailed information on the specificities of the test systems used.<sup>40 41</sup> There is no doubt that good occupational hygiene practice is the most effective risk management strategy. But, it has to be assumed that the introduction of new enzymes might increase the risk of allergy in the absence of appropriate preventive measures.<sup>5</sup>

Our findings indicate that new sources of enzymes, as well as genetically engineered enzymes, are posing potential health risks. Owing to the current knowledge, the genetically modified enzymes have the same sensitising potential as traditional enzymes but require assays directed at the new/changed enzyme rather than its native form. The regulation of enzymes in legislation depends on their use and not on the health risk.<sup>6</sup> The assessment of allergenicity should be mandatory for all new products. No reports may indicate no tests rather than no effect.<sup>33</sup> Enzymes should be tested like any other potentially hazardous chemical.

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**Competing interests** LTB, ES, PSB and XB declare that they have no financial or personal relationship with any organisation or person that would inappropriately influence their work or to have any other conflict of interest within 3 years of beginning the work submitted. ES had consultant arrangements with some of the companies whose workers have been tested within the study. XB and PSB testified in occupational litigation cases on behalf of plaintiffs. LTB is co-chairing the task force 'Immunological Methods in Occupational Settings' from the German Society for Environmental and Occupational Medicine, DGAUM, and XB is chairing the study group 'Allergic Disorders' of the European Society for Environmental and Occupational Medicine, EOMSociety.org.

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## REFERENCES

- Longo MA, Sanromán MA. Production of food aroma compounds: microbial and enzymatic methodologies. *Food Technol Biotechnol* 2006;44:335–53.
- Li C, Sun J, Li T, et al. Chemical and enzymatic synthesis of a library of 2-phenethyl esters and their sensory attributes. *Food Chem* 2014;154:205–10.
- Christen P, López-Munguía A. Enzymes and food flavor: a review. *Food Biotechnology* 1994;8:167–90.
- FDA. Office of Food Additive Safety—New Enzyme notifications. 2006. <http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-foods-gen/documents/document/ucm269075.pdf>
- Green BJ, Beezhold DH. Industrial fungal enzymes: an occupational allergen perspective. *J Allergy (Cairo)* 2011;2011:682574.
- Office for Official Publications of the European Communities. Collection of information on enzymes. 2002. <http://ec.europa.eu/environment/archives/dansub/pdfs/enzymerepcomplete.pdf>
- Palmer KN. The effect of an aerosol detergent in chronic bronchitis. *Lancet* 1957;272:611–13.
- Baur X, Fruhmann G, Haug B, et al. Role of *Aspergillus* amylase in baker's asthma. *Lancet* 1986;1:43.
- Hendricks MH. Measurement of enzyme laundry product dust levels and characteristics in consumer use. *J Am Oil Chem Soc* 1970;47:207–11.
- Bruce CF, Dunn E, Brotherton R, et al. Methods of measuring biologically active enzyme dust in the environmental air of detergent factories. *Ann Occup Hyg* 1978;21:1–20.
- Baur X. Enzymes as occupational and environmental respiratory sensitizers. *Int Arch Occup Environ Health* 2005;78:279–86.
- Brisman J, Nieuwenhuijsen MJ, Venables KM, et al. Exposure-response relations for work related respiratory symptoms and sensitisation in a cohort exposed to alpha-amylase. *Occup Environ Med* 2004;61:551–3.
- Vanhanen M, Tuomi T, Tiikkainen U, et al. Sensitisation to enzymes in the animal feed industry. *Occup Environ Med* 2001;58:119–23.
- Vanhanen M, Tuomi T, Nordman H, et al. Sensitization to industrial enzymes in enzyme research and production. *Scand J Work Environ Health* 1997;23:385–91.
- Brisman J, Järholm B, Lillienberg L. Exposure-response relations for self reported asthma and rhinitis in bakers. *Occup Environ Med* 2000;57:335–40.
- Cullinan P, Harris JM, Newman Taylor AJ, et al. An outbreak of asthma in a modern detergent factory. *Lancet* 2000;356:1899–900.
- Vanhanen M, Tuomi T, Tiikkainen U, et al. Risk of enzyme allergy in the detergent industry. *Occup Environ Med* 2000;57:121–5.
- Sander I, Raulf-Heimsoth M, Siethoff C, et al. Allergy to *Aspergillus*-derived enzymes in the baking industry: identification of beta-xylosidase from *Aspergillus niger* as a new allergen (Asp n 14). *J Allergy Clin Immunol* 1998;102:256–64.
- Elberling J, Linneberg A, Dirksen A, et al. Mucosal symptoms elicited by fragrance products in a population-based sample in relation to atopy and bronchial hyper-reactivity. *Clin Exp Allergy* 2005;35:75–81.
- Hannu TJ, Riihimäki VE, Piirilä PL. Reactive airways dysfunction syndrome from acute inhalation of a dishwasher detergent powder. *Can Respir J* 2012;19:e25–8.
- Brant A, Upchurch S, van Tongeren M, et al. Detergent protease exposure and respiratory disease: case-referent analysis of a retrospective cohort. *Occup Environ Med* 2009;66:754–8.
- van Rooy FG, Houba R, Palmen N, et al. A cross-sectional study among detergent workers exposed to liquid detergent enzymes. *Occup Environ Med* 2009;66:759–65.
- Fink MJ, Rudroff F, Mihovilovic MD. Baeyer-Villiger monooxygenases in aroma compound synthesis. *Bioorg Med Chem Lett* 2011;21:6135–8.
- Li C, Sun J, Fu C, et al. Synthesis and evaluation of odour-active methionyl esters of fatty acids via esterification and transesterification of butter oil. *Food Chem* 2014;145:796–801.
- Smit G, Smit BA, Engels WJ. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiol Rev* 2005;29:591–610.
- Williams AG, Withers SE, Brechany EY, et al. Glutamate dehydrogenase activity in lactobacilli and the use of glutamate dehydrogenase-producing adjunct *Lactobacillus* spp. cultures in the manufacture of cheddar cheese. *J Appl Microbiol* 2006;101:1062–75.
- Aasen TB, Burge PS, Henneberger PK, et al. Diagnostic approach in cases with suspected work-related asthma. *J Occup Med Toxicol* 2013;8:17.
- Baur X, Sigsgaard T, Aasen TB, et al. Guidelines for the management of work-related asthma. *Eur Respir J* 2012;39:529–45.
- Deckwart M, Carstens C, Webber-Witt M, et al. Development of a sensitive ELISA for the detection of casein-containing fining agents in red and white wines. *J Agric Food Chem* 2014;62:6803–12.
- Budnik LT, Preisser AM, Permentier H, et al. Is specific IgE antibody analysis feasible for the diagnosis of methylenediphenyl diisocyanate-induced occupational asthma? *Int Arch Occup Environ Health* 2013;86:417–30.
- Baur X, Budnik LT, von Kirchbach G. Allergic asthma caused by exposure to bacterial alpha-amylase Termamyl(R). *Am J Ind Med* 2013;56:378–80.
- Baur X, Huber H, Degens PO, et al. Relation between occupational asthma case history, bronchial methacholine challenge, and specific challenge test in patients with suspected occupational asthma. *Am J Ind Med* 1998;33:114–22.
- Elms J, Robinson E, Mason H, et al. Enzyme exposure in the British baking industry. *Ann Occup Hyg* 2006;50:379–84.
- O'Connor TM, Bourke JF, Jones M, et al. Report of occupational asthma due to phytase and beta-glucanase. *Occup Environ Med* 2001;58:417–19.
- National Institute of Health. NIH guidelines for the diagnosis and management of food allergy in the United States. Report of the NIAID-Sponsored Expert Panel, 2010. NIH Publication no. 11-7700. <http://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/TipsandArticlesonDeviceSafety/ucm109367.htm>
- Ladics GS, Fry J, Goodman R, et al. Allergic sensitization: screening methods. *Clin Transl Allergy* 2014;4:13.
- Johnsen CR, Sorensen TB, Ingemann Larsen A, et al. Allergy risk in an enzyme producing plant: a retrospective follow up study. *Occup Environ Med* 1997;54:671–5.
- Lipińska-Ojrzanowska A, Świerczyńska-Machura D, Tymoszek D, et al. Occupational asthma in female factory worker resulting from exposure to savinase in dishwashing tablets—a case study. *J Occup Health* 2013;55:318–21.
- Caballero ML, Gómez M, González-Muñoz M, et al. Occupational sensitization to fungal enzymes used in animal feed industry. *Int Arch Allergy Immunol* 2007;144:231–9.
- Larsen AI, Johnsen CR, Frickmann J, et al. Incidence of respiratory sensitisation and allergy to enzymes among employees in an enzyme producing plant and the relation to exposure and host factors. *Occup Environ Med* 2007;64:763–8.
- Basketter DA, Kruszewski FH, Mathieu S, et al. Managing the risk of occupational allergy in the enzyme detergent industry. *J Occup Environ Hyg* 2015;12:431–7.