

Safety assessment of enzyme-containing personal cleansing products: Exposure characterization and development of IgE antibody to enzymes after a 6-month use test

Christopher K. Kelling, PhD, Robert G. Bartolo, PhD, Keith D. Ertel, PhD,
Laurence A. Smith, PhD, Debra D. Watson, and Katherine Sarlo, PhD
Cincinnati, Ohio

Background: Enzyme-containing personal cleansing products were being considered for the consumer market. Although enzymes have been marketed safely for many years as ingredients in laundry products, their use in a personal cleansing application represented a new type of exposure for consumers that was not supported by the historical safety data. An exposure assessment and additional safety data would be needed before marketing to ensure consumer safety.

Objective: The work in this paper was designed to evaluate the potential for inhalation exposure to the enzyme during use of this new product while showering. Then a clinical trial was conducted to determine whether or not the level, duration, and routes of exposure encountered during use of this product would induce a Type I sensitization response to the enzyme.

Methods: Exposure was assessed during normal showering activities by collecting air samples with both high volume and personal samplers and quantitating enzyme levels with an ELISA. To assess the potential for sensitization, panelists were asked to use a prototype protease-containing bar product for all personal cleansing tasks and to keep a use diary reporting any associated symptoms. Physical and dermatologic examinations and skin prick tests with enzyme were conducted before the test commenced and at 2-month intervals.

Results: Exposure assessment results showed that airborne enzyme levels were primarily dependent on the concentration of the enzyme in the personal cleansing product. Mean values for total airborne enzyme protein ranged from 5.7 to 11.8 ng/m³ when enzyme concentration, time of use, and measurement technique remained constant. After 6 months of at-home product use, four of 61 test subjects using the enzyme-containing bar had positive skin prick test responses when tested with the enzyme. The skin prick test data were supplemented with serologic analyses, which detected IgE specific for the protease enzyme. None of these subjects showed any clinical symptoms indicative of allergic reaction.

Conclusion: The ability of enzymes to induce development of allergic antibodies in this study led to the conclusion that this prototype enzyme-containing personal cleansing bar

would represent an inappropriate use of enzymes in a consumer product application. The likelihood of both induction of an immunologic response and subsequent elicitation of allergy symptoms in a small but significant fraction of the user population was high. This finding resulted in the decision to halt further development of this prototype. (*J Allergy Clin Immunol* 1998;101:179-87.)

Key words: Enzyme, proteases, inhalation exposure, IgE-mediated hypersensitivity, allergy

Enzymes have been used safely in laundry products for many years. Enzymes provide unique cleaning benefits with good chemical efficacy and excellent environmental safety profiles. With the exception of a few isolated cases of allergic reactions among consumers exposed to enzyme powders in very dusty laundry products in the late 1960s,¹⁻³ no consumers have been known to have allergic antibodies to enzymes caused by the use of enzyme-containing laundry products. On the other hand, consumers have developed allergy to papain when this enzyme was incorporated into products that brought it into intimate contact with a mucosal surface. One example was development of allergy to papain used in contact lens cleaning solutions.^{4,5} Another example was the development of allergy in a cosmetologist who handled papain powder in cosmetics.⁶ Therefore there have been situations where enzymes caused allergy in consumers.

Detergent enzymes are recognized occupational allergens. In the late 1960s, when enzyme powders were first incorporated into detergents, high levels of worker exposure in detergent manufacturing facilities resulted in cases of IgE-mediated hypersensitivity reactions.^{7,8} As a result, commercial enzyme producers developed technology to deliver enzymes to products in much less dusty forms, and the detergent industry implemented strict occupational controls for allowable airborne exposure.⁹ Since these controls have been in place, reports of adverse health effects and development of IgE antibodies in workers have been greatly reduced. This history of safe use of enzyme-containing laundry products has prompted consideration of the incorporation of detergent enzymes into other types of products. Even so, given the recognized potential of enzymes as occupational respiratory allergens¹⁰⁻¹³ and proposed new uses

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Reprint requests: Katherine Sarlo, PhD, The Procter & Gamble Company, Miami Valley Laboratories, P.O. Box 538707, Cincinnati, OH 45253-8707.

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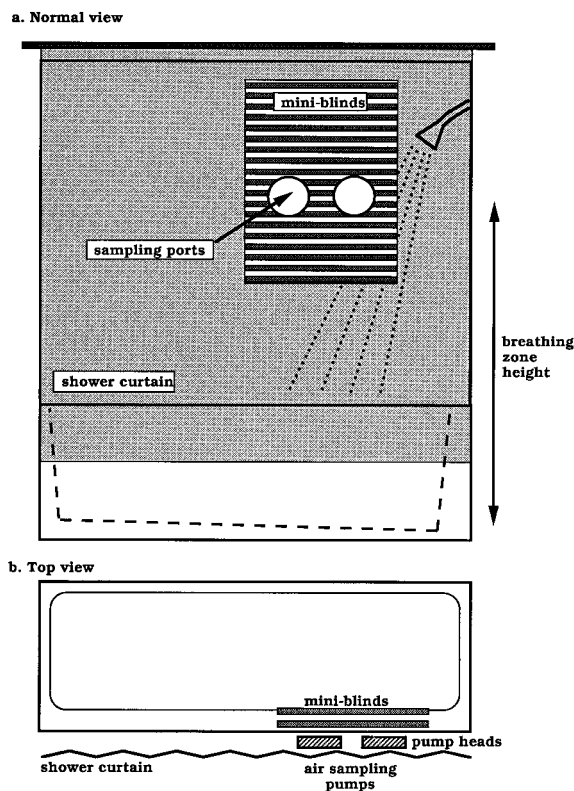


FIG. 1. An illustration of the shower enclosure set-up used in measurement of airborne enzyme levels with high volume samplers. The shower enclosure consisted of a standard tub and shower curtain. For some protocols, an extension was taped in place above the shower curtain to seal the area between the shower curtain and the ceiling. High volume samplers were mounted at the sampling ports with the pump heads extended 1 inch inside the shower enclosure. Two sets of mini-blinds that were hung in front of the pump heads eliminated splashing onto the sampler filter pad.

for enzymes in consumer products, it was important to fully understand the level of consumer exposure that could result from any potential new application of enzymes. Then one should determine if such a level could lead to respiratory sensitization under normal use conditions.

The new product application considered in this assessment was the inclusion of proteolytic enzymes in personal cleansing products. Recently, it has been suggested that the performance of these products might be enhanced by the inclusion of proteolytic enzymes.¹⁴ It was recognized that this application represented a potential new source of exposure to enzymes for the consumer above and beyond that associated with use of laundry products. For example, there is the potential for the enzyme to become aerosolized in the shower with subsequent inhalation, as well as mucosal and dermal exposure. Therefore careful evaluation of the inhalation exposure levels during use and the consumer practices influencing these exposures were critical to the overall safety assessment. Once the exposures were measured,

the important next step was to determine if it would be safe to market this new product application when air concentration of enzymes and routes of exposure go beyond those previously established as safe.

The threshold limit value for subtilisin is 60 ng/m³.¹⁵ The Procter & Gamble Company uses a more stringent voluntary occupational exposure guideline of 15 ng/m³.^{13, 16} We have data to show that workers who have allergic antibody to a particular enzyme can work without allergic symptoms in an environment when enzyme levels in the detergent manufacturing facilities are below 15 ng/m³.^{16, 17} Our data also suggest that the majority of sensitizations (development of allergic antibody) to enzymes occur when exposures exceed 15 ng/m³ (unpublished data, 1985-1997). Frequency, duration, and magnitude of exposures to enzyme proteins from personal cleansing products were shown to be substantially less than those occurring in an occupational setting. Therefore a 6-month at-home pilot use test with concurrent monitoring for signs of IgE-mediated hypersensitivity was initiated. Individuals with a documented history of seasonal allergic rhinitis were enrolled in the study.

METHODS

Test materials

The enzyme used was a *Bacillus subtilis* B-derived serine protease supplied by Genencor International (San Francisco, Calif.). This particular enzyme has been used safely in nationally marketed laundry products for over 5 years (unpublished observations). The enzyme is available in liquid and granular forms. The ratio of active protein to total protein content varies between forms and between enzyme lots. Relative levels for liquid lots used were 3.4% to 3.6%:6.8% to 7.2% (active:total). For the single lot of granular enzyme used to provide partial activity for the 1.0% active bar described below, this relationship was 4.1%:13.4%. Test products were made by adding the appropriate amount of the protease enzyme preparation to the base products. For test bars, enzyme was added just before amalgamation at levels sufficient to achieve 1.0%, 0.2%, and 0.1% proteolytically active enzyme. These levels corresponded to 2.8%, 0.4%, and 0.2% total enzyme protein (active plus inactive). The control product was a prototype bar containing a sodium cocoyl isethionate-based surfactant system and was very similar in overall composition to products that have been marketed safely for a number of years.

Test subjects: exposure assessment

Subjects that participated in the exposure assessment studies were normal healthy volunteers. All subjects were given full explanations of the risks and benefits of the procedures and signed an informed consent statement. Risk assessment criteria supporting the test protocols were reviewed by the sponsor's institutional review board. Specific test protocols were reviewed and approved by a 10-member advisory group composed of safety professionals, including two physicians. Before study commencement, each subject was given a physical examination, spirometry test, and a skin prick test to confirm that the subject was in generally good health and was not sensitized to the enzyme being used in the studies. Subjects wore swimming attire for all experiments.

Subjects were assigned randomly to the various test products or control product (i.e., product without enzyme). Several

subjects participated in multiple trials. In some experiments subjects were asked to wear respirators (North Series 7700 half mask respirators with HEPA filter cartridges; North Safety, Inc., Cranfinance R.I.) and were given appropriate training on the proper use and fit of these devices. The subjects showering with products containing lower enzyme concentrations (0.2% or less) were permitted to dispense with the respirators. Respirators were still required for subjects using products with higher enzyme levels.

Experimental design: exposure assessment

Two different showering protocols were used. For the controlled time experiments, subjects were asked to perform three 2-minute showers with the test product. Two minutes was chosen as a representative amount of time for a consumer to accomplish the shower task. Two sampling pumps were used concurrently. Air samples were collected onto the same two filter pads for all three showers. For the ad-lib use experiments, subjects were asked to shower as they normally would at home. The amount of product used was determined by weighing each bar at the beginning of the trial and again at the end. The shower enclosure within the test bathroom facility consisted of a standard tub, a removable shower head that could be used in either a fixed or hand-held manner, and a shower curtain. The volume of the shower enclosure was 3.2 m³, and the volume of the test bathroom facility (not including the shower enclosure) was 23 m³. Holes were cut in the shower curtain at a height of 5 feet (the approximate height of the breathing zone), and air samplers were mounted outside the shower enclosure with the filter assemblies protruding one inch through the holes into the shower area. To minimize splashing onto the collection filters, two sets of mini-blinds with nonparallel slats were hung inside the shower curtain in front of the air samplers (Fig. 1). The effectiveness of this set-up for prevention of splashing was confirmed by conducting a showering experiment with the sampling pumps off and analyzing sample filters to confirm that there was no detectable enzyme. After each subject completed a trial, the room was thoroughly vented, the shower enclosure and mini-blinds were cleaned, and a new shower curtain was installed in preparation for the next subject. During showering, the water flow rate was between 190 and 120 ml/sec, and the water temperature was 92° to 110° F. For the controlled time experiments, one side of the shower curtain and the area above the shower curtain (i.e., between the shower curtain and the ceiling) were sealed to minimize air exchange between the shower enclosure and the rest of the room. Sampling was done only while showers were in progress. For the ad-lib use experiments, the shower curtain was positioned and closed normally by the subject with no particular attempt to control airflow. Enzyme levels were measured in the shower enclosure during showering as described above. To measure enzyme levels in the room, an additional two air samplers were placed approximately 3 feet from the front of the shower curtain and were allowed to run during the shower and immediately after showering while the subjects performed their normal post-shower habits (e.g., towel drying and drying hair).

Sample collection

Air samples were collected with General Metal Works P-2000, Handi-Vol high volume air samplers (General Metal Works, Inc., Cleves, Ohio) equipped with airflow meters and 4 inch round filter pad holders containing Whatman GFC grade glass microfibre filters. These samplers were the method of choice for these experiments because they have been used for

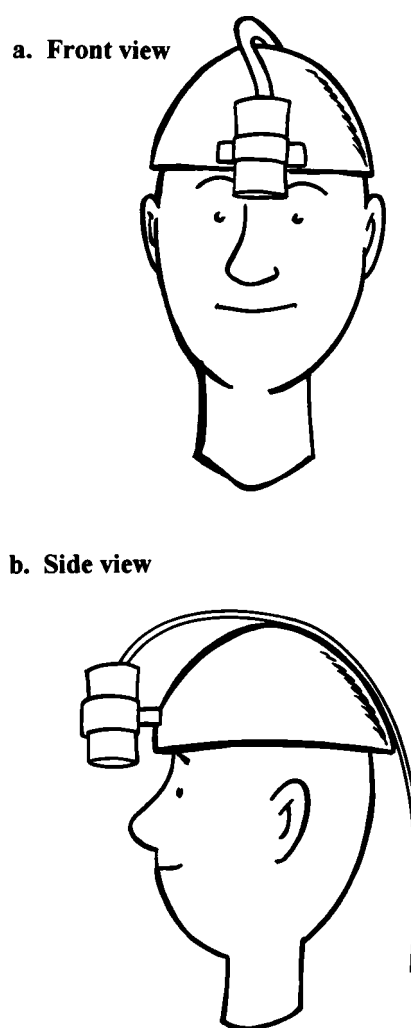


FIG. 2. An illustration of the personal sampling apparatus used in measurement of airborne enzyme levels in the breathing zone. The personal sampler was affixed to a hard hat such that the sampling port was as close as possible to the breathing zone.

over 30 years to monitor airborne enzyme levels in detergent manufacturing facilities. A large body of internal data and some external data are available on average airborne enzyme levels detected with these samplers and on corresponding health status (skin prick test reactivity and lung function analysis) of workers exposed to these levels.^{16, 17} The airflow rate of the Handi-Vol sampler is generally between 0.35 and 0.65 m³/min. The high moisture content of the air being sampled in these experiments caused airflow to vary slightly more than normal, resulting in airflow rates of 0.27 to 0.7 m³/min. The total volume of air sampled during a given trial was determined by measuring airflow at frequent time intervals and summing intermediate volumes (time elapsed for interval $\times$ airflow [m³/min] for that interval). In some experiments personal samplers were used. The personal sampler consisted of a 37 mm Nuclepore air sampling cassette (SKC, Inc., Eighty Four, Pa.) with Whatman GFC glass microfibre filters (Whatman Int., Ltd., Madstone, England) of the appropriate size. This was attached to a Dawson High Volume Air Sampler (Dawson Associates, Inc., Lawrenceburg, Ind.). These devices have a maximum airflow of 0.02 m³/min. The sampling cassettes

TABLE I. Comparison of airborne enzyme levels with different prototypical bar products

Experiment no.	Enzyme protein concentration in product (%)	Airborne concentration (ng/m ³ ± SD)		Product used in 2-minute shower (gm)	Number of trials
		Hi volume sampler	Personal sampler		
1	2.8	183 ± 142	ND	10.2 ± 5.1*	21
	0.4	15.7 ± 8.4‡	ND	6.0 ± 5.9§	14
	0.2	11.4 ± 7.8‡	ND	9.1 ± 4.4	8
2	0.2	5.7 ± 2.9	ND	8.7 ± 4.9	10
	0.2	6.7 ± 4.9	3.5 ± 2.1†	7.4 ± 3.8	10
3	0.2	11.8 ± 2.4		ND	5
	(shower stall)				
	0.2 (room)	4.3 ± 3.8		ND	5

The amount of bar product use was determined by weighing the bar before and after completion of three 2-minute showers and dividing this amount by 3. In experiment 3, all sampling was conducted with the Hi volume sampler.

ND, = Not determined.

*Product use is based on 14 trials.

†One value was at the lower detection limit. For the purposes of statistics, this value was assigned the numerical value of the lower limit of detection (1.6 ng/m³).

‡Significantly different from the group using 2.8% ($p < 0.001$).

§Significantly different from the groups using 2.8% and 0.2% ($p < 0.001$ and $p < 0.05$, respectively).

were taped to hard hats worn by the test subjects during showering. The sampling cassette was positioned a few inches above and in front of the nose and mouth (Fig. 2). For these experiments, the air flow of the personal sampling apparatus was between 0.014 and 0.015 m³/min.

ELISA for analysis

Enzyme concentrations were quantitated with an ELISA. The ELISA was developed by Battelle Laboratories (Columbus, Ohio) for use in manufacturing facilities. This assay measures total enzyme protein (active plus inactive) and has been described in detail elsewhere.¹⁸ Briefly, microwell plates were precoated with 100 µl of a 2 µg/ml solution of affinity-purified rabbit antibody to the enzyme and 1% bovine serum albumin. Enzyme was extracted from the air-sampling filters by incubating in 25 ml of extraction buffer (a NaCl/Tris buffer containing sodium thiosulfate and calcium chloride). Next, 100 µl of the extracted samples were added to the microwells along with 50 µl of NaCl/Tris buffer. After 15 minutes of incubation at 37° C, 50 µl of alkaline phosphatase conjugate of the rabbit anti-enzyme antibody were added. After 2 hours of incubation, 100 µl of wash buffer and 10 µl of the chromogenic substrate (para-nitrophenyl phosphate) were added to each microwell and incubated for an additional 30 min. The reaction was stopped by adding 100 µl of 7.5% ethylenediamine tetraacetic acid. The absorbance at 405 nm was determined on a BioTek plate reader (BioTek, Winooski, Vt.). All samples were run in triplicate and were not diluted unless they were expected to be above the detection range of the assay. Standard curves were conducted with each assay, with additional high and low controls included at the end of each separate microwell plate. The detection range of the assay was 1.9 to 190 ± 0.5 ng/m³ at an air sampling flow rate of 0.67 m³/min. For experiments with the personal samplers, the method was modified to increase sensitivity by decreasing the volume of the extraction buffer from 25 to 5 ml, increasing the concentration of the antibody used to coat the wells from 2 µg/ml to 5 µg/ml, increasing the volume of coating antibody from 100 µl to 150 µl, and extending the incubation time after addition of extracted samples and before addition of the chromogenic substrate to the

microwells by 1 hour. The detection range of this assay was 1.7 to 17 ± 0.7 ng/m³ at an air sampling flow rate of 0.015 m³/min.

Test subjects: pilot use test

Male and female volunteers between the ages of 18 and 65 who reported a history of seasonal allergic rhinitis in a detailed medical screening questionnaire were recruited for the study. All subjects were given a full explanation of the risks and benefits of the procedures and signed an informed consent statement. All aspects of the protocol and informed consent were approved by the clinical site's institutional review board. Before study commencement, each subject underwent examination by a board-certified dermatologist to ensure that they were in good health and had clinically normal skin. Each subject was screened by skin prick testing with the enzyme used in the test product to confirm the absence of allergic antibodies to the enzyme. Serum samples were collected from each subject for future serum immunoglobulin analysis. A total of 108 subjects were enrolled in the study and were randomly assigned to the test ($n = 65$) and control ($n = 43$) groups. Subjects were unaware of their group assignment. Those in the test group were supplied with test products containing the enzyme throughout the 6-month study. Those in the control group were supplied with control products (identical product without the enzyme). During the study, each subject was asked to substitute this product for their usual product for all personal cleansing tasks and to not allow other members of the household to use the product. Each subject was asked to maintain a diary detailing the date and frequency of product use, any noticeable respiratory or other health-related symptoms, and any changes in skin condition. Subjects returned every 2 weeks to receive a fresh supply of their assigned product and to report any observations on product performance, including potential adverse effects on skin or other health effects. In addition, every 2 months subjects underwent dermatologic evaluation and skin prick testing with the enzyme of interest. Examiners were not aware of the group assignments of the subjects. Blood sampling for serum immunoglobulin analysis was done only on subjects who had a positive skin prick test response to the enzyme.

Skin prick tests

The test enzyme solution was prepared for use in skin prick tests by diluting with a 50/50 mixture of sterile saline and glycerol to a concentration of 50 µg protein/ml. The solution was stored at 4° C until needed. The test enzyme, the positive histamine control (1:1000 dilution of histamine sulfate in saline and glycerol), and the negative saline control were tested at the start of the study, at the 2- and 4-month evaluation points, and at the 6-month completion point of the study. The enzyme and enzyme concentration were identical to those used in the current medical surveillance program used at Procter & Gamble detergent manufacturing facilities.¹⁶ Tests were performed with the method described by Pepys.¹⁹ A drop of the test or control material was placed on the volar surface of the forearm and a slight prick of the surface epidermis was made using a sterile 26-27 gauge needle. The test site was observed after 15 minutes for the presence of a wheal and flare reaction. Resulting reactions were measured, and the sizes of the wheal and flare reactions at the widest points were recorded. Any positive reactions to the test antigen were confirmed by repeat testing on the opposite arm. A test was considered positive if it exhibited a wheal at least 3 mm greater than the negative control and a flare that was larger than the wheal.

Analysis of sera

Semiquantitative analysis of serum samples for the presence of enzyme-specific IgE was conducted with the Kabi Pharmacia Diagnostics' CAP System RAST FEIA fluoroimmunoassay (Uppsala, Sweden). The method was carried out according to the manufacturer's directions. This assay was developed specifically for the 6-month use test. ImmunoCAPs with the protease enzyme conjugated to the cellulose carrier were custom-made by Pharmacia. Sera from three individuals known to have positive skin prick test responses through occupational exposure were used to develop the reference standards. These were used to construct a standard curve corresponding to the manufacturer's standard curve for an allergen-specific IgE anti-IgE system. Normal pooled human sera and nonspecific high IgE sera (500 to 2000 IgE units/ml) selected from a patient population served as negative controls. For the assay, control sera and sera from subjects with positive skin prick test results were incubated with the immunoCAPs, followed by rabbit anti-human IgE-β-galactosidase. A development solution of 4-methylumbelliferyl-β-galactoside was then added and allowed to develop for 10 minutes before adding the stop solution. The fluorescence was then measured in a FluoroCount 96 (Pharmacia, Uppsala, Sweden) and compared with the standard curve to determine the concentration of allergen-specific IgE (expressed in kilounits of antibody/liter or kUA/L) and the RAST class for each test serum. The assay had a measuring range of 0.35 to 100 kUA/L in undiluted serum. In this study the level of reaction was designated as *absent* (RAST class 0) if the fluorescence of the test serum was lower than that of the 0.35 kUA/L standard (on the basis of the cut-off for positive and negative results with the standard serum), *low* (RAST class I) if the fluorescence fell between that of the 0.35 and 0.7 kUA/L standards, *moderate* (RAST class II) if the fluorescence was between the 0.7 and 3.5 kUA/L standards, *high* (RAST class III) if between the 3.5 and 17.5 kUA/L standards, and *very high* (RAST classes IV to V) if greater than the 17.5 kUA/L standard. The division into RAST classes was done on the basis of fluorescence values and IgE units of the manufacturer's standard, which was run in parallel with the enzyme assay.

TABLE II. Skin prick test results with test enzyme

	Baseline		2 Months		4 Months		6 Months	
	pos	neg	pos	neg	pos	neg	pos	neg
Test group	0	65	0	65	0	64‡	4	57§
Control group	0	43	1*	41†	0	42	0	38§

Subjects in the test group were using the control soap product containing 0.2% enzyme. Those in the control group were using product with no enzyme.

pos, Positive; neg, negative.

*This subject completed the study and was negative at the 4- and 6-month evaluations.

†One subject in the Control Group failed to return for the 2-month evaluation. This subject could not be contacted for follow up.

‡One subject dropped out of the Test Group for personal reasons before the 4-month evaluation.

§Two subjects (one from the test group and one from the control group) dropped out of the study because of scheduling conflicts before the 6-month evaluation. One subject in the control group was withdrawn from the study after the 4-month evaluation when it was discovered that this subject was concurrently participating in a nonrelated personal cleansing study. Four subjects (two from the test group and two from the control group) failed to return for the 6-month evaluation.

Diagnostic patch test

The patch test method used in this study was a modification of the standard diagnostic patch test protocol used by the test facility. Subjects were patch tested through a semi-occlusive patch in the paraspinal region of the back. Test samples included a 0.5% solution of the test product (control soap with enzyme), a 0.5% solution of the control product (control soap without enzyme), and 0.1% and 0.2% solutions of sodium lauryl sulfate as irritant controls. All dilutions (wt/vol) were prepared with tap water as the diluent. Tap water was also used as a negative control. For each patch, 0.2 ml of the test or control sample was applied to a Professional Products patch (consisting of a Webril pad covered with Blenderm tape). Single patches were used for all test materials with the exception of the test product (control soap with enzyme). Two patches of this material were used. One of the two test product patches was removed after 1 hour, and the site was scored immediately and 24 hours after patch removal. All other patches were removed after 24 hours and scored 1 hour and 24 hours after patch removal. Sites were scored for erythema using a four point scale: 0 = no visible erythema, 1 = mild erythema, 2 = moderate erythema, and 3 = severe erythema. Any evidence of edema, spreading, or weeping was also noted.

Statistical methods

For both aerosol levels and use data, comparisons were made between group means by using Wilcoxon's Sum of Ranks Test.²⁰ In addition, to determine if aerosol levels were dependent on product use, scatter plot analyses were conducted and correlation coefficients were computed for the test groups as indicated in the Results section.²¹

RESULTS

Airborne enzyme concentrations in the shower enclosure

Experiments were conducted to determine the airborne enzyme concentrations reached during showering while making use of bar products containing different

TABLE III. Skin prick test responses

Subject no.	Test material	Initial	2 months	4 months	6 months	
					Test	Confirmation
34	Test enzyme	0	0	0	8 (25)*	5 (16)
	Histamine	10 (35)	3 (7)	9 (17)	7 (14)	15 (7)
	Saline	0	0	0	0	0
43	Test enzyme	0	0	0	6 (11)	6 (9)
	Histamine	6 (10)	3 (5)	5 (8)	5 (9)	6 (9)
	Saline	0	0	0	0	0
60	Test enzyme	0	0	0	5 (15)	4 (25)
	Histamine	7 (20)	6 (14)	4 (12)	3 (30)	ND
	Saline	0	0	0	0	ND
62	Test enzyme	0	0	0	3 (15)	3 (30)
	Histamine	5 (10)	3 (8)	4 (9)	5 (30)	ND
	Saline	0	0	0	0	0

ND, Not done.

*The skin prick test result is recorded as the size in millimeters of the wheal (flare) at the largest points.

concentrations of enzyme. Each subject was asked to take three 2-minute showers. Results are shown in Table I, *experiment 1*. Airborne enzyme levels decreased proportionately as the concentration of enzyme in the product decreased. The highest enzyme concentration in the product produced significantly higher airborne enzyme levels than the two lower concentrations. Parallel measurements were made by using both the high volume sampler positioned normally at the shower curtain and the personal sampler affixed to a hard hat worn by the test subject during showering. The results are shown in Table I, *experiment 2*. In this experiment, the airborne enzyme levels were monitored for half the subjects by using only the high volume sampler. For the other half of the subjects, the two sampling methods were used simultaneously. There was no difference between the high volume sampling data for both groups. The personal sampler data showed airborne levels that were not significantly different than those obtained with the high volume sampler. The mean product use for the group wearing the personal samplers was no different than that of the groups that were not wearing personal samplers.

Airborne enzyme concentrations outside the shower enclosure

Airborne enzyme levels in the bathroom outside the shower enclosure were measured in ad-lib use experiments where no special precautions were taken to confine the airborne enzyme inside the shower enclosure. Table I, *experiment 3* shows those results. Levels in the room were about 2.5-fold lower (4.3 ± 1.7 ng/m³) than those in the shower enclosure itself (11.8 ± 2.4 ng/m³).

Development of enzyme-specific IgE

Table II shows the results of skin prick tests with the protease enzyme on both the test and control groups after 2, 4, and 6 months of at-home use of the product.

None of the subjects in the test group had positive test results at 2 or 4 months. However, by 6 months four of the 61 subjects completing the study in the test group had positive skin prick test responses to the protease enzyme. Table III shows the wheal and flare measurements of the skin prick tests conducted on these four individuals throughout the study.

In the control group one individual had a weak skin prick test response (3 mm wheal and 4 mm flare) at 2 months (Table II). Because of a laboratory error, the confirmatory skin prick test was not conducted, and the subject continued in the study. This subject had negative reactions to the test antigen at 4 and 6 months. No other individual in the control group showed any reaction to the enzyme at any other evaluation time point.

Sera from the four individuals who had positive skin prick test responses at the 6-month time point were analyzed for the presence of protease-specific IgE in the Pharmacia CAP System and compared with sera collected from the same individuals before test commencement. Table IV shows the fluorescence values and IgE concentrations (kUA/L) of the positive and negative control sera. The negative control sera provided fluorescence values that correlated with IgE concentrations below the reference standard cut-off of 0.35 kUA/L. The undiluted positive control serum A contained 55.25 kUA/L IgE (RAST class IV or V). Dilutions of this sample resulted in a corresponding decrease in the concentration of IgE and a drop in RAST class. Calculation of the concentration of IgE and corresponding RAST class in the diluted samples from the positive controls correlated with the concentration of IgE and corresponding RAST class in the undiluted sample. Table V shows the results of the analyses of the four test sera from the subjects with positive skin prick test responses. All four subjects were negative for the presence of protease-specific IgE before the start of the study (less than 0.35 kUA/L). After 6 months of at-home

TABLE IV. Serologic analysis of subjects with positive skin prick test responses: Characterization of positive and negative control sera

Sample	Fluorescence	IgE concentration (kUA/L $\pm$ SD)	RAST class	Calculated IgE concentration*	RAST class
Control A					
Undiluted	16148 $\pm$ 452†	55.25 $\pm$ 8.1	IV-V		
1:3	8796 $\pm$ 190†	20.24 $\pm$ 2.2	IV-V	61.35	IV-V
1:15	2836 $\pm$ 76.4†	4.66 $\pm$ 0.06	III	69.9	IV-V
1:75	777 $\pm$ 82†	0.90 $\pm$ 0.14	II	67.5	IV-V
1:150	456 $\pm$ 15†	0.44 $\pm$ 0.04	I	66	IV-V
Control B					
Undiluted	3859 $\pm$ 108†	6.90 $\pm$ 0.22	III		
1:5	1010 $\pm$ 7.1†	1.65 $\pm$ 0.007	II	8.3	III
1:10	615 $\pm$ 17.6†	0.79 $\pm$ 0.28	I-II	7.9	III
Control C					
(undiluted)	244‡	<0.35	0		
Normal sera					
(undiluted)	235 $\pm$ 20.5†	<0.35	0		
High IgE sera					
(undiluted)	202 $\pm$ 1.4†	<0.35	0		

*IgE concentration was calculated by multiplying the average IgE units (interpolated from the standard curve) by the dilution factor.

†Average of two determinations.

‡Single determination.

use of the protease-containing personal cleansing bar, they developed antibody levels that were above baseline and fell into RAST Class I to RAST Class III or IV.

Respiratory symptoms

None of the four individuals who had IgE to the enzyme (test subjects 34, 43, 60, and 62) or other individuals in the test or control groups reported any respiratory symptoms throughout the course of the study. No respiratory symptoms were observed during the 2-, 4-, or 6-month examinations.

Dermal symptoms

Several subjects in the test and control groups reported symptoms such as redness, itching, and dryness. In two instances subjects reported exacerbation of acne. One subject developed what appeared to be Type IV dermatitis approximately 2 months after the start of the study. However, diagnostic patch test results were negative, suggesting that this reaction was not indicative of contact dermatitis caused by the test material.

Two of the four individuals who had positive responses to skin prick tests reported skin reactions during the course of the study. Subject 34 reported dry, itchy patches on the legs beginning after approximately 3 months of test product use and appearing periodically throughout the remainder of the study. After approximately 5 months of test product use, Subject 62 reported itching and blotching on her face that occurred immediately after washing.

DISCUSSION

In this study we have shown that individuals exposed to proteolytic enzyme in a personal cleansing product

developed IgE antibody to the enzyme after 4, but before 6, months of exposure to the product. None of the sensitized individuals developed allergic symptoms. The dermal reactions observed in the study were irritant in nature and not indicative of Type I hypersensitivity reactions to the enzyme. The predominant symptoms (redness, itching, and dryness) were common for the cold weather conditions that were prevalent during the course of this study. The exposure assessment performed before this pilot clinical test with the high volume sampler showed airborne enzyme concentrations below the threshold limit value of 60 ng/m³ and near our internal manufacturing occupational exposure guideline of 15 ng/m³. We believe that exposures that exceed the 15 ng/m³ level are responsible for the occupational sensitizations in detergent manufacturing sites.¹⁶ After considering these exposure data and the short duration of exposure (as compared with 8-hour occupational exposures), we conducted the pilot clinical study with the expectation that no subjects would be sensitized. The results from this study now lead us to believe that these sensitizations and their rapid onset were caused by the unique exposure from use of the personal cleansing product and could not have been predicted from our occupational experience. The test subjects were exposed to the enzymes in a very moist environment and were intentionally applying the product to hydrated skin and mucous membranes. It was also possible that lather containing enzyme protein was splashed into the nasal cavities, eyes, and other mucosal surfaces. The relative contributions of each of these exposures to the sensitizations observed could not be addressed by this study but should be considered in the overall safety assessment by any manufacturer considering the development of

TABLE V. IgE antibody in baseline and 6 month sera from subjects with positive skin test responses

Subject no.	Baseline			6 Months		
	Fluorescence	IgE Concentration (kUA/L $\pm$ SD)	RAST class	Fluorescence	IgE Concentration (kUA/L $\pm$ SD)	RAST class
34	147 $\pm$ 20†	<0.35	0	416 $\pm$ 63*	0.39 $\pm$ 0.05	I
43	151 $\pm$ 19‡	<0.35	0	1873 $\pm$ 59‡	2.77 $\pm$ 0.10	II
60	143 $\pm$ 16†	<0.35	0	2172 $\pm$ 322*	3.3 $\pm$ 0.50	III-IV
62	150 $\pm$ 0†	<0.35	0	1023 $\pm$ 103*	1.3 $\pm$ 0.17	II

*Average of two determinations.

†Average of three determinations.

‡Average of four determinations.

similar products. Therefore incorporation of enzymes into personal care products must be carefully evaluated before marketing.

The data from our exposure assessment experiments showed that the concentration of enzyme in the product was clearly related to the airborne levels in the shower. Although not presented, a prototype liquid soap matrix resulted in higher airborne concentrations of enzyme as compared with the bar. This indicates that the form of the product can also affect aerosolization. Conversely, the use of a hand-held shower head versus a fixed shower head had no effect on aerosolization of the enzyme-containing personal care product (data not shown). The comparison between data collected with the personal sampler versus the high volume sampler was made after the clinical study to place the unexpected results from this test into perspective. The intent was to determine whether protein levels immediately next to the skin and in the breathing zone were substantially higher than those detected by the high volume sampler, which was positioned at the shower curtain. Our hypothesis was that the shower spray impinged on the surface of the skin, creating a microenvironment with higher aerosolized enzyme concentrations in the immediate vicinity of the source of aerosolization and that a sharp concentration gradient existed, with the highest levels very close to the skin (and in the breathing zone) and lower concentrations in other more distant regions of the shower stall (e.g., where the high volume sampler was positioned). This was not the case. Measurements conducted with the personal samplers placed directly in the breathing zone yielded airborne enzyme levels that were comparable to those detected with the high volume sampler.

As stated earlier, we concluded that the dermal reactions observed in this study were irritant in nature. With the exception of subjects 34 and 62, the individuals who reported dermal reactions had negative skin prick test responses throughout the course of the study. We also believe that allergy can be ruled out as the cause of the skin reactions in subjects 34 and 62. The dermal symptoms for subject 34 were reported after 3 months of product use. Because this subject had a negative skin prick test response at 4 months, the onset of dermal symptoms occurred before the development of IgE. The dermal symptoms reported by subject 62 began after the

4-month negative skin prick test response and before the 6-month positive response. The subject reported that the symptoms she experienced would subside if she immediately applied make-up. This would not be consistent with a Type I hypersensitivity reaction, which would not be affected by the application of make-up. However, this would be consistent with weather-related dryness and irritation because facial make-up usually has some moisturizing properties that may ameliorate sensations associated with dryness.

The possibility that Type I hypersensitivity can be induced through dermal exposure is controversial. Recent reports indicate that allergic reactions to proteins in latex can be elicited by dermal contact, confirming the ability of these allergens to penetrate the skin barrier under some circumstances. However, the mechanism for induction of the allergic response to latex proteins is not fully understood. Although respiratory exposure is believed to be the primary route accounting for these reactions, cutaneous and mucosal exposure may also play a role.²²

Other evidence indicates that dermal exposure alone would not be sufficient to account for the results observed in this shower study. In several geographic locations around the world (e.g., Philippines, India, and Latin America), hand scrubbing all laundry with detergents containing enzymes is the normal practice²³ (unpublished observations, 1990-1997). In a recent evaluation of several hundred atopic Filipino consumers who were exposed to an enzyme-containing detergent bar product and product solutions during daily rigorous handwashing conditions for at least 2 years, there was no evidence that any of these subjects had allergic antibody to enzymes as assessed by skin prick tests with the enzyme in question.²³ These data support our conclusion that intimate exposure to mucosal surfaces was most likely the primary cause of the development of IgE in the four subjects in this study.

It only became apparent at the 6-month time point that these four subjects had positive skin prick test reactions to the enzyme. All four individuals had negative skin prick test reactions at 4 months. This finding is consistent with the papain experience in contact lens solutions in which allergy became apparent only after 1 year of exposure.⁴ This emphasizes the need to conduct

studies of sufficient duration when considering the possibility of allergic reactions to these enzymes. A study of shorter duration would not have revealed the potential for developing allergic antibodies to these materials with this particular type of exposure.

The absence of symptoms typical of Type I allergy among these four individuals during showering with the enzyme-containing product is consistent with experience from the manufacturing setting. Occasionally, workers will develop positive skin prick test responses that are picked up on routine screening.^{16, 17} These individuals are able to work with no evidence of symptoms in plants operating below the 15 ng/m³ control limit.^{16, 17} Rare instances of respiratory reactions in the workplace can be traced to unusual events such as failure to comply with guidelines on wearing protective equipment when exposure is expected to be above 15 ng/m³. Nevertheless, the potential for consumers to become subclinically sensitized and subsequently to experience clinically significant symptoms with ongoing use of this personal cleansing product could not be ruled out.

One can compare exposure to enzyme in this personal cleansing product with known safe exposures to enzymes in laundry products. Work by Hendricks²⁴ found that consumers were exposed to 0.27 µg of detergent dust each time a laundry product was poured into a washing machine. Furthermore, Hendricks found that less than 0.05% of this dust was enzyme stock material, containing 5% enzyme protein. Therefore consumer exposure to enzyme protein was calculated to be 0.0067 ng total protein. Assuming that this protein is released into 1 m³ over a washing machine, the theoretical concentration would be 0.0067 ng/m³. Even if it is assumed that the release was into 0.1 m³, the theoretical concentration (0.067 ng/m³) would still be several orders of magnitude below airborne enzyme concentrations reported from these shower studies.

To our knowledge, this is the first clinical study to show that exposure to enzymes through use of a personal cleansing product led to the development of allergic antibodies. This study serves as an example of the need to consider potential exposure sources or routes when developing a safety assessment for enzymes in new product forms and to confirm the assessment with an appropriate clinical study. Clinical studies should be conducted when exposures are greater than levels of consumer exposure known to be safe. As a result of this use study, a decision was made not to proceed with further development of these prototype enzyme-containing personal cleansing products.

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