

Respiratory pathophysiologic responses

Proteolytic detergent enzymes enhance the allergic antibody responses of guinea pigs to nonproteolytic detergent enzymes in a mixture: Implications for occupational exposure

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A guinea pig intratracheal test was developed to assess the respiratory allergenicity of enzymes used in the detergent industry. Information gained from this test was used in a process for setting operational exposure guidelines to protect worker health. Mixtures of enzyme proteins were given to guinea pigs once per week for 10 weeks to determine whether there were interactions among enzymes that affected the induction of antibody responses to each enzyme in the mixture. Passive cutaneous anaphylaxis antibody titers against each enzyme were measured in sera. Mixtures of two or three enzymes always consisted of a protease (Alcalase, Savinase; Novo Industri A/S) with an α -amylase (Termamyl; Novo Industri A/S), a lipase (Lipolase; Novo Industri A/S), or both. Control animals were exposed to single enzymes. The antibody titers to Termamyl and Lipolase were significantly greater in animals dosed with the protease-containing mixtures as compared with control animals dosed with a single enzyme. Antibody titers to the protease were unchanged in the presence of additional enzymes in the mixture. Complete inactivation of protease activity abrogated the enhanced antibody response to Lipolase. Inhalation exposure of guinea pigs to a mixture of Alcalase and Lipolase also resulted in higher antibody titers to Lipolase as compared with animals exposed by inhalation to Lipolase alone, showing that the enhanced response was not due to intratracheal delivery of antigen to the respiratory tract. These results show that proteolytic enzymes in a mixture enhance antibody responses to other enzymes. This should be considered when defining exposure guidelines for protease-containing enzyme mixtures. (*J Allergy Clin Immunol* 1997;100:480-7.)

Key words: Enzymes, enzyme mixtures, guinea pig models, allergic responses

The existence of occupational respiratory allergic reactions to enzymes in detergents has been known since they were first described by Flindt¹ and Pepys et al.² over 25 years ago. These responses were noted shortly after the start of extensive manufacture of enzyme-containing

Abbreviations used

DFP:	Diisopropyl fluorophosphate
GPIT:	Guinea pig intratracheal test
HAR:	Homocytotropic antibody response
HMI:	Hexylmethyleneisocyanate
OD:	Optical density
PBS:	Phosphate-buffered saline
PCA:	Passive cutaneous anaphylaxis

detergents, and the original observations were substantiated by others within a short time.³⁻⁸ As a result of these observations, enzyme manufacturers developed less dusty formulations. Dust control measures and exposure guidelines were instituted in enzyme detergent manufacturing facilities. On the basis of our manufacturing experience with the serine protease Alcalase, we implemented dust and aerosol control measures as part of overall operating guidelines for our manufacturing sites. These operating guidelines include (1) engineering controls to minimize dust and aerosol generation during normal operation and peak exposure situations, (2) product formulations that minimize dust and aerosol generation, (3) limits on the level of enzyme in formula, (4) establishment of exposure guidelines for air monitoring purposes, and (5) employee education and training to minimize personal exposure. As a result of these actions, the frequency of allergic sensitization to enzymes has been minimized, and enzyme-mediated allergic symptoms have become a rare event.⁹⁻¹²

A guinea pig intratracheal test (GPIT) was developed to assess the relative allergenic potential of new enzymes when compared with the reference allergen, Alcalase.¹³ Alcalase was used as the reference enzyme allergen because the bulk of our human experience has been with this enzyme, and a threshold limit value of 60 ng of protein/m³ was established for this enzyme.¹⁴ We have successfully used this model to rank enzymes as more potent than, less potent than, or equipotent to Alcalase. Prospective evaluation of skin prick test reactions among workers exposed to these different enzymes is consistent with the ranking obtained in the guinea pig model.^{15, 16} Therefore the GPIT model is considered an appropriate

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TABLE I. Study designs

	Alcalase	Savinase	Lipolase	Termamyl	HMI-Alcalase	DFP-Alcalase
Study 1: binary mixture						
Group 1	1.0 µg		0 µg			
Group 2	1.0 µg		0.25 µg			
Group 3	0 µg		0.25 µg			
Group 4	0.3 µg		0 µg			
Group 5	0.3 µg		0.08 µg			
Study 2: binary mixture						
Group 1	0.3 µg	0 µg	0 µg			
Group 2	0.3 µg	0 µg	0.08 µg			
Group 3	0 µg	0.3 µg	0.08 µg			
Group 4	0 µg	0 µg	0.08 µg			
Study 3: tertiary mixture						
Group 1	0.3 µg		0 µg	0 µg		
Group 2	0.3 µg		0.1 µg	0 µg		
Group 3	0.3 µg		0.1 µg	0.05 µg		
Group 4	0.3 µg		0 µg	0.05 µg		
Group 5	0 µg		0 µg	0.05 µg		
Study 4: inactive enzyme						
Group 1	0.3 µg		0.08 µg		0 µg	0 µg
Group 2	0 µg		0.08 µg		0.3 µg	0 µg
Group 3	0 µg		0.08 µg		0 µg	0.3 µg
Group 4	0 µg		0.08 µg		0 µg	0 µg

animal model with which to evaluate the allergenic potential of enzymes used by the detergent industry.

Until recently, factory workers were exposed to one and occasionally two enzymes during the manufacture of detergents. However, with the introduction of multiple enzyme classes in detergent formulas, workers can be exposed to several different enzymes at the same time. To assure the continued safe use of enzymes in detergents, the GPIT model was used to evaluate the allergic response to enzyme allergens in a mixture. The data from these studies indicate that proteases enhance antibody responses to other enzymes. Therefore occupational exposure guidelines must take this interaction into consideration when protease-containing enzyme mixtures are used, and careful control of exposure to enzyme mixtures in manufacturing facilities is needed to control allergy to enzymes.

METHODS

Animals

Female Hartley guinea pigs (Charles River Laboratories, Portage, Mich.) ranging in weight from 300 to 400 gm were quarantined for 2 weeks before placement in studies. Animals were randomized into groups according to body weight before initiation of the experiments and were housed individually in stainless steel, wire-bottom cages. Water and food (Purina Guinea Pig Chow, Purina Mills, Richmond, Ind.) were provided ad libitum. Room temperature was controlled at 67° to 79° F, and relative humidity was maintained at 45% to 76%. A light-dark cycle of 12 hours per phase was maintained throughout the experiments. National Institutes of Health guidelines for the care and use of laboratory animals were followed.

Test materials

Detergent enzymes (Alcalase, Savinase, Lipolase, and Termamyl) were supplied by Novo Industri A/S (Bagsvaerd, Denmark). The protein concentrations of these enzymes, as determined by Kjeldahl nitrogen analysis, were 35.4%, 43.1%, 2.0%, and 4.6%, respectively. All enzymes were stored at 4° C. Separate batches of Alcalase were inactivated with either hexylmethyleneisocyanate (HMI; Aldrich Chemical, Milwaukee, Wis.) or diisopropyl fluorophosphate (DFP; Sigma Chemical, St. Louis, Mo.) as previously described.¹⁷ The protein concentrations of HMI- and DFP-Alcalase were 55.4% and 14.7%, respectively. HMI-Alcalase retained 15% of its original activity, whereas DFP-Alcalase retained no measurable activity. The inactivated samples were lyophilized and stored at 4° C. The detergent used in these studies was formulated at Procter & Gamble and consisted primarily of anionic surfactants, nonionic surfactants, silicate builders, and a perborate bleach.

Intratracheal exposures

Exposures of animals were done as described previously.¹³ Briefly, restrained, unanesthetized animals (10 or 15 per group) were intratracheally dosed once a week for 10 consecutive weeks with 100 µl of a solution containing 250 µg of detergent in saline plus appropriate levels of the different enzyme proteins. All solutions were on ice, and the mixtures were prepared immediately before dosing. These doses were delivered near the tracheal bifurcation by inserting a 2-inch, 18-gauge, ball-tipped gavage needle into the trachea and injecting the dose during inspiration. This method of dosing, when performed by qualified personnel, has been deemed by several institutional animal care and use committees to cause no more than momentary distress to the animal. Immediately after exposure, animals were observed for signs of respiratory distress such as periodic diaphragmatic spasms or retractions. The more severe the response, the greater was the number of retractions be-

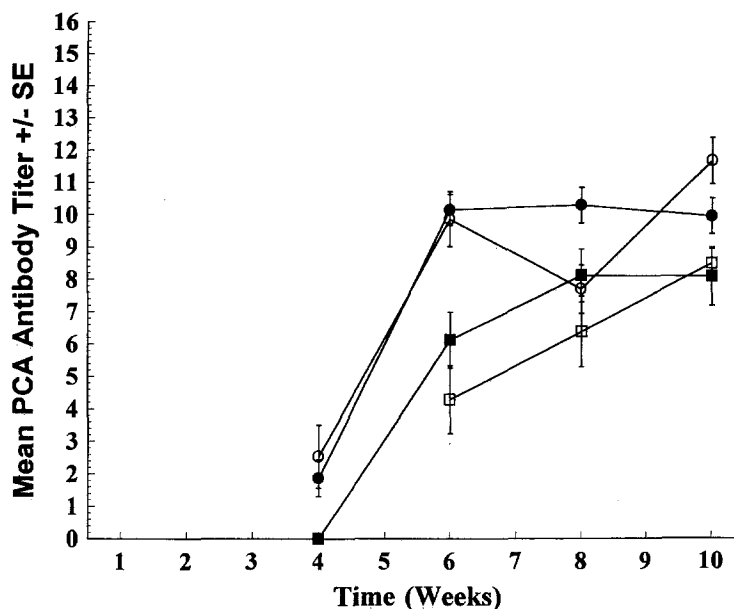


FIG. 1. PCA antibody titers to Alcalase in serum from animals exposed to 1.0 μg of Alcalase (filled circles), 0.3 μg of Alcalase (filled squares), 1.0 μg of Alcalase combined with 0.25 μg of Lipolase (open circles), or 0.3 μg of Alcalase combined with 0.08 μg of Lipolase (open squares).

tween normal breaths.¹³ Because of the low level of protein used in these studies, severe immediate-onset respiratory reactions were not provoked, and the group sizes were not altered over the course of the studies.

Inhalation exposures

Two groups of eight guinea pigs each were exposed to aerosol sprays of Lipolase alone (500 $\mu\text{g}/\text{m}^3$) or a combination of Alcalase and Lipolase (2000 $\mu\text{g}/\text{m}^3$ and 500 $\mu\text{g}/\text{m}^3$, respectively) in whole body glass plethysmographs for 20 minutes per day for 5 consecutive days (a similar regimen has been shown to sensitize guinea pigs to proteins).¹⁸ Seven, 14, and 21 days after the last of the five exposures, animals were re-exposed to the appropriate atmosphere for 20 minutes. Enzyme aerosol sprays were generated by using separate Lovelace nebulizers (In-Tox Products, Albuquerque, N.M.). Enzyme atmospheres were diluted with different volumes of filtered, dry air to achieve the targeted concentrations in the inhalation system. Enzyme concentrations were determined by collecting chamber air onto 47 mm A/E glass-fiber filters (Gelman Co., Ann Arbor, Mich.) and eluting the protein into physiologic saline solution. The recovered protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with standard curves developed with Alcalase or Lipolase. In addition, proteolytic activity and lipolytic activity were periodically checked with the appropriate assays (paranitroanilide method for protease¹⁹ and pancreatic substrate method for lipase; Sigma).

Serum collection

Sera from ether-anesthetized animals were obtained by retroorbital bleeding. Sera were collected 24 hours before intratracheal exposure at weeks 4, 5, 6, 8, and 10 or 1 week after the 7-, 14-, and 21-day inhalation exposures. All sera were heat-inactivated at 56°C for 45 minutes and stored frozen at -20°C until use.

Passive cutaneous anaphylaxis (PCA) assays

Circulating IgG₁ homocytotropic antibodies in sera from animals exposed intratracheally or through inhalation were measured in a 4-hour PCA test as previously described.¹³ Naive guinea pigs were intradermally injected with sera diluted 1:4 to 1:16,384 in physiologic saline solution. Normal serum (1:4) and saline solution served as controls. Four hours later, the animals were lightly anesthetized with ether and injected intracardially with 2 ml/kg of body weight of a physiological saline solution containing 1% Evans blue dye (Sigma) and 100 μg of protein/ml of the appropriate enzyme. The highest dilution of serum showing a positive response (bluing at the injection site) was the end titer. The IgG₁ antibody titer (homocytotropic or PCA antibody) was expressed as the logarithm to the base 2 of the reciprocal of the end-point dilution (i.e., 2 to 14, representing the 1:4 to 1:16,384 dilution range).

ELISA

Circulating anti-Lipolase IgG antibodies in sera from inhalation-exposed animals were detected by an indirect ELISA. Immulon II U-bottom 96-well plates (Dynatech Labs, Alexandria, Va.) were coated overnight at 4°C with 100 μg of protein per well of Lipolase in carbonate buffer. The plates were washed four times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween) and postcoated with 100 μl of 10% Carnation skim milk in PBS-Tween for 2 hours at room temperature. The plates were washed four times with PBS-Tween, and 100 μl of guinea pig sera (diluted 1:200) was added to triplicate wells and incubated for 1 hour at room temperature. The plates were washed four times, and 100 μl of a 1:700 dilution of alkaline phosphatase-conjugated rabbit anti-guinea pig IgG antisera (ICN Immunobiologics, Lisle, Ill.) was added to each well and incubated for 1 hour at room temperature. After washing four times, 100 μl of substrate, 1 mg/ml p-nitrophenyl phosphate (Sigma) prepared in diethanolamine buffer (Aldrich), was added to each well. The plates were

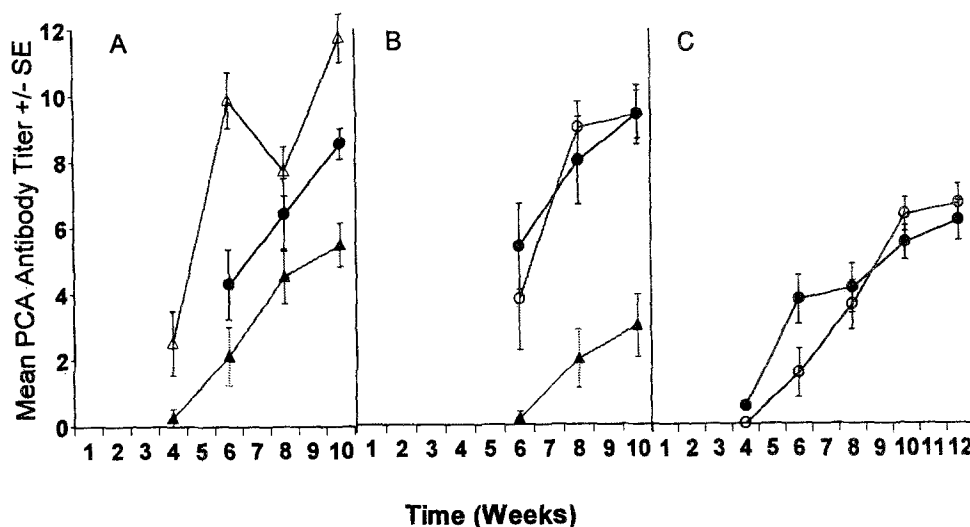


FIG. 2. PCA antibody titers to Lipolase in serum from animals exposed to (A) 0.25 µg of Lipolase (filled circles), 1.0 µg of Alcalase combined with 0.25 µg of Lipolase (open triangles) or 0.3 µg of Alcalase combined with 0.08 µg of Lipolase (filled triangles); (B) 0.08 µg of Lipolase (filled triangles), 0.3 µg of Alcalase combined with 0.08 µg of Lipolase (open circles), or 0.3 µg of Savinase combined with 0.08 µg of Lipolase (filled circles); (C) Alcalase combined with Lipolase (open circles) or Alcalase, Lipolase, and Termamyl (filled circles).

incubated for 30 minutes at room temperature, and the optical density (OD) of each well was measured at 410 nm with an MR-600 Dynatech ELISA reader (Dynatech Labs, Alexandria, Va.).

GPIT experimental design

Because proteolytic enzymes are the major enzymes in laundry detergents, the enzyme mixtures always consisted of a constant level of protease (1.0 µg or 0.3 µg/100 µl dose) with varied levels of nonprotease enzyme (e.g., 0.25 µg, 0.1 µg, 0.08 µg, or 0.05 µg/100 µl dose). The ratios of protease protein to nonprotease protein in the test mixtures were based on the proposed ratio of these various enzymes in laundry products. PCA antibody titers to each enzyme in a mixture were compared with the titers generated by animals exposed to the enzyme alone, as well as to the antibody titers generated by animals exposed to the reference enzyme, Alcalase. Table I outlines the study designs.

Statistical analysis

The PCA data were statistically analyzed by using Bartlett's test of homogeneity of variance.²⁰ If this test result was not significant, comparisons were based on the least significant criterion. If Bartlett's test result was significant, Wilcoxon's rank-sum test was used. These statistical tests were conducted at a 5%, two-sided risk level.

RESULTS

Alcalase and Lipolase in a binary mixture

Homocytotropic antibody responses (HARs) to Alcalase were not affected in animals exposed to Lipolase with 1.0 or 0.3 µg of Alcalase (Fig. 1). The HARs to Lipolase were significantly enhanced in animals exposed to 0.25 µg of Lipolase in the presence of 1.0 µg of Alcalase as compared with the HARs of animals exposed to 0.25 µg of Lipolase at weeks 6, 8, and 10 (Fig.

2, A). In addition, the HARs of animals exposed to 0.08 µg of Lipolase with 0.3 µg of Alcalase were nominally greater than the titers from animals exposed to 0.25 µg of Lipolase alone at weeks 6, 8, and 10. The number of animals with symptoms and the severity of the symptoms were not different among the animals exposed to Alcalase alone or the mixtures of Alcalase and Lipolase (data not shown).

Protease, Lipolase, and Termamyl in binary and tertiary mixtures

The effects of Alcalase and a second serine protease (Savinase) on the HARs to Lipolase are shown in Fig. 2, B. The HARs to Lipolase from animals exposed to a mixture of 0.3 µg of Alcalase and 0.08 µg of Lipolase or a mixture of 0.3 µg of Savinase and 0.08 µg of Lipolase were significantly greater than the HARs to Lipolase from animals exposed to 0.08 µg of Lipolase alone at weeks 6, 8, and 10. There were no differences between the anti-Lipolase antibody titers of animals exposed to Lipolase in the presence of either Alcalase or Savinase. The addition of 0.05 µg of Termamyl to a mixture of 0.3 µg of Alcalase and 0.1 µg of Lipolase had no effect on the enhanced HAR to Lipolase (Fig. 2, C).

The number of animals with symptoms and the severity of symptoms from animals exposed to the mixtures of Alcalase and Lipolase; Savinase and Lipolase; or Alcalase, Lipolase, and Termamyl were not different from those of animals exposed to Alcalase alone or Savinase alone (data not shown).

Fig. 3 shows the HARs to Termamyl in sera from animals exposed to 0.05 µg of Termamyl alone or with Alcalase or with a mixture of Alcalase and Lipolase. The

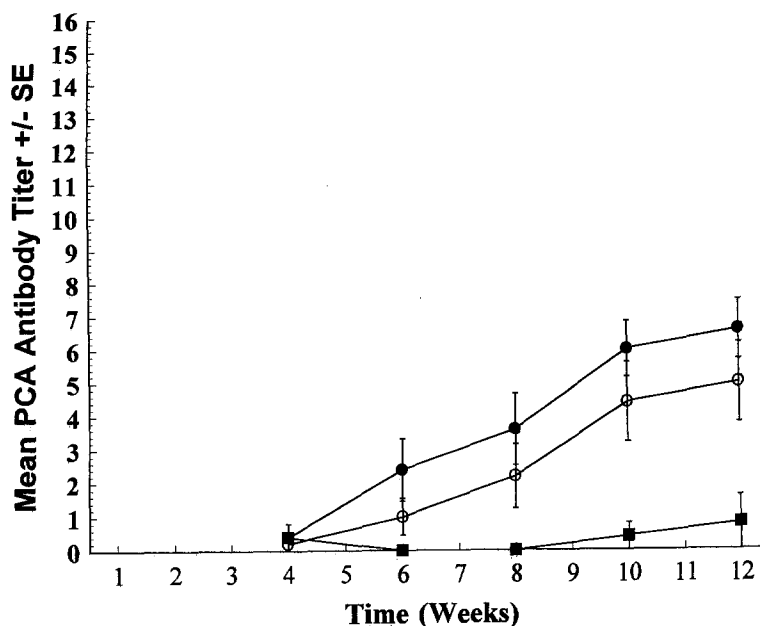


FIG. 3. PCA antibody titers to Termamyl in serum from animals exposed to 0.05 µg of Termamyl (filled squares); 0.3 µg of Alcalase combined with 0.05 µg of Termamyl (open circles); or a mixture of 0.3 µg of Alcalase, 0.1 µg of Lipolase, and 0.05 µg of Termamyl (filled circles).

TABLE II. Percent of animals with allergic IgG1a antibody to Lipolase: Inhalation exposure to Lipolase alone or to the combination of Alcalase and Lipolase

Exposure group	Week 1	Week 2	Week 3
Lipolase	0%	0%	12%
(mean titer $\pm$ SEM)	(0)	(0)	(8 $\pm$ 3.2)
Alcalase and Lipolase	0%	25%	38%
(mean titer $\pm$ SEM)	(0)	(8 $\pm$ 2.1)	(48 $\pm$ 8.6)

antibody titers to Termamyl in sera from animals dosed with the binary or tertiary enzyme mixtures were significantly greater than the antibody response of animals dosed with Termamyl alone. There were no significant differences between the HARs to Termamyl from animals exposed to Termamyl in binary versus tertiary enzyme mixtures.

Inactivated Alcalase and Lipolase in a binary mixture

Fig. 4 shows the antibody titers to Lipolase in sera from animals exposed to Lipolase alone, a mixture of Lipolase and active Alcalase, a mixture of Lipolase and partially inactive Alcalase (HMI-Alcalase), and a mixture of Lipolase and fully inactive Alcalase (DFP-Alcalase). Those animals exposed to Lipolase with HMI-Alcalase had significantly higher anti-Lipolase titers at weeks 8 and 10 than animals exposed to Lipolase alone. However, these titers were significantly lower than the anti-Lipolase titers of animals exposed to a mixture of

Lipolase and active Alcalase. There were no differences between anti-Lipolase titers of animals exposed to Lipolase alone and those exposed to Lipolase in the presence of DFP-Alcalase.

Inhalation exposure to Lipolase and Alcalase in a binary mixture

Animals were exposed to an aerosol spray of Lipolase alone (500 µg/m³) or an aerosol spray of a mixture of Alcalase (2000 µg/m³) and Lipolase (500 µg/m³). Table II shows the percentage of responding animals (and mean titer) with allergic antibody to Lipolase after the 7-, 14-, and 21-day inhalation "boost" exposure. A greater number of animals responded faster and with higher PCA antibody titers to Lipolase when also exposed to Alcalase. Table III shows the mean IgG anti-Lipolase antibody OD readings of sera obtained after the 21-day "boost" exposure. Sera from animals exposed to both Lipolase and Alcalase had significantly higher IgG activity (OD readings) in response to Lipolase when compared with sera from animals exposed to Lipolase alone. In addition, these ODs were significantly higher than the ODs obtained with baseline sera at these dilutions.

DISCUSSION

The results of these studies clearly showed that in a guinea pig model of respiratory allergy, proteolytic enzymes enhanced the PCA antibody response to non-proteolytic enzymes of animals exposed to enzyme allergen mixtures. The activity of the protease was key to the enhanced antibody responses as demonstrated by partial abrogation of the enhancement by partially inactivated protease and total abrogation of the enhancement by

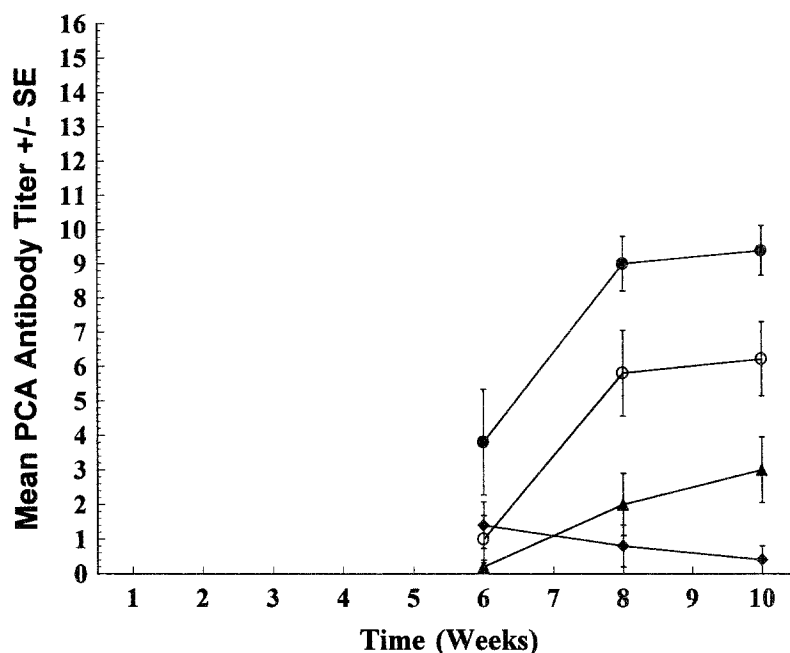


FIG. 4. PCA antibody titers to Lipolase in serum from animals exposed to 0.08 μ g of Lipolase (filled triangles), 0.3 μ g of Alcalase combined with 0.08 μ g of Lipolase (filled circles), 0.3 μ g of HMI-Alcalase combined with 0.08 μ g of Lipolase (open circles), or 0.3 μ g DFP-Alcalase combined with 0.08 μ g of Lipolase (filled diamonds).

TABLE III. Anti-Lipolase IgG antibody in sera from animals exposed through inhalation to Lipolase alone or a mixture of Alcalase and Lipolase

Animal no.	IgG* (Lipolase alone)		Animal no.	IgG* (Alcalase and Lipolase)	
	Baseline	After exposure		Baseline	After exposure
1	0.064	0.097	9	0.064	0.913
2	0.062	0.187	10	0.094	0.461
3	0.053	0.075	11	0.094	0.233
4	0.047	0.068	12	0.073	0.833
5	0.124	0.180	13	0.077	0.087
6	0.071	0.130	14	0.114	0.142
7	0.047	0.063	15	0.057	0.310
8	0.070	0.045	16	0.096	0.339
Mean	0.067	0.109		0.084	0.415
SEM	0.009	0.020		0.007	0.108

*IgG antibody activity was defined as the OD at a 1:200 dilution of serum.

totally inactivated protease. The implication of these findings for the occupational setting in detergent manufacturing sites is that operational guidelines designed to control exposure must take into account the interaction among the enzyme allergens (proteases and nonproteases) to continue to minimize sensitization and eliminate the clinical symptoms of allergy. We have shown that the GPIT is an appropriate animal model to use for evaluating the relative allergenic potency of enzymes used in the detergent industry.¹⁶ The skin prick test responses of exposed human beings to several different enzymes followed what was predicted from the animal model.^{15, 16} This correlation between the animal and

human responses to enzymes gives us the confidence to use this model for new situations (i.e., enzyme mixtures) and extrapolate the findings to human beings.

The results of several GPITs showed that the allergic antibody responses to Lipolase or Termamyl were enhanced when animals were exposed to these enzymes in the presence of the protease, Alcalase. Savinase, another serine protease that is immunologically distinct from Alcalase and enzymatically different from Alcalase,^{12, 19, 23} also enhanced the allergic antibody response to Lipolase. These data showed that protease-mediated enhancement was not unique to Alcalase. The antibody response to Alcalase was not affected by the presence of

Lipolase, Termamyl, or both, indicating that the interaction was driven by the protease and not by other enzymes in the mixture.

The antibody response to Alcalase was comparable in animals exposed by inhalation or by intratracheal instillation, which suggests that intratracheal instillation is a valid alternative for the introduction of allergen to the respiratory tract.¹³ However, we questioned whether intratracheal bolus instillation of enzyme mixtures was the reason for the protease-enhanced HARs to Lipolase. Inhalation exposure of animals to a mixture of Alcalase and Lipolase resulted in an enhanced antibody response to Lipolase as compared with exposure to aerosol sprays of Lipolase alone. Therefore the method of delivery does not appear to play a role in this phenomenon.

To begin to understand the mechanism of protease-mediated enhancement of HARs to other proteins, we examined the role of proteolytic activity in this phenomenon. The HARs to Lipolase were significantly enhanced in animals exposed to the partially inactivated mixture of Alcalase and Lipolase. However, the titers were not as high as those from the animals exposed to Lipolase with the active protease. The partially inactivated Alcalase retained 15% of its proteolytic activity. Complete inactivation resulted in no enhancement of the HARs to Lipolase. Therefore proteolytic activity plays a role in the enhanced HARs to other enzymes in the mixture.

We can speculate about how proteolytic activity acts to enhance the HAR. The proteolytic action may compromise the mucosal surface, altering permeability and allowing greater accessibility of the other enzymes to the mucosal surface, which in turn leads to greater accessibility to immune cells. The association between environmental insults (e.g., cigarette smoke and air pollutants) and sensitization supports the theory that changes in mucosal permeability in the respiratory tract can lead to sensitization and clinical symptoms to inhaled allergens.²⁴⁻³² Some of the major allergenic proteins of common aeroallergens (e.g., house dust mite) are proteases.³⁴⁻³⁷ A recent review of the biochemistry of allergens showed that many common and occupational allergens are hydrolytic enzymes.³⁸ The activity of these proteins may allow them, as well as other associated proteins, to penetrate the tissue and be accessible to immune cells.

Another way in which protease may act to enhance the HAR to other proteins is by partial digestion of the accompanying protein, leading to the generation of antigenic peptides. These peptides may be easier to endocytose or bind to specific B cells or antibody-bearing, antigen-presenting cells, which in turn stimulate T cells. This has been theorized to lead to a more robust or diversified immune response to antigens.³⁹ We are actively investigating these possibilities.

What are the implications of these findings to the occupational setting in detergent manufacturing facilities? From our experience, we believe that operational guidelines must be established for enzyme mixtures

containing protease because the guidelines established for Alcalase alone may not be applicable to the mixtures. A downward adjustment of exposure guidelines for nonproteolytic enzymes should occur when such exposures are accompanied by simultaneous exposure to proteolytic enzymes. Careful control of enzyme levels in formula is a key factor in controlling exposure at the work site. More extensive testing of enzyme mixtures should provide the data that will allow us to establish operational guidelines for each nonprotease enzyme introduced to a manufacturing site. Under these conditions of exposure, we predict that the rate of sensitization (skin test conversion without symptoms) to these enzymes will not be different from the recent experience with Alcalase and Savinase.¹⁶ In addition, under these exposure conditions, we predict that the onset of clinical symptoms will remain a rare event. Confirmation of these predictions will arise from vigilant monitoring of occupationally exposed workers. Continued investigation of the mechanism of multiple enzyme interactions will help apply these findings to human beings and may contribute to the overall understanding of occupational allergy to enzyme allergen mixtures.

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