

Respiratory Allergenicity of Detergent Enzymes in the Guinea Pig Intratracheal Test: Association with Sensitization of Occupationally Exposed Individuals¹

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A guinea pig intratracheal test was used to set occupational operating guidelines for new enzyme proteins used in the detergent industry. In these studies, animals were intratracheally dosed with different levels of enzyme protein and sera from the animals were titrated for allergic antibody to the enzyme. The amount of antibody produced to an enzyme was compared to the amount of antibody produced to the same protein doses of Alcalase, for which effective operating guidelines exist. These comparisons were used to determine if a new enzyme was more potent, less potent, or equivalent to Alcalase; operating guidelines were then established for the new enzyme. Termamyl was about 10-fold more potent than Alcalase and the protease subtilisin B was shown to be less potent. Another protease, Savinase, was shown to be equivalent in potency to Alcalase. The operating guidelines for Termamyl were adjusted lower, whereas the operating guidelines for the proteases were set the same as that of Alcalase. Under these conditions, we would predict that sensitizations to new enzymes would be comparable to or lower than the sensitizations to Alcalase. Prospective evaluation of skin prick test data of factory workers showed that sensitizations to Termamyl and Savinase were similar to sensitizations to Alcalase. The sensitizations to subtilisin B were lower than those to Alcalase. During this time period (7 years), only three respiratory incidents (rhinitis) were reported, demonstrating that employees with positive skin prick tests can continue to work. These comparisons indicate that the guinea pig intratracheal test is a good animal model for evaluating enzymes as respiratory allergens and that the data generated can be used to set operating guidelines for occupational allergens. © 1997 Society of Toxicology.

Occupational allergic antibody-mediated respiratory reactions to enzyme-containing detergent products were first de-

scribed by Flindt (1969) and Pepys *et al.* (1969). Since that time, more than 25 years of detergent industry experience has provided much information on understanding and managing the effects to humans from exposure to enzyme materials (Pepys *et al.*, 1973, 1985; Weill *et al.*, 1974; Zetterstrom, 1977). Control of occupational exposure to respirable enzyme dusts has greatly reduced the incidence of immediate hypersensitivity responses to enzymes and the occurrence of enzyme-associated occupational asthma among detergent workers is now a rare event (Juniper and Roberts, 1984; Flood *et al.*, 1985; Sarlo *et al.*, 1990). In addition, a survey of several thousand consumers has shown that the use of enzymes in laundry products is safe for consumer use (Pepys *et al.*, 1973). Based upon much of these historical data, the American Conference of Governmental and Industrial Hygienists (ACGIH) developed a threshold limit value (TLV) in the workplace for subtilisin A (Alcalase) at 60 ng protein/m³ (ACGIH, 1990). Based upon our manufacturing experience with Alcalase, we have implemented dust and aerosol control measures as part of overall operating guidelines for our detergent manufacturing sites. These operating guidelines include (1) engineering controls to minimize dust and aerosol generation during normal operation and during peak exposure situations, (2) changes in enzyme physical form and product formulations that minimize dust and aerosol generation, (3) limits on individual enzyme levels in the complete detergent formula to reflect the potency of each enzyme, (4) the establishment of exposure guidelines for air monitoring purposes, and (5) employee education and training to minimize personal exposure. To ensure the continued safe manufacture of enzyme-containing laundry products, operating guidelines for new enzymes have been developed. The overall goal of these guidelines is to minimize new occupational sensitizations (skin prick test conversions) to enzymes and to reduce significantly the risk of allergic symptomatology.

A guinea pig intratracheal (GPIT) test was developed to allow us to evaluate all new enzymes for their potential to induce the formation of allergic antibodies (Ritz *et al.*, 1993). The allergenic potency of new enzymes was compared to

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our known reference allergen, Alcalase, on a protein weight basis. Alcalase was chosen as the reference allergen (or benchmark) since the bulk of our human experience has been with this enzyme, a TLV was established by the ACGIH, and we developed internal operating guidelines to control exposure to this enzyme. By comparing the amount of allergic antibody produced to a new enzyme with the amount of allergic antibody produced to equiprotein amounts of Alcalase, we determined if the new enzyme was more potent than, less potent than, or equivalent to Alcalase. Operating guidelines for the new enzyme were then developed based upon the potency differential extrapolated from the dose-response vs time curves. More potent enzymes require more stringent operating guidelines. Since engineering controls are at an optimum for dust control, exposure guidelines are driven by limits on the level of enzyme in formula. The more potent the enzyme, the less of that enzyme in detergent product. In this report, we describe how the GPIT test distinguishes among several enzymes and, by the use of these results, how operating guidelines were adjusted for each enzyme. In addition, we show how the human response (defined as skin prick test conversions) to each enzyme followed what would be predicted from the guinea pig model. Therefore, the guinea pig model can be used to set control guidelines and has predictive value for the development of specific IgE antibody responses in humans under controlled conditions of exposure to enzymes during the manufacture of detergents. Because of the link between the guinea pig and human responses to these enzymes, we believe this model represents a novel approach to risk assessment of protein allergens.

MATERIALS AND METHODS

Animals. Female Hartley strain guinea pigs (Charles Rivers) weighing from 300 to 400 g were quarantined for 2 weeks prior to placement in the study. Animals were randomized by body weight into equal-sized dose groups ($n = 10/\text{group}$). Animals were individually housed in stainless steel, wire-bottom cages, and water and food (Purina guinea pig chow) were provided *ad libitum*. Air temperature was controlled at 67–79°F, relative humidity at 45–76% and each day consisted of 12 hr of fluorescent light followed by 12 hr of dark. The NIH guidelines for the care and use of laboratory animals were followed throughout the course of these studies.

Test materials. All enzymes were produced by different *Bacillus* species. The detergent enzymes Alcalase (serine protease), Savinase (serine protease), and Termamyl (amylase) were pure commercial preparations supplied by Novo Industries A/S (Bagsvaerd, Denmark). The protein concentrations of Alcalase, Savinase, and Termamyl were 35, 21, and 22%, respectively. The detergent enzyme subtilisin B (serine protease), a commercial preparation supplied by Genencor International (South San Francisco, CA), contained 5% protein. Protein levels were determined by Kjeldahl nitrogen analysis before and after trichloroacetic acid precipitation. All enzymes were stored at 4°C. Protease enzymes were inactivated with either hexylmethylene isocyanate (HMI) (Aldrich Chemicals, Milwaukee, WI) or diisopropyl fluorophosphate (DFP) (Sigma Chemical Co., St. Louis, MO) as previously described (Hartley *et al.*, 1959). The inactivated proteases were lyophilized and stored at 4°C. Enzyme inactivations were confirmed

by the lack of digestion of gelatin-coated film (Guntelberg and Offensen, 1954).

Intratracheal (IT) exposures. Restrained, unanesthetized guinea pigs (10 per group) were intratracheally dosed with 0.1 ml of enzyme protein in saline once per week for 10 or 12 consecutive weeks as previously described (Ritz *et al.*, 1993). All enzymes were dosed on an equiprotein basis and the doses were delivered near the tracheal bifurcation by inserting a 2-in., 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 being dosed, each animal was observed for signs of respiratory distress as demonstrated by periodic diaphragmatic spasms or retractions. Doses of enzyme were chosen to minimize the severity of respiratory distress (no anaphylaxis) and to maintain the group size throughout the course of the study.

Serum collection. Sera were obtained from ether-anesthetized animals via retroorbital bleeding. Sera were collected 24 hr before IT exposure at weeks 4, 5, 6, 8, and 10 (weeks 3 and 12 in one study). All sera were heat inactivated at 56°C for 30 min and stored frozen at –20°C until use.

Passive cutaneous anaphylaxis (PCA) assay. Circulating IgG1 allergic antibodies in sera collected from IT-exposed animals were measured in a 4-hr PCA test as previously described (Ritz *et al.*, 1993). Naive guinea pigs were shaved and depilated with Neet (Whitehall Laboratories, NY). The next day, the animals were intradermally injected with 100- μl volumes of test sera diluted from 1:4 to 1:16,384 in physiological saline. Normal serum (1:4) and saline served as controls. Four hours after the intradermal injections, the recipient animals were lightly ether-anesthetized and injected intracardially with 2 ml/kg body wt of a physiological saline solution containing 1% Evans blue dye (Sigma) and 1 mg protein/ml of inactivated enzyme (inactivated proteolytic enzymes must be used for intracardial injection to eliminate widespread vascular damage). The highest dilution of serum showing a positive response (bluing at the dermal injection site) was designated as the endpoint titer. The IgG1 antibody titer was expressed as the logarithm to the base 2 of the reciprocal of the endpoint dilution (i.e., 2 to 14, representing the 1:4 to 1:16,384 dilution range).

Microimmunodiffusion (MID) analysis. MID analysis was used to detect enzyme-specific precipitating antibodies in order to evaluate the overall immunogenic properties of each enzyme. Commercially prepared immunodiffusion plates were used (Immuno-plate, Hyland Laboratories). Peripheral wells were filled with 0.3, 0.1, or 0.03% enzyme protein; the center well was filled with undiluted guinea pig sera. Hyperimmune rabbit antisera were used on each plate as positive controls. The plates were incubated at 4°C for 24 hr, soaked in physiological saline for 24 hr, and stained with 0.1% thiazine red R (ICN, NY) in 5% acetic acid (Sigma) for 15 min. The plates were decolorized with two 1-hr rinses in 5% acetic acid followed by a 2-hr immersion in a 1% glycerol (Aldrich) saline solution. The number of animals with precipitating antibodies detectable by this procedure was counted at each evaluated time point and the data were expressed as the percentage of responding animals.

How GPIT test data are used to rank-order enzymes. The homocytotropic IgG1 antibody data were the primary data used to compare enzymes to Alcalase. Precipitating antibody data from the MID were used to supplement the IgG1 antibody data when making the comparison to Alcalase. The comparisons between Alcalase and the test enzyme were often made during the early part of the study (i.e., weeks 4, 5, and 6) when the differences in antibody titers were more apparent. Test enzymes were considered more potent than Alcalase or less potent than Alcalase if significant differences between antibody titers were observed at more than one dose level and at more than one time point. Consistency of differences between antibody titers at more than one dose comparison is critical for determining if a test enzyme was more potent or less potent than Alcalase. A second approach to evaluate potency differences was to compare dose-response curves at

selected time points. A less potent enzyme would have a dose-response curve shifted to the right of the dose-response curve for Alcalase. A more potent enzyme would have a dose-response curve shifted to the left of the dose-response curve for Alcalase. The potency relationship to Alcalase should be apparent at more than one time point. A comparison of the amount of Alcalase or new enzyme protein required to attain one-half the maximum response was used to calculate a potency difference between the enzymes.

Study group and exposures. Employees of The Procter & Gamble Company liquid and granule detergent manufacturing sites were evaluated for sensitization to detergent enzymes using a skin prick test method as part of our ongoing surveillance program. The test was performed annually. Also, employees were interviewed by occupational physicians regarding potential work-related symptoms. This program was reviewed by an institutional review board. In this report, sensitization was defined as a positive skin prick test with or without clinical symptomatology (symptoms were rarely observed). As a consequence of normal manufacturing procedures, employees had been exposed to low levels of enzyme containing detergent dusts or aerosols over a consecutive 3- to 6-year time period. All employees worked in different sections of the manufacturing facility on a rotation schedule. The average turnover rate for each site was less than 5% per year (mostly due to retirement).

Total dust or aerosol levels were controlled to less than 1 mg/m³ of air. Airborne levels of detergent dust and enzyme were determined by using a 2-hr high-volume air sample measurement in selected work areas. Direct activity measurements of proteolytic enzyme activity were made as previously described (Rothgeb *et al.*, 1988) and targeted to 0.3 µg active enzyme/m³ of air (which translates to 15 ng enzyme protein/m³ of air). Direct activity measurements of Termamyl were not possible due to interference by indigenous amylases. However, Termamyl airborne levels were controlled by limiting amylase levels in a detergent product also containing a protease to at least one-sixth to one-tenth the concentration of protease in finished product and tracking airborne levels of protease. The exposure conditions in both manufacturing sites were constant from year to year.

Skin prick test and antigens. Powdered enzymes were obtained from the manufacturers, sterilized via irradiation, aseptically weighed, and placed in sterile vials for storage. Each vial was reconstituted on the day of skin testing with 6 ml of a sterile 50% glycerol in physiological saline solution to obtain a 500 µg/ml enzyme protein solution. Glycerol-saline was used as a vehicle control and histamine phosphate at a 1:1000 dilution was used as a positive control on each subject.

Employees were evaluated once per year for sensitization to enzymes by the skin prick test. Sensitization was defined as a positive skin prick test response with or without clinical symptomatology. One drop (20 µl) of enzyme solution, vehicle control, or histamine was placed on the volar aspect of the forearm. Using a sterile 27-gauge needle inserted through the drop, the underlying superficial epidermis was gently lifted. One needle was used per skin site and discarded. Immediately after pricking, each skin site was blotted dry. After 10 to 15 min, the skin sites were evaluated for wheal and flare responses. A significant skin prick test response was defined as a wheal, at least 3 mm larger than the wheal of the vehicle control, surrounded by a flare. Employees that were skin prick test positive were retested to confirm the response. Skin prick test-positive asymptomatic employees were allowed to continue to work in the detergent manufacturing site.

Expression of skin prick test data. Employees that were skin prick test positive to an enzyme were included in the prevalence pool of total sensitized workers to a specific enzyme. Once an employee developed a skin prick test-positive response to the enzyme, no subsequent skin prick testing to that specific enzyme occurred in the following years. Yearly skin prick tests with other enzymes continued as long as the individual remained skin test negative to these other enzymes. The prevalence rate was calculated by dividing all previously positive employees plus newly positive employees by the total number of exposed employees. The participation rate in the

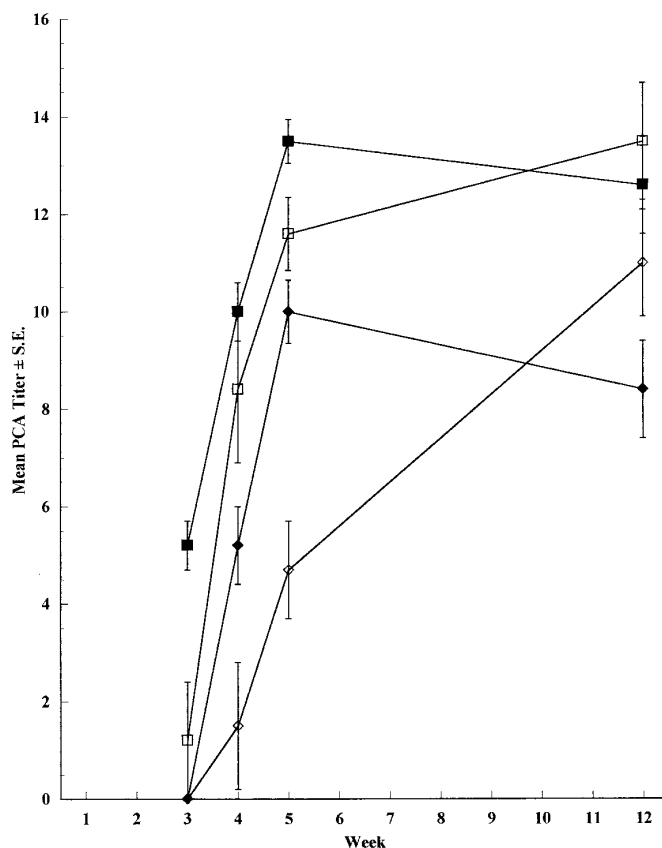


FIG. 1. Allergic antibody titers in sera from animals intratracheally dosed with 10 µg Termamyl (■), 1.0 µg Termamyl (◆), 10 µg Alcalase (□), or 1.0 µg Alcalase (◇).

skin prick test program averaged 99% at both granule and liquid detergent facilities.

Statistical analysis. Guinea pig PCA data were expressed as the mean PCA titer ± the standard error. These data were statistically analyzed using Bartlett's test of homogeneity of variance (Snedecor and Cochran, 1980). If this test was not significant, comparisons were based on the least significant difference criterion. If Bartlett's test was significant, Wilcoxon's rank sum test was used. These statistical tests were conducted at a 5%, two-sided risk level. In addition, standard regression analysis techniques were used to evaluate the significance of the dose-allergic antibody responses when appropriate (Draper and Smith, 1981).

RESULTS

Termamyl vs Alcalase Antibody Titers

The IgG1 antibody titers of guinea pigs dosed with 10 µg of Termamyl protein were significantly greater than the titers to 10 µg of Alcalase protein at week 3 only (Fig. 1). The antibody titers to 1 µg Termamyl protein were significantly greater than the titers to 1 µg Alcalase protein at weeks 4 and 5 but not different from titers to 10 µg Alcalase protein at weeks 3 and 5. No differences were observed at week 12. In a separate experiment in which 0.1-µg doses were

TABLE 1
Percentage of Animals Responding with Serum Precipitating Antibody to Enzyme Antigens

Enzyme and dose	Week 4 (%)	Week 5 (%)	Week 6 (%)	Week 8 (%)	Week 10 (%)	Week 12 (%)
Termamyl vs Alcalase						
0.1 μ g Termamyl	0	0	—	0	20	20
1.0 μ g Termamyl	20	40	—	100	100	100
0.1 μ g Alcalase	0	0	—	0	0	0
1.0 μ g Alcalase	0	0	—	0	40	50
Savinase vs. Alcalase						
1.0 μ g Savinase	—	—	0	0	14	—
3.0 μ g Savinase	—	—	0	20	60	—
10.0 μ g Savinase	—	—	5	55	80	—
1.0 μ g Alcalase	—	—	7	30	70	—
3.0 μ g Alcalase	—	—	37	92	100	—
10.0 μ g Alcalase	—	—	60	100	100	—
Subtilisin B vs Alcalase						
0.3 μ g subtilisin B	—	—	0	0	0	—
1.0 μ g subtilisin B	—	—	0	0	0	—
3.0 μ g subtilisin B	—	—	0	10	20	—
0.3 μ g Alcalase	—	—	0	0	33	—
1.0 μ g Alcalase	—	—	0	10	33	—
3.0 μ g Alcalase	—	—	10	33	50	—

compared, IgG1 antibody to the amylase was detected in sera from 20% of the animals at week 8; no antibody to the protease was detected (data not shown). MID analysis was also conducted to evaluate the overall immunogenic profile of Termamyl compared to Alcalase. Twenty percent of the animals dosed with 0.1 μ g Termamyl made precipitating antibody by week 10; none of the animals dosed with 0.1 μ g Alcalase made precipitating antibody (Table 1). Animals intratracheally dosed with 1 μ g Termamyl had precipitating antibody at weeks 4 and 5 and 100% of the animals had antibody by week 8. Conversely, animals dosed with 1 μ g Alcalase did not have detectable precipitating antibody until week 10. Based on the PCA and MID data, Termamyl was judged to be approximately 10-fold more potent than Alcalase. The operating guideline for Termamyl was adjusted downward from the guideline for Alcalase by limiting the amount of amylase in formula.

Savinase vs Alcalase Antibody Titers

Figure 2 shows the IgG1 antibody titers of animals intratracheally dosed with 1, 3, or 10 μ g Savinase or Alcalase protein. IgG1 antibody titers to both enzymes were not significantly different at the 10- μ g dose level at any time. The titers to Savinase were lower than the titers to Alcalase at the 3- μ g dose level at week 8 only. The titers to Savinase were lower than the titers to Alcalase at the 1- μ g dose level at weeks 5 and 6. A significant dose response was observed for both Alcalase and Savinase at all time points. Figures 3A–3C show the dose–response curves to Savinase and

Alcalase at weeks 4, 5, and 6, respectively. The calculated doses of Alcalase or Savinase to yield a response that were one-half the maximum antibody response at week 4 were 1.4 and 1.5 μ g, respectively. The calculated potency difference between Alcalase and Savinase was 0.93. Similar comparisons at weeks 5 and 6 resulted in calculated potency differences of 0.89 and 0.9, respectively. The percentage of animals dosed with 1, 3, or 10 μ g Savinase with precipitating antibody lagged behind those animals with antibody to Alcalase; this was observed for both the number of animals with antibody and for the kinetics of the response (Table 1). Based on these data, Savinase was judged to be equivalent to or slightly less allergenic than Alcalase. The operating guideline for Savinase was kept the same as the guideline for Alcalase and the amount of Savinase in formula was the same as the level of Alcalase in formula.

Subtilisin B vs Alcalase Antibody Titers

IgG1 antibody titers to 3 μ g Alcalase were significantly higher than the titers to 3 μ g subtilisin B at weeks 5, 6, and 8, whereas titers to 1 μ g Alcalase were greater than titers to 1 μ g subtilisin B at weeks 6 and 8 (Fig. 4). IgG1 antibody titers to 0.3 μ g Alcalase were significantly greater than titers to 0.3 μ g subtilisin B at weeks 6, 8, and 10. The antibody titers to 1 μ g Alcalase were not different from the titers to 3 μ g subtilisin B at weeks 4, 5, and 8; the anti-Alcalase titers were greater than the subtilisin B titers at week 6. Similarly, anti-Alcalase antibody titers at the 0.3- μ g dose level were not different from anti-subtilisin B antibody titers

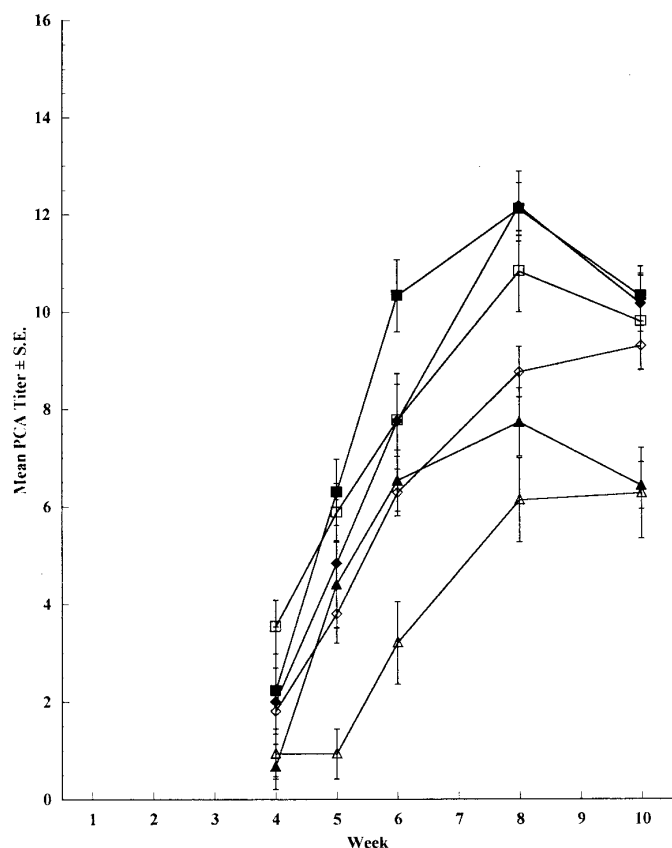


FIG. 2. Allergic antibody titers in sera from animals intratracheally dosed with 10 μ g Savinase (□), 3.0 μ g Savinase (◇), 1.0 μ g Savinase (△), 10 μ g Alcalase (■), 3.0 μ g Alcalase (◆), or 1.0 μ g Alcalase (▲).

at the 1- μ g dose level. A significant dose response to Alcalase was obtained at weeks 5, 6, 8, and 10, whereas a significant dose response to subtilisin B was only obtained at weeks 8 and 10. Figures 5A–5C show the dose–response curves to Alcalase and subtilisin B at weeks 6, 8, and 10, respec-

tively. The calculated doses of Alcalase vs subtilisin B to yield a response that was one-half the maximum antibody response at week 8 were 0.45 and 1.3 μ g, respectively. The calculated potency difference between Alcalase and subtilisin B was 2.8. A similar comparison at week 10 resulted in a calculated potency difference of 2.9. No precipitating antibody to subtilisin B was found at any time point in sera from animals dosed with 0.3 or 1 μ g subtilisin B protein, whereas precipitating antibody to Alcalase was found in sera from animals dosed with these protein levels of Alcalase (Table 1). At the 3- μ g doses, there were fewer animals with precipitating antibody to subtilisin B than to Alcalase. Based on these data, the subtilisin B was judged to be approximately threefold less potent than Alcalase. The operating guideline for subtilisin B was conservatively kept the same as the guideline for Alcalase and the level of this enzyme in formula did not exceed the level of Alcalase in formula.

Skin Prick Test Responses—Granule Detergent Facility

Table 2 shows the prevalence of employees sensitized to Savinase or Alcalase in a granule detergent manufacturing site during a period that ranged from 1986 to 1991. Protein levels of the enzymes were similar in the products that contained either Savinase or Alcalase (see formula index) and exposures to either enzyme were comparable from year to year. Savinase was used for 5 years and Alcalase was used for 3 years. Approximately 250 employees were skin prick tested during each year of testing. Approximately 5% of the total employees were sensitized to Savinase. Comparably, 3.6% of employees were sensitized to Alcalase during this time period. During the entire production period, the workforce remained essentially symptom free with only three isolated cases of rhinitis reported for this production period.

Skin Prick Test Responses—Liquid Detergent Facility

Table 2 shows the prevalence of employees skin prick test positive to Alcalase, Termamyl, or subtilisin B at a liquid

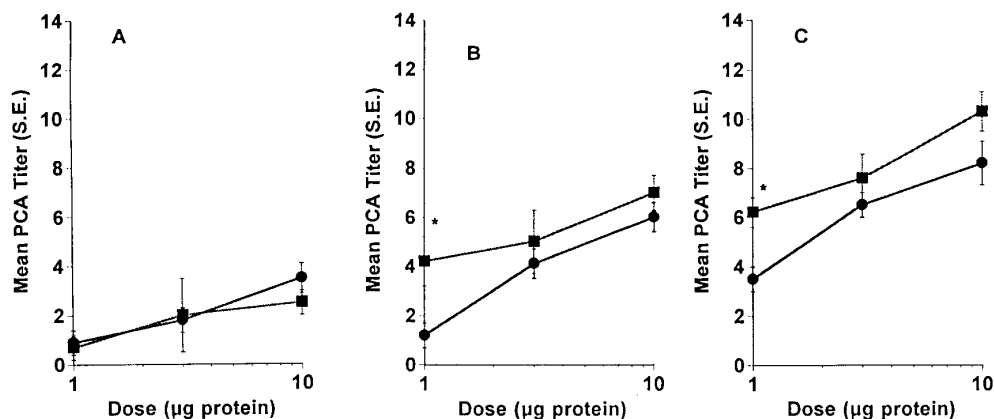


FIG. 3. Comparison of the allergic antibody response to Alcalase (■) and Savinase (●) at (A) week 4, (B) week 5, and (C) week 6.

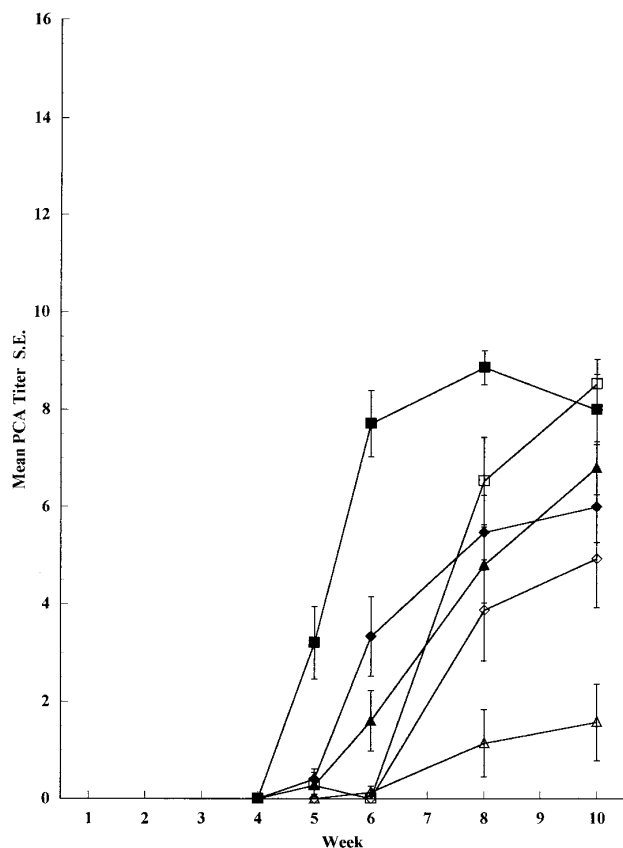


FIG. 4. Allergic antibody titers in sera from animals intratracheally dosed with 3.0 µg subtilisin B (□), 1.0 µg subtilisin B (◇), 0.3 µg subtilisin B (△), 3.0 µg Alcalase (■), 1.0 µg Alcalase (◆), or 0.3 µg Alcalase (▲).

detergent manufacturing site during a period that ranged from 1986 to 1991. Approximately 150 employees were skin prick tested each year. The amount of Alcalase protein in product was comparable to the amount of subtilisin B protein in product (see formula index, Table 2). The amount of

Termamyl protein in product was kept at one-sixth to one-tenth the protein level of protease in product (see formula index, Table 2). As with the granule manufacturing site, the exposure conditions did not vary from year to year. Termamyl was used over the 6-year period, Alcalase was used over a 5-year period, and subtilisin B was used over a 4.5-year period. Approximately 11.6% of the total employees were skin test positive to Alcalase over the production period. Similarly, only 11.3% of total employees were skin test positive to the amylase. The total percentage of employees skin prick test positive to subtilisin B was 6.7%, lower than that observed for Alcalase and Termamyl. No symptoms were noted among this population during the entire production period.

DISCUSSION

In this study, we have shown that the GPIT test is an appropriate animal model for evaluating the relative allergenic potency of various enzymes used in the detergent industry. The serine protease Alcalase is used as the benchmark enzyme allergen in this test since an established TLV and internal operating guidelines exist for this enzyme. Therefore, operating guidelines can be developed for new enzymes based upon how these protein allergens compare against the benchmark allergen in the animal model. These guidelines are established before the introduction of new enzymes into the manufacturing facilities. Through the use of the skin prick test, we can monitor the prevalence rates of sensitization (skin prick test positive with or without clinical signs) to each enzyme in the workplace. Historically, our experience with Alcalase use in the United States and Europe indicates that we can minimize the number of new skin prick test-positive responses per year by compliance with the product design guidelines established from the data from the GPIT test and by manufacturing dust control, engineering, and emphasis on safe work practices.

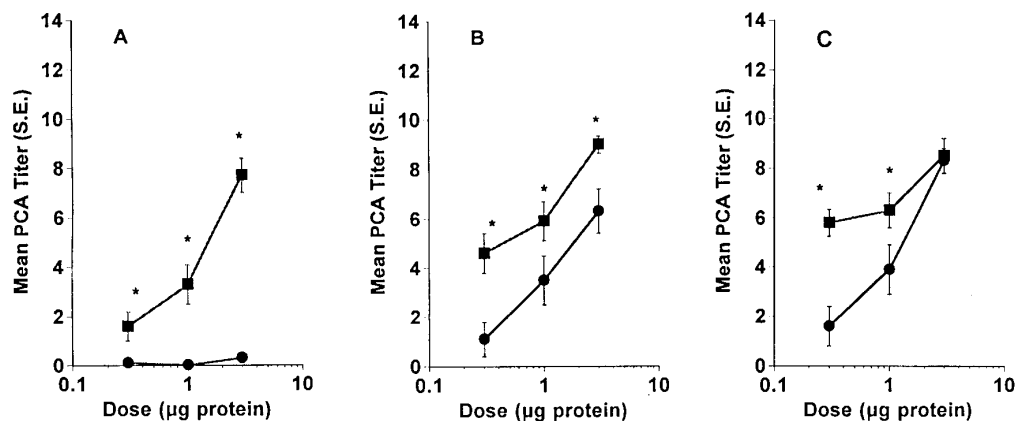


FIG. 5. Comparison of the allergic antibody response to Alcalase (■) and subtilisin B (●) at (A) week 6, (B) week 8, and (C) week 10.

TABLE 2
Percentage of Total Employees at a Liquid or Granular Detergent Manufacturing Site Skin Prick Test Positive to Enzymes and Comparison with Enzyme Potency

	% SPT (+)	Potency vs Alcalase	Formula index ^a
Granular detergent site			
Alcalase	3.3	1	1
Savinase	5.2	1	1
Liquid detergent site			
Alcalase	11.6	1	1
Termamyl	11.3	10	0.1 to 0.16
Subtilisin B	6.7	0.3	1

^a The formula index shows the amount of enzyme allowed in product relative to the amount of Alcalase allowed in product.

Since the animals are repetitively dosed with enzyme protein over a 10- to 12-week time period, the IgG1 antibody titers to all the enzymes tend to plateau toward the end of the study. Therefore, comparisons between Alcalase and the test enzyme were often made during the early part of the study (i.e., weeks 4, 5, and 6) when the differences in antibody titers were more apparent. The MID data were used to gauge the overall immunogenic profile of an enzyme compared to Alcalase and to help further position the potency difference between enzymes. The IgG1 antibody data were primarily used to evaluate allergenic potency. We were confident that we were measuring homocytotropic IgG1 antibody since heat treatment of the sera did not affect the PCA responses (unpublished observations). Although not shown, the onset of immediate respiratory symptoms in the guinea pigs was associated with the appearance of allergic antibody.

Termamyl was shown to be more allergenic (PCA data) and more immunogenic (MID data) than Alcalase in the GPIT test. By looking at the limited dose–response relationship at selected time points, Termamyl was deemed to be approximately 10-fold more potent than Alcalase. For this enzyme, the PCA antibody results suggested a 10-fold difference and the MID antibody data solidified this position. The operating guidelines for the amylase were adjusted downward from the guidelines for Alcalase to reflect the more potent allergenic profile of Termamyl. Exposure to Termamyl was primarily controlled by limiting the amount of Termamyl protein placed in detergent product so that exposure to this enzyme would always be at least 6- to 10-fold less than exposure to Alcalase.

Evaluation of the serine protease Savinase in the GPIT test revealed that this enzyme was less immunogenic than Alcalase (based upon precipitating antibody data) but was roughly equivalent to Alcalase as an allergen since both proteins induced equivalent IgG1 antibody titers at two of the three equiprotein doses. Also, the calculated potency

differences from the PCA antibody dose–response curves were close to 1.0. Therefore, the operating guidelines for Alcalase were applied to Savinase and the amount of Savinase allowed into detergent product was the same as the amount of Alcalase in detergent product. Evaluation of subtilisin B in the GPIT test revealed that this enzyme was both less immunogenic and less allergenic than Alcalase. By examining the dose–response relationship between subtilisin B and Alcalase at weeks 8 and 10, it was concluded that this protease was about threefold less allergenic than Alcalase. The kinetics and magnitude of the antibody response to Alcalase were faster and greater than the response to subtilisin B for each protein dose. Rather than relax the operating guidelines for subtilisin B to reflect the less allergenic profile of the enzyme, the operating guidelines for Alcalase were conservatively applied to subtilisin B. The amount of subtilisin B in detergent product was the same as the amount of Alcalase in product. Under these conditions, we expected the number of sensitizations to subtilisin B to be lower than that obtained with Alcalase. For Savinase, we expected sensitizations to be comparable to or lower than those obtained with Alcalase.

Via prospective evaluation of skin prick test results of workers in the liquid detergent manufacturing site, we found that the total number of workers SPT positive to Termamyl were comparable to the total number of workers SPT positive to Alcalase. Exposure to Termamyl was lower than exposure to Alcalase since Termamyl was put into detergent product at one-sixth to one-tenth the level of protease. Since there was an even distribution of enzyme protein throughout the liquid product (unpublished data), we assumed that Termamyl was present in the air at one-sixth to one-tenth the level of protease. We believe that the downward adjustment of the guidelines for Termamyl (to compensate for the more allergenic nature of this enzyme) led to the similarities between the numbers of sensitizations to Termamyl vs Alcalase. Evaluation of skin prick test responses of workers exposed in the early 1970s to Alcalase and this amylase showed greater sensitizations to amylase (unpublished observations; Bernstein *et al.*, 1994). Also, more workers remained prick test positive to amylase compared to Alcalase once exposure to these enzymes stopped in the mid to late 1970s (Gilson *et al.*, 1976). Based on the data from the GPIT test along with the clinical information on factory workers exposed to these enzymes in the 1970s, we believe that if the overall operating guidelines for Termamyl were not adjusted downward, we would have observed more sensitizations to the amylase compared to Alcalase.

Conversely, the total number of workers SPT positive to subtilisin B was lower than that observed for Alcalase even though employees were exposed to these two enzymes over similar time intervals and the amount of subtilisin B protein used by the manufacturing site was comparable to the

amount of Alcalase protein used by the same site. Subtilisin B was considered to be less immunogenic and allergenic than Alcalase in the guinea pig test, but the operating guidelines for this enzyme were kept the same as the guidelines for Alcalase. We believe that the fewer sensitizations to subtilisin B reflect the less allergenic nature of this enzyme compared to Alcalase.

During the 7-year time period, no adverse respiratory incidents (upper airway or asthma) were reported (self-reported plus physician interviews) in the liquid detergent manufacturing site. This demonstrates that sensitized (skin prick test positive) employees can continue to work in the manufacture of enzyme-containing detergents and not experience allergic symptoms as long as the allergen levels are controlled within the established exposure guidelines.

Since Savinase was considered to be equivalent to Alcalase in terms of its ability to induce allergic antibody in the guinea pig test, the operating guidelines for Alcalase were adopted for Savinase. The total number of SPT-positive responses to Savinase was similar to the number of SPT-positive responses to Alcalase in the granule detergent manufacturing site. The amount of Savinase protein used was similar to the amount of Alcalase protein used by the site. During this 7-year time period, only three isolated incidents of rhinitis were reported among the workforce; asthmatic events did not occur. The three isolated rhinitis incidents were linked with accidental exposure to high levels of enzyme dust. This experience points to the importance of controlling exposure to enzyme in order to eliminate respiratory symptoms in a sensitized workforce.

Based upon the data generated in the GPIT test on four different enzymes, we can rank the enzymes as most potent to least potent as follows: Termamyl > Alcalase = Savinase > subtilisin B. We would predict, under the operational guidelines established for these enzymes, that the number of sensitizations to each enzyme should be equivalent to or lower than the number of sensitizations to Alcalase. Prospective evaluation of skin prick test responses among workers exposed to the different enzymes followed the predictions outlined from the guinea pig test. There were fewer sensitizations observed to subtilisin B compared to Alcalase even though the amount of subtilisin B protein used at the detergent manufacturing site was comparable to the amount of Alcalase protein used by the same facility. There were comparable numbers of sensitizations to Termamyl compared to Alcalase. Also, there were comparable numbers of sensitizations to Savinase compared to Alcalase. The results described in this paper show an association between the human and guinea pig allergic antibody responses to these enzyme allergens. This gives us confidence in using the guinea pig model to evaluate the relative allergenic potential of enzymes and to predict how humans will respond to these materials under established occupational exposure conditions. Since

we have a data base in occupationally exposed humans with four different enzymes, we are confident that this model can be used to evaluate new classes of potentially allergenic enzymes (e.g., lipases, cellulases) as well as other proteins. Since enzymes are being used or are being considered for use by other industries (e.g., textiles, food, cosmetics), the learnings from the detergent industry can be applied to these other industries (Brennan, 1996; Krawczyk, 1997). Also, we believe this model and approach can be used to assess the safety of other allergenic proteins. For example, we have tested protein extracts from guar gum and found these proteins to be at least an order of magnitude less allergenic than Alcalase and poorly immunogenic (unpublished observation). The scarcity of reported occupational allergic responses to guar linked with the high volume of use by various industries (e.g., food, textiles, oil drilling) helps to support the data from the animal model (Anon., 1995). Therefore, we believe this model represents a novel and appropriate approach for evaluating proteins as occupational allergens. For the detergent industry and other enzyme-using industries, we predict that, with the proper operating guidelines achieved by formulation controls and engineering controls, occupational allergic sensitization and symptomatology to new classes of enzymes will be minimal and similar to the recent sensitization experiences with Alcalase, Savinase, subtilisin B, and Termamyl.

REFERENCES

- American Conference of Governmental Industrial Hygienists (1990). Threshold Limit Values and Biological Exposure Indices, p. 38. Cincinnati, OH.
- Anonymous (1995). This year's guar crop starting to look strong. *Chem. Market. Rep.* **248**, 14.
- Bernstein, D. I., Bernstein, I., Gaines, W. G., Stauder, T., and Wilson, E. R. (1994). Characterization of skin prick testing responses for detecting sensitization to detergent enzymes at extreme dilutions: Inability of the RAST to detect lightly sensitized individuals. *J. Allergy Clin. Immunol.* **94**, 498–507.
- Brennan, M. B. (1996). Enzyme discovery heats up. *C&EN* **74**, 31–33.
- Draper, N. R., and Smith, H. (1981). *Applied Regression Analysis*, 2nd ed., pp. 8–38. Wiley, New York.
- Flindt, M. L. H. (1969). Pulmonary disease due to inhalation of derivatives of *Bacillus subtilis* containing proteolytic enzyme. *Lancet* **1**, 1177–1181.
- Flood, D. F. S., Blofeld, R. E., Bruce, C. E., Newitt, J. I., Juniper, C. P., and Roberts, D. M. (1985). Lung function, atopy, specific hypersensitivity, and smoking of workers in the enzyme detergent industry over 11 years. *Br. J. Ind. Med.* **42**, 43–50.
- Gilson, J. C., Juniper, C. P., Mortin, R. B., and Weill, H. (1976). Biological effects of proteolytic enzyme detergents. *Thorax* **31**, 621–634.
- Guntelberg, A. V., and Offesen, M. (1954). Purification of the proteolytic enzyme from *B. subtilis*. *Comp. Rend. Lab. Carlsberg Sor. Chim.* **29**, 36–48.
- Hartley, B. S., Haughton, M. A., and Sanger, F. (1959). The amino acid sequence around the reactive serine of elastase. *Biochim. Biophys. Acta* **34**, 243–244.

- Juniper, C. P., and Roberts, D. M. (1984). Enzyme asthma: Fourteen years' clinical experience of a recently prescribed disease. *J. Soc. Occup. Med.* **34**, 127–132.
- Krawczyk, T. (1997). Enzymes: Gaining momentum and branching out. *Inform* **8**, 6–14.
- Pepys, J., Longbottom, J. L., Hargreave, F. E., and Faux, J. (1969). Allergic reactions of the lungs to enzymes of *Bacillus subtilis*. *Lancet* **1**, 1181–1184.
- Pepys, J., Wells, I. D., D'Souza, M. F., and Greenberg, M. (1973). Clinical and immunological responses to enzymes of *Bacillus subtilis* in factory workers and consumers. *Clin. Allergy* **3**, 143–160.
- Pepys, J., Mitchell, J., Hawkins, R., and Malo, J. L. (1985). A longitudinal study of possible allergy to enzyme detergents. *Clin. Allergy* **15**, 101–115.
- Ritz, H. L., Evans, B. L. B., Bruce, R. D., Fletcher, E. R., Fisher, G. L., and Sarlo, K. (1993). Respiratory and immunological responses of guinea pigs to enzyme containing detergents: A comparison of intratracheal and inhalation modes of exposure. *Fundam. Appl. Toxicol.* **21**, 31–37.
- Rothgeb, T. M., Goodlander, B. D., Garrison, P. H., and Smith, L. A. (1988). The raw material, finished products and dust pad analysis of detergent proteases using a small synthetic substrate. *J. Am. Oil Chem. Soc.* **65**, 806–810.
- Sarlo, K., Clark, E. D., Ryan, C. A., and Bernstein, D. I. (1990). ELISA for human IgE antibody to Subtilisin A (Alcalase): Correlation with RAST and skin test results with occupationally exposed individuals. *J. Allergy Clin. Immunol.* **86**, 393–399.
- Snedecor, G. W., and Cochran, W. G. (1980). *Statistical Methods*, 7th ed., pp. 93–98. Iowa State Univ. Press, Ames, IA.
- Weill, H., Waggenpaek, C., DeRoven, T., and Ziskind, M. (1974). Follow-up observations of workers exposed to enzyme detergents. *Am. N.Y. Acad. Sci.* **221**, 76–85.
- Zetterstrom, O. (1977). Challenge and exposure test reactions to enzyme detergents in subjects sensitized to subtilisin. *Clin. Allergy* **7**, 355–363.