

Antigenic and Allergenic Characterization of the Enzymes Alcalase and Savinase by Crossed Immunoelectrophoresis and Crossed Radioimmunoelectrophoresis

L.G. Arlian^a, D.L. Vyszynski-Moher^a, J.A. Merski^b, H.L. Ritz^b, T.L. Nusair^b, E.R. Wilson^b

^a Department of Biological Sciences, Wright State University, Dayton, Ohio; ^b Procter & Gamble Co., Cincinnati, Ohio, USA

Abstract. Alcalase and savinase, produced by *Bacillus* species, are proteolytic enzymes that are used in laundry products and are known to cause respiratory allergy. Antigenic and allergenic characteristics of alcalase and savinase and their potential cross-reactivity were evaluated using crossed immunoelectrophoresis and crossed radioimmunoelectrophoresis. Alcalase exhibited two distinct antigens; one electropositive and one electronegative. The electropositive antigen exhibited some retrograde anodic mobility when coupled with antiserum components. Savinase exhibited one electropositive and two electronegative antigens. The antigens of the two enzymes were clearly different from each other, the three savinase antigens exhibiting greater electrophoretic mobility than the two alcalase antigens. In crossed radioimmunoelectrophoresis studies, only the electropositive antigen of alcalase, its retrograde complex, and the electropositive antigen of savinase bound IgE from the sera of individuals who were skin test positive to one or both enzymes. No evidence of cross-reactivity was observed in heterologous and tandem crossed immunoelectrophoresis studies and heterologous microimmunodiffusion reactions.

Introduction

Alcalase (subtilopeptidase A) and savinase are proteolytic enzymes derived from nonpathogenic strains of *Bacillus* species. They are used in household laundry products to enhance stain removal and general cleaning performance. Shortly after introduction into laundry products in the late 1960s, it was recognized that many detergent workers developed IgE-mediated allergy to alcalase following exposure to high levels of airborne enzyme dust [1-5]. Savinase has been more recently used in laundry products. Since both alcalase and savinase are proteases produced by the same bacterial genus, the question of immunologic cross-reactivity has been raised [6]. Using the radioallergosorbent test (RAST), Dor et al. [6] reported elevated levels of IgE to savinase in a small number of detergent workers who had no known prior industrial exposure to this enzyme. To explain their findings they proposed allergenic cross-reactivity between test enzymes, since cross-contamination

of the enzymes was considered to be inappreciable. In the present study, antigenic and allergenic characteristics of alcalase and savinase, including their potential cross-reactivity, were evaluated by crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE).

Materials and Methods

Enzyme Preparation

Specially purified savinase and alcalase were supplied by Novo Industri (A/S Bagsvaerd, Denmark). Based on Kjeldahl nitrogen analysis, alcalase and savinase contained 44.4 and 21.2% protein, respectively. Solutions of the enzymes were made by dissolving 2.4 mg/ml of either the alcalase or savinase sample in glass-distilled water. Though most of these enzyme solutions were used within 1-2 h, all were used within 24 h of preparation. The stability of the enzyme solutions was confirmed by CIE and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Identical CIE and sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles were obtained when enzyme solutions refrigerated for 24 h and fresh enzyme solutions were compared.

Rabbit Antisera

Antisera to alcalase and savinase were produced in New Zealand white rabbits. For immunization, alcalase or savinase was dissolved in 0.9% NaCl at a concentration of 2 mg of enzyme protein per milliliter. The enzyme solutions were emulsified with equal volumes of Freund's complete adjuvant (Calbiochem®; Calbiochem, La Jolla, Calif., USA).

Initially each rabbit received a 0.5-ml injection of the enzyme/adjuvant emulsion in each shoulder. A 1-ml intravenous booster injection containing 1 mg protein/ml in saline was given to each rabbit for 4 consecutive days, beginning 26 days after the initial injections. Rabbits were exsanguinated 41–44 days after the initial injections. All sera were individually analyzed by microimmunodiffusion, and high-titer sera were pooled and frozen.

Human Sera

Six sera, designated A–F, from individuals who were skin-prick test positive to alcalase, were used to perform CRIE. Five of these sera, B–F, were from individuals who were also skin-prick test positive to savinase. Individual A was not skin-prick tested with savinase. The 6 individuals from whom sera were obtained worked in a detergent plant where alcalase was manufactured and were presumably exposed to enzyme-containing dust. They had no known industrial exposure to savinase, since this enzyme had not been introduced into this plant. Three sera from plant workers who had negative skin-prick tests to both alcalase and savinase served as controls.

CIE and CRIE

CIE and CRIE were performed by methods similar to those previously described [7–13], except that the antigen well was placed in the center of the first-dimension gel strip (1 × 5 cm) in order to accommodate cathodic and anodic migration of antigens. For homologous CIE, 10 µl of a solution containing 24 µg of enzyme was horizontally electrophoresed in the first dimension with Svendsen's buffer at 200 V for 20 min in 1% agarose gel at 16°C. For second-dimension electrophoresis, the homologous cathodic gels (5 × 4.5 cm) required 3 ml of the agarose solution which contained 125–250 µl of rabbit antiserum raised to the enzyme used in the first-dimension gel. The anodic gels (5 × 1.5 cm) required 1 ml of agarose which contained 21–66 µl of the same rabbit antiserum. Following second-dimension electrophoresis, the gels were stained with Coomassie brilliant blue R250.

Heterologous CIE gels were run in the same way, except that the antiserum used in the second-dimension gel was produced to the other enzyme being studied (e.g., alcalase versus antisavinase). For tandem CIE, two separate wells were punched 4 mm apart in the center of the first-dimensional agarose gel. Enzyme solutions were placed in the wells, allowed to diffuse for 30 min, and then the wells were filled with agarose. The gels were then electrophoresed in the first and second dimensions as for regular CIE gels.

CRIE was performed by incubating unstained CIE gels overnight in 15–20 ml of RAST A buffer containing 600–850 µl of human sera. The gels were then washed in RAST B buffer and incubated overnight in RAST C buffer containing ¹²⁵I-radiolabeled antihuman IgE (1.3–1.5 million cpm; Kallestad). The compositions of RAST A, B, and C buffers were as previously described [7, 8]. After incubation, the gels were washed, pressed, and blown dry. This was followed by autoradiography at –80°C using metal cassettes, tungsten enhancement screens, and Kodak X-Omat RP film.

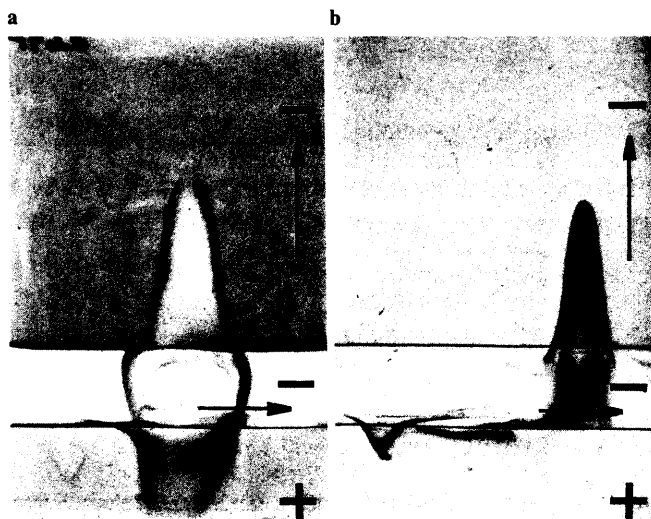


Fig. 1. CIE patterns. **a** Alcalase extract reacted with rabbit antialcalase serum. **b** Savinase reacted with rabbit antisavinase serum.

To estimate the relative amount of specific IgE for each enzyme in each human serum, equal volumes of each serum were incubated with identical individual, homologous CIE gels. The standard CRIE procedure was then followed. The amounts of radiostaining observed on the autoradiograms at the sites corresponding to the precipitin lines of the CIE gels and their exposure times were used to estimate the relative amount of alcalase- or savinase-specific IgE in each human serum sample.

Microimmunodiffusion Assays

Radial immunodiffusion was performed on pattern B immunoplates (Hyland Laboratories, Costa Mesa, Calif., USA) with a central well and 5 peripheral wells 7 mm from the central well. All wells were 2.5 mm in diameter. Five microliters of rabbit antisavinase or antialcalase hyperimmune serum and the enzyme preparations were used in the central and peripheral wells, respectively. Alcalase and savinase preparations were placed in alternate wells (in clockwise direction), starting with enzyme that matched the antiserum and reacted with either antisavinase or antialcalase.

Results

Detection of Antigens in Extracts of Alcalase and Savinase by CIE

The CIE reaction of alcalase with antialcalase built in rabbits resulted in the formation of one large, prominently stained electropositive and one small, weakly stained electronegative antigen-antibody precipitin complex (fig. 1a). In addition, under the electroendosmotic and pH conditions used in these experiments, once some of the cathodically migrating anti-

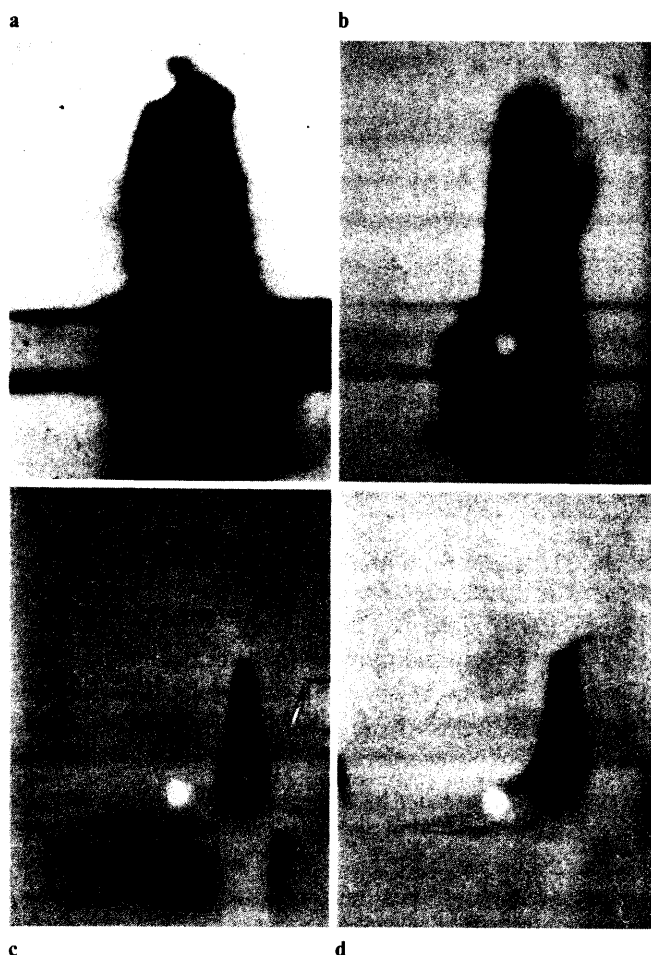


Fig. 2. **a, b** Representative autoradiographs (4 days exposure) of the CIE patterns of alkalase reacted with antialcalase for reference sera A and C show intense and moderate radiostaining, respectively. **c, d** Representative autoradiographs (14 days exposure) of the CIE pattern of savinase reacted with antisavinase for reference sera A and C showing very low or low radiostaining, respectively.

gen became bound in an antigen-antibody complex and possibly with other antiserum components, a net change in the charge resulted. These antigen complexes then migrated toward the anode (retrograde migration). As a result, stained gels exhibited a diffuse antigen-antibody precipitate on the anodic side of the gel opposite the large, well-defined cathodic antigen-antibody precipitin peak. Similar retrograde mobility was previously reported for components of subtilopeptidase A, the major component of alkalase [9]. Had a charge change not occurred, the antigen would have migrated in the opposite direction during 1st-dimension electrophoresis. CIE gels in which savinase

was reacted with antisavinase rabbit serum exhibited one large, prominently stained electropositive and two (one prominently stained and one weakly stained) electronegative antigen-antibody precipitin complexes (fig. 1b). The three savinase antigens migrated faster in the first dimension than the two alkalase antigens. Electrophoresis of either savinase or alkalase into gels containing serum of nonimmunized control rabbits resulted in no visible precipitates.

Detection of Alkalase and Savinase Allergens by CRIE

CRIE studies were performed with six individual human sera to determine if their IgE reacted with the alkalase or savinase precipitin complexes. Autoradiograms of the homologous alkalase reaction exhibited radiostaining only at the electropositive antigen-antibody precipitate and the retrograde migrating, modified antigen complex (fig. 2a, b). The relative intensity of radiostaining was high in autoradiograms made from gels reacted with serum A or B, moderate when made from the gel reacted with serum C, and low for autoradiograms made from gels reacted with serum D, E, or F (table 1).

Radiostaining was observed only at the electropositive precipitin complex for savinase (fig. 2c, d). Of the 6 reference sera, 4 exhibited IgE binding to the savinase antigen. Based on the intensity of radiostaining on the autoradiograms, the savinase-specific IgE levels were generally lower than the alkalase-specific IgE levels in the six sera (table 1). Specifically, autoradiograms made from gels reacted with serum C (fig. 2d) or serum F gave low reactions to savinase compared to alkalase, while autoradiograms made from gels reacted with serum A (fig. 2c) or serum B had very low levels of radiostaining. Comparatively, the radiostaining of the savinase precipitin complex noted on the autoradiograms made from gels reacted with serum D or with serum E was equivalent to that of the controls. Control sera from 3 individuals who were skin-prick test negative to both enzymes were negative or gave only insignificant radiostaining after more than 21 days of autoradiographic exposure in CRIE studies with both alkalase and savinase.

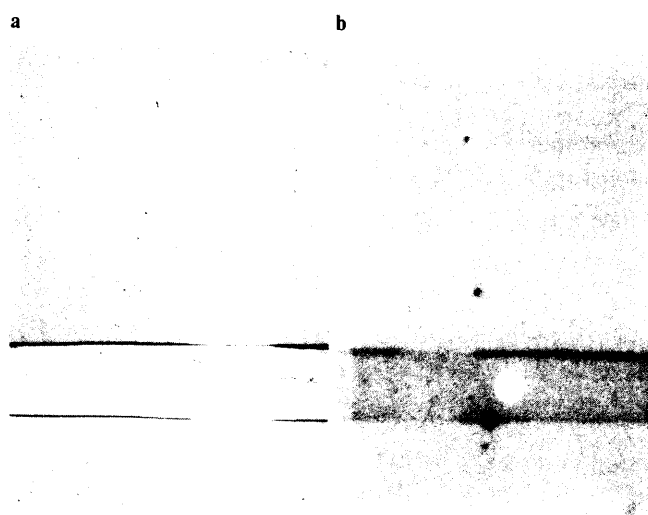
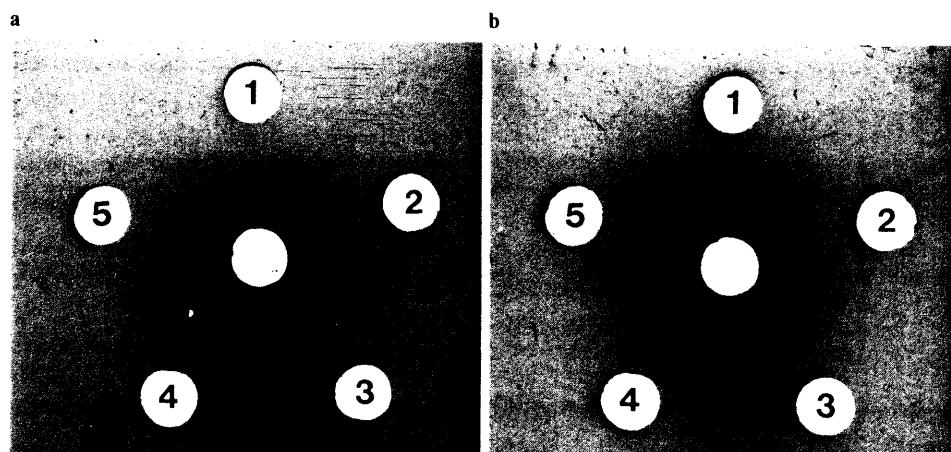
Heterologous CIE and CRIE

To evaluate the potential cross-reactivity between the alkalase and savinase used in this study, heterologous CIE and CRIE studies were also performed. Heterologous CIE of savinase, electrophoresed into a

Table 1. Relative levels of IgE to alcalase and savinase in six human sera

Human serum	Intensity of radiostaining ^a		Skin-prick test responsiveness	
	alcalase	savinase	alcalase	savinase
A	++++	+	positive	not done
B	++++	+	positive	positive
C	+++	++	positive	positive
D	++	—	positive	positive
E	++	—	positive	positive
F	++	++	positive	positive
Control (n = 3)	—	—	negative	negative

^a All CIE reactions were incubated in labelled human anti-IgE with the same activity (¹²⁵I ≈ 1.4 million cpm) before autoradiography. ++++ = High (at 4 days); +++ = moderate (at 4 days); ++ = low (at 11 days); + = very low (at 14 days); — = no radiostaining (at 14–21 days).

**Fig. 3.** **a** CIE pattern of savinase reacted with rabbit antialcalase. **b** Autoradiograph (CRIE) of the CIE pattern with reference serum A.**Fig. 4.** **a** Microimmunodiffusion patterns of rabbit antialcalase (center well) reacted with (clockwise): (1) 0.10% alcalase; (2) 0.80% savinase; (3) 0.10% alcalase; (4) 0.20% savinase, and (5) 0.10% alcalase. **b** Rabbit antisavinase (center well) reacted with (clockwise): (1) 0.13% savinase; (2) 0.80% alcalase; (3) 0.13% savinase; (4) 0.20% alcalase, and (5) 0.13% savinase.

second-dimension gel containing antiserum produced to alcalase, resulted in a clear area in the Coomassie blue stained gel (fig. 3a). This clear area was similar in shape and position to the electropositive precipitate that resulted from reaction of savinase with antiserum to savinase (see figure 1b). Therefore, to determine that no weak nonstaining precipitate had formed, CRIE was performed using the sera from the 6 skin test positive individuals and 3 negative controls. These gels gave no radiostaining following incubation with any of the six human sera (fig. 3b) or the

three control sera. Each serum had been demonstrated to be moderately or strongly positive to alcalase in homologous CIE gels (table 1). Likewise, human sera A–C and F previously showed that they contained IgE directed against the electropositive savinase antigen.

Microimmunodiffusion

Microimmunodiffusion was performed using either antisavinase or antialcalase in the center well and different quantities of both alcalase and savinase in

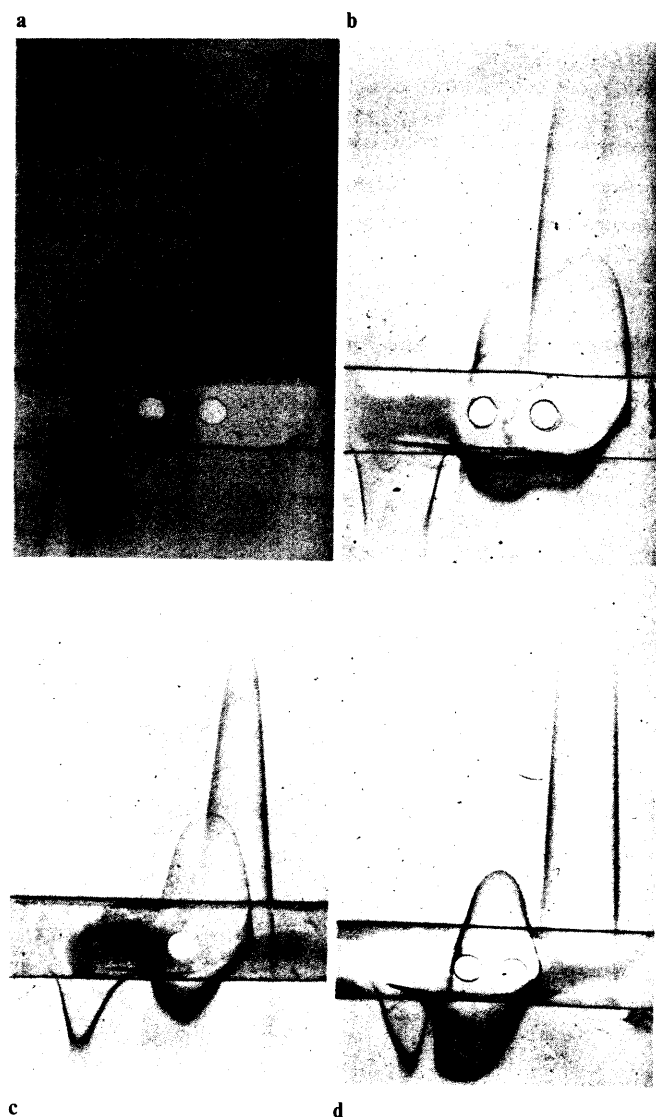


Fig. 5. a Tandem CIE pattern of savinase in both wells reacted with an antisavinase and antialcalase mixture. b Tandem CIE pattern of an alkalase/savinase mixture in both wells reacted with a mixture of antisavinase and antialcalase. c CIE pattern of an alkalase/savinase mixture reacted with an antisavinase and antialcalase mixture. d Tandem CIE pattern of alkalase and savinase in separate wells reacted with an antiserum mixture containing both antialcalase and antisavinase.

the peripheral wells (fig. 4). A single precipitin line formed with the reaction of antialcalase and alkalase, while two precipitin lines formed with the reaction of antisavinase and savinase. The precipitates were completely fused when adjacent wells contained the same enzyme preparation, indicating that the enzyme preparations in these wells were antigenically identical.

No precipitate formed between an antiserum and noncorresponding enzyme preparation, nor were the precipitates of adjacent homologous reactions distorted. This technique did not resolve all of the antigens visualized by CIE.

Assessment of Cross-Reactivity between Alkalase and Savinase by Tandem CIE

To confirm the results of the heterologous CIE and microimmunodiffusion studies, that the two enzymes were not cross-reactive, a series of tandem reactions using several combinations or mixtures of alkalase and savinase was performed. Some were designed to simulate cross-reactivity. In tandem CIE studies crossing, fusion, or interference of antigen peaks are the primary criteria used to assess if antigens are completely different, identical, or share some determinants.

In one series of reactions, the first-dimension gels contained either savinase or alkalase in both separate tandem wells and were electrophoresed as usual. For each enzyme two different antisera were used in the second-dimension gels. In one case savinase was reacted with an antiserum mixture containing both antialcalase and antisavinase (fig. 5a), and in a second case it was reacted with only antisavinase. Both of the savinase tandem gels resulted in only double savinase precipitate peaks with adjacent overlapping legs that were fused with no interference, as expected (fig. 5a). There was no evidence of an alkalase peak. In a similar experiment, alkalase in tandem was reacted with an antialcalase/antisavinase mixture and with antialcalase alone. Two alkalase precipitin peaks were formed with both the antialcalase/antisavinase combination and antialcalase alone in the second-dimension gels, with no evidence of a savinase precipitate. The adjacent legs of the alkalase peaks fused in both cases with no evidence of interference.

In a second set of tandem reactions, a mixture of alkalase and savinase was placed in both wells and then electrophoresed into an antisavinase/antialcalase mixture. This combination resulted in double precipitin peaks for both enzymes (fig. 5b). The adjacent, overlapping legs of the precipitin peaks of the same enzyme fused, while those of alkalase clearly crossed without interference with those of savinase (fig. 5b), then a mixture of savinase and alkalase in one well was reacted with a mixture of antialcalase and antisavinase. This also resulted in clearly defined

and unfused alcalase and savinase precipitin peaks (fig. 5c).

In a final tandem experiment, alcalase and savinase in separate wells were reacted with an antialcalase and antisavinase mixture. Normal alcalase and savinase antigen-antibody peaks were formed. No fusion of the alcalase and savinase peaks was observed that would indicate cross-reactivity (fig. 5d).

Discussion

In this study, CIE and CRIE were used to investigate the antigenic and allergenic properties of the proteolytic enzymes alcalase and savinase because these techniques allow clear visualization of individual antigens in antigen mixtures. Homologous CIE demonstrated that alcalase had one electropositive (with a reverse migrating antigen-antibody complex) and one electronegative antigenic component, while savinase had one electropositive and two electronegative antigenic components. The respective components for each enzyme are distinct from each other, as indicated by the differences in their electrophoretic mobility.

Our finding that the alcalase material contained two clearly distinct, antigenically different components was in contrast to a previous report [9] showing that alcalase (subtilopeptidase A) was a multiple isoenzyme system in which different forms of the antigen exhibited partial antigenic identity. In that previous report, CIE analysis of subtilopeptidase A showed that it contained one major and one minor electropositive antigen, each consisting of three and two isozymes, respectively. Interbatch heterogeneity and retrograde antigen mobility had been noted also. Our homologous CIE results did not show the different isozyme forms for similar antigens as previously noted (as evidenced by fused CIE peaks). It is not likely that the antigens visualized by CIE in this study were isozymes, since they exhibited opposite electrophoretic mobility, and homologous CIE gels did not lead to precipitin peak fusion.

Some of the differences between the results of our analysis and those of previous studies may be attributed to interbatch heterogeneity in the enzyme that had been previously reported [9], use of different buffers for the CIE that gave different antigen mobility, or the fact that the current bacterial strains produce a more homogeneous product. We have confirmed the

heterogeneity in other batches of alcalase we have analyzed by CIE which contained three distinct antigens in contrast to the two for the batch used for this study. CRIE analysis of the alcalase antigenic components showed that only the electropositive antigen with its retrograde migrating antigen complex bound significant levels of human IgE and is thus allergenic. Likewise, only the electropositive antigen of savinase was allergenic and bound IgE. For both enzymes, the electronegative antigens were nonallergenic or too weakly allergenic to be detected by the reference sera tested.

Heterologous and tandem CIE and microimmunodiffusion were used to determine if the two extracts contain common or cross-reacting antigens. In particular, CIE was used because, unlike RAST, it allows for the visualization of individual antigens and the possible interaction of common antigenic determinants in extracts containing multiple antigens. Heterologous CIE reactions of each enzyme with antiserum produced to the other enzyme resulted in no antigen-antibody precipitates. When individual enzymes were used in both wells of tandem gels and electrophoresed against homologous antiserum, double antigen-antibody precipitates of identity with fused adjacent legs were formed, as expected [10, 11]. When mixtures of the enzymes were used in both wells of a tandem gel or in a single well and reacted with a single antiserum or an antiserum mixture, double, fused peaks of the individual enzymes were also formed. However, there was no fusion or interference of overlapping adjacent legs between precipitin peaks of the two different enzymes. Likewise, heterologous immunodiffusion reactions resulted in no precipitates, and there was no distortion of precipitates formed by adjacent homologous reactions. However, not all antigenic components of both enzymes could be seen with this technique. The results of both CIE and immunodiffusion indicated that the two extracts contained different antigen molecules and that these molecules did not share common antigenic determinants. Therefore, alcalase and savinase are not immunologically cross-reactive.

It appears likely that the positive skin-prick tests and IgE-binding reactions to savinase observed in the detergent plant workers used in this study and who had no known exposure to the enzyme at the workplace resulted from contamination of one enzyme with another and/or sensitization to savinase outside the workplace. If the alcalase used in detergents in the

late 1960s and 1970s contained a low level of savi-nase, workers could have become sensitized to both enzymes during periods of high exposure. Though bacterial selection and culture and manufacturing processes are designed to keep commercial detergent enzymes pure, it has been confirmed by enzyme producers that some contamination may occur. In addition, *Bacillus* species are common soil bacteria, so it is possible that individuals were exposed to savinase outside the workplace.

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Received: August 14, 1989

Accepted after revision: November 17, 1989

Correspondence to: Dr. L.G. Arlian
Department of Biological Sciences
Wright State University
Dayton, OH 45435 (USA)