

Respiratory pathophysiological responses

Diagnostic tests in enzyme allergy

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Background: Allergies to enzymes occur frequently in enzyme manufacturing plants. In epidemiologic studies the number of exposed subjects with symptoms is considerably higher than the number of sensitizations as assessed by skin tests or RAST. It was the aim of this study to evaluate the sensitivity and specificity of skin prick tests, enzyme allergosorbent test, and immunoprint with enzymes by using the results of bronchial provocation tests as the "gold standard."

Methods: We performed 82 bronchial provocation tests with nine different enzymes in 42 chemical plant workers, all of whom had reported work-related symptoms. All tests with the exception of bronchial provocation tests were also performed in 10 atopic and 10 healthy control subjects.

Results: Thirteen workers showed positive results to bronchial provocation tests (50% or greater fall in specific airway conductance) with an enzyme at a maximal concentration of 10 mg/ml. Equivocal test results, that is, tests with a 30% or greater and less than 50% fall in specific airway conductance were seldom. Skin prick tests with nondialyzed aqueous enzyme extracts at a concentration of 10 mg/ml yielded a sensitivity of 100% and a specificity of 93%. Control subjects had no cutaneous reactions to enzymes. Discrepancies between skin tests and bronchial provocation tests occurred in 5 of 82 tests, all with a positive skin test and a negative bronchial provocation test. False-positive skin tests were considered more likely in four subjects and a false-negative bronchial challenge more likely in one case. Enzyme allergosorbent test was 62% sensitive and 96% specific, and atopic control subjects showed positive results in two cases. Results of immunoprint with amylase showed one main band at pH 3.4; this band was also found in exposed subjects without further signs of sensitization, but was not found in control subjects. All but one person with positive results to bronchial provocation tests with enzymes showed bronchial hyperresponsiveness. We identified 13 subjects with bronchial hyperresponsiveness, but without occupational allergy: five of these had sensitizations to nonoccupational allergens and the remainder showed bronchial hyperresponsiveness without any detectable cause.

Conclusions: Occupation-related symptoms were not indicative of occupational allergy. Bronchial provocation tests and skin prick tests with nondialyzed aqueous enzyme extracts were appropriate techniques for the diagnosis of enzyme allergy. (*J ALLERGY CLIN IMMUNOL* 1993;92:264-77.)

Key words: Enzymes, allergy, asthma, diagnosis, enzyme allergosorbent test, bronchial provocation test, skin test

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Immediate-onset asthma and rhinoconjunctivitis have been described in bakers and in industrial workers occupationally exposed to enzymes. Bakers demonstrate sensitization mainly to starch-cleaving enzymes and rarely to proteolytic enzymes.¹ In contrast, workers in pharmaceutical industries,²⁻⁷ in kitchens, meat-processing plants,^{4,8} and chemical plants⁹⁻¹² (mostly laundry detergent industries¹³⁻¹⁷) are sensitized to proteolytic enzymes of microbial, plant, or animal origin, with

Abbreviations used

BPT:	Bronchial provocation tests
EAST:	Enzyme allergosorbent test
IEF:	Isoelectric focusing
NCM	Nitrocellulose membrane
PRU:	Phadebas RAST units
sG _{aw} :	Specific airway conductance
sR _{aw} :	Specific airway resistance
TBS:	TRIS-buffered saline (pH 7.4)
TBS-Tw:	TBS plus 0.05% Tween 20
TBS-Tw-M:	TBS-Tw plus 0.5% nonfat dry milk

few exceptions.^{11, 13, 18} A number of enzyme preparations were identified as causes of IgE-mediated allergic symptoms: pectinase,¹¹ α -amylase,^{13, 19} cellulase,¹⁸ trypsin,^{9, 20} papain,^{21, 22} bromelain,^{2, 21} subtilisins,^{13, 14, 23} flaviastase,²⁴ pepsin,⁵ and pancreatic enzymes.^{25, 26}

Cross-sectional or longitudinal surveys of industrial settings have been confined mostly to the detergent industry. These studies demonstrated a high allergenic potential of proteolytic enzymes. In one study, 49 out of 98 workers exposed to proteolytic enzymes had lower-respiratory-tract symptoms.²⁷ In another survey 57 (21%) of 271 exposed workers had cutaneous reactivity with a *Bacillus subtilis*-derived enzyme preparation.²⁸ In two detergent manufacturing plants 45% and 52% of subjects in the high-exposure groups had cutaneous reactivity with enzymes.¹⁴

Initial studies were performed in the late sixties or early seventies. At that time consumers of enzyme-containing washing powder were also reported to be sensitized to enzymes.^{29, 30} Subsequently it was demonstrated in the detergent industry that the number of sensitized subjects can be reduced by process modification measures such as encapsulation of enzyme preparations.³¹ However, also in the 1980s high numbers of workers with sensitization to papain were reported.^{4, 8} A recent survey of a pharmaceutical plant revealed positive results to a skin prick test for α -amylase in 26 of 83 subjects.¹⁹

Skin prick tests, RAST, and specific bronchial provocation tests (BPT) have been used to show sensitization and occupational asthma caused by enzymes. Skin testing and RAST have a wide range of sensitivity and specificity in cross-sectional studies for identification of workers with symptoms: skin test results were positive in 23% of 91 papain workers with symptoms.⁸ The RAST was positive to enzyme allergens in 33% of six

Bacillus subtilis-exposed workers with symptoms.¹⁵ Others found positive skin test results with proteolytic enzymes in 72% of 49 exposed workers with symptoms.²⁷ Baur et al.⁴ reported 13 subjects with symptoms among 29 workers exposed to papain, 10 with positive RAST and 12 with positive skin test results, thus yielding a sensitivity of 77% for RAST and 92% for skin tests.

RAST and skin tests probably have higher specificity than sensitivity: positive skin tests were rarely found in control subjects.^{32, 33} The number of false-positive skin reactions can be reduced by the use of more diluted extracts, that is, 1 mg/ml or less.³² False-positive RAST results in control subjects were reported in few studies.^{32, 33}

Specific BPT have never been applied as the "gold standard" of diagnosis in enzyme allergy. Also BPT have been performed in a limited number of subjects and mostly with only one enzyme. One investigation of 83 α -amylase-exposed pharmaceutical workers demonstrated positive bronchial reactions in 6 of 14 subjects.¹⁹ The low sensitivity of BPT was attributed to exposure to further enzymes not being tested. Several case reports of immediate asthmatic reactions after inhalation of different enzyme preparations, mostly proteases, have been published. Such observations have been made for papain,^{3, 4, 22} pepsin,⁵ amylase,^{34, 35} subtilisins,^{23, 36} bromelain,² trypsin,^{9, 20} and cellulase.¹⁸

There have been few methodic investigations of RAST with enzymes. Inhibition experiments with papain coupled to the solid phase revealed inhibition with fluid-phase papain. Inhibitory rates were the same when the enzymatic activity of the fluid phase was blocked with chemicals.²¹ Others reported nonspecific interference of RAST with proteases and thus recommended inactivation of protease activity when protease-containing allergen extracts are used in RAST.³⁷

Because a high percentage of false-positive symptoms among subjects with enzyme exposure could be anticipated from results of previous studies, in the present investigation we used the results of BPT as the gold standard for identifying enzyme allergies. There is good evidence to believe that BPT are the most specific tests for enzyme allergies; positive tests in control subjects have not been reported.^{4, 5, 9, 18-20, 23, 38} Almost all subjects with a positive BPT result also exhibited both positive skin test and positive RAST results.^{4, 5, 18, 36} Thus sensitivity of the latter tests can be assumed to be far higher when BPT is used instead of symptoms as the diagnostic standard.

It was the aim of this study to evaluate the sensitivity and specificity of specific skin prick testing and enzyme allergosorbent test (EAST) techniques for diagnostic and epidemiologic purposes. EAST is a modification of the Phadebas RAST (Pharmacia, Freiburg, Germany). This was performed with BPT serving as the gold standard and with the use of different enzyme preparations and simple standard procedures.

METHODS

Subjects

A total of 46 workers from an enzyme manufacturing plant were referred to the pulmonary department between November 1989 and December 1990 by the Ministry of Social Welfare for a medical evaluation. These workers gave histories of asthma, chronic cough, rhinitis, or conjunctivitis. All had reported work-related symptoms in a cross-sectional survey that had been taken from November 1988 to April 1989.³⁹ Thirty-one were actually exposed (20 production workers, 4 laboratory assistants, 7 workmen with occasional exposure to enzymes) and 15 subjects had been removed from enzyme exposure because they were thought to have an occupational disease (12 production workers, 3 laboratory assistants). Tests were performed 46 (1 to 120) months after these subjects had been removed from exposure.

Of the 46 subjects 42 underwent BPT with both methacholine and at least one enzyme. No BPT with enzymes were performed in four subjects: two of these had airway obstruction (FEV_1 in percent of inspiratory vital capacity 59% and 24%), one inhaled only rice flour beetle as an occupational antigen, and one had hypersensitivity pneumonitis. For control purposes we investigated 10 patients from our outpatient clinic with a history of allergy and a positive skin prick test result to at least one common allergen. Also 10 healthy members of our clinical staff with negative skin test results to common allergens were examined. Control subjects had no occupational exposure to enzymes and did not use meat tenderizers. No subject had taken any medication for at least 48 hours before the tests, and neither steroids nor antihistamines were administered for at least 4 weeks. All subjects signed informed consent statements for all tests.

Test protocol

Data about occupational exposure time, smoking history, symptoms, and the time between the onset of occupational exposure to enzymes and the occurrence of symptoms (sensitization period), together with a personal or family history of bronchitis, asthma, or any type of allergic diseases, were recorded with a questionnaire. Erythrocyte sedimentation rate, white and red blood cell count, total serum IgE, EAST with enzymes, electrocardiogram, roentgenogram of the chest, spirometry, and skin test results with enzymes and common

allergens were recorded from all subjects. Control subjects did not undergo BPT. EAST-inhibition experiments were performed with sera of two workers sensitized to enzymes and with that of control subjects. All sera were examined by immunoprint with amylase, but not with other enzymes.

Skin prick tests

Modified skin prick tests were performed on the volar part of the forearm with 18 common allergens (grass and tree pollen, animal dander, and house-dust mites) and with 19 molds, soy bean, rice flour beetle, and wheat and rye flour (Allergopharma, Reinbek, Germany, and Bencard, Neuss, Germany). Tests were also performed with enzymes handled by the subjects at work (Röhm, Darmstadt, Germany)). We used the raw materials provided by the plant and did not adjust to a certain protein concentration. The protein concentrations of the 100 mg/ml enzyme extracts were measured; proteases from animal (4.7 mg/ml), plant (23.8 mg/ml), and *Aspergillus niger* (11.8 mg/ml) and bacterial origin (3.2 mg/ml) were assayed, as well as amylase from *Aspergillus oryzae* (44.9 mg/ml), pectinase (11.3 mg/ml), cellulase from *Aspergillus niger* (13.3 mg/ml), amyloglucosidase from *Aspergillus niger* (25.6 mg/ml), and hemicellulase (36.4 mg/ml). Tests were performed as dose-response curves with concentrations of 1, 10, 20, 50, and 100 mg/ml. Enzymes were dissolved in 0.9% NaCl for 16 hours, and phenol 0.4% and glycerol 50% were added. Extracts for all tests (with the exception of IEF) were filtered through filter paper and a bacterial filter. The stock solution was stored at 4° C for not longer than 4 weeks. Dilutions of the stock solutions were performed with NaCl 0.9%, phenol 0.4%, and glycerol 50%. Control tests were done with NaCl 0.9% and histamine 1 mg/ml. Tests were performed in duplicate on the ventral forearms with number 20 needles. The histamine equivalent concentration (i.e., the enzyme concentration that caused a wheal of the size of the histamine control or greater) was calculated with a Hewlett Packard 41C calculator (Hewlett Packard, Frankfurt, Germany) from individual dose-response curves. A positive skin test result was defined as a wheal diameter greater than or equal to the histamine control with the 10 mg/ml enzyme extract or the standard allergen solution.

Lung function and BPT

Lung function tests (FEV_1 , inspiratory vital capacity, specific airway resistance [sR_{aw}]) were performed with a volume-constant body plethysmograph (Jaeger, Würzburg, Germany). Measuring conditions were chosen as recommended by Quanjer.⁴⁰ Bronchial hyperresponsiveness was assessed with methacholine (Aldrich, Steinheim, Germany No. A1.800-1). A stock solution of 50 mg/ml in 0.9% NaCl was used. It was stored at 4° C for not longer than 2 weeks and dilutions were done freshly. All tests were performed between 9 and 12 AM. The sR_{aw} was recorded 1 minute after 10 breaths

TABLE I. Anamnestic data of exposed subjects ($n = 42$) and control subjects ($n = 20$)

	Symptoms	Smoking	Pack-years	BPT positive (decrease in $sG_{aw} \geq 50\%$)	BPT negative (decrease in $sG_{aw} < 50\%$)
Subjects' workplace					
Actually exposed					
Production	A, R, C	Smo	30	Papain	Amylase, hemicellulase
Production	A, R, C	Smo	50	Pectinase	Amylase, hemicellulase
Production	A, R, C	ExS	8	Amylase	ND
Production	A	ExS	63	Amylase	Hemicellulase
Production	A, R	Smo	14	Cellulase	Amylase, hemicellulase
Laboratory	A, R	NoS	0	Amyloglycosidase	ND
Previously exposed					
Production	A, R, C	ExS	7	Bacterial protease	Amylase
Laboratory	A, R, C	NoS	0	Amylase	ND
Confection	A, R, S	ExS	6	Amylase	Hemicellulase
Workman	A, R, S	ExS	1	Amylase	ND
Laboratory	A, R, C, S	ExS	3	Cellulase	Amylase
Production	A, R	Smo	15	Hemicellulase	Amylase
Production	A, C, S	ExS	7	Hemicellulase	Amylase
Total ($n = 13$)	A: 13, R: 11, C: 7, S: 4	Smo: 4, ExS: 7, NoS: 2	7 (0-63)	13 Tests (7 enzymes)	11 Tests (2 enzymes)
BPT negative ($n = 29$)	A: 16, R: 19, C: 11, S: 7	Smo: 11, ExS: 11, NoS: 7	10 (0-50)	—	58 Tests (9 enzymes)
Atopic controls ($n = 10$)	A: 7, R: 9, C: 4	Smo: 2, ExS: 1, NoS: 7	ND	ND	ND
Nonatopic controls ($n = 10$)		Smo: 1, ExS: 0, NoS: 9	ND	ND	ND

A, Asthma; R, rhinitis; C, conjunctivitis; Smo, smoker; ExS, ex-smoker; ND, not done; NoS, nonsmoker; S, skin lesions.

of each methacholine concentration (0.5, 1, 5, and 25 mg/ml). Inhalation of methacholine was done with a jet nebulizer (Heyer, Bad Ems, Germany). An estimate of the amount of aerosol released from the nebulizer was made by weighing the aerosol reservoir. These measurements were used for the calculation of provocation doses.⁴¹ PD_{50} was calculated from the linear curve between the two data points of the cumulative dose-response curve adjacent to the 50% fall in specific airway conductance (sG_{aw}) by interpolation with a Hewlett Packard 41C calculator. Bronchial hyperresponsiveness was defined as a 50% or greater fall in sG_{aw} with a provocation dose of less than 1 mg.

The same enzymes as used in the skin tests were used for BPT. The number of tests is given in Table I. The enzyme was chosen because of exposure and skin test results. In the case of a positive reaction with the first enzyme, a second BPT was performed, primarily with amylase or hemicellulase. These were also the main enzymes used in the subjects with negative skin test results. Extracts contained 0.4% phenol, but no glycerol. The inhalation procedure was the same as for methacholine. Inhalation of the enzymes was per-

formed with 10 breaths of freshly prepared 10-fold dilutions of the 100 mg/ml solutions in intervals of 10 minutes. In accordance with nonspecific hyperresponsiveness and anamnestic data, BPT started with enzyme concentrations of 10^{-4} to 5 mg/ml. Individual dose-response curves were obtained and $PD_{50}sG_{aw}$ was calculated as for methacholine. A positive reaction was defined as a fall in sG_{aw} of 50% or more with enzyme concentrations of 10 mg/ml or less (highest concentration used for inhalation). Time-response studies were performed by measuring sR_{aw} at 0, 10, 20, 30, and 60 minutes after the inhalation of the highest enzyme concentration. Peak flow measurements were recorded for 5 hours with a Mini-Wright peak flowmeter (Airmed, London, England). Measurements for longer periods were not possible because subjects left the clinic after that time.

Total serum IgE

IgE levels were measured with Phadezym PRIST (Pharmacia). Measurements were performed in duplicate according to the manufacturer's directions.

EAST

Cyanogen bromide-activated cellulose discs were provided by Allergopharma. Enzymes were diluted to a concentration of 20 µg/ml with incubation buffer (0.05 mol/L phosphate-buffered saline, pH 7.4, 0.05% NaN₃, 0.9% NaCl, and 0.3% human serum albumin). Ten discs per milliliter were incubated for 16 hours at 4° C in the incubation buffer. Enzyme discs were washed twice with 0.1 mol/L NaHCO₃ and incubated in 0.1 mol/L NaHCO₃ and 0.05 mol/L ethanolamine for 3 hours at 4° C. Then they were washed once with 0.1 mol/L NaHCO₃ and three times with sodium acetate buffer 0.1 mol/L, pH 4.0. They were stored in incubation buffer at -20° C for not longer than 4 weeks. Further analysis was performed with Phadezym RAST according to the manufacturer's directions. Results were expressed in Phadebas RAST units (PRU). A positive reaction was defined as 0.35 PRU or more (RAST class I or higher).

EAST inhibition

We performed inhibition experiments with the sera of two clinically sensitized workers with high binding in the EAST. One subject had cutaneous reactivity to papain, but not to amylase. The other was sensitized to both amylase and papain. In addition, two control sera with high titers of specific IgE to nonoccupational allergens were used: the first subject was highly sensitized to grass and birch pollen and not to house-dust mite and the second was allergic to mites without pollen sensitization. Two enzymes (papain and amylase), grass pollen, and *Dermatophagoides pteronyssinus* (Pharmacia) coupled to paper discs were used as solid phase. The same allergens were used as inhibitors; grass pollen and house-dust mite extracts were provided by Allergopharma. Fifty microliters of serum was incubated for 30 minutes at 20° C with 50 µl of diluted allergen (1, 0.1, 0.001, and 0.0001 mg/ml) or NaCl 0.9 mg/ml and NaN₃ 0.05 mg/ml. EASTs were performed as already described except that the serum samples preincubated with inhibitor were added to the tubes instead of serum alone. Percent inhibition was calculated as follows:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{Binding with inhibitor} \cdot 100}{\text{Binding without inhibitor}} \right)$$

Protein measurements

Protein was measured according to a modified Lowry method.⁴² Briefly, 99 ml of Lowry 1 solution (20 mg/ml Na₂CO₃ and 0.27 mg/ml sodium-potassium tartrate in 0.1N NaOH) was mixed with 1 ml of Lowry 2 solution (1.56 mg/ml CuSO₄). Samples (100 µl) were incubated with 10 µl of 2 mg/ml sodium desoxycholate (all reagents from Merck, Darmstadt, Germany) for 15 minutes at 20° C. One milliliter of cold trichloroacetic acid (Boehringer, Mannheim, Germany) was added, and after incubation for 15 minutes the samples were centrifuged at 8000 g for 45 minutes at 8° C. The samples

were decanted and 1 ml of the mixture of Lowry 1 and 2 solution was added. After a washing step the samples were incubated with 100 µl of Lowry 3 solution (Folin Ciocalteu reagent, Merck) for 30 minutes at 20° C in the dark. The extinction was measured at 740 nm.

Immunoprint

IEF was performed with a modified method according to Haas et al.⁴³ with the use of agarose thin-layer gels of 0.23-mm thickness that contained 0.9% agarose IEF (Pharmacia, No. 17-0468-01), 12% d(-)-sorbitol (Merck, No. 7759), 3% polyampholytes for IEF, pH 3 to 10, and 1% Servalytes, pH 4 to 6 (Serva, Heidelberg, Germany, Nos. 42940 and 42904). The cathode strip was soaked in 1 mol/L NaOH and the anode strip (Electro-focusing strips, Pharmacia, No. 18-1004-40) in 0.05 mol/L H₂SO₄.⁴⁴ Amylase was diluted to 0.75% and passed twice through a folded filter (Schleicher & Schüll, Dassel, Germany, No. 311645). Ten-microliter aliquots of the sample were applied per 5-mm slot. After completion of the electrophoretic separation one part of the gel was stained in Coomassie blue solution (0.5% CuSO₄, 0.05% Coomassie Brilliant Blue G-250 [Serva, No. 42655], 0.05% Crocein Scarlet [Serva, No. 17598], 25% isopropyl alcohol, 10% acetic acid) and destained under visual guidance for a few seconds in 18% isopropyl alcohol and 9% acetic acid. For immunoprint analysis the focused enzymes were transferred to the nitrocellulose membrane (NCM, 0.45 µm pore size, Bio-Rad, Munich, Germany No. 162-0115) by means of a modified print technique.⁴⁵ Then the NCM was incubated at 20° C for 1 hour either in TRIS buffered saline (TBS: 100 mmol/L TRIS/HCl, 100 mmol/L NaCl, 2.5 mmol/L MgCl₂; 0.02% NaN₃) with Tween 20 (Merck, No. 822184⁴⁶) (TBS-Tw) for subsequent India ink staining or for subsequent antibody detection in TBS-Tw with non-fat dry milk blocker (TBS-Tw-M: Bio-Rad, Munich, Germany, No. 170-6404). A part of the NCM, which was saturated in TBS-Tw, was incubated for 16 hours in 0.1% Fount India ink (Pelikan, Hannover, Germany, No. 518-22 A 143) and 0.75% acetic acid in TBS-Tw. Antibody detection was performed with a modified method according to Leary et al.⁴⁷ and Reese et al.⁴⁸ The NCM strips were incubated with 4 ml of diluted sera (1:40 in TBS-Tw-M) for 16 hours. Then they were incubated with 4 ml of a 1:2000 dilution (TBS-Tw-M) of biotin-labeled ε-chain specific goat antihuman IgE (Medac, Hamburg, Germany, No. H 15715) for 3 hours. The strips were then incubated with a 1:800 dilution (TBS-Tw-M) of alkaline phosphatase-conjugated streptavidine (Dianova, Hamburg, No. 016050084) for 15 minutes. They were stained with chromogen substrate solution (0.016% 5-bromo-4-chloro-3-indolyl phosphate, Serva, No. 15257, and 0.033% nitro blue tetrazolium chloride, Serva, No. 30550, diluted with the pH 9.5 buffer) at 37° C for 10 minutes in the dark. A band intensity scale, ranging from 0 to 4 according to Bernstein et al.⁴⁹ was used to semiquantitate the antibody

responses. Because there was one main band at pH 3.4, only this band was considered.

Statistical analysis

Mean values, if not indicated otherwise, are medians. The time-response curve for BPT was drawn as a Box-and-Whisker plot with medians, maximal and minimal values, and upper and lower quartiles. Differences between subjects were compared by the two-tailed U test (Wilcoxon-Mann-Whitney). For both tests a 5% level of significance was assumed. For the calculation of sensitivity, specificity, and positive and negative predictive values we used the results of BPT of exposed workers or ex-workers; tests of control subjects were not considered.

RESULTS

Actually exposed workers

Six (19%) of 31 workers actually exposed to allergens who had work-related symptoms showed a fall of sG_{aw} of at least 50% after inhalation of the enzyme. One person who did not inhale an enzyme because of obstructive airways disease had no signs of allergy. Another subject was allergic to both rice flour beetle (positive results of BPT) and enzymes as evidenced by skin tests and EAST. The remaining 23 subjects did not have a 50% fall in sG_{aw} after inhalation of enzymes. Thus the prevalence of occupational asthma among actually exposed subjects with symptoms was 22.6% (7 of 31).

Ex-workers

Seven (46.7%) of 15 formerly exposed workers had positive BPT results in response to at least one enzyme. Six subjects showed negative BPT results with enzymes: one of these had a positive EAST (without a cutaneous reaction) and five were hyperreactive to methacholine. Two ex-workers did not undergo BPT with enzymes: one worker had clinical signs of hypersensitivity pneumonitis and the second, showing airways obstruction, was considered allergic to enzymes as evidenced by positive results of skin tests and EAST to various enzymes. Because the time interval between the end of exposure and the actual study was longer in subjects with a positive BPT result (58 months, range 12 to 120 months) than in those with a negative result (34 months, range 1 to 84 months), false-negative BPT results because of longer periods without exposure were improbable. The prevalence of enzyme allergy was 53.3% (8 of 15) for workers who had been removed from enzyme exposure because of work-related symptoms.

Total

Thirteen of the 42 subjects who underwent BPT had a 50% or greater fall in sG_{aw} after inhalation of the enzyme. Eleven tests with a second enzyme showed negative results in these subjects. Positive bronchial reactions were identified with papain, bacterial protease, pectinase, amylase, cellulase, amyloglucosidase, and hemicellulase. It should be emphasized that the number of tests varied between enzymes with amylase ($n = 31$), hemicellulase ($n = 29$), and papain ($n = 9$) being the most frequently used extracts. Allergies to further occupational substances were rare: one subject allergic to enzymes was also sensitized to flours. Sensitization to occupational molds was found in one subject without enzyme allergy (positive skin test result for *Aspergillus*). Six subjects with enzyme sensitization were sensitized also to molds, almost exclusively to *Aspergillus* species. There were no differences in parameters (history, skin tests, EAST, nonspecific hyperresponsiveness) between actually exposed subjects and ex-workers.

The time-response curve of the subjects with positive BPT results showed a typical immediate reaction. Severe asthmatic reactions occurred rarely, but baseline values were not reached 1 hour after the end of the enzyme inhalation (Fig. 1, top). No dual or late reactions were observed during the 5 hours after inhalation of the highest enzyme concentration. The concentrations that elicited a 50% fall in sG_{aw} ranged from 0.001 to 5 mg/ml. The small numbers of positive BPT results did not allow a comparison of provocation doses for the different enzymes; descriptive analysis did not show any evident differences. Fourteen of 69 negative BPT (11 tests in 13 BPT-positive subjects and 58 tests in 29 BPT-negative subjects) showed an equivocal reaction (fall in sG_{aw} 30% or greater, but less than 50%). Equivocal reactions were observed with amylase and hemicellulase, but not with other enzymes, and occurred with one exception in persons with nonspecific bronchial hyperresponsiveness. Five equivocal test results occurred in subjects considered allergic to a further enzyme, leaving nine equivocal test results in six subjects considered not allergic to enzymes, all with a negative EAST and one with a positive skin test result. Moderate clinical symptoms after inhalation of the enzyme were recorded in three subjects with equivocal test results and no signs of sensitization. Thus irritant effects of the aqueous extracts were not important.

Most affected individuals were production

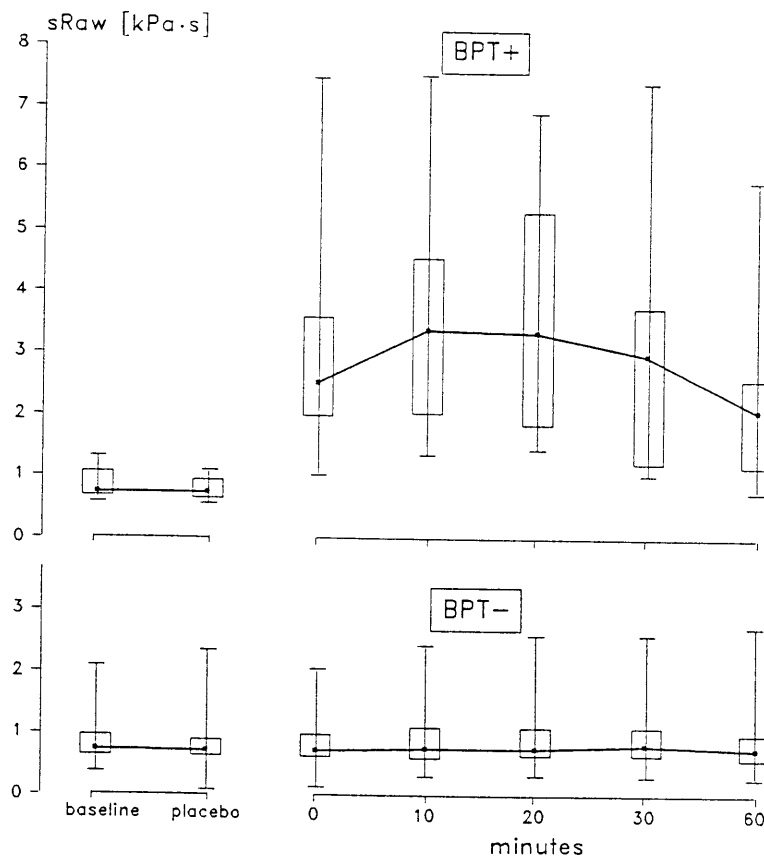


FIG. 1. Time-response curve of positive ($n = 13$, top) and negative ($n = 69$, bottom) BPT results with seven enzymes.

workers (Table I). All had asthma, 87% had rhinitis, 46% had conjunctivitis, and 31% had skin lesions. Of workers with a positive BPT result 85% were smokers or ex-smokers, as were 72% of those with negative BPT results. When smoking habits were expressed as pack-years, there was no difference between the groups. Positive BPT results occurred with seven different enzymes, primarily starch-cleaving enzymes. Cutaneous sensitizations to more than one enzyme were found in 10 subjects. Interestingly, all these subjects had sensitizations to both proteases and starch-cleaving enzymes.

Occupational exposure time was similar in both groups (100 months, range 19 to 370 months, in subjects with positive BPT results versus 120 months, range 9 to 437 months in those with negative BPT results). The period between start of exposure and first symptoms was the same (59 months, range 1 to 384, versus 61 months, range 1 to 360).

Six subjects with positive BPT results (46%), but only three with negative BPT results (10%),

gave a history of allergy in their family. A history of allergy before employment was reported by two subjects with positive BPT results (15%) and by three with negative BPT results (10%). In both groups we identified eight subjects with positive results on skin tests to common allergens. Smokers and ex-smokers were more frequent among those with positive BPT results (85% versus 76%). Thus there was a trend for atopic subjects and smokers to acquire enzyme allergy more often.

Bronchial hyperresponsiveness was present in 12 of the 13 subjects with positive BPT results and in 17 of the 29 with negative BPT results (Table II). Allergy to nonoccupational allergens as the cause of hyperresponsiveness was detectable in only five subjects with negative BPT results. In four subjects we found positive skin reactions to enzymes that were not used in BPT. False-negative BPT results might be suspected in these subjects; however, EAST was found positive in only one of them. Eight subjects had bronchial hyperresponsiveness without any detectable cause.

TABLE II. Synopsis of parameters

	PD ₅₀ sG _{aw} enzymes (mg)	PD ₅₀ sG _{aw} methacholine (mg)	SPT enzymes (+/-)	SPT standard (+/-)	EAST enzymes (+/-)	Total IgE (U/ml)
Results in subjects with positive BPT						
Actually exposed						
Papain	0.001	0.660	+	-	+	56
Pectinase	4.716	0.460	+	+	-	152
Amylase	0.004	1.229	+	+	+	54
Amylase	1.359	0.241	+	-	-	187
Cellulase	0.095	0.183	+	-	-	25
Amyloglycosidase	0.067	0.641	+	-	+	34
Previously exposed						
Bacterial protease	0.396	0.043	+	+	+	1000
Amylase	0.090	0.041	+	+	-	74
Amylase	1.709	0.878	+	+	-	497
Amylase	0.063	0.281	+	+	+	1000
Cellulase	0.013	0.445	+	+	+	384
Hemicellulase	8.702	0.516	+	+	+	28
Hemicellulase	0.328	0.145	+	-	+	172
Total (n = 13) positive test results*	13	12	13	8	8	7
BPT negative (n = 29)	-	17	2	8	1	7
positive test results						
Atopic controls (n = 10)	ND	ND	0	10	4	10
positive test results						
Nonatopic controls (n = 10)	ND	ND	0	0	0	1
positive test results						

Note: Numbers given indicate the number of tests, not the number of subjects.

SPT, Skin prick tests; ND, not done.

*Positivity criteria: PD₅₀sG_{aw} (methacholine): 1 mg or less; skin prick tests enzymes greater than or equal to histamine control with the 10 mg/ml enzyme extract; skin prick tests standard: greater than or equal to histamine control; EAST: 0.35 PRU or greater; IgE: 100 U/ml or more.

Skin prick tests

Cutaneous sensitizations were demonstrated with all enzymes (Fig. 2) and occurred mostly as sensitizations to more than one enzyme (10 subjects). One subject was allergic to all enzymes. All subjects with positive BPT results had a positive skin reaction with the corresponding enzyme (Table II). When all BPT (n = 82) were considered, we calculated a 100% sensitivity and a 93% specificity of skin testing with a maximal concentration of 10 mg/ml (Table III). None of the atopic and nonatopic control subjects had cutaneous reactivity to enzymes.

Calculating our results with skin prick tests (at a concentration of 10 mg/ml) used as the gold standard, BPT were 100% specific and 72.2% sensitive: five of 82 BPT results were negative despite a positive skin test result with the corresponding enzyme. False-negative BPT results

could be suspected in three subjects who were allergic to further enzymes; however, only one person also had a positive EAST with the corresponding enzyme. The remaining two subjects had a positive skin test result with one enzyme without any other positive test result. Thus false-negative BPT results were probably seldom, although this could not be excluded in a small number of tests (as was the case with false-positive skin tests when BPT was used as standard).

Total serum IgE

Total serum IgE level was elevated in 54% of subjects with positive BPT results and in 24% of those with negative BPT results. The difference between the groups was statistically significant (p < 0.05). Both sensitivity and specificity were too low to use total serum IgE as a diagnostic tool (Table II).

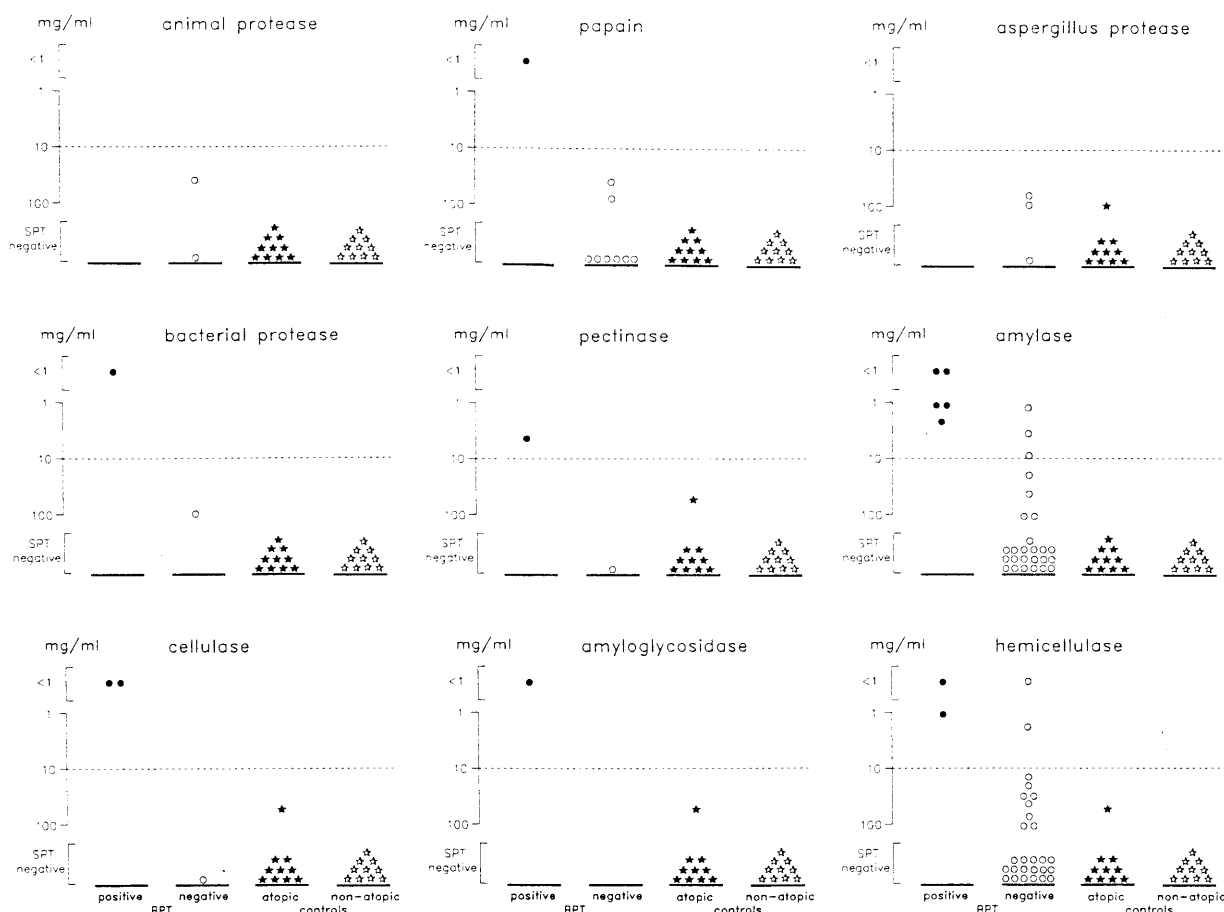


FIG. 2. Skin prick test results with enzymes for subjects who underwent BPT with corresponding enzyme and for control subjects. X-axis represents enzyme concentration for wheal diameter of size of histamine control. BPT-positive: fall in sG_{aw} 50% or greater, $n = 13$ tests, solid dots; BPT-negative: fall in sG_{aw} less than 50%, 69 tests, open dots; atopic controls: $n = 10$, solid stars; nonatopic controls: $n = 10$, open stars. SPT, Skin prick test.

TABLE III. Dose-response relationship of skin prick tests

Enzyme concentration (mg/ml)	No. BPT positive ($n = 13$)	No. BPT negative ($n = 69$)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
100	13	23	100	67	36	100
50	13	14	100	79	48	100
20	13	8	100	88	62	100
10	13	5	100	93	72	100
1	9	3	70	96	75	96

Results of BPT and the skin test results with the corresponding enzymes were used for calculation.

EAST and EAST inhibition

A positive EAST result could be demonstrated with seven enzymes (Fig. 3). Eight subjects with positive BPT results had a positive EAST result with the corresponding enzyme. Among 69 nega-

tive BPT results we found only three positive EAST results with the corresponding enzyme. Two of the false-positive EAST results occurred in subjects who had a positive BPT result with a second enzyme (one had a positive EAST result

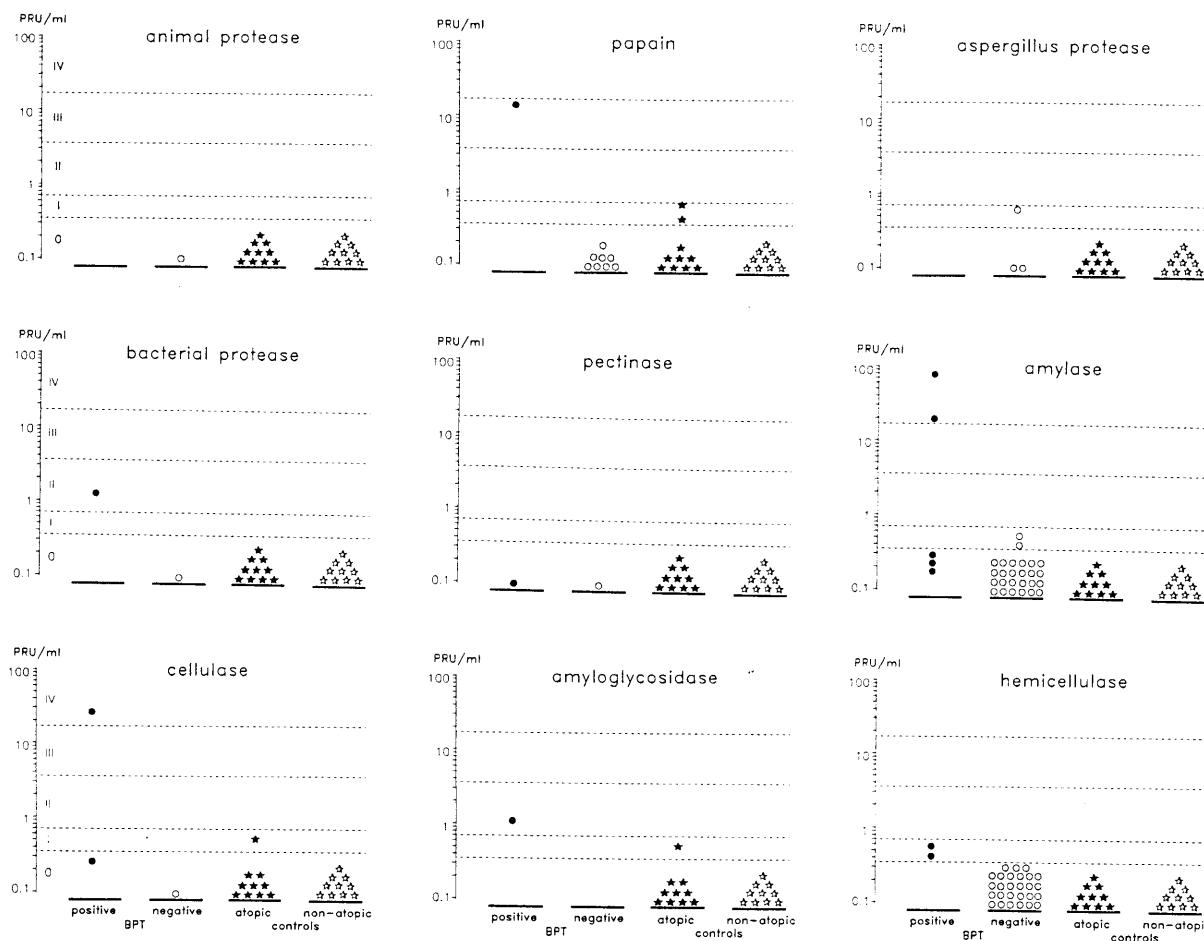


FIG. 3. EAST results with enzymes (groups and enzymes as in Fig. 2). RAST classes were drawn with dotted lines.

and a positive skin test result). We calculated a sensitivity of only 62% and a specificity of 96%. Positive EAST results in two control subjects were found with papain (two tests), cellulase, and amyloglycosidase. These results were found exclusively in the atopic control group and these subjects were classified as being in RAST class I. Both persons were sensitized to birch pollen; the second person was also sensitive to grass pollen. Five control subjects had pollen sensitizations without specific IgE to enzymes. Thus low binding in the EAST with enzymes should be interpreted with caution in individuals sensitized to pollen.

For inhibition experiments, two samples of sera of workers allergic to enzymes were used (Fig. 4). In the homologous systems there were 70% and 69% inhibition rates and cross-reactivity was not present between enzymes. Enzymes as inhibitors did not affect inhibition in the mite or grass pollen systems with the exception that papain in its highest concentration inhibited binding to the

grass pollen disc. This effect could be because of cross-reactivity of papain and grass pollen, which has also been described by others.²¹

Immunoprint

Immunoprint assay was performed only with amylase. A total of 31 BPT were performed with this enzyme; five tests were positive. These results showed very dark bandings in three cases; the other results were considered dark and moderate. Eighteen of the 26 subjects with a negative BPT result with amylase showed bandings (Fig. 5). The arithmetic mean intensity score was 3.4 for subjects with a positive BPT result with amylase ($n = 5$) and 1.58 for those with negative BPT results ($n = 26$). Among the 18 subjects with negative BPT results with bandings were six persons who had a positive BPT result with a second enzyme (the arithmetic mean score of these six persons was 2.33). All results for control subjects, atopic and nonatopic, showed no bandings. Thus immuno-

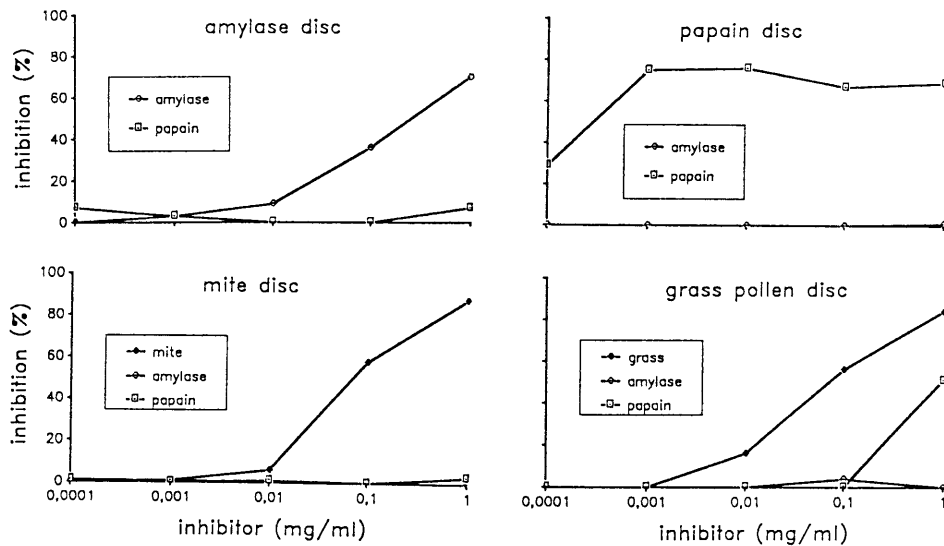


FIG. 4. EAST inhibition tests. Allergen coupled to cellulose discs is given for enzymes (*top graphs*) and control antigens (*bottom graphs*). For each of these allergens, serum of a subject allergic to this substance was used. Inhibitors are given in the boxes of each graph.

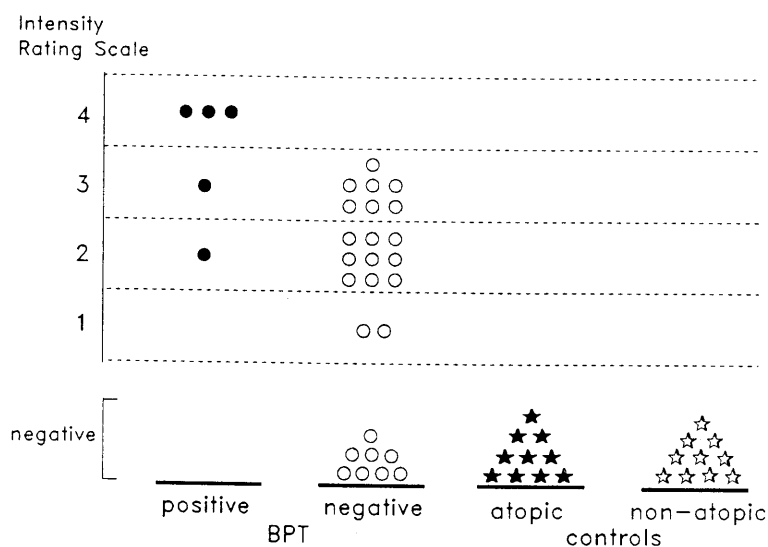


FIG. 5. Immunoprint analysis with amylase (groups as in Fig. 2). Rating scale was graded 1 (weak), 2 (moderate), 3 (dark), and 4 (very dark). Only one main band at pH 3.4 was considered as quantitatively most important.

print assays gave positive reactions in exposed subjects without further signs of sensitization, but not in control subjects without exposure.

DISCUSSION

It is difficult to interpret epidemiologic studies without standardization of the diagnostic techniques. It is obvious from several cross-sectional studies in enzyme-manufacturing plants that there were more subjects with symptoms than subjects with sensitizations as assessed by means of skin

tests or RAST.^{4, 8} This difference may be a result of inappropriate methods, further unknown specific allergens, or toxic or irritant effects of enzyme dust. Because BPT with enzymes have never been reported to have positive results in control subjects, we established BPT as the gold standard and evaluated sensitivity and specificity of skin prick tests, EAST, and immunoprint.

Among 46 enzyme-exposed subjects with symptoms we identified a total of 15 (32.6%) with enzyme allergy (13 positive BPT results and 2

positive skin test results in persons who were not challenged). Among 31 actually exposed subjects, enzyme allergy was present in 22.6%.

With the enzyme extracts used in this study we found almost no nonspecific skin test reactions at a concentration of 10 mg/ml (specificity of 93%, no positive results in control subjects). Specificity was poorer with higher concentrations; similar results were reported in an earlier study.³² We found a 100% sensitivity of skin prick tests with nonstandardized enzyme extracts. The high sensitivity is remarkable considering the protein content varied widely between the extracts. Thus skin prick tests are the diagnostic tool of first choice for suspected enzyme allergy.

Sensitivity of EAST was poor (62%). We did not block the enzymatic activity, thus interference of the enzymes with the assay cannot completely be ruled out,³⁷ although preincubation of serum from subjects allergic to mite or pollen with enzymes did not inhibit binding. Specificity of the EAST was high (96%), however some control subjects allergic to pollen also had positive reactions. Cross-reactivity of papain and grass pollen can be assumed by the results of the EAST inhibition experiments and by studies of others.²¹ Interestingly, two of four positive EAST results in control subjects were found with starch-cleaving enzymes (the two others with papain), which indicated cross-reactivity between enzymes and pollen antigens. Although we used no pure enzyme preparations, the sensitization to more than one enzyme and the sensitization to both proteolytic and starch-cleaving enzymes in most subjects is highly suggestive of cross-reactivity between enzymes, independent of their enzymatic activity.

Immunoprint studies were performed with the amylase extract. We did not detect any bandings in studies in control subjects, but even when only dark and very dark bandings were used, specificity of this method was poor (73%). Sensitivity was higher, but of the five positive results, one had only moderate bandings. It would be of interest to do longitudinal surveys to evaluate the clinical implication of these findings, that is, to find out whether "sensitization" as assessed by means of immunoprint is more than a marker of exposure. The difference in the mean intensity score between groups might be caused by a higher exposure of those with positive results.

Alternatively, with the use of skin prick tests (at a concentration of 10 mg/ml) as the gold standard, BPT were 100% specific, but only 72.2% sensitive. The low sensitivity was because of five positive

skin test results in subjects with a negative BPT result. A false-negative BPT result was suspected in one case because of a clearly positive EAST result and an equivocal BPT result with the enzyme. There were no further data that supported the hypothesis of false-negative BPT results in the remaining subjects. Because all these subjects showed bronchial hyperresponsiveness, there was no better prediction of enzyme allergy with the use of methacholine challenge and skin test instead of skin test alone.

In this highly selected group, equivocal reactions (defined as fall in sG_{aw} of more than 30% and less than 50%) occurred infrequently in subjects without positive results of skin test or EAST with enzymes. Our data give indirect evidence that inhalation of the aqueous extracts used in this study does not induce relevant immediate bronchoconstriction without sensitization.

In a recent study, only 21 of 47 workers in a pharmaceutical setting who had symptoms were considered sensitized to α -amylase.¹⁹ For the others sensitizations to further enzymes were hypothesized. Our study indicates that occupation-related symptoms in enzyme-processing plants are not confined exclusively to occupational sensitizations. We are convinced that further occupational allergens were not overlooked inasmuch as (1) we used materials that were handled by the subjects at work, (2) we chose the enzyme for the BPT according to both anamnestic data and skin test results, (3) we used a large spectrum of further, possibly occupational allergens as flours, molds, insects, mites, and so on, and (4) only one subject (of 105 actually exposed subjects) was found to have skin sensitization to enzymes when 30 enzymes were used in the cross-sectional study, as compared with nine enzymes in the present study.

Bronchial hyperresponsiveness to methacholine was common in our study population, with 29 (69%) of 42 tests considered to be positive. Thirteen subjects with bronchial hyperresponsiveness, but without occupational sensitizations, reported work-related symptoms. It is impossible to conclude from our data whether there might be a certain potential for enzyme-dust exposure to induce nonspecific bronchial hyperresponsiveness without sensitization. This has to be addressed by a longitudinal controlled survey. However, irritant effects of enzyme dust have to be considered inasmuch as about one third of workers with work-related symptoms had bronchial hyperresponsiveness without sensitization to occupational allergens.

We have provided data that will allow a better interpretation of symptoms, skin tests, EAST, and immunoprint assays in subjects exposed to various enzymes. We suggest that studies on occupation-related asthma should address the problem of a valid standard. If there are discrepancies between symptoms and results of tests, BPT should be considered to standardize further techniques. For enzyme allergy both BPT and skin prick test were appropriate diagnostic tests.

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