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# New occupational allergen in a pharmaceutical industry: serratial peptidase and lysozyme chloride

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**Background:** Serratial peptidase and lysozyme are often used as anti-inflammatory agents. There have been very few documented cases of occupational allergy caused by these substances. We report a case of a pharmaceutical industry worker who developed occupational asthma and rhinitis caused by both serratial peptidase and lysozyme chloride.

**Objective:** It is important to alert physicians to the possibility of occupational asthma when dealing with workers in the pharmaceutical industry.

**Method and Result:** The patient had strong positive responses to peptidase and lysozyme extracts on skin-prick tests. Bronchoprovocation tests showed a dual asthmatic response to peptidase and an early asthmatic response to lysozyme. Serum specific IgE antibodies to peptidase and lysozyme were detected by enzyme-linked immunosorbent assay (ELISA). In order to further characterize the allergenic component of these extracts, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting studies were also performed. More than ten components ranging from 7.3 to 83.1 kD were found in peptidase extracts, and two IgE binding components (67, 10.9kD) were detected within the lysozyme extracts.

**Conclusion:** These findings suggest that inhalation of peptidase and lysozyme can induce IgE-mediated bronchoconstrictions in an exposed worker.

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## INTRODUCTION

There have been many reports of occupational asthma induced by biologic enzymes inhaled in the course of work in the pharmaceutical industry.<sup>1-7</sup> An IgE-mediated mechanism has been implicated in most cases.<sup>2-7</sup> To the best of our knowledge, however, occupational asthma caused by two different enzymes, peptidase and lysozyme, has not been reported in a pharmaceutical industry worker. The peptidase (60 kD) in this study is a proteolytic enzyme derived from *Serratia* spp. and a drug mainly composed of this enzyme has been used as an anti-inflammatory agent. The lysozyme (lysozyme chlo-

ride, 14.5 kD) is also used as anti-inflammatory agent in this country. We report herein a case of occupational rhinitis and asthma, which is the first case of dual sensitization to peptidase and lysozyme powder in a single worker handling these powders in a pharmaceutical company. The diagnosis was confirmed by bronchoprovocation tests. Furthermore, serum specific IgE antibodies were detected and their IgE binding components were identified.

## SUBJECTS AND METHODS

### Case Summary

The patient was a 23-year-old female worker, who was admitted to the Allergy Clinic of Ajou University Hospital, Suwon, Korea. Three years earlier, she had been employed at a pharmaceutical company and her job responsibilities included handling drug powders on a daily basis for the man-

ufacture of tablets. She complained of profuse rhinorrhea and sneezing over the previous 26 months, and she had started to cough and experience shortness of breath in the last year. These symptoms were more severe at night after handling lysozyme and peptidase powders during the day, but improved when she was on vacation. On admission, physical examination showed clear breathing sound without wheezing.

She was a non-smoker and a skin prick test survey revealed negative immediate and late responses to 80 common inhalant allergens (Bencard, Bedford, UK), suggesting non-atopic status. Immediate and late skin responses were observed with peptidase and lysozyme extracts. Not one among five atopic and five non-atopic controls had a positive response to these extracts. Total IgE level by paper radioimmunosorbent test was 877 IU/mL. The total eosinophil count was 1,365/mm<sup>3</sup>. A chest radiography revealed no abnormal findings.

Methacholine bronchial challenge test showed a positive response at 2.5 mg/mL without significant change in the subsequent methacholine bronchial challenge test administered after the bronchoprovocation test. She gave written informed consent as regulated by Ajou University Hospital, Suwon, Korea.

### Preparation of Peptidase and Lysozyme Extracts

Peptidase and lysozyme powders were obtained from the patient's workplace. They were extracted with phosphate-buffered saline [(PBS, pH 7.5), 1:5 wt/vol] at 4°C for one hour. The supernatants were used for the bronchoprovocation tests at a concentration of

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1:10 wt/vol For the skin prick test, the 1:5 wt/vol extracts were mixed with an equal amount of sterile glycerine.

Some of the supernatant was dialyzed (cut-off molecular weight was 6,000 D) against 4 L of distilled water at 4 °C for 48 hours, and lyophilized at -70 °C.

#### *Bronchoprovocation Test with Peptidase and Lysozyme*

Airway responsiveness to methacholine was tested according to the method described previously.<sup>8</sup> Bronchoprovocation tests were performed according to standard procedure in occupational asthma studies.<sup>9</sup> Normal saline was administered from a nebulizer 646 connected to a dosimeter (Devilbiss Co; USA). The subject was asked to breathe the aerosol for two minutes with tidal breathing. This was followed by antigen extracts at concentrations ranging from 1:1000 wt/vol to 1:10 wt/vol. The pulmonary function test including forced expiratory volume in one second (FEV<sub>1</sub>) and FEF<sub>25-75</sub> were measured with a spirometer (Chest, Japan) before and ten minutes after each inhalation. Then, the FEV<sub>1</sub> and FEF<sub>25-75</sub> were measured every ten minutes during the first hour, and pulmonary function tests were performed every hour for seven hours after the challenge.

#### *ELISA for Specific IgE Antibodies to Peptidase and Lysozyme Extracts*

The presence of specific IgE antibodies to peptidase and lysozyme were determined by ELISA. Microtitre plates (Dynatech, USA) were first coated with 100 µL of peptidase (5 µg/well) and lysozyme (12.5 µg/mL) extracts, respectively, and left at 4 °C overnight. Each well was washed three times with 0.05% Tween phosphate buffered saline (PBS-T) and the remaining binding sites were blocked by incubation with 250 µL of 10% newborn calf serum for one hour at room temperature. The wells were then incubated for two hours at room temperature with 100 µL of either the patient's serum (undiluted) or control sera from ten patients who showed

negative skin prick test responses to common inhaled allergens as well as to peptidase and lysozyme. After washing three times with PBS-T, 100 µL of 1:1000 vol/vol biotin-labeled goat anti-human IgE antibody (Sigma Co. USA) was added to the wells and incubated for two hours at room temperature. The wells were then washed three times with PBS-T and incubated with 1:1000 vol/vol streptavidin-peroxidase (Sigma Co, USA) for 30 minutes before another washing step followed by incubation with 100 µL ABTS (2,2'-azino-bis-3-ethyl-benzthiazoline sulfonic acid in a citrate phosphate buffer) for ten minutes at room temperature. The reaction was stopped by the addition of 50 µL 2 M sodium azide and the absorbance was read at 410 nm by an automated microplate reader. All assays were performed in triplicate.

#### *SDS-PAGE and Immunoblotting*

Peptidase (10 mg/mL) and lysozyme (10 mg/mL) were dissolved in a buffer (0.5 M tris-HCl, pH 6.8, 2.5 mL, glycerol 2.0 mL, 10% wt/vol SDS 4.0 mL, 0.1% bromophenol blue 0.5 mL and distilled water 1.0 mL) and boiled. Ten microlitres of standard marker (6–200 kDa) (Novex, San Diego, CA, USA) and two antigen solutions were added to a Novex precast tris-glycine gradient gel (4% to 20% acrylamide) for the separation of antigens. Electrophoresis was performed with a Novex Mini-cell for 90 minutes at 125 constant voltage. The gel was fixed and stained with Coomassie brilliant blue. For immunoblotting, the gel was soaked in a transfer buffer (12 mM tris, 96 mM glycine in 20% methanol) for ten minutes. Electroblotting was carried out for 90 minutes at 200 mA using a Novex western transfer apparatus. The blotted nitrocellulose membrane was then treated with a 0.5% tris-bovine serum albumin solution for one hour to block non-specific protein binding. The patient's serum and control sera were diluted to 1:2 vol/vol with a Tween-tris buffered saline (TBS-T buffer: tris 10 mM, NaCl 150 mM, 0.05% Tween-80).

Each membrane was then incubated with the patient's and control sera for two hours at room temperature. It was washed with 0.1% triton in TBS-T for ten minutes, and followed by washes with 0.5% triton in TBS-T for ten minutes, and 0.5% NaCl in TBS-T for a further ten minutes. Biotin-conjugated anti-human IgE antibody (Serotec #G 3918, UK) was diluted to 1:200 with TBS-T and used as a second antibody solution. The membranes were incubated with the second antibody for one hour at room temperature and washed again as above. As a substrate solution, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Co, USA) was applied to the membrane until positive bands appeared. After the reaction, the membranes were washed with distilled water and observed.

## RESULTS

#### *Bronchoprovocation Test with Peptidase and Lysozyme*

As shown in Figure 1, there was no significant variation of FEV<sub>1</sub> on the control day when the subject was exposed to the diluent. A dual asthmatic reaction was noted after inhalation of 1:10 wt/vol peptidase extracts (Fig 1a). Figure 1b shows the early asthmatic reaction to the bronchoprovocation test using 1:100 wt/vol lysozyme extracts. The second methacholine bronchial challenge test done 24 hours after the peptidase-bronchoprovocation test yielded similar results in airway hyperresponsiveness to methacholine as the first test (from 2.5 to 2.3 mg/mL). When bronchial challenge tests with these extracts were performed on two house dust mite-sensitive asthmatic patients, respectively, they showed negative responses.

#### *Detection of Specific IgE Antibodies to Peptidase and Lysozyme Extracts*

Figure 2 shows the specific IgE binding to peptidase and lysozyme extracts in the patient's serum and control sera. In comparison to control results,

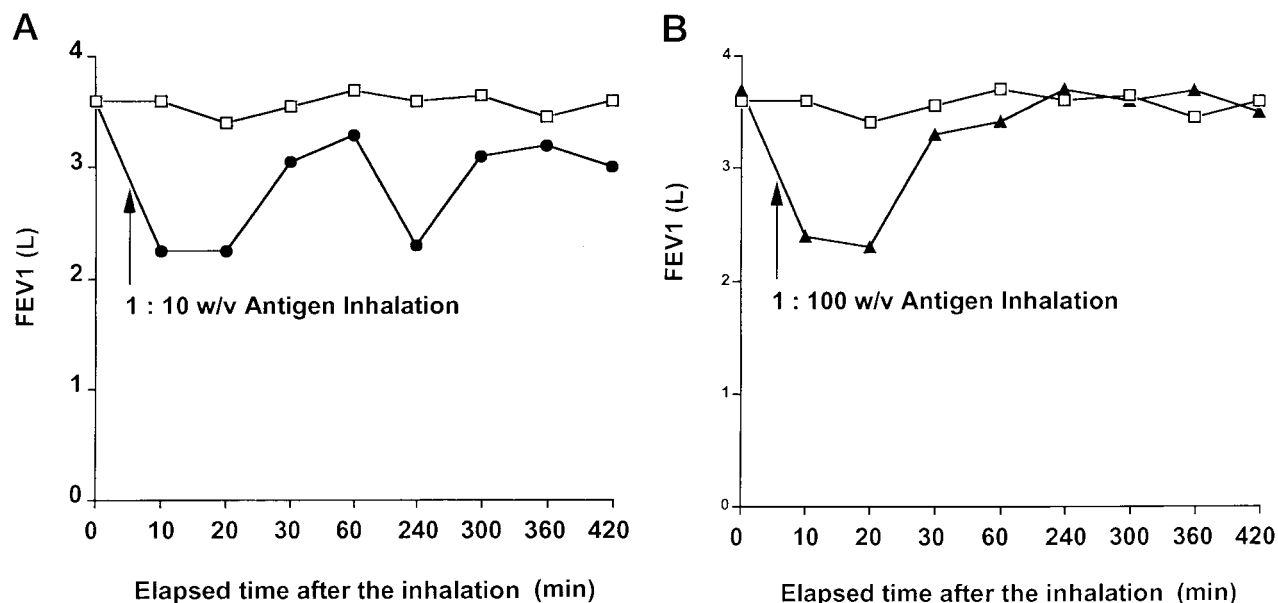


Figure 1a. The result of bronchoprovocation test with serratal peptidease extracts. -□- diluent and -●- peptidease. Figure 1b. The result of bronchoprovocation test with lysozyme extracts. -□- diluent and -▲- lysozyme.

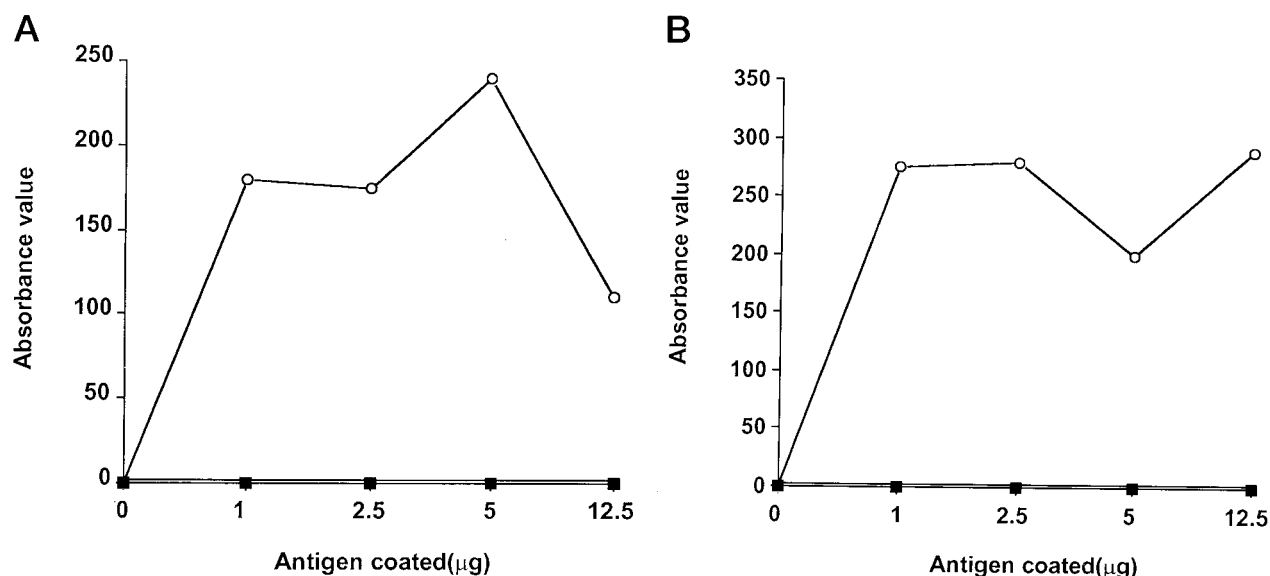


Figure 2. Specific IgE bindings to peptidease (2a) and lysozyme (2b) extracts in sera of the patient and control according to antigen concentrations. -○- patient and -■- control.

highly specific IgE bindings to peptidease (Fig 2a) and lysozyme (Fig 2b) extracts were noted in the patient's serum. Figure 3 demonstrates the inhibition results of peptidease ELISA and lysozyme ELISA with the additions of these two extracts (Fig 3a: peptidease ELISA inhibition test, Fig 3b: ly-

sozyme ELISA inhibition test).

Significant inhibitions in peptidease ELISA inhibition tests were noted with serial additions of peptidease extracts, but not with lysozyme extracts. Similarly, significant inhibitions in lysozyme ELISA inhibition tests were noted with serial addition of lysozyme

extracts, but not with peptidease extracts.

#### Gel Electrophoresis

In order to determine the protein components of peptidease and lysozyme extracts, the extracts were analyzed by a 4% to 20% gradient SDS-PAGE. Fig-

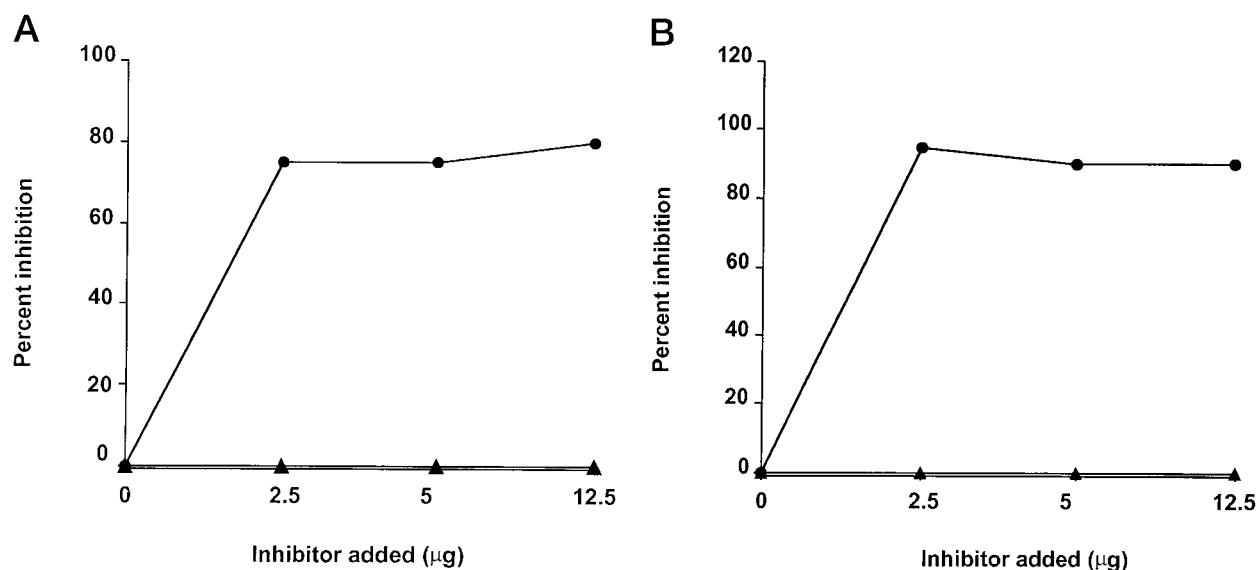


Figure 3. The inhibition results of peptidase ELISA (3a) and lysozyme ELISA (3b) with addition of peptidase and lysozyme extracts -●- peptidase and -▲- lysozyme.

ure 4a shows that the peptidase extracts resolved into nine bands ranging from 6 to 64.1 kD and the lysozyme extracts into two bands (10.9, 15.1 kD).

#### Immunoblot Analysis

Figure 4b shows the binding of specific IgE in patient's (P) and control sera (C) on a blotted nitrocellulose membrane. With peptidase extracts, there were more than ten bands bound to IgE antibody with molecular weights ranging from 7.3 to 83.1 kD.

With lysozyme extracts, two IgE binding components with molecular weights of 10.9 and 67.0 kD, respectively, were noted.

#### Discussion

While there have been many reports that biologic enzymes can cause occupational asthma in exposed workers working in the pharmaceutical industry,<sup>1-7</sup> our review of the literature failed to locate any cases of occupational asthma caused by two different enzymes, peptidase and lysozyme in a

single worker. In this study, the patient had clinical presentation of occupational asthma and rhinitis due to high-molecular-weight agents, specifically serratial peptidase and lysozyme chloride, as confirmed by bronchoprovocation tests.

The pathogenetic mechanism of occupational asthma caused by peptidase and chloride may be IgE-mediated reactions since skin prick tests with these two extracts showed immediate and late positive responses. Furthermore, IgE antibodies specific to each substance were detected by ELISA; the ELISA inhibition results suggested specificity of IgE binding to these antigens, as specific inhibitions were obtained with various dilution of homologous antigens but not with other extracts. The immunoblotting study also showed more than 10 IgE binding components within peptidase and two in lysozyme extracts. When the bronchial challenge test was performed on two house dust mite-sensitive asthmatic patients with similar levels of airway hyperresponsiveness to methacholine, they showed no response. This indicates that the bronchoconstrictive response in this study's patient was a

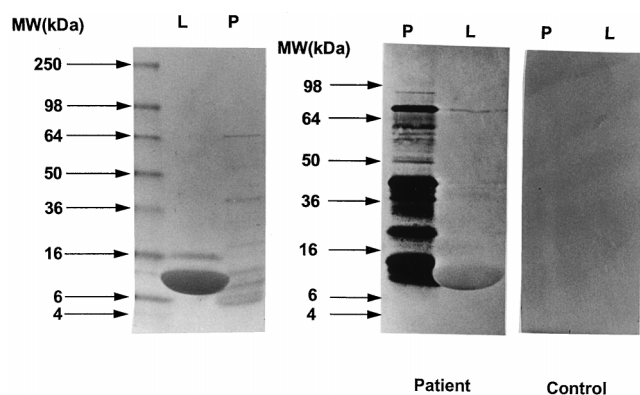


Figure 4. Four to twenty percent gradient SDS-PAGE (left) of peptidase (P) and lysozyme (L) extracts and immunoblotting (right) of peptidase and lysozyme extracts in patient (P) and control sera (C). M: marker protein.

specific one. These results suggested that both peptidase and lysozyme can induce IgE-mediated bronchoconstriction in an exposed patient.

Several authors argue the important role of atopy in the development of IgE sensitization to occupational allergens with high molecular weight.<sup>10-12</sup> In our case, both peptidase and lysozyme antigens must be of macromolecular size because compounds with a molecular weight of less than 6000 D were excluded by the dialysis membrane we used. The patient was non-atopic, showing all negative responses to common inhalant allergens and was a non-smoker. There were three other workers who had asthmatic symptoms in her company, possible from the same cause. Confirmative diagnostic studies could not be performed in those three workers since they refused to be studied.

In conclusion, this study demonstrates that commonly used drug powders, serratial peptidase and lysozyme chloride, can induce occupational asthma in an exposed employee working in the pharmaceutical industry. Evidence is presented to indicate that

IgE-mediated immunologic mechanisms may be involved.

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