

In vitro studies of degradation of birch and timothy pollen allergen preparations by human duodenal juice

R. EINARSSON,¹ B. RENCK¹ & E. TAUDORF²

¹Pharmacia AB, Uppsala, Sweden, and ²Bispebjerg Hospital, Copenhagen, Denmark

The hydrolysis of birch and timothy pollen allergen preparations by duodenal juice from an adult volunteer was studied *in vitro* with the aid of rocketimmunoelectrophoresis, radiorocketimmunoelectrophoresis and RAST-inhibition. The results achieved with all methods were similar. The duodenal juice had the capacity to hydrolyse both the grass and tree pollen proteins, and after 30 min preincubation only 10% of the original birch and timothy allergenic activity remained. Heat treatment of the duodenal juice completely destroyed the proteolytic activity (i.e. no hydrolysis occurred) while protease inhibitors only marginally prevented the hydrolysis.

Key words: degradation; duodenal juice; pollen allergens.

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Birch and timothy pollen are inhalant allergens which commonly cause rhinitis and/or allergic asthma in many patients. Many efforts have been devoted to the study of the antigenic/allergenic composition, to identify and characterize individual components and to establish the stability by applying various physico-chemical and immunological methods (1, 3, 11).

Oral hyposensitization with different pollens has been used for many years in the treatment of allergic asthma and rhinoconjunctivitis (7, 10). However, the efficacy of the treatment has not been convincingly shown as most of the studies have been open and uncontrolled. The allergens were administered mostly as a powder or an aqueous solution without any control of stability. Only two controlled clinical studies (5, 8) showed effect of oral immunotherapy, both in birch pollinosis. Although if the allergens were administered in enterosoluble tablets in these studies (5, 8), the stability of the allergens at the intestinal mucous surface was not investigated.

The aim of this investigation was to study the degradation of birch and timothy pollen al-

lergens in human duodenal juice under *in vitro* conditions. The time-dependent change in allergenic activity was assayed by RAST-inhibition, rocket immunoelectrophoresis and radiorocket-immunoelectrophoresis.

MATERIAL AND METHODS

Human duodenal juice was collected from a volunteer (a 21-year-old male medical student, non-alcoholic and healthy) by placing a probe in the intestine of the fasting subject. The position of the probe was checked by X-ray and by determination of pH of the intestinal fluid. After a standard meal of Babymint® A (Ferrosan) (a mother's milk substitute consisting of proteins, carbohydrate, maize oil, milk fat and vitamins), the intestinal duodenal juice was collected by aspiration and frozen directly at -80° to conserve the proteolytic activity. An aliquot of intestinal juice was heat-treated directly after collection at +70°C for 10 min in a waterbath, to destroy the proteolytic activity (tested with timothy pollen extracts), followed by freezing at -80°C. This study was

approved by the Ethics Committee of the University of Copenhagen.

Crude birch and timothy pollen allergen extracts were prepared by extracting the pollen source material in 0.125 mol/l ammoniumbicarbonate buffer for 16 h at 4°C, extraction ratio 1/10 (w/v). After filtration to remove solid material the extracts were freeze-dried. The lyophilised pollen extracts were dissolved in distilled water to a stock concentration of 1.8 mg dw/ml. The total protein content of birch and timothy pollen allergen extracts was determined by amino acid analysis (6). Equal volumes of allergen extract and normal or heat-treated duodenal juice were mixed and incubated at +37°C for various time periods. Aliquots were withdrawn for analysis after 0, 5, 15, 30, 60 and 120 min, respectively. The incubation was stopped by freezing the allergen extracts prior to analysis.

A panel of various protease inhibitors (Phenyl methylsulfonyl fluoride (Sigma), Epsilon-amino caproic acid (Kabi-Vitrum), Iodoacetamide (Fluka) and Aprotinin (Sigma)) was used to study the kinetics of the proteolytic degradation *in vitro*.

Electroimmunoassay (rocketimmuno-electrophoresis) was carried out according to Laurell (4) on plastic film (Gelbond, Pharmacia Fine Chemicals) in 1% Agarose A. Five µl of each sample was added to the wells followed by electrophoresis into the rabbit antibody containing agarose (3.4 µl antiserum/cm² gel) for 20 h, 2 V/cm at 15°C.

The immunoprecipitates on the gelplates were visualized by Coomassie Brilliant Blue R 250. Radiorocketimmuno-electrophoresis (2) was carried out by incubating the washed plates with serum pools from birch and timothy pollen-sensitive individuals according to the previously described procedure for CIE/CRIE analysis (9). The plate was washed and incubated with ¹²⁵I-anti-IgE isotope (Phadebas RAST® Isotope reagent) at a concentration of 72 000 cpm/ml for another 15 h. The plate was washed, dried and placed on X-ray photographic film for autoradiography.

RAST-inhibition was used to determine the total allergenic activity of birch and timothy pollen extracts (12).

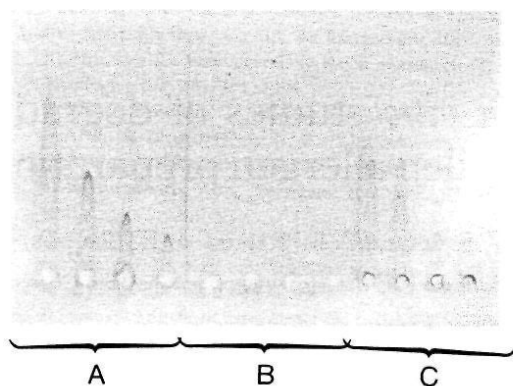


Fig. 1. Electroimmunoassay of timothy pollen proteins. Running time 20 h, 2 V/cm. Stained with Coomassie Brilliant Blue R-250.

- A. Timothy reference extract dissolved in distilled water dilution of reference 1/1 (16.8 µg protein), 1/2, 1/4 and 1/8.
- B. Timothy reference extract (conc., see A) incubated in normal duodenal juice at 20°C for 30 min.
- C. Timothy reference extract (conc., see A) incubated in heat-treated (+ 70°C, 10 min) duodenal juice at 20°C for 30 min.

RESULTS AND DISCUSSION

Fig. 1 illustrates the hydrolysis of the timothy pollen proteins after protein staining of the formed rockets. The timothy proteins are rapidly and nearly completely hydrolysed in normal duodenal juice. The change in the peak heights in rocketimmuno-electrophoresis directly shows that a significant degradation of the allergen occurs in duodenal juice, as the basic principle of this technique is a quantitative estimation of proteins by precipitation with the corresponding antibodies. The heat-treated duodenal juice causes very little degradation of the timothy proteins as the peak heights are comparable to the peaks of the timothy reference allergen preparation dissolved in water and analysed in parallel. However, there is some minor change within the main anodal protein peaks as the shape of the precipitates is changed and, furthermore, they are not so distinct in the heat-treated sample. Similar effects were seen with birch proteins (protein dilution 9.2–1.1 µg) preincubated for 30 min at 20°C in normal intestinal juice, except that no cathodal migration was observed. Further, the heat-

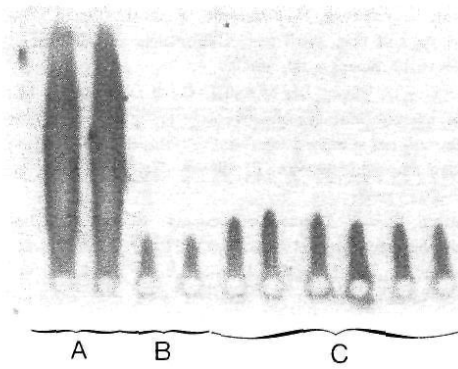


Fig. 2. Radiorocketimmuno-electrophoresis analysis of a birch pollen allergen reference preparation (1.1 μ g protein). Visualized after radiostaining with ^{125} I-anti IgE.

- A. Birch reference extract reconstituted in distilled water.
 B. Birch reference extract incubated in normal duodenal juice at 20°C for 15 min.
 C. Birch reference extract incubated in normal duodenal juice for 15 min at 20°C in the presence of variable concentration of Aprotinin (0.1, 0.5, 1.0, 2.5, 5.0 and 10 mg/ml).

treated duodenal juice caused a slight reduction in the rocket peak heights for birch pollen extracts in contrast to the timothy pollen allergens, indicating that a small amount of proteinases remained in the heat-treated duodenal juice.

Fig. 2A demonstrates the alteration in birch

allergenic activity measured by radiorocket-immuno-electrophoresis. The birch reference extract dissolved in distilled water exhibits pronounced rockets with intense radiostaining, indicating a high degree of specific IgE-binding to the birch allergens. The birch allergen preparation preincubated in normal duodenal juice for 15 min at 20°C gives very small peaks (Fig. 2B) in the electro-immunoassay, indicating a high degree of degradation. However, some allergenic activity still remains as the birch proteins in the rockets still bind specific IgE antibodies.

Fig. 2 also demonstrates the effect of a protease inhibitor on the hydrolysis of birch pollen allergens caused by duodenal juice. Aprotinin (0.1–10 mg) was added to the duodenal juice prior to incubation with birch allergens. The radiorocket analysis reveals only slight inhibition of the birch allergen degradation. Other protease inhibitors (phenylmethylsulfonyl-fluoride, epsilon-aminocaproic acid, jodoacetamide or mixture of them) were also investigated separately or as a mixture of all four inhibitors, in the presence of birch allergens, but the inhibition capacity of the proteolytic activity (endopeptidases in the duodenal juice) was very low. Similar results were obtained with timothy pollen allergens in the presence of the protease inhibitors (data not shown).

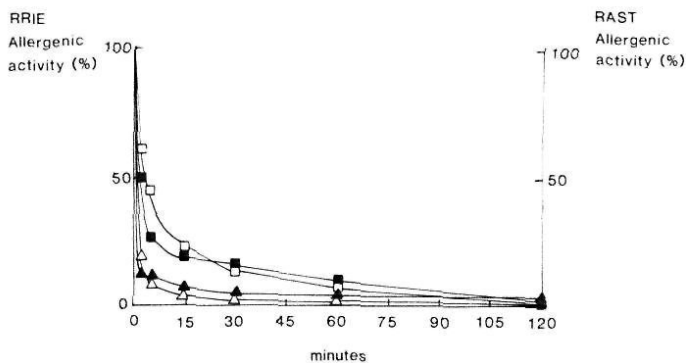


Fig. 3. Allergenic activity of birch pollen and timothy pollen allergen extracts (1.8 mg dw) incubated at 37°C for variable time periods (0–120 min). The allergenic activity was assayed by RAST and radiorocketimmuno-electrophoresis (RRIE).

- — □ RAST timothy
 ■ — ■ RRIE timothy
 △ — △ RAST birch
 ▲ — ▲ RRIE birch

Fig. 3 compares the time-dependent hydrolysis of birch and timothy pollen allergen preparations in duodenal juice. Aliquots of the pollen preparations were preincubated for various time periods at 37°C and after that directly frozen at -70°C.

All samples were analysed in parallel with radiorocketimmuno-electrophoresis and RAST inhibition to minimize experimental differences. The degradation of the birch allergen preparations appears to be somewhat more rapid during the initial phase of hydrolysis. The time required to hydrolyse 50% of the pollen allergen preparations in normal duodenal juice at 37°C was 4 min for timothy and 2.5 min for birch. Already after 15 min, more than 90% of the birch allergenic activity was destroyed. In contrast, the timothy allergens seemed to be more resistant to the initial rapid hydrolysis, but after 60 min preincubation in normal duodenal juice the allergenic activity was drastically reduced and well below 10%. This study shows definitely that native pollen allergen preparations do not survive intact in duodenal juice for a longer period of time. Obviously, even if allergen preparations are administered in enterosoluble tablets or capsules instead of the classical administration as a powder or an aqueous solution to obtain a high concentration of undigested allergens at the intestinal mucous, it is not certain that the native allergens will be available for absorption undegraded.

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Address

Dr. Roland Einarsson
Pharmacia AB
S-751 82 Uppsala
Sweden

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