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Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure

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Abstract Stability under digestion is thought to be an important prerequisite determining the allergenicity of food proteins. To test this hypothesis, two important allergenic plant-derived foods were selected for this investigation. Protein extracts from roasted peanuts and unprocessed (native) hazelnuts were digested by a static, two-step in vitro procedure with commercial enzyme tablets containing peptic and pancreatic enzymes, respectively. The food extracts were subjected to gastric digestion for 2 h followed by a 45-min treatment under duodenal conditions. Undigested control samples and the two digests were investigated by analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), by SDS-PAGE immunoblotting, by an enzyme allergosorbent test (EAST) with human IgE, and by a rat basophil leukaemia (RBL) cell mediator release assay that depends on specific IgE raised in mice. Peanut proteins appeared to be more stable under digestion than hazelnut proteins, as shown by electrophoresis. These results were also underlined by immunoblot data. The gastric digest from peanut contained various protein fragments that were detected by antibodies from a peanut-specific rabbit antiserum and by IgE from patients allergic to peanuts. These immunoblot reactivities decreased strongly after subsequent pancreatic digestion. In the gastric digest from hazelnuts, a rabbit antiserum with a broad reactivity against native hazelnut proteins exclusively recognized small protein fragments of <15 kDa. This serum showed no binding to blots of the pancreatic digest. Sera from hazelnut-allergic patients presented IgE reactivities to an 18-kDa major allergen with homology to major tree-pollen allergens, to a minor allergen of 12 kDa, and to multiple bands >30 kDa in native hazelnut extract. No binding was observed with these sera on blot strips of

the two digests prepared from hazelnut extract. Under the native conditions of EAST, both digests from peanuts strongly reacted with human IgE. Their IgE binding capacity persisted at a level of about 50% when compared to undigested peanut. In the case of hazelnuts the IgE reactivity of the untreated samples was reduced to <10% by both gastric and combined gastric/duodenal digestion for a serum pool prepared from four patients and sera from three additional participants. By contrast, a constantly high immunoreactivity of the hazelnut digests was detected with serum from one patient. The results of the EAST were confirmed by dose-related mediator release experiments performed with RBL cells passively sensitized with allergen-specific murine IgE. As a whole, our results indicated that the EAST and the RBL cell assay are superior to immunoblotting for the immunologic testing of digests. In accordance with clinical observations, the allergenicity of peanut proteins was very persistent during digestion, whereas the native birch-pollen-related hazelnut allergens appeared to be relatively labile under identical conditions.

Key words Allergens · Peanut · Hazelnut · Digestion · Enzyme allergosorbent test · IgE immunoblotting

Introduction

In contrast to inhalant allergens which sensitize atopic individuals via the mucosa of the respiratory tract, food allergens have to pass through the gastrointestinal tract to reach the immunologically active gut mucosa. Since intact proteins or their larger fragments are required for processing by antigen-presenting cells, a considerable degree of stability under digestion may be necessary for food allergens to initiate sensitization by the oral route. Astwood et al. [1] presented data which indicated an exceptional stability of food allergens under

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digestion in simulated gastric fluid containing pepsin. By contrast, a variety of non-allergenic common plant proteins and proteins which had been introduced into genetically modified food plants were all rapidly degraded within seconds or minutes. However, these data were obtained with purified native proteins in the absence of characteristic constituents of a food matrix, and without testing the IgE reactivity of the digests prepared from allergens. Thus, matrix influences as well as effects caused by processing and preparation of the foods were not taken into consideration. Moreover, basic studies revealed that the use of single enzymes may underestimate digestibility, and that *in vitro* digestibility systems which contain a broad spectrum of digestive enzymes should give a better simulation of the *in vivo* process [2]. Regarding allergenicity, conclusions should not be drawn until immunological tests have been included in a study. Moreover, it has to be mentioned that the specific features and the concentrations of the molecular structures responsible for sensitization via the gastrointestinal tract remain unknown. Processes mimicking digestion may help to identify the proteins or peptides that cause sensitization and the compounds responsible for triggering symptoms. These are important steps towards achieving a better understanding of the pathomechanisms of classic food allergy. The *in vitro* procedure used in this study was originally developed to study baker's yeast allergens [3] and has been applied to celery allergens in a previous investigation [4]. The model is based on commercial gastric and duodenal enzyme tablets used for pharmaceutical purposes. In the case of celiac, a strong degradation of the whole protein pattern occurred after gastric treatment that was further enhanced by pancreatic digestion. Surprisingly, extended immunochemical tests indicated a considerable degree of residual allergenicity even after 45 min under gut conditions [4].

Proteins from peanut and hazelnut are important allergens even when they are present in small amounts in heated and processed foods. Multiple-IgE-binding proteins have been detected in peanuts [5], including the major allergens Ara h 1 and Ara h 2 [6, 7]. Peanut allergy mainly occurs independently of pollen allergy. By contrast, allergic reactions to hazelnut are almost exclusively restricted to patients with birch-pollen allergy. This phenomenon is caused by IgE cross-reactivity between the 18-kDa major hazelnut allergen and Bet v 1, the major birch-pollen allergen [8; unpublished results]. Important minor IgE-binding proteins in hazelnuts are the 14-kDa plant pan-allergen, profilin, and partly thermostable 43-kDa and 48- to 50-kDa glycoproteins [8, -9; unpublished data].

The aim of this study was to investigate the influence of digestion on the allergenic potential of two important plant foods capable of eliciting severe allergic reactions. Two steps of *in vitro* digestion were followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein staining, SDS-PAGE immunoblotting with animal antibodies and sera

from food-allergic patients, an enzyme allergosorbent test (EAST) for specific IgE under native conditions, and mediator release experiments with rat basophil leukaemia (RBL) cells passively sensitized with murine IgE. As in our previous work, the results indicated that despite strong degradation of the protein pattern, allergenic activity can persist in specific, digested preparations.

Materials and methods

Food extracts. Roasted peanuts were ground and extracted as described previously [10]. Protein extract from native hazelnut was prepared with phosphate-buffered saline (pH 7.4, 4 °C, 4 h) as previously reported [11]. Samples were stored freeze-dried at -20 °C until use.

Sera and antibodies. A serum pool from three patients and serum from one additional patient suffering from severe peanut allergy were kindly supplied by Dr. W.-M. Becker (Borstel Research Center, Borstel, Germany). Sera from individual patients with an oral allergy to hazelnut and birch pollinosis and additional sera from patients with a peanut allergy were taken from the in-house collection of the Paul-Ehrlich-Institut (PEI). The criteria for selection were sensitization patterns to specific allergens as described in the text. Control sera from patients without peanut and hazelnut allergies were also taken from the in-house collection. Rabbit antisera against peanut protein and hazelnut protein (corylin) and rabbit pre-immune serum were purchased from Riedel-de Haën (Seelze, Germany). Monoclonal antibodies (mabs) directed against the major peanut allergen, Ara h 1, were donated by Dr. W.-M. Becker. Murine sera containing IgE against roasted peanuts and birch pollen extract, respectively, were raised in Balb/c or CBA/J mice as described previously [4, 12].

***In vitro* digestion.** The model digestions were performed by a two-step procedure according to the rationale described for protein extract from celery tuber [4] using a modification of the protocol originally described by Kortekangas-Savolainen et al. [3]. Briefly, gastric and duodenal conditions were simulated by using commercial enzyme tablets. The enzyme activities of the two preparations were determined by the manufacturers according to the German pharmacopeia [13]. The activities were calculated as Federation Internationale Pharmaceutique (FIP) units. The tablets are pharmaceuticals and are recommended as food supplements for individuals with reduced pepsin production or those who suffer from a reduced function of the pancreas. The gastric tablets (Enzynorm; Nordmark, Hamburg, Germany) contained 350 mg porcine stomach extract/tablet, corresponding to 62.5 mg pepsin and 40 protease units. The gastric solution consisted of one Enzynorm tablet in 100 ml of 0.15 M sodium chloride. Festal tablets (Cassela-Med, Köln, Germany) consisting of porcine pancreas extract, with proteases, amylases and lipases, were used to mimic duodenal conditions. Each of these tablets contained 6000 FIP units of pancreatic lipases, 4500 FIP units of pancreatic amylase and 300 FIP units of pancreatic proteases. Pancreas proteases consist of trypsin, chymotrypsin, elastase, endopeptidases, exopeptidases, and carboxypeptidases A and B [14]. The duodenal solution was prepared by dissolving one Festal tablet in 100 ml of 0.15 M sodium chloride. Two hours were taken as an average time for stomach passage [4]. Forty-five minutes were selected for duodenal digestion since the major proportion of protein digestion takes place within this time when pure protein extracts are treated under intestinal conditions. An almost complete degradation of yeast allergens was obtained within this period [3]. Five milligrams of protein from hazelnut or peanut extract in 5 ml of 0.01 M phosphate-buffered saline was treated with 1 ml of gastric

solution at 37°C and a pH of 1.5 for 2 h. Subsequently, the pH was adjusted to 7.0, 2 ml of duodenal solution was added, and the sample was digested at 37°C for 45 min. Both incubations were carried out under gentle agitation. An acidic buffer control was performed with the same amount of protein (pH 1.5, 2 h). After termination of the digestion by freezing with liquid nitrogen, all samples were freeze-dried and stored at -80°C until used.

Electrophoresis and immunoblotting. Electrophoresis and immunoblotting were carried out as previously described [15], with minor modifications. Briefly, the extracts were separated by discontinuous SDS-PAGE under reducing conditions, using 5% (w/v) stacking gels (1.0 mm, T=5.0%, C=2.6%) (T=total percentage of acrylamide monomers, C=percentage of the crosslinker relative to T) and 13% (w/v) resolving gels (T=12.8%, C=2.7%). In order to compare the results with those of the original samples, the digested preparations were loaded on the gels according to the protein content of the undigested samples. For subsequent silver staining, 5 µg/cm of total protein was applied to analytical slots. After loading 25 µg/cm of protein on 5.5-cm preparative slots the separated extracts were transferred onto nitrocellulose (NC) membranes by semi-dry blotting. Membranes were blocked with 100 mM TRIS/HCl, pH 7.4, containing 100 mM sodium chloride, 2.5 mM magnesium chloride (TBS) and 0.3% (v/v) Tween 20. Cut NC strips (4 mm) were either stained for total protein with India ink or used for immuno-detections. All sera and antibodies were diluted in TBS containing 0.05% (v/v) Tween 20 (TBS-Tween). The total incubation volume was 1 ml per NC strip. The strips were washed three times for 5 min with TBS-Tween between all incubation steps. The following dilutions were used: human sera, 1:6.7; rabbit anti-peanut, 1:10000; rabbit anti-hazelnut, 1:5000; rabbit pre-immune serum was applied at an identical dilution to the specific rabbit antiserum used in the same experiment. Mabs were used at 1 µg/ml. Human IgE was detected with monoclonal anti-IgE alkaline phosphatase conjugate (4 h, 1:1000; Pharmingen, Hamburg, Germany). Bound IgG from rabbit antisera was detected with goat anti-rabbit biotin conjugate (Dako, Hamburg, Germany), diluted at 1:2500 for 1 h. For recognition of mabs, rabbit anti-mouse IgG/IgM-biotin (Dianova, Hamburg, Germany) was applied at 1:2500 for 1 h. Biotinylated secondary antibodies were subsequently incubated with streptavidin alkaline phosphatase conjugate (Caltag; Medac, Hamburg, Germany) at a dilution of 1:3000 for 30 min. Enzymatic staining was performed with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate using a commercial staining kit (Biorad, Munich, Germany).

Enzyme allergosorbent test. Hazelnut and peanut extracts were adjusted to a total protein concentration of 250 µg/ml and covalently coupled to cyanogen-bromide-activated filter paper disks as described for apples and extracts from other plant foods [16, 17]. After enzymatic digestion, the hydrolysates were subjected to the same procedure. The concentrations for coupling corresponded to the protein amount of the untreated samples. The allergen disks were incubated overnight with 50 µl human serum at room temperature. After washing, the amount of IgE specifically bound to the immobilized allergens was semi-quantitatively determined by a commercial EAST (Allergopharma, Reinbek, Germany), using the calibration system of the manufacturer. The results were expressed as EAST classes or as kU/l (class 0, no specific IgE; class 4, very high content of specific IgE corresponding to >17.5 kU/l). All samples were analysed as duplicates.

RBL cell mediator release assay. A murine in vitro model of the type I allergic reaction was applied to estimate the biological activity of the allergen preparations [4, 12]. The assay is based on a culture of permanently growing RBL cells. The subline RBL-2H3 (DSMZ, Braunschweig, Germany) that was selected for maximal mediator release was used in all experiments. These cells represent functional analogues of mucosa mast cells and can be passively sensitised with murine and rat IgE, but not with human IgE. Specific crosslinking of cell-bound IgE by allergens results in

dose-related mediator release at allergen concentrations in the range of pg/ml to mg/ml. This event is very close to one basic mechanism of the human type I allergic reaction and is similar to the histamine release assay with basophils from allergic humans. Pooled sera of at least four responding mice (criterion: at least 30% mediator release at an allergen concentration of 0.1 µg/ml and a serum dilution of 1:50) with IgE against birch-pollen extract or roasted peanut were diluted 1:50 or 1:100 prior to passive sensitization. Permanently growing RBL-2H3 cells were harvested in the stationary phase, transferred at 10⁵ cells/well to 96 well-cell culture plates (Nunc, Wiesbaden, Germany) and cultivated for 18 h at 37°C in modified Eagle's medium (MEM) containing 5% fetal calf serum (FCS). The attached cells were passively sensitised for 1 h with pooled murine antiserum dilutions in Eagle's MEM (5% FCS). After sensitization, the cells were washed 5 times with Tyrode's buffer. For crosslinking and degranulation, the RBL cells were incubated with 100 µl of serial dilutions of allergen extracts or digests in Tyrode's buffer in a humidified atmosphere at 37°C for 1 h. The specific mediator release was assayed by measurement of the β -hexosaminidase activity. Enzymatic activity in the supernatant was detected by hydrolysis of *p*-nitrophenyl-*N*-acetyl β -D-glucosaminide for 1 h. Absorbance was measured at 405 nm. The total release was quantified by adding the buffer containing 1% Triton X-100. Controls were run with Tyrode's buffer without allergen to measure spontaneous release, and by stimulation of the sensitized cells with peanut extract. Results were expressed as % of the total release after subtraction of the spontaneous release.

Results and discussion

Study design

Extracts from native hazelnuts and roasted peanuts were selected for the digestion experiments. This decision was based on clinical and in vitro observations: roasted peanuts are regularly capable of inducing allergic reactions in sensitized patients, whereas in birch-pollen-allergic patients with adverse reactions to hazelnuts the IgE reactivity of roasted hazelnuts is strongly decreased to a relatively low level of persistent activity [11]. Moreover, there are no reports about patients who react more strongly to roasted hazelnuts when compared to the unprocessed (native) food. The digestion conditions were chosen on the basis of literature data. The procedure applied in this study led to the destruction of the majority of baker's yeast allergens [3] and strongly degraded bovine serum albumin in our optimization experiments (results not shown). In contrast to other studies, we wanted to apply a broad spectrum of assays to test the antibody reactivity and the allergenic potential of the digests. These tests included antibody binding after denaturing pretreatment of the samples for SDS-PAGE immunoblotting, and with the allergen extracts and digests applied in physiological buffers and thus tested under native conditions (EAST). Moreover, we used an assay for cross-linking properties with cell-bound IgE (RBL cell mediator release assay). In general, all protein amounts were calculated on the basis of the protein content of the undigested allergen extracts in order to compare the results directly.

SDS-PAGE and immunoblotting

Peanut extract and hazelnut extract were subjected to analytical SDS-PAGE followed by silver staining. The results obtained with peanut extract are shown in Fig. 1. Identical protein patterns were obtained for undigested peanut extract (lane 1) and an acidic buffer/temperature control (not shown), indicating that the pH changes did not cause protein degradation. After 2 h under gastric conditions, peanut proteins of >30 kDa, including the major allergen Ara h 1 of 66 kDa, were strongly degraded, and several fragment bands became visible <25 kDa (Fig. 1, lane 2). As shown in lane 3 Fig. 1, degradation was further enhanced after 45 min under gut conditions, leading to strongly degraded material of ≤ 16 kDa. The pancreatic

enzymes appeared as strong bands at approximately 30 kDa (Fig. 1, lane 5). When hazelnut extract was subjected to the same experiment, again, no buffer-related effects occurred (result not shown). Compared to undigested hazelnut (Fig. 2, lane 1), all protein bands >14 kDa were destroyed by the gastric treatment (lane 2), and a strong band at about 12 kDa was further reduced by the pancreatic treatment (lane 3). Thus, hazelnut proteins were notably more susceptible to digestion than peanut proteins.

The results of the immunoblot experiments with peanut extract are depicted in Fig. 3. The controls showed no evidence for non-specific binding. The rabbit antiserum against peanut protein recognized multiple bands in a broad mass range in both extracts from roasted peanut (Fig. 3A, lane r) and the gastric digest (Fig. 3B), but showed very little binding to several smaller fragments after subsequent duodenal treatment

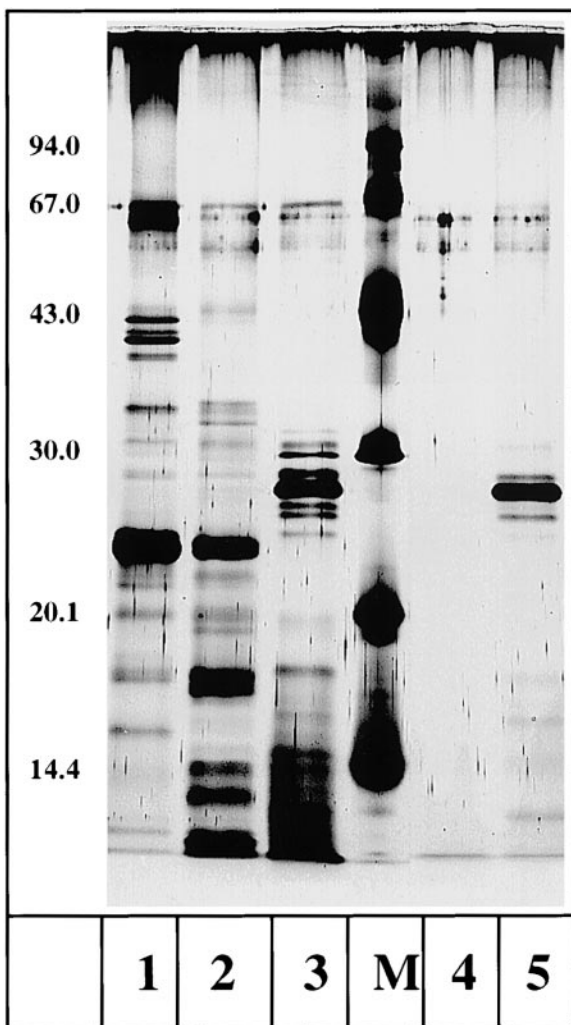


Fig. 1 Degradation of peanut extract by the model digestion monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent silver staining. 1 Undigested peanut extract, 2 peanut extract after 2 h of gastric treatment, 3 peanut extract after 2 h of gastric treatment followed by 45 min of pancreatic digestion, 4 gastric enzyme solution, 5 pancreatic enzyme solution, M molecular weight marker (14.4, 20.1, 30, 43, 67, 94 kDa)

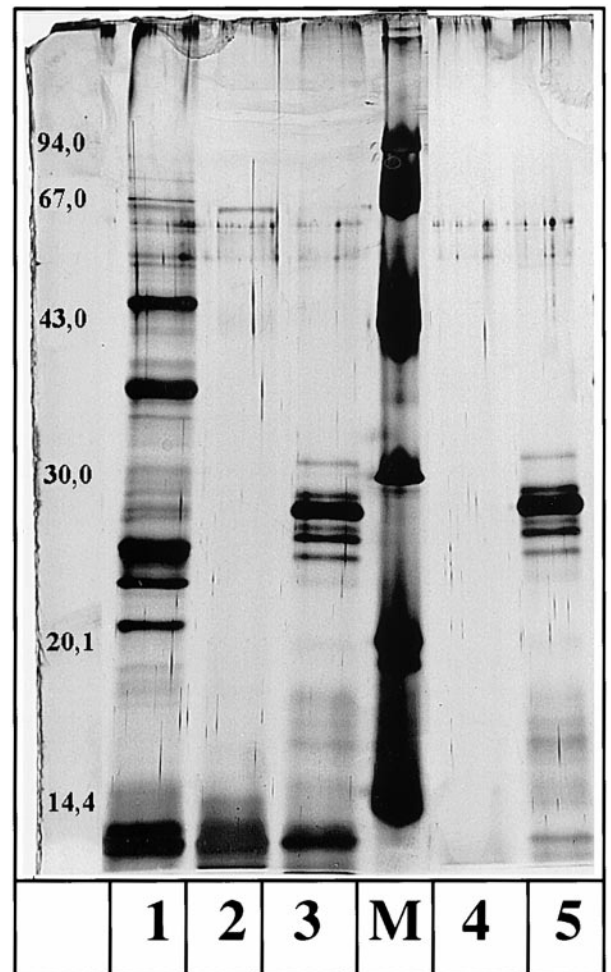


Fig. 2 Degradation of hazelnut extract by the model digestion monitored by SDS-PAGE and subsequent silver staining. 1 Undigested hazelnut extract, 2 hazelnut extract after 2 h of gastric treatment, 3 hazelnut extract after 2 h of gastric treatment followed by 45 min of pancreatic digestion, 4 gastric enzyme solution, 5 pancreatic enzyme solution, M molecular weight marker (14.4, 20.1, 30, 43, 67, 94 kDa)

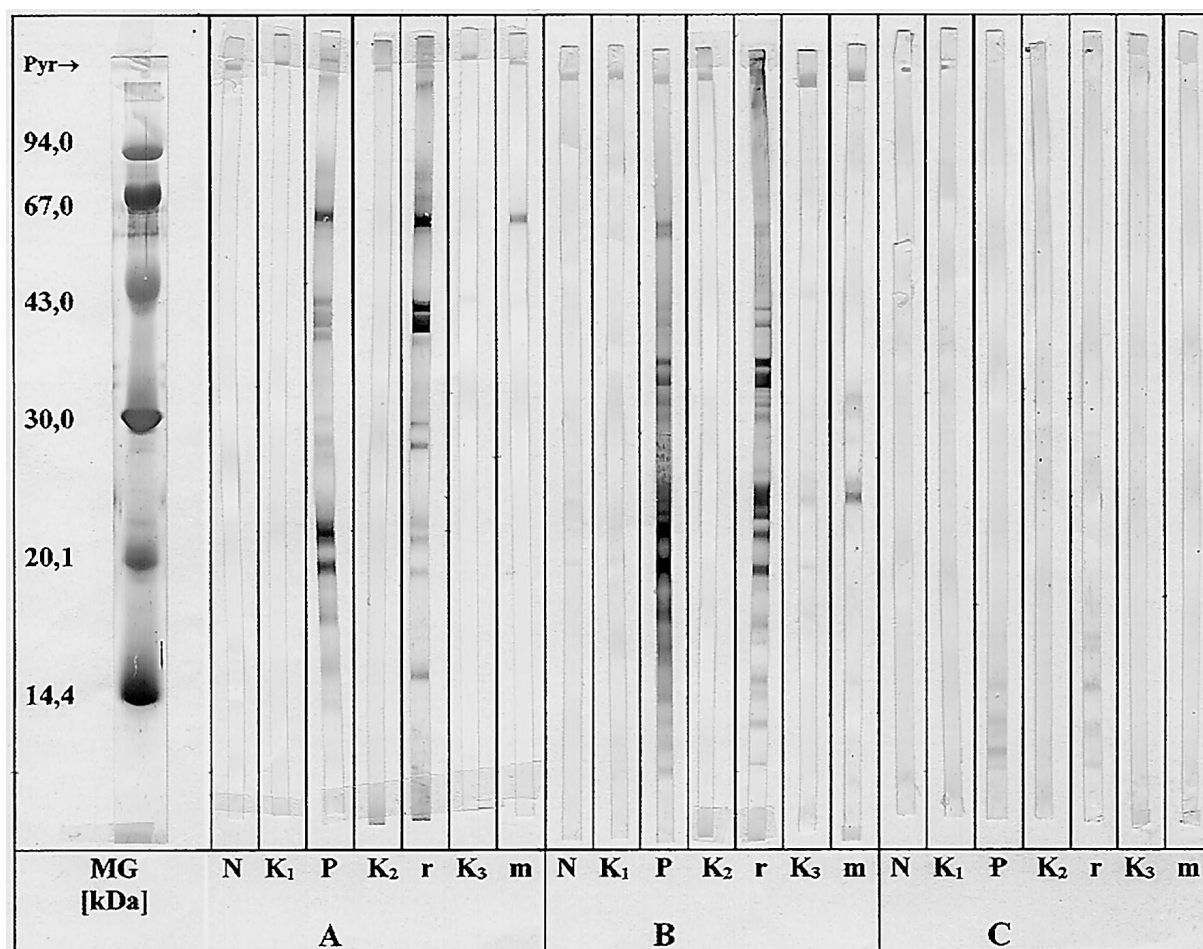


Fig. 3 Results of immunoblotting analyses of peanut extract before digestion (**A**), after gastric treatment (**B**), and after combined gastric and pancreatic treatment (**C**). *N* Non-allergic control, *K*₁ serum from an allergic patient without peanut allergy, *P* serum pool from 3 peanut-allergic patients, *K*₂ serum from a non-immunized rabbit, *r* rabbit antiserum against peanut, *K*₃ monoclonal antibody against *Mycobacterium paratuberculosis* (control), *m* monoclonal antibody-PN-t against the major peanut allergen, Ara h 1

(Fig. 3C). The major allergen, Ara h 1, was recognized by the specific mab, PN-t, at approximately 66 kDa in untreated extract (Fig. 3A, lane m). After gastric treatment, this band disappeared, but the epitope of PN-t was detected on a fragment at about 23 kDa (Fig. 3B). The mab did not bind to any fragments after combined gastric and pancreatic digestion (Fig. 3C). As indicated by the reaction pattern of the pooled serum from peanut-allergic patients, also shown in Fig. 3, multiple IgE reactivities persisted after gastric treatment (Fig. 3B, lane P) which were further reduced to three very weak bands < 14 kDa after subsequent incubation of the extract under gut conditions (Fig. 3C). Degradation of hazelnut extract was monitored with rabbit antiserum against corylin (Fig. 4A, B; lane 7). The rabbit antibodies bound to multiple bands in the untreated sample (Fig. 4A), whereas a reduced reactivity to several small

fragments remained detectable after simulating stomach conditions; this reactivity disappeared after subsequent pancreatic treatment (Fig. 4B). The IgE reactivity of native hazelnut was investigated with sera from four hazelnut-allergic patients with different sensitization patterns (Fig. 4A, lanes 2–5). The Bet-v-1-related major allergen was identified at 18 kDa by the serum applied in lane 2 (Fig. 4A). The IgE reactivity of all patient sera to hazelnut was almost completely destroyed by the first step of digestion (Fig. 4B). In summary, the results of immunoblotting were in accordance with the apparent digestion of the protein pattern as indicated by SDS-PAGE and subsequent silver staining. Peanut protein appeared to be more resistant to enzymatic hydrolysis, which corresponded to a stronger immunoreactivity of the digests from peanut when compared to hazelnut treated under identical conditions.

Enzyme allergosorbent test

After immobilization on activated paper disks, the overall IgE binding capacity of the undigested extracts and the digested preparations were investigated by EAST. Allergen disks of the peanut preparations were incubated with the serum pool from three peanut-allergic

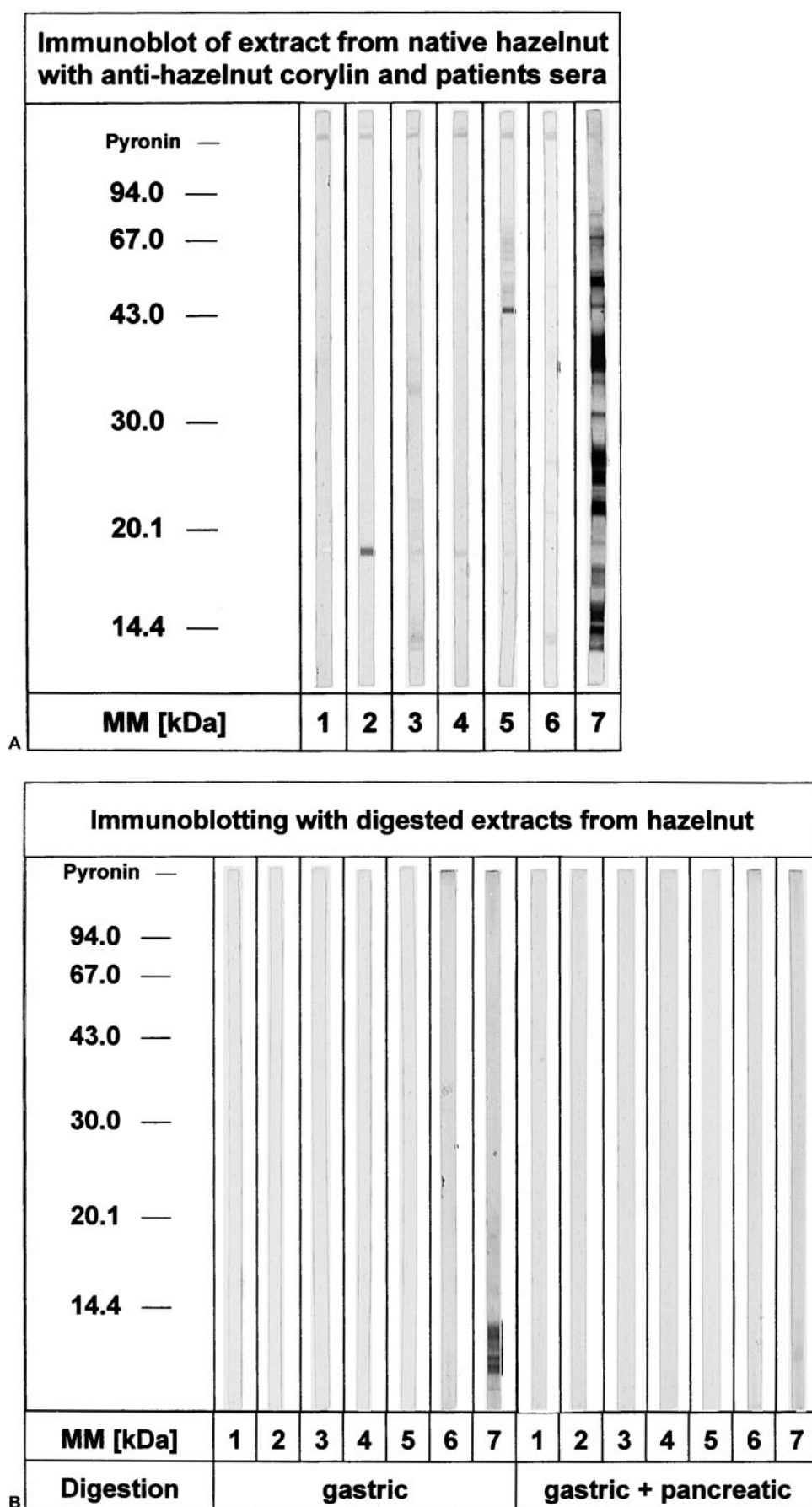
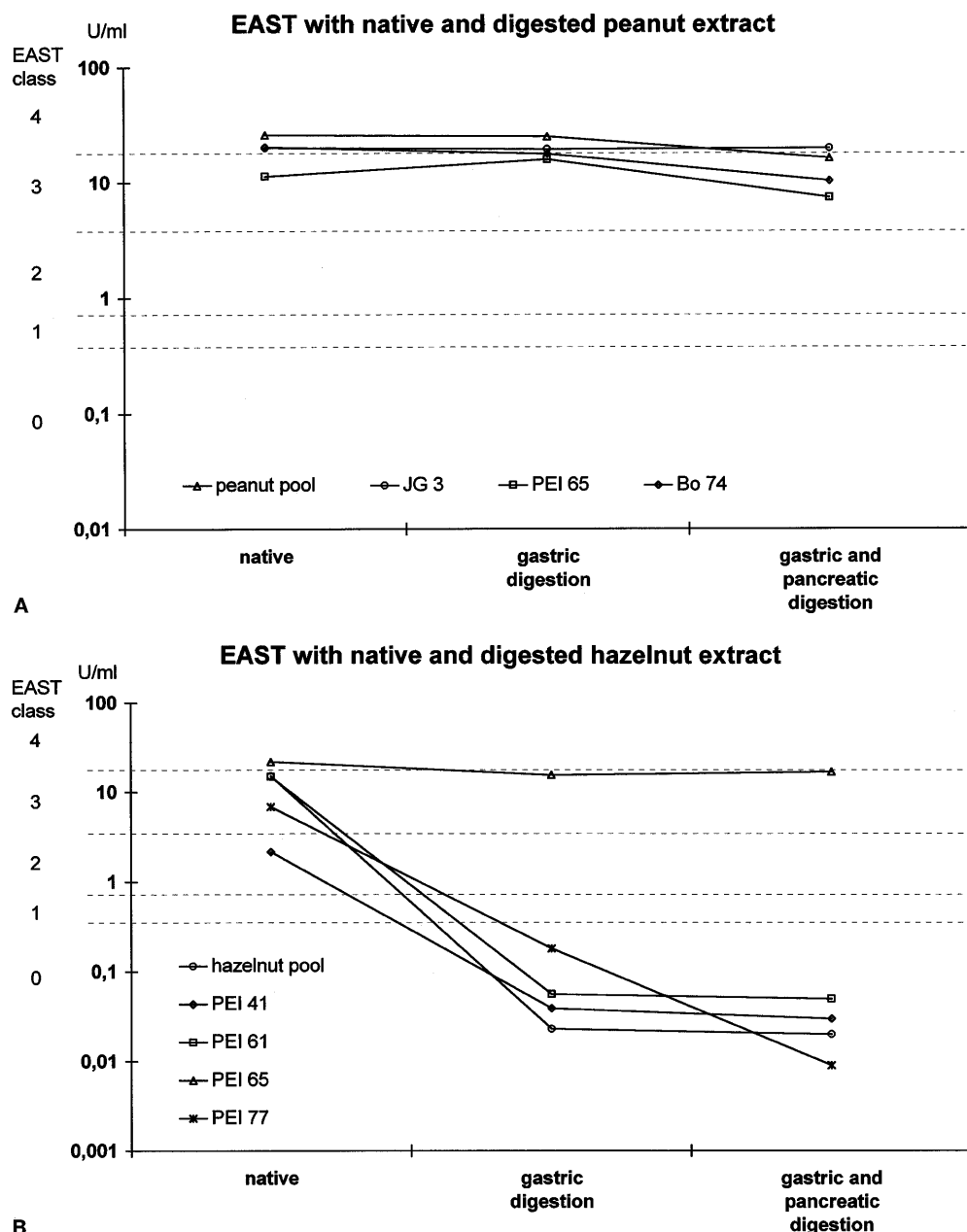


Fig. 5 Determination of the reactivity of untreated (native) and digested peanut (A) and hazelnut (B) extracts by enzyme allergosorbent test (EAST) using sera from patients with allergies to the specific foods. The peanut pool was prepared from equal amounts of serum from three peanut-allergic patients and the hazelnut pool was from four patients with an oral allergy to hazelnuts presenting IgE against the 18-kDa major hazelnut allergen. Paul-Ehrlich-Institute patient 61 (PEI 61) had an additional sensitization against the minor allergen, profilin. PEI 65 had IgE specific for a 12-kDa minor hazelnut allergen. PEI 41 and PEI 77 showed IgE binding to carbohydrate determinants of multiple glycoproteins with masses of >30 kDa. For allergen patterns see also Fig. 4A. To facilitate easy recognition of data from individuals, patient symbols are connected by lines, these do not represent mathematical regressions



gic patients and sera from three individual patients with adverse reactions to peanuts and strong serological sensitizations. As shown in Fig. 5A, the gastric digest reacted strongly with IgE from all four human sera. This immunoreactivity was similar or even stronger (patient PEI 65) than with the peanut extract before digestion. Surprisingly, despite a very weak reactivity of the pancreatic digest determined by immunoblotting, this sample strongly bound to IgE from the human sera under the native conditions of EAST. This may be explained by a negative effect of the denaturing conditions of SDS-PAGE prior to immunoblotting, or by the presence of small IgE-reactive fragments generated from peanut proteins which were below the separation range of the electrophoretic system. The latter interpre-

tation would be in accordance with results of Burks et al. [18], who determined a minimal epitope size of six sequential residues on the major peanut allergen, Ara h 1.

Hazelnut extract was investigated by the same assay with pooled serum from four patients whose IgE antibodies were mainly directed against the Bet-v-1-related major allergen, and with sera from individual patients with additional IgE specificities against multiple bands ≥ 30 kDa (PEI 41, PEI 77), against hazelnut profilin of approximately 14 kDa (PEI 61), and against a 10- to 12-kDa allergen (PEI 65) that did not cross-react with birch pollen allergens in immunoblot-inhibition experiments (unpublished data) (Fig. 5B; for allergen identification also see Fig. 4A). The serum pool had an EAST

class of 3 (>15.2 kU/l) to undigested hazelnut which decreased drastically to class 0 for both digests. The reactivity of three sera with EAST classes 2 (PEI 41) and 3 (PEI 61, PEI 77) against native hazelnut was reduced to class 0 after the first step of digestion. In contrast, IgE from serum PEI 65 with reactivity to a minor allergen in the region of 10–12 kDa reacted strongly with native hazelnut as well as with the two digests (Fig. 5B). These results were remarkably different from those obtained with peanut extract. Firstly, they indicated the lability of the Bet-v-1-related major allergen, of profilin and of multiple allergens of higher masses to digestion. In the case of the major allergen, this may have reflected cross-reactive sensitization to hazelnut via inhalation of birch pollen. Bet v 1 and its related allergens in foods are known to contain conformational epitopes which are destroyed by heating of the food and enzymatic cleavage [19–21]. Secondly, IgE which is directed against plant food allergens of >30 kDa is known to be directed against specific carbohydrate determinants on glycoproteins which are highly stable during heating and processing of foods [19, 20]. Interestingly, IgE from sera PEI 41 and PEI 77 which was directed against such epitopes did not react with the digested hazelnut samples. This result may have been caused by the presence of glycosidases in the protease preparations or by the acidic pH during gastric digestion.

Taken together, the EAST data confirmed the higher stability of peanut proteins under conditions of the gastrointestinal tract. Moreover, the results clearly identified the EAST as the more sensitive system for testing IgE reactivities of digests. Hence, we suggest that this simple immunoassay should be routinely applied in studies investigating the influence of digestion on food allergens. Finally, the higher lability of the majority of hazelnut allergens during digestion was confirmed by the results of the EAST. This lability may be related to a lack of stability of the Bet-v-1-related major allergen. However, as evidenced by the results obtained with serum PEI 65, even hazelnut digests may contain strong residual allergenic activity for patients who are sensitized to specific minor allergens with a higher degree of stability.

RBL cell mediator release assay

The IgE crosslinking potential of the digests was investigated by the RBL cell mediator release assay with murine IgE. Since this assay mimics allergen-specific degranulation of mast cells and basophils, a positive result may be taken as evidence that an allergen is also able to trigger symptoms in sensitized patients. The preparations from peanuts were investigated with IgE from pooled serum from five mice immunized with whole peanut extract (Fig. 6A). All three peanut samples caused a strong dose-related mediator release of the sensitized cells, whereas no release was triggered by

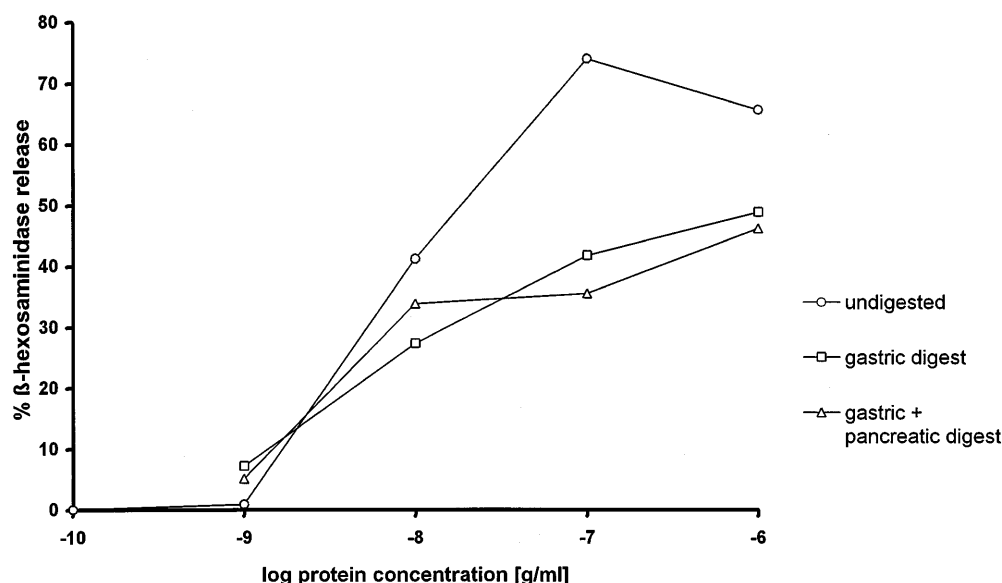
the pure enzyme preparations that were applied as controls. In comparison to the undigested sample, the allergenic potential of the two digests determined in this biological system was reduced to approximately 70%. Thus, in the case of peanut, the data obtained by the RBL cell assay corresponded closely to those obtained by EAST with human IgE, and indicated similar IgE reactivities for both the gastric and the gastric/pancreatic digest. Since hazelnut allergy is mainly induced by cross-reactive respiratory sensitization to birch pollen, and pollen-independent hazelnut allergy is very rare, we sensitized the RBL cells with murine IgE against birch pollen extract prior to investigating the mediator release capacity of the hazelnut preparations. As shown in Fig. 6B, prior to digestion, hazelnut extract caused a cross-reactive mediator release with anti-birch-pollen IgE. No release was triggered by the digested hazelnut preparations. Thus, the lability of the birch-pollen-related allergen of hazelnut, which is recognized by IgE from the majority of hazelnut-allergic patients, was confirmed by the biological assay.

Conclusions

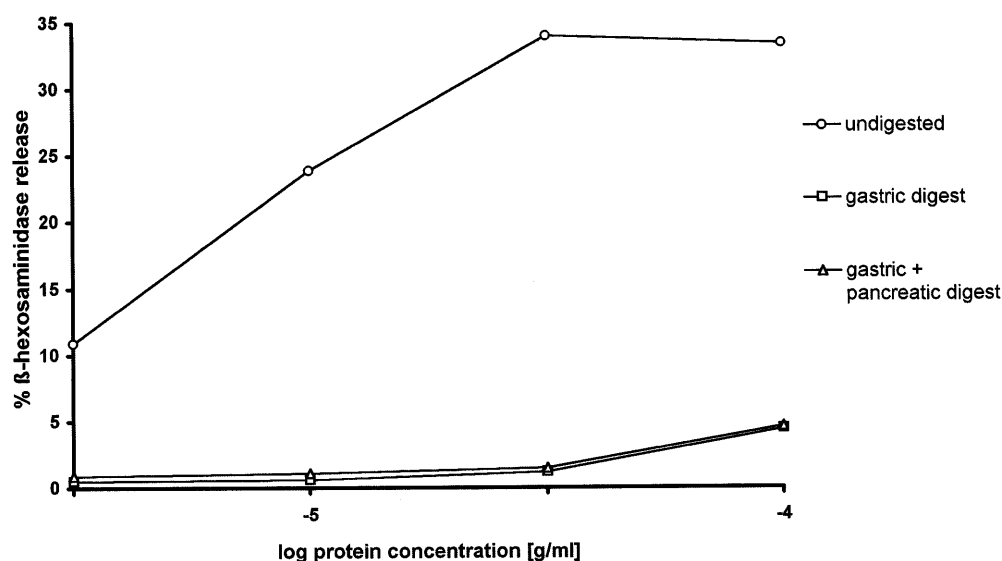
Stability under digestion is thought to be an important requirement for the allergenicity of food proteins. This stability may be important for both sensitization of the immune system after penetration of the gastric mucosa and for the elicitation of gastrointestinal symptoms of a food allergy. A validated, static two-step in vitro digestion procedure [3, 4] was applied in this study using periods of 2 h and 45 min for gastric and duodenal digestion, respectively. Thus, our results are not directly comparable to those of Astwood et al. [1] who treated purified proteins with simulated gastric fluid for 60 min, and monitored degradation by multiple sampling during this period. However, our results clearly indicated that immunological assays are required to investigate the potential allergenicity of food proteins instead of monitoring degradation of bands by analytical electrophoresis. Even immunoblotting as applied in this study seemed to be inappropriate for sufficiently sensitive testing of the digests. This conclusion may be underlined by the results obtained with peanut extract. Although the immunoreactivity on blots was reduced to a minimal level after duodenal digestion, both the EAST with IgE from peanut-allergic patients and the mediator release assay with cells passively sensitized by murine IgE indicated a strong allergenicity of this digest. Moreover, the latter assay may be preferred because it mimics one basic mechanism of the type I allergic reaction, i.e. mediator release from sensitized cells caused by crosslinking of allergen-specific, cell-bound IgE. The generation of small IgE-reactive proteolytic fragments <10 kDa and below the range of separation of our electrophoretic system would be a conclusive explanation for the inappropriate sensitivity of immunoblotting. The separation range could be extended to smaller

A

Fig. 6A,B Investigation of the dose-related IgE crosslinking capacity of untreated extracts and the digested preparations by the mediator release assay with rat basophil leukaemia 2H3 cells passively sensitized with murine IgE raised against the specific foods. **A** sensitization by pooled murine serum raised against peanut proteins (1:100) and dose-related stimulation by peanut preparations, **B** sensitization by pooled murine serum raised against birch pollen extract (1:100) and dose-related stimulation by hazelnut preparations. No release was triggered by the pure enzyme preparations at concentrations present in the digested samples



B



peptides by the application of tricine SDS-PAGE [22]. However, in the case of celery, no evidence for small IgE-binding peptides arose from the results of ultrafiltration experiments [4]. Nevertheless, apart from the estimation of the overall allergenicity which was the subject of this study, the molecular structures of the allergenic degradation products generated by the model digestions remain to be determined.

Interestingly, the major hazelnut allergens were remarkably more labile during the model digestions than the peanut allergens. This observation may have reflected the route of sensitization: since hazelnut allergy mainly occurs in patients allergic to tree pollen subse-

quent to the development of pollinosis, and the major allergens from both birch pollen and hazel pollen are known to cross-react with IgE against the major hazelnut allergen [8], it is very likely that respiratory sensitization to major pollen allergens such as Bet v 1 from birch pollen induces cross-reactive sensitization to hazelnuts. Stability under digestion is not important for this process. In contrast, peanut allergy is not closely related to pollen allergy, and sensitization may be caused by consumption of this food.

Assessing the allergenicity of new proteins in foods derived from genetically modified plants will be an important challenge in the area of food allergy research.

Although we have shown that the investigation of digests by immunoassays is required to obtain reliable results, we have to bear in mind that sera from volunteers who are allergic to these new proteins are not available. In our opinion, antibodies raised in animals may serve as useful tools to monitor the influence of digestion on the immunoreactivity of novel food proteins. Since the RBL cell mediator release assay has been shown to yield results close to those seen with humans [12], elicitation of an IgE response in specific mice strains and subsequent mediator release experiments with immune cells passively sensitized with these antibodies may be helpful in solving the problem of allergy assessment of these new food proteins and their digests. In the case of peanut allergens, this assay may also assist in the identification of IgE-crosslinking allergen fragments generated by digestion after chromatographic separation of digests.

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References

1. Astwood JD, Leach JN, Fuchs RL (1996) *Nature Biotechnology* 14:1269–1273
2. Porter DH, Swaisgood HE, Catignani GL (1984) *J Agric Food Chem* 32:334–339
3. Kortekangas-Savolainen O, Savolainen J, Einarsson R (1993) *Clin Exp Allergy* 23:587–590
4. Jankiewicz A, Baltes W, Bögl KW, Dehne LI, Jamin A, Hoffmann A, Hausteine D, Vieths S (1997) *Food Agric Immunol* 9:203–217
5. Uhlemann L, Becker WM, Schlaak M (1993) *Z Ernährungswiss* 32:139–151
6. Burks AW, Williams LW, Helm RN, Connaughton C, Cockrell G, O'Brian T (1991) *J Allergy Clin Immunol* 88:172–179
7. Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brian T, Helm RN (1992) *J Allergy Clin Immunol* 90:962–969
8. Hirschwehr R, Valenta R, Ebner C, Ferreira F, Sperr WR, Valent P, Rohac M, Rumpold H, Scheiner O, Kraft D (1992) *J Allergy Clin Immunol* 90:927–936
9. Vocks E, Borga A, Szliska C, Seifert B, Burow G, Boreli S (1993) *Allergy* 48:168–172
10. Holzhauser T, Dehne LI, Hoffmann A, Hausteine D, Vieths S (1998) *Z Lebensm Unters Forsch* 206:1–8
11. Vieths S, Hoffmann A, Holzhauser T, Müller U, Reindl J, Hausteine D (1998) *Allergy (Suppl 46)* 53:65–71
12. Hoffmann A, Jamin A, May S, Hausteine D, Vieths S (1997) *Food Agric Immunol* 9:309–325
13. Hartke K (ed) (1986) *Deutsches Arzneibuch* 9. Ausgabe, Band 3: Monographien M-Z. Wissenschaftl VlgGes Stuttgart, Germany
14. Schmidt RF, Thews G (eds) (1990) *Physiologie des Menschen*, 24th edn. Springer, Berlin Heidelberg, New York
15. Vieths S, Schöning B, Baltes W (1992) *Food Agric Immunol* 4:181–197
16. Vieths S, Schöning B, Brockmann S, Aulepp H (1992) *Dtsch Lebensm Rundsch* 88:239–243, 273–279
17. Vieths S, Jankiewicz A, Schöning B, Aulepp H (1994) *Allergy* 49:262–271
18. Burks AW, Shin D, Cockrell G, Stanley JS, Helm RM, Bannon G A (1997) *Eur J Biochem* 245:334–339
19. Jankiewicz A, Baltes W, Bögl KW, Dehne LI, Jamin A, Hoffmann A, Hausteine D, Vieths S (1997) *J Sci Food Agric* 75:359–370
20. Jankiewicz A, Aulepp H, Altmann F, Fötisch K, Vieths S (1998) *Allergo J* 7:87–95
21. Schou C (1995) *Clin Exp Allergy* 25:10–14
22. Schagger H, Jagow G von (1987) *Anal Biochem* 166:368–379