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***In Vitro* Study of the Gastrointestinal Stability of Celery Allergens**

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The gastrointestinal stability of proteins and allergens from native celery roots was studied by a simple two-step *in vitro* procedure. Commercial enzyme tablets that contained peptic and pancreatic enzymes were used to simulate gastric and duodenal conditions respectively. The influence on the whole protein pattern was monitored by SDS-PAGE. The antigenicity and allergenicity of the three known important allergenic structures of celery were investigated with IgG from two rabbit antisera, IgE from sera of allergic patients with known specificity for individual allergens, and by a mediator release assay utilizing allergen-specific polyclonal murine IgE. The following allergens were studied: (i) Api g 1, a 16-kDa celery protein which shares high sequence identity with the major birch pollen allergen, Bet v 1, (ii) celery profilin that belongs to a group of ubiquitous 'plant pan allergens' and (iii) ubiquitous carbohydrate determinants present in many glycans of plant proteins in the mass range of 35–90 kDa. SDS-PAGE indicated degradation of many proteins under gastric conditions, but immunoblotting showed only a weak influence on the antigenicity and IgE reactivity of the allergens. The protein degradation was clearly enhanced after 45 min of pancreatic digestion. To a weaker degree, all allergens were again recognized on immunoblots. The presence of a considerable residual allergenic activity which seems to resist even duodenal conditions was confirmed by the determination of human IgE specific for the digests using an Enzyme Allergosorbent Test. In addition, competitive IgE inhibition assays and measuring the celery-induced mediator release of permanently growing RBL-2H3 cells that had been passively sensitized with celery-specific murine IgE showed similar results. The generation of small IgE binding peptides (≤ 3 kDa) was tested by ultrafiltration experiments. The whole IgE binding capacity was present in the retentates even after duodenal digestion. We concluded that, like many other allergens from foods and other sources, the allergens of native celery are relatively stable under

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gastrointestinal conditions and that the residual allergenic activity is mainly due to undigested allergens.

Keywords: Food allergy, celery allergens, enzymatic digestion, IgE, immunoblotting, RBL-cell mediator release assay

INTRODUCTION

Celery is a common vegetable in human nutrition. It is consumed either in fresh salads, cooked, fermented or as a spicing ingredient of many processed foods. Celery roots (celeriac) are important elicitors of food allergies which can cause severe symptoms in allergic patients (Wüthrich & Dietschi, 1985; Pauli *et al.*, 1988; Wüthrich, 1993; André *et al.*, 1994). Allergy to celery is closely linked with specific pollen allergies, in particular to birch pollen and mugwort pollen. This phenomenon is mainly due to three distinct structures being recognized by cross-reactive IgE of pollen-allergic patients. These structures have been intensively characterized over the past years: (i) Api g 1 (allergen 1 from *Apium graveolens*), a 16-kDa protein, cross-reacts with Bet v 1, the major birch pollen allergen (Vieths *et al.*, 1995a), and other allergens from birch pollen related fruits and vegetables (Ebner *et al.*, 1995; Schöning *et al.*, 1995). Api g 1 shares a high sequence identity of approximately 40% with Bet v 1, and both allergens probably belong to a group of plant 'pathogenesis-related proteins' (Breiteneder *et al.*, 1995; Schöning *et al.*, 1995). (ii) The 15-kDa protein profilin has recently been identified as another important celery allergen (Vallier *et al.*, 1992; Vieths *et al.*, 1995a). Since profilins are involved in the regulation of actin polymerization and participate in signal transduction pathways, they are present in almost all eucaryotic cells and are ubiquitous allergens in plant (van Ree *et al.*, 1992; see also reviews of Martinez *et al.*, 1995; Vieths *et al.*, 1996a,b). (iii) Multiple allergen bands in the mass range 35–90 kDa seem to carry ubiquitous cross-reactive carbohydrate determinants (CCD) (van Ree & Aalberse, 1993; Vieths *et al.*, 1994a). α 1,3-Fucose and β 1,2-xylose residues of plant N-glycans are thought to be important constituents of the epitope structures (Tretter *et al.*, 1993; van Ree & Aalberse, 1993; Petersen *et al.*, 1996).

The allergenic activity of celery is known to contain a thermostable component (Wüthrich *et al.*, 1990). We have recently demonstrated that residual IgE binding capacity can be present in the vegetable after application of several thermal and non-thermal technological processes and that the stabilities of the known celery allergens during these treatments are different (Jankiewicz *et al.*, 1996, 1997). The activity of Api g 1 was rapidly destroyed by microwave treatment whereas the two other structures were more resistant to the application of heat.

However, nothing is known about the gastrointestinal stability of these celery allergens. Although the role of the gastrointestinal tract in the sensitization to food allergens and the development of allergic symptoms is not well understood, many strong food allergens are known to resist enzymatic digestion (Houben & Penninks, 1996; Vieths *et al.*, 1996c). The stability against proteolytic enzymes is thought to be an important criterion that determines allergenicity, or the sensitizing properties of, for example, genetically engineered food proteins (Astwood & Fuchs, 1996). Moreover, tryptic digests of β -lactoglobulin have been shown to bind specific IgE and there is evidence that some peptides derived from milk and wheat proteins carry such new epitopes (Huang *et al.*, 1985; Kushimoto & Aoki, 1985; Ferguson & Watret, 1988). Finally, the IgE-mediated recognition of allergens in the gastrointestinal tract may be important in atopic dermatitis (Kortekangas-Savolainen *et al.*, 1993).

The present *in vitro* study was undertaken to investigate the gastrointestinal stability of celery allergens by a simple procedure that has, with some modifications, previously been

used to analyze the digestibility of β -lactoglobulin (Haddad *et al.*, 1979), flour components (Sutton *et al.*, 1982) and recombinant proteins from transgenic plants (Astwood & Fuchs, 1996). The exact procedure applied in this investigation has been used to study the stability of baker's yeast allergens under gastric and intestinal conditions (Kortekangas-Savolainen *et al.*, 1993). Our results obtained with celery suggest that residual allergenic potency can be present in this vegetable after passage through the stomach and even the duodenum.

MATERIALS AND METHODS

Enzymatic Digestion

Allergen extract from native celery roots was prepared as described (Jankiewicz *et al.*, 1996, 1997). Gastric and duodenal conditions were simulated by commercial enzyme tablets, as described by Kortekangas-Savolainen *et al.* (1993). The enzyme activities of the two preparations were determined by the manufacturers according to the German pharmacopoeia (Hartke, 1986). The activities were calculated as FIP units (Federation International Pharmaceutique). The tablets are pharmaceuticals and are recommended as food supplements for people with reduced pepsin production or for those who suffer from a reduced function of the pancreas. The procedure applied here is thought to be suitable for a general approach to study suspected food allergens (Kortekangas-Savolainen *et al.*, 1993).

The gastric tablets (Enzynorm; Nordmark, Hamburg, Germany) contained 350 mg of porcine stomach extract per tablet, corresponding to 62.5 mg of pepsin and 40 protease units. The gastric solution consisted of one Enzynorm tablet in 100 ml of 0.15 M-NaCl. Festal tablets (Cassella-Med, Köln, Germany) consisting of porcine pancreas extract, with proteases, amylases and lipases, were used to mimic duodenal conditions. Compared to other mammals such as cattle, porcine pancreatin is known to contain an enzyme pattern relatively close to that in humans (Hartke, 1986). Each of these tablets contained 6000 FIP units of pancreatic lipases, 4500 FIP units of pancreatic amylases and 300 FIP units of pancreatic proteases. Pancreas proteases consist of trypsin, chymotrypsin, elastase, endopeptidases, exopeptidases and carboxypeptidases A and B (Schmidt & Thews, 1990). The activities of these enzymes are not assayed separately by the official German method. The duodenal solution was prepared by dissolving one Festal tablet in 100 ml of 0.15 M-NaCl. The time schedule as well as the pH value were close to the major passage times obtained by a complex continuous model, which is thought to be close to *in vivo* conditions (Houben & Penninks, 1996). Two hours were taken as an average time for stomach passage. This period is twice as long as the gastric digestion used in the allergy assessment of proteins from transgenic food plants (Astwood & Fuchs, 1996). 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. Due to Kortekangas-Savolainen *et al.* (1993), an almost complete degradation of yeast allergens was obtained within this period.

The procedure was as follows. Protein (5 mg) from native celery roots in 5 ml of 0.01 M-phosphate-buffered saline (PBS) was treated with 1 ml of gastric solution at 37°C and a pH of 1.25 for 2 h. Subsequently, the pH was adjusted to 6.8, 2 ml of duodenal solution were 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 celery protein (pH 1.25, 2 h). After adjusting the pH to 7.4, all samples were freeze dried and stored at -20°C until used. Aliquots of the reconstituted samples were subjected to ultrafiltration using disposable centricon microconcentrators (Amicon, Witten, Germany) with a cut-off of 3 kDa. After five washings with distilled water, the filtrates and retentates were freeze dried and reconstituted to the original volume either with PBS (retentate) or distilled water (filtrate) prior to use.

Polyclonal Antisera

Api g 1 was immuno-detected by means of a rabbit antiserum (aBv) which had been raised against Bet v 1 (Allergopharma, Reinbek, Germany). Due to the structural similarities of the two allergens, this serum strongly cross-reacted with Api g 1 (Schöning *et al.*, 1995; Jankiewicz *et al.*, 1996; Vieths *et al.*, 1996c). A rabbit antiserum against celery profilin (aCp) (kindly supplied by Dr P. Deviller, Lyon, France) was used to detect antigenic profilin in celery extract and the digested preparations (Vallier *et al.*, 1992; Vieths *et al.*, 1995a; Jankiewicz *et al.*, 1996).

Patients' Sera

All sera were taken from birch pollen allergic patients with pollen-related food allergies at the Department of Dermatology and Allergology, Hospital Borkum Riff, Germany (Head Physician: Dr H. Aulepp). Most of the patients were also sensitive to grass pollen and/or weed pollen (for further data, see Jankiewicz *et al.*, 1996). Sera with increased levels of IgE specific for celery (EAST class ≥ 2 , see later) were used as probes to characterize the immunoreactivity of celery allergens during *in vitro* digestion. Serum from a non-atopic individual was used as a negative control. The allergen specificity of human IgF was analyzed by comparative immunoblot testing with the rabbit antibodies. IgE binding to CCD was determined by a glycan ELISA which was kindly performed by Dr F. Altmann, Austria, Vienna (Tretter *et al.*, 1993; Petersen *et al.*, 1996). Results of these experiments were confirmed as follows. (i) Pre-incubation of sera with 10–15 μg of purified Bet v 1 prior to immunoblotting deleted IgE binding to Api g 1 but not to other celery allergens (Vieths *et al.*, 1995a). (ii) Immunoblotting with profilin that was purified by poly-L-proline affinity chromatography resulted in a typical double band at 15–16 kDa (Vallier *et al.*, 1992; Vieths *et al.*, 1995a). (iii) IgE binding to carbohydrate determinants on multiple allergen bands was destroyed by incubating blot strips in 0.1 M-NaIO₄, pH 5.0, for 2 h as described (Vieths *et al.*, 1994a, 1995a).

Electrophoresis and Immunoblotting

Electrophoresis and immunoblotting were carried out as previously described (Vieths *et al.*, 1992a, 1995a). Briefly, the extracts were separated by discontinuous SDS-PAGE according to the method of Lämmli (1970), using 5% (w/v) stacking gels (1.0 mm, T = 5.0%, C = 2.6%) and 13% (w/v) resolving gels (T = 12.8%, C = 2.7%) where T = total acrylamide and C = cross-linker. The protein load on the gels was 18 $\mu\text{g cm}^{-1}$ on 5.5-cm preparative slots. After separation, the extracts were transferred on to nitrocellulose (NC) membranes by semi-dry blotting (Khyse-Andersen, 1984). Cut NC-strips (0.4 mm) were either stained for total protein with India ink or used for immuno-detections. The latter were performed by incubating one NC-strip in 1.0 ml of diluted serum. The sera were diluted as follows: human sera: 1:6.7; Rabbit aBv: 1:2000; aCp: 1:5000. Immuno-detections were carried out by an amplified system that is based on a biotinylated tertiary antibody and a subsequent streptavidin horseradish peroxidase conjugate incubation (Vieths *et al.*, 1992a, 1994b). Bound enzymatic activity was stained with 3,3',5,5'-tetramethylbenzidine. In addition, pure recombinant Api g 1 (Biomay, Linz, Austria) was used for immunoblot inhibition in selected experiments.

Determination of Specific IgE

Extract of native celery roots was adjusted to a total protein concentration of 12 $\mu\text{g ml}^{-1}$ and covalently coupled to cyanogen bromide-activated filter paper disks as described for apples and extracts from other plant foods (Vieths *et al.*, 1992b, 1994a, 1995b). After enzymatic digestion, the celery hydrolysates as well as the filtrates and retentates of the ultrafiltration experiments were subjected to the same procedure. The concentrations for coupling corresponded to the protein amount of the untreated samples. The allergen disks

were incubated with 50 μ l of human serum overnight at room temperature. After washing, the amount of IgE specifically bound to the immobilized allergens was semi-quantitatively determined by a commercial Enzyme Allergosorbent Test (EAST) (Allergopharma, Reinbek, Germany), using the calibration system of the manufacturer. The results were expressed as EAST classes or as kU l^{-1} (class 0: no specific IgE; class 4: very high content of specific IgE corresponding to 17.5 kU l^{-1}). To distinguish more precisely EAST classes were expressed with one decimal place (Vieths *et al.*, 1994b, 1995b).

IgE Inhibition Assays

A quantitative estimation of the IgE binding potency of the celery preparations was derived from a competitive ELISA inhibition assay. Patient sera were diluted to an optical density (OD) of approximately 70% of the EAST signal (usually 1:2–1:10) in 0.05 M-Tris buffer, pH 7.4, 0.15 M-NaCl, 0.3 g l^{-1} bovine serum albumin (BSA) and 0.1% (v/v) Tween 20 (TBS/BSA/Tween). A 10-fold dilution series of the inhibitor extracts was prepared in five steps in TBS/BSA/Tween. Volumes of 50 μ l of diluted serum and 50 μ l of inhibitor solution were incubated with an allergen disk overnight at room temperature. Subsequently, the EAST was continued according to the standard procedure. The results were expressed as % inhibition. Extract potencies were quantitatively compared by estimating the protein concentration responsible for a 50% inhibition of the IgE binding to the solid phase (C_{50}) from the inhibition graphs. In the case of the digests, these C_{50} values were calculated on the basis of the initial protein content of the untreated samples. Control inhibitions were performed with ovalbumin and extract from low-fat milk at a single protein concentration of $250 \text{ } \mu\text{g ml}^{-1}$. Generally, all determinations were run in duplicate.

Rat Basophil Leukaemia 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 (Hoffmann *et al.*, 1997a,b). The assay is based on a culture of permanently growing rat basophil leukaemia cells. The subline RBL-2H3 that was selected for maximal mediator release (kindly supplied by Dr I. Pecht, Weizmann Institute, Rehovot, Israel) (Barsumian *et al.*, 1981) was used in all experiments. These cells represent functional analogues of mucosa mast cells and can be passively sensitized with murine and rat IgE, but not with human IgE. Dose-related allergen-specific mediator release can be obtained from sensitized cells at allergen concentrations in the range of pg ml^{-1} . 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. We have optimized a procedure for raising murine IgE specific for important major allergens such as phospholipase A_2 from honeybee venom, Fel d 1 from cat dander or Bet v 1 from birch pollen. The mediator release in the following assay has been shown to be very specific and allowed the low detection thresholds mentioned earlier (Hoffmann *et al.*, 1997a, b). The procedure was also tested for allergenic foods such as apple, peanut and celery tuber (Hoffmann *et al.*, 1997c). In this case, the IgE response was found to be comparable to that obtained in the majority of allergic humans.

Briefly, Balb/c or CBA/J mice were injected four or five times with 5 μ l of allergen extract containing 1 μg of protein adsorbed to 5 mg of $\text{Al}(\text{OH})_3$ in 0.5 ml PBS at intervals of 2 weeks. Final bleeding was performed 10 days after the last immunization. Sera of at least four responding mice (criterion: at least 30% mediator release at an allergen concentration of $0.1 \text{ } \mu\text{g ml}^{-1}$ and a serum dilution of 1:50) were pooled.

The development of reaginic antibodies was monitored directly by the mediator release assay. For isotype controls, mediator release experiments were run with native and heat-inactivated serum to distinguish between IgE and IgG, and confirmed by stimulation with anti-mouse IgG and anti-mouse IgE. It was shown that the reaginic sera always contained IgE (Hoffmann *et al.*, 1997a). Subsequently, the assay was performed as follows.

After raising specific IgE against extract from native celery in Balb/c mice, a serum pool was prepared from at least four responding mice. Permanently growing RBL-2H3 cells were harvested in the stationary phase, transferred at 10^5 per 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% of fetal calf serum (FCS). The attached cells were passively sensitized for 1 h with pooled murine reagenic antiserum, diluted 1:65 in MEM, 5% FCS. After sensitization, the cells were washed three times with Tyrode's buffer. For cross-linking and degranulation, the RBL cells were incubated with 100 μ l of serial allergen extract dilutions in a humidified atmosphere at 37°C for 1 h. The specific release was assayed by measurement of β -hexosaminidase activity, which is present in the same granula as histamine (Schwartz *et al.*, 1981). 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 in % of the total release after subtraction of the spontaneous release (Hoffmann *et al.*, 1997a,b).

RESULTS AND DISCUSSION

SDS-PAGE and Immunoblotting

According to the procedure originally described for yeast allergens, the influence of digestion on extract from native celery roots was monitored by SDS-PAGE and immunoblotting (Kortekangas-Savolainen *et al.*, 1993). After electrophoretic separation, the celery proteins were transferred to NC and the protein pattern was visualized with India ink (see India ink lanes of Figure 1). When compared to the native sample (not shown), no changes in the protein pattern were observed in the control preparation that was subjected to the whole procedure without adding the enzymes. The undigested control contained at least 50 distinct protein bands in the mass range 10–70 kDa (Figure 1). Gastric treatment resulted in a reduction of bands in the mass range above 43 kDa. Subsequent duodenal treatment led to a stronger degradation of the whole protein pattern except some bands below 20 kDa and at 38 kDa.

The antigenicity of Api g 1 and celery profilin was tested with the rabbit antisera aBv and aCp respectively. On the basis of the initial protein content, gastric treatment decreased the intensity of the Api g 1 band recognized by aBv, whereas profilin was recognized by aCp in the peptic digest with approximately the same intensity as before. A further decrease of antigenicity of both allergens resulted when the celery extract was subsequently subjected to duodenal treatment (Figure 1).

Similar experiments were undertaken with patients' sera which contained IgE specific for Api g 1 (serum 196), profilin (serum A21) and multiple bands at higher molecular masses (HMM) that were due to the recognition of CCD (serum 064). The latter serum contained additional IgE antibodies against Api g 1 at 16 kDa. The selection of sera from individual patients was based on the results of previous experiments (Jankiewicz *et al.*, 1997). In the case of thermal and non-thermal processing of celery, immunoblot testing was performed with a total number of 25 sera. It was shown that, unless some individual variation, data of allergen stability obtained from single patients seem to be representative. Sera from typical patients of this group were selected for the present study. Moreover, sera of patients with an exclusive monosensitization to one celery allergen were required to test the stability of allergens by IgE inhibition in the native EAST system. Since such sera are difficult to obtain, the number of patients was not increased.

In the case of Api g 1 and profilin, the immunoblot results were in accordance with those of the rabbit antisera. The reactivity of serum 064 to the HMM bands was only weakly influenced by peptic treatment, but decreased clearly after application of pancreatic

enzymes (Figure 1). However, a weak reactivity to all three allergenic structures survived even after 45 min at gut conditions. In the pancreatic digest, Api g 1 split into a double band at approximately 16 kDa (sera 196 and 064) and an additional faint band occurred at 40 kDa. The possibility that these bands were due to epitopes not present on native Api g 1 was tested by IgE inhibition experiments as described in the following. No antibody binding occurred with the serum of a non-atopic individual (results not shown). Antigenic degradation products were not recognized by the rabbit antibodies.

Determination of Specific IgE

To investigate whether the digestion resulted in a loss of the total IgE binding activity, native celery, the control extract and both hydrolysates were coupled to activated filter paper disks using coupling dilutions that corresponded to the protein content before digestion. When the allergen disks were used for semi-quantitative EAST analyses of the patients' sera, equally high capacities of IgE binding were measured for all preparations (Table 1). Since serum 064 showed an additional reactivity to Api g 1 on immunoblots (Figure 1), this serum was replaced by Bo 153 which exclusively recognized multiple bands at HMM and also presented IgE binding to CCD in a glycan ELISA (Jankiewicz *et al.*, 1997). The results of the EAST were not in complete agreement with the immunoblotting results. To test the hypothesis whether the clearly detectable IgE binding capacity of the digests was due to IgE-reactive small proteolytic fragments at the solid phase, the samples were crudely fractionated by ultrafiltration. An aliquot of each sample was passed through a microconcentrator containing ultrafiltration membranes with an exclusion limit of 3 kDa. After lyophilization, retentates and filtrates were diluted to the initial volume and again used to prepare allergen disks. This procedure was chosen to analyze the proportion of the IgE reactivity present in both fractions and to relate the data to the activity of the whole digest. The results of the subsequent EAST determinations with the above sera revealed EAST classes between 2.2 and 3.6 in the HMM fraction of native celery and the peptic digest. After combined gastric and duodenal digestion, EAST classes of 1.6–2.1 were measured in the same fraction. In all cases, no IgE binding activity could be detected in the low molecular mass fraction. All EAST determinations were negative (<class 1) with the non-atopic control serum. Comparison with the IgE binding to the whole digests (Table 1) revealed weakly reduced reactivity of the filtrates, which is most probably caused by adsorptive losses of proteins or peptides. We concluded that the remaining allergenic potency detectable after digestion is most probably due to intact allergens or fragments of molecular masses above 3 kDa. However, it could not be excluded that small immuno-reactive peptides could not be immobilized at the solid phase and have been overlooked by assaying both the whole digests and the fractions.

IgE Inhibition Assays

Dose-related EAST inhibition experiments with sera of known specificity were used to obtain a more quantitative relation of the immunologic activity of the hydrolysates and the

TABLE 1. Determination of specific IgE to native celery and celery after enzymatic digestion by EAST

Serum	Allergen specificity	Native celery	pH/T control	Peptic digest (gastric)	Pancreatic digest (gastric + duodenal)
196	Api g 1	2.8	2.5	2.5	2.7
A21	Profilin	3.7	3.4	2.6	3.8
Bo 153	CCD	2.5	2.5	2.2	2.1

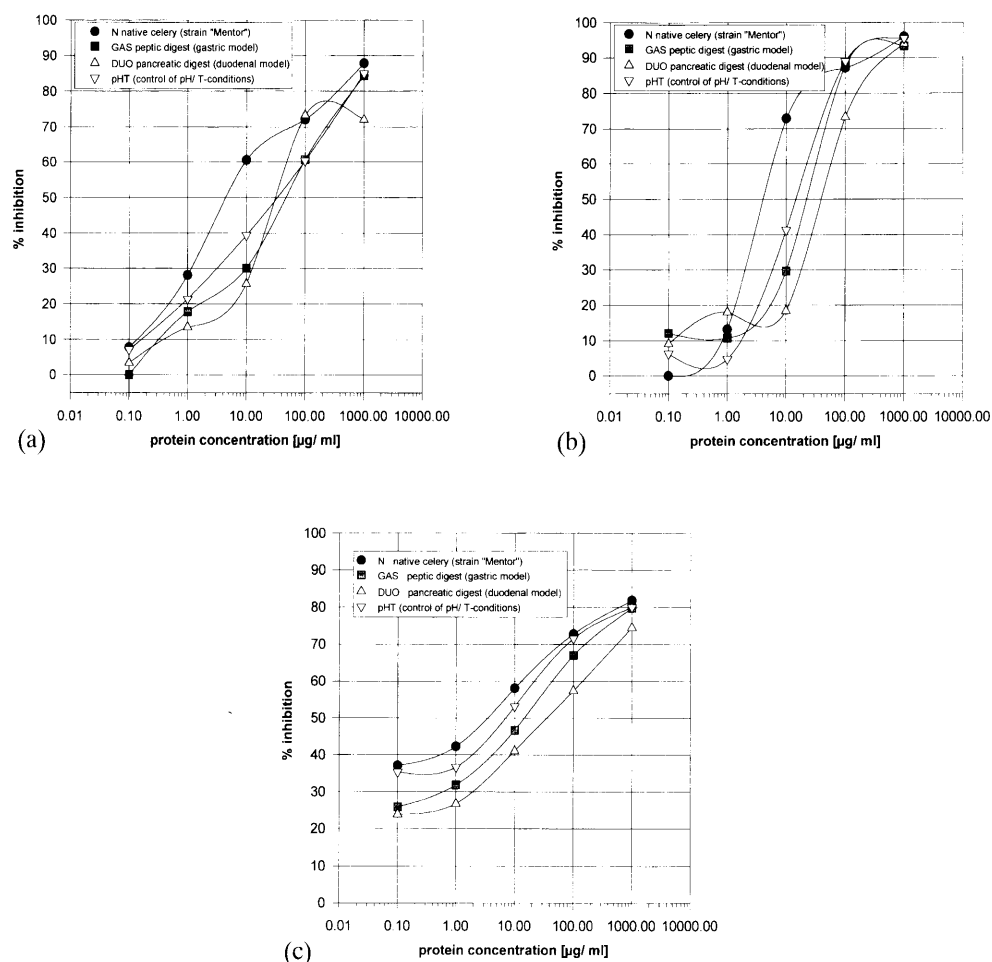


FIG. 2. Results of dose-related inhibition of IgE binding to allergen disks with native celery allergens. The sera were diluted 1:2.5. Extracts from native celery roots and the digested preparations were used as the inhibitors as indicated in the figure. Results are expressed as % inhibition. Controls were performed with 250 mg ml⁻¹ of ovalbumin and the same amount of protein from low-fat milk as described in the text. (a) Serum 196 with IgE exclusively directed against Api g 1. (b) Serum A21 from a patient monosensitized to profilin. (c) Serum of patient Bo 153 whose IgE antibodies are directed against carbohydrate structures on glycoproteins.

control sample to the immunoreactivity of native celery (Figures 2(a)–2(c)). The calculated 50% inhibition concentrations of all experiments reflecting the inhibition potency are presented in Table 2. Native celery, control-treated celery and the gastric digest almost completely inhibited IgE binding to the native celery allergens at the initial protein concentration of 1000 μg ml⁻¹, and this strong inhibition occurred with IgE against Api g 1, profilin and CCD respectively. In the case of sera Bo 153 (Figure 2(c), CCD) and 196 (Figure 2(a), Api g 1), pancreatic digestion reduced the maximal inhibition capacity from 80 and 85 to 75% respectively. However, the C₅₀ values indicated changes of the inhibition potency (Table 2). To some extent, a decrease of the reactivity was related to the control treatment of celery extract in the absence of enzymes. In the case of profilin (serum A21, Figure 2(b)), for example, the pH/T control had only approximately one-third of the inhibition potency of the native extract, and the potency was reduced to 50% for serum

TABLE 2. Results of dose-related inhibition of IgE binding to allergen disks with native celery allergens. The allergenic potencies of the preparations were estimated by calculation of the protein concentration responsible for a 50% inhibition of the antibody binding to the solid phase (C_{50})

Serum	Specificity	Inhibitor	C_{50} ($\mu\text{g/ml}$ of protein)
196	Api g 1	Native Celery	30
		pH/T control	34
		Peptic digest	48
		Pancreatic digest	200
A21	Profilin	Native Celery	4.0
		pH/T control	14
		Peptic digest	22
		Pancreatic digest	40
Bo 153	CCD	Native Celery	3.4
		pH/T control	7.0
		Peptic digest	15
		Pancreatic digest	36

Control inhibitions were performed with $250 \mu\text{g ml}^{-1}$ of ovalbumin (Ova) and the same amount of protein from low-fat milk. These resulted in the following levels of inhibition (%): serum 196: Ova 0%, milk 1%; serum A21: Ova 14%, milk 17%; serum 153: Ova 28%, milk 31%.

Bo 153 (Figure 2(c)). However, with all three sera the inhibition potency was further reduced by gastric treatment and again by the subsequent duodenal digestion. The differences which occurred between IgE inhibition and immunoblotting in the case of the native extract and the pH/T control may be due to the destruction of conformational epitopes by the denaturing sample treatment of the native extract prior to SDS-PAGE. Such epitopes may also be affected by the control treatment. In contrast to the enzymatic degradation of the protein antigens, one would not expect an influence of the digestion on the inhibition potency of CCD. This was, however, observed to some extent in the present experiments (Figure 2(c)). Explanations for this could be the involvement of parts of the peptide backbone which are sensitive to digestion in the epitope structure, or the cleavage of glycans by pancreatic enzymes. Controls with $250 \mu\text{g ml}^{-1}$ of ovalbumin and protein from low-fat milk led to weak inhibitions below 20% for sera 196 and A21. In the case of serum Bo 153, ovalbumin caused 28% inhibition and low-fat milk 31% inhibition of the IgE binding to celery allergens. Since this serum contained carbohydrate-specific IgE, these results are most probably due to cross-reacting carbohydrate moieties in the control solutions.

Further inhibitions were performed to test the hypothesis that the 16-kDa double band and the band at 40 kDa that were stained by IgE from serum 196 after intestinal digestion were due to epitopes of native celery allergens. Allergen disks with native celery and both digests were incubated with $50 \mu\text{l}$ of serum 196 diluted as earlier and $50 \mu\text{g}$ of protein from native celery. Ovalbumin was again used as a negative control. The inhibitions measured with allergen disks of native celery, gastric digest and duodenal digest were 94, 95 and 92% respectively. The control inhibitions with ovalbumin were below 10% with all three allergen disks. Moreover, blot strips with separated duodenal digest were incubated with serum 196 and after pre-incubation of the same serum with $100 \mu\text{g}$ of protein from native celery and $10 \mu\text{g}$ of recombinant Api g 1. In both experiments, the IgE binding to the new bands at 16 kDa and 40 kDa was completely inhibited (results not shown). Taken together, these results indicated that all epitopes of the new bands were present in undigested celery and on its major allergen. The band at 40 kDa may thus be due to aggregates of Api g 1 and/or its digestion products.

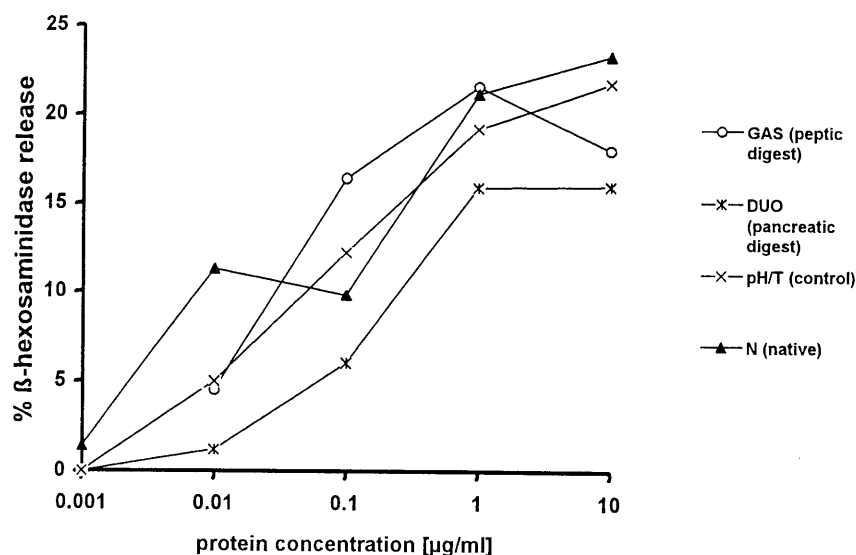


FIG. 3. Dose-related mediator release of RBL cells sensitized with a murine reagenic antiserum against extract from native celery roots. Prior to sensitization, the serum was diluted 1:65. Stimulator extracts as indicated in the figure. The results are expressed as % of the total release after subtraction of spontaneous release. No release was measured after stimulation with peanut extract as the negative control.

Mediator Release Assay

Cross-linking of allergen-specific IgE on the surface of mast cells is one basic event of the type I allergic reaction. The subsequent mediator release is responsible for eliciting symptoms in allergic patients. For ethical reasons, and due to the problems of recruiting a sufficient number of allergic volunteers, only a strictly limited number of experiments to test this mechanism can be performed in humans or *in vitro* with cells of allergic patients. Thus, a murine model was developed which is based on a permanent culture of RBL-2H3 cells (Hoffmann *et al.*, 1997a). These cells were passively sensitized with a serum pool of mice which had been immunized with extract from native celery to generate allergen-specific IgE. Due to the low amount of serum obtainable from individuals, pooled murine sera had to be applied to allow the screening of an extended range of samples with a standardized procedure. Subsequent to sensitization, the ability of triggering a specific mediator release was tested with the digested and undigested celery extracts. The stimulator protein concentration ranged from $0.001 \mu\text{g ml}^{-1}$ to $10 \mu\text{g ml}^{-1}$, calculated on the basis of the content of the undigested native sample. The protein concentrations were not increased to higher levels to avoid non-specific release effects observed in initial optimization experiments. After triggering, the mediator release was determined by measuring the activity of β -hexosaminidase in the cell supernatants. As shown in Figure 3, all four celery preparations had a high allergenic activity in this model system. With respect to the inherent variation of the cell culture system, a clear decrease of the activity was only observed after combined gastric and pancreatic treatment (indicated by 'DUO' in Figure 3).

CONCLUDING REMARKS

The results of the present investigation indicate that allergenic activity can be present in native celery roots after treatment with gastric enzymes, and can even survive the initial phase of subsequent duodenal digestion. Four immunological methods were applied: immunoblotting, determination of allergen-specific IgE by EAST, a competitive IgE

inhibition assay, and testing the cross-linking capacity of allergens by a rodent RBL cell mediator release assay. The data obtained by these methods showed some variations which are most probably due to specific features of the test systems. However, the following general conclusions can be drawn from our experiments:

- The whole protein pattern was obviously degraded by the model digestion, first under gastric conditions, and more pronounced during subsequent pancreatic treatment.
- Irrespective of whether the sample was digested or not, a high allergenic activity, or the presence of active IgE binding allergens, was indicated by all four methods. This activity was related to all three allergenic structures of celery, namely Api g 1, profilin and carbohydrate determinants.
- As expected, the pancreatic digest contained the weakest activity, whereas no concordant differences were obtained for the gastric digest, the acidic buffer control and native celery.

However, the proteolytic experiments described here were performed as a simple two-step procedure that is different from the continuous process in the human gastrointestinal tract. Despite this drawback, several aspects indicate that the results are also important for humans. First, the conditions are comparable to those used by Astwood and Fuchs (1996) for allergy assessment of recombinant proteins. The experimental details applied by these authors are based on the United States pharmacopoeia. In their study, exclusive gastric digestion for a maximum of 60 min was suitable to obtain a complete degradation of non-allergenic proteins and a weak reduction of several allergens due to the results of SDS-PAGE. Moreover, Kortekangas-Savolainen *et al.* (1993) described an almost complete loss of immunoreactivity of yeast allergens by the procedure that was applied in the present study. Therefore, we feel that our results on celery can at least be related to those obtained in similar models for other allergenic and non-allergenic proteins.

Extract from native celery roots was selected for the model experiments. Since celery is consumed either native or after application of heat, this decision needs to be explained. Celery belongs to the allergenic foods which almost exclusively affect pollen-allergic patients. The common view about this phenomenon of pollen-related food allergies is that the inhalation of pollen allergens initiates the primary immunogenic stimulus (Dreborg, 1988; Ebner *et al.*, 1991; Vieths *et al.*, 1995b; Schwedler *et al.*, 1996). This stimulus might be enhanced or modified by the consumption of native plant foods containing cross-reactive allergens. If oral uptake of heat-denatured celery could lead to the patients' sensitization, one would expect to identify some celery-allergic patients who are not or only weakly sensitized against pollen allergens. Moreover, in some patients the IgE reactivity to heated celery should exceed the antibody binding to native celery proteins. However, this was neither observed in our experiments with sera of 46 celery-sensitized patients (Jankiewicz *et al.*, 1996) nor described in the literature. The heat-stable allergenic component of celery hence represents a residual activity, but is not due to neoallergens or new allergenic determinants that are created by the heating process (Jankiewicz *et al.*, 1997). Consequently, as the worst case, the ingestion of native celery was simulated in our gastrointestinal model. Since the allergenicity of celery is strongly reduced by thermal processes of food technological treatment for the majority of patients (Jankiewicz *et al.*, 1997), it would, in our opinion, not be useful to perform a similar series of labour-intensive experiments with heated celery roots.

In contrast to the majority of proteins which were present in the celery extract, the allergenic proteins were obviously relatively stable under the applied conditions. This is concordant with the view that many food allergens and other allergenic proteins resist, to some extent, the hydrolysis by proteolytic enzymes (Astwood & Fuchs, 1996; Houben & Penninks, 1996; Vieths *et al.*, 1996c). On the other hand, many non-allergenic plant proteins have been found to be degraded within seconds or minutes under gastric conditions (Astwood & Fuchs, 1996). In contrast to the findings of the latter authors, neither

immunoblotting nor separation by ultrafiltration led to the identification of immunoreactive peptide fragments in our experiments.

The findings of the present study are contrary to those of Kortekangas-Savolainen *et al.* (1993), who applied the same model on baker's yeast allergens. They reported a strong degradation of most of the IgE binding components. However, allergenicity was exclusively tested by immunoblotting. Since the allergen bands were clearly weaker after combined gastric and duodenal treatment in our study, restriction to immunoblotting would have led to an underestimation of allergenicity. In addition to the denaturing conditions of the electrophoresis, these differences may also be due to a higher sample capacity of the solid phase of the EAST or to lower detection limits of the EAST and the RBL cell assay. On the other hand, immunoblotting is the only suitable technique that supplies data about the stability of specific allergens when working with crude protein extracts from foods. We therefore propose not only to choose immunoblotting for testing the overall allergenic activity in such experiments. As shown in the present study, a panel of immunological methods that are based on divergent test principles will improve the reliability of the results.

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