

# Differentiating Cross-Reacting Allergens in the Immunological Analysis of Celery (*Apium graveolens*) by Mass Spectrometry

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**Celery is acknowledged as a major food allergen in Europe, and mandatory labeling for preprocessed foods has been implemented. However, no methods for the specific detection of celery protein in foods have been published. In the present study, a sandwich celery ELISA using polyclonal anticelery antibodies for capture and detection was developed and validated. The method has an LOD of 0.5 mg/kg in buffer; however, it is applicable only for the screening of food products because of extensive cross-reactivity with potato and carrot proteins. Using nanoLC–ion-trap MS/MS, a number of proteins in the three vegetable species were identified as candidates for causing cross-reactions due to amino acid sequence homologies. Among others, a novel patatin (Sola t 1)-like protein was detected in celery and a flavin adenine dinucleotide binding domain-containing protein (Api g 5)-like protein was identified in carrot. The utility of triple-quadrupole MS/MS for specific and quantitative analysis of celery, potato, and carrot allergens was evaluated using whole protein extracts. Several unique precursor ion-to-product ion transitions were determined for each species, suggesting the feasibility of developing an MS-based screening method to specifically detect celery allergens in foods.**

Celery (*Apium graveolens*) root, tuber (celeriac), and stick are consumed as raw or cooked vegetables. Because of its aromatic flavor, celery is also used as a spice in soups, salads, and sausages.

Allergy to celery is one of the most frequent food allergies in many European countries. Sensitization to celery often develops in relation to birch and/or mugwort pollinosis (1, 2). If the cross-reactivity becomes clinically manifest as oral allergy syndrome (OAS), it is often termed “birch-mugwort-celery syndrome” or “celery-carrot-mugwort-spice” syndrome (3, 4). Symptoms are often of the immediate type, ranging from relatively mild reactions to anaphylactic shock.

Allergic reactions occur predominantly to raw celery and less frequently to cooked celery. Response to celery spice is comparable to the raw celery (5, 6). Threshold doses for local and systemic symptoms have been determined in two double-blind of placebo-controlled food challenges (DBPCFC) with fresh and cooked whole celery (2, 6) at about 0.7–2.7 and 7.5–31 g, respectively. Celery spice elicited systemic reaction at doses as small as 0.2–5.9 g.

Four celery allergens have been previously described and characterized (4, 7–10). The heat-labile Api g 1 is a 16 kDa birch pollen-related, pathogenesis-related protein 10 (PR-10), which is recognized by 59–80% of celery allergic patients (4, 6, 11, 12). It represents about 20% of total soluble proteins in celery tuber (10). Two different isoforms have been cloned and expressed in vitro to obtain recombinant protein for diagnostics (7, 13).

Celery profilin Api g 4 was allergenic for 23–50% of patients in DBPCFC (4, 6, 14). The 14 kDa heat-stable panallergen profilin has been identified as a cause for extensive cross-reactivity in many plant species and seems to

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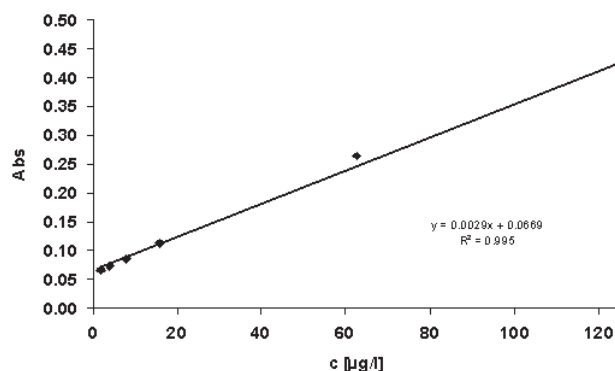


**Figure 1. Representative linear seven-point calibration curve of the celery sandwich ELISA, including SDs and regression parameters, based on the standard curve obtained using celery protein standard in assay buffer.**

be especially important in patients with the birch-mugwort-celery syndrome (8, 15).

The glycoprotein Api g 5, occurring in two isoforms with 53 and 57 kDa, contains cross-reactive carbohydrate determinants, which have been identified as major IgE-binding moieties (16, 17). The allergenicity of Api g 5, a flavin adenine dinucleotide (FAD)-binding domain-containing protein, seemed to depend on the glycosylation level and was completely abolished by chemical deglycosylation. However, thermal treatment did not significantly decrease IgE-binding (6, 10, 12). In patient studies, Api g 5 showed IgE-reactivity in 55% of the cases (4).

Api g 3, a chlorophyll-binding protein, was isolated from a cDNA library by screening with sera of celery-allergic patients (18). However, the relevance has not yet been demonstrated.

Celery is often used in prepacked foods, especially as a dried seasoning. Because inadvertent consumption is a considerable risk for allergic consumers, mandatory labeling of celery in the ingredients list of manufactured foods was implemented in Europe in 2005 (19). Specific and sensitive detection methods are required to test for compliance with labeling regulations. Although a number of DNA-based methods have been published and commercialized (20, 21), there are presently no protein-based immunoassays available.

Therefore, it was the aim of this study to evaluate the applicability of a newly developed ELISA for the detection of celery in foods, to elucidate potential interferences from cross-reacting proteins by proteomic analysis, and to investigate the use of MS as a confirmatory method.

## Materials and Methods

### Celery Extraction

Celery root powder (Toro A/S, Bergen, Norway), used as spice in processed foods, was homogenized in 50 mM Tris-glycine–1 mM dithioerythritol (DTE; Sigma Chemicals

Co., St. Louis, MO), pH 8.7 (w/v, 1:5), extracted overnight at 45 °C in a shaking water bath (OLS 200, Grant, Cambridge, UK) and centrifuged for 40 min at 4 °C and 15 000 g (J2-MC, Beckman Instruments, Palo Alto, CA). Proteins in the supernatant were precipitated with 65% ammonium sulfate (Merck, Whitehouse Station, NJ), at room temperature (RT) under continuous stirring, and centrifuged (30 min, 15 000 g, 4 °C). The pellet was resuspended in 50 mM Tris–1 mM DTE (pH 8.0) and dialyzed against water in a dialysis tube with 6–8 kDa pore size (Spectra/Por, Spectrum Medical Industries, Los Angeles, CA) and freeze-dried (Heto, Allerød, Denmark). The protein was dissolved and resuspended in phosphate-buffered saline (PBS), pH 7.4. Total protein content was determined by Lowry Protein Assay (BioRad Laboratories, Hercules, CA). The concentrated extract was used for rabbit immunization and as celery protein standard in immunological analysis. Aliquots stored at –20 °C were stable for at least 3 months.

### Extraction of Food Samples

Food samples for specificity testing were obtained in local retail stores. Some samples were analyzed both raw and after cooking for 20 min in water. Several processed foods were a gift from the Swedish National Food Administration (Uppsala, Sweden). The samples were homogenized in a mechanical blender (Retsch GmbH & Co., Haan, Germany) and extracted (w/v, 1:5) with 0.1 M Tris–0.5 M glycine, pH 8.7, as described above. After centrifugation (39 200 g, 25 min, 4 °C), the supernatants were filtered through glass wool, if required, and the total protein content was determined. The samples were stored in aliquots at –20 °C. They were stable for at least 3 months.

### Production, Purification, and Labeling of Anticelery Antibody

A polyclonal antiserum was raised against concentrated celery root powder extract by injecting a female Chinchilla rabbit subcutaneously with 1.5 mL of a diluted (1:1 Freund's complete adjuvant) 200 µg/mL protein solution. After a second immunization (1.5 mL, 1:1 Freund's incomplete adjuvant, week 4), and a third dose (50 µL, week 5), the rabbit was finally bled in week 10, and anticelery serum was obtained by centrifugation. The specificity and titer of the antiserum were monitored throughout the immunization period using noncompetitive ELISA.

The crude serum was desalted on PD-10 columns (Amersham Biosciences, Uppsala, Sweden), diluted 1:10 in 20 mM sodium phosphate buffer (pH 7.0; binding buffer), and purified by using a HiTrap r-Protein A FF-column (Amersham Biosciences) as previously described (22). The purified antibody had a concentration of 3 mg/mL. It was stored in aliquots at –80 °C until use.

For covalent conjugation with biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt (Sigma), the buffer was changed to 0.05 M carbonate-bicarbonate buffer, pH 9, by dialysis (3.5 kDa cut off membrane; Pierce, Rockford, IL) overnight at 4 °C. The conjugation reaction and

**Table 1. Validation parameters of the celery sandwich ELISA in several different matrixes**

| Matrix                     | Assay buffer | Taco mix <sup>a</sup> | Swedish sausage <sup>a</sup> | Dressing <sup>a</sup> | Pasta sauce <sup>a</sup> |
|----------------------------|--------------|-----------------------|------------------------------|-----------------------|--------------------------|
| Standard curve ( $R^2$ )   |              |                       |                              |                       |                          |
| Lin. reg. 1.95–125 g/L     | 0.993        | 0.968                 | 0.995                        | 0.980                 | 0.994                    |
| Pol. reg. 1.95–500 g/L     | 0.998        | 0.989                 | 0.996                        | 0.982                 | 0.999                    |
| LOD, g/L                   | 4.7          | 11.2                  | 7.3                          | 12.0                  | 16.5                     |
| mg/kg food                 | 0.5          | 1.1                   | 0.7                          | 1.2                   | 1.7                      |
| LOQ, g/L                   | 20.4         | 57.9                  | 29.1                         | 64.9                  | 38.8                     |
| mg/kg food                 | 2.0          | 5.8                   | 2.9                          | 6.5                   | 3.9                      |
| Accuracy, % recovery + SEM |              |                       |                              |                       |                          |
| 0.8 mg/kg                  | 86 ± 19      | 53                    | 80                           | 195                   | 93                       |
| 6.3 mg/kg                  | 109 ± 10     | 124                   | 112                          | 85                    | 109                      |
| Precision                  |              |                       |                              |                       |                          |
| Intra-assay variance (%CV) |              |                       |                              |                       |                          |
| 0.4 mg/kg                  | 13.2         | —                     | —                            | —                     | —                        |
| 4.1 mg/kg                  | 4.3          | —                     | —                            | —                     | —                        |
| Interassay variance (%CV)  |              |                       |                              |                       |                          |
| 0.4 mg/kg                  | 232          | —                     | —                            | —                     | —                        |
| 4.1 mg/kg                  | 16           | —                     | —                            | —                     | —                        |

<sup>a</sup> Matrix sample extracts were diluted 1:200;  $n = 1$  experiment.

conjugate purification were performed as described (22). The total protein concentration was measured and the biotinylated anticelery antibody was stored at  $-20^{\circ}\text{C}$  until use. The final concentration was 1.2 mg/mL.

### Celery Sandwich ELISA

The sandwich ELISA procedure was developed according to a previously established protocol (23), with minor modifications. Microtiter plates were coated with 100  $\mu\text{L}$ /well coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) containing 5  $\mu\text{g/mL}$  purified anticelery antibody. Celery protein standard and samples were added in assay buffer [PBS containing 1% bovine serum albumin (BSA)], and bound antigen was detected by adding 100  $\mu\text{L}$ /well biotinylated anticelery antibody diluted 1:10<sup>5</sup> in assay buffer, followed by incubation with 100  $\mu\text{L}$ /well horseradish peroxidase (HRP)-streptavidin conjugate (Zymed, San Francisco, CA) diluted 1:10<sup>5</sup> in assay buffer.

### Assay Validation: Specificity, LOD, LOQ, Accuracy, and Precision

The specificity of the anticelery antibody was assessed in cross-reactivity studies using extracts from vegetables, fruits, and typical food ingredients in processed foods. Before ELISA analysis, samples were diluted 1:20 (v/v) in assay buffer. Measured celery equivalents were compared to the total protein contents of the extracts, and retrieval rates were calculated.

The LOD for the celery sandwich ELISA was calculated from eight experiments as the mean of the measured content of duplicate buffer blank samples plus 3 times the SD of the mean value. The LOQ was calculated as the buffer blank mean value plus 10 times the SD of the mean value. Additionally, the LODs and LOQs in four complex food matrixes (taco mix, Swedish sausage, dressing, and pasta sauce) were estimated in the same way on the basis of one matrix-assisted standard curve for each food.

The accuracy of the celery sandwich ELISA was evaluated by performing recovery studies using assay buffer and four food matrixes, which were spiked with the celery standard protein at concentrations equivalent to 0.8 and 6.3 mg/kg celery protein in food. Recovery rates and standard errors of the means were determined for assay buffer from 14 independent experiments. The recoveries from different foods were estimated by one experiment in each case.

The precision of the celery sandwich ELISA was estimated for assay buffer at concentrations equivalent to 0.4 and 4.1 mg/kg celery protein in food. The intra-assay variance was determined from repeated analyses in one assay as the mean CV. The interassay variance was determined as the mean CV of duplicate analyses in eight independent experiments.

### Gel Electrophoresis and Immunoblot

Protein extracts (5  $\mu\text{g}$ ) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using 4–12% bis-tris pre-cast gels (Invitrogen, Carlsbad, CA; 22). Gels were either stained with

**Table 2. Specificity of the celery sandwich ELISA**

| Food/ingredient         | Scientific name                 | Protein content, mg/mL <sup>a</sup> | Celery protein equivalents, % <sup>b</sup> | Immunoblot <sup>c</sup> |
|-------------------------|---------------------------------|-------------------------------------|--------------------------------------------|-------------------------|
| Celery protein standard | <i>Apium graveolens</i>         | 6.0                                 | 100                                        | ++                      |
| Celery root powder      | <i>Apium graveolens</i>         | 1.3                                 | 5                                          | ++                      |
| Celeriac, raw           | <i>Apium graveolens</i>         | 0.9                                 | >100                                       | ++                      |
| Celeriac, cooked        | <i>Apium graveolens</i>         | 0.4                                 | >100                                       | +                       |
| Potato, raw             | <i>Solanum tuberosum</i>        | 1.8                                 | 9                                          | ++                      |
| Potato, cooked          | <i>Solanum tuberosum</i>        | 0.9                                 | >100                                       | +                       |
| Carrot, raw             | <i>Daucus carota</i>            | 0.7                                 | >100                                       | ++                      |
| Carrot, cooked          | <i>Daucus carota</i>            | 0.3                                 | >100                                       | (+)                     |
| Parsnip, raw            | <i>Pastinaca sativa</i>         | 1.7                                 | 41                                         | ++                      |
| Parsley, fresh          | <i>Petroselinum crispum</i>     | 3.9                                 | 18                                         | ++                      |
| Parsley, dried          | <i>Petroselinum crispum</i>     | 25                                  | 1                                          | (+)                     |
| Caraway                 | <i>Carum carvi</i>              | 12                                  | 5                                          | +                       |
| Hazelnut, raw           | <i>Corylus avellana</i>         | 39                                  | 6                                          | +                       |
| Fennel, raw             | <i>Foeniculum vulgare</i>       | 40                                  | 4                                          | ++                      |
| Fennel seeds            | <i>Foeniculum vulgare</i>       | 11                                  | 2                                          | ND                      |
| White cabbage, raw      | <i>Brassica oleracea capit.</i> | 1.3                                 | 19                                         | ND                      |
| Morello cherry          | <i>Prunus avium</i>             | 5.5                                 | 12                                         | ND                      |
| Common dill, fresh      | <i>Anethum graveolens</i>       | 3.4                                 | 12                                         | ND                      |
| Common dill, dried      | <i>Anethum graveolens</i>       | 29                                  | 2                                          | ND                      |
| Radish, raw             | <i>Raphanus sativus</i>         | 1.1                                 | 7                                          | ND                      |
| Soybean, raw            | <i>Glycine max.</i>             | 31                                  | 6                                          | ND                      |
| Ginger                  | <i>Zingiber officinale</i>      | 30                                  | 4                                          | ND                      |
| Aniseed                 | <i>Pimpinella anisum</i>        | 17                                  | 3                                          | ND                      |
| Almond, raw             | <i>Prunus dulcis</i>            | 19                                  | 3                                          | ND                      |
| Chervil                 | <i>Anthriscus cerefolium</i>    | 3.4                                 | 2                                          | ND                      |
| Garden lovage           | <i>Levisticum officinale</i>    | 14                                  | 1                                          | ND                      |
| Cinnamon                | <i>Cinnamomum zeyanicum</i>     | 56                                  | 1                                          | ND                      |

<sup>a</sup> Following extraction of 2 g food with 10 mL buffer.<sup>b</sup> Recovery of celery standard protein equivalents.<sup>c</sup> Cross-reaction with anticelery antibody in immunoblot. Immunoblot signal intensities: ++ = strong; + = medium; (+) = weak; ND = not detected.

SimplyBlue™ SafeStain (Invitrogen) or the separated proteins were electrophoretically transferred onto nitrocellulose membranes. For immunoblot analyses, membranes were blocked with Tris-buffered saline containing 0.05% Tween-20 (TBST) and 1% BSA for 1 h, following incubation with the purified anticelery antibody (1:10<sup>4</sup> diluted in TBST) overnight at 4 C. Signals were developed using goat anti-rabbit IgG HRP conjugate (1:5000) and tetramethyl benzidine solution (both from Zymed).

#### Sample Preparation for MS Analysis

Protein bands of interest were excised from SDS-PAGE gels and the gel slices were placed in microfuge tubes, sliced into pieces, and destained by shaking with acetonitrile (ACN)/50 mM NH<sub>4</sub>HCO<sub>3</sub> (50:50, v/v) at RT for 10 min. The liquid was discarded, and the procedure was repeated twice. In

the case of persistent blue color, the gel was treated with 100 mM NH<sub>4</sub>HCO<sub>3</sub> at 50 C for 10 min. After drying in a Speed-Vac centrifuge (Heto) for approximately 10 min, proteins in the gel were reduced with 1.5 mg/mL DTE in 25 mM NH<sub>4</sub>HCO<sub>3</sub> at 56 C for 1 h in a heating block (Dri-Block, Techno, Duxford, UK). The incubation mixture was cooled to RT, and the supernatant was discarded after centrifugation at 13 000 g for 2 min (Eppendorf GmbH, Hamburg, Germany).

Proteins were alkylated by incubation with 10 mg/mL iodoacetamide (Sigma) in 25 mM NH<sub>4</sub>HCO<sub>3</sub> at RT for 45 min in the dark under shaking (Shuttler MTS 4I, IKA-Werke GmbH, Staufen, Germany). The supernatant was discarded and the gel pieces were washed once with 100 mM NH<sub>4</sub>HCO<sub>3</sub> and twice with ACN/50 mM NH<sub>4</sub>HCO<sub>3</sub> (50:50, v/v), each time

**Table 3. Celery protein content in different food samples measured by celery sandwich ELISA**

| Food                 | Labeled with celery | Celery protein, mg/kg | Possible cross-reacting ingredients |
|----------------------|---------------------|-----------------------|-------------------------------------|
| Oriental stew        | No                  | 90.6                  | Potato starch                       |
| Air-dried sausage    | No                  | 10.8                  | —                                   |
| Bread                | No                  | <LOD                  | —                                   |
| Meatballs            | No                  | <LOD                  | —                                   |
| Spiced dip           | No                  | 106                   | Potato starch                       |
| Ready-to-eat pap     | Yes                 | 8.8                   | Carrot, parsnip                     |
| Sea salt with herbs  | Yes                 | 17.9                  | Parsley, lovage                     |
| Sweet and sour sauce | Yes                 | 21.2                  | Carrot                              |
| Vegetable/meat soup  | Yes                 | 35.8                  | Carrot, parsnip                     |
| Vegetarian beefsteak | Yes                 | 309                   | Carrot, potato, parsnip, parsley    |
| Mixed herbs sauce    | Yes                 | 28.4                  | —                                   |
| Vegetable soup       | Yes                 | 143                   | Carrot, potato starch               |

for 10 min at RT under shaking. Gel pieces were dried by Speed-Vac centrifugation.

Proteins were digested *in situ* in the gel pieces by adding a few microliters of 0.1 g/mL trypsin solution (Trypsin Gold MS grade, Promega, Madison, WI), which had been prepared by diluting 1 mg/mL trypsin in 50 mM acetic acid with 25 mM  $\text{NH}_4\text{HCO}_3$ . The gel pieces were covered with ACN/10 mM  $\text{NH}_4\text{HCO}_3$  (10:90, v/v) and incubated at 37 °C overnight.

The tryptic protein fragments were collected from the supernatant. Additionally, two extractions were subsequently performed by shaking the gel pieces with ACN/1% formic acid (10:90, v/v) for 10 min at RT and then ACN/1% formic acid (50:50, v/v), each time collecting the supernatant. Finally, the volume of the pooled supernatants was reduced to about 7 L in a Speed-Vac centrifuge.

The tryptic peptides were mixed with 0.3 L concentrated formic acid and 0.1% trifluoroacetic acid to a final volume of 15 L.

#### *NanoLC-IonTrap MS/MS*

Tryptic celery peptides were analyzed by reversed-phase nanoscale LC nanoelectrospray ion-trap MS (nanoLC-ion trap MS/MS) on an Agilent 1100 HPLC system using a 75 m 15 cm column (Agilent Technologies, Palo Alto, CA) and an Agilent LC/MSD Trap XCT Plus (Agilent Technologies) mass spectrometer. Peptides were eluted using a gradient of solvent A (0.1% formic acid) and solvent B (90% ACN, 0.1% formic acid) at a flow rate of 300 nL/min. The gradient was ramped from 3 to 8% solvent B in 1 min, then from 8 to 45% solvent B in 85 min, and finally to 90% B in 5 min. After a 10 min hold, solvents were returned to initial conditions for equilibration.

#### *Protein Identification*

The Spectrum Mill database search algorithm (Agilent Technologies) was used to search the NCBI nr and SwissProt databases, using the taxonomy filter for Other Green Plants.

Parameters used for the search included monoisotopic mass, a peptide mass tolerance of 2.4 Da, and a fragment ion mass tolerance of 0.7 Da. Furthermore, tryptic peptides were allowed two missed cleavages, and carbamidomethylation of cysteine was selected as a fixed modification. Spectrum Mill scores above 13, with peptide scores above 10 and a scored intensity of 70%, were used as a cut off for initial hit validation.

#### *Triple-Quadrupole MS/MS*

Whole protein extracts [10 g/mL in methanol–water (60:40) with 0.1% formic acid] were infused (3 mL/h) with a syringe pump (Harvard Apparatus, Holliston, MA) into a Sciex API4000 triple-quadrupole mass spectrometer with positive turbo ionspray source controlled by the Analyst software (Version 1.3.1; Applied Biosystems, Foster City, CA). Initially, positive ion, full-scan mode was used to select protonated molecular ions of interest, and then multiple reaction monitoring (MRM) was used to detect characteristic precursor ion-to-product ion transitions. The declustering potential was set to 30 V, the entrance potential to 10 V, the interface temperature to 500 °C, the ionspray voltage to 4500 V, and the collision energy to 25 eV.

## **Results and Discussion**

### *Properties of the Celery Standard Protein*

Industrial celery root powder was used as source material for the preparation of celery standard protein and the generation of polyclonal anticelery antibodies. By prolonged extraction under moderate basic conditions and temperate heating, a celery protein fraction was obtained that was depleted of heat-labile Api g 1 (5, 12). On a control gel, the 16 kDa band disappeared and only diffused peptide fragments were visible <3 kDa (data not shown). Considering the cross-reacting potential with other Bet v 1-homologous proteins in many plants (1, 24, 25), the removal of Api g 1 was intended to increase the specificity of the anticelery antibody.

**Table 4. Allergens in celery, carrot, and potato (Protein Database entries)**

| Species | Allergen              | MW, kDa         | Type                                               | Accession | Ref. <sup>a</sup> |
|---------|-----------------------|-----------------|----------------------------------------------------|-----------|-------------------|
| Celery  | Api g 1 isoforms      | 16.3            | Bet v1-related, ribonuclease                       | P49372    | III               |
|         |                       |                 |                                                    | P92918    |                   |
|         | Api g 3               | 28.1            | Chlorophyll-ab binding                             | P92919    |                   |
|         | Api g 4               | 14.3            | Profilin, actin-binding                            | Q9XF37    | II                |
|         | Api g 5               | 9.4 (fragment)  | FAD-linked oxidoreductase                          | P81943    | VI                |
| Carrot  | Api g LTP             |                 | Lipid transfer protein                             | —         | I                 |
|         | Dau c 1 isoforms      | 16.0–16.5       | Bet v1-related, ribonuclease                       | O04298    | III               |
|         | Dau c 3               | 12.5            | Nonspecific LTP                                    | P27631    | I                 |
|         | Dau c 4               | 14.4            | Profilin, actin-binding                            | Q8SAE6    | II                |
|         | Dau c Cyp             | ~20             | Cyclophilin                                        | —         |                   |
| Potato  | Sola t 1              | 42.6            | Patatin                                            | P15476    | V                 |
|         | Sola t 2              | 20.6            | Aspartic protease inhibitor, cathepsin D inhibitor | P16348    |                   |
|         | Sola t 3 isoforms     | 21.0 (fragment) | Cystein protease inhibitor                         | O24383    |                   |
|         |                       | 24.7            |                                                    | P20347    |                   |
|         | Sola t 4              | 24              | Cerine protease inhibitor                          | P30941    | IV                |
|         | Sola t 8              | 14.1            | Profilin, actin-binding                            | Q38HU3    | II                |
|         | Sola t gluc. isoforms | 37.0 (fragment) | Glucan endo-1,3-beta-glucosidase                   | P52400    |                   |
|         |                       | 39.8            |                                                    | P52401    |                   |
|         |                       | 36.2 (fragment) |                                                    | P52402    |                   |
|         |                       | 36.4 (fragment) |                                                    | Q8GUR3    |                   |
|         |                       | 37.7 (fragment) |                                                    | Q8GUR4    |                   |

<sup>a</sup> Cross-reference to Figures 2 and 3.

The total protein concentration of the celery standard protein was 6 mg/mL.

#### *Characteristics of the Celery Sandwich ELISA*

The standard curve of the assay was linear in the range of 1.95–125 g/L, corresponding to 0.2–12.5 mg/kg celery protein in food (Figure 1). Samples containing higher celery protein concentrations had to be additionally diluted. Using second-degree polynomial regression on the ELISA standard curve resulted in a working range of 1.95–500 g/L or 0.2–50 mg/kg food (Table 1).

The celery sandwich ELISA was validated in buffer and four typical foods (Table 1). The standard curves obtained in the respective matrixes showed similar performances. However, the LODs and LOQs obtained were 2–4 times higher than for assay buffer. The recoveries were within acceptable error margins ( $\pm 25\%$ ) for all matrixes at a celery concentration above the respective LODs. In contrast, for two of the five matrixes analyzed, accuracy was insufficient at a concentration slightly below the LOD. The precision of the celery sandwich ELISA was only determined in assay buffer. Intra-assay variance was low, even for celery concentrations below the LOD, whereas interassay variance reached acceptable values only above the LOD.

#### *Specificity and Cross-Reacting in the Celery Sandwich ELISA*

Celery contains a number of cross-reacting allergens belonging to protein families with many Homologous in different fruits and vegetables (14). The aim of the celery sandwich ELISA, however, was to specifically detect celery protein in multicomponent foods. Therefore, extensive cross-reactivity testing was performed using protein extracts from a large number of different food ingredients. Test samples were treated according the protocol for food products, resulting in a final dilution of 1:100 in the ELISA. As a result, protein concentrations in the specificity testing were, for many ingredients, much higher than what could be expected in multicomponent food products. The cross-reactivities in the celery sandwich ELISA observed for certain seeds, nuts, spices, or herbs are therefore greatly overestimated and probably of little significance.

After prescreening more than 60 different plant and several animal species using a noncompetitive celery ELISA (data not shown), 36 species were analyzed in the celery sandwich ELISA and on immunoblot, some of them additionally after previous cooking. Cross-reactivity was not detected for parsley root (*Petroselinum hortense*), broccoli (*Brassica oleracea silv.*), cauliflower (*B. oleracea*), turnip

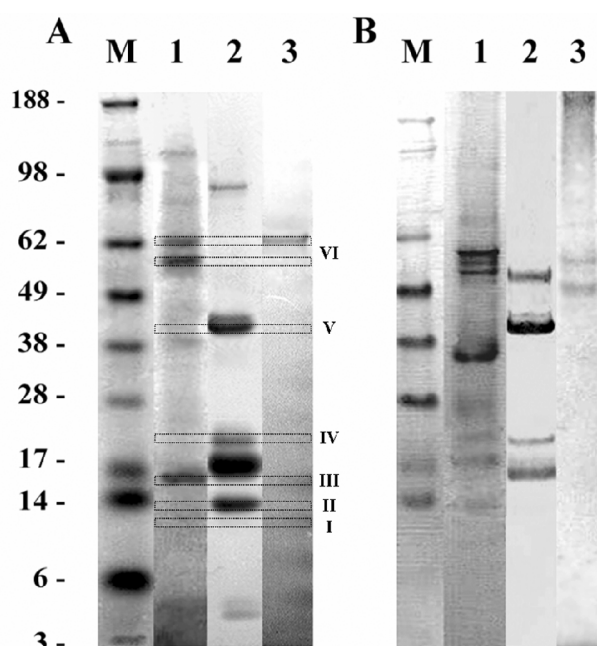


**Figure 2.** Gel (A) and immunoblot (B) of celery, carrot, and potato extracts using purified polyclonal anticelery antibody. M: molecular weight marker, protein molecular weights (kDa) are indicated on the left side of the gel; 1: celery; 2: potato; 3: carrot. The boxes I–VI indicate proteins with homologous amino acid sequences identified by proteomic analysis.

(*B. napobrassica*), paprika (*Capsicum annum*), leech (*Allium porrum*), yellow onion (*A. cepa*), common bean (*Phaseolus vulgaris*), garden pea (*Pisum sativum*), mustard (*Sinapis alba*), sunflower seeds (*Helianthus annuus*), pine seeds (*Pinus pinea*), peanut (*Arachis hypogaea*), pear (*Pyrus communis*), apple (*Malus domestica*), wheat flour (*Triticum aestivum*), beef (*Bos tauris*), and chicken (*Gallus domesticus*).

Accordingly, celery could be differentiated from these food ingredients by using the polyclonal anticelery antibody. Considering that, in the sera of patients with allergy to celery, IgE cross-reactivity to paprika (26), apple (4, 27), pear and peanut (15, 28), and sunflower seeds (29) had been reported, the absence of such unspecificity in the celery sandwich ELISA was promising for the feasibility of the assay.

However, for 18 other plants, cross-reactivities were detected in the ELISA, and for seven of them, additionally by immunoblot (Table 2). Some of the species reaching the highest recoveries of celery protein equivalents are like celery members of the *Apiaceae* family, e.g., carrot, parsnip, parsley, dill, and aniseed, but also botanically are not closely related plants; e.g., potato, white cabbage, and morello cherry reacted considerably with the anticelery antibody.

Allergenic cross-reactivities between celery and carrot (1, 30, 31), potato (27, 31, 32), parsnip (31),

cherry (28, 33), parsley (34), and dill (35) have been previously reported in detail. Therefore, it is reasonable to expect that a polyclonal anticelery antibody would show similar characteristics, even if it was raised against an Api g 1-depleted fraction of celery root extract.

Excluding ingredients that are generally used only in small amounts in prepacked foods or are rarely used in the same products as celery, the cross-reactivities to carrot, potato, and parsnip seemed to be the most relevant. It was concluded that the celery sandwich ELISA was not suited to specifically detect celery in the presence of these three vegetables. Even affinity purification with polymer-bound carrot and potato protein could not remove the cross-reactivity to these plants (data not shown).

The applicability of the celery sandwich ELISA is thus limited to the prescreening of food products and the identification of celery-free samples, and any positive results would have to be verified by confirmatory methods such as PCR or MS.

#### Detection of Celery Proteins in Foods

A number of prepacked foods were analyzed using the celery sandwich ELISA (Table 3). From the five food products without celery in their ingredient lists, two negative and two positive results were obtained. The food products with the positive results contained potato starch, which could cross-react and deliver false-positive determinations due to potato protein residues in the starch. In a sample of air-dried sausage, however, a small amount of celery was detected in the absence of interfering proteins. Presumably, celery spice had been added to the sausage as a condiment, making consumption potentially dangerous for celery-allergic consumers.

While celery sandwich ELISA was suitable for the detection of unlabeled celery protein traces, the application was less successful for food products with celery as a declared ingredient. Six of the seven foods tested contained cross-reacting vegetables, e.g., carrot and potato; thus, the quantitation of the celery content by sandwich ELISA was impossible, leaving real-time PCR as a possible alternative (20, 21).

#### Identification of Celery, Potato, and Carrot Proteins on Gel and Immunoblot

Celery, potato, and carrot contain several proteins with IgE-binding activities. Their allergenic potential and biochemical and molecular biological characteristics have been reported in a number of publications, and they have been entered into the protein databases (Table 4). In the present study, celery, carrot, and potato protein extracts were separated by gel electrophoresis and analyzed by immunoblot. The gel (Figure 2A) showed a number of major protein bands. Api g 1 (17 kDa) and Api g 5 (53 and 57 kDa) were most prominent in the celery extract, while Api g 4 (14 kDa) was less visible. On immunoblot using the polyclonal anticelery antibody (Figure 2B), distinct signals were only visible for Api g 5 and two proteins at 52 and 36 kDa, possibly identical to putative latex Hev b 13-like lipolytic esterase (40 kDa; 10).

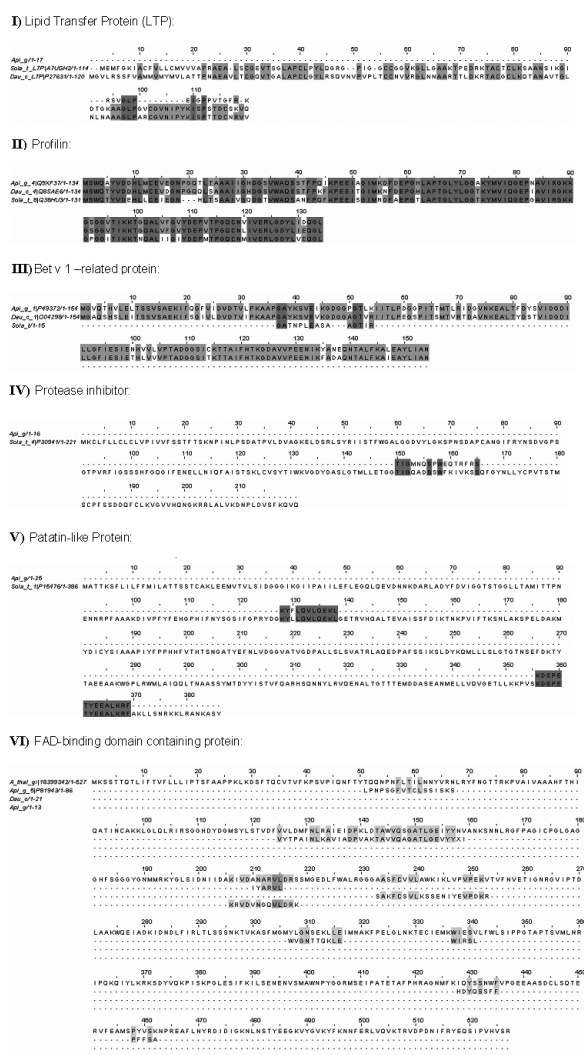


**Figure 3. Protein sequence alignment (Cluster Alignment by T-Coffee; [www.tcoffee.org](http://www.tcoffee.org)) of homologous allergens in celery, potato, and carrot. Novel, putative cross-reacting allergens were identified by nanoLC-ion-trap MS/MS, database searching, and BLAST homology analysis. The grey scales indicate levels of amino acid homologies: dark grey: 100% identity; medium grey: 66% identity; light grey: 50% identity.**

The limited binding pattern of the polyclonal antibody was likely the result of using a celery extract for immunization, in which heat-labile and low-molecular proteins had been reduced by thermal treatment and fractionation. However, the high-molecular allergens are likely to be present in manufactured food products and were also primarily targeted in a study investigating IgE binding to native celeriac extracts using sera from DBPCFC-positive patients (4).

In potato extract, the main tuber storage protein, patatin Sola t 1 (36), a 43 kDa lipid acyl hydrolase, produced an intensely stained band on gel (Figure 2). Additionally, the protease inhibitor-like potato allergens Sola t 2 (16 kDa),

Sola t 3 (18 kDa), and Sola t 4 (20 kDa; 37), as well as the potato profilin Sola t 8 (14 kDa; 32) were clearly visible (Table 4). Cross-reaction of several potato proteins with the anticelery antibody was observed on blot. Patatin exhibited the most intense signal, whereas binding to the protease inhibitors was weaker. Additionally, a protein of about 50 kDa was detected, which might be identical to one discovered in extracts of thermally treated potatoes in a study on food processing and allergenicity (38). It was also shown that the IgE-binding capacity of potato allergens decreased only slightly by heating processes, although patatin and the protease inhibitor undergo conformational changes and loss of enzyme activity at temperatures exceeding 28 C (39). Interestingly, cooked potato cross-reacted in the celery sandwich ELISA even more strongly than raw potato extract (Table 2).

The carrot extract produced only faintly visible protein bands on gel: three distinct signals between 55 kDa and 66 kDa, and several diffuse bands in the 10–20 kDa range (Figure 2). This is consistent with previously published results (25) demonstrating that the heat-labile, birch pollen-related major carrot allergen Dau c 1 (17 kDa) might degrade during sample extraction (40). Only high-molecular-weight carrot proteins showed cross-reactivity with the anticelery antibody, which is comparable to reported IgE-binding patterns observed in patients with carrot allergy (41). In parallel to what has been found for celery Api g 5, cross-reactive carbohydrate determinants on carrot glycoproteins have been confirmed to be essential for antibody-binding (16, 25, 42).

### Sequence Homologies of Potential Cross-Reacting Proteins

Plant food allergens can be classified into families and superfamilies on the basis of their structural and functional properties (43). In the present study, proteins from celery, potato, and carrot were excised from an SDS-PAGE gel (Figure 2), trypsinized, and identified using nanoLC-ion-trap MS/MS. Protein database searching was used to assign several of the known allergens to bands (Table 4). Additionally, amino acid sequence homologies were used to elucidate novel, potentially cross-reacting proteins.

In the celery extract, a peptide with sequence homology to the family of plant nonspecific lipid transfer proteins (LTP) was identified and compared to the known LTPs from potato and carrot (Figure 3). LTPs are ubiquitous defense proteins of low molecular weights (9–10 kDa) that exhibit extreme thermal and proteolytic resistance and allergenicity (44). Although the degrees of sequence identity between LTP from different plant species range from 30 to 90%, consistent and extensive IgE cross-reactivity has been demonstrated (6). Celery LTP has been postulated in one study as cross-reactive to apple LPT (45), and there is an entry for Api g LTP in the allergen database, although a sequence has not yet been published.

Plant profilins are small cytosolic proteins (12–15 kDa) involved in actin cytoskeleton regulation (46). They are

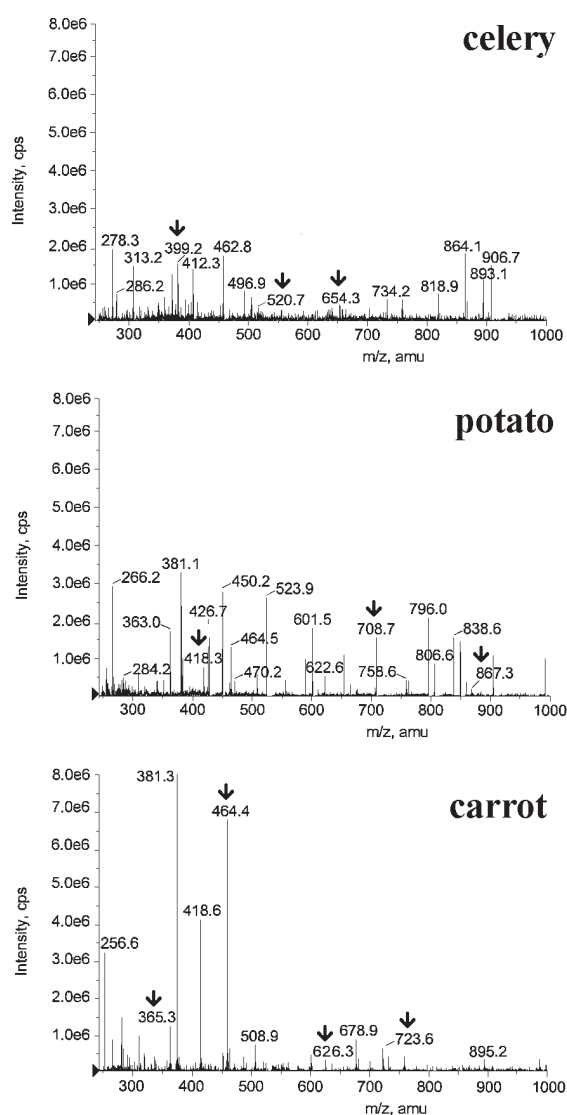


**Figure 4.** Protonated ion spectra (Q1) of celery, potato, and carrot protein extracts obtained by positive ion electrospray MS on a Sciex API 4000 triple-quadrupole mass spectrometer. Peaks indicated by arrows were identified as suitable precursor ions and, after fragmentation, product ions were selected for subsequent MRM experiments.

highly conserved, with 70–85% sequence identity between different species (43), and account for substantial cross-reactivity between pollen and raw foodstuffs. The profilins from celery (Api g 4; 15), potato (Sola t 8; 38), and carrot (Dau c 4; 41) have been extensively studied and their sequences have been published (Figure 3). However, profilins are relatively sensitive to heat denaturation and gastric digestion. They are therefore of low abundance in processed foods and considered minor food allergens (43). Profilins did not reveal antigenicity to the anticelery antibody on immunoblot (Figure 2).

Food allergy to certain fruits and vegetables is directly associated with birch pollinosis, manifesting itself as OAS.

The broad cross-reactivity between the major birch pollen allergen Bet v 1 and homologous allergens from the large family of pathogenesis-related proteins PR-10 is primarily responsible for this correlation (4). PR-10 food allergens were described in fruits and vegetables belonging to the plant families *Rosaceae* (apple and stone fruits), *Fabaceae* (peanut and soybean), and *Apiaceae* (celery and carrot; 47). The major celery allergen, Api g 1, shares 40% amino acid sequence identity with Bet v 1 (10). However, Api g 1 and the carrot allergen Dau c 1 are 81% homologous (24), facilitating extensive cross-reaction. The P-loop region, containing amino acids 46 to 52 in Api g 1, has been identified as an important IgE-binding epitope (48). In the present study, a potato peptide with homology to the P-loop region was detected which might have affinity to the anticelery antibody. This peptide may be derived from a member of the PR-10-like STH-2 plant defense proteins (Accession Nos. P17641 and P17642; 47, 49). For a STH-2 from pea, 46% sequence identity with Bet v 1 has been shown (50), suggesting that these proteins may be candidates for cross-reactivity.

Potato protease inhibitors have been identified as minor allergens (37). The analysis of a celery protein of the same molecular weight as the serine protease inhibitor Sola t 4 revealed considerable homology in a peptide sequence, which might explain the relatively strong affinity of the anticelery antibody to the 20 kDa potato protein on immunoblot (Figure 2).

The patatin storage protein Sola t 1, accounting for 40% of total soluble protein in tubers, has been described as the major allergen in potato (36). Latex (*Hevea brasiliensis*) Hev b 7 is the only homologous plant allergen that has been identified thus far (51). However, in the study presented here, two peptides were recovered from a celery protein that showed a high degree of homology (24/25 amino acids) to Sola t 1 (Figure 3). Therefore, the presence of a patatin-like protein in celery seems to be likely and would explain the observed cross-reactivity of Sola t 1 with the anticelery antibody (Figure 2).

The celery glycoprotein Api g 5, an oxygen-dependent FAD-linked oxidoreductase involved in stress response mechanisms, contains MUXF<sup>3</sup> and MMXF<sup>3</sup>, two complex N-linked glycans with alpha-1,3-fucose and beta-1,2-xylose residues (9, 42). Asparagine-linked oligosaccharides are abundant in plants, but occur with large quantitative differences. The MMXF<sup>3</sup>-structure has been found in nonallergenic proteins, in pollen allergens from grass and latex (42), and additionally in Api g 5 and Lyc e 2, a -fructofuranosidase from tomato (*Lycopersicon esculentum*; 52). Currently, only an 86 amino acid-long fragment of the Api g 5 sequence has been published (17). Therefore, a homologous FAD-binding protein (GI:18399342) from thale cress (*Arabidopsis thaliana*) containing 527 amino acids (59.05 kDa) was used for the alignment analysis in the present study (Figure 3). Comparison with Api g 5 revealed seven homologous regions. A peptide that was identified in the celery extract showed

significant sequence homology (11/13 amino acids) with a peptide from the thale cress FAD-binding protein.

From the carrot preparation, the three high-molecular-weight proteins that displayed affinity to the polyclonal anticelery antibody (Figure 2) and serum IgE (41) were analyzed. One peptide of 21 amino acids with considerable sequence homology to the thale cress FAD-binding protein was identified (Figure 3), supporting the hypothesis that carrot might contain glycoproteins carrying cross-reactive carbohydrates. The potato extract included a 50 kDa potato protein cross-reacting with the anticelery antibody (Figure 2), in which three peptides with sequence homologies to Lyc e 2 were found (data not shown). Because tomato and potato both belong to the *Solanaceae* family, the existence in potato of a related N-linked glycoprotein with a MMXF<sup>3</sup> motif seems plausible.

### Differentiation by Specific Ion Mass Transitions

The ambiguity of positive results from the celery sandwich ELISA due to cross-reactivity among celery, potato, and carrot proteins makes confirmation by alternative methods essential. PCR analysis of celery is well-established; however, the method is DNA-based and does not detect the causative agent for food allergy. Therefore, MS of food proteins is an interesting new technique, providing novel insights that may be important for future diagnostic and therapeutic efforts. Several quantitative MS-based methods have been published. Due to its high sensitivity and specificity, the most promising approach appears to be triple-quadrupole MS/MS with MRM (53).

In the present study, undigested extracts of celery, potato, and carrot were infused directly into the source of a triple-quadrupole MS. Mass spectra of precursor ions are plotted for the three species, and ions selected for fragmentation are indicated with arrows (Figure 4). In subsequent MS/MS experiments, specific precursor ion-to-product ion transitions were determined for each food extract based upon the MS/MS fragmentation patterns: In celery, the transitions  $m/z$  399.2  $m/z$  301.4,  $m/z$  520.7  $m/z$  399.5 and  $m/z$  777.6, as well as  $m/z$  654.3  $m/z$  637.2 were selected; in potato, the transitions  $m/z$  418.3  $m/z$  325.1,  $m/z$  257.6 and  $m/z$  163,  $m/z$  708.7  $m/z$  365.4, as well as  $m/z$  867.3  $m/z$  675.7 were found; and in carrot, the transitions  $m/z$  365.3  $m/z$  203.3,  $m/z$  464.4  $m/z$  122.8,  $m/z$  626.3  $m/z$  122.8, as well as  $m/z$  723.6  $m/z$  381.4 were the most dominant.

These results suggest that MS/MS may be a promising method for the specific and quantitative detection of celery, potato, and carrot proteins. Possibly time-consuming sample preparation may be omitted. However, the present experiments have to be considered preliminary. It remains to be seen how a fully developed method using LC/MS/MS for real, matrix-containing food samples would perform.

### Conclusions

Cross-reactivity between anticelery antibodies and common food ingredients frustrates the development of

ELISA methods for the detection of celery in food products. The sandwich celery ELISA developed in this study showed acceptable performance regarding validation parameters; however, extensive cross-reactions with potato, carrot, and parsnip limited the application to food prescreening, making it necessary to confirm positive results by an alternative method. The proteomic analysis of celery, potato, and carrot extracts revealed considerable amino acid sequence homologies within different protein families. Apart from known cross-reacting allergens like profilins and Bet v 1-related proteins, the data suggested the presence of a novel Api g 5-like glycoprotein in carrot, a Sola t 4-like protease inhibitor in celery, and a Sola t 1-like protein in celery. Preliminary experiments for the development of a confirmatory MS method revealed specific fragmentation patterns in the three immunologically cross-reacting plant species.

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