

# Molecular and Immunological Characterization of a Wheat Serine Proteinase Inhibitor as a Novel Allergen in Baker's Asthma<sup>1</sup>

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IgE-mediated sensitization to wheat flour belongs to the most frequent causes of occupational asthma. A cDNA library from wheat seeds was constructed and screened with serum IgE from baker's asthma patients. One IgE-reactive phage clone contained a full-length cDNA coding for an allergen with a molecular mass of 9.9 kDa and an isoelectric point of 6. According to sequence analysis it represents a member of the potato inhibitor I family, a group of serine proteinase inhibitors, and thus is the first allergen belonging to the group 6 pathogenesis-related proteins. The recombinant wheat seed proteinase inhibitor was expressed in *Escherichia coli* and purified to homogeneity. According to circular dichroism analysis, it represented a soluble and folded protein with high thermal stability containing mainly  $\beta$ -sheets, random coils, and an  $\alpha$ -helical element. The recombinant allergen showed allergenic activity in basophil histamine release assays and reacted specifically with IgE from 3 of 22 baker's asthma patients, but not with IgE from grass pollen allergic patients or patients suffering from food allergy to wheat. Allergen-specific Abs were raised to localize the allergen by immunogold electron microscopy in the starchy endosperm and the aleuron layer. The allergen is mainly expressed in mature wheat seeds and, despite an ~50% sequence identity, showed no relevant cross-reactivity with allergens from other plant-derived food sources such as maize, rice, beans, or potatoes. Recombinant wheat serine proteinase inhibitor, when used in combination with other specific allergens, may be useful for the diagnosis and therapy of IgE-mediated baker's asthma. *The Journal of Immunology*, 2008, 180: 7451–7460.

Allergic sensitization to wheat flour components is one of the most frequent causes of occupational asthma (1, 2). Support for the assumption that baker's asthma is a true occupational disease comes from the finding that the prevalence of sensitization to bakery-associated allergens is ~10-fold higher in flour-exposed persons as compared with control populations without flour exposure (3). Furthermore, it has been possible to establish threshold values for wheat flour that are known to cause bronchial asthma in sensitized persons (4). Approximately 1–10% of bakery workers develop IgE Abs against wheat flour allergens and flour-induced asthma and/or rhinitis (1, 5). Anecdotal reports from antiquity indicate that Roman slaves used as millers and bakers suffered from baker's asthma (6), and the first scientific description

of baker's asthma originated from Ramazzini in 1700 as cited by Bonnevie (7). A first systematic investigation on flour allergy was conducted already in 1933 by Baggoe (8). Other early reports focused on the description of cases of baker's asthma followed by mechanistic studies demonstrating the importance of IgE-mediated mechanisms in baker's asthma (9–12). Thereafter, several attempts were made to characterize the disease-eliciting flour allergens by immunochemical methods, radioallergosorbent test (RAST)<sup>3</sup> technology, immunoblotting, and recently by molecular cloning techniques (13–18).

Today, only a few wheat flour allergens have been identified and characterized (19–21). They include the members of the  $\alpha$  amylase inhibitor family (22), acyl-CoA oxidase, peroxidase (23), fructose-bisphosphate aldolase, and recently thioredoxins (18). However, the great majority of the soluble wheat flour allergens have not yet been identified (24). Furthermore, the question is open as to whether it is possible to identify allergens that can be used for selective diagnosis and eventually treatment of the various wheat-induced manifestations of allergy, such as baker's asthma, food allergy, and pollinosis (25).

Cross-reactivity between grass pollen allergens and wheat seed allergens has been described, and there is evidence that patients suffering from baker's asthma and IgE-mediated food allergy to wheat may recognize different allergens that may be used for the differential diagnosis of baker's asthma, food allergy to wheat flour, and grass pollen allergy (26). In this context it has been

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<sup>3</sup> Abbreviations used in this paper: RAST, radioallergosorbent test; CD, circular dichroism; PR, pathogenesis related; RBL, rat basophil leukemia.

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Table I. Demographic, clinical, and serological characteristics of patients suffering from baker's asthma<sup>a</sup>

| Patients | Age<br>(Years) | Sex | Asthma | Occupational<br>Exposure | IgE Concentration (kUA/L) |       |         |                     | SPT to<br>Grass<br>Pollen | Total IgE<br>(kU/L) | PC20 (mg/ml) |       |
|----------|----------------|-----|--------|--------------------------|---------------------------|-------|---------|---------------------|---------------------------|---------------------|--------------|-------|
|          |                |     |        |                          | Wheat                     | Rye   | Soybean | Fungal<br>α-Amylase |                           |                     |              |       |
| 1        | 35             | M   | +      | +                        | 74.2                      | 20.7  | ND      | 0.63                | +                         | 163                 | +            | 0.29  |
| 2        | 29             | M   | +      | +                        | 18                        | 7.9   | 6.1     | 1.5                 | +                         | 271                 | +            | 2     |
| 3        | 36             | M   | +      | +                        | 1.66                      | 1.24  | <0.35   | <0.35               | —                         | 353                 | +            | 0.31  |
| 4        | 39             | M   | +      | +                        | 25.8                      | 15.6  | 1.36    | 18.6                | +                         | 1387                | +            | 0.25  |
| 5        | 54             | M   | +      | +                        | <0.35                     | <0.35 | <0.35   | 3.21                | —                         | 30.1                | ND           |       |
| 6        | 35             | M   | +      | +                        | 24.3                      | 39.6  | 5.73    | 2.29                | +                         | 416                 |              | +     |
| 7        | 28             | M   | +      | +                        | 2.58                      | 3.77  | <0.35   | 5.39                | +                         | 248                 | +            | 0.25  |
| 8        | 27             | M   | +      | +                        | 7.86                      | 7.4   | 3.19    | 32.2                | +                         | 1773                | +            | 0.47  |
| 9        | 24             | M   | —      | +                        | 2.35                      | 2.32  | 1.62    | ND                  | +                         | 204                 | +            | 0.09  |
| 10       | 60             | M   | +      | +                        | 3.44                      | 4.08  | <0.35   | 1.15                | +                         | 73.3                | +            | 0.44  |
| 11       | 22             | M   | +      | +                        | 3                         | 2     | <0.35   | <0.35               | —                         | 2509                | +            | 3.38  |
| 12       | 26             | M   | +      | +                        | >100                      | >100  | 10.7    | 7.63                | +                         | 321                 | +            | 0.5   |
| 13       | 54             | M   | +      | +                        | 2.48                      | 0.6   | 0.71    | <0.35               | —                         | 480                 | +            | 0.12  |
| 14       | 60             | M   | +      | +                        | 31.8                      | 31.1  | ND      | <0.35               | +                         | 629                 | +            | 0.15  |
| 15       | 27             | M   | +      | +                        | 5.06                      | ND    | ND      | <0.35               | +                         | 278                 | +            | 0.079 |
| 16       | 42             | F   | +      | +                        | 3.71                      | 2.71  | 1.69    | <0.35               | +                         | 271                 | +            | 0.187 |
| 17       | 54             | M   | +      | +                        | 1.68                      | 1.13  | <0.35   | <0.35               | —                         | 79.2                | +            | 16    |
| 18       | 59             | M   | +      | +                        | <0.35                     | <0.35 | <0.35   | 5                   | —                         | 673                 | +            | 0.23  |
| 19       | 26             | M   | +      | +                        | 74.6                      | 58.4  | <0.35   | <0.35               | +                         | 17.7                | +            | 1.16  |
| 20       | 43             | M   | +      | +                        | 13.8                      | 26.9  | 0.77    | <0.35               | +                         | 538                 | +            | 0.25  |
| 21       | 34             | F   | +      | +                        | 2.05                      | 1     | <0.35   | <0.35               | +                         | ND                  | +            | 0.5   |
| 22       | 41             | F   | +      | +                        | 1.77                      | 0.75  | <0.35   | <0.35               | +                         | 23.4                | +            | 0.23  |

<sup>a</sup> kU/L, kilounit Ag per liter; SPT, skin prick test; PC20, methacholine inhalation challenge; ND, not done.

reported that  $\omega$ -5 gliadin and certain peptides thereof may be specific tools for the diagnosis of wheat-dependent, exercise-induced anaphylaxis, a distinct form of common food allergy induced by the combination of food ingestion, physical exercise, and/or aspirin intake (27, 28).

Herein we constructed a cDNA library from wheat seeds and screened this expression cDNA library with IgE Abs from patients suffering from baker's asthma. One of the IgE-reactive clones coded for a novel wheat allergen that, according to sequence analysis, belongs to a family of serine proteinase inhibitors that occur throughout the plant and animal kingdom. The recombinant expression, purification, physicochemical and immunological characterization, and immunoelectron microscopic localization of the wheat serine proteinase inhibitor-like allergen are reported. Recombinant wheat serine proteinase inhibitor reacted specifically with serum IgE from baker's asthma patients and hence may be used for the diagnosis and treatment of this disease.

## Materials and Methods

### Biological materials, patients' sera, and Abs

Wheat seeds from *Triticum aestivum* L. cv. Michael were obtained from the Österreichische Agentur für Gesundheit und Ernährungssicherheit and

planted in a glasshouse. Immature seeds were harvested 7, 10, 15, 20, 25, 30, and 35 days after the onset of pollination directly into liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. Wheat pollen was obtained from Allergon. Rice, maize, beans, and potatoes were bought at a local market. Recombinant Phl p 1, Phl p 5, Phl p 7, and Phl p 12 were purchased from BIOMAY, and human serum albumin was obtained from Behring.

Sera were obtained from 22 patients suffering from baker's asthma. Baker's asthma was diagnosed on the basis of a positive case history, IgE specific for wheat and rye flour as determined by the CAP-FEIA system (Phadia), and included specific inhalation challenge tests for confirmation of a clinically relevant sensitization (29). Demographic, clinical, and serological data for these patients are summarized in Table I. Additionally, serum from a nonallergic individual, sera from four patients suffering from food allergy to wheat, and sera from four grass pollen-allergic patients without baker's asthma but with serum IgE reactivity to wheat and rye flour were included in the experiments (Table II). Sera from grass pollen-allergic patients had been analyzed for total serum IgE levels and IgE-specific for timothy grass pollen by the CAP-FEIA system, and patients with food allergy to wheat were characterized as described previously (30). The specificity of the clone 10-derived allergen for baker's asthma was confirmed by testing additional sera from 20 celiac disease patients, 119 food allergic patients, 23 grass pollen-allergic patients, and 25 baker's asthma patients by gene chip analysis (C. Constantin and R. Valenta, unpublished data).

Specific rabbit Abs against the clone 10-derived allergen were raised by immunization of a rabbit in monthly intervals with purified clone 10-derived allergen (200  $\mu\text{g}$  per injection) using Freund's complete adjuvant (once) and IFA (twice) (Charles River Laboratories). Preimmune serum

Table II. Demographic, clinical, and serological characteristics of patients suffering from food allergy to wheat (F1–F4) and grass pollen allergy (G1–G4)<sup>a</sup>

| Patients | Age (Years) | Sex | RC | AD | Asthma | IgE Concentration (kU/L) |      | Total IgE (kU/L) |
|----------|-------------|-----|----|----|--------|--------------------------|------|------------------|
|          |             |     |    |    |        | Wheat                    | Rye  |                  |
| F1       | 34          | F   | —  | +  | +      | 18.6                     | 13.4 | >2000            |
| F2       | 34          | M   | +  | —  | —      | 3.1                      | 3.08 | 155              |
| F3       | 24          | F   | —  | +  | —      | 1.3                      | ND   | 336              |
| F4       | 15          | F   | —  | +  | —      | 5.91                     | 5.3  | 915              |
| G1       | 45          | M   | +  | —  | +      | 1.76                     | 2    | 175              |
| G2       | 39          | M   | +  | —  | +      | 11.1                     | 10.3 | 401              |
| G3       | 55          | F   | —  | —  | —      | 3.16                     | 1    | 157              |
| G4       | 54          | M   | +  | —  | +      | ND                       | ND   | 1528             |

<sup>a</sup> F, Food allergy; G, grass pollen allergy; RC, rhinoconjunctivitis; A, atopic dermatitis; kU/L, kilounit Ag per liter; ND, not done.

## Clone #10 Sequence:

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1   ATG AGC CCT GTG GTG AAG AAG CCG GAG GGA GGG AAC ACC 13
    M  S  P  V  V  K  K  P  E  G  G  N  T
40  GAT ACT GGT GAC CAT CAC AAC CAG AAG ACG GAG TGG CCA 26
    D  T  G  D  H  H  N  Q  K  T  E  W  P
79  GAG TTG GTG GGG AAG TCG GTG GAG GAG GCC AAG AAG GTG 39
    E  L  V  G  K  S  V  E  E  A  K  K  V
118 ATT ATG CAG GAC AAG TCA GAG GCA CAG ATC GTA GTT CTA 52
    I  M  Q  D  K  S  E  A  Q  I  V  V  L
157 CCG GTG GGG ACA ATT GTG ACC ATG GAA TAT CGA ATC GAC 65
    P  V  G  T  I  V  T  M  E  Y  R  I  D
196 CGT GTC CGC CTC TTT GTT GAC AGT CTC GAC AAA ATT GCC 78
    R  V  R  L  F  V  D  S  L  D  K  I  A
235 CAG GTC CCC AGG GTC GGC tagcaagcttaagatctagcctgctcct 84
    Q  V  P  R  V  G
281 agcgtatatgtatcgtggcttgataatctcttcttgatatagcaagattga

333 gatatatagatcatatacaataagagttgatgcattgaaagtgaattgataa

385 tagaataagtcagagagcgcgtataaaaaaaaaa

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**FIGURE 1.** Nucleotide and deduced amino acid sequence of the clone 10-derived allergen. Coding and noncoding regions are in uppercase and lowercase letters, respectively; the start (ATG) and stop codons are underlined. Amino acids of the potato inhibitor I family signature are printed with gray background. Left-hand numbers are for the nucleotides and right-hand numbers are for the amino acids. The sequence has been submitted to the GenBank under the accession number (EU051824).

was obtained from the rabbit before immunization. For control purposes, a rabbit immune serum specific for a house dust mite allergen and rabbit antiserum specific for wheat profilin were used.

#### Construction of a *λgt11* cDNA library from wheat seeds

Total RNA was extracted according to Yeh et al. (31) from wheat seeds, harvested 25 days after the onset of pollination, and stored at  $-80^{\circ}\text{C}$ . Next, the RNA pellet was dissolved in guanidinium isothiocyanate buffer (4 M guanidinium isothiocyanate, 0.83% (v/v) 3 M sodium acetate (pH 6), and 11 mM  $\beta$ -ME) and purified by cesium chloride density gradient ultracentrifugation (32). Poly(A)<sup>+</sup> RNA was isolated by oligo(dT) cellulose affinity chromatography (Nucleo Trap mRNA from Machery-Nagel), and double-stranded cDNA was synthesized with a cDNA synthesis kit (cDNA synthesis system from Roche Diagnostics). After methylation with *Eco*RI methylase (New England Biolabs), *Eco*RI linkers (New England Biolabs) were added to the cDNA. Linkered cDNA was digested with *Eco*RI (Roche Diagnostics). Digested linkers were removed with a Nick column (Pharmacia Biotech) and the cDNA was ligated into *λgt11* arms (Stratagene). The ligation product was packed in vitro (Gigapack III Gold cloning kit, Stratagene) resulting in a *λgt11* expression cDNA library with  $2.43 \times 10^6$  PFU.

#### Isolation and characterization of IgE-reactive clones from a wheat seed cDNA library

*E. coli* Y1090 were infected with  $7 \times 10^5$  PFU of recombinant phages and immunoscreened with serum IgE of four patients (nos. 1, 2, 4, and 12) suffering from baker's asthma as described (33). These sera were selected for screening because they showed high levels of wheat-specific IgE (i.e., RAST class  $\geq 4$ ) and reacted to a wide range of wheat allergens when tested by immunoblot. Fifteen IgE-reactive phage clones were selected for further recloning and their DNA was PCR-amplified using Platinum PCR SuperMix (Invitrogen) with *λgt11* primers and sequenced (MWG Biotech). The obtained sequences were compared with sequences submitted to the GenBank database at the National Center for Biotechnology Information (NCBI). Multiple sequence alignment was performed using the GenBank database at the NCBI. For amino acid sequence identities, the ClustalW multiple alignment tool was used. A motif search was conducted with the PROSITE tool of the ExPASy proteomics server; for amino acid composition, the ProtParam tool of ExPASy was used. Solvent accessibility and secondary structure prediction were calculated using the PROT software from the Columbia University Bioinformatics Center. A phylogenetic tree was reconstructed based on the amino acid sequence of the clone 10-derived allergen and homologous proteins using multiple alignment and phylogenetic tree reconstruction software provided by the Max Planck Institute for Molecular Genetics.

#### Expression and purification of the clone 10-derived recombinant allergen

The coding region of the clone 10 cDNA was amplified by PCR using the following primer pair: forward 5'-CATATGAGCCCTGTGGTGAA

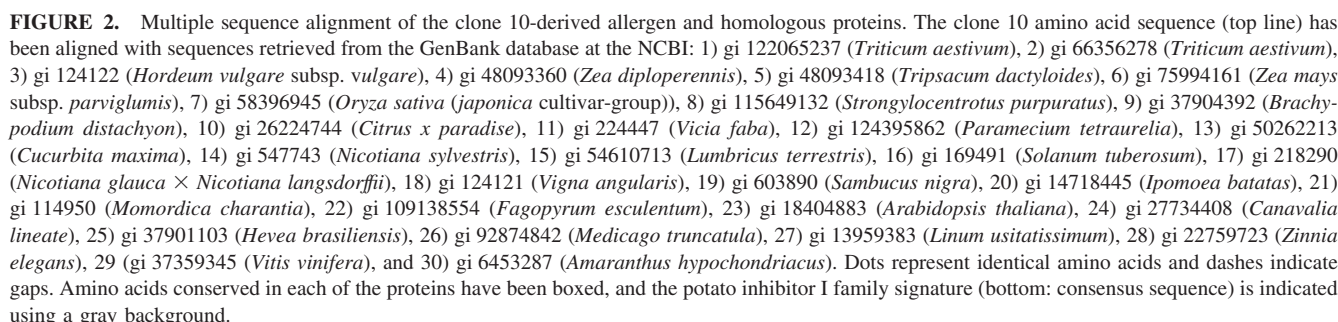
GAAGCCGAGGGA-3' and reverse, 5'-GAATTCCTTAGTGATGGT GATGGTGATGGCCGACCCTGGGGAC-3' (MWG Biotech). The PCR product contained *Nde*I (italics) and *Eco*RI (underlined) restriction sites and a hexahistidine tag-encoding sequence (boldface). The PCR product was subcloned into an AccepTor Vector (Novagen) and sequenced again (MWG Biotech). Next, the insert was cut out of the AccepTor Vector with *Nde*I and *Eco*RI (Roche Diagnostics), gel-purified, and subcloned into the expression plasmid pET 17b (Novagen). The DNA sequence was confirmed by sequencing both DNA strands (Microsynth). The pET 17b-clone 10 construct was transformed into *E. coli* BL21 (DE3) (Stratagene) and grown in Luria broth (17) medium containing 100 mg/L ampicillin at  $37^{\circ}\text{C}$  to an OD (600 nm) of 0.8–1. Protein expression was induced by addition of isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and then growing the bacteria for an additional 3 h. Bacteria were harvested by centrifugation and homogenized in 25 mM imidazole (pH 7.5), 0.1% (v/v) Triton X-100 with an Ultra-Turrax (IKA). DNA was digested by addition of DNase I, stirred for an additional 10 min at  $20^{\circ}\text{C}$ , and the reaction was stopped with 200  $\mu$ l of 5 M NaCl and then centrifuged at  $4^{\circ}\text{C}$  ( $6000 \times g$ , 20 min). Most of the clone 10-derived allergen was found in the insoluble fraction of the bacterial extract. Clone 10-derived allergen was purified from the inclusion body-containing pellet under denaturing conditions using Ni-NTA resin affinity columns according to the QIAexpressionist handbook (Qiagen). Fractions containing the recombinant allergen were pooled and dialyzed against 10 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.5). The protein concentration was determined with a Micro BCA protein assay kit (Pierce).

#### ELISA

Clone 10-derived allergen was dissolved in PBS at a concentration of 5  $\mu\text{g/ml}$  and coated on ELISA plates (Nunc Maxisorb). After blocking with 1% (w/v) BSA in PBS, 0.05% (v/v) Tween 20 (PBST), plates were incubated with sera diluted 1/50 in PBST, 0.5% (w/v) BSA for measurement of IgG1, IgG2, IgG3, and IgG4 as described (34). Bound Abs were detected by incubating first with monoclonal mouse anti-human IgG subclass Abs (BD Biosciences) diluted 1/1000 in PBST, 0.5% (w/v) BSA, and then with a horseradish-peroxidase-coupled sheep anti-mouse antiserum (GE Healthcare) diluted 1/2000 in PBST, 0.5% (w/v) BSA as previously described (30). All determinations were performed as duplicates, and results are expressed as mean values.

#### Protein extracts, SDS-PAGE, and immunoblots

SDS-protein extracts from mature and immature wheat seeds, rice (*Oryza sativa*), maize (*Zea mays*), common bean (*Phaseolus vulgaris*), and potato (*Solanum tuberosum*) were prepared by homogenization of 3 g of tissue in 32 ml of sample buffer (6) and subsequent boiling for 10 min. To remove insoluble particles, the extracts were centrifuged at  $10,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ , and the supernatants were stored in aliquots at  $-20^{\circ}\text{C}$ . Additionally, a PBS-protein extract from wheat seeds was prepared as previously described (30). *Triticum aestivum* pollen (500 mg) was extracted at  $4^{\circ}\text{C}$  overnight in 5 ml PBS, 2 mM EDTA, 1 mM PMSF. After centrifugation for 1 h at  $13,000 \times g$  at  $4^{\circ}\text{C}$ , the protein concentration of the supernatant was



For IgE dot blot experiments, 100 ng of recombinant clone 10-derived allergen and recombinant grass pollen allergens Phl p 1, Phl p 5, Phl p 7, and Phl p 12, as well as 3  $\mu$ g of wheat pollen extract and 2  $\mu$ g of mature wheat seed PBS extract, were dotted onto a nitrocellulose membrane. The nitrocellulose strips were blocked with buffer A and exposed to patients sera at a 1/10 dilution in buffer A overnight at 4°C. Bound IgE Abs were detected with <sup>125</sup>I-labeled anti-human IgE Abs (RAST RIA, Demeditec Diagnostics) diluted 1/20 in buffer A overnight at room temperature and visualized by autoradiography using Kodak X-OMAT films with intensifying screens at -70°C.

CD measurements were performed with purified clone 10-derived allergen (in H<sub>2</sub>O) at a protein concentration of 0.1 mg/ml on a Jasco J-810 spectropolarimeter using a 0.2-cm path length rectangular quartz cuvette. Far-UV CD spectra were recorded from 190 to 260 nm with 0.5-nm resolution at a scan speed of 50 nm/min and resulted from the average of three scans. Results are expressed as the mean residue ellipticity ( $\theta$ ) at a given wavelength. Temperature scans were performed according to a step-scan procedure, where the sample was heated from 25 to 95°C with a heat rate of 2°C/min and cooled back to 25°C at the same rate. Every 5°C, continuous wavelength spectra were recorded with the specified parameters. Additionally, temperature scans were recorded at 215 nm with a step resolution of 0.5°C. Results are expressed as the molar mean residue ellipticity ( $\theta_{\text{MRE}}$ ) at a given wavelength. The final spectra were corrected by subtracting the corresponding baseline spectrum obtained under identical conditions. The secondary structure content of clone 10-derived allergen was calculated using the secondary structure estimation program CDSSTR (37).

Table III. Percentage amino acid sequence identities<sup>a</sup>

| Clone | Clone (%) |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-------|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|       | 10        | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | 23  | 24  | 25  | 26  | 27  | 28  | 29  | 30  |
| 10    | 100       | 96  | 94  | 85  | 54  | 49  | 49  | 52  | 43  | 43  | 35  | 50  | 43  | 41  | 32  | 35  | 33  | 32  | 34  | 31  | 38  | 35  | 36  | 32  | 46  | 31  | 30  | 30  | 34  | 35  | 28  |
| 1     |           | 100 | 97  | 86  | 55  | 50  | 50  | 53  | 44  | 44  | 35  | 50  | 44  | 41  | 32  | 36  | 33  | 32  | 34  | 31  | 38  | 35  | 36  | 32  | 46  | 31  | 30  | 30  | 34  | 35  | 28  |
| 2     |           |     | 100 | 84  | 52  | 48  | 47  | 50  | 44  | 43  | 33  | 46  | 41  | 40  | 30  | 35  | 33  | 30  | 32  | 31  | 37  | 35  | 34  | 30  | 43  | 28  | 31  | 28  | 32  | 35  | 27  |
| 3     |           |     |     | 100 | 55  | 50  | 50  | 53  | 44  | 44  | 38  | 50  | 44  | 43  | 30  | 40  | 29  | 30  | 32  | 31  | 35  | 35  | 37  | 34  | 43  | 32  | 34  | 31  | 35  | 35  | 30  |
| 4     |           |     |     |     | 100 | 86  | 97  | 76  | 35  | 62  | 33  | 50  | 41  | 40  | 27  | 47  | 31  | 37  | 37  | 36  | 38  | 42  | 33  | 41  | 44  | 32  | 34  | 30  | 32  | 36  | 30  |
| 5     |           |     |     |     |     | 100 | 87  | 73  | 37  | 60  | 33  | 50  | 40  | 40  | 30  | 44  | 30  | 38  | 45  | 41  | 41  | 45  | 34  | 42  | 44  | 32  | 37  | 33  | 34  | 38  | 25  |
| 6     |           |     |     |     |     |     | 100 | 73  | 34  | 60  | 33  | 48  | 40  | 40  | 29  | 45  | 33  | 36  | 45  | 36  | 40  | 44  | 33  | 41  | 43  | 34  | 35  | 31  | 32  | 38  | 30  |
| 7     |           |     |     |     |     |     |     | 100 | 36  | 69  | 38  | 46  | 40  | 36  | 36  | 55  | 29  | 35  | 35  | 33  | 33  | 33  | 38  | 33  | 40  | 30  | 30  | 27  | 35  | 35  | 27  |
| 8     |           |     |     |     |     |     |     |     | 100 | 32  | 41  | 37  | 46  | 40  | 34  | 47  | 34  | 34  | 37  | 38  | 32  | 40  | 35  | 34  | 36  | 26  | 35  | 37  | 34  | 38  | 37  |
| 9     |           |     |     |     |     |     |     |     |     | 100 | 30  | 43  | 35  | 34  | 30  | 39  | 28  | 26  | 28  | 28  | 30  | 33  | 28  | 28  | 33  | 26  | 26  | 23  | 31  | 31  | 24  |
| 10    |           |     |     |     |     |     |     |     |     |     | 100 | 43  | 40  | 52  | 40  | 34  | 39  | 39  | 31  | 46  | 42  | 54  | 49  | 54  | 46  | 51  | 54  | 57  | 68  | 56  | 51  |
| 11    |           |     |     |     |     |     |     |     |     |     |     | 100 | 37  | 46  | 41  | 45  | 35  | 40  | 69  | 43  | 48  | 41  | 37  | 40  | 79  | 38  | 37  | 30  | 38  | 38  | 33  |
| 12    |           |     |     |     |     |     |     |     |     |     |     |     | 100 | 35  | 37  | 46  | 41  | 37  | 34  | 31  | 31  | 34  | 34  | 35  | 40  | 35  | 32  | 34  | 34  | 34  | 32  |
| 13    |           |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 37  | 38  | 37  | 35  | 41  | 44  | 40  | 40  | 41  | 38  | 47  | 44  | 43  | 35  | 41  | 44  | 37  |
| 14    |           |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 25  | 58  | 88  | 25  | 36  | 38  | 39  | 36  | 42  | 40  | 37  | 41  | 37  | 43  | 39  | 38  |
| 15    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 26  | 31  | 36  | 31  | 34  | 41  | 37  | 35  | 49  | 34  | 37  | 31  | 38  | 32  | 35  |
| 16    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 57  | 27  | 32  | 41  | 42  | 43  | 42  | 35  | 34  | 41  | 36  | 46  | 43  | 38  |
| 17    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 25  | 40  | 42  | 41  | 37  | 44  | 38  | 38  | 44  | 37  | 44  | 42  | 35  |
| 18    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 34  | 47  | 39  | 36  | 40  | 80  | 38  | 34  | 31  | 37  | 38  | 31  |
| 19    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 37  | 58  | 50  | 50  | 46  | 47  | 52  | 53  | 52  | 57  | 42  |
| 20    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 41  | 39  | 35  | 47  | 35  | 34  | 37  | 43  | 42  | 35  |
| 21    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 50  | 55  | 44  | 47  | 57  | 48  | 56  | 55  | 50  |
| 22    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 49  | 40  | 49  | 47  | 55  | 53  | 43  | 65  |
| 23    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 43  | 52  | 50  | 49  | 56  | 51  | 51  |
| 24    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 41  | 40  | 33  | 38  | 40  | 35  |
| 25    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 44  | 50  | 40  | 50  | 45  |
| 26    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 53  | 59  | 58  | 47  |
| 27    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 58  | 47  | 60  |
| 28    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 59  | 61  |
| 29    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 | 45  |
| 30    |           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 100 |

<sup>a</sup>Sequences numbered 1–30 follow organism specifications as in Fig. 2.

Human rat basophil leukemia (huRBL) assay

For the quantification of IgE Ab-mediated, immediate-type reactions, huRBL cell mediator release assays were performed. RBL cells (clone RBL-703/21) transfected with the human FcεRI (38) were cultured in RPMI 1640 medium supplemented with 5% FCS, 4 mM L -glutamine, and 1 mg/ml G418 sulfate. Cells were harvested after incubation with trypsin/EDTA, washed, resuspended in culture medium, and the cell concentration was adjusted to 2 × 10<sup>6</sup> cells/ml. Fifty-microliter aliquots of the cell solution were added to the wells of a 96-well flat-bottom microplate (cell density was 1 × 10<sup>5</sup> cells/well). Human sera were diluted 1/10 in culture medium, added to the cells, and incubated overnight at 37°C, 7% CO<sub>2</sub>, at 95% relative humidity. Medium was removed and the plates were washed three times with 200 μl/well of Tyrode's buffer + 0.1% BSA. For IgE cross-linking, 100 μl of clone 10-derived allergen or rPhl p 1 (0.3 μg/ml) diluted in Tyrodes's buffer containing 50% D<sub>2</sub>O and 0.1% (w/v) BSA was added to the cells. For spontaneous release, Tyrode's buffer without protein was added to the wells. Total release was determined by addition of Tyrode's buffer containing 10% Triton X-100. After incubation at 37°C, 7% CO<sub>2</sub>, and 95% relative humidity for 1 h, cells were harvested by centrifugation and 50 μl supernatant was transferred to a new plate, and 50 μl assay solution (0.1 M citric acid or sodium citrate (pH 4.5) and 160 μM 4-methyl umbelliferyl-*N*-acetyl-β-D -glucosaminide) per well was added. After another 1-h incubation, the reaction was stopped by adding 100 μl glycine buffer (0.2 M glycine, 0.2% NaCl (pH 10.7)) to each well. Fluorescence was measured at λ<sub>ex</sub> of 360 nm/λ<sub>em</sub> of 465 nm in a fluorescence microplate reader. Spontaneous release was determined from control wells that had not been lysed by Triton X-100. Specific release was calculated using the formula: (Sample – spontaneous/Total – spontaneous) × 100.

Immunogold electron microscopy

Dry grains of wheat were cut into small pieces (cubes of ~0.5 mm size) using a sharp razor blade. To preserve the dry state of the cells, the cubes were anhydrously fixed in acrolein vapor for 5 days at room temperature. They were transferred at room temperature for 1 day to dimethoxypropane (DMP) for removing any residual water and were embedded into Lowicryl K4M resin using ascending series of DMP-ethanol and ethanol-monomeric

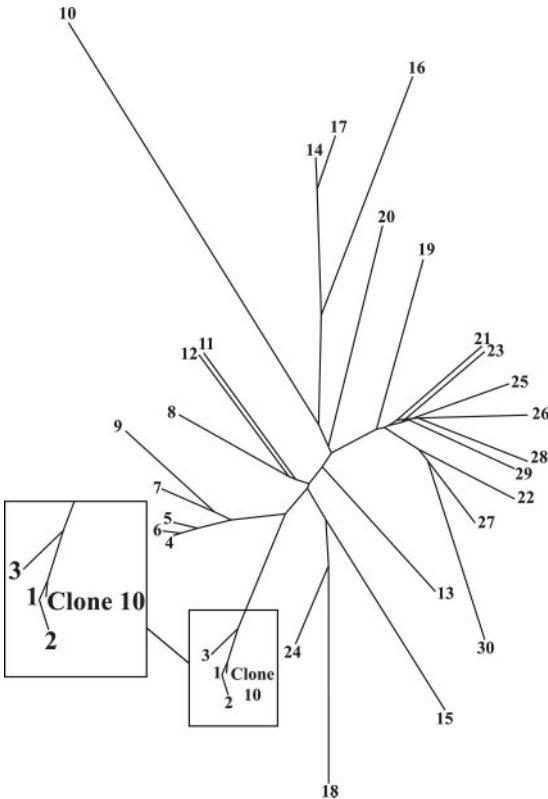


FIGURE 3. Phylogenetic tree based on the amino acid sequence similarities between the clone 10-derived allergen and homologous proteins. Numbers 1–30 correspond to the database entry codes and organism specifications as in Fig. 2. Distances indicate the relationship of the proteins to each other.

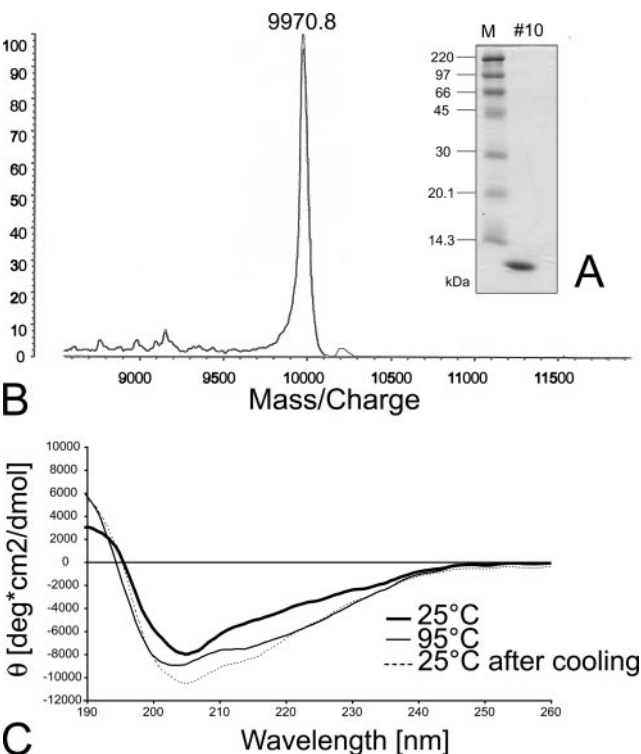


**FIGURE 4.** Characterization of purified serine proteinase inhibitor-like allergen. **A**, Coomassie brilliant blue-stained SDS-PAGE containing purified clone 10-derived allergen. A molecular mass marker (kDa) is shown on the left side. **B**, Mass spectroscopy of the purified clone 10-derived allergen. The mass/charge ratio is shown on the x-axis, and the intensity is displayed on the y-axis as a percentage of the most intensive signal obtained in the investigated mass range. **C**, Far-UV CD analysis of the purified clone 10-derived allergen. The spectra are expressed as mean residue ellipticities ( $\theta$ ) (y-axis) recorded at 25°C (bold line), 95°C (regular line), and 25°C after cooling (dotted line) at given wavelengths (x-axis).

Lowicryl K4M resin as intermediate stages. Polymerization was performed at -35°C.

Ultrathin sections were cut from both peripheral and central grain tissues and placed on silver grids for immunolabelling procedures.

Labeling for clone 10-derived allergen was performed in a moist chamber at room temperature (PBS buffer + 1% (w/v) BSA (pH 7.4), Tris buffer + 1% (w/v) BSA (pH 8.2)) as follows: 1) 5% (w/v) BSA in PBS buffer, 15 min; 2) rabbit anti-wheat protein 10 Abs and preimmune Abs, diluted 1/35 in PBS buffer, 2 h; 3) PBS buffer, 5 min, Tris buffer, twice for 5 min; 4) goat anti-rabbit IgG Abs coupled to colloidal gold particles of 10 nm size (BioCell) diluted 1/20 in Tris buffer; and 5) Tris buffer, 1 × 5 min, distilled water, twice for 5 min.

Sections were stained using uranyl acetate (5 min) and lead citrate (10 s).

Samples were analyzed with a transmission electron microscope EM 410 (FEI).

**Results**

*Isolation and characterization of a wheat cDNA coding for a serine proteinase inhibitor-like allergen*

A wheat seed cDNA expression library was screened with IgE Abs from four patients suffering from baker's asthma. Six different IgE-reactive clones were isolated and expressed in *E. coli*. The open reading frame of the cDNA of the IgE-reactive clone 10 contained 262 nucleotides coding for an 84-amino acid polypeptide (Fig. 1). A molecular mass of 9.4 kDa and an isoelectric point (pI) of 6.08 was calculated according to the deduced amino acid sequence for the clone 10-derived allergen. The analysis of amino acid composition showed a high content of valin residues (15.5%) and absence of cysteine residues. According to computer-aided secondary structure analysis, the clone 10-derived allergen consists mainly of random coils and  $\beta$ -sheets and one  $\alpha$ -helical domain. According to solvent accessibility calculations, almost 80% of the amino acids are solvent exposed. A search for sequence motifs revealed the presence of one potential casein kinase II phosphorylation site (amino acid 32), two N-terminal myristoylation sites (amino acids 11 and 55), and a potato inhibitor I family signature (Fig. 1: amino acids 25–36 are shaded in gray). No glycosylation site was found in the sequence.

The comparison of the clone 10-derived amino acid sequence with sequences deposited in the NCBI database showed that the allergen is almost identical to a *Triticum aestivum* subtilisin-chymotrypsin inhibitor (WSC1) precursor (accession no. gi 122065237) and to *T. aestivum* WSC1 proteinase inhibitor (accession no. gi 66356278) and exhibits significant sequence homologies with a group of serine proteinase inhibitors occurring in plants and animals (Fig. 2). These serine proteinase inhibitors constitute a family that is designated potato inhibitors I and are characterized by a typical consensus sequence pattern (Fig. 2: gray box) that is conserved in each of the proteins. The serine proteinase inhibitors belonging to the potato inhibitor I family are small proteins of 60–90 amino acids that lack disulfide bonds and contain only a single inhibitory site. The sequences of wheat serine proteinase inhibitors show a significant degree of sequence conservation to proteinase inhibitors of other monocotyledonic plants such as barley (*Hordeum vulgare*), maize (*Zea mays*, *Zea diploperennis*), gamma grass (*Tripsacum dactyloides*), and rice (*Oryza sativa*), as well as dicotyledonic plants and worms (Table III). Table III displays the percentages of sequence identities between each of

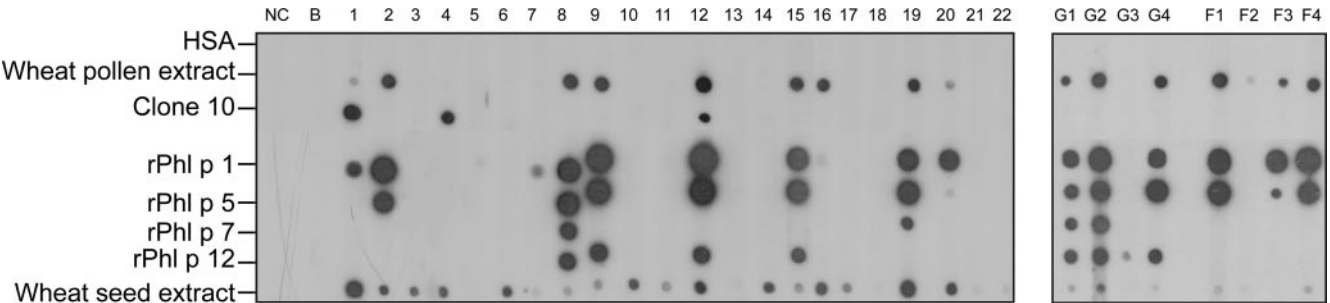


**FIGURE 5.** IgE reactivity of patients suffering from baker's asthma, grass pollen allergy, and food allergy. Purified clone 10-derived allergen, human serum albumin, rPhl p 1, rPhl p 5, rPhl p 7, rPhl p 12, wheat pollen extract, and wheat seed extract were dotted onto nitrocellulose membrane strips and incubated with sera from 22 baker's asthma patients (1–22), 4 grass pollen-allergic patients sera (G1–G4), 4 sera from patients suffering from food allergy to wheat (F1–F4), one nonallergic individual (NC), and buffer without addition of serum (B). Bound IgE Abs were detected with <sup>125</sup>I-labeled anti-human IgE Abs and visualized by autoradiography.

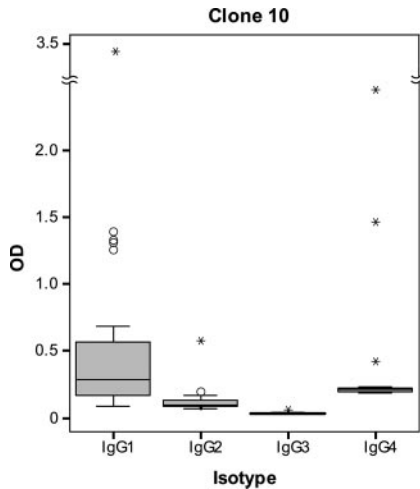


**FIGURE 6.** Box plot representation of IgG subclass reactivities to clone 10-derived allergen. IgG1–4 subclass reactivities to the clone 10-derived allergen were determined by ELISA for patients suffering from baker’s asthma ( $n = 22$ ) and are displayed as box plots where 50% of the values are within the boxes and nonoutliers are between the bars. The lines within the boxes indicates the median values. Circles are outliers and asterisks represent extreme values.

the serine proteinase inhibitors, and the phylogenetic tree constructed in Fig. 3 illustrates the relationships between the proteins as distances from each other. The other five IgE-reactive clones isolated by screening the cDNA expression library encode for thioredoxin h, glutathione transferase, 1-Cys-peroxyredoxin, profilin, and dehydrin according to homology with sequences deposited in the NCBI database (C. Constantin and R. Valenta, unpublished data).

*Expression, purification, and physicochemical characterization of the serine proteinase inhibitor-like allergen*

The clone 10-derived allergen was expressed in *E. coli* BL21 (DE3) with a C-terminal hexahistidine tag. Approximately 25 mg/L liquid culture of serine proteinase inhibitor-like allergen could be purified by nickel chromatography (Fig. 4A). MALDI-TOF analysis of purified recombinant protein 10 resulted in a mass peak of 9970.8 Da (Fig. 4B). The far-UV CD spectrum of purified recombinant clone 10-derived allergen (Fig. 4C) indicates that the protein is folded and contains a considerable amount of  $\beta$ -sheets and a low  $\alpha$ -helical content. The spectrum is characterized by a minimum at 204 nm and a maximum at 190 nm. Secondary structure analysis using the program CDSSTR with the reference dataset 7 yielded 8%  $\alpha$ -helix, 23%  $\beta$ -sheets, 14%  $\beta$ -turns, and 53% random coils. The normalized root mean square difference value of 0.033 demonstrated a good fit between the calculated and the experimentally derived spectra. Upon heating to 95°C, a slight shift of the minimum of the CD spectrum (from 204 to 201 nm) was observed, indicating a partial denaturation of the protein. Upon cooling to 25°C, the protein refolded (Fig. 4C). However, the minimum at 204 nm was lower than before heating, which suggests a rearrangement of the  $\beta$ -sheets. In summary, the spectra observed during the temperature scan point to a high thermal stability of the clone 10-derived allergen.

*Recombinant clone 10-derived allergen is a new serine proteinase inhibitor-like allergen from wheat*

Purified clone 10-derived allergen was tested for IgE reactivity using sera from patients suffering from baker’s asthma, grass pollen allergy, and food allergy to wheat by dot blot analysis (Fig. 5).

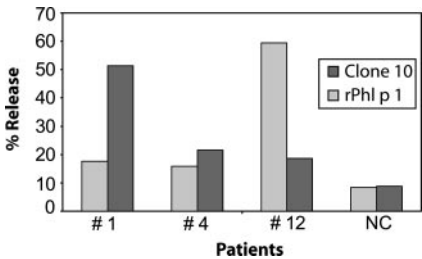


**FIGURE 7.** Allergenic activity of the clone 10-derived allergen. RBL cells were loaded with serum IgE from three baker’s asthma patients (nos. 1, 4, and 12) or with serum of a nonallergic patient (NC) and then challenged with recombinant clone 10-derived allergen or timothy grass pollen allergen rPhl p 1. The mean  $\beta$ -hexosaminidase releases are shown on the y-axis as percentages of total release after subtraction of percentages for spontaneous release.

The recombinant clone 10-derived allergen reacted with IgE Abs from 3 (nos. 1, 4, and 12) out of 22 patients suffering from baker’s asthma (13.6%). Patient no. 1, who had only low IgE levels specific for the major wheat allergen,  $\alpha$ -amylase inhibitor, showed strong IgE reactivity to the serine proteinase inhibitor-like allergen. Interestingly, the clone 10-derived allergen was exclusively recognized by IgE Abs from patients with baker’s asthma but not by patients suffering from IgE-mediated food allergy to wheat or by patients suffering from grass pollen allergy. Several sera from each of the three patients groups showed IgE reactivity to recombinant timothy grass pollen allergens due to cosensitization to grass pollen. The specific IgE reactivity of the clone 10-derived allergen by baker’s asthma patients was confirmed in a protein chip analysis using an additional 20 sera from celiac disease patients, sera from 119 patients suffering from wheat-induced food allergy, 23 sera from grass pollen-allergic patients, sera from the 22 baker’s asthma patients tested in Fig. 5, and an additional 3 sera from baker’s asthma patients (C. Constantin and R. Valenta, unpublished data). None of the patients who suffered from wheat-induced food allergy, celiac disease patients, or grass pollen-allergic patients had IgE Abs specific for the clone 10-derived allergen. The analysis of IgG subclass reactivities to the clone 10-derived allergen in the group of baker’s asthma patients showed the presence allergen-specific IgG1 and to a lower extent of allergen-specific IgG4 levels, with both being indicative of a  $Th_2$  response, whereas no relevant IgG2 and IgG3 reactivities specific for the clone 10-derived allergen were detected (Fig. 6). No reciprocal associations between IgE and IgG responses to the clone 10-derived allergens were noted.

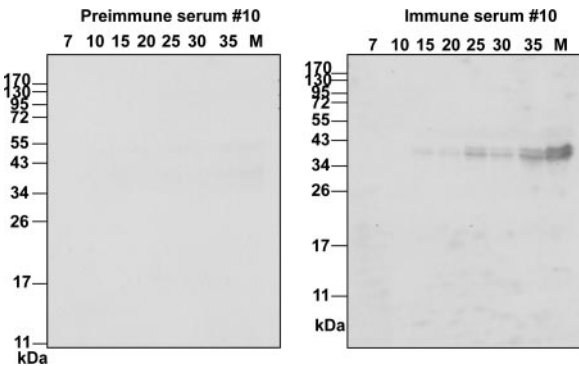


**FIGURE 8.** Expression of the serine proteinase inhibitor-like allergen in seeds during seed maturation. Nitrocellulose-blotted wheat extract from immature (days 7, 10, 15, 20, 25, 30, and 35) and mature (M) wheat seeds were probed with rabbit Abs specific for the clone 10-derived allergen and, for control purposes, with the corresponding preimmune serum. Molecular masses are indicated on the left side in kilodaltons.

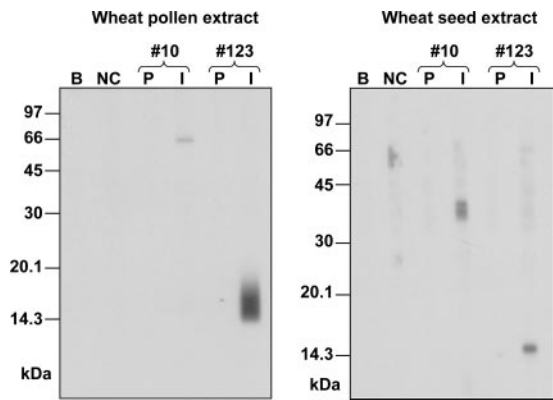


**FIGURE 9.** Identification of the clone 10-derived allergen (I: #10), Abs specific for wheat profilin (I: #123), in wheat pollen and seed extracts. Nitrocellulose blotted extracts were probed with rabbit Abs specific for the clone 10-derived allergen, Abs specific for wheat profilin, for a mite allergen (NC), or with buffer without addition of rabbit Abs (B). The corresponding preimmune sera are referred as P no. 10 and P no. 123, respectively. Bound IgG Abs were detected with  $^{125}$ I-labeled donkey anti-rabbit Abs and visualized by autoradiography. Molecular mass markers (in kilodaltons) are indicated on the left.

To study the allergenic activity of IgE Abs specific for the serine proteinase inhibitor-like allergen, RBL cells expressing the human Fc $\epsilon$ RI were loaded with serum IgE from patients with and without specific IgE Abs and subsequently exposed to the allergen (Fig. 7). RBL cells loaded with serum IgE from patient no. 1 showed the strongest degranulation upon allergen exposure (51% of total  $\beta$ -hexosaminidase release). A lower degranulation was obtained with RBL cells that had been loaded with IgE from patients nos. 4 and 12 (22 and 19%, respectively), which corresponded with the intensity of IgE recognition in the dotblots (Fig. 5). Almost no degranulation was observed when RBL cells were loaded with serum from a nonallergic person (Fig. 7: NC). The major timothy grass pollen allergen rPhl p 1 induced strong degranulation in RBL cells loaded with serum IgE from patient no. 12 and mild degranulation when RBL cells were loaded with sera from patients nos. 1 and 4 (Fig. 7). In fact, patients nos. 1, 4, and 12 suffered also from grass pollen allergy (Table I).

*The serine proteinase inhibitor-like allergen accumulates in wheat seeds during maturation*

Rabbit Abs specific for clone 10-derived allergen were used to investigate the expression of the protein during wheat seed maturation (Fig. 8). Nitrocellulose sheets containing extracts from wheat seeds collected at different time points of seed maturation

were probed with specific rabbit Abs and the preimmune Ig. The clone 10-specific Abs reacted with a protein of 40 kDa, representing a tetramer of the serine proteinase inhibitor-like allergens (Fig. 8) (39). The expression of the protein became detectable in 15-day-old seeds and continued to increase during further maturation of seeds (Fig. 8). No immunoreactivity was found when the blots were incubated with the preimmune Ig from the same rabbit (Fig. 8).

*The serine proteinase inhibitor-like allergen is preferentially detected in wheat seeds*

When we compared pollen and seeds, we found that the serine proteinase inhibitor-like allergen is preferentially expressed in seeds (Fig. 9), whereas only a weak signal was obtained at ~65 kDa in wheat pollen extract. The panallergen profilin was detected with rabbit anti-wheat seed profilin Abs in wheat seeds and pollen (Fig. 9: no. 123). No reactivity was found with preimmune Ig (Fig. 9).

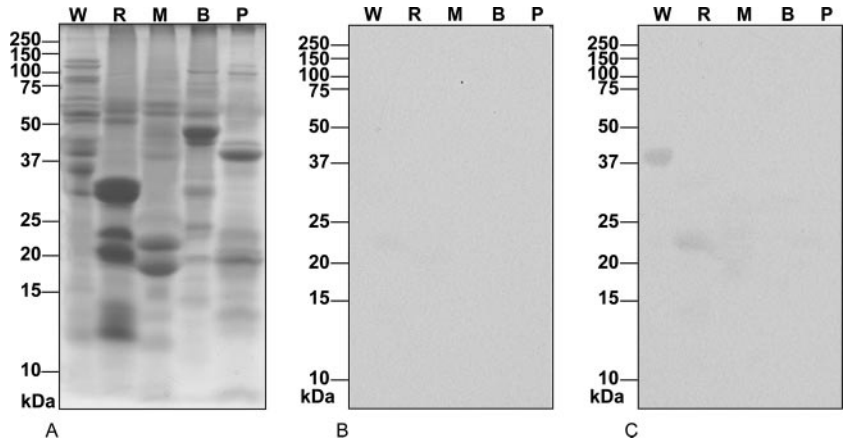
Next, we used the serine proteinase inhibitor-like allergen-specific Abs to search for cross-reactive structures in rice, maize, the common bean, and potato for which homologous proteins have been described with a sequence identity of 50% (bean), 49% (maize, rice), and 33% (potato) (Table III).

The serine proteinase inhibitor-like allergen was detected again as tetramer in wheat seeds; a band of ~23 kDa was detected in rice, but no reactivity was found in maize, the common bean, or potato (Fig. 10C), although comparable amounts of each extract had been subjected to SDS-PAGE (Fig. 10A). No reactivity was observed when the blots were incubated with the preimmune Ig (Fig. 10B).

Localization of the serine proteinase inhibitor-like allergen in the aleurone layer and between starch granules of a wheat grain by immunogold electron microscopy.

Fig. 11A shows an ultrathin section through a wheat grain at low magnification in the transmission electron microscope. Three major morphological components of the grain are visible: an outward multilayered fruit and seed coat (C), the aleuron layer (AL), and the beginning of the voluminous interior of the grain, the starchy endosperm (SE). The rectangle in Fig. 11A marks an area comparable to the area shown in Fig. 11B. Fig. 11B displays the border between an aleuron cell and the adjoining starchy endosperm at higher magnification. The aleuron cell is filled with aleuron grains (AG) (protein vacuoles), which are surrounded by small lipid vesicles (L). Both components are embedded in the cytoplasmic matrix of the cell. The starchy endosperm consists of starch granules with variable sizes that are closely packed, just leaving small interspaces of amorphous cytoplasmic material. The rectangles indicate areas shown in high magnification in Fig. 11, C and D and E and F, respectively. Fig. 11C shows the

**FIGURE 10.** Detection of the serine proteinase inhibitor-like allergen in extracts from wheat seeds, rice, maize, bean, and potato. A, Coomassie blue-stained gel containing extracts from wheat (W), rice (R), maize (M), common bean (B), and potato (P). B, Nitrocellulose blotted extracts were exposed to rabbit preimmune serum and C, rabbit Abs specific for the clone 10-derived allergen. Molecular masses (in kilodaltons) are indicated on the left.



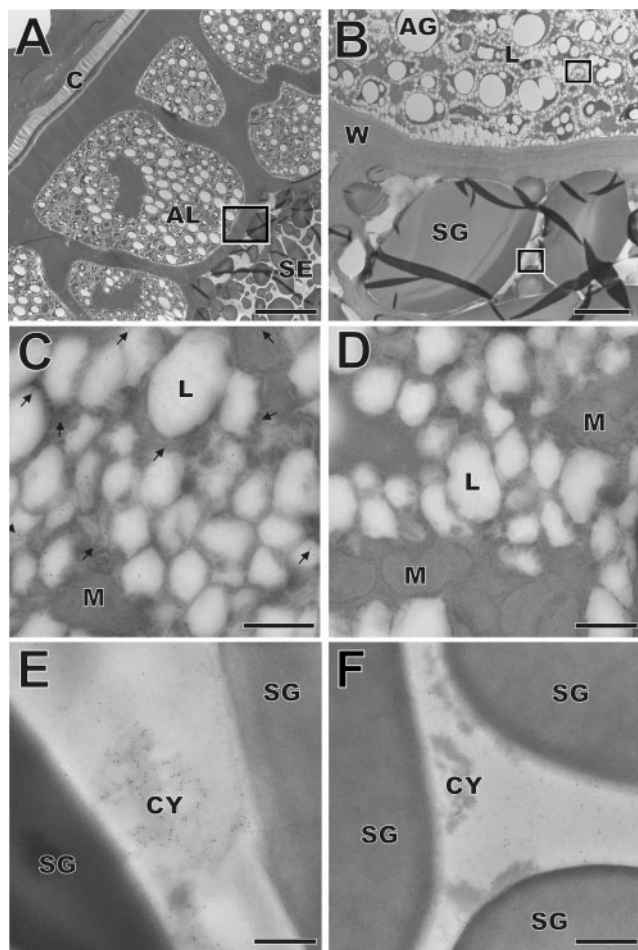


**FIGURE 11.** Localization of clone 10-derived allergen by transmission immunogold electron microscopy in a wheat seed. *A* and *B*, Cross section of a wheat grain at low (*A*) and high (*B*) magnification. *A*, Fruit and seed coat (*C*), aleuron layer (*AL*), and the beginning of the starchy endosperm (*SE*). The rectangle in *A* indicates an area comparable to the area shown in *B*, that is, the border between the aleuron layer and starchy endosperm. The rectangles in *B* indicate areas shown in high magnification in *C* and *D* and *E* and *F*, respectively. *C* and *D*, Detail of a wheat seed aleurone cell probed with rabbit anti-clone 10-derived Ig (*C*) or preimmune Ig (*D*). *E* and *F*, High magnification micrograph of the starchy endosperm after immunogold localization of wheat protein 10 with rabbit anti-clone 10-derived Ig (*E*) or preimmune Ig (*F*). Bound rabbit Abs were detected with a gold-conjugated goat anti-rabbit Ig antiserum (gold particles = black dots). Arrows point to colloidal gold particles. The bars represent: *A*, 20  $\mu$ m; *B*, 5  $\mu$ m; *C–F*, 0.5  $\mu$ m. *AG*, aleuron grain; *AL*, aleuron layer; *C*, multilayered fruit and seed coat; *CY*, cytoplasmic materials; *L*, lipid body; *M*, mitochondrion; *SE*, starchy endosperm; *SG*, starch grain; *W*, cell wall.

localization of clone 10-derived allergen in an aleuron cell using Abs raised against wheat protein 10. Gold particles (arrows) indicate the presence of wheat protein 10 predominantly in the cytoplasmic matrix between cell organelles but also in the peripheral parts of the lipid vesicles (*L*). In the starch region, wheat protein 10 is associated with the amorphous cytoplasmic material (40) between the starch granules (*SG*) (Fig. 11*E*). Control experiments with preimmune Abs showed a very low degree of non-specific labeling (Fig. 11, *D* and *F*).

## Discussion

Baker's asthma is one of the most important and long-known occupational diseases based on an IgE-mediated mechanism (2). The disease is mainly caused by allergens present in wheat flour. Some of

these allergens, such as fungal  $\alpha$ -amylase, are derived from contaminants or additives (41), whereas others represent genuine wheat proteins or glycoproteins (18, 22, 23, 42). At present, only few genuine wheat allergens have been identified, and it is not yet clear whether they are specific for baker's asthma or whether they are also recognized by patients suffering from wheat-associated food allergy or grass pollen allergy (26). For example, profilin, a highly conserved cytoskeletal protein, occurs as a cross-reactive allergen in wheat seeds as well as in pollen and numerous other allergen sources (43, 44).

To identify wheat allergens that are associated with baker's asthma we have constructed a cDNA library from wheat seeds selected at a maturation stage where allergens recognized by baker's asthma patients were expressed. This cDNA library was screened with serum IgE from patients with clinically confirmed baker's asthma (29) to isolate phage clones containing cDNAs coding for allergens associated with baker's asthma. One clone (i.e., clone 10) contained a complete cDNA that coded for an allergen specifically recognized by 13.5% of patients suffering from baker's asthma. There are several allergens that are more frequently recognized by baker's asthma patients such as  $\alpha$ -amylase from *Aspergillus oryzae*, acyl-CoA oxidase, and peroxidase, but clone 10-derived allergen is an allergen with high specificity for baker's asthma (19).  $\omega$ -5 gliadin was identified as an allergen with high specificity for patients suffering from wheat-dependent, exercise-induced anaphylaxis. According to Matsuo and colleagues, 80% of patients suffering from wheat-dependent, exercise-induced anaphylaxis showed IgE-reactivity to  $\omega$ -5 gliadin in dot blot analyses (27). Sequence analysis of the clone 10-derived allergen unambiguously identified the allergen as a serine proteinase inhibitor of the potato inhibitor I family (45). The clone 10-derived allergen contained the consensus sequence that is the typical signature of the members of this proteinase inhibitor I family (45). Furthermore, the clone 10-derived allergen showed the typical architecture and length ( $\sim$ 9 kDa) of the potato I inhibitors and exhibited significant end-to-end sequence identities with other members of this protein family (Fig. 2). The clone 10-derived allergen therefore belongs to the potato inhibitor I family, which together with other serine protease inhibitors, cysteine protease inhibitors, metalloprotease inhibitors, and aspartic protease inhibitors are important elements of the plant defense response to insect predation and nematode infection and therefore are referred to as pathogenesis-related (PR) proteins (46, 47). PR proteins are induced by environmental stress, wounding, and infection, and their expression is regulated by several signal pathways (47). Interestingly, many important allergens, such as the major birch pollen allergen, Bet v 1, and several fruit, vegetable, and latex allergens belong to the PR proteins (47). However, as yet, no member of the group of PR6 proteins consisting mainly of protease inhibitors such as the potato inhibitor I has been identified (47). The clone 10-derived serine proteinase inhibitor-like allergen thus is the first allergen described for the PR6 family. It is quite possible that PR proteins, in particular proteinase inhibitors, frequently become allergenic because they represent stable proteins that can survive in the environment and in digestion in the host tissues. In this context, we found that the clone 10-derived allergens represented a folded protein and preserved fold even when exposed to high temperatures. The clone 10-derived allergens bound specifically IgE Abs from baker's asthma patients, but not from patients allergic to wheat food or grass pollen. The serine proteinase inhibitor-like allergen was highly expressed in mature wheat seeds, but only weak immunoreactivity was found in pollen and in other plant food sources, suggesting that this allergen may be rather specific for wheat seeds. A rabbit antiserum raised against the clone 10-derived allergen showed besides weak reactivity to a 23-kDa structure in rice no

relevant cross-reactivity with maize, bean, and potato. However, using recombinant clone 10-derived allergen for IgE inhibition studies, no cross-reactive structure could be detected in rice (data not shown). The latter result is interesting because serine proteinase inhibitors of the potato inhibitor I family with significant sequence homology to the clone 10-derived allergens occur in various plants and even in animals, but the sequence homology seems to be too low to account for a relevant immunological cross-reactivity.

The wheat-derived serine proteinase inhibitor was in fact specifically recognized by serum IgE from patients with baker's asthma but showed no IgE reactivity when tested with sera from patients suffering from food allergy to wheat, celiac disease, or grass pollen allergy. It is therefore possible that the clone 10-derived allergen together with other wheat allergens can be used to establish diagnostic tests that allow to specifically identify patients suffering from IgE-mediated baker's asthma and to discriminate these patients from allergic patients with food or pollen allergy. Ultimately it may even be considered to develop strategies for specific immunotherapy based on the clone 10-derived allergens and other relevant wheat allergens for the treatment of baker's asthma.

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