

Food and drug reactions and anaphylaxis

IgE binding of the recombinant allergen soybean profilin (rGly m 3) is mediated by conformational epitopes

Hans-Peter Rihs, PhD,^a Zhiping Chen, PhD,^a Franziska Ruëff, MD,^b Amd Petersen, PhD,^c Peter Rozynek, MSc,^a Heike Heimann,^a and Xaver Baur, MD^a Bochum, Munich, and Borstel, Germany

Background: Soybean proteins are constituents of a number of food products and represent a panel of potential allergens. Thus far, little is known about the molecular characteristics of soybean allergens.

Objective: The aim of this study was to identify the soybean profilin by PCR-based complementary (c)DNA cloning and to elucidate its allergenic characteristics.

Methods: Highly degenerate profilin-specific primers were used to identify, by means of PCR, 2 soybean profilin isoforms (GmPRO1 and GmPRO2) by using soybean cDNA as a target. One isoform (GmPRO1) with a length of 394 bp corresponding to 131 amino acid residues was subcloned and expressed in fusion with the maltose-binding protein. Moreover, 3 overlapping recombinant soybean profilin fragments comprising amino acid residues 1-65, 38-88, and 50-131 were also prepared as maltose-binding protein fusion proteins. IgE-binding reactivity of the recombinant proteins and the cross-reactivity of soybean profilin with birch profilin was studied by immunoblotting, enzyme-linked allergosorbent assays (EASTs), and competitive inhibition experiments by using serum samples from 13 soybean-sensitized subjects.

Results: Results of immunoblot analysis, EAST, and EAST-inhibition experiments indicate the presence of profilin in soybean extract. The recombinant soybean profilin (rGly m 3) was recognized by IgE in 9 (69%) of the 13 sera tested. Only the full-length rGly m 3 was able to bind with IgE antibodies, whereas the 3 soybean profilin fragments did not show significant binding reactivity, indicating that the IgE binding to rGly m 3 depends on the integrity of a conformational structure, which was not present in the overlapping profilin fragments. The rGly m 3 cross-reacted with birch pollen profilin (Bet v 2), and the IgE binding to Bet v 2 could be inhibited by rGly m 3.

Conclusions: rGly m 3 represents a new soybean allergen with well-characterized primary sequence, and its IgE-binding reactivity is mediated by conformational epitopes. (*J Allergy Clin Immunol* 1999;1293-1301.)

Key words: Recombinant profilin, soybean profilin, IgE, conformational epitope, soybean allergy

Soybean proteins are important constituents of a number of food products and may cause hypersensitivity reactions in susceptible individuals, especially in children with atopic dermatitis.¹ Occupational asthma associated with exposure to soybean proteins has been reported.²⁻⁴ Airborne soy protein allergens have also been identified as the major causal agent of epidemic asthma outbreaks in Spain. Between 1981 and 1987, 26 such asthma outbreaks occurred in Barcelona, affecting about 680 patients with asthma and causing 24 deaths.⁵⁻⁸ Similar epidemic cases were also reported in Cartagena, Spain^{9,10} and in New Orleans in the 1950s and 1960s.¹¹ Serologic studies^{8,12} detected specific IgE antibodies to soybean proteins in almost all of the serum samples from patients with epidemic asthma, indicating an IgE-mediated pathogenesis in subjects with soybean-induced asthma.

For diagnostic purposes, the whole soybean extract is used. However, soybean proteins represent a panel of potential allergens. By using immunoblotting, allergens were identified in the molecular mass range of 8 to 90 kd.^{3,13-17} As yet little is known about the molecular characteristics of allergens that are responsible for the allergic reactions. Recently, Helm et al¹⁷ could show that an IgE-binding soybean protein named Gly m Bd 30K is identical with the soybean 34-kd seed vacuolar protein P34, a thiol protease of the papain family. Two additional allergens of the soybean hull, the 7.5-kd Gly m 1 and the 8-kd Gly m 2, both associated with the asthma outbreaks in Cartagena and Barcelona, have been partially characterized.¹⁸⁻²⁰ Moreover, in a number of studies^{3,13-15,21,22} the allergenicity of the individual soybean allergens according to immunoblotting patterns was quite controversial. Several proteins with molecular weights between 14 and 60 kd in soybeans were suggested as major allergenic protein components, which, however, could be ascertained in individual experiments only. Different sensitization histories of individual patients may be one of the reasons for this discrepancy, but the application of nonstandardized soybean protein extracts as allergen sources for experiments might be the major cause for getting different results.

In the present study highly degenerated primers²³ were

From ^athe Research Institute for Occupational Medicine (BGFA) at the Ruhr-University, Bochum; ^bthe Dermatological Clinic, Ludwig-Maximilians-University, Munich; and ^cthe Research Centre Borstel, Borstel.

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Reprint requests: Hans-Peter Rihs, PhD, BGFA, Buerkle-de-la-Camp-Platz 1, 44789 Bochum, Germany.

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TABLE I. Clinical data and total and specific IgE levels of the selected patients with and without food-adverse reactions

Sub- jects*	Age (y)	Sex	Total IgE (kU/L, CAP)	Food- associated symptoms	Soybean- specific IgE (kU/L, CAP)	rGly m 3- specific IgE (kU/L, EAST)	rGly m 3 (1-65)- specific IgE (kU/L, EAST)	rGly m 3 (38-88)- specific IgE (kU/L, EAST)	rGly m 3 (50-131)- specific IgE (kU/L, EAST)	Birch pollen- specific IgE (kU/L, CAP)	rBet v 2- specific IgE (kU/L, CAP)
1	28	F	433	AE, AN†	2.76	11.14	0.98	0.51	<0.35	25.60	8.20
2	26	F	>2000	AE, AN‡	4.01	3.68§	<0.35§	<0.35§	<0.35§	4.84	22.30
3	27	F	>2000	AE	34.70	2.73	<0.35	<0.35	0.38	>100	22.50
4	31	F	>2000	AE	9.51	7.63	<0.35	<0.35	<0.35	79.70	34.50
5	54	F	>2000	AE	7.08	ND	ND	ND	ND	ND	22.30
6	39	F	>2000	AE	22.00	1.79	ND	ND	ND	19.80	2.46
7	42	M	>2000	AE, AS	43.00	0.40	ND	ND	ND	>100	1.12
8	34	M	448	AE	0.51	0.50	<0.35	<0.35	<0.35	3.66	2.15
9	18	F	140	AE	2.16	0.61	<0.35	<0.35	<0.35	1.45	1.77
10	36	M	1597	AE	3.43	<0.35	ND	ND	ND	2.93	<0.35
11	6	F	ND	AE	3.82	<0.35	ND	ND	ND	1.34	<0.35
12	57	F	171	AE	2.82	<0.35	ND	ND	ND	90.60	<0.35
13	33	F	320	AE	5.52	<0.35	ND	ND	ND	10.10	<0.35
14	52	F	356	—	<0.35	1.11	ND	ND	ND	11.22	5.21
15	29	F	170	—	0.63	3.35	ND	ND	ND	23.20	4.33
16	51	M	563	—	1.07	14.75	ND	ND	ND	97.60	21.30

AE, Atopic eczema; AN, anaphylaxis (†to carrot, celery, banana, avocado, parsley; ‡to kiwi, nuts, fish); AS, asthma, ND, not determined.

*No. 1-13, Patients with food-adverse reactions and soybean-specific IgE antibodies; No. 14-16, birch pollen-sensitive patients without food-adverse symptoms.

§Serum was at a 1:2 dilution.

||rGly m 3 elicited positive test results only by immunoblot (Fig 4, lane 7).

Abbreviations used

EAST: enzyme-linked allergosorbent assay
MBP: Maltose-binding protein

used to identify the soybean profilin gene. The recombinant soybean profilin, as well as its 3 long, overlapping protein fragments, were then produced in C-terminal fusion with the maltose-binding protein (MBP). Their IgE-binding abilities were studied by immunoblotting and enzyme-linked allergosorbent assays (EASTs) with IgE antibodies from soybean-sensitized patients. Our results demonstrate that the soybean profilin was a new allergen in soybean with a fully characterized primary sequence, and the conformational epitopes on the soybean profilin were necessary for the efficient mediation of IgE binding. The new soybean profilin allergen was named rGly m 3 in accordance with the World Health Organization guidelines of allergen nomenclature.²⁴

METHODS

Human sera

Serum samples were obtained from 13 patients (10 female and 3 male patients; age range, 6-57 years) who were suspected of having food-induced allergies and showed positive IgE antibodies to soy proteins, as determined by using the Pharmacia CAP system (Pharmacia-Upjohn, Uppsala, Sweden). They were all highly atopic and had atopic dermatitis. Food-associated symptoms were found to be atopic eczema, anaphylaxis, and/or asthma (Table I). According to case histories, soybean components were suggestive allergens in all subjects, but no double-blind, placebo-controlled food challenges

were performed. In one patient an oral provocation test was performed and led to a degeneration of the atopic eczema. The concentrations of specific IgE antibodies to soybean were found to be 0.51 to 43 kU/L in sera. In addition, IgE antibodies were found to celery (n = 10), potato (n = 10), hazelnut (n = 9), egg white (n = 8), cow milk proteins (n = 6), and peanut (n = 6). Two groups of sera were taken as controls. In the first group serum samples from 18 individuals allergic to birch pollen (8 female and 10 male subjects; age range, 20-65 years) without food allergies were used to investigate the potential cross-reactivity of rGly m 3 with the recombinant birch profilin rBet v 2. An additional 6 serum samples obtained from nonallergic individuals served as negative controls.

PCR of the complementary DNA

Soybean profilin-specific amplification was carried out in a 50-μL reaction mixture containing 1 ng of soybean complementary (c)DNA (isolated from Glycine max, var Resnik, 10-day-old seedlings; Quick-Clone, Clontech, Palo Alto, Calif), 50 mmol/L KCl, 10 mmol/L TRIS-HCl (pH 9.0), 2.0 mmol/L MgCl₂, 0.01% (wt/vol) gelatin (Sigma, Deisenhofen, Germany), 500 μmol/L of each deoxynucleotide triphosphate, 25 pmol of each of the primers PRO52 (5'-ATGTCNTGGCARGCNTAYGT-3') and PRO33 (5'-ARTADCCYTGTNCTDATNAGRTART-3'),²³ and 2.5 U of Taq Polymerase (Life Technologies, Eggenstein, Germany). Reaction products were obtained in a thermal cycler (Biometra, Göttingen, Germany) with an initial denaturation step (95°C for 3 minutes) and a total of 35 cycles of denaturation (95°C for 1 minute), annealing (57°C for 1 minute), and extension (72°C for 2 minutes) followed by 10 minutes at 72°C.

Subcloning and sequencing of the PCR products

The 394-bp PCR product was end-filled, phosphorylated, and subcloned into *Sma*I-digested and dephosphorylated pUC18 (Pharmacia, Freiburg, Germany) to ensure the PCR products. Clones

were characterized by restriction analysis and by nonradioactive sequencing on an ABI 310 sequencer (Applied Biosystems, Weiterstadt, Germany). PCR products were also subcloned after end-filling and phosphorylation and followed by preligation with an adapter molecule (5'-ACGTAGGATCCAC-3') and a final *AflIII*-*Bam*HI digest into *Nco*I-*Bgl*III-digested pQE60 (Qiagen, Hilden, Germany). Sequencing analysis with QIAexpress sequencing primers (Qiagen) revealed that the PCR products correspond to open reading frames coding for 2 isoforms of soybean profilin named GmPRO 1 and GmPRO 2. Because GmPRO 2 carries an uncomfortable *Hind*III restriction site at the 5' end of the cDNA (Fig 1, nucleotides 11-16) and has a high amino acid sequence homology with GmPRO1 (98%), the characterization of the allergenic properties were exclusively investigated with the GmPRO 1 isoform and 3 of its overlapping fragments.

For the efficient expression of the recombinant soybean profilin in *Escherichia coli* TB-1, the complete GmPRO 1 sequence was subcloned after primer-mediated adaptation into the expression vector pMAL-c2 downstream from the *malE* gene coding for the MBP.

Five hundred nanograms of GmPRO 1 DNA were used as the target for the amplification with primer pair MAL-S1 (5'-ACCGGTCTCCATGTCSTGGCATGC) and PRO331 (5'-GGGAATTCAGTAGCCYTGTCGATGAGGTARTC-3') by using the same PCR conditions as described above, with the exception that the annealing temperature was 60°C. After a *Bsa*I digest, an end-filling reaction, and a final *Eco*RI digest, the insert was subcloned into *Xmn*I-*Eco*RI-digested pMAL-c2 (New England Biolabs, Schwalbach, Germany) yielding pMALGmPRO 1, which was able to overexpress a 56.8-kd MBP-soybean profilin fusion protein in the presence of 0.3 mmol/L isopropyl thiogalactose in the *E coli* host TB-1 (New England Biolabs).

To investigate the IgE-binding properties of soybean profilin in different parts of the protein, we amplified the soybean DNA of pMALGmPRO 1 with primer pairs A/B (5'-pATGTCCTGGCAGGCGTATGTCG/5'-GGGAATTCACAATCCAGTTGGAGCAAG-3), C/D (5'-GGGTCGACTTCCCTCAGTTCAAACCTGAGG-3'/5'-CCGAATTCAACCTTCTTCCCTCGAATGACGAC-3'), and E/PRO331 (5'-CGGATATCATGAATGACTTTAATGAGCC-3'/PRO331), yielding PCR products corresponding to soybean profilin amino acid residues 1-65, 38-88, and 50-131. After treatment with *Eco*RI (A/B), *Hinc*II-*Eco*RI (C/D), and *Eco*RV-*Eco*RI (E/PRO331), all 3 fragments were separately subcloned into *Xmn*I-*Eco*RI-digested pMAL-c2 vector, yielding the 3 different malE-soybean profilin fragments pMALGmPRO 1 (1-65), pMALGmPRO 1 (38-88), and pMALGmPRO 1 (50-131), expressing the fusion proteins MBP-rGly m 3 (1-65), MBP-rGly m 3 (38-88), and MBP-rGly m 3 (50-131). After verification by sequencing, all constructs were overexpressed in *E coli* TB-1 by using the growing conditions described below.

Expression and purification of MBP-rGly m 3 and its fragments

One liter of LB medium containing 100 µg/mL (wt/vol) ampicillin and 2 mg/mL (wt/vol) glucose was inoculated with 10 mL of fresh recombinant *E coli* cells from an overnight culture and incubated at 37°C for 2 hours on an laboratory shaker (New Brunswick, Nuertingen, Germany). Expression of the fusion proteins was induced by shaking in the presence of 0.3 mmol/L isopropyl thiogalactose (Roth, Freiburg, Germany) for 4 hours at 37°C. *E coli* cell lysates containing the expressed fusion protein were prepared by sonification in an ice-water bath with a Branson sonifier-250 (8 intervals of 20 seconds on level 4 followed by a time stop of 30 seconds) followed by centrifugation at 10,000g for 30 minutes. The supernatant was loaded onto a 1.5 × 20-cm column filled with a 7-mL bed volume of amylose (New England Biolabs). Equilibration,

washing, and elution were performed as recommended by the supplier's protocol (New England Biolabs).

The rGly m 3 was cleaved from MBP by incubation of affinity-purified fusion protein with proteinase Factor Xa (MobiTech, Goettingen, Germany) dissolved in 100 mmol/L PBS (pH 7.6) at a Factor Xa/fusion protein ratio of 1:20 (wt/wt) for 48 hours at room temperature under smooth shaking on a vortexer. For the MBP-rGly m 3 fusion protein, but not for its 3 fragments, 10% SDS (wt/vol) were added up to a final concentration of 0.004% (vol/vol).

N-terminal sequence analysis

The blotted proteins on a polyvinylidene difluoride membrane (Pall, Dreieich, Germany) were visualized with Coomassie brilliant Blue R-250 stain (Roth). Protein bands of interest were excised from the blotting matrix and analyzed on a 476A Protein-Sequencer (Applied Biosystems). Routinely, 7 to 10 Edman degradation cycles were performed.

Preparation of soybean extract

Soybean extract was prepared from commercial available yellow soybeans (Henselwerk GmbH, Magstadt, Germany) by mixing 5 g of the pulverized raw material in 12 mL of TRIS-buffered saline. The mixture was stirred for 30 minutes at 4°C, transferred into Eppendorf caps, and centrifuged at 40,000g for 1 hour at 4°C. The lipid-free part of the solution was filtered with a 0.45-µm syringe filter. Finally, the protein concentration was determined by Bradford assay by using BSA as a reference protein and was found to be 60.5 mg/mL.

Electrophoresis and immunoblotting

One-dimensional SDS-PAGE and immunoblot analysis of fusion proteins, as well as their Factor Xa cleavage products, were performed by using 100 µg of rGly m 3 or 400 µg of soybean extract on a 10% NuPAGE Bis-TRIS gels (NOVEX, San Diego, Calif) under reducing conditions, as described previously.²⁵ As a control, sera from 6 nonallergic individuals were used in all experiments.

Immunoblot inhibition of IgE binding to profilin in soybean extract

For immunoblot inhibition, 100 µL of a pooled serum comprising 6 sera from subjects showing IgE antibodies to soybean proteins, as well as to the MBP-rGly m 3, and 100 µL of 3 individual sera of subjects showing specific IgE-binding properties were diluted 1:10 and preincubated with 50 µg of MBP-rGly m 3 by shaking for 3 hours at room temperature. The serum samples were then reacted with soybean extract-blotted membrane strips, as described above. The presence of natural profilin in the soybean extract was detected by comparison of the immunoblotting results with those obtained by the immunoblot inhibition assays. In case the soy profilin is involved in the soybean extract, the IgE binding to this natural profilin should be inhibited by preincubation of the serum samples with soluble MBP-rGly m 3-recombinant soy profilin.

Determination of specific IgE antibodies

IgE antibodies to the whole soybean allergens were determined by using the Pharmacia CAP-system. The detection of specific IgE antibodies to recombinant soybean profilin and its fragments in patients allergic to soybeans was examined by using EASTs.²³ To prepare the solid-phase antigen, cellulose disks (Schleicher und Schuell, Dassel, Germany) were activated with BrCN, and the rGly m 3 or its fragments were then coupled as a fusion protein (0.5 mg protein/mL) onto the activated disks in 0.1 mol/L sodium bicarbonate buffer (pH 8.4). After washing and treatment with 0.1 mol/L diethylamine, the antigen disks were incubated with 50 µL of each

FIG 1. Nucleotide sequences and deduced amino acid sequences of profilin isoforms GmPRO 1 and GmPRO 2 from soybean cDNA. The numeric order starts with the open reading frame of soybean cDNA. The coding regions are denoted by the use of capital letters up to the last nucleotide of primer PRO33 (T in position 394). The translation initiation codon ATG is underlined. Only nucleotide substitutions relative to GmPRO 1 are shown. A *dot* indicates that the nucleotide or the amino acid residue is identical with that shown for GmPRO 1. The male-GmPRO 1 hybrid finishes with the following nucleotide sequence: 5'-³⁹¹TAC TGA ATT CGG TCC TCT AGA GTC CAG CTC CAG GCA AGC-3'. Letters in *italics* (GA) stem from the 3-primer PRO331 described in the "Methods" section. Amino acid residues are shown as a 1-letter code.

using EAST, as described above. In much the same manner, the ability of rGly m 3 fragments to inhibit the IgE binding to the complete rGly m 3 and the cross-inhibition of the IgE-binding to Bet v 2 were also studied. Results are expressed as the percentage inhibition of the IgE reaction in the absence of inhibitor. Considering that the EAST is a semiquantitative assay system, we regard the results as significant when the inhibition of the IgE binding is greater than 20%.

RESULTS

Amplification of soybean profilin cDNA by PCR and sequence analysis

The PCR amplification of soybean cDNA with degenerate primers produced a single 394-bp fragment. The

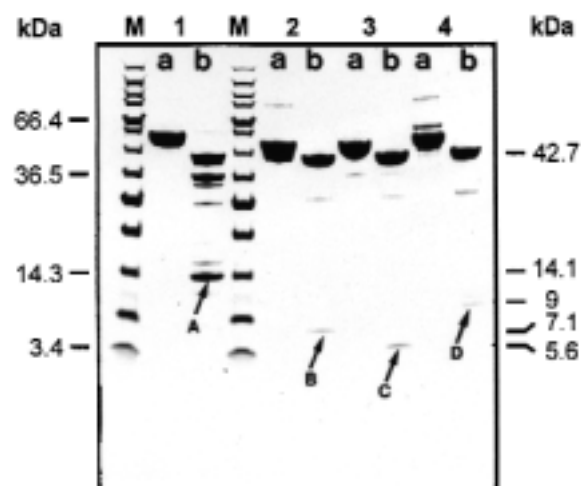


FIG 2. SDS-PAGE analysis of MBP fusion proteins of soybean profilin that were purified by means of affinity chromatography on an amylose resin before (a) and after (b) cleavage with Factor Xa. M, Molecular mass marker; 1, MBP-rGly m 3; 2, MBP-rGly m 3 (1-65); 3, MBP-rGly m 3 (38-88); 4, MBP-rGly m 3 (50-131). Bands A-D correspond to the target proteins after Factor Xa cleavage: A, 14.1 kd of rGly m 3; B, 7.1 kd of rGly m 3 (1-65); C, 5.6 kd of rGly m 3 (38-88); D, 9 kd of rGly m 3 (50-131).

PCR product was modified by an adapter fragment to permit subcloning into the expression vector pQE60. Two different cDNAs (GmPRO 1: European Molecular Biology Laboratory accession number AJ223982; GmPRO 2: European Molecular Biology Laboratory accession number AJ223981) were obtained by this procedure. The nucleotide sequences and the corresponding deduced amino acid sequences of Gm PRO 1 and GmPRO 2 are shown in Fig 1. Both carried an open reading frame coding for 131 amino acid residues with a predicted average molecular mass of 14.1 kd and theoretical isoelectric point of 4.4 and 4.6, respectively. High amino acid identity (98%) between the sequence of GmPRO 1 and GmPRO 2 was observed. The latter isoform differed only in 3 amino acid positions from the primary structure of GmPRO 1 (D¹⁴→G, A³⁴→L, P¹²²→L). Analysis of the amino acid sequence homology of 11 different plant profilins revealed values between 88% (*Phaseolus vulgaris*) and 69% (one *Arabidopsis thaliana* isoform). The homology of soybean profilin to the well-characterized birch profilin Bet v 2 was 73%.

Expression, purification, and analysis of soybean profilin rGly m 3

For the efficient expression of soybean profilin rGly m 3 and its fragments, a subcloning into the pMAL vector system (New England Biolabs) was necessary. Because the isoform GmPRO 2 is nearly identical with its GmPRO 1 counterpart and carries a *Hind*III restriction site near its N-terminus (Fig 1, nucleotides 11-16), which is uncomfortable for subcloning experiments, the GmPRO 1 clone was exclusively used for the expression of the MBP-rGly m 3 fusion protein and its correspond-

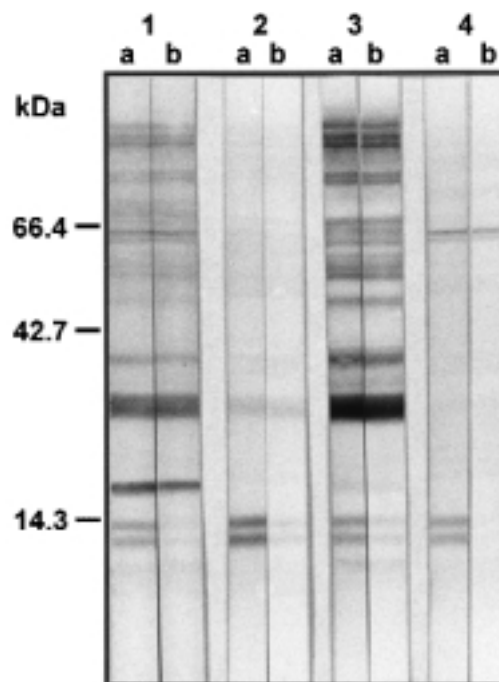


FIG 3. IgE immunoblot inhibition of rGly m 3 in natural soybean protein extract. Crude soybean extract (400 µg per gel) from yellow soybeans separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes were immunodetected with a pooled (1) and 3 individual sera (2-4), which were preincubated with buffer (a) or 50 µg of rGly m 3 (b). An almost complete inhibition of a 14-kd double band in the soybean protein extract is visible in all 4 cases.

ing fragment molecules. To obtain information about the IgE-binding properties of rGly m 3 in different parts of the protein, 3 long overlapping fragments with overhangs of 22 and 38 amino acid residues were chosen for expression. The expression of the insert cDNA fragment for the full-length soybean profilin generated a fusion protein, MBP-rGly m 3, with an estimated molecular mass of 56.8 kd (Fig 2), corresponding to the mass of 14.1 kd for the rGly m 3 plus the mass of 42.7 kd for the MBP. The yield of affinity-purified recombinant MBP-rGly m 3 fusion protein was 8 mg/L (mean of 3 isolations), according to the protein analysis by the Bradford method. The mean yields of the purified recombinant fusion fragments were 1 mg/L for the 48.9 kd of MBP-rGly m 3 (1-65), 3 mg/L for the 48.3-kd MBP-rGly m 3 (38-88), and 2.5 mg/L for the 51.7-kd MBP-rGly m 3 (50-131), respectively. The isolated fusion proteins had a purity of more than 95%, as shown by SDS-PAGE (Fig 2).

Factor Xa cleavage and N-terminal microsequencing of rGly m 3

The rGly m 3, as well as its fragment molecules, can be cleaved from the carrier protein MBP by Factor Xa digest. In the case of rGly m 3, Factor Xa digest was performed in the presence of 0.004% SDS and yielded 2 prominent bands corresponding to the 42.7-kd MBP carrier protein and the 14.1-kd soybean profilin of rGly m 3

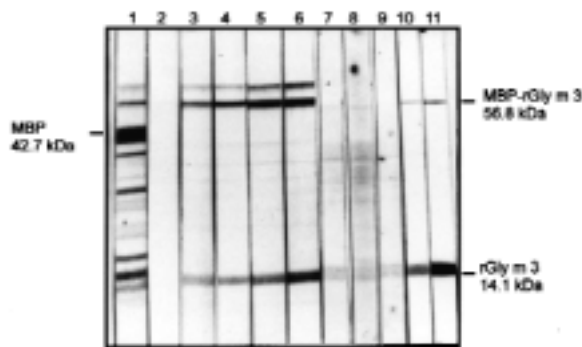


FIG 4. IgE-immunoblotting analysis of the fusion proteins of rGly m 3 and its fragments after cleavage with enzyme Factor Xa. *Lane 1*, Coomassie staining of the MBP-rGly m 3 protein after Factor Xa digestion; *lane 2*, negative pooled serum comprising 6 sera from patients not allergic to soybean proteins; *lanes 3-10*, 8 sera of soybean-sensitive individuals (dilutions of 1:5, 1:5, 1:5, 1:10, 1:5, 1:10, 1:10, and 1:10) showing rGly m 3-specific IgE binding; *lane 11*, positive pooled serum comprising 7 of 9 sera showing IgE binding to soybean profilin rGly m 3 (1:5 dilution).

(Fig 2). In contrast, the soybean profilin fragments were all cleaved in the absence of SDS and yielded fragments with a molecular mass of 7.1 kd for rGly m 3 (1-65), 5.6 kd for rGly m 3 (38-88), and 9 kd for rGly m 3 (50-131), as shown in Fig 2 (*lanes 2b through 4b*). The efficiency of the cleavage was up to 90% for all constructs. However, when the cleavage time exceeds 48 hours, an unspecific cleavage of MBP by the Factor Xa could be seen, which resulted in some protein bands in the gel in the range of 15 to 36 kd. The N-terminal sequence of the rGly m 3 was analyzed and confirmed by Edman degradation. A 2-dimensional gel electrophoresis of rGly m 3 was also carried out, which confirmed the theoretically ascertained isoelectric point value of 4.4 (data not shown).

Detection of natural profilin in soybean extract

To confirm that profilin is present in the natural soybean extract, we performed IgE immunoblot inhibition assays with a pooled sera and 3 single sera (Fig 3, *lanes 2-4*), which showed rGly m 3-specific IgE antibodies. After preincubation of sera with 50 μ g of MBP-rGly m 3, a significant inhibition of IgE binding to a double band of the blotted soybean extract in the area of 14 kd could be observed (Fig 3) in all 4 serum samples tested, whereas the other IgE-binding bands were not affected. These results indicate clearly that the profilin is present in the protein extract of commercially available soybean food.

Immunologic characterization of rGly m 3 and its fragments

The allergenicity of the rGly m 3 was first assessed by immunoblot analysis by using Factor Xa-cleaved MBP-rGly m 3 fusion protein and serum IgE antibodies from 12 individuals allergic to soybean and a pooled serum. A pooled serum sample comprising 6 sera of subjects without soybean allergies served as the negative control. rGly

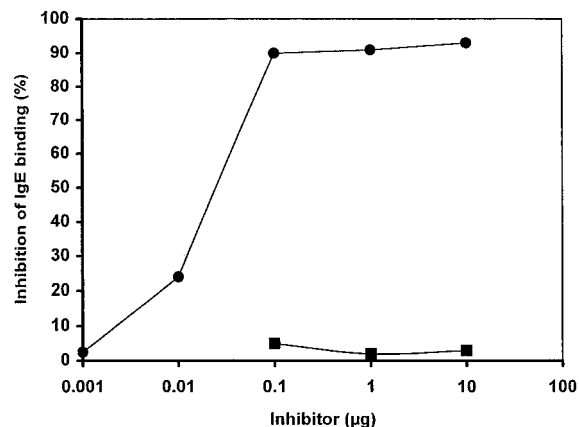


FIG 5. Autoinhibition of IgE binding to solid-phase MBP-rGly m 3 by EAST inhibition assay by using soluble MBP-rGly m 3 (*circles*) and MBP alone (*squares*) as inhibitor and the pooled serum as IgE source.

m 3 was recognized by IgE antibodies in 8 single sera and the positive pooled serum. Fig 4 shows these positive IgE-binding results. No IgE-binding reactivity to MBP or to the negative control serum was observed.

In contrast to the full-length rGly m 3, none of the 3 fragment molecules of rGly m 3 showed detectable IgE-binding reactivity on the blots when the same immunoblot experiments were carried out (data not shown).

To confirm the observations of the IgE immunoblot experiments, we then coupled the recombinant MBP-soybean profilin rGly m 3, as well as its fragments, separately onto CNBr-activated cellulose disks. Three sera showing IgE antibody binding to soybean profilin in the immunoblot experiments were randomly selected and tested by using EAST. As shown in Table I, only the complete recombinant soybean profilin was able to bind with IgE antibodies. Only 2 sera showed a weak IgE binding to 1 and 2 of the rGly m 3 fragments, respectively. The results of the competitive inhibition assays shown in Fig 5 confirm the IgE-binding specificity to rGly m 3. The IgE-binding reactivity to solid-phase rGly m 3 could be blocked by preincubation of serum samples with the soluble rGly m 3. The inhibition was dose related, and a 50% inhibition of IgE binding was achieved by adding less than 0.1 μ g of MBP-rGly m 3. The MBP alone showed no inhibition reactivity, indicating that the IgE antibodies in sera were specifically bound to the rGly m 3 molecule.

In addition, our results obtained by inhibition assays showed that the rGly m 3 fragments had no significant IgE-binding reactivity when they were used individually or in a mixture to compete with solid-phase rGly m 3 (Table II), whereas the full-length rGly m 3 fusion protein was able to block up to 99% of IgE binding to the solid-phase MBP-rGly m 3 by 3 individual serum samples tested. These results suggest that the rGly m 3 lost its IgE-binding reactivity when the allergen molecule was disrupted by expressing in 3 long fragments, even though the fragment parts have a long overlapping sequence over the break-off point.

TABLE II. Inhibition (%) of IgE antibody binding to rGly m 3 and Bet v 2 by MBP fusion proteins of soybean profilin rGly m 3 and its individual fragments and fragment mixtures

Sera*	rGly m 3	rGly m 3 (1-65)	rGly m 3 (38-88)	rGly m 3 (50-131)	rGly m 3 (1-65) + rGly m 3 (38-88) + rGly m 3 (50-131)	rGly m 3 (1-65) + rGly m 3 (38-88)	rGly m3 (1-65) + rGly m 3 (50-131)	rGly m 3 (38-88) + rGly m 3 (50-131)
rGly m 3 as solid-phase antigen								
2°	99	0	11	0	37	4	0	5
3	91	0	0	18	4	0	2	0
4	98	12	8	19	25	6	6	0
rBet v 2 as solid-phase antigen								
15	100	5	0	5	15	0	0	0
16	100	5	0	0	33	29	30	22

*Sera numbers match those in Table I.

Cross-reactivity of rGly m 3 with birch profilin Bet v 2

Because rGly m 3 shows 73% of identical amino acid residue with the well-characterized recombinant birch profilin rBet v 2, we studied the cross-reactivity between these 2 kinds of profilins. By means of the CAP method, 9 of 13 sera from individuals with soybean allergy showing IgE to rGly m 3 were found to have specific IgE antibodies also to rBet v 2. None of the 13 sera showed IgE binding only to one of the 2 recombinant profilins. In a group of 18 birch pollen-sensitive individuals without histories of soybean allergy, 3 (17%) were found to have specific IgE to rBet v 2, and all 3 sera also showed IgE binding to rGly m 3 in the EAST (Table I). Two of these 3 sera were tested in the EAST-inhibition assay. As shown in Table II, the IgE-binding to rBet v 2 could be completely inhibited by preincubating the sera with 10 µg of soluble MBP-rGly m 3. However, by using single fragments or fragment mixtures of MBP-rGly m 3 as inhibitor, only a slight inhibition (up to 30%) was observed. These results suggest that rGly m 3 and rBet v 2 share IgE-binding epitopes, and these epitopes exist as a discontinuous form.

DISCUSSION

Profilin is a ubiquitous 12- to 15-kd protein initially isolated from bovine spleen, with the ability to bind actin monomers and to regulate the polymerization of actin filaments.²⁶ The property of profilin to bind specific IgE in human sera was initially described for birch profilin by Valenta et al.²⁷ Because of its high allergic incidence, the plant profilin has been reported as a pan-allergen in pollens and vegetable foods.^{27,28}

In the present study we report the construction, expression, and purification of soybean profilin (rGly m 3) by means of recombinant techniques. Our immunoblot and EAST experiments revealed an unexpectedly high prevalence of IgE antibodies to rGly m 3. About 69% (9/13) of our sera from food-sensitized individuals who showed soybean-specific IgE exhibited positive IgE anti-

bodies to MBP-rGly m 3. Previous studies^{14,15,29} showed a very different frequency (between 1.4% and 65.2%) of IgE reactivity to the individual soybean proteins among soybean-sensitized patients. The incidence of individual IgE-binding proteins in soybean reported varied significantly from each other. In all these studies, however, nonstandardized soybean extracts were used as allergen sources, and the reported frequencies were based only on immunoblot experiments.

The observation that profilin in soybean is recognized by IgE antibodies from most serum samples studied is of great interest because the frequency of IgE reactivity to some well-characterized plant profilins is quite low; for example, the prevalence of seropositive IgE to birch profilin Bet v 2 has hitherto been considered to be about 20% in all patients allergic to birch pollen.^{28,30} In the case of olive pollen profilin, a prevalence of 24% was described.³² Nevertheless, Vallverdu et al³² recently described the profilin of the *Euphorbiaceae* plant *Mercurialis annua* (Mer a 1) as a major allergen, showing an incidence of up to 59% for IgE reactivity in their patient sera tested.³³

The route of allergic sensitization to soybean profilin (eg, allergen inhalation or ingestion) is unknown. Although the natural profilin could be demonstrated to exist in soybean extract, and it also binds to IgE antibodies in tested sera; the clinical relevance of the positive IgE to rGly m 3, however, needs to be further investigated. Recent studies³⁴ showed a poor correlation between the level of food-specific IgE antibodies and the severity of the allergic reactions in double-blind, placebo-controlled food challenges, especially in subjects suspected of having allergy to soybean. The optimal cutoff of specific IgE antibodies has been suggested to be 5 kU/L.³⁴ Thus far, no data are available to explain why such a high specific IgE value, which may be clinically irrelevant, was found in subjects.

The fact that all tested sera that showed IgE to rGly m 3 also had IgE antibodies to rBet v 2 and that the IgE binding to rBet v 2 could be completely inhibited by preincubation with rGly m 3 indicates that rGly m 3 and rBet v 2 had the common IgE-binding epitopes. These

results raise the questions of whether the IgE to birch pollen profilin may be responsible for the IgE binding to rGly m 3 and which profilin is the primary cause for the sensitization to rGly m 3. Although the answer to these questions remain open in our study, the possible cross-reactivity of different environmental allergens with certain proteins in soybean should be considered by interpreting the IgE levels in connection with soy allergy. Further studies are necessary to evaluate the clinical relevance of rGly m 3 sensitivity in more detail.

The IgE-epitope binding to Bet v 2 has been studied recently by Fedorov et al.³⁵ They probed an expression library carrying random fragments of birch pollen profilin cDNA smaller than 300 bp by using serum IgE from a single allergic patient. Three linear IgE-binding epitopes on the Bet v 2 molecule were described. Although rGly m 3 shows 73% of amino acid residue identity to Bet v 2, we did not observe a corresponding sequential IgE epitope in rGly m 3 fragments when we compared their binding results with those of full-length rGly m 3. The 3 recombinant soybean profilin fragments showed no IgE-binding reactivity in the immunoblotting experiments when tested with a pooled serum, including 6 of 9 sera showing soybean profilin-specific IgE binding neither as MBP-fusion protein nor after the Factor Xa digest (data not shown). Further studies by using EAST and EAST inhibition (Tables I and II) confirmed that only the full-length rGly m 3 was able to bind IgE antibodies efficiently. The 3 fragments of rGly m 3 showed no significant IgE-binding reactivity, even though they all had a long overlapping sequence over the break-off point, and they were also used as mixture in the competitive assays. From these results we conclude that the conformational epitopes on the soybean profilin were necessary for the mediation of IgE antibody binding.

The use of well-characterized protein fragments with T-cell epitopes for specific immunotherapy of type I allergy has been recently proposed as a possible new tool instead of using natural allergen extracts.^{36,37} Allergen fragments with or without strongly reduced IgE reactivity and covering a large repertoire of T-cell epitopes are suitable candidates because anaphylactic side effects might be significantly reduced or completely prevented, as recently proposed for the major birch allergen Bet v 1, which was cut into 2 nonallergenic compartments by genetic engineering.³⁷ Both fragments had the property to induce the proliferation of human Bet v 1-specific T-cell clones but showed no IgE-binding reactivity.

Because the disruption of the tertiary structure of rGly m 3 also led to a loss of the IgE-binding activity, future experiments should include T-cell proliferation studies to determine which of the fragment molecules are able to induce a T-cell response.

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