

Occurrence of IgE Antibody-Recognizing N-Linked Glycan Moiety of a Soybean Allergen, Gly m Bd 28K

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Key Words

Glycan moiety, N-linked · Anti-horseradish peroxidase antibody · IgE antibody · Soybean allergen · Gly m Bd 28K

Abstract

Background: It has been reported that N-linked glycan moieties of glycoproteins function as IgE-reactive determinants. Gly m Bd 28K, a soybean allergen, was a glycoprotein with glycan moieties, which are supposed to be the Man₃GlcNAc₂ backbone with the β1→2 xylose and α1→3 fucose branches. The purpose of the present study was to examine the IgE-binding ability of the glycan moiety of Gly m Bd 28K in the binding reaction with patients' sera. **Methods:** A peptide containing the glycan moiety was prepared from Gly m Bd 28K by digestion with lysyl endopeptidase. The binding site of the glycan moiety was determined by amino acid sequence analyses. The glycan moiety of the allergen was characterized using anti-horseradish peroxidase antibody (anti-HRP) recognizing the N-linked glycan moieties of glycoproteins. The binding of patients' IgE antibodies with their glycan moi-

ety was examined by an immunostaining technique using the glycopeptide and its deglycosylated peptide derived from Gly m Bd 28K. **Results:** The binding site of the glycan moiety in Gly m Bd 28K was shown to be its Asn20 residue. Gly m Bd 28K did react with anti-HRP and the sera of soybean-sensitive patients, but the binding of IgE antibodies was inhibited by the preincubation with anti-HRP. Moreover, the glycopeptide also reacted with the sera of soybean-sensitive patients, but its deglycosylated peptide did not react with any IgE antibodies of patients' sera. **Conclusions:** The specific IgE antibodies recognizing the N-linked glycan moieties of Gly m Bd 28K and other glycoproteins with homologous glycan moieties occur in the sera of soybean-sensitive patients. It was indicated that the N-linked glycan moieties such as that of Gly m Bd 28K may be one of the common IgE-reactive determinants distributed in various plant food proteins.

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Introduction

Soybean is an important plant protein source for Asian people, but it is one of three major allergenic foods in Japan, the other two being cow's milk and hen's eggs. We studied the allergenic components in soybean and found approximately 15 protein species which are specifically recognized by IgE antibodies in the sera of soybean-sensitive patients with atopic dermatitis [1]. Three of them, named Gly m Bd 68K (α -subunit of β -conglycinin), Gly m Bd 30K (soybean oil-body-associated protein) and Gly m Bd 28K, are identified as the major allergenic proteins in soybean [1]. However, Gly m Bd 28K isolated as a glycoprotein [2] has not been characterized in detail, with regard to its immunochemical properties and the allergenic significance of the glycan moiety.

Many allergenic proteins in vegetable foods, insects and pollens are glycoproteins with N-linked glycan moieties, which are associated with their immunoreactivities [3–11]. The oligosaccharide moiety of phospholipase A₂ was reported to be an IgE determinant site of a major allergen from honeybee venom [12]. Among the inhalant pollen allergens, Batanero et al. [13, 14] also reported that its N-linked glycan moiety of Ole e 1, a major olive pollen allergen, is connected with the binding to IgE antibodies of the sera. On the pollen and food allergens, it was reported that allergen molecules contain cross-reactive carbohydrate determinants abbreviated as CCDs [15–19]. However, little is known about the allergenicity of the glycan moieties of glycoprotein as food allergens.

Recently, during the course of an investigation on the structure of allergenic proteins in foods, we also observed that glycoproteins with N-linked glycan moieties including Gly m Bd 28K are recognized by the sera of soybean-sensitive patients, suggesting that the glycan moieties of the glycoproteins may be a common cross-reactive antigenic site.

The present study aimed at elucidating the IgE-binding activity of the glycan moiety of Gly m Bd 28K using the sera of soybean-sensitive patients. Furthermore, the other glycoproteins, horseradish peroxidase (HRP), bromelain, ascorbate oxidase and ovalbumin were examined with regard to binding to IgE antibodies in the patients' sera.

Materials and Methods

Patients' Sera

The sera of soybean-sensitive patients were selected according to the case histories and the score of radioallergosorbent test (RAST) as described previously [1]. A serum sample from a non-soybean-allergic individual, who had no case history and a negative RAST score to soybean, was used as a control.

Glycoproteins

Pineapple stem bromelain (EC 3.4.22.32), ovalbumin and HRP (EC 1.11.1.7) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Ascorbate oxidase (EC 1.10.3.3) from *Cucurbita pepo* was obtained from Boehringer-Mannheim (Mannheim, Germany).

Purification of Gly m Bd 28K

Gly m Bd 28K was purified from defatted soybean flakes (Glycine max, mixture of IOM varieties) from Fuji Oil (Osaka, Japan) according to the method described previously [2].

Isolation of Glycopeptide from Gly m Bd 28K

The isolation of the glycopeptide was performed as described below. Gly m Bd 28K was digested with lysyl endopeptidase (Promega, USA) in 0.5 M Tris-HCl buffer, pH 8.0, for 20 h at 30°C. The ratio of the protease to Gly m Bd 28K was 1:100 (w/w). The peptides in the hydrolysate were fractionated using high-performance liquid chromatography (HPLC). For the isolation of the glycopeptide(s), the hydrolysate was applied on a μ BONDASPHERE C₄ column (0.39 $\times$ 15 cm, Waters, Japan). The peptides were eluted with a linear gradient of acetonitrile (0–60%) including 0.05% trifluoroacetic acid. Peptides in the eluate were monitored by absorbance at 215 nm. The eluate was collected at an interval of 2 min and each fraction was evaporated to dryness by a centrifugal evaporator. The dried materials were dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl (TBS) and an aliquot of each solution was applied directly on a nitrocellulose membrane (Bio-Rad, USA). The fraction containing glycopeptide(s) with an N-linked glycan moieties was detected by immunostaining using anti-HRP as described below. Moreover, the glycan(s) was also detected by the phenol-sulfuric method [20]. The glycan-containing fraction was further subjected to HPLC with a μ BONDASPHERE C₁₈ column (0.39 $\times$ 15 cm, Waters, Japan) to purify the glycopeptide. The glycopeptide was eluted by a linear gradient of acetonitrile (0–70%) including 0.05% trifluoroacetic acid. The glycopeptide was used in the following experiments.

Preparation of the Deglycosylated Peptide

The isolated glycopeptide was deglycosylated with almond glycopeptidase A (Seikagaku Kogyo, Japan) in 0.1 M sodium citrate buffer, pH 5.0, at 30°C for 5 h. The deglycosylated peptide in the hydrolysates was purified by HPLC with a μ BONDASPHERE C₄ column in the same manner as described above.

Analysis of the N-Terminal Amino Acid Sequence and Glycosylation Site

The purified glycopeptide and deglycosylated peptide were subjected to N-terminal amino acid sequencing using a Hewlett Packard protein sequencer (C1000A).

Electrophoresis and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15% polyacrylamide gels according to the method of Laemmli [21]. The proteins on the gel were stained with 0.1% Coomassie brilliant blue R-250 dissolved in methanol/acetic acid/water (3:1:6, by volume). For the immunoblotting analysis, the proteins separated on the gel were electrophoretically transferred

onto a nitrocellulose membrane in the same manner as described previously [1].

The N-linked glycan moieties of the glycoproteins were examined by the immunostaining method using rabbit polyclonal anti-HRP antibodies (Sigma Chemical Co., St. Louis, Mo., USA) recognizing N-linked glycan moieties with the $\beta 1 \rightarrow 2$ xylose and/or $\alpha 1 \rightarrow 3$ fucose branches [13, 22–25].

IgE antibodies were analyzed by an immunoblotting technique as follows. Glycoproteins were electrophoresed as described above and electroblotted onto a nitrocellulose membrane. The membrane was divided into several strips and blocked with 1% BSA in TBS for 1 h, after which the glycoproteins on the membranes were incubated with the patient's serum diluted 1:4 with TBS containing 1% BSA for 2 h at room temperature and then at 4°C for 20 h. The strips were washed with TBS 3 times, thereafter the immunocomplexes were incubated with ^{125}I -labeled anti-human IgE (Pharmacia Diagnostics, Sweden) at room temperature for 20 h. The complexes were visualized by autoradiography as described previously [1].

Inhibitory Effect of Anti-HRP on the Reaction of Glycoproteins with Patients' IgE Antibodies

After the electrophoresis of Gly m Bd 28K and other glycoproteins on a polyacrylamide gel, they were electroblotted onto a nitrocellulose membrane, which was then cut into several pieces as described above. After blocking, their glycoproteins blotted on the strips were preincubated with or without anti-HRP in TBS containing 1% BSA and 0.05% Tween 20 (buffer A) at room temperature, and the ratio of anti-HRP to the glycan moieties of the glycoproteins was 50-fold by molar basis. After washing, the glycoproteins on the strips were immunoblotted using individual sera of soybean-sensitive patients, and the immunocomplexes with IgE antibodies were detected by autoradiography as described above.

Binding of IgE Antibodies to the Glycan Moiety of Gly m Bd 28K

To confirm the presence of glycan-specific IgE antibodies, equal amounts of the glycopeptide and its deglycosylated peptide prepared from Gly m Bd 28K were dot-blotted directly on nitrocellulose membranes. Then, the membranes were immunostained with the sera of soybean-sensitive patients as described above. Thereafter, the IgE antibodies bound to the peptides were detected using ^{125}I -labeled anti-human IgE in the same manner as described above. Intact Gly m Bd 28K was used as a positive control. One set of the membranes was subjected to an immunoblotting analysis with the anti-HRP to examine the presence of the glycan moiety of Gly m Bd 28K.

Results

Isolation of the Glycopeptide and Its Deglycosylated Peptide

To determine the glycan binding site(s) of Gly m Bd 28K, the allergen was hydrolyzed with lysyl endopeptidase and the peptide fragments in the hydrolysates were isolated in the same manner as described above by HPLC. By immunostaining with anti-HRP and phenol-sulfuric acid reaction, a glycopeptide fragment was detected only in the peak at a retention time of 18.8 min (fig. 1a). This

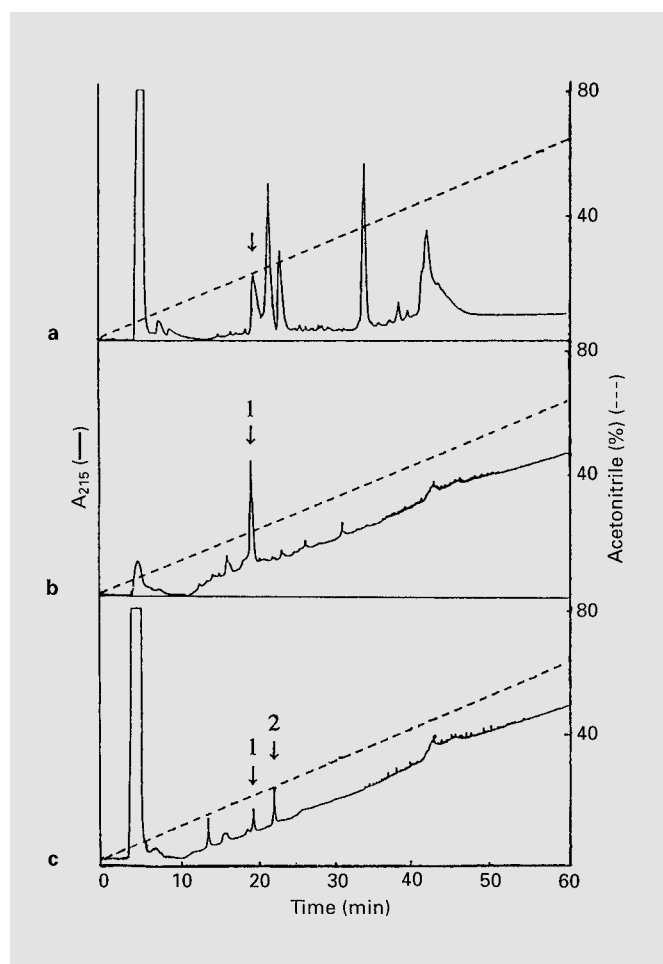


Fig. 1. HPLC elution patterns of the peptides obtained from Gly m Bd 28K. **a** Elution profile of the peptides obtained from Gly m Bd 28K by digestion with lysyl endopeptidase. **b** That of the isolated glycopeptide. **c** That of the glycopeptide treated with almond glycopeptidase A. 1 = Isolated glycopeptide; 2 = deglycosylated peptide.

finding suggests that the allergen has only one glycan binding site on the protein molecule. The glycopeptide was purified by rechromatography on HPLC (fig. 1b). Both the glycopeptide and its deglycosylated peptide obtained with glycopeptidase A hydrolysis were subjected to HPLC with the same column. The intact glycopeptide was eluted at a retention time of 18.8 min by HPLC (fig. 1b), whereas a peak of its deglycosylated peptide was eluted at a retention time of 22.0 min (fig. 1c). The deglycosylated peptide was not shown to bind to anti-HRP by dot-blotting analyses. These findings show that the glycan moiety was removed from the glycopeptide by treatment with glycopeptidase A.

Table 1. N-Terminal amino acid sequences of Gly m Bd 28K, the glycopeptide and its deglycosylated peptide

Peptide		Sequence		
(A)	Gly m Bd 28K	1	10	20
		FHDDEGGDKKSPKSLFLMSXSTR---		
(B)	Isolated glycopeptide	SLFLMSXSTRVFK		
(C)	Deglycosylated peptide	SLFLMSDSTRVFK		
X in the sequence represents the amino acid residue determined by N-terminal amino acid sequencing.				

Table 2. Structures of the glycan moieties of the representative N-linked glycoproteins

Glycoprotein	N-linked glycan
HRP [22]	Manα1-6 Manα1-3 Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAcβ1-N.Asn Xylβ1-2 Fucα1-3
Bromelain [26]	Manα1-6 Manα1-3 Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAcβ1-N.Asn Xylβ1-2 Fucα1-3
Ascorbate oxidase [27]	Manα1-6 Manα1-3 Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAcβ1-N.Asn Xylβ1-2
Ovalbumin [28]	Manα1-6 Manα1-3 Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAcβ1-N.Asn

Glycosylation Site of Gly m Bd 28K
Both the glycopeptide and its deglycosylated peptide were subjected to the analysis of the N-terminal amino acid sequence. The sequence of the purified glycopeptide was determined to be SLFLMSXSTRVFK (table 1, B), in which the 7th amino acid residue (X) from its N-terminus could not be detected. This sequence corresponded to the N-terminal amino acid sequence of Gly m Bd 28K from Ser14 to Lys23, as reported previously [2] (table 1, A). The sequence of the deglycosylated peptide was shown to be SLFLMSDSTRVFK (table 1, C). These findings suggested that the unknown residue (X) in the glycopeptide is Asn with an N-linked glycan moiety. The Asn residue on an amino acid sequence of Asn-X-Thr (or Ser) in proteins is known to be an N-glycosylated site. Together with the findings obtained by digestion with glycopeptidase A,

the glycan moiety should bind to the Asn20 residue of Gly m Bd 28K.

Binding of Anti-HRP to N-Linked Glycoproteins

The glycan moieties of HRP, bromelain, ascorbate oxidase and ovalbumin have been well characterized (table 2) [22, 26–28]. The above representative glycoproteins were separated by SDS-PAGE, stained with Coomassie brilliant blue (fig. 2a) and immunoblotted with anti-HRP (fig. 2b) specific to the N-linked glycan moiety with the β 1→2 xylose and/or α 1→3 fucose structures. Gly m Bd 28K, bromelain and ascorbate oxidase were stained by anti-HRP as was HRP, suggesting that these proteins are cross-reactive with anti-HRP. Ovalbumin, which did not contain the structure of the β 1→2 xylose or α 1→3 fucose branch, was not recognized by anti-HRP. Since the amino

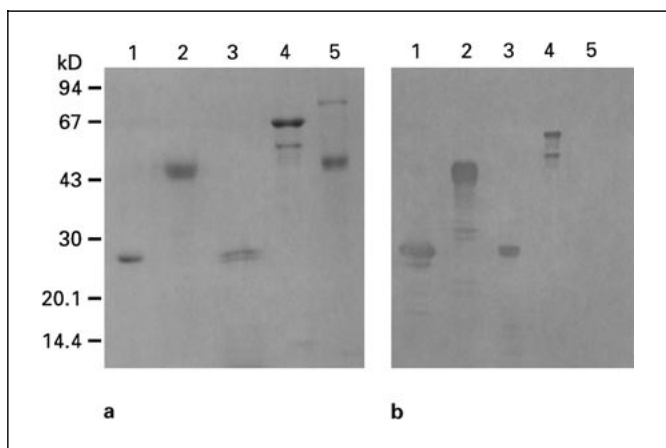


Fig. 2. SDS-PAGE and immunostaining of glycoproteins with anti-HRP. The glycoproteins (containing 100 pmol of oligosaccharide) were applied on two polyacrylamide gels and SDS-PAGE was done in the same manner as described in the text. The proteins on one gel were stained with Coomassie brilliant blue R-250 (**a**). The proteins on the other gel were electroblotted onto a nitrocellulose membrane, and the glycoproteins on the membrane were immunostained with anti-HRP (**b**). The molecular masses of the standard proteins are indicated on the left. Lane 1 = Gly m Bd 28K; lane 2 = HRP; lane 3 = bromelain; lane 4 = ascorbate oxidase; lane 5 = ovalbumin.

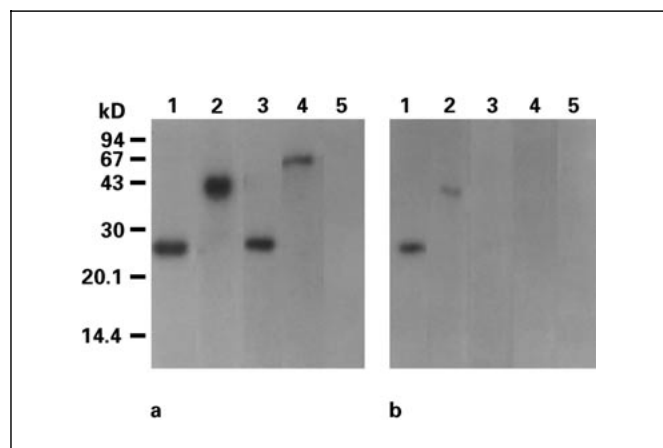


Fig. 3. Immunostaining of the glycoproteins with the serum of a soybean-sensitive patient. The glycoproteins (containing 100 pmol oligosaccharide) were separated by SDS-PAGE on a polyacrylamide gel. After electrophoresis, the proteins were immunoblotted with the serum of a soybean-sensitive patient as described in the text. **a** Membranes treated with buffer A and then immunostained with the patient's serum. **b** Membranes treated with buffer A containing anti-HRP and then immunostained with the patient's serum. The molecular masses of the marker proteins are indicated on the left. Lane 1 = Gly m Bd 28K; lane 2 = HRP; lane 3 = bromelain; lane 4 = ascorbate oxidase; lane 5 = ovalbumin.

acid sequences of Gly m Bd 28K, bromelain and ascorbate oxidase are completely different from that of HRP, their polypeptide chains may not be involved in the binding to anti-HRP. This confirms that anti-HRP recognizes the N-glycan moiety common among these glycoproteins with the $\beta 1 \rightarrow 2$ xylose and/or $\alpha 1 \rightarrow 3$ fucose structures.

Binding of Glycoproteins with the Serum of a Soybean-Sensitive Patient

The binding of IgE antibody to the representative glycoproteins was examined using the serum of a soybean-sensitive patient. The serum of the soybean-sensitive patient recognized HRP, bromelain and ascorbate oxidase, but not ovalbumin (fig. 3a). However, the binding activities of Gly m Bd 28K, HRP, bromelain and ascorbate oxidase to IgE antibody in the serum of the soybean-sensitive patient were markedly reduced when the membranes were preincubated with anti-HRP and then the proteins on the membranes were immunoblotted with the serum (fig. 3b).

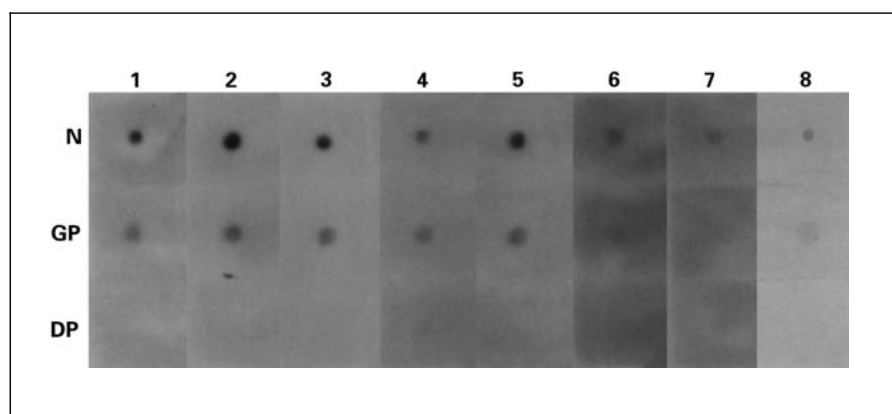
Inhibitory Effect of Anti-HRP on Binding Reaction between Gly m Bd 28K and Patients' IgE Antibodies

The effect of anti-HRP on the binding reaction between Gly m Bd 28K and IgE antibodies of the patients was examined in the 7 soybean-sensitive patients, described in the legend of figure 4, and 1 nonallergic patient. In the 7 soybean-sensitive patients tested, preincubation of Gly m Bd 28K with anti-HRP reduced the IgE-binding activity against the sera of 5 patients, but did not show an effect on the IgE-binding activity using those of the other 2 patients (data not shown). However, the serum of the control patient with no allergic symptom against soybean did not recognize Gly m Bd 28K (data not shown).

Reactivity of the Glycopeptide with the Sera of Soybean-Sensitive Patients

To confirm that the glycan moiety of the allergen is involved in its allergenicity, the IgE-binding reaction of the glycopeptide and its deglycosylated derivative with the sera of the above-mentioned patients were investigated. As shown in figure 4, the glycopeptide prepared from Gly m Bd 28K was strongly recognized by the sera of the soybean-sensitive patients 1–5 (see lanes 1–5), where-

Fig. 4. Immunostaining of Gly m Bd 28K, the glycopeptide and its deglycosylated peptide with the sera of soybean-sensitive patients. The proteins and peptides blotted onto nitrocellulose membranes were treated with patients' sera. N = Gly m Bd 28K (0.05 nmol); GP = glycopeptide (1.4 nmol); DP = deglycosylated peptide (1.4 nmol); lane 1–7 = immunoblots with patients' sera; lane 8 = immunoblot with anti-HRP as a control.



as it reacted weakly with the serum of patient 6 (lane 6), and did not react with the serum of patient 7 (lane 7). The deglycosylated peptide was not recognized by any of the patients' sera and anti-HRP (lane 1–8).

Discussion

A number of investigations on the antigenicity or allergenicity of the glycan moieties of glycoproteins have been reported. The binding of IgE antibodies in the sera of allergic patients to the N-linked glycan moieties of glycoproteins in some pollens has been demonstrated [14, 17, 18, 29]. Garcia-Casado et al. [30] purified 14 members of the α -amylase inhibitor family of wheat and barley and showed that the inhibitor proteins bind to IgE antibodies in the sera of patients with wheat allergy with different affinities. Among them, the glycan moieties of the glycoproteins were revealed to be involved in their binding to IgE antibodies in the sera of patients. Recently, IgE antibodies specific for CCDs were reported to exist in the sera of celery-allergic patients [31]. Since the glycan moieties share a common structure, these findings suggested that the glycan moieties could be responsible for the cross-reactivities observed between pollens and plant foods.

Gly m Bd 28K is an allergenic glycoprotein and is proposed to contain a typical N-linked glycan moiety of $\text{Man}_3\text{GlcNAc}_2$ with the $\beta 1 \rightarrow 2$ xylose and $\alpha 1 \rightarrow 3$ fucose branches on the basis of the analysis of its monosaccharide composition [2]. The glycan moiety of the allergen has been examined using the sera of 7 patients sensitive to soybean. The glycopeptide obtained from the allergen was observed to be strongly reactive with the sera of 5 patients, but weakly reactive with those of 2 other patients.

Moreover, its deglycosylated peptide was shown to be non-reactive with the sera of the 7 patients. These findings demonstrate that the glycan moiety of Gly m Bd 28K is involved in the binding of IgE antibodies in the sera of the soybean-sensitive patients. The fact that the binding reaction between Gly m Bd 28K and patients 6 and 7 was not inhibited by anti-HRP treatment indicates that the sera of patients 6 and 7 have a very small or negligible amount of IgE antibodies recognizing the glycan moiety of Gly m Bd 28K. In other words, they would mainly contain the IgE antibodies specific to the peptide backbone as epitope sites. The involvement of the glycan moiety in Gly m Bd 28K is consistent with the report of Batanero et al. [14] that the allergenicity of the glycoprotein in olive pollens may be connected with its N-linked glycan.

Bromelain and ascorbate oxidase are the above-mentioned N-linked glycan moieties with the $\beta 1 \rightarrow 2$ xylose and/or $\alpha 1 \rightarrow 3$ fucose branches. As expected, these proteins were shown to be reactive with the anti-HRP, but ovalbumin was not recognized by the anti-HRP. Furthermore, bromelain and ascorbate oxidase were reactive with the sera of soybean-sensitive patients, but ovalbumin was not recognized by the sera. The IgE antibodies in the sera were also shown to bind to Gly m Bd 28K and its glycopeptide, but did not react with its deglycosylated peptide. These findings suggest that IgE antibodies, which recognize the glycan moieties of the glycoproteins, may occur in the sera of soybean-sensitive patients. However, the epitopic structure of the glycan moiety still has not been clearly elucidated. Two possibilities concerning the epitopic structure of the glycan moiety may be proposed. One possibility is that the epitope may consist of only its glycan moiety. The other possibility is that the epitope may be constructed from both the glycan moiety and part of the

polypeptide moiety. Further examination of these two possibilities should be done in detailed studies on the binding reaction between the glycan moiety and IgE antibodies in the sera of soybean-sensitive patients.

Food-specific IgE antibodies bind to high-affinity FcεRI receptors on mast cells, basophils, macrophages, monocytes, lymphocytes, eosinophils and platelets. When food allergens pass through mucosal barriers and reach IgE antibodies on mast cells or basophils, mediators elicit symptoms of immediate hypersensitivity histamine releases (type 1 IgE-mediated reactions). For the induction of type 1 IgE-mediated reactions, it is a prerequisite that allergens which meet IgE antibodies on the cells possess more than two epitopes [32]. RAST has been used in the diagnosis of food allergies for the identification of allergic foods. This method detects IgE antibodies in the sera of patients, recognizing allergens in the allergic foods. As

described above, the IgE antibodies in the sera of the soybean-sensitive patients recognized bromelain and ascorbate oxidase as well as Gly m Bd 28K. Similar phenomena were observed by Mari et al. [33] using the serum of a patient sensitive to pellitory and rye grass. However, these glycoproteins were shown to have no response to the patient sensitive to pellitory and rye grass by skin prick tests [33]. These observations suggest that only the detection of the IgE antibodies specific for allergens may result in an incorrect diagnosis of food allergy. CCDs commonly distributed on glycoproteins may act only as monovalent antigens which do not induce IgE-mediated reactions. Accordingly, it must be carefully examined whether the glycan moiety plays a significant role in the allergenicity of Gly m Bd 28K. The clinical relevance of the CCD-directed IgE antibodies in soybean-allergic patients is presently being investigated.

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