Note

Investigation of Multiple Forms of Tri a Bd 27K, a Major Wheat Allergen, by Immunoblotting Analysis

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Tri a Bd 27K, a major wheat allergen, is a glycoprotein. Tri a Bd 27K was found to occur in multiple forms by two-dimensional polyacrylamide gel electrophoresis and immunoblotting with a monoclonal antibody against the allergen. Furthermore, it was found that only Tri a Bd 27K components, which have N-linked glycan moieties with fucose residues, bound to IgE antibodies in the sera of wheat-sensitive patients.

Key words: Tri a Bd 27K; wheat allergen; multiple form; N-linked glycan moiety; fucose residue

Wheat is a major crop that is utilized in many processed foods and wheat allergy is a very serious world-wide problem. Wheat is also known as the allergy-inducing food, followed by egg and milk in Japan. Therefore, wheat is an allergenic food that must be expressed as “specific raw materials” in processed foods. The intake of wheat induces food allergic symptoms such as asthma, and entities responsible for the symptoms have been identified as α-amyase inhibitors as a representative allergen of baker’s asthma.1 Gliadins have been found to be involved in IgE-mediated reactions to ingested wheat in wheat-dependent exercise-induced anaphylaxis.2 Moreover, the gluten fraction of wheat is also related to celiac disease,3 but no overview of the allergens in wheat has been done. Hence, we investigated IgE-binding proteins in wheat by immunoblotting with the sera of 30 wheat-sensitive patients.4 Fifteen allergens were found as candidates in allergenic patients, and among them, Tri a Bd 17 K, Tri a Bd 27 K, and Tri a Bd 36 K were found to be major allergens.5 Furthermore, Tri a Bd 17 K and Tri a Bd 36 K were identified as α-amyase inhibitor CM16/CM17 and a peroxidase respectively.4,5 Tri a Bd 27 K is the most predominant allergen of the above-mentioned allergens. Tri a Bd 27 K is contained in the albumin fraction of wheat.4 We isolated this allergen from wheat and indicated that it is a glycoprotein highly homologous with a γ-interferon-inducible thiol reductase.6 However, characterization of Tri a Bd 27 K as an allergenic protein molecule including the property of its glycan moieties has not been done well, and the allergenicity of the allergen has never been investigated. Just recently, a monoclonal antibody (mAb) against recombinant Tri a Bd 27 K (rTri a Bd 27 K) expressed in Escherichia coli was prepared as a probe to detect the allergen in wheat, and was characterized.7 In the present study, we examined the forms of Tri a Bd 27 K in wheat by immunoblotting with the mAb, and elucidated the components of their glycan moieties.

The albumin fraction of wheat was prepared from wheat flour (Triticum aestivum L., c.v. Chinese Spring) in the manner described earlier.6 Furthermore, a wheat protein sample containing high concentration of Tri a Bd 27 K was prepared from the above-mentioned fraction by a modification of the previous method.6 Briefly, proteins in that fraction were fractionated with ammonium sulfate (30–70% saturated), and the proteins in the precipitate obtained by centrifugation were dialyzed against 20 mM Tris–HCl buffer, pH 8.0, and applied onto a Q-Sepharose 4B column. The proteins on the column were eluted with 20 mM sodium chloride in the same buffer. Finally the proteins were dialyzed against 10 mM sodium phosphate buffer, pH 7.4.

The proteins in the albumin fraction and the wheat protein sample, including a high concentration of Tri a Bd 27 K, were subjected to immunoblotting analysis with the mAb prepared against rTri a Bd 27 K in a previous study.7 The proteins were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) on the combination of a short/long instruction immobiline dry strip, pH 3–10 NL, which is composed of a non-linear gradient from pH 3 to pH 10, as the first-dimensional electrophoresis, with a 15% polyacrylamide gel as the second one, and were transferred onto a nitrocellulose membrane, as described previously.8 Subsequently, the proteins on the membrane were immunoblotted with mouse mAb, 1G11, against rTri a Bd 27 K in the manner described in the previous paper.7

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Abbreviations: mAb, monoclonal antibody; rTri a Bd 27 K, recombinant Tri a Bd 27 K; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; AAL, lectin of Aleuria aurantia; ABEE, 4-aminobenzoic acid ethyl ester; pI, isoelectric point; PVDF, polyvinylidene difluoride
Detection of the glycan moiety was performed using a G. P. Sensor kit (Honen, Tokyo) and lectin of "Aleuria aurantia" (AAL), as our previous studies. Moreover, the components of sugar compounds were examined as described below. The respective spots on a gel corresponding to the proteins separated by 2D-PAGE were cut off, and then the proteins on the gel pieces were electrophoto metrically removed. The proteins were hydrolyzed with 4 M trifluoroacetic acid, and then the monosaccharides released were labeled with a 4-aminobenzoic acid ethyl ester (ABEE) glycan composition analysis kit (Honen, Tokyo) according to the manufacturer’s instructions. Authentic monosaccharides, N-acetylgalactosamine, N-acetylgalactosaminic, mannose, glucose, galactose, xylose, and fucose (Wako, Tokyo), were also treated in the manner described above. The ABBE-labeled monosaccharides were analyzed by high performance liquid chromatography with μBondasphere C18 using 0.2 M borate buffer (pH 9.0) containing 5% acetonitrile. The ABBE-labeled monosaccharides were detected with a fluorescent detector (Ex 305 nm, Em 360 nm) and were identified by comparing them with the retention times of the authentic compounds.

For immunoblotting with IgE antibodies in the sera of three patients sensitive to wheat, RAST scores 1, 3, and 4 were obtained from International Enzyme (Fallbrook, CA) and the National Hospital Organization at the Minami-Okayama Medical Center (Okayama, Japan). The sera of a mite-sensitive patient who had a high level of IgE antibodies and a healthy person from the above-mentioned center were used as controls. The experiments with the above sera were done in accordance with ethical standards as formulated in the Helsinki Declaration. The sera were diluted to 1/5 and used in the subsequent experiments. Immunoblotting with the sera was performed in the manner described in a previous paper. By immunoblotting with the mAb against rTri a Bd 27K, several spots with different isoelectric point (pI) values and different molecular masses were detected in the immunoblots of the proteins in the albumin fraction (Fig. 1). When the wheat protein sample including a high concentration of Tri a Bd 27K was subjected to 2D-PAGE using a large slab gel, as described in footnote to Fig. 1, the proteins in the parts enclosed with the circles in Fig. 1 were separated into clear distinct spots. The proteins in the circles positive to the mAb were separated into 14 spots. In order to determine whether these proteins, proteins 1–14, were Tri a Bd 27K, the N-terminal amino acid sequences of them were determined. Proteins 1–14 were separated on a gel and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane, cut off, and subjected to N-terminal amino acid sequencing. Standard protein markers are shown on the left side of panel A.

Fig. 1. 2D-PAGE of the Wheat Albumin Fraction and Its Immunoblot with the mAb against rTri a Bd 27K.

Proteins (100 μg) in the albumin fraction obtained from wheat were separated by 2D-PAGE, as described in the text. The proteins on the slab gel were electroblotted to a nitrocellulose membrane and the membrane was stained with Ponceau S (A), and then immunostained with the mAb against rTri a Bd 27K (B). The spots corresponding to the proteins positive to the mAb are enclosed with a solid circle. In order to separate more clearly the protein spots enclosed with the circle, 2D-PAGE of the wheat proteins was done with larger slab gels (160 × 160 mm) than the ones (106 × 100 mm) shown in the inside square of each panel. The proteins corresponding to spots 1–14 indicated with arrows were electroblotted onto a PVDF membrane, cut off, and subjected to N-terminal amino acid sequencing. Standard protein markers are shown on the left side of panel A.
multi-genomes of wheat might explain the occurrence of multiple forms of the allergen. Alternatively, the occurrence of Tri a Bd 27K components with different pI values must be due to carbamylation which is induced by the modification of Lys and/or Arg residues and deamidation of Asn and/or Gln residues during electrophoresis. This possibility was described in a previous paper. 8)

We have reported that purified Tri a Bd 27K from wheat is a glycoprotein with L-fucose residues. 6) On the basis of that study, the proteins detected as above were examined with regard to their glycan moieties. The staining patterns obtained with the G. P. Sensor kit and AAL are shown in Fig. 2. Proteins 1–6, 8, and 12, indicated in Fig. 2B, were stained with a G. P. Sensor kit, which is used to detect the glycan moieties of glycoproteins. Among them, proteins 1–6 were also stained with AAL specific to L-fucose residue (Fig. 2C) in the manner described in a previous paper, 6) suggesting that these proteins contain the structures of their glycan moieties similar to that of the allergen purified previously. 6) Furthermore, we examined the components of the sugar compounds in proteins 1–6, 8, and 12 which were detected with the G. P. Sensor kit. N-Acetylgalactosamine and fucose were detected in only proteins 1–6. In contrast, although galactose, mannose, glucose, and xylose were detected in all of proteins analyzed, N-acetylgalactosamine and fucose were not detected in proteins 8 and 12. Moreover, no N-acetylgalactosamine was detected in any of the proteins. Therefore, these monosaccharides detected in proteins 8 and 12 are unlikely to be N- and O-linked glycoproteins. The manner by which the sugar components bind to the polypeptide region of the proteins remains unsolved. These observations suggest that the differences in the molecular masses of the Tri a Bd 27K components are explained by the presence of glycan moieties in the components or by the sugar composition of their glycan moieties.

Finally, the IgE-binding capability of the proteins recognized by the mAb against rTri a Bd 27K was investigated. The sera of the three wheat-sensitive patients used in the previous study 6,7) were examined in following experiments. The IgE antibodies in the serum of the representative patient with a RAST score of 4 gave six intensive spots (Fig. 3B), although no proteins were detected in immunoblots with the sera of the mite-allergic patient with a high level of IgE antibody and the non-allergic patient (Fig. 3C, D). All of the positive spots were found to correspond to Tri a Bd 27K, proteins 1–6. Similar patterns were observed in the immunoblots of the sera of the other two wheat-sensitive patients. Among the Tri a Bd 27K multiple forms, only proteins 1–6 stained with fucose-specific lectin were found to be positive to the sera of the three patients. In the previous study, rTri a Bd 27K was not recognized by the IgE antibodies in the sera of any wheat-sensitive patients. 6) The Tri a Bd 27K components are glycoproteins containing N-linked glycan moieties, as described above, but rTri a Bd 27K, expressed in Escherichia coli, is not glycosylated. This suggests that the N-linked glycan moieties are involved in the binding of IgE antibodies in the sera of the patients sensitive to Tri a Bd 27K. Furthermore, we have reported the relationship between N-linked glycan moieties of soybean allergens and IgE-binding, and have proposed that the fucose or xylose residues in N-linked glycan moieties play important roles in binding

Fig. 2. Detection of the Glycan Moieties in the Proteins Positive to the mAb.

A wheat protein sample (100 μg) was separated by 2D-PAGE with larger slab gels and electrophoetblotted onto nitrocellulose membranes, as described in the footnote to Fig. 1. After staining of the membranes with Ponceau S (A), proteins containing glycan moieties were detected with a G. P. Sensor kit (B) or stained with AAL (C). The arrows represent the protein spots of the Tri a Bd 27K components positive to G. P. Sensor and AAL.

Fig. 3. Immunoblots of Wheat Proteins with the Sera of a Representative Wheat-Sensitive Patient, a Mite-Sensitive Patient, and a Non-Allergic Patient.

A wheat protein sample (100 μg) was subjected to 2D-PAGE and electrophoetblotted onto nitrocellulose membranes, as described in the footnote to Fig. 1. Then the membranes were stained with Ponceau S (A), and then immunostained with the serum of a representative wheat-sensitive patient (B), with that of a mite-sensitive patient who had a high level of IgE antibodies (C), or with that of a non-allergic patient (D), as described in the text. Arrows 1–6 represent the protein spots of Tri a Bd 27K components positive to IgE antibodies.
to IgE antibodies.\textsuperscript{8,11} Together with these results, the N-linked glycan moieties with the fucose residue of Tri a Bd 27K are responsible for the binding to IgE antibodies in the sera of wheat-sensitive patients.

In conclusion, the present findings indicate that Tri a Bd 27K in wheat occurs in multiple forms. It was indicated that among these, only the Tri a Bd 27K components having N-linked glycan moieties with fucose residues are involved in the binding to IgE antibodies in the sera of wheat-sensitive patients.

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References