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Note

## IgE-reactive 60 kDa Glycoprotein Occurring in Wheat Flour

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**A new IgE-reactive glycoprotein with a molecular size of 60 kDa was isolated from wheat flour. The N-terminal amino acid sequence of the protein was LDPDESEXVTRYFRIR. The 8th amino acid residue would have been Asn to which the peroxidase-type glycochain was attached. The IgE-binding activity of the glycoprotein was rendered negligible by the enzymatic treatment applied for hypoallergenic flour production.**

**Key words:** wheat allergen; allergenic glycoprotein; peroxidase type glycochain

An IgE-mediated hypersensitive response to wheat has long been an important public health problem. While the  $\alpha$ -amylase inhibitor<sup>1)</sup> and gliadin<sup>2)</sup> have been identified as allergens occurring in wheat, we have found that glutenin was allergenic for most patients allergic to wheat<sup>3)</sup> and have elucidated a Gln-Gln-Gln-Pro-Pro motif as the IgE-binding epitope.<sup>4-6)</sup> Based on this epitope structure, a practical method has been proposed to produce a hypoallergenic flour by the enzymatic treatment of flour.<sup>7-9)</sup> The effectiveness of this hypoallergenic product has been immunologically evaluated with clinically satisfactory results.<sup>10)</sup> More recently, we have reported the isolation and identification of a novel  $\beta$ -1,4-linked mannoglucan as a possible allergen occurring in wheat flour.<sup>11)</sup> Unlike proteinaceous allergens, mannoglucan would be very stable in the body, possibly acting as a remaining allergen to cause a longer-lasting allergic reaction. Thus, the existence of such a non-proteinaceous allergen would explain why wheat allergy is difficult to recover from.

As another non-proteinaceous allergen, the glycochain of the wheat  $\alpha$ -amylase inhibitor has also been reported to react with IgE antibodies in patients with baker's asthma.<sup>12)</sup> In addition, Asn-linked

glycochains have received recent attention in studies on the cross-reactivity between pollen, insects, and food allergens.<sup>13,14)</sup> Garcia-Casado *et al.*<sup>13)</sup> have reported that the presence of a  $\beta$ 1 $\rightarrow$ 2 xylosyl residue, which was attached to  $\beta$ -linked mannose of the glycochain core in bromelain and peroxidase, constituted an IgE-reactive determinant.

The aim of this present study is to examine IgE-reactive glycoproteins in wheat flour and to clarify whether any new glycoproteins exist or not.

Soft flour (*Triticum aestivum*), commercially named Cleopatra, was presented by Showa Sangyo Co. (Japan). The flour (10 kg) was extracted with 10 mM sodium dihydrogenphosphate (20 liters), and to the extract was added ammonium sulfate at 50% saturation and pH 7.0. The precipitate was dialyzed against running water. The non-diffusible fraction was centrifuged at 7,500  $\times$  g for 20 min to obtain a precipitated fraction. This fraction was rinsed twice with water and then dissolved in a 10 mM acetate buffer (pH 4.5, 50 ml) containing 0.5 M NaCl. The solution was, after being diluted with water (500 ml), loaded into a CM-cellulose (Wako Pure Chemical Ind.) column (5  $\times$  5 cm). Elution was conducted with a 10 mM acetate buffer (pH 4.5, 100 ml) containing 0.5 M NaCl. The eluate was dialyzed against running water and then submitted to DEAE-cellulose (Wako Pure Chemical Ind.) column chromatography, using a column of 5  $\times$  5 cm and eluting solvent of the 10 mM phosphate buffer (pH 6.0) containing 0.5 M NaCl. The eluate was exhaustively dialyzed against distilled water. All the procedures were carried out in a cold chamber. A 1/10 part of the crude fraction thus obtained was lyophilized. SDS-PAGE was carried out as reported by Laemmli,<sup>15)</sup> using 10–20% gradient gel (Daiichi Pure Chemicals) at 40 mA for 1 h. Proteins in the gel were electrotransferred to a PVDF membrane (Bio Rad) at 25 V for 90 min at

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**Abbreviations:** BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; CM, carboxymethyl; DEAE, diethylaminoethyl; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBS-Tween, PBS containing 0.05% Tween 20; PVDF, polyvinylidene difluoride; TFA, trifluoroacetic acid; UTH, 0.1 M Tris-HCl (pH 8.6) containing 4 M urea

ambient temperature, using Dunn's carbonate buffer solution (pH 9.9) containing 0.004% SDS. The same procedure was conducted three times. One of the membranes was stained with CBB R-250 (Daiichi Pure Chemicals). Another membrane was blocked with PBS containing 5% skim milk and 1% BSA, and then immunoassayed by using the serum of a wheat-sensitive allergic patient (patient MC, International Bioscience). Alkaline phosphatase-conjugated goat anti-human IgE (Kirkegaard & Perry Laboratories) was used as the secondary antibody, and the immune complexes were visualized by applying an Immun-Star substrate kit (Bio Rad). The 3rd membrane was submitted to an immunoassay with rabbit anti-horseradish peroxidase (Sigma Chemical Co., P-7899) as the primary antibody which recognizes peroxidase-type Asn-linked glycochains.<sup>14)</sup> Alkaline phosphatase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) was used as the secondary antibody. Figures 1-A and 1-C show that one unknown protein with IgE-binding activity was detected at about 60 kDa (asterisked band). This protein reacted with the anti-peroxidase antibody (Fig. 1-B), indicating that it possessed Asn-linked glycochain(s). The bands at about 40 kDa and 16 kDa were probably from peroxidases<sup>16)</sup> and  $\alpha$ -amylase inhibitors,<sup>1)</sup> respectively.

The remainder of the crude fraction was subjected to the first HPLC run in a reversed-phase column (Shodex RSpak RP18-415,  $4.6 \times 150$  mm) at 20°C. A linear gradient of 0.1% TFA-acetonitrile (0 to 10% in acetonitrile concentration) was used as the eluting solvent, and UV at 220 nm was used for detection. Each peak in Fig. 2-A was assayed for IgE-binding activity by ELISA with the MC serum. Each well of a 96-well microtiter plate (Nalge Nunc International) was coated overnight at 4°C with the fraction (about 1  $\mu$ g of solid) dissolved in a UTH buffer solution (100  $\mu$ l) and then blocked with 2% BSA (Sigma Chemical Co.) in PBS (200  $\mu$ l) at 37°C for 1 h. The serum diluted with a 1000-fold volume of PBS (100  $\mu$ l) was added and incubated at 37°C for 2 h. This procedure was followed by the addition of biotinylated anti-human IgE (Kirkegaard & Perry Lab.) and streptoavidin-peroxidase conjugate (Boehringer Mannheim). The plate was developed at room temperature after the addition of *o*-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.016%) in a citrate-phosphate buffer (pH 5.0). The absorbance at 490 nm was measured with a microplate reader (Bio-Rad, Model 550), the plate being washed 5 times with PBS-Tween between each step. Two peaks marked with a and b were bound to IgE from the wheat-allergic patient. The electrophoretic patterns of peaks a and b indicate that their molecular sizes were 60 kDa and 40 kDa, respectively. Peak a was further purified by a second HPLC run by modifying the solvent system in an

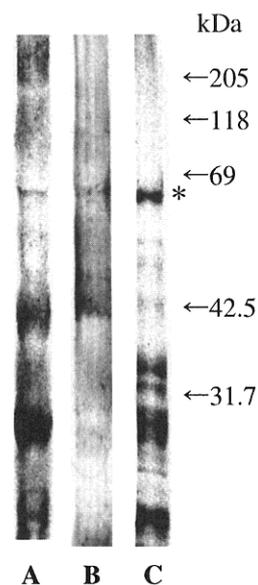


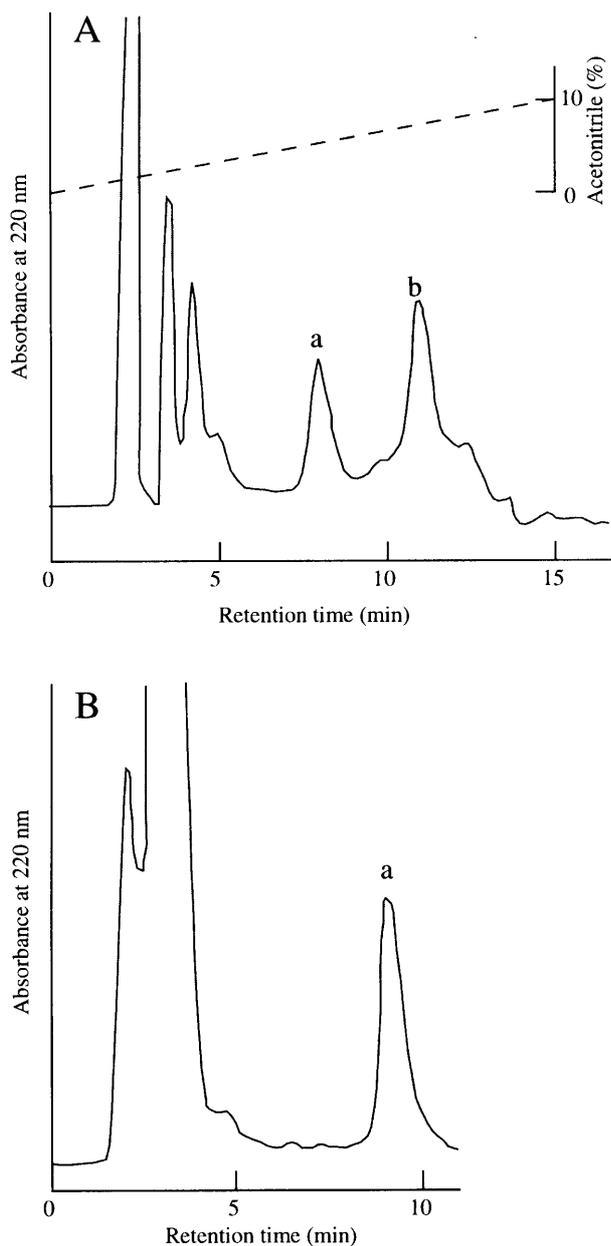
Fig. 1. SDS-PAGE Patterns of the Active Fraction Obtained by Ammonium Sulfate Precipitation and Ion-exchange Chromatography.

A, detected by using the serum from a patient; B, detected by using the anti-peroxidase antibody; and C, stained with CBB. A mixture of rabbit muscle myosin (205 kDa), *E. coli*  $\beta$ -galactosidase (118 kDa), bovine serum albumin (69 kDa), rabbit muscle aldolase (42.5 kDa), and bovine carbonic anhydrase (31.7 kDa) was used to provide molecular size markers.

isogratic manner with 0.1% TFA. Peak a in Fig. 2-B obtained from the second run showed IgE-binding activity.

The N-terminal amino acid sequence of the compound was determined by auto-Edman degradation with an amino acid sequencing system (Hewlett Packard, G1005A), the determined N-terminal structure being LDPDESEXVTRYFRIR. The 8th amino acid residue would have been Asn to which a glycochain was attached. The glycoprotein reacted both with the anti-horseradish peroxidase IgG antibody and the sera from 5 patients allergic toward wheat at Kansai Medical University Hospital. The relative ELISA values against the IgG antibody and the sera were in the range of 0.7–1.1 when the concentration of each of the 60 kDa and 16 kDa (control) proteins was set to an absorbance at 220 nm of 0.01. The amino acid sequence similarity between the peptide fragment and other naturally occurring proteins was checked by using a sequence database (ver. 3.0.0, ProteoMetrics). No similarity was apparent between the sequence of the 60 kDa glycoprotein and any other protein, including wheat allergens. Thus, the glycoprotein was newly identified.

When the 60 kDa glycoprotein was hydrolyzed with cellulase (from *Trichoderma viride*, 86.8 units/mg of solid, Amano Pharmaceutical Co.) at pH 5 and 50°C and then by actinase (250 Tyr units/mg of solid, Kaken Pharma Co.) at pH 7 and 40°C as reported in the previous paper,<sup>9)</sup> the ELISA value



**Fig. 2.** Reversed-phase Chromatography of the Active Fraction (A) Obtained by Ion-exchange Chromatography and of the First HPLC Eluate (B, peak a in Fig. 2-A).

dropped below the detection limit when using the serum from wheat-allergic patient MC. This result supports the notion that the two-step enzymatic process using cellulase and actinase would be effective for decomposing the IgE-reactive structure of the 60 kDa glycoprotein and for the production of hypoallergenic wheat flour.<sup>9)</sup>

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