Isolation and Characterization of a Novel Polysaccharide As a Possible Allergen Occurring in Wheat Flour

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A new polysaccharide with a molecular weight of 5.0 × 10^4 was isolated as a possible wheat allergen from a water-soluble fraction of flour by affinity chromatography and gel filtration. The isolated polysaccharide was found to be a possible wheat allergen, as it bound specifically to IgE antibodies in the sera of patients allergic to the water-soluble fraction of flour. Chemically, the sugar moiety of the polysaccharide consisted of α-glucose and β-mannose with a 1,4-linkage in a molar ratio of 4.5:1. Since this mannoglucon was thought to be stable in our body, it would act as a remaining allergen to cause a long-lasting allergic reaction to wheat flour.

Key words: wheat allergy; allergen; polysaccharide; mannoglucon

IgE-mediated hypersensitive responses to wheat have long been an important public health problem. While α-amylase inhibitor10 and gliadin11 have been identified as allergens occurring in wheat, we found that glutenin was allergenic for most patients allergic to wheat12 and have elucidated a Gln-Gln-Gln-Pro-Pro-Pro motif as the IgE-binding epitope.4) Based on this epitope structure, a practical method has been proposed to produce a hypoallergenic flour using food-processing proteases.6) The effectiveness of this hypoallergenic product is being immunologically evaluated and has already shown clinically satisfactory results.8)

In the meantime, it remains unclear whether a non-proteinaceous constituent in wheat also acts as an allergen. Unlike proteinaceous allergens, some non-proteinaceous substances would be more 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 treat. The aim of this study is to isolate a polysaccharide allergen from a water-soluble fraction of wheat flour and to clarify its chemical structure and immunological properties. We report here the isolation and identification of a novel β-1,4-linked polysaccharide as a possible allergen occurring in wheat flour.

Materials and Methods

Reagents. Soft flour (Triticum aestivum), commercially named Cleopatra, was presented by Showa Sangyo Co. (Japan). Cellulase (EC 3.2.1.4; 9.8 U/mg) from Penicillium ficurciulius was purchased from Sigma Chemical Co. (U.S.A.), and a pyridylaminated-glucose (PA-Glc) oligomer mixture, ranging from PA-Glc3 to PA-Glc11, was purchased from Takara Shuzo Co. (Japan). Glucosaminan of Amorphophallus konjac origin was obtained from a commercial source, and the other reagents were all of reagent grade.

Sera. Peripheral blood samples were prepared from eight wheat-allergic patients with atopic dermatitis as previously reported.4,5) The specific IgE titers of these patients were 17.7, 16.2, 12.6, 11.0, 6.9, 4.3, 3.8, and 1.4 U/ml as estimated by RAST. Based on ELISA, four of the eight patients were found to be sensitive to a water-soluble fraction of wheat flour; thus, their sera were used for inhibition ELISA after appropriate dilution.

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Abbreviations: ConA, concanavalin A; DEAE, diethylaminoethyl; ELISA, enzyme-linked immunosorbert assay; FT-IR, Fourier transform infrared absorption; Glc, glucose; GNA, Galanthus nivalis plant lectin; Man, mannose; NMR, nuclear magnetic resonance; ODS, octadecyl silica; PA-, pyridylaminated; RAST, radioallergosorbent test; TFA, trifluoroacetic acid
Inhibition ELISA. Inhibition ELISA was performed as previously described. Briefly, soft flour (10 g) was suspended in cold water (50 ml), and stirred for 30 min. The suspension was centrifuged at 5,000 × g for 20 min, and the supernatant was boiled for 2 min to inactivate the enzymes. The solution was centrifuged again under the same conditions, and the supernatant was freeze-dried. This water-soluble fraction (0.5 mg) was dissolved in PBS (1 ml), and the resulting solution (100 μl) was coated on a microplate overnight at 4°C. Unoccupied sites in the wells were saturated by incubating with 200 μl of bovine serum albumin (2%, Sigma Chemical Co.) at 37°C for 1 h. Next, a freeze-dried test sample was dissolved in PBS-0.05% Tween 20, and the resulting solution (80 μl) was preincubated with a serum sample (40 μl) at 25°C for 1 hr. Aliquots (100 μl each) of the treated serum were used as the antibody for ELISA, and untreated serum was used as a control. This procedure was followed by the addition of bivalent anti-human IgE (Kirkegaard & Perry Lab., U.S.A.), streptavidin-peroxidase conjugate (Boehringer Mannheim, Germany), and α-phenylenediamine (Wako Pure Chemical Industries, Japan). The wells were washed between each addition with PBS Tween 20 (0.05%). After the reaction, the OD value was read by a plate reader, using a 490 nm filter. A test sample was judged to be allergenic if it inhibited antigen-antibody binding by more than 10%.

Extraction and anion-exchange column chromatography. Soft flour (100 kg) was suspended in cold water (200 l) and stirred for 30 min. The suspension was then centrifuged at 5,000 × g for 20 min, and the resulting supernatant was boiled for 2 min to inactivate the enzymes. The solution was centrifuged again under the same conditions, and the supernatant (4 l for each run) was subjected to chromatography in a DEAE-cellulose column (14 × 10 cm; Pharmacia Biotech, Sweden). Sodium dihydrogenphosphate and magnesium chloride (final concentration of 10 mM each) was added to the unretained fraction. The mixture was adjusted to pH 7.0 and then submitted to ConA affinity chromatography.

ConA-agarose affinity chromatography. The DEAE-cellulose unretained fraction was subjected to chromatography in a ConA-agarose column (5 × 2 cm; Wako Pure Chemical Industries), eluting with a 10 mM magnesium chloride-10 mM phosphate buffer (pH 7.0) at an appropriate flow rate. The ConA-agarose-adsorbed fraction was eluted with 0.1 M methyl α-D-mannoside in a 10 mM magnesium chloride-10 mM phosphate buffer (pH 7.0), and the eluate was dialyzed against cold water. The non-diffusible fraction was subjected to HPLC in a ConA-agarose-packed column (8.0 × 50 mm; ACA-894, Showa Denko, Japan). Elution was with the same solvent system at a flow rate of 1 ml/min, and peaks were detected by UV at 220 nm. The ConA-agarose-adsorbed polysaccharide fraction obtained was dialyzed against cold water, and then subjected to gel-filtration HPLC.

Gel-filtration HPLC. The sample was loaded into two gel filtration columns (7.8 × 300 mm each; TSK-gel G3000PWXL, Tosoh Co., Japan) connected in series, and eluted with distilled water at a flow rate of 1 ml/min. Peaks were detected by UV at 220 nm. Pullulan P-20, P-50, and P-100 (Showa Denko) of Mw 2.28 × 10^6, 4.73 × 10^6 and 1.12 × 10^6, respectively, were used as molecular weight markers, detection being made with a refractive index detector (Labo System, RI-98 Scope).

Instrumental analyses. The IR spectrum was recorded with a micro-FT-IR spectrometer (JASCO, MFT-2000) for KBr disks. For the NMR analysis, the wheat polysaccharides and A. konjac glucosann were dissolved in D₂O. NMR spectra were recorded at 30°C with a Bruker AMX500 spectrometer (1H at 500 MHz) equipped with an H/C/N triple-resonance probe. Chemical shifts (δ) in ppm were determined relative to the external standard [2,2,3,3-D₄] sodium 3-(trimethylsilyl) propanoate in D₂O (0.00 ppm). For the 1D 1H spectra, 282 (wheat polysaccharide) and 27 (A. konjac glucosann) free induction decays were accumulated in 32 K data points for each spectral width of 8,064 Hz at 500.135 MHz. Exponential multiplication (LB = 0.2) was performed prior to Fourier transformation.

GNA affinity chromatography. The polysaccharide was applied to a GNA-Sepharose column (1.2 × 1.2 cm, EY Laboratories; U.S.A.), washed with a 10 mM magnesium chloride-10 mM phosphate buffer (pH 7.0) at an appropriate flow rate, and eluted with 0.1 M methyl α-D-mannoside in the 10 mM magnesium chloride-10 mM phosphate buffer (pH 7.0). The eluate was dialyzed against cold water, and the non-diffusible fraction was submitted to gel-filtration HPLC prior to an analysis of the sugar composition.

Analysis of the sugar composition. The polysaccharide allergen (approximately 2 μg) was completely hydrolyzed with 2 M TFA at 110°C for 2 hr. The sugar composition of the hydrolysate was analyzed by HPLC in an anion-exchange column (4.0 × 250 mm; CarboPac PA1, Dionex, U.S.A.) according to the method of Shiomi et al.9

Cellulase treatment. The polysaccharide allergen was treated with cellulase in distilled water at 37°C for 4 hr. The reaction mixture was applied to an ODS column (Sep-pak C₁₈, Waters, U.S.A.) and eluted with distilled water to remove cellulase. The eluate
(the oligosaccharide fraction) was freeze-dried. Pyridylation of the obtained oligosaccharides was carried out according to the method of Hase et al.\(^{10}\) 

**Sephadex G-15 column chromatography.** The PA-oligosaccharides were subjected to chromatography in a Sephadex G-15 column (2.5 \(\times\) 60 cm, void volume of 80 ml; Pharmacia Biotech), eluting with 10 mM ammonium bicarbonate at a flow rate of 50 ml/hr. PA-Glc\(_{15}\), PA-Glc\(_{10}\), PA-Glc\(_5\), and PA-Glc\(_1\) were used as molecular weight markers and detected with a fluorescence spectrophotometer (RF-1500, Shimadzu, Japan) at excitation and emission wavelengths of 320 and 360 nm, respectively.

**Results**

The water-soluble fraction of wheat flour was first subjected to DEAE-cellulose column chromatography to remove the proteinaceous substances. The unretained fraction was then subjected to ConA-agarose column chromatography. The adsorbed fraction was eluted with 0.1 M methyl \(\alpha\)-D-mannoside, and found to be allergenic by ELISA. This allergenic fraction was rechromatographed by HPLC in a ConA-agarose packed column, resulting a fraction (asterisked in Fig. 1) to which all four patients were sensitive. This ConA-agarose-adsorbed fraction was purified by gel filtration HPLC to obtain the asterisked fraction (Fig. 2) with allergenicity in a yield of approximately 1 mg from 100 kg of flour.

The mean molecular weight was estimated to be approximately \(5.0 \times 10^4\) (Fig. 2).

ConA is a specific adsorbent with an affinity for Man- and/or Glc-containing polysaccharides and glycoproteins. To clarify whether the allergenic compound consisted of polysaccharide or glycoprotein, it was examined by IR spectrometry. As shown in Fig. 3, the IR spectrum of the allergenic compound suggested the presence of OH groups based on the absorption at 3400 cm\(^{-1}\), with no characteristic absorption for amide groups (1550 cm\(^{-1}\) and 1650 cm\(^{-1}\)) being apparent. Therefore, the compound appeared to consist mainly of polysaccharide.

While it is well known that the non-starchy polysaccharides occurring in wheat are mainly arabinoxylans, \(\beta\)-glucans, and mannan, the occurrence of mannogluclan in wheat remains unclear.\(^{11-14}\) Thus, to clarify the possibility that this might be a mixture of glucan and mannan or a hetroglycan containing glu-
cose and mannose as component sugars, the isolated polysaccharide allergen was subjected to chromatography in a GNA column.\textsuperscript{15} As a result, the allergen bound to GNA-Sepharose and was eluted with 0.1 M methyl \(\alpha\)-D-mannoside. When the GNA-Sepharose-adsorbed polysaccharide was subjected to gel filtration chromatography as described in Fig. 2, it was eluted as a single peak with the same elution time as that shown in Fig. 2. It was also confirmed that even after GNA-Sepharose chromatography, the GNA-Sepharose-adsorbed polysaccharide retained its IgE-binding ability (Table 1). The polysaccharide was hydrolyzed with 2 M TFA, and the sugar composition of the hydrolysate was analyzed by HPLC. The result revealed that the polysaccharide consisted of Glc and Man in a molar ratio of 4:4:1. No other common sugars such as xylose, galactose, fucose, \(N\)-acetyl glucosamine, or \(N\)-acetyl galactosamine were detected. These results indicate that the polysaccharide allergen was a pure mannanogluca.

Heteropolysaccharides containing Man and Glc are commonly found in tubers of *Amorphophallus* sp., seeds of iris, and bulbs of certain orchids and lilies.\textsuperscript{16} Using *A. konjac* glucomannan as a control polysaccharide, the \(1^H\)-NMR spectrum of the wheat polysaccharide antigen was recorded (Fig. 4). The spectrum of the polysaccharide was similar to that of *A. konjac* glucomannan. The data also support that the wheat polysaccharide consisted mainly of polysaccharide.

The polysaccharide allergen was treated with cellulase, and the resulting oligosaccharides were pyridylaminated to enable detection by fluorescence. The pyridylaminated oligosaccharides were chromatographed on a gel filtration column. As shown in Fig. 5, the polysaccharide was converted to oligosaccharides by hydrolysis with cellulase, suggesting that the polysaccharide had \(\beta\)-1,4-glycosidic linkages.

### Discussion

Food allergies are the hypersensitivity caused by the continuous ingestion of constituents called allergens which are, in most cases, proteins. However, we have found that some wheat-allergic patients are still sensitive to hypoallergenic wheat flour in which the proteins have been almost completely hydrolyzed by proteases (data not shown). This suggests that non-proteinaceous substances in wheat flour may also be allergenic. Such non-proteinaceous allergen(s) would explain why wheat allergy is difficult to treat. This study has aimed to provide answers to this question of social importance.

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**Table 1.** Inhibition ELISA of the Wheat Polysaccharide Allergen (A) and (B)

<table>
<thead>
<tr>
<th>Patient</th>
<th>(%) inhibition</th>
<th>Dilution</th>
<th>(%) inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>29</td>
<td>(\times 1)</td>
<td>90</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>(\times 5)</td>
<td>32</td>
</tr>
<tr>
<td>C</td>
<td>24</td>
<td>(\times 25)</td>
<td>13</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td></td>
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</tbody>
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(A) The water-soluble fraction of wheat flour (0.5 mg/ml) was used as the antigen. Each individual serum sample (40 \(\mu\)l) was incubated at 25°C for 1 h with the polysaccharide solution (80 \(\mu\)l, \(A_{280}\) = 0.5), and aliquots were taken for use as the antibody in the assay. The percentage inhibition value was calculated by using intact serum as a control. Details of the conditions are described in the "Materials and Methods" section.

(B) Pooled serum (40 \(\mu\)l) from the four patients was preincubated with the polysaccharide solution (80 \(\mu\)l, \(A_{280}\) = 2.5). This polysaccharide solution was diluted with 4- or 24-fold of PBS Tween 20 (0.05%) and assayed by the same method (\(\times 5\), \(\times 25\)).

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**Fig. 4.** \(1^H\)-NMR (500 MHz, 50°C) Spectra of the Polysaccharide Allergen (A) and *Amorphophallus konjac* Glucomannan (B).

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**Fig. 5.** Sephadex G-15 Chromatogram of the Cellulase-treated PA-oligosaccharide from the Polysaccharide Allergen.

The pyridylaminated oligosaccharides were subjected to Sephadex G-15 column chromatography (2.5 × 60 cm, void volume (\(V_0\) = 80 ml). The elution positions of PA-Glc\(_{16}\), PA-Glc\(_{10}\), PA-Glc\(_{5}\), and PA-Glc\(_3\) are shown by arrows. Detection was made with a fluorescence spectrophotometer at excitation and emission wavelengths of 320 and 360 nm, respectively.
We report here that wheat contained a novel polysaccharide allergen with linear β-1,4 linkages composed of Glc and Man in a molar ratio of 4.4:1. While some studies have shown the presence of arabinoxylan and arabinogalactan in water extracts of wheat flour,17,18 our report is the first that clearly demonstrates the occurrence of a mannogluca in wheat flour. It remains unclear why the mannogluca obtained in this study had UV absorption, but we assume that it was associated with some contaminants that were not essentially involved in IgE-binding.

While the orally administered mannogluca allergen would be excreted because of its indigestible nature, it could be absorbed by the inhalation of wheat flour. In this case, it would not be degraded, and would remain longer in the body as a remaining allergen. This would be the probable reason why patients sensitive to the water-soluble fraction of wheat flour are found to possess mannogluca-specific IgE antibodies.

As for the epitope structure of the wheat allergens, the glycan moieties of the wheat α-amylase inhibitor have been reported to play a critical role in the reactivity with IgE antibodies in patients with baker’s asthma.19 In addition, Asn-linked glycans have received recent attention in studies on cross-reactivity between pollen, insects, and food allergens.20,21 Moreover, Dudler et al.22 have reported that glycosylated protein-dependent T cells in allergic patients responded to the bee venom allergen. It remains unclear whether these glycoprotein allergens containing Asn-linked glycan(s) and the wheat mannogluca can be recognized by the common IgE-antibodies.

In general, allergenic proteins induce the production of IgE antibodies, resulting in an allergic disease. The differentiation of B lymphocytes to IgE-producing plasma cells requires interaction with activated Th cells. The helper activity is dependent on the recognition of T cell epitopic peptides presented by MHC molecules on the antigen-presenting cells.23 The classical concept is that T cell recognition is confined to antigen-derived peptides, whereas polysaccharides cannot induce a T cell response. It would be interesting to know whether T cells recognize the mannogluca allergen directly. Or else, it could trigger an allergic reaction when the mannogluca-IgE complex causes the release of histamine and other chemical mediators. Elenkov et al.24 have recently suggested the possibility that the secretion of histamine itself potentiates Th2-mediated allergic reactions. We are also undertaking T cell-recognition and histamine-release assays on the isolated mannogluca allergen, since work along this line would be of both immunological and clinical importance in developing practical countermeasures against the world-wide social problem, wheat allergy.

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References