Mini-Review:
Identification of Wheat Allergens

Soichi TANABE

Faculty of Applied Biological Science, Hiroshima University, Japan

SUMMARY
Several research groups have identified IgE-binding wheat proteins associated with baker's asthma or atopic dermatitis. This review pinpoints our attempts at the structural identification of wheat allergens using sera from atopic dermatitis patients with definite wheat allergy. Two thirds of patients tested were gluten-sensitive. An IgE-binding peptide was isolated from the chymotryptic hydrolysate of gluten. The primary structure of the peptide was determined as (Ser-Gln-Gln-(Gln-)Pro-Pro-Phe). The peptide originated from low-molecular-mass glutenin. The epitope in the allergenic peptide was Gln-Gln-Gln-Pro-Pro. The N-terminal glutamine and the two proline residues were essential for epitopic function as determined by the IgE-binding potencies of synthetic peptides. In the meantime, a new IgE-binding 60-kDa glycoprotein and a mannogluccan were identified as potential allergens. Other wheat allergens, for example, alpha-amylase inhibitors, are also discussed.
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KEYWORDS
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Correspondence:
Ass. Prof. Soichi Tanabe, PhD
Faculty of Applied Biological Science, Hiroshima University
1-4-4 Kagamiyama, Higashi-Hiroshima
Hiroshima 739-8528, Japan
Phone: +81-824-24-7932
Fax: +81-824-22-7067
eMail: stanabe@hiroshima-u.ac.jp

Soichi Tanabe
INTRODUCTION

Wheat seeds are composed of four protein classes, such as 1) water-soluble albumins, 2) salt-soluble globulins, 3) ethanol-soluble gliadins, and 4) urea, detergent, or KOH-soluble glutenins. Wheat gluten is a complex of gliadins and glutenins. Gluten is the elastic rubbery protein that binds the dough in breads and other bakery products.

Hypersensitivity responses to wheat have long been an important public health problem. The adverse reactions to wheat flour develop three different phenomena, enteropathy (diarrhea), asthma and atopic dermatitis.

Gluten-sensitive enteropathy is called "celiac disease", and is caused by ingestion of the gliadin fraction (Sturgess et al. 1994). The reported prevalence of this disease is 1:300 to 1:1000 in European countries (Troncone et al. 1992). The amino acid sequences responsible for the disease have been characterized, and the minimum epitope structures were found to be Pro–Ser-Gln-Gln and Gln-Gln-Gln-Pro (Sturgess et al. 1994). Although the disease is mediated by T-lymphocyte-driven immunological activation in the gastrointestinal mucosa (Trier 1991, Marsh 1992, Troncone et al. 1996), the levels of total and wheat-specific IgE antibodies of celiac patients are usually not elevated (Mietens et al. 1971, Hodgson et al. 1976, Bahna et al. 1980). Therefore, in the general context of considering "allergy" synonymous with IgE-mediated hypersensitivity, celiac disease should not be classified as an allergic disorder (Bahna 1996).

The inhalation of wheat flour also often causes baker’s asthma (Amano et al. 1998), a typical occupational allergic disease that has been known since ancient Roman times. Extensive studies identified some proteins as allergens associated with asthma. Among them, alpha-amylase inhibitors (AI) from the globulin fraction were identified as major allergens (Gómez et al. 1990, Sánchez-Monge et al. 1992, Armentia et al. 1993, Amano et al. 1998). The IgE-binding epitope structures of an AI (known as the 0.28 wheat AI) were determined (Walsh & Howden 1989), and, acyl-CoA oxidase (Posch et al. 1995, Weiss et al. 1997), peroxidase (Sánchez-Monge et al. 1997), and fructose-bisphosphate aldolase (Weiss et al. 1997) were identified as other allergens.

The other wheat-associated phenomenon is skin inflammation, atopic dermatitis, that develops shortly after cereal-based products are ingested, usually resulting in eruption and itching. Wheat allergens associated with atopic dermatitis are so heterogeneous that many attempts had been made internationally to identify them. However, little information had been available on the molecular structure of major allergens associated with atopic dermatitis when our group started to carry out a systematic experiment (Varjonen et al. 1994, Varjonen et al. 1995, Watanabe et al. 1995, Tanabe et al. 1996). Afterwards, some groups identified several more wheat allergens (Sandiford et al. 1997, Kusaba-Nakayama et al. 2000, Sander et al. 2001, Takizawa et al. 2001).

In our first experiment, wheat flour proteins were divided into salt-soluble and salt-insoluble (gluten) fractions (Table 1). The allergenicity of each fraction was evaluated by means of enzyme-linked immunosorbent assay (ELISA), using sera of atopic patients allergic to wheat; most patients were found to be sensitive to gluten. Thus, we first tried to determine a major epitope structure of gluten responsible for atopic dermatitis (Watanabe et al. 1995).

The second approach aimed at identifying IgE-binding wheat proteins containing carbohydrate moieties (Watanabe et al. 2001), since glycosylated subunits of the alpha-amylase-inhibitor family were shown to have enhanced IgE-binding capacity (Sánchez-Monge et al. 1992).

In a further investigation we sought to evaluate the IgE-binding properties of the polysaccharide fraction of wheat flour (Tanabe et al. 2000).
Table 1: Allergicities of salt-soluble and -insoluble (gluten) fractions of wheat flour. Wheat flour proteins were divided into salt-soluble and gluten fractions in the usual manner, and subjected to ELISA. IgE-binding is expressed in arbitrary units based on the absorbance at 490 nm.

<table>
<thead>
<tr>
<th>Type of patient</th>
<th>Patient No.</th>
<th>Salt-soluble Fraction</th>
<th>Gluten Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive to salt-soluble fraction</td>
<td>1</td>
<td>0.18</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.15</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&gt;2.0</td>
<td>0.07</td>
</tr>
<tr>
<td>Sensitive to gluten fraction</td>
<td>4</td>
<td>&lt;0.05</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.05</td>
<td>0.23</td>
</tr>
<tr>
<td>Sensitive to both fractions</td>
<td>6</td>
<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&gt;2.0</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>&gt;2.0</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>&gt;2.0</td>
<td>&gt;2.0</td>
</tr>
</tbody>
</table>

1 IgE-BINDING TO AMINO ACID SEQUENCE BASED STRUCTURES OF GLUTENIN

1.1 IgE-BINDING OF CHYMOTRYPIC PEPTIDES FROM LOW-MOLECULAR-MASS GLUTENIN

Since gluten was insoluble in aqueous media, it was hydrolyzed with alpha-chymotrypsin to obtain soluble peptide fragments with allergenicity. Food allergens are often characterized by their high stability against digestive enzymes, with their epitope structures remaining unchanged (Taylor et al. 1987). Thus digested peptides derived from the gluten fraction were expected to be still capable of IgE-binding. The resulting hydrolytic reaction product was centrifuged, and the supernatant was subjected to gel filtration and reversed-phase HPLC. The allergenicity of the fractionated elute was evaluated by ELISA, and the peak with the highest allergenicity was subjected to a primary structure determination.

The primary structure of the purified compound was a 30-mer peptide and determined to be (Ser-Gln-Gln-Gln-(Gln-Pro-Pro-Phe)). This allergenic peptide showed high similarities (almost 90%) to low-molecular-mass glutenin precursors (Pitts et al. 1988, Colot et al. 1989). Therefore, we concluded the peptide originated from low-molecular-mass glutenin. Similarities of about 70% were also obtained between the sequence of the allergenic peptide and those of the low-molecular-mass glutenin precursors from durum wheat (Cassidy & Dvorak 1990, D'Ovidio et al. 1992.). Surprisingly, a high degree of similarity (53.6%) was also found between the allergenic peptide and a Saccharomyces cerevisiae protein (Rasmussen 1994). Thus, it should be noted that wheat allergic patients are also suspected to be sensitive to yeast used for bread making.

The repeated sequence in allergenic peptides such as (Ser-Gln-Gln-Gln-(Gln-Pro-Pro-Phe)) may be favourable for cross-linking IgE antibodies and triggering the release of chemical mediators from mast cells in our body. There exists a very famous allergen, that is cod allergen (allergen M) which contains three homologous IgE-binding tetrapeptides in the residues 41-64 (Elsayed et al. 1982).

1.2 ANALYSIS OF IgE-BINDING EPITOPEs WITH SYNTHETIC PEPTIDES

In order to identify IgE-binding epitopes on the 30-mer amino acid sequence, peptides listed in Table 2 were synthesized according to the solid phase method. The N-terminal amino acid of each peptide was acetylated to mimic the condition under which each peptide existed in intact form. The allergenicity of each peptide was evaluated by ELISA (Tanabe et al. 1996). As shown in Table 2-A, (Ser-Gln-Gln-Gln-(Gln-Pro-Pro-Phe)) and Ser-Gln-Gln-Gln-(Gln-Pro-Pro-Phe) bound to IgE almost equally. There was no difference between the relative ELISA values of Ser-(Gln)₄
-Pro-Pro-Phe and Ser-(Gln)-Pro-Pro-Phe. These data suggest that the Ser-Gln-Gln-Gln-Pro-Pro-Phe motif is involved in binding to IgE antibodies.

To examine which amino acid residues in the motif are essential for binding to IgE, we replaced each constituent amino acid residue by Gly. When any of the asterisked amino acid residues in the sequence Ser-Gln*-Gln-Gln-Pro*-Pro*-Phe was replaced, the ELISA value dropped below the limit of detection (Table 2-B). These amino acid residues are therefore thought to be indispensable for IgE-binding. Tables 2-B and C also show that Gln-Gln-Gln-Pro-Pro, which lacks the N- and C-terminals of Ser-Gln-Gln-Gln-Pro-Pro-Phe, gave an ELISA value equal to that obtained with the full peptide.

We further examined which amino acid residues of Gln-Gln-Gln-Pro-Pro are essential for binding to IgE (Table 2-C) and found that the N-terminal glutamine residue and the two proline residues are essential. It was thus concluded that the IgE-binding epitope of the allergenic peptide comprised Gln-X-Y-Pro-Pro, where X and Y were replaceable amino acid residues. Indeed the inhibition ELISA assay showed that Ac-Gln-Gln-Gln-Pro-Pro bound to wheat-specific IgE in the serum of patients. To analyze the binding between Ac-Gln-Gln-Gln-Pro-Pro and IgE antibody, Fukushi et al. (1998) made the first NMR analysis of Ac-Gln-Gln-Gln-Pro-Pro. Their data showed that the configurations of the amide bonds of the peptide backbone were all-trans.

As in Table 2-C, the ELISA value obtained with Gln-Gly-Gln-Pro-Pro was lower by almost 30% than that obtained with Gln-Gln-Gln-Pro-Pro, and the value with non-acetylated Gln-Gln-Gln-Pro-Pro was almost

### Table 2: IgE-binding abilities of synthetic peptides

Peptides were synthesized according to the solid phase method. The peptide-bound multipins in a solid state were subjected to ELISA using sera of wheat allergic patients. Amino acids are denoted in the single-letter code. (Tanabe et al. 1996)

<table>
<thead>
<tr>
<th>(A) Peptide</th>
<th>Relative ELISA value</th>
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<tbody>
<tr>
<td>Ac-SQQQPPF</td>
<td>1.0</td>
</tr>
<tr>
<td>Ac-SQQQPPF</td>
<td>1.1</td>
</tr>
<tr>
<td>Ac-SQQQPPF</td>
<td>1.1</td>
</tr>
<tr>
<td>Ac-SQQQPFF</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B) Peptide</th>
<th>Relative ELISA value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-GQQQPPF</td>
<td>1.1</td>
</tr>
<tr>
<td>Ac-SQQQPPF</td>
<td>nd</td>
</tr>
<tr>
<td>Ac-SQQQPPF</td>
<td>0.8</td>
</tr>
<tr>
<td>Ac-SQQQPPF</td>
<td>1.0</td>
</tr>
<tr>
<td>Ac-SQQQGPF</td>
<td>nd</td>
</tr>
<tr>
<td>Ac-SQQQGPF</td>
<td>nd</td>
</tr>
<tr>
<td>Ac-SQQQPPG</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(C) Peptide</th>
<th>Relative ELISA value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-QQQPP</td>
<td>0.9</td>
</tr>
<tr>
<td>Ac-GQQPP</td>
<td>nd</td>
</tr>
<tr>
<td>Ac-OQQPP</td>
<td>0.7</td>
</tr>
<tr>
<td>Ac-OQQPP</td>
<td>1.0</td>
</tr>
<tr>
<td>Ac-OQQPP</td>
<td>nd</td>
</tr>
<tr>
<td>Ac-OQQPP</td>
<td>nd</td>
</tr>
<tr>
<td>QQPP</td>
<td>0.6</td>
</tr>
</tbody>
</table>
half of that with acetylated Gln-Gln-Gln-Pro-Pro. From these data, the second glutamine residue of Gln-Gln-Gln-Pro-Pro and acetylation of the N-terminal amino group are both advantageous for binding to IgE. Also, recombinant low-molecular-mass glutenin, which contained many Gln-Gln-Gln-Pro-Pro motifs, were expressed in *Escherichia coli* by a pET vector system and confirmed its IgE-binding ability (Maruyama et al. 1998).

2 IgE-BINDING TO STRUCTURES CONTAINING ASN-LINKED GLYCOCHAINS

Wheat alpha-amylase inhibitors (AIs) have been studied as allergens for over 20 years. There is a family of wheat AIs with a number of differing monomeric, dimeric and tetrameric proteins. As described above, IgE-binding epitope structures of AI 0.28 have already been determined on the amino acid sequence (Walsh & Howden 1989). Moreover, a glycan moiety of one subunit from the tetrameric AI (CM16) was also capable of IgE-binding from sera of patients with baker’s asthma (Sánchez-Monge et al. 1992). Although, other AIs, such as AI 0.19, AI 0.28, and AI 0.53, were also reported as allergens, involvement of glycans in allergic responses has not been fully proven. As for the AI, James et al. (1997) showed that AI was an allergen for both asthma and wheat allergy.

In addition, Asn-linked glycochains have received recent attention in the studies on cross-reactivity between pollen, insects, and food allergens (Batanero et al. 1996, Garcia-Casado et al. 1996). Garcia-Casado et al. (1996) reported that the presence of a beta-1,2-xylosyl residue, which was attached to the beta-linked mannose of the glycochain core in bromelain and peroxidase, constituted an IgE-reactive determinant. Indeed, we previously reported that patients sensitive to salt-soluble fraction of wheat flour cross-reacted to bromelain (Tanabe et al. 1997). We thus examined IgE-binding glycoproteins in wheat flour and clarified whether any new glycoprotein occurred or not (Watanabe et al. 2001).

Wheat flour was extracted with 10 mM sodium dihydrogenphosphate followed by addition of ammonium sulfate to 50% saturation at pH 7.0. The precipitate was dialyzed against running water, and then dissolved with 10 mM acetate buffer (pH 4.5) containing 0.5 M NaCl. The solution was submitted to Carboxymethyl-(CM)-cellulose and DEAE-cellulose column chromatography. The IgE-binding crude fraction thus obtained was lyophilized. SDS-PAGE was carried out using a 7.5% gel, and proteins in the gel were electrotransferred onto a PVDF membrane. The same procedure was repeated three times. One of the membranes was immunoassayed using sera of wheat-sensitive allergic patients (Figure 1, lane A). Another membrane was submitted to immunodetection with a rabbit anti-HRP (horse radish peroxidase) as a primary antibody, which recognizes peroxidase type N-linked glycochains (Batanero et al. 1996) (lane B). The other membrane was stained non-specifically with coomassie blue (CBB R-250) (lane C). There was one unknown IgE-binding protein in all three lanes detected at about 60 kDa (asterisked band). The protein reacted with the anti-peroxidase antibody (lane B), indicating that it contained N-linked glycochain(s). The reactivity of the glycan moiety in the 60 kDa allergen is under investigation. Bands at about 40 kDa and 16 kDa are probably peroxidase (Sánchez-Monge et al. 1997) and AIs (James et al. 1997, Sánchez-Monge et al. 1997), respectively.

![Figure 1: Wheat flour proteins separated by SDS-PAGE.](image_url)
The N-terminal amino acid sequence of the asterisked band was determined to be LDPDESEXVTTRYFRIR. The 8th amino acid residue would be Asn to which a glycochain attaches. The glycoprotein reacted both with the anti-horse radish peroxidase IgG antibody and sera from several wheat-allergic patients. The amino acid sequence similarity between the peptide fragment and other naturally occurring proteins was checked using a sequence database. As a result, no similarity was obtained between the sequence of the 60 kDa glycoprotein and any other proteins including wheat allergens. Thus, the glycoprotein was identified as a new wheat allergen.

### 3 IgE-BINDING TO POLYSACCHARIDE STRUCTURES: MANNOGLUCAN

In the meantime, it remained 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 our 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. Next, we aimed to isolate a polysaccharide allergen from a water-soluble fraction of wheat flour and to clarify its chemical structure and immunological properties (Tanabe et al. 2000).

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 affinity column chromatography and gel filtration HPLC to isolate the fraction with IgE-binding activity. The mean molecular mass was estimated to be approximately 50,000 kDa.

ConA is a specific adsorbent with an affinity for mannose (Man)- and/or glucose (Glc)-containing polysaccharides and glycoproteins. To clarify whether the IgE-binding compound consisted of polysaccharide or glycoprotein, it was examined by IR spectrometry. The IR spectrum of the allergenic compound suggested the presence of OH-groups, with no characteristic absorption for amide groups being apparent. Therefore, the compound appeared to consist mainly of polysaccharide. The polysaccharide was hydrolyzed with 2 M TFA (trifluoroacetic acid), 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, while no other common sugars such as xylose, galactose, fucose, N-acetyl glucosamine, or N-acetyl galactosamine were detected. Furthermore the polysaccharide allergen was converted to oligosaccharides by hydrolysis with cellulase, suggesting that the polysaccharide had beta-1,4-glycosidic linkages.

Judging from our detailed analysis, the polysaccharide was a novel allergen with linear beta-1,4 linkages composed of Glc and Man. While some studies have shown the presence of arabinoxylan and arabinoxylooligosaccharides in water extracts of wheat flour, our report was the first that clearly demonstrated the occurrence of mannoglucon in wheat flour (Tanabe et al. 2000).

The IgE-binding ability of wheat mannoglucon was confirmed by inhibition ELISA. The water-soluble fraction of wheat flour was coated on a microplate. Separately, patients’ sera were incubated with wheat mannoglucon. After blocking unoccupied sites with bovine serum albumin (BSA), the preincubated sera were used as the antibody for ELISA, and untreated sera were used as controls. This procedure was followed by the addition of biotinylated anti-human IgE, streptavidin-peroxidase conjugate, and o-phenylenediamine. As a result, wheat mannoglucon inhibited antigen-antibody binding by approximately 30% in all (four) patients allergic to the water-soluble fraction of wheat flour.

While the orally administered mannoglucon 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 mannoglucon-specific IgE antibodies.
As I described, there are three classes of allergens in wheat flour: 1) proteins such as alpha-amylase inhibitors, low-molecular-mass glutenin, acyl-CoA oxidase, peroxidase, fructose-bisphosphate aldolase, and so on, 2) Asn-linked glycochains in alpha-amylase inhibitor, peroxidase, and newly found 60 kDa protein (however it should be noted that the reactivity of the glyc can moiety in these glycoprotein has not been fully proven), and 3) polysaccharide (mannoglu can). The knowledge of allergens will contribute to the countermeasures against the worldwide social problem, intractable wheat allergy. For example, hypoallergenic rice and wheat flour have been produced for patients by our group. Such products should be of great benefit to patients as was reported for hypoallergenic rice in Nature with the title "Japan explores the boundary between food and medicine" (Swinbanks & O'Brien 1993).

Moreover, recent reports indicate that strategies aiming specific immunotherapy at the level of specific T cells are promising (Secrist et al. 1993, Bellinghausen et al. 1997, Ebner et al. 1997). We aim at the identification of T cell epitope structures of food allergens.

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