

Identification of major wheat allergens by means of the *Escherichia coli* expression system

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(Received 23 February/16 April 1998) – EJB 98 0260/3

Wheat proteins were fractionated into salt-soluble, glutenin-rich, and gliadin-rich fractions. Reactivities of these protein fractions with sera of patients with wheat-associated allergies were examined under various conditions. The relative reactivity of the fractions was generally in the order glutenin-rich > gliadin-rich > salt-soluble fractions, although their reactivities were variable among patients and among the reaction conditions, indicating that the kind, the number and the epitope of allergens were variable among patients. To identify major allergens, α -, γ - and ω -gliadin, and low-molecular-mass (LMM)- and high-molecular-mass (HMM)-glutenin genes were expressed in *Escherichia coli* by means of a pET vector. Recombinant gliadins and glutenins were partially purified on the basis of the solubilities of prolamins and glutelin. The partially purified recombinant proteins were reacted with the patients' sera. LMM glutenin containing many Gln-Gln-Gln-Pro-Pro motifs, which was identified to be IgE-binding epitope [Tanabe, S., Arai, S., Yanagihara, Y., Mita, H., Takahashi, K. & Watanabe, M. (1996) *Biochem. Biophys. Res. Commun.* 219, 290–293], exhibited the highest reactivity. The next highest reactivities were observed on α -gliadin and γ -gliadin, which had not been identified as allergens.

Keywords: wheat; allergen; low-molecular-mass glutenin; gliadin; heterologous expression.

Wheat is one of the foods most commonly responsible for allergic reactions in children and adults. This is considered to be a serious problem globally, since wheat is the most consumed crop in the world. As a step for development of hypoallergenic wheat, identification of proteins responsible for allergenicity of wheat was attempted by some groups [1–5]. Wheat proteins are classified into two fractions: salt-soluble (albumin and globulin) and salt-insoluble (glutenin and gliadin) fractions [6]. Ikezawa et al. [5] reported that a major wheat allergen occurs in a salt-soluble fraction. However, Watanabe et al. [1] found that most patients with wheat allergy were sensitive to gluten and identified the allergen to be a low-molecular-mass (LMM) glutenin subunit containing a number of a pentapeptide repeat motif, Gln-Gln-Gln-Pro-Pro, which was identified to be IgE-binding epitope [2, 4]. One of the reasons for the discrepancy in the reports from the two groups may be the difference in major allergens among patients. In addition to this, we have to consider the structural characteristics of wheat proteins. Generally, glutenins and gliadins exhibit anomalous relative mobilities (slower migrations than expected from their real molecular masses) on

SDS/PAGE [6], indicating that they have a rigid structure. Therefore, the reaction conditions used for patient's serum with salt-insoluble proteins must influence their reactivities.

It is known that glutenins and gliadins exhibit extreme molecular heterogeneity [6]. Accordingly, the use of recombinant DNA techniques for the identification of a major allergen of wheat and its residues involved in allergenic determinants is preferred to the traditional procedures [7, 8]. In this study, we analyzed reactivities of recombinant wheat proteins with the sera of patients with wheat-associated allergies under various reaction conditions to identify a major allergen.

MATERIALS AND METHODS

Fractionation of proteins from wheat flour. 10 g wheat hard flour (1CW) provided by Chiba Flour Milling Co. was added to 50 ml 1% (mass/vol.) NaCl. After stirring for 30 min at room temperature, the suspension was centrifuged at 18 000×g for 20 min at 4°C. The resultant supernatant was dialyzed against distilled water (pH 8.0), lyophilized and used as a salt-soluble fraction. Wheat flour (30 g) was mixed with 15 ml distilled water to form a dough. The dough was washed thoroughly by 1% NaCl solution and H₂O with hand-kneading to remove salt-soluble proteins and starch. The gluten ball (10 g) thus obtained was homogenized and stirred in 600 ml 0.1 M acetic acid at 4°C for overnight. After centrifugation at 8500×g for 30 min at 4°C, the supernatant was mixed with 1400 ml of ice-cold ethanol and was adjusted to pH 7.0. After storing at 4°C overnight, the solution was centrifuged. The resultant supernatant and precipitate were concentrated and lyophilized, respec-

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Abbreviations. LMM, low molecular mass; HMM, high molecular mass.

Note. The nucleotide sequence data presented here are available from the DDBJ/EBI/GenBank database under accession numbers D84341 (α -gliadin), D78133 (γ -gliadin), AB007763 (LMM glutenin 1), AB007764 (LMM glutenin 2) and AB008497 (LMM glutenin 3).

Note. N. Maruyama and K. Ichise contributed equally to this work.

Table 1. Radioallergosorbent-test and radioimmunosorbent values.

Patient	Age	Radioallergosorbent test U/ml	Radioimmunosorbent test IU/ml
A	19	2.37	n.d.
B	44	0.39	300
C	27	0.44	239
D	33	0.38	411
E	29	1.15	1052
F	28	4.84	972
G	3	5.56	n.d.
H	4	29.5	4400
I	1	11.8	1900
J	1	19.6	n.d.
Control	45	<0.34	19

tively, and used as gliadin-rich and glutenin-rich fractions, respectively.

Immunoassay using wheat proteins. Ten serum samples with significant radioallergosorbent-test scores against wheat proteins were selected from sera of patients with known clinical histories of allergies. Informed consent was obtained from all donors or their parents. The control serum was obtained from a healthy adult volunteer who had no clinical history of allergic diseases. The scores of radioallergosorbent tests and radioimmunosorbent tests of ten serum samples were listed in Table 1. The salt-soluble, the gliadin-rich and the glutenin-rich fractions were dissolved in 0.1 M Tris/HCl, pH 8.0, 2, 4 or 8 M urea and 2% (mass/vol.) SDS with/without 0.1 M 2-mercaptoethanol to give protein concentrations of 2 µg/µl. In addition, the latter two protein fractions were dissolved in 70% (by vol.) ethanol, 0.1 M acetic acid and 10 mM aluminium lactate with/without 2-mercaptoethanol. Since glutenins are not soluble in ethanol without a reducing agent, the glutenin-rich fraction was not dissolved in ethanol without 2-mercaptoethanol. Proteins in the samples were measured according to the method of Bradford [9]. Undissolved materials were removed by centrifugation. 2 µl each of the dissolved protein solutions were spotted on a nitrocellulose membrane. After treating the membrane with appropriately diluted human sera (1:30), the human IgE that reacted with the membrane-bound antigens were detected using a goat anti-(human IgE) Ig conjugated to alkaline phosphatase (Sigma) [10–12]. The intensity of the colour of the spot was estimated by densitometry using transmitted light and an NIH image program. The intensities derived from non-specific binding of the human antibodies of control serum and anti-human IgE to wheat proteins were subtracted from those from patients' sera.

Genomic DNA cloning by PCR and construction of expression plasmids. The coding sequences of α -, γ - and ω -gliadin and HMM-glutenin and LMM-glutenin genes were isolated by PCR. Oligonucleotides used as primers for PCR were designed based on the nucleotide sequences reported for α -gliadin (M11076), γ -gliadin (M16064), HMM glutenin (X12928 for x and y types and X12929 for the repetitive domain of the y type), a major LMM glutenin (X13306) and a minor LMM glutenin (X07747) to have a *Bam*HI site at the 3' end (Table 2). The wheat genomic DNA was isolated from shoots (seeds of 1CW were donated by Dr Igarashi of Chiba Flour Milling Co.) by the cetyltrimethylammonium bromide nucleic-acid-extraction procedure [13]. PCR were conducted on template wheat DNA with reagents supplied in a kit form (Perkin-Elmer) in a DNA thermal cycler (Perkin-Elmer). A PCR cycle consisted of denaturation at 98°C for 20 s, and annealing and extension at 68°C for 3 min. After 30 PCR cycles, the reaction mixtures were kept at 72°C for 10 min and the products were separated by electrophoresis

on a 1.2% (mass/vol.) agarose gel and purified using glass powder as directed (Takara). Details for ω -gliadin will be described elsewhere. The products were verified by the dideoxynucleotide sequencing method [14].

After blunting using a blunting kit (Takara), restriction by *Bam*HI and phosphorylation by T4 polynucleotide kinase, individual DNA fragments were inserted between the *Bam*HI and filled-in *Nco*I sites of pET-21d or pET-24d (Novagen). The start codon ATG was located in the filled-in *Nco*I site of the vector. The resultant plasmids were used to transform *Escherichia coli* HB101. Several clones containing in-frame constructs for each gene were selected by the dideoxynucleotide sequencing method [14]. Nucleotide sequences of the inserts of such in-frame constructs were determined by sequencing using primer walking or the subcloning of restriction fragments.

Expression and detection of gliadins and glutenins from *E. coli*. *E. coli* BL21 (DE3) cells harboring individual expression plasmids (pEW α for α -gliadin, pEW γ for γ -gliadin, pEW ω for ω -gliadin, pEWHx for HMM glutenin x-type, pEWHy for HMM glutenin y-type, pEWHyR for the repetitive domain of HMM glutenin y-type, pEWL1 and pEWL2 for major LMM glutenins and pEWL3 for a minor LMM glutenin) were cultured in 3 ml terrific broth medium containing 50 µg/ml carbenicillin for pET-21d or kanamycin for pET-24d. At $A_{600}=0.6$, expression was induced by the addition of isopropyl thio- β -D-galactopyranoside to 1 mM. After cultivation at 25°C for 24 h, cells were harvested by centrifugation. The cells were analyzed by SDS/PAGE using 11% (mass/vol.) acrylamide [15] as described previously [16]. Translational products were identified based on their expected sizes.

Partial purification of the expressed gliadins and glutenins. *E. coli* BL21(DE3) cells harboring individual expression plasmids from 300 ml medium cultured as described above were disrupted by sonication in 20 vol. 10 mM aluminium lactate, 0.1 M 2-mercaptoethanol with/without 2 M urea at 4°C. The expressed proteins were extracted by incubation at 25°C for overnight. The supernatant was neutralized and kept overnight at 4°C. The pellet after centrifugation was analyzed by SDS/PAGE and for allergenicity.

Determination of the N-terminal amino acid sequence. The N-terminal amino acid sequences of the individual expressed proteins were determined using a gas-phase protein sequencer (Applied Biosystems, Model 477A) according to the procedure of Matsudaira [17], as described previously [10, 11].

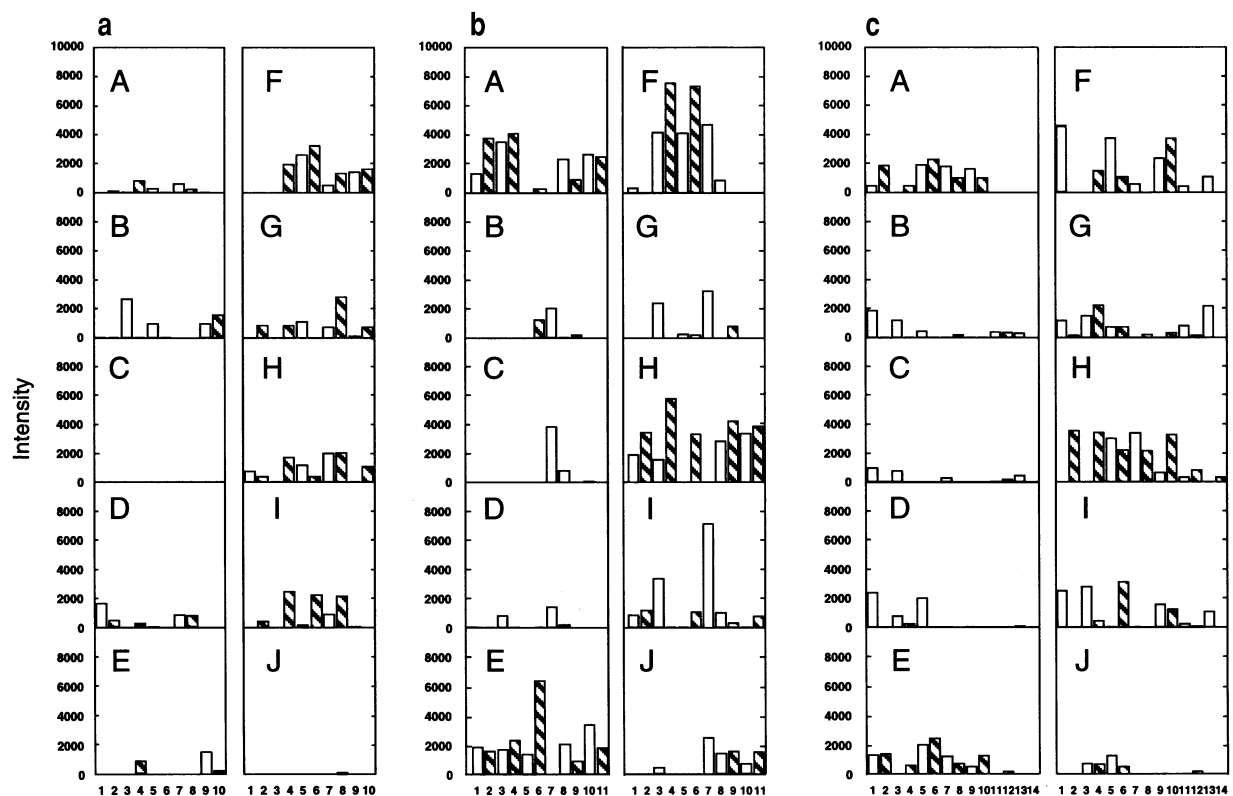
Immunoassay using the recombinant gliadins and glutenins. The pellets of partially purified recombinant gliadins and glutenins were dissolved in 10 mM aluminium lactate, 0.1 M 2-mercaptoethanol and 2 M urea (buffer A), or 0.1 M Tris/HCl pH 8.0, 8 M urea and 0.1 M 2-mercaptoethanol (buffer B) to give 1 µg/µl recombinant proteins. 2 µl each of the protein solutions was spotted on a nitrocellulose membrane. The proteins on the membrane were reacted with human sera and detected by a goat anti-(human IgE) conjugated to alkaline phosphatase as described above.

RESULTS

Allergenicity of wheat protein fractions. Wheat proteins were fractionated into salt-soluble, gliadin-rich and glutenin-rich fractions based on their solubility. To consider the effect of their structural characteristics on the reactivity with patients' sera, the fractionated proteins were dissolved in various solutions. As a native state, the salt-soluble fraction was dissolved in 1% NaCl, and the gliadin-rich and the glutenin-rich fractions were in 70% ethanol, 0.1 M acetic acid, 10 mM aluminium lactate with/with-

Table 2. Oligonucleotides used for the amplification of genes encoding wheat storage proteins. The genes coding wheat storage proteins were amplified by using the forward and reverse primers for each subunit.

Sequence (5'→3')	Primer
GTTAGAGTTCAGTGCCAC	α -gliadin forward
CGCGGATCCTTCTCAGTTAGTACCGAAG	α -gliadin reverse
AATATCCAGGTCGACCTAGCGCCAAGTAC	γ -gliadin forward
CGCGGATCCAGTTTTTCATTGGCCACCAATGCC	γ -gliadin reverse
GAAGGTGAGGCTCTGAGCAACTACAGTGTGAG	HMM glutenin forward
CGCGGATCCTATCACTGGCTGGCCGACAATGC	HMM glutenin reverse
GAGACCACGCCACTGCAGCAACTCCAACAAGG	HMM yRepeat forward
CGCGGATCCTATCACTGGCTGGCCGACAATGC	HMM yRepeat reverse
AGATGCATCCCTGGTTTGGAGAGACCATGGCAG	major LMM glutenin forward
CGCGGATCCTTATCAGTAGGCACCAACTCC	major LMM glutenin reverse
GCAAGTGCCGTTGCGCAAATTTTC	minor LMM glutenin forward
CGCGGATCCTTTTCTTATCAGTAGACACC	minor LMM glutenin reverse

**Fig. 1. Reactivities of wheat protein fractions with sera from patients.** The reactivities of the salt-soluble (a), glutenin-rich (b), and the gliadin-rich (c) fractions with sera from patients A–J were determined by densitometry. The fractionated proteins were dissolved in various solutions. (a) 1 and 2, 1% NaCl; 3 and 4, 0.1 M Tris/HCl pH 8.0, 2 M urea; 5 and 6, 0.1 M Tris/HCl pH 8.0, 4 M urea; 7 and 8, 0.1 M Tris/HCl pH 8.0, 8 M urea; 9 and 10, 2% (mass/vol.) SDS; 11 and 12, 10 mM aluminium lactate. (b) 1 and 2, 0.1 M Tris/HCl pH 8.0, 4 M urea; 3 and 4, 0.1 M Tris/HCl pH 8.0, 8 M urea; 5 and 6, 2% (mass/vol.) SDS; 7, 70% (by vol.) ethanol; 8 and 9, 0.1 M acetic acid; 10 and 11, 10 mM aluminium lactate. (c) 1 and 2, 0.1 M Tris/HCl, pH 8.0, 2 M urea; 3 and 4, 0.1 M Tris/HCl, pH 8.0, 4 M urea; 5 and 6, 0.1 M Tris/HCl, pH 8.0, 8 M urea; 7 and 8, 2% (mass/vol.) SDS; 9 and 10, 70% (by vol.) ethanol; 11 and 12, 0.1 M acetic acid; 13 and 14, 10 mM aluminium lactate. Open bars, 2-mercaptoethanol (0.1 M) was added; hatched bars, 2-mercaptoethanol not added.

out 2-mercaptoethanol. The glutenin-rich fraction dissolved in ethanol containing 2-mercaptoethanol was opalescent. As a denatured state, all fractions were dissolved in 2% SDS and 0.1 M Tris/HCl pH 8.0, containing urea. To attain various denatured states, the concentration of urea was changed. The solubility of the glutenin-rich fraction in 0.1 M Tris/HCl, 2 M urea without 2-mercaptoethanol was very low.

Reactivities of wheat protein fractions with patients' sera (Fig. 1) demonstrated that the reactivity of each fraction was variable among patients and among the reaction conditions. The

reactivity of the salt-soluble fraction was much lower in 1% NaCl than those under denaturing conditions (Fig. 1a). The reactivity of the glutenin-rich fraction dissolved in ethanol containing 2-mercaptoethanol was high with many patients examined here. However, it is likely that the high reactivity is due to incomplete solubility (opalescence) of the glutenin-rich fraction in this condition. If so, the reactivity of the glutenin-rich fraction was generally higher in the denatured state than in the native state, although sera of patients A, E and H also exhibited fairly high reactivities in the native state (Fig. 1b). The high reactivities

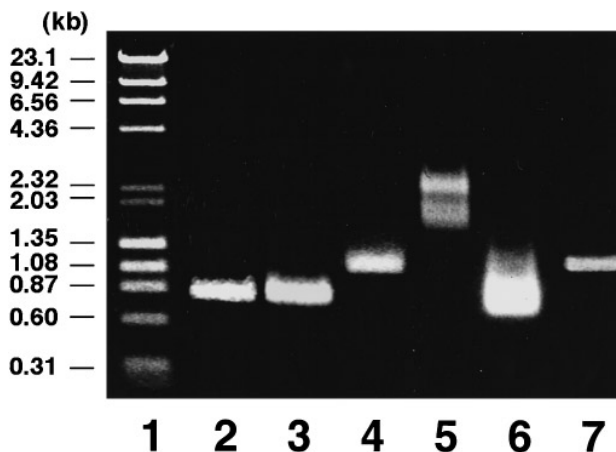


Fig. 2. Agarose gel electrophoresis of PCR products of the genes encoding wheat storage proteins. The analysis of PCR products were performed on a 1.2% agarose gel in Tris/acetate buffer. Lane 1, size standards; lane 2, α -gliadin; lane 3, γ -gliadin; lane 4, ω -gliadin; lane 5, HMM glutenin; lane 6, a major LMM glutenin; lane 7, a minor LMM glutenin. Numbers on the left indicate molecular sizes (bp).

were variable between the denaturants and their concentrations. The characteristics of the reactivity of the gliadin-rich fraction were very similar to that of the glutenin-rich fraction (Fig. 1c). The effect of 2-mercaptoethanol on the reactivity of each fraction was variable among the patients and the reaction conditions. Comparison of the reactivities of individual protein fractions in conjunction with the abundance ratio of each fraction in wheat seeds (salt-soluble/glutenin/gliadin = 1.5:4:4.5) [18] indicates that relative reactivity of each fraction can be generally ordered as follows: glutenin rich > gliadin rich $\gg$ salt soluble. This result is consistent with that obtained by Watanabe et al. [1].

Cloning and sequencing of genes encoding glutenins and gliadins. For identification of proteins responsible for the reactivities of the glutenin-rich and the gliadin-rich fractions, preparation of homogeneous proteins by expression of the corresponding DNA in *E. coli* is a powerful method. Since the cloned genes encoding glutenins and gliadins do not contain an intron [19], we attempted to clone genomic genes encoding glutenins and gliadins using PCR technology. For PCR reactions, we employed the primers listed in Table 2, which were designed based on the nucleotide sequences extracted from GenBank. Since Watanabe and coworkers [4] reported that Gln-Gln-Gln-Pro-Pro is an IgE-binding epitope, we designed primers for a major LMM glutenin containing a few Gln-Gln-Gln-Pro-Pro motifs and for a minor LMM glutenin containing many Gln-Gln-Gln-Pro-Pro motifs. The primers for HMM glutenin were common to x and y types.

PCR products of about 800 bp for α -gliadin, 800 bp for γ -gliadin, 1000 bp for ω -gliadin, 2300 bp and 1900 bp for x and y types of HMM glutenin, and 900 bp and 1100 bp for LMM glutenin, respectively, were obtained (Fig. 2). The resultant fragments were inserted into pET-21d or pET-24d to construct expression plasmids. Partial nucleotide sequences ($\approx$ 400 bp) of five clones for each construct were determined from the 5' ends, indicating that all clones for each construct were the same as each other, except clones for the major LMM glutenin. For the major LMM glutenin, two sequences were observed. Nucleotide sequences of one clone for α -, γ - and ω -gliadins, and a minor LMM glutenin (LMM glutenin 3), and two clones for a major LMM glutenin (LMM glutenins 1 and 2) were determined. More than 60% of nucleotide sequences of HMM-glutenin x-type

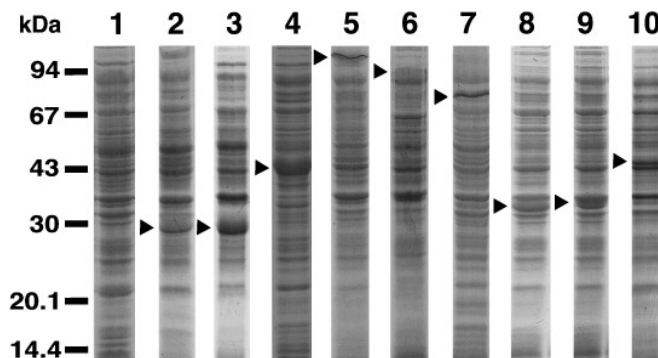


Fig. 3. SDS/PAGE analysis of the expressed proteins from individual expression plasmids. The *E. coli* strain BL21(DE3) cells harboring individual expression plasmids were analysed by SDS/PAGE in 11% gels. Lane 1, pET-21d; lane 2, pEW α ; lane 3, pEW γ ; lane 4, pEW ω ; lane 5, pEWHx; lane 6, pEWHy; lane 7, pEWHyR; lane 8, pEWL1; lane 9, pEWL2; lane 10, pEWL3. The numbers on the left denote molecular masses. The arrowheads indicate the positions of the expressed proteins.

(HMM-x) and y-type (HMM-y) genes were determined, indicating that HMM-x and HMM-y correspond to 1Ax2 and 1Dy10, respectively. The gene for ω -gliadin cloned here coded a species containing a repeat motif of PQQPFPQQ with a molecular mass of about 40 kDa. Each gliadin and glutenin species has a characteristic domain structure and a characteristic distribution of cysteine residues [6, 20]. The amino acid sequences deduced from the nucleotide sequences of individual gliadin and glutenin genes determined here satisfied all these characteristics. LMM glutenins 1 and 2 and LMM glutenin 3 contained 2 and 15 Gln-Gln-Gln-Pro-Pro motifs, respectively.

Expression and partial purification of recombinant gliadins and glutenins. Expression of gliadins and glutenins in *E. coli* BL21(DE3) cells harboring individual expression plasmids cultured in test tubes was assessed by SDS/PAGE (Fig. 3). The densitometric scanning of the gels indicates that the expression levels of α -, γ - and ω -gliadins, LMM glutenins and HMM-x were about 10–15% and about 5% of the total *E. coli* proteins, respectively, compared with the gels of the total proteins from the cells harboring the expression vector pET-21d alone. Since the expression level of HMM-y was very low, we attempted the expression of only the central repetitive domain of y-type (HMM-yRepeat) using the primers listed in Table 2 and obtained an expression level of about 5%. All gliadins and glutenins were expressed at such levels using pET-21d except LMM glutenin 3. pET-24d was suitable for LMM glutenin 3.

For large-scale culture, the expression levels of HMM glutenins were lowered to around 1–2%, but those of others did not vary. Extractions of expressed gliadins and glutenins from the cells were attempted with 70% ethanol. Although 80% ω -gliadin and 70% HMM-yRepeat were soluble, others were completely insoluble (data not shown). Addition of 2-mercaptoethanol (to 0.1 M) to 70% ethanol solubilized 60% α -gliadin, but not other protein. Then, we attempted to solubilize proteins using 10 mM aluminium lactate, 0.1 M 2-mercaptoethanol. α -Gliadin, ω -gliadin, HMM-x and HMM-yRepeat were almost completely soluble, but the other protein required the addition of 2 M urea for complete solubilization. All the recombinant gliadins and glutenins precipitated dominantly by neutralization, resulting in purities of about 90% (α -, γ - and ω -gliadins) (Fig. 4), 70% (LMM glutenins 1, 2 and 3), although the purities of HMM glutenins were low: about 10% (HMM-x) and 35% (HMM-yRepeat). The lower purity of HMM glutenins may be

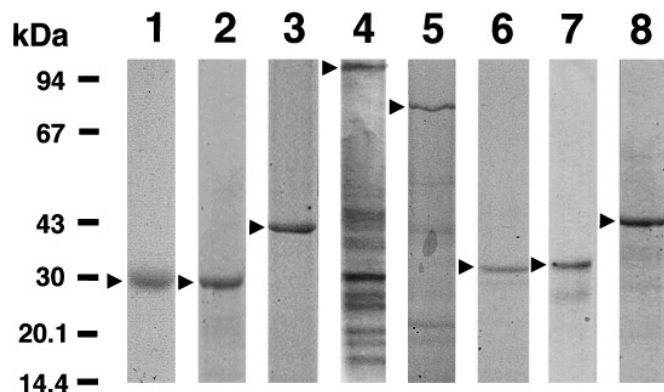


Fig. 4. SDS/PAGE analysis of the partially purified recombinant wheat storage proteins. The expressed wheat proteins were partially purified on the basis of their intrinsic solubilities and applied to SDS gels. Lane 1, α -gliadin; lane 2, γ -gliadin; lane 3, ω -gliadin; lane 4, HMM-x; lane 5, HMM-yRepeat; lane 6, LMM glutenin 1; lane 7, LMM glutenin 2; lane 8, LMM glutenin 3. The numbers on the left denote molecular masses. The arrowheads indicate the positions of the expressed proteins.

due to lower expression levels (1–2%) than the others (10–15%).

To verify the identities of the recombinant proteins, their N-terminal amino acid sequences were determined. The sequences determined coincided with those predicted from the nucleotide sequences of individual genes. The N-terminal methionine was retained in γ -gliadin, ω -gliadin, HMM-x, HMM-yRepeat and LMM glutenins 1 and 2, but cleaved in LMM glutenin 3. For α -gliadin, cleaved and non-cleaved forms were obtained. These phenomena are consistent with the proposal of Sherman et al. [21] that the specificity of methionine aminopeptidase is determined by the size of the side chain of the penultimate residue.

Allergenicity of recombinant gliadins and glutenins. Reactivities of recombinant gliadins and glutenins with patients' sera are shown in Fig. 5. Recombinant proteins were dissolved in buffer A or B. Five of ten patients (patients E, F, G, H and I) exhibited highest reactivity with LMM glutenin 3, which contains 15 Gln-Gln-Pro-Pro motifs, dissolved in buffer B. Patients D, G, H and I exhibited fairly high reactivity with α - and/or γ -gliadins (especially dissolved in buffer A), although all patients except H exhibited no reactivity with ω -gliadin. Generally reactivity of patients with LMM glutenins 1 and 2 was very low. Some patients exhibited reactivity with HMM glutenins. However, there is a possibility that the reactivity with HMM glutenin is an artifact, because the samples of HMM glutenin contained a large amount of *E. coli* proteins, and the reactivities of sera with *E. coli* proteins were also variable (data not shown). The expression system of HMM glutenins should be improved for the precise evaluation of their reactivity.

These results obtained here were consistent with those shown in Fig. 1 and those of Watanabe et al. [1], supporting the proposal by Watanabe et al. [2] and Tanabe et al. [4] that a major allergen of wheat is LMM glutenin containing many Gln-Gln-Gln-Pro-Pro motifs and suggesting that some patients' sera recognize α - and/or γ -gliadins as an allergen.

DISCUSSION

Characteristics of recombinant gliadins and glutenins. ω -Gliadin and HMM-yRepeat expressed in *E. coli* were pretty soluble in 70% ethanol. By the addition of 2-mercaptoethanol to 70%

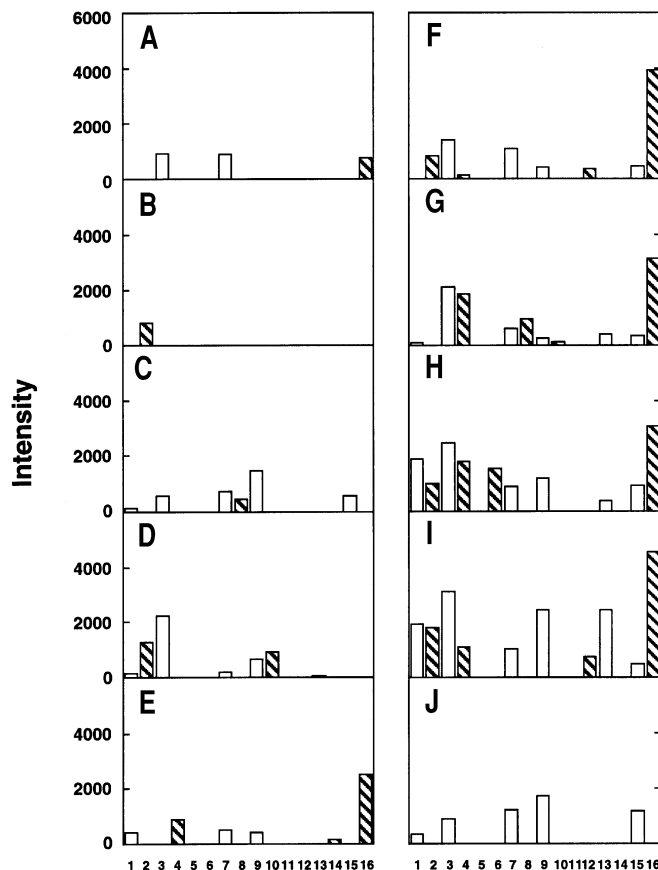


Fig. 5. Reactivities of recombinant gliadins and glutenins with patients' sera. The reactivities of α -gliadin (1 and 2), γ -gliadin (3 and 4), ω -gliadin (5 and 6), HMM-x (7 and 8), HMM-yRepeat (9 and 10), LMM glutenin 1 (11 and 12), LMM glutenin 2 (13 and 14) and LMM glutenin 3 (15 and 16) with sera from patients (A–J) were determined by densitometry. Open bars, 2-mercaptoethanol (0.1 M) was added; hatched bars, no 2-mercaptoethanol added.

ethanol, α -gliadin became fairly soluble. In addition, HMM-x were very soluble in 10 mM aluminium lactate containing 0.1 M 2-mercaptoethanol. γ -Gliadin and LMM glutenins 1, 2 and 3 required the presence of 2 M urea for solubilization. The differences in solubility in various solutions might be explained by the number and the topology of cysteine residues as follows. The number of cysteine residues in ω -gliadin, HMM-yRepeat, α -gliadin, HMM-x, γ -gliadin and LMM glutenins were 0, 2, 5, 6, 8 and 8, respectively. Thus, the number of cysteine residues is strongly correlated to the solubility in 70% ethanol and 10 mM aluminium lactate with/without 2-mercaptoethanol and 2 M urea. In this case, gliadins and glutenins containing many cysteine residues (i.e. γ -gliadin and LMM glutenins) should have mixed disulfide bonds, induced by the incorrect conformations, that are different from the inherent bonds. In contrast, ω -gliadin and HMM-yRepeat expressed in *E. coli* can form proper conformations. These results are fundamentally consistent with those obtained with C-hordein [22], γ -gliadin [23], LMM glutenin [24] and HMM glutenin [25]. The presence of 2 M urea might induce slight unfolding of γ -gliadin and LMM glutenins, resulting in the approach of 2-mercaptoethanol to disulfide bonds. These observations and speculations suggest that solubilities of gliadins and glutenins are related to conformation and are more critical in ethanol than in acid, and that the *E. coli* expression system is suitable for the investigation of the structural factors determining the characteristics of prolamins.

High-level expression of α -, γ - and ω -gliadins, LMM glutenins 1 and 2, HMM-x and HMM-yRepeat was attained by using pET-21d. However, LMM glutenin 3, which contains 15 Gln-Gln-Gln-Pro-Pro motifs, required pET-24d for high-level expression. We observed often such a phenomena. Although the reason is not clear, it is necessary to change the expression vector if high-level expression is not obtained with a certain expression vector.

Allergenicity of wheat proteins and the prospect for hypoallergenicity. Although the reactivity of fractionated proteins with sera from patients allergic to wheat was variable under different reaction conditions, it was generally higher under denaturing conditions than under non-denaturing conditions, especially those of glutenins and gliadins (Fig. 1). The effect of reducing agents was also variable under the different reaction conditions. These results suggest that allergenicity of wheat proteins should be evaluated in their denatured states and under several reaction conditions with and without reducing agents. This variability is probably due to a rigid structure of glutenins and gliadins. The observation that LMM glutenin 3 dissolved in buffer B exhibited higher reactivity than the protein in buffer A is consistent with the proposal above.

The variability of the reactivities of fractionated proteins with sera from patients allergic to wheat among patients and under different reaction conditions (Fig. 1) indicates that the type, the number and the epitope of allergens are variable among patients, although LMM glutenin 3, which contains 15 Gln-Gln-Gln-Pro-Pro motifs is probably the major allergen. Therefore, methods for developing hypoallergenic wheat products should not be limited to only a certain protein. From this view point, the method using proteases developed by Watanabe et al. [1, 26] and Tanabe et al. [3] is excellent. Although these authors succeeded in developing hypoallergenic noodles and bread [1, 3, 26], flour treated with protease must differ from the original flour. Gliadins and glutenins are important for the formation of a continuous proteinaceous network (gluten), conferring the unique physicochemical properties such as elasticity and viscous flow to doughs [20]. The structural characteristic (β -spiral) of HMM glutenin may be important for bread-making quality [27]. Therefore, developing an alternative method is desired.

Genetic engineering could be an alternative method. Recently, reduction of the amount of 14–16-kDa allergenic proteins of rice by an antisense RNA method was successful [28]. The reduction of LMM glutenins containing many Gln-Gln-Gln-Pro-Pro motifs can be achieved similarly without disturbing functionality of gluten, because such LMM glutenins are minor components and are not known to contribute to functionality. In addition, reduction of allergenicity of LMM glutenin by altering their primary structures is possible. However, complete reduction of allergenicity of wheat is not attained only by reduction of the amount of such LMM glutenins. Since some patients exhibit reactivities to α -gliadin and/or γ -gliadin, reduction of allergenicity of these gliadins is also required. Complete reduction of these gliadins by antisense RNA method is not desired, because these gliadins are major components and important for extensibility [19]. It is necessary to elucidate epitopes of α -gliadin and γ -gliadin using the *E. coli* expression system established here, and to reduce their reactivities with patients' sera by alteration of their primary structures.

The Gln-Gln-Gln-Pro-Pro peptide constitutes an IgE-binding epitope [4]. However, it is known that the sequences Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro are common for coeliac-active gliadin peptides [29]. The motif Gln-Gln-Gln-Pro is contained in the IgE-binding epitope Gln-Gln-Gln-Pro-Pro in LMM glutenins. The reason LMM glutenins contain many Gln-Gln-Gln-

Pro-Pro motifs but do not participate in coeliac disease is unclear. The recombinant system established here is suitable for investigation of these points.

This work was supported in part by grants from Program for Promotion of Basic Research Activities for Innovative Biosciences (to S. U.) and The Iijima Memorial Foundation of Food Science and Technology (to T. S. and S. U.).

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