



Isolation and Molecular Cloning of a Major Wheat Allergen, Tri a Bd 27K

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Received July 14, 2008; Accepted August 29, 2008; Online Publication, January 7, 2009

[doi:10.1271/bbb.80485]

Tri a Bd 27K is the predominant allergen in wheat. In the present study, this allergen was purified to homogeneity from wheat flour. The N-terminal amino acid sequences of the purified allergen and the peptides obtained by its digestion, with trypsin were determined, and the allergen was shown to be a glycoprotein with an Asn-linked sugar moiety containing fucose residues.

A cDNA encoding the allergen was obtained by polymerase chain reaction (PCR). The cDNA codes for a protein of 203 amino acid residues, with a molecular mass of 22,803 Da, that has two tentative sites glycosylated at Asn residues. Homology analysis suggested that the allergen might belong to a family of γ -interferon-inducible thiol reductases. The cDNA was expressed as a fusion protein with glutathione S-transferase in *Escherichia coli*. However, unlike the allergen purified from wheat, recombinant Tri a Bd 27K was not immunoblotted with IgE antibodies in the serum of a wheat-sensitive patient.

Key words: wheat; allergen; Tri a Bd 27K; cDNA cloning; N-linked glycan moiety

Wheat is the most widely consumed crop in the world, but it is a representative allergenic food. Currently the number of patients suffering from food allergy is increasing, and, particularly, wheat allergy is considered to be a serious problem globally.

In the Western European countries, bakers' asthma, an immediate-type allergic response to the inhalation of cereal flours, is an important occupational disease among persons working in the baking and milling industries. Most of the bakers with asthma have IgE antibodies specific to wheat or rye flour,^{1,2)} and the components of these flours are considered to be the most relevant allergens. Investigation of the allergens responsible for this inhalation-induced hypersensitivity has been carried out. The major allergens responsible for bakers' asthma, contained in the salt-soluble fraction of wheat, have been found to be α -amylase inhibitors.^{3,4)} Weiss *et al.*,⁵⁾ Posch *et al.*,⁶⁾ and Sander *et al.*⁷⁾ found that other allergens connected with bakers' asthma occur in the water/salt-soluble fraction (albumins and globulins)

of wheat proteins. Nine allergens with molecular masses of 14–18, 26–28, and 35 kDa, were identified as α -amylase/protease inhibitors, acyl-CoA oxidase and fructose-bisphosphate aldolases from wheat, barley, maize, and rice by two-dimensional gel electrophoresis, immunoblotting, and N-terminal amino acid sequencing.^{5,6)} Lipid transfer proteins (LTPs) have been reported to be important allergens in maize⁸⁾ and barley,⁹⁾ and Pastorello *et al.*¹⁰⁾ recently found that LTP is a new allergen occurring in the water/salt-soluble fraction of wheat.

The proteins in the salt-insoluble fraction (gliadins and glutenins) of wheat flour have been found to be recognized by IgE antibodies in the sera of patients with bakers' asthma.^{11–13)} Maruyama *et al.*¹⁴⁾ elucidated by means of the *Escherichia coli* expression system that low-molecular weight glutenin, α -gliadin and γ -gliadin are other relevant allergens in wheat. They found that low-molecular mass glutenin containing many QQQPP motifs, which had been identified to be IgE-binding epitope,¹⁵⁾ exhibited the highest reactivity. The gluten fraction of wheat, composed of gliadins and glutenins, is also known to be responsible for celiac disease, which is considered not to be connected with food allergy. Furthermore, ω -gliadins have been found to be involved in IgE-mediated reactions to ingested wheat in wheat-dependent exercise-induced anaphylaxis.^{16,17)}

Recently, in Japan, patients suffering from allergic responses to wheat products have increased in number as the traditional eating habits of Japanese has changed to a Westernized style. In order to reveal the overall view of the allergenicity of wheat, we fractionated the proteins in wheat and examined in detail the IgE-binding proteins in each fraction using immunoblotting with the sera of 30 wheat-sensitive patients.^{18,19)} Fifteen allergens were identified and, among them, Tri a Bd 17K, Tri a Bd 27K, and Tri a Bd 36K were found to be major allergens.¹⁸⁾ Tri a Bd 17K was identified as α -amylase inhibitors, CM16 and CM17, with Asn-linked sugar chains.¹⁸⁾ Tri a Bd 36K has peroxidase activity and is highly homologous with barley peroxidase BP1,²⁰⁾ which indicates that Tri a Bd 36K is a peroxidase.²¹⁾ Sanchez-Monge *et al.*²²⁾ reported this allergen

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Abbreviations: AAL, *Aleuria aurantia* lectin; Con A, concanavalin A; GST, glutathione S-transferase; IPTG, isopropyl- β -thio-D-galactopyranoside; LB, Luria-Bertani; LTP, lipid transfer protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; rTri a Bd 27K, recombinant Tri a Bd 27K; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

previously, but it has not been characterized well. Tri a Bd 27K, with a molecular mass of 27 kDa, is the predominant allergen of the above-mentioned allergens in wheat.¹⁸⁾ This allergen is likely to be identical with the allergenic protein that was reported to be detected on a two-dimensional gel electrophoresis by Posch *et al.*⁶⁾ Although they determined the 13 N-terminal amino acid residues of the allergen,⁶⁾ further investigation of the allergen has never been done. Hence, it is important that the allergen be well characterized.

In the present paper, we describe the isolation, characterization and molecular cloning of Tri a Bd 27K. The expression and allergenicity of a recombinant protein for the allergen as a fusion protein with glutathione *S*-transferase in *Escherichia coli* are also reported.

Materials and Methods

Materials. The materials used were obtained as follows: Alkphos direct labeling reagents, oligonucleotide primers, Q-Sepharose 4B, phenyl-Sepharose CL-4B, PBE 94 and Polybuffer 74 (Pharmacia-Amersham, Uppsala, Sweden); Amicon PM10 membrane (Millipore, Co., USA); Biotin-Lectin Set and G.P.SENSOR kit (Honen, Tokyo); and nitrocellulose and PVDF membranes (Bio-Rad, CA).

Isolation of Tri a Bd 27K. Wheat flour was the gift of Okumoto Flour (Osaka), where wheat grains (*Triticum aestivum* L., c.v. Chinese Spring) were blended and powdered. The albumin fraction of the wheat proteins was prepared from wheat flour (450 g), as described previously.²¹⁾ The albumin proteins were fractionated with solid ammonium sulfate. The precipitate formed between 30–70% ammonium sulfate was centrifuged, dissolved in 20 mM Tris–HCl buffer, pH 8.0 (buffer A), and dialyzed overnight at 4 °C against the same buffer. The dialysate was applied to a Q-Sepharose 4B column (2.5 × 24 cm), and then after washing with buffer A, the proteins on the column were eluted with a linear gradient of 0–0.3 M sodium chloride in the same buffer. The allergen-containing fractions, in which the allergen was confirmed to occur by SDS–PAGE and immunoblotting with a patient's serum, were combined, concentrated by ultrafiltration with an Amicon PM10 membrane, and dialyzed against 10 mM imidazole–HCl buffer (pH 7.0). The dialysate was applied to a PBE 94 column (1.0 × 30 cm) equilibrated with the above imidazole–HCl buffer. The proteins adsorbed on the column were eluted with Polybuffer 74 (pH 4.0). The fractions containing the allergen were pooled and concentrated by the ultrafiltration as described above. Solid ammonium sulfate was added to the concentrate to a final concentration of 1.5 M, and the solution was applied to a phenyl-Sepharose CL-4B column (1.0 × 9 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.4) containing 1.5 M ammonium sulfate. The column was washed with the same buffer, and the proteins were eluted with a linear gradient of 1.5–0 M ammonium sulfate in 10 mM sodium phosphate buffer (pH 7.4). Tri a Bd 27K was eluted with 10 mM sodium phosphate buffer (pH 7.4). The protein concentration was determined with a Protein Assay kit (Bio-Rad, CA.) using bovine serum albumin as the standard protein.

Patient's sera. The serum of a patient (patient 1) sensitive to wheat (RAST class 4) was obtained from International Enzyme (CA). The sera of two wheat-allergic patients with RAST values of 3 and 1 (patients 2 and 3) were received from National Hospital Organization at the Minami-Okayama Medical Center (Okayama). The patient's sera were confirmed to recognize the above-mentioned major allergens and were used in subsequent experiments. The experiments were done in accordance with ethical standards of the Helsinki Declaration.

SDS–PAGE and immunoblotting. Protein samples were analyzed by SDS–PAGE on a 12% polyacrylamide gel under reducing conditions by the method of Laemmli.²³⁾ For immunostaining, the proteins separated on the gel were electroblotted onto a nitrocellulose membrane. The proteins on the membrane were stained with

Ponceau S. After destaining with 1% acetic acid and washing with 10 mM NaOH, the membrane was incubated overnight with the patient's sera (diluted 1:5), and the IgE antibodies bound to the allergen were reacted with biotin-labeled anti-human IgE antibody (diluted 1:1000) (Seikagaku Kogyo, Tokyo), and then with horseradish peroxidase-labeled streptavidin (diluted 1:5000, Roche Diagnostics, Tokyo). The immunocomplexes on the membrane were detected in the manner described in the previous paper.²⁴⁾

Carbohydrate analysis of Tri a Bd 27K. The purified Tri a Bd 27K was subjected to SDS–PAGE, and then the allergen on the gel was electroblotted on a nitrocellulose membrane. In order to detect its glycan moiety, the membrane was divided into several strips, and one of the strips was subjected to the PAS test using a G.P.Sensor kit. Briefly, the strips were blocked with 10 mM Tris–HCl-buffered saline (pH 7.4) containing 0.05% Tween 20 for 10 min at room temperature and washed gently with water. Then, after the protein on the strip was reacted with 10 mM NaIO₄, the strip was washed with 10 mM phosphate-buffered saline, pH 7.4 (PBS), and incubated with biotin-labeled hydrazide diluted with PBS. The strip was incubated with horseradish peroxidase (HRP)-labeled streptavidin. After washing, the avidin-biotin complex on the strip was visualized with 0.75% 4-chloro-1-naphthol and 0.3% H₂O₂ in 50 mM Tris–HCl buffer (pH 7.3).

The sugar compounds in the carbohydrate moieties were examined with various biotin-labeled lectins.

Amino acid sequencing of Tri a Bd 27K. The purified allergen was subjected to SDS–PAGE to determine its N-terminal amino acid sequence. The allergen on the gel was electroblotted on a PVDF membrane, and then the N-terminal amino acid sequence of the allergen on the membrane was determined by automated Edman degradation with a Hewlett Packard G1005A Protein Sequencing System (Palo Alto, CA). In order to elucidate the internal sequences of the protein, the purified Tri a Bd 27K in the gel was digested with trypsin. The resulting peptides were isolated by reversed-phase HPLC as described previously,²⁴⁾ and their amino acid sequences were determined.

Preparation of mRNA and cDNA synthesis. Developing cotyledons of wheat (*Triticum aestivum*) were obtained from Research Institute for Bioresources, Okayama University. Total RNA was prepared from the developing wheat cotyledons. The cotyledon samples were ground to powder in a mortar filled with liquid nitrogen and homogenized in an extraction buffer containing vanadyl ribonucleoside complexes (Invitrogen, CA) as ribonuclease inhibitors and 5% SDS. The extract obtained by 10-min centrifugation at 4 °C was mixed vigorously with an equal volume of phenol/chloroform and centrifuged. The crude RNA in the aqueous solution was obtained as a pellet by precipitation with ethanol. These treatments were repeated several times to yield total RNA, and then mRNA was purified from the total RNA using a mRNA Isolation kit (Roche Diagnostics, Tokyo) according to the manufacture's instructions.

In order to confirm the occurrence of the mRNA for Tri a Bd 27K, Northern-blot analysis was carried out with the above mRNA fraction in the manner described previously.²⁵⁾

3'-RACE- and 5'-RACE-ready first-strand cDNAs were synthesized using 1.0 µg of mRNA with a SMART™ RACE cDNA amplification kit (Clontech Laboratories, CA) according to the user's manual.

Polymerase chain reaction (PCR)-based cloning of Tri a Bd 27K cDNA. The amino acid sequences (Table 1) of the peptides obtained by Edman degradation were used in the preparation of three sense degenerate sequences (TR1, TR2, TR3) and two antisense degenerate sequences (TR4 and TR5). The sequences for the primers are shown in Table 2. The first-strand cDNA as a template was subjected to PCR amplification, and after purification of the fragment obtained, the DNA fragment was inserted into pGEM-T Easy vector included in a TA cloning kit (Promega, Madison, WI) and cloned. Based on the sequence of the DNA fragment, two oligonucleotides, the sense sequence for IYYESLCPYS (TR6, 5'-ATCTACTACGAGTCGCTGT-GCCCCCTACT-3') and the antisense sequence for YGRQTAE LVP (TR7, 5'-GCACGAGCTCAGCTGTCTGTCTCCCAT-3'), were synthesized. PCR amplification of the 3'-end of the cDNA coding

Expression of a recombinant Tri a Bd 27K in E. coli. The cDNA encoding the mature Tri a Bd 27K protein was amplified by PCR using the primers with the *Bam*H I and *Eco*R I sites. TR8 (5'-AGCCTAGGATCCG-GGGATGTGGCAACGGGCA-3') and TR9 (5'-TCATCAGAATTCCGATCAAAAT-CGGCCGTACACATC-3') were used as the sense sequence for GDVATGR and as antisense sequence for DVGRL. The PCR product was cloned into pGEM-T Easy vector in the manner described above. After confirming the sequence of the clone obtained, the inserted cDNA fragment was digested with *Bam*H I and *Eco*R I and isolated by electrophoresis on an agarose gel. The fragment isolated was ligated into pGEX-6P-1 expression vector to construct expression vector pGEX/Tri 27. In order to obtain a fusion protein of Tri a Bd 27K with glutathione *S*-transferase (GST), a recombinant plasmid, pGEX/Tri 27, was transformed in *E. coli* BL21, and clones producing the recombinant Tri a Bd 27K (rTri a Bd 27K) were examined according to the method described previously.²⁶⁾

The N-terminal amino acid sequence of the purified Tri a Bd 27K was determined by Edman degradation. In order to obtain further information on the internal amino

Peptide	Sequence
(A) Tri a Bd 27K	¹ GDVATGRGSEK ¹⁰ VHVAIYYESLC ²⁰ PY SVRFV ³⁰ A---
(B) Peptide 1	¹ FVANHL ⁸ FK
(C) Peptide 2	¹ QTAELVPPHQFV ¹² ---
(D) Fragment I	¹ GDVATGRGSEK ³⁵ VHVAIYYESLC ³⁵ PY SVRFVANHLFK
[(A) + (B)]	

Peptide	Primer sequence	Primer
<i>Fragment 1</i>		
¹ GDVATGR ⁷ sense	5'-GGN*GA(T/C)GTI**GCN*ACI** GGN*(A/C)G-3'	TR1
⁸ GSEKVHV ¹⁴ sense	5'-GGN*(T/A)(C/G)N*GA(A/G)AA(A/G)GTI**CA(T/C)GT-3'	TR2
¹⁰ EKVHVAI ¹⁶ sense	5'-GA(A/G)AA(A/G)GTI**CA(T/C)GTN*GCN*AT-3'	TR3
²⁹ VANHLFK ³⁵ antisense	5'-TT(A/G)AAN*A(A/G)(A/G)TG(A/G)TTI**GCN*AC-3'	TR4
<i>Peptide 2</i>		
⁶ VPPHQFV ¹² antisense	5'-AC(A/G)AA(T/C)TG(A/G)TGI**GGN*GCN*AC-3'	TR5

N*, A/C/G/T; I**, Inosine

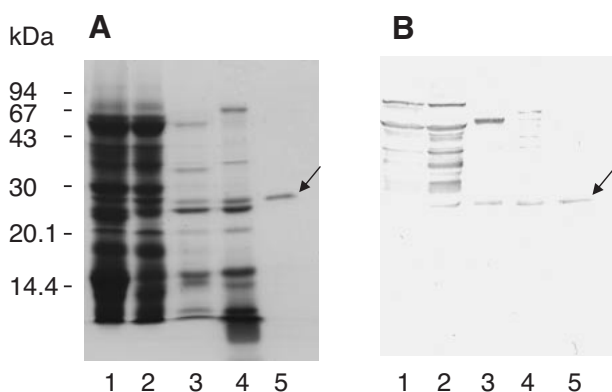


Fig. 1. SDS-PAGE and Immunoblot of Tri a Bd 27K Obtained from Wheat Flour at the Various Purification Steps.

The proteins obtained at each step were electrophoresed on a 15% polyacrylamide gel. The proteins on the gel were electroblotted onto a nitrocellulose membrane. The membrane was stained with Ponceau S (A), and after destaining as described in the text, it was immunoblotted with the serum of wheat-sensitive patient (B). Lane 1, albumin fraction; lane 2, proteins in the precipitate formed between 30% and 70% saturation with solid ammonium sulfate; lane 3, proteins obtained by Q-Sepharose chromatography; lane 4, proteins obtained by chromatofocusing; lane 5, the allergen purified by phenyl-Sepharose chromatography. The arrows in lane 5 show the position corresponding to Tri a Bd 27K.

acid sequence of the allergen, the purified allergen was digested with trypsin. Two peptides (1 and 2) were isolated by reversed-phase HPLC, and their N-terminal amino acid sequences were analyzed as described above. The N-terminal amino acid sequences of the allergen and its two peptides, obtained from the allergen, are summarized in Table 1. The amino acid sequence of peptide 1 was found to partly overlap the N-terminal sequence of Tri a Bd 27K, indicating that fragment I was composed of the 35 N-terminal amino acid residues.

Characterization of Tri a Bd 27K

After SDS-PAGE and electroblotting on a nitrocellulose membrane, the membrane was divided into several strips. The 27-kDa allergenic protein on the strip was examined with regard to its glycan moiety using the PAS test. The protein was found to be positive to the PAS test (Fig. 2C), suggesting that a glycan moiety is linked with it. The sugar compounds in the glycan moiety of Tri a Bd 27K on the strips were examined with biotin-conjugated lectins. As shown in Fig. 2D, the glycan moiety of the 27-kDa protein reacted with lectins Con A and AAL (*Aleuria aurantia*), indicating that the allergen has a glycan moiety containing D-mannose- and L-fucose residues.

cDNA cloning and deduced amino acid sequence of Tri a Bd 27K

Based on the amino acid sequences of the N-terminal peptide of Tri a Bd 27K and its tryptic peptides, several oligonucleotides were synthesized and used as primers in the amplification of cDNA fragments encoding the allergen. A cDNA fragment with a nucleotide sequence of 461 bp was amplified by PCR with the first-strand cDNA as the template, which was prepared using 3'-RACE and primers TR1 and TR5. Northern-blot analysis with the 461-bp DNA fragment as a probe indicated that the mRNA for Tri a Bd 27K had a nucleotide sequence of approximately 1.0 kb (Fig. 3). When the first-strand cDNA as a template was amplified by PCR

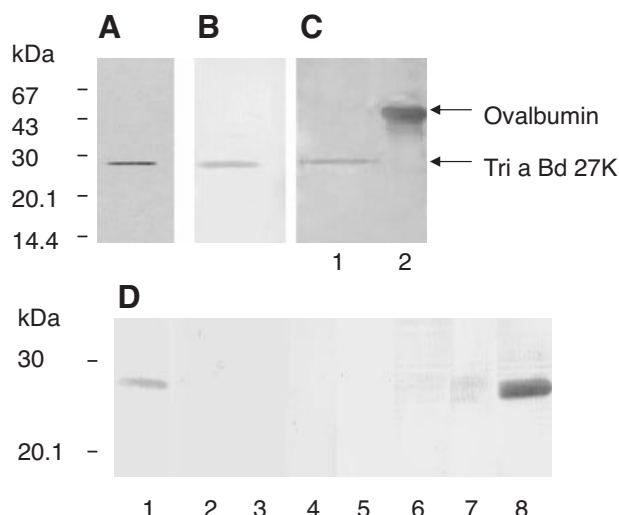


Fig. 2. Analyses of the Sugar Compounds in the Glycan Moiety of the Purified Allergen by the PAS Test and Staining with Various Lectins.

Purified Tri a Bd 27K was electrophoresed on a 15% polyacrylamide gel. The gel was subjected to electroblotting onto a nitrocellulose membrane and the membrane was cut into several strips. One of the strips was stained with Ponceau S (A). Others were immunoblotted with the serum of the wheat-sensitive patient (B), or stained with G.P.SENSOR kit (C) or with various lectins (D). In panel C, lanes 1 and 2 represent the results of the PAS test with the purified allergen and with ovalbumin used as a standard glycoprotein respectively. The figures in panel D express reactivity to the different lectins used: Lane 1, Con A; lane 2, *Pisum sativum* agglutinin; lane 3, *Lens culinaris* agglutinin; lane 4, phytohemagglutinin-E; lane 5, *Arachis hypogaea* agglutinin; lane 6, *Ricinus communis* agglutinin; lane 7, *Ulex europaeus* lectin; lane 8, AAL.

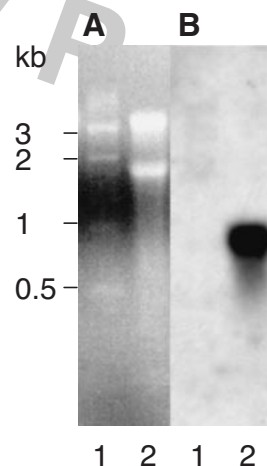


Fig. 3. Northern-Blot Analysis of the mRNA for Tri a Bd 27K.

mRNA (2.9 μ g) obtained from the developing cotyledons of wheat was electrophoresed on a denaturing 1.4% agarose gel. The mRNA on the gel was visualized by staining with ethidium bromide (A), after which the mRNA was transferred to a nylon membrane. The mRNA on the membrane was hybridized overnight with the AP-labeled probe and visualized as described in the text (B). Lane 1, RNA ladder as a standard marker; lane 2, mRNA prepared from cotyledons of wheat.

with a 5'-RACE kit, no PCR-product was obtained. This indicates that the nucleotide sequence with the open reading frame coding for the whole amino acid sequence of Tri a Bd 27K might be difficult to be determined. However, when PCRs with a 3'-RACE kit, 3'-RACE ready cDNA as a template and two pairs of primers (TR6/UPM and TR1/TR7) were done, two DNA fragments with nucleotide sequences of 750 bp and

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1  GGGGATGTGGCAACGGGACAGGCGAGGAGGTGCACGTGGCAATCTACTACGAGTCGCTGTGCCCTTAC
   G D V A T G R G S E K V H V A I Y Y E S L C P Y
73  TCGGTCCGCTTCGTGGCGAACCCACCTCTTCAAGGCCTACAGGACGGCCTGCTCGACGCCGCAATCTCACC
   S V R F V A N H L F K A Y R D G L L D A A N L T
145 CTGCTCCCTATGGCAACGCGTGGTCCGCAATGACGGCACCATCTCTGTGTCAGCATGGCCCCGAGGAATGC
   L V P Y G N A V V R N D G T I S C Q H G P E E C
217 CTTCTCAACACCGTGGAGGCTTGCAGCATCGACGCTGGCCGACGTGAAGGTGCATCTCGGCTTCATTATAC
   L L N T V E A C A I D A W P D V K V H L G F I Y
289 TGCCTGTGCGACCTGGTGTGAAGAACAAGCACCGGGAGTGGGAGTCGTCTCCAGAAACGGGGCTTGAC
   C V S D L V L K N K H R E W E S C F Q K Q G L D
361 CCAAGCCAGTCACGGAGTGTACAAGGGCGAGCGTGGCCACAATCTATCGCTCGAGTATGGGAGACAGACA
   P K P V T E C Y K G E R G H N L S L E Y G R Q T
433 GCTGAGCTCGTGCCTCCTCACCAGTTGCTTCCATGGGTGGTGGTCGACGGCAAGCCGCTCTACAACGATTAC
   A E L V P P H Q F V P W V V V D G K P L Y N D Y
505 GGGAACTTCAAAGCTTACATCTGCAAGGCTACAAGGGTATCCCTCTCGAGGCGTGTGCAAGCCCTGGGC
   G N F K A Y I C K A Y K G Y P L L E A C R S L G
577 CTGGAGGCACACGACGATGTGTACGGCCGACTTTGATCGTGATGATGATGATGCCATCGCCAAGATTAGAT
   L E A H D D V Y G R L ***
649 GGTCCTGCCGGATGGTGATAATAATGATTGATAAATTTATGTTCTCTCGGGGATCATACATCTGAACATA
721 GGGCAGTTCAATCTCTCTGTTAAGGAATTTAAATCAAGTGGTTGTGCTCTAAAAA
793 AAAAAA798

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Fig. 4. Nucleotide Sequence of cDNA Encoding the Allergen and Its Deduced Amino Acid Sequence.

The deduced amino acid sequence is shown under the nucleotide sequence of the cDNA. Underlining of the amino acid sequence represents the amino acid residues of the purified allergen as determined by automated Edman degradation. Asterisks show the stop codon of the cDNA. Two arrows express the putative asparagine residues to be glycosylated.

450 bp, were amplified (Fig. 4). By combination of the two sets of sequence data obtained from the plasmids with the two DNA fragments, a 798-bp cDNA was established (Fig. 4). The nucleotide sequence established has been registered accession no. AB085212. As shown in Fig. 4, the cDNA was found to consist of a 609-bp open reading frame and a termination codon at 610, followed by a 158-bp 3'-untranslated region and a 28-bp polyadenylation tail. The open reading frame was found to encode a polypeptide with a molecular mass of 22,803 Da, composed of 203 amino acids. The chemically determined amino acid sequences of the peptides obtained from the allergen were shown to be contained in the amino acid sequence deduced from the cDNA. Possible glycosylation sites (NXS/T) were found at positions Asn66 and Asn135. This finding is compatible with the above observation obtained with the G.P.SENSOR kit, ConA, and AAL, but which site of the allergen is glycosylated remains unknown. The pI value of Tri a Bd 27K was calculated from the amino acid sequence to be 6.05.

We surveyed proteins homologous with the deduced amino acid sequence of the cDNA in the SWISSPROT data base using FASTA. The amino acid sequence of the allergen, deduced from the cDNA cloned, was shown to exhibit high homology with a number of proteins. Most of the homologous proteins are those deduced from the DNA sequences obtained by genomic analysis, which have not been reported to occur in any organism. Of the homologous proteins, putative uncharacterized proteins such as the γ -interferon-inducible lysosomal thiol reductase family protein in *Oryza sativa* ssp. japonica,²⁷⁾ the ARATH F10K1.21 protein in *Arabidopsis thaliana* (accession no. AC067971), and the protein²⁸⁾ which has been found in humans and identified as a functional protein are aligned with the allergen, which exhibits high homology (61.2%, 52.9%, and 37.7% identity, respectively) with these deduced amino acid sequences (Fig. 5).

Expression and purification of rTri a Bd 27K

An expression vector, pGEX/Tri 27, was constructed to express cDNA encoding Tri a Bd 27K in *E. coli* under the control of the *lac* promoter. The nucleotide sequence of the cDNA inserted into the expression vector was confirmed to be identical with that of the

original cDNA by nucleotide sequencing. *E. coli* BL21 cells with the vector were cultured as described in "Materials and Methods." When *E. coli* BL21, harboring the expression vector, was cultured in the absence of IPTG, no fusion protein of the 27-kDa protein with GST with a molecular mass of 26 kDa was detected by SDS-PAGE followed by staining with Ponceau S, but the fusion protein was observed to be expressed when *E. coli* was cultured in the presence of 0.1 mM IPTG (Fig. 6).

rTri a Bd 27K as the GST fusion protein was estimated to account for about 10% of the total protein in the *E. coli* cells expressing Tri a Bd 27K (Fig. 6, lane 1), but a large portion of the fusion protein was shown to be expressed in inclusion bodies. Hence, we isolated the inclusion bodies from *E. coli* lysate and they were used in the following experiments as purified rTri a Bd 27K. The rTri a Bd 27K obtained was subjected to SDS-PAGE and to immunoblotting with the serum of patient 1 (Fig. 7). As shown in Fig. 7B, Tri a Bd 27K partially purified from wheat flour was clearly immunoblotted with sera of three patients sensitive to wheat, whereas the rTri a Bd 27K isolated from *E. coli* was not detected with these sera. Of the sera, the serum of patient 1 was confirmed to recognize the sugar moieties of the two allergens in soybean, Gly m Bd 28K²⁹⁾ and the 23-kDa peptide.²⁵⁾

Discussion

We have found out 15 allergens in wheat by immunoblotting with the sera of 30 patients sensitive to wheat, and among them, Tri a Bd 27K was shown to be the predominant allergen.¹⁸⁾ In the present study, this allergen was purified from wheat flour by various chromatographies, and the 35 N-terminal and 12 internal amino acid residues of the allergen were determined. Posch *et al.*⁶⁾ reported the occurrence of a new allergen with a molecular mass of 27 kDa by two-dimensional PAGE, and its N-terminal amino acid sequence consisting of 15 amino acid residues. The sequence of the 27-kDa protein revealed by Posch *et al.* was shown to be included in that of Tri a Bd 27K as described above, suggesting that Tri a Bd 27K is identical to the allergen detected by Posch *et al.*⁶⁾ By the PAS test and glycan analysis, the allergen was found to be a glycoprotein

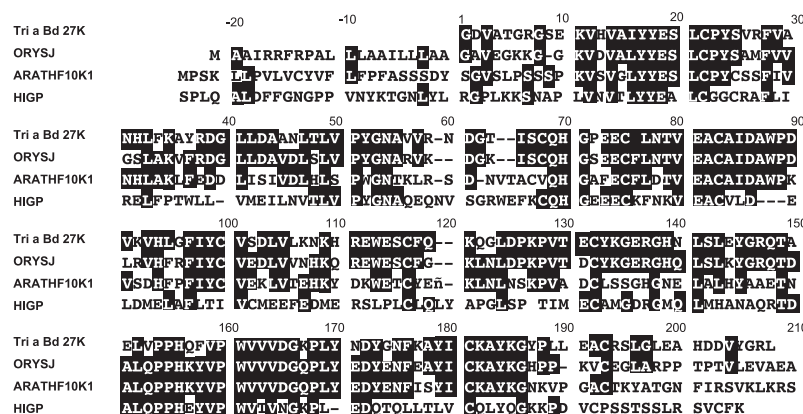


Fig. 5. Alignment of the Deduced Amino Acid Sequence of Tri a Bd 27K with Those of the Three Homologous Proteins.

Shaded amino acid residues express amino acid residues identical between two or three of the four proteins or all of the proteins. The sequences of the γ -interferon-inducible lysosomal thiol reductase family protein (ORYSJ), the ARATH F10K1.21 protein (ARATHF10K1), and human γ -interferon-inducible thiol reductase (HIGP) were from *Oryza sativa* ssp. japonica²⁶⁾ *Arabidopsis thaliana* (accession no. AC067971), and *Homo sapiens*,²⁷⁾ respectively.

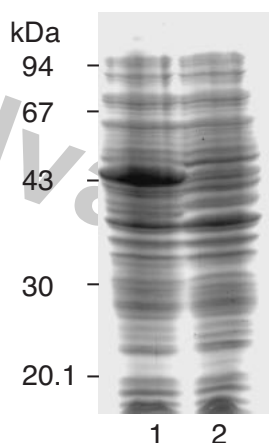


Fig. 6. SDS-PAGE of rTri a Bd 27K Expressed as a Fusion Protein with GST in *E. coli* BL21.

E. coli BL21 harboring expression vector pGEX/Tri 27 was cultured for 3 h in the absence of IPTG, as described in the text, after which a further 7-h incubation of the bacteria in the presence of IPTG was done. Bacteria cultured without and with IPTG were collected by a 15-min centrifugation at $10,000 \times g$, and the pellet was suspended in the sample buffer for SDS-PAGE and heated for 10 min at 100°C . After centrifugation, the supernatant was subjected to SDS-PAGE. Lane 1, lysate obtained from bacteria cultured in the presence of IPTG; lane 2, lysate obtained from bacterial culture in the absence of IPTG.

with mannose residues and fucose residues, indicating that it is an N-linked glycoprotein.

We prepared mRNA from developing cotyledons of wheat as the first step in the cloning of cDNA encoding Tri a Bd 27K. After synthesis of first-strand cDNA from the mRNA obtained, we tried to amplify a cDNA fragment by PCR with various degenerate primers prepared on the basis of the amino acid sequence determined, and a 461-bp DNA fragment was obtained with primers TR1 and TR5. Northern-blot analysis with the DNA fragment as a probe showed that the size of the mRNA for Tri a Bd 27K was approximately 1.0 kb (Fig. 3). Then, cloning of a cDNA encoding the allergen was tried by PCR with a 3'-RACE kit and a 5'-RACE kit. PCR product was obtained with the 5'-RACE kit. However, when PCR was carried out using the 3'-RACE kit, two DNA fragments were amplified and a 798-bp nucleotide sequence was established by overlapping

those of the two DNA fragments. The established DNA fragment was shown to contain a 609-bp open reading frame between 1 and 609, encompassing 203 amino acid residues, a 161-bp 3'-untranslated region, and a polyadenylation tail. As shown in Fig. 4, the amino acid sequence deduced from the DNA fragment contained all of the chemically determined amino acid sequences of the peptides. This finding indicates that the cDNA encoded the purified allergen, and it means that the allergen was synthesized from its precursor by elimination of a small segment from the N-terminus. The molecular mass of the protein for the cDNA was calculated to be 22,803 Da, but that of the allergen purified from wheat was shown to be 27 kDa by SDS-PAGE. The deduced amino acid sequence of the allergen was shown to have two glycosylation sites. Furthermore, sugar analysis revealed that the allergen was an Asn-linked glycoprotein. These observations suggest that the difference between the molecular mass of the predicted protein as deduced from the cloned cDNA and, that of the purified allergen is to be explained by the occurrence of a glycan moiety, but the exact location of the glycan moiety on the allergen remains unsolved.

We surveyed proteins homologous with the deduced amino acid sequence of the allergen in the SWISSPROT data base. The allergen showed high homology with a number of proteins. Most of the homologous proteins identified were predicted proteins revealed by genomic analysis and have never been demonstrated to occur in any organisms but human γ -interferon-inducible thiol reductase²⁸⁾ has been found to be expressed as a protein that has thiol reductase activity. As shown in Fig. 5, the allergen exhibited high overall homology with the γ -interferon-inducible lysosomal thiol reductase family protein in *Oryza sativa* ssp. japonica (61.2% identity),²⁷⁾ the ARATH F10K1.21 protein in *Arabidopsis thaliana* (52.9% identity), and human γ -interferon-inducible thiol reductase (37.7% identity).²⁸⁾ These findings suggest that it might belong to the family of γ -interferon-inducible thiol reductases. High homology of the allergen with human γ -interferon-inducible thiol reductase suggests that it has enzyme activity. The active motif of the enzyme was found to be sequence 46–49 (CGGC), in which two Cys residues are essential for enzyme activity. However, sequence 22–25 (CPYS)

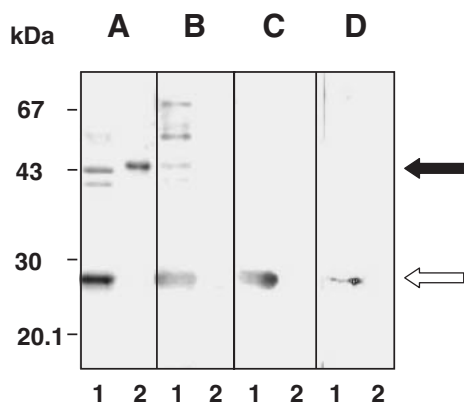


Fig. 7. SDS-PAGE and Immunoblot of the Allergen Purified from Wheat Flour and Tri a Bd 27K Expressed as a Fusion Protein in *E. coli* BL21.

Natural Tri a Bd 27K was partially purified from wheat flour essentially in the manner described in the text, except that the allergen purified by chromatofocusing with a PBE column was further subjected to Sephacryl S-200 chromatography instead of phenyl-Sepharose chromatography, and rTri a Bd 27K was prepared as described in the text. Natural Tri a Bd 27K (lane 1) and rTri a Bd 27K (lane 2) were separated on a 12% polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. The membrane was stained with Ponceau S (A) and immunoblotted with sera of patient 1 (B), patient 2 (C), and patient 3 (D) in the manner described in the footnote to Fig. 1. Lane 1, purified Tri a Bd 27K; lane 2, rTri a Bd 27K. The open arrow expresses the band corresponding to natural Tri a Bd 27K, and the solid arrow represents that corresponding to rTri a Bd 27K.

of the allergen corresponds to the active motif and Cys-49 of the enzyme, responsible for thiol reductase activity changes to Ser-25 of the allergen. This suggests that the allergen does not have thiol reductase activity.

For further elucidation of the allergenicity of Tri a Bd 27K, an expression vector, pGEX/Tri 27, was constructed using a pGEX-6P-1 expression vector and the PCR product prepared from the cDNA cloned, as described in "Materials and Methods," and transformed into *E. coli* BL21. When cells harboring the expression vector were cultured in the presence of IPTG, the allergen was expressed as a fusion protein with glutathione *S*-transferase (Fig. 6). As shown in Fig. 7, the allergen purified from wheat flour was found to be recognized by the sera of the three wheat-sensitive patients, but the rTri a Bd 27K expressed in *E. coli* was observed to be immunoblotted not at all or weakly with the same sera. Since the recombinant protein is expressed without any glycation, the difference between the binding of the IgE antibodies to the purified allergen and the recombinant protein must be due to the occurrence of the glycan moiety of the allergen, which indicates that its glycan moiety is connected with the binding to IgE antibodies in the sera of the allergic patients. Similar phenomena have been observed with regard to glycan moieties of various plant glycoallergens.^{3,18,25,29-31} The sugar moieties of the allergens were very similar to that of horseradish peroxidase.³⁰ In the present study, sugar analysis with various lectins indicated that fucose residues were present in the glycan moiety of Tri a Bd 27K in addition to mannose residues, suggesting that the allergen has a glycan moiety similar to that of peroxidase. The above observations confirm that the glycan moiety can serve as a common epitope.^{30,31} If an allergen that has only a glycan moiety as an epitope has more than two glycan moieties, it

crosslinks two IgE antibodies attached to cells such as mast cells and induces an allergic reaction. This also suggests that patients, the sera of which recognize the allergens with the above glycan moiety as a common epitope, are sensitive to any foods including other glycoproteins with a glycan moiety similar to the common epitope, even if the patients have never had the foods, and that the occurrence of IgE antibodies against the common epitope would result in a wrong diagnosis, in cases in which the antibodies are not involved in the allergic responses, but the mechanism of the interaction of their glycan moieties with IgE antibodies in the sera of patients has never been characterized. Further elucidation of the binding of the glycan moieties of glycoallergens to IgE antibodies should be done.

In a previous study,¹⁸ Tri a Bd 27K was to be the allergen recognized most frequently with the sera of wheat-sensitive Japanese patients. It was also observed to occur in wheat by Posch *et al.*⁶ On the other hand, Pastorello *et al.*¹⁰ stated that many allergens including a peptide with a molecular mass of 27 kDa occur in the albumins/globulins, gliadins and glutenins of wheat, but did not consider Tri a Bd 27K a prominent allergen. They reported that the sera of Italian patients recognized the LTP in wheat, but that those of Swiss and Danish patients did not recognize it.¹⁰ On the basis of these observations, they proposed that the difference in the IgE recognition pattern is due to differences in geographical areas and in age groups. The above-mentioned difference in IgE reactivity to Tri a Bd 27K confirms the hypothesis proposed by Pastorello *et al.*¹⁰

Acknowledgments

This study was supported by a grant from the Elizabeth Arnold Fuji Foundation and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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