

Sandwich Enzyme-linked Immunosorbent Assay System for Micro-detection of the Wheat Allergen, Tri a Bd 17 K

Hiromi YAMASHITA,[†] Masumi KIMOTO, Miki HIEMORI, Misako OKITA, Kazuhiko SUZUKI, and Hideaki TSUJI

Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University, Soja, Okayama 719-1197, Japan

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Seven monoclonal antibodies (mAbs) against the wheat allergen, Tri a Bd 17 K, were prepared to obtain mAbs suitable for a sandwich enzyme-linked immunosorbent assay (sandwich ELISA) for determination of the allergen. Two of the mAbs strongly immunoblotted the allergen purified from wheat flour. However, only one (1G11) of them was found to be suitable for sandwich ELISA. Epitope mapping against mAb-1G11 on the allergen showed that the mAb recognized the peptide containing Lys-38 and Gln-39 of the allergen. We developed a sandwich ELISA method consisting of *Aleuria aurantia* lectin for fixing the allergen and 1G11 as the first antibody that enabled 4–4,000 ng/well of the allergen to be determined.

Key words: wheat allergen; Tri a Bd 17 K; monoclonal antibody; sandwich ELISA; lectin

Egg, milk, wheat, buckwheat and shrimp are well known as representative allergy-inducing foods in Japan. The allergens in the former two foods have been actively characterized.^{1,2)} While the allergens in wheat have been reported,^{3,4)} its overall features have never been elucidated. In a previous study, we examined the wheat allergens by using sera from 65 wheat-sensitive patients, found 15 antigens recognized by IgE antibodies in the patients' sera, and specified Tri a Bd 16 K, 17 K, 27 K, 31 K, 37 K and 47 K as the major wheat allergens.⁵⁾ Furthermore, one of these major wheat allergens, Tri a Bd 17 K, has been identified as α -amylase inhibitor CM16 with an Asn-linked sugar chain.⁵⁾ Interestingly, most of the IgE antibodies in the sera of patients sensitive to Tri a Bd 17 K seemed to bind mainly to its sugar moiety.⁵⁾ It is very important for patients suffering from wheat allergy to develop hypoallergenized wheat, because all wheat and wheat products must be removed from their diets to avoid the severe responses induced by

ingesting wheat. It is therefore important to establish a method for the detection of wheat allergens in order to develop hypoallergenized wheat products.

In the present study, we prepared mAbs against Tri a Bd 17 K and characterized them. Furthermore, a sandwich enzyme-linked immunosorbent assay (ELISA) method for highly sensitive determination of Tri a Bd 17 K in wheat products was developed.

Materials and Methods

Reagents. The materials used in this work were obtained from the following sources: mouse myeloma cell line, P3U1 (Rikagaku Institute, Japan), Balb/c mice (Japan Clea, Japan), bovine fetal serum, sterilized L-glutamine, and sterilized streptomycin/penicillin mixture (MA Bioproducts, U.S.A.), RPMI1640 (Nissui Pharmaceutical Co., Japan), isopropyl 1-thio- β -D-galactopyranoside (IPTG; Nippon Gene Co., Japan), aminopterin (Sigma, U.S.A), polyethylene glycol 4000 (Merck, Germany), mouse monoclonal antibody isotyping kit (Amersham International Plc, Amersham, U.K.), *Aleuria aurantia* lectin (AAL; Seikagaku Corporation, Japan) nitrocellulose membrane (Bio-Rad, U.S.A.), goat peroxidase-conjugated anti-mouse IgG (Funakoshi, Japan), protein A-Sepharose 4B and marker proteins with low molecular weights for gel electrophoresis (Amersham Pharmacia Biotech, U.K.). A plasmid (pUC118-CM16) encoding α -amylase inhibitor CM16⁶⁾ was presented by Dr. M. F. Gautier (National Agriculture Institute, France). Tri a Bd 17 K was purified in the same manner as described previously.⁵⁾ The other reagents were of analytical grade and commercially available.

Preparation of mAbs. Female Balb/c mice (8 week-old) were intraperitoneally injected with an emulsion of complete Freund's adjuvant and purified

[†] To whom correspondence should be addressed. Fax: +81-866-94-2150; E-mail: yamashit@fhw.oka-pu.ac.jp

Abbreviations: ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; AAL, *Aleuria aurantia* lectin; IPTG, 1-thio- β -D-galactopyranoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis

Tri a Bd 17 K (10 µg/mouse). At intervals of two weeks, the emulsion of incomplete Freund's adjuvant and the allergen was intraperitoneally administered to the mice two times. Two weeks later, the mice were boosted with the allergen in phosphate-buffered saline (PBS). Four days later, the spleens were removed from the mice. The spleen cells were fused with P3U1 myeloma cells in the presence of 50% polyethylene glycol 4000. The selection and cloning of hybridomas producing mAbs against the allergen were done in the same manner as that described in a previous report.⁷⁾ The mAbs were obtained by purification from ascites in the mice intraperitoneally injected with the established hybridomas, using a protein A-Sepharose 4B column.

This study was conducted under the control of guidelines from Okayama Prefectural University, and under the Law and Notification of the Japanese government.

ELISA. Fifty µl of Tri a Bd 17 K (0.5 µg/ml) in PBS was put into each well of a 95-well microplate at 4°C and left overnight, after which the allergen solution was removed from the wells and the wells incubated with 1% bovine serum albumin in PBS containing 0.05% Tween 20 (PBS-T) at 37°C. After 1 hr, the wells were reacted with mAbs at 37°C for 1 hr. After washing three times with PBS-T, the immunocomplexes in the wells were incubated with peroxidase-conjugated anti-mouse IgG (0.5 µg/ml) at 37°C. After a 1-hr incubation, the wells were washed three times with PBS-T, and the immunocomplexes were visualized by the peroxidase reaction with a cocktail consisting of *O*-phenylenediamine and H₂O₂ in a 0.1 M citrate buffer (pH 5.0). The absorbance of each reaction mixture in the wells was measured at 490 nm with a Bio-Rad microplate reader.

Sandwich ELISA. The wells of a 96-well microplate were coated with AAL (1 µg/ml, 50 µl/well) and left overnight at 4°C. The wells were blocked for 1 hr with PBS-T containing 1% bovine serum albumin and then incubated with 50 µl of Tri a Bd 17 K (0.5 µg/ml) in PBS-T containing 1% bovine serum albumin for 1 hr at 37°C. After three washes with PBS-T, the wells were incubated with 1 µg/ml of mAbs for 1 hr at 37°C. After washing three times with PBS-T, peroxidase-conjugated anti-mouse IgG (0.5 µg/ml) was added to the wells, and the mixture incubated at 37°C. After a 1-hr incubation, the wells were washed three times with PBS-T, and the immunocomplexes were visualized by the peroxidase reaction with a cocktail consisting of *O*-phenylenediamine and H₂O₂ in a 0.1 M citrate buffer (pH 5.0). The absorbance of each reaction mixture in the wells was measured at 490 nm with the microplate reader.

Immunoblot. Proteins in the samples were separated on 15% polyacrylamide gel by the method of Laemmli.⁸⁾ After the electrophoresis, the proteins on the gel were electroblotted on to nitrocellulose membranes. After blocking the membranes with 1% bovine serum albumin in a 20 mM Tris-HCl buffer (pH 7.3) containing 0.9% NaCl and 0.05% Tween 20 (TBS-T) at room temperature for 1 hr, the membranes were incubated with mAbs (2 µg/ml) at room temperature. After 1 hr, the membranes were washed with TBS-T and then reacted with peroxidase-conjugated anti-mouse IgG (0.5 µg/ml) at room temperature for 1 hr. After washing with TBS-T, the immunocomplexes on the membranes were detected by reacting with 4-chloro-1-naphthol and H₂O₂. To detect the IgE antibodies in the patient's sera, the membranes were treated with biotinylated anti-human IgE and peroxidase-conjugated avidin instead of peroxidase-conjugated anti-mouse IgG just mentioned.

Expression of recombinant α -amylase inhibitor CM16 and its recombinant peptide fragments as fusion proteins with glutathione *S*-transferase. Recombinant α -amylase inhibitor CM-16 and its several recombinant peptide fragments were expressed as fusion proteins with glutathione *S*-transferase by using expression vector pGEX-6P-1 (Amersham Pharmacia Biotech, U.K.). The fusion proteins contained three additional amino acid residues at the N-terminal of the inhibitor and its peptide fragments.⁹⁾ Briefly, the DNA fragments encoding sequences 1–38, 39–78, 79–116, 1–78, 39–116 and 1–116 were prepared by the polymerase chain reaction (PCR) with primers 1 (5'-CCCCTGGGATCCATCGGCAATGAAGATTGCACC-3') and 2 (5'-CCCACAGAATTCTACTTGGCGAGGTACGGCGA-3'), primers 3 (5'-GTACCTCGGATCCAGCAGTGCTGTGGGGAGCT-3') and 4 (5'-TGCACGAATTCCTAGCATCCGGGGAGTTCCATGA-3'), primers 5 (5'-CTCCCCGGATCCCCTAGGGAGGTGCAGATGGA-3') and 6 (5'-ACCCGGGAATTCTCTAGCTCCACTGAGACT-3'), primers 1 and 4, primers 3 and 6, and primers 1 and 6, respectively. PCR was conducted at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min in 30 cycles. The reaction was completed by a further 10-min incubation at 72°C. The DNA fragment formed was digested with *Bam*HI and *Eco*RI and inserted it into the cloning site of pGEX-6P-1 that had been digested with *Bam*HI and *Eco*RI (pGEX-6P-1/CM16). *Escherichia coli* JM109 was transformed with constructed pGEX-6P-1/CM16. *E. coli* were grown by LB culture at 37°C overnight and then further cultured in the presence of IPTG for an additional 7 hr. The cell pellet was collected by centrifugation and solubilized with 62.5 mM Tris-HCl (pH 6.8) containing 5% 2-mercaptoethanol, 2% SDS and 20% glycerol. For purification of the fusion proteins, the

lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The parts of the gel containing the fusion proteins were cut out, and the proteins were electrophoretically eluted from the gel. These fusion proteins were concentrated and used as the purified fusion proteins in the present study.

Results and Discussion

Four female Balb/c mice were immunized with purified Tri a Bd 17 K as described in the Materials and Methods section. Hybridomas producing mAbs against the allergen were detected by ELISA with the allergen coated on the wells of microplates. The hybridomas were established three times by the limiting dilution method, using a feeder layer of thymus cells obtained from 5-week-old female Balb/c mice. Finally, seven mAbs (1G11, 3A12, 2B1, 5G12, 2A6, 1C5, and 6G5) against the allergen were obtained. When the isotypes of the mAbs were examined with a mouse monoclonal antibody isotyping kit (Amersham), all of the mAbs were found to have κ light chains and γ_1 heavy chains. Each of the mAbs was found to strongly bind to the allergen on the well of the microplate (Fig. 1). However, immunoblotting revealed the crossreactivity of these mAbs to be different from one another (Fig. 2(B)) as compared with the results obtained by ELISA. Two mAbs, 1G11 and 3A12, immunoblotted the allergen more strongly than the others. The allergen contains an Asn-linked sugar chain with a fucosyl residue.^{5,10} Most of the patients susceptible to allergenicity with Tri a Bd 17 K were found to have IgE antibodies binding mainly to its sugar moiety.^{5,10} Purified Tri a Bd 17 K used in this study was stained with the fucose-specific lectin, AAL, and also immunoblotted by the patient's serum (Figs. 2(C) and 2(D)).

In our previous study,⁵ Tri a Bd 17 K was identified as α -amylase inhibitor CM16 with an Asn-linked sugar chain. During the purification of the allergen, it had been observed that the albumin fraction in wheat proteins contained another type of CM16 α -amylase inhibitor which was less reactive with AAL.⁵ The preparation of partially purified Tri a Bd 17 K showed two main bands by SDS-PAGE (Fig. 3(A)). The upper band indicates a CM16 protein with a fucosyl residue reactive to AAL, which had the same mobility as that of purified Tri a Bd 17 K, while the lower band is another type of CM16 less reactive to AAL. Both of the proteins corresponding to the bands were immunoblotted by the mAbs, (Fig. 3(B)); however, only the upper band was stained with AAL and also immunoblotted with a patient's serum (Figs. 3(C) and 3(D)).

As described next, only one of the mAbs was found to be a suitable candidate for the probe to be utilized for sandwich ELISA. Therefore, we examined the epitope against mAb-1G11 on the allergen

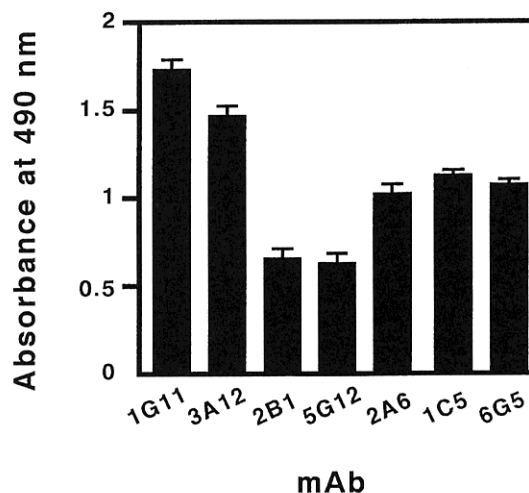


Fig. 1. Crossreactivity of the mAbs with Tri a Bd 17 K Adsorbed to the Wells of a Microplate.

ELISA was done as described in the Materials and Methods section. The crossreactivity of each mAb with Tri a Bd 17 K was measured in triplicate. The absorbance of the background for each mAb was subtracted from each value, and the arithmetic mean $\pm$ SD is given.

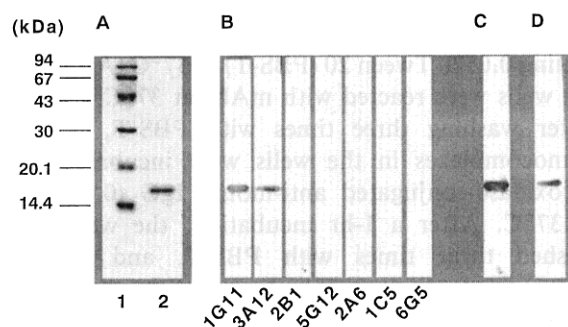


Fig. 2. SDS-PAGE of Purified Tri a Bd 17 K (A), and Its Reactivity with mAbs (B), AAL (C) and a Patient's Serum (D).

After SDS-PAGE, the marker proteins (lane 1) and purified Tri a Bd 17 K (lane 2) on the gel were stained with Coomassie Brilliant Blue R250 (A). Tri a Bd 17 K electroblotted on to a nitrocellulose membrane was divided into several strips, and the allergen on each strip was immunoblotted with each of the mAbs indicated in the figure (B). One of them was stained with AAL (C), and another was immunoblotted with a patient's serum (D).

by using the allergen and its peptide fragments expressed as fusion proteins with glutathione *S*-transferase in *E. coli* JM109. We have previously elucidated that the N-terminal amino acid sequence of Tri a Bd 17 K was EDCTPWM and identified it as CM16.⁵ Analysis of the cDNA encoding the full length of CM16 showed that the open reading frame of the cDNA coded for a preprotein of CM16 which eliminated a signal peptide of 27 amino acids to form the mature protein, CM16. According to the sequence of this mature protein, the peptide fragments were designed and expressed as fusion proteins (Fig. 4). Without IPTG, *E. coli* JM109 harboring

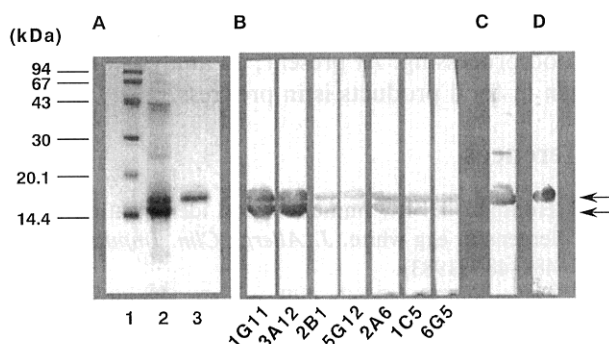


Fig. 3. SDS-PAGE of Partially Purified Tri a Bd 17 K (A), and Its Reactivity with mAbs (B), AAL (C) and a Patient's Serum (D).

After SDS-PAGE, the marker proteins (lane 1), partially purified (lane 2), and purified Tri a Bd 17 K (lane 3) on the gel were stained with Coomassie Brilliant Blue R250 (A). Partially purified Tri a Bd 17 K electroblotted on to a nitrocellulose membrane was divided into several strips. The allergen on each strip was immunoblotted with each of the mAbs indicated in the figure (B). One of them was stained with AAL (C), and another was immunoblotted with a patient's serum (D). The upper and lower arrows respectively indicate Tri a Bd 17 K with a fucosyl residue and the same protein without a fucosyl residue.

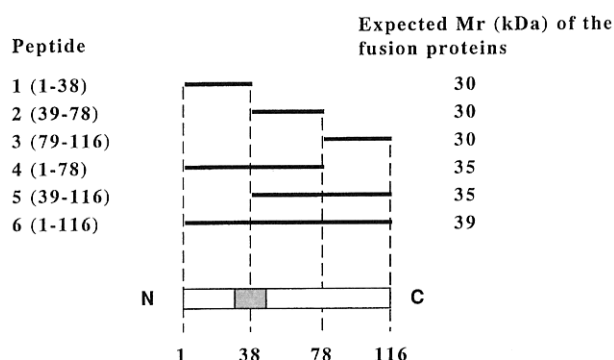


Fig. 4. Tentative Location of the Epitope against mAb-1G11 on Tri a Bd 17 K.

The DNA fragments encoding peptide sequences 1–38, 39–78, 79–116, 1–78, 39–116 and 1–116 of CM16 were prepared by PCR, and expression vectors of those peptide fragments were constructed by using pGEX-6P-1 as described in the Materials and Methods section.

pGEX-6P-1/CM16 did not yield the fusion protein of CM16 with glutathione *S*-transferase, but after incubating with IPTG, the fusion protein was found to be accumulated in a high concentration. The fusion protein with various peptide fragments of CM16 could be expressed in the same manner as that described for the case with CM16. In order to locate the epitope against mAb-1G11 on the allergen, the peptide fragments expressed as fusion proteins were carefully investigated with regard to their crossreactivity with mAb-1G11 by immunoblotting analyses. As shown in Fig. 5, peptide 4 and peptide 6, whole CM16, were immunoblotted with mAb-1G11, suggesting two hypotheses that its epitope may be located

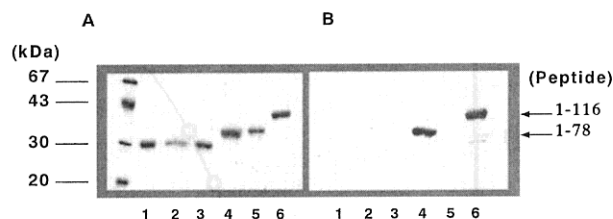


Fig. 5. Analysis of the Epitope against mAb-1G11 on the Allergen.

(A) Ponceau staining of fusion proteins blotted on the membrane from SDS-PAGE gel. Various peptide fragments of α -amylase inhibitor CM16 were expressed as fusion proteins with glutathione *S*-transferase in *E. coli* JM109. The fusion proteins were purified as described in the text. (B) Immunoreactivity of mAb-1G11 to the fusion proteins blotted on the membrane. Lane 1, peptide 1–38; lane 2, peptide 39–78; lane 3, peptide 79–116; lane 4, peptide 1–78; lane 5, peptide 39–116; lane 6, CM16 (sequence 1–116). The standard protein markers are shown on the left.

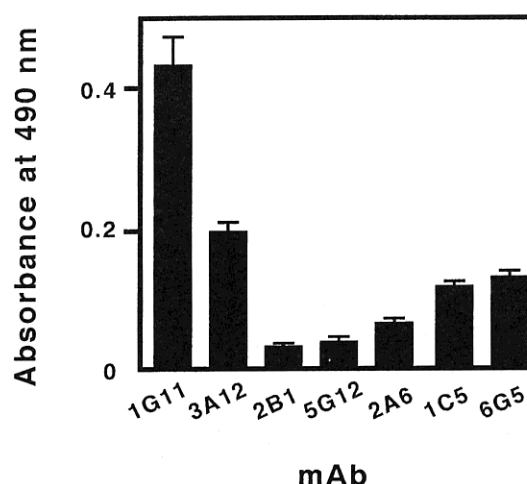


Fig. 6. Crossreactivity of the mAbs with Tri a Bd 17 K Bound to AAL Lectin on the Wells of a Microplate.

Sandwich ELISA was done as described in the Materials and Methods section. The crossreactivity of each mAb with Tri a Bd 17 K was measured in triplicate. The absorbance of the background for each mAb was subtracted from each value, and the arithmetic mean $\pm$ SD is given.

ed on the sequence corresponding to the region including Lys-38 and Gln-39, or that mAb-1G11 may recognize the peptide structure of 1–78 containing Lys-38 and Gln-39. Further investigation will be needed to resolve these possibilities.

In order to determine the Tri a Bd 17 K contents in wheat products, a selective and sensitive method for the analysis of allergens in wheat needs to be established. The method requires two probes specific to the allergen. We examined a probe which is suitable for fixing the allergen on wells of a microplate and found that AAL was the best ligand for this. Next, a probe suitable to detect the allergen trapped by the fixing ligand, AAL, coated on the wells of a microplate was screened. Figure 6 shows that the

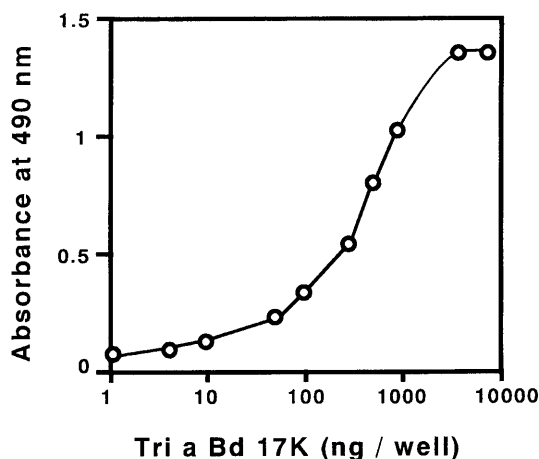


Fig. 7. Dose-response Curve of Tri a Bd 17 K by Sandwich ELISA.

Serially diluted Tri a Bd 17 K (1 ng to 8 μ g/well) was measured by sandwich ELISA using mAb-1G11 and AAL as described in the Materials and Methods section.

reactivity of mAbs to the allergen was different for each. The reactivity of mAb-1G11 was the strongest of all mAbs tested, showing mAb-1G11 to be the most suitable probe for the sandwich ELISA assay to detect Tri a Bd 17 K (Fig. 6).

Serially diluted Tri a Bd 17 K samples (1 ng to 8 μ g/well) were measured as described in the Materials and Methods section. The standard dose-response curve for quantification of Tri a Bd 17 K revealed a characteristic sigmoidal shape when plotted on a semi-logarithmic scale (Fig. 7). The results show that the sensitivity, defined as the antigen concentration that produces a significant positive signal, was 4 ng/well and that this sandwich ELISA method using AAL and mAb-1G11 was capable of determining a Tri a Bd 17 K concentration of 4–4,000 ng/well.

As already described, mAb-1G11 can be utilized for sandwich ELISA in combination with AAL as the other probe for detecting the allergen, Tri a Bd 17 K, in wheat products. This sandwich ELISA method is highly sensitive and specific to the allergen. Therefore, the system provides a useful tool in determining the allergen content in wheat products for labeling allergy-eliciting foods. Furthermore, the method can be utilized as a powerful tool for evaluating whether

wheat flour or wheat proteins may be supplemented in food processing. At present, an analysis of the allergen in food products is in progress.

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