



Note

Preparation and Epitope Mapping of a Monoclonal Antibody against Tri a Bd 27K, a Major Wheat Allergen

Nobuko KOMIYAMA, Miki HIEMORI, Masumi KIMOTO, Makiko SUZUKI, Hiromi YAMASHITA, Yoshitaka TAKAHASHI, and Hideaki TSUJI[†]

Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University, 111 Kuboki, Soja, Okayama 719-1197, Japan

Received April 1, 2009; Accepted May 17, 2009; Online Publication, September 7, 2009

[doi:10.1271/bbb.90224]

We produced a monoclonal antibody (mAb) as a probe for detection of Tri a Bd 27K, a major wheat allergen. The mAb recognized the allergen purified from wheat flour, and the epitope on the allergen to the mAb was determined to be amino acid sequence ¹⁵⁴VPWVVVDGKPL¹⁶⁴ of Tri a Bd 27K. Of the amino acid residues on the epitope, the amino acid residues responsible for the binding to the mAb were found to be W156, D160, G161, and P163.

Key words: wheat; allergen; Tri a Bd 27K; epitope structure; monoclonal antibody

In Japan, patients suffering from allergic responses to foodstuffs have increased in number, and this has become a serious social problem. Wheat is one of the most widely consumed crops in the world, and it is utilized in the production of various foods. However, it is also known a major allergy-eliciting food, and it ranks as the main allergy-inducing food following egg and milk in Japan.

In Western European countries, bakers' asthma, an immediate-type allergic response to the inhalation of cereal flours, is a major occupational disease among persons working in the baking and milling industries, allergens responsible for bakers' asthma, contained in the salt-soluble fraction of wheat, has been found to be α -amylase inhibitors.^{1,2)} In addition to this allergen, several other allergens have been identified,^{3,4)} but, the allergens in wheat have not yet been characterized well.

We have carried out systematic studies of wheat allergens by immunoblotting with the sera of patients sensitive to wheat, and have found 15 allergens in wheat.^{5,6)} Among these, Tri a Bd 17K, Tri a Bd 27K, and Tri a Bd 36K were found to be the major allergens. Tri a Bd 17K and Tri a Bd 36K have been identified as an α -amylase inhibitor and a peroxidase respectively.^{5,7)} Recently, we isolated Tri a Bd 27K from wheat, and cloned a cDNA encoding it.⁸⁾ It allergen was found to be an unknown Asn-linked glycoprotein with 203 amino acid residues, the molecular mass of which of was 22,803 Da.⁸⁾ A sugar moiety of the allergen was perhaps be involved in the binding to IgE antibodies in the sera of patients sensitive to wheat.⁸⁾ A similar phenomenon

was observed in the case of a soybean allergen, Gly m Bd 28K.⁹⁾ It is necessary to produce a probe detecting the allergen for further elucidation of its allergenicity and development of hypoallergenic products of wheat.

In the present study, we produced a monoclonal antibody (mAb) against Tri a Bd 27K as a probe, and we report a structural characterization of the epitope of the allergen to mAb and its usefulness as a probe.

In order to obtain the fusion protein of Tri a Bd 27K with glutathione *S*-transferase (GST), an expression vector, pGEX/Tri 27, was transformed in *Escherichia coli* JM109, and the recombinant Tri a Bd 27K (rTri a Bd 27K) was expressed as the fusion protein, as described previously.⁸⁾ rTri a Bd 27K was used as an immunogen. Immunization of 8-week old BALB/c female mice, cloning of hybridoma cells, and the production of mAbs against the recombinant protein were carried out as described as previously.¹⁰⁾ rTri a Bd 27K obtained and native Tri a Bd 27K purified from wheat flour were analyzed by SDS-PAGE by the method of Laemmli.¹¹⁾ For immunoblotting, the proteins separated on the gel were electroblotted onto a nitrocellulose membrane. The proteins on the membrane were immunoblotted with the serum of a wheat-sensitive patient with a RAST score of 4 or the mAbs, as described in the previous paper.⁸⁾

To elucidate the epitope on the allergen to a selected mAb, peptide fragments of the allergen were expressed in *E. coli* JM109 as fusion proteins with GST, as described above. The reaction of the mAb with the fragments obtained was examined in detail by an immunoblotting technique. Subsequently, designed peptide fragments with 10 and 11 amino acid residues prepared on a SPOTS membrane by peptide synthesis were obtained from Sigma-Genosys (St. Louis, MO). The sequences of the peptide fragments are summarized in Fig. 3B. We examined the reactivity of the peptides on the membrane by immunostaining with the mAb.

Several 8-week old female mice were immunized with the above-mentioned recombinant protein, and the spleen cells in the mice were fused with P3NS1 myeloma cells, as described previously.¹⁰⁾ Five hybridoma producing the mAb against the allergen were established. All of the mAbs were found to recognize the

[†] To whom correspondence should be addressed. Tel/Fax: +81-866-94-2143; E-mail: htsuji@fhw.oka-pu.ac.jp

Abbreviations: mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; rTri a Bd 27K, recombinant Tri a Bd 27K; GST, glutathione *S*-transferase

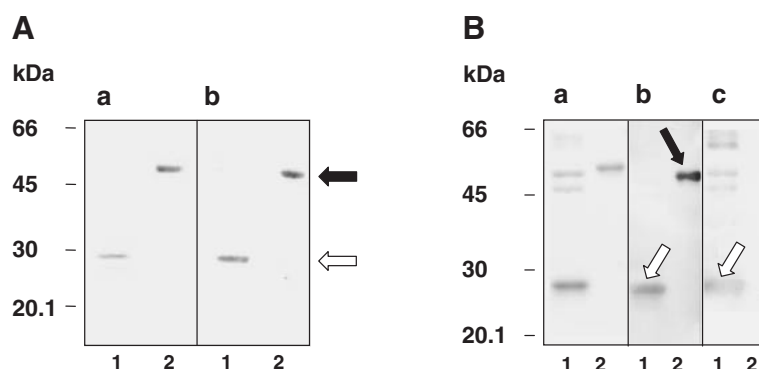


Fig. 1. SDS-PAGE and Immunoblot of Tri a Bd 27K Purified from Wheat Flour and rTri a Bd 27K and Tri a Bd 27K in the Extract of Wheat.

Tri a Bd 27K purified from wheat flour and the extract of wheat were obtained as described previously.⁸⁾ rTri a Bd 27K was prepared as described in the text. The extract of wheat was prepared by extraction with 10% NaCl followed by fractionation with solid ammonium sulfate, as described in a previous paper.⁸⁾ The purified and recombinant allergens and the extract of wheat were electrophoresed on 15-% gels as described in the text. Then the proteins on the gel were electroblotted onto nitrocellulose membranes. The proteins on the membranes were stained with Ponceau S (a). After washing with 1% acetic acid and destaining with 10mM NaOH, the native Tri a Bd 27K and rTri a Bd 27K were immunoblotted with the mAb (b) and with the serum of a wheat-sensitive patient (c). A, SDS-PAGE and immunoblot of native Tri a Bd 27K. Lane 1, the purified Tri a Bd 27K; lane 2, rTri a Bd 27K. B, SDS-PAGE and immunoblot of rTri a Bd 27K and Tri a Bd 27K in the wheat extract. Lane 1, Tri a Bd 27K in the wheat extract; lane 2, rTri a Bd 27K. The open and closed arrows represent bands corresponding to the purified and recombinant allergens respectively.

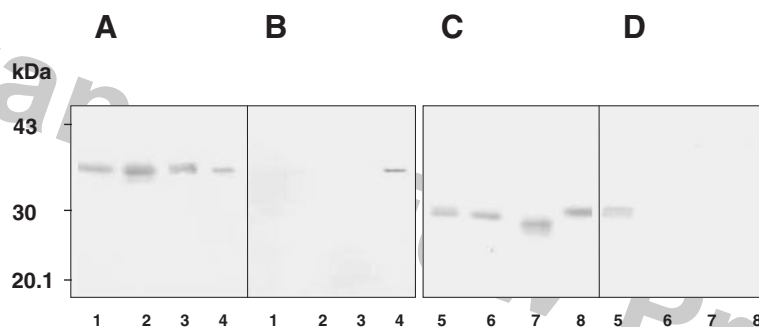


Fig. 2. SDS-PAGE and Immunoblot of the Fragments of rTri a Bd 27K.

The fragments of the allergen were expressed as fusion proteins with GST in *E. coli* JM109, as described in the text. Each fragment was separated on a 15-% polyacrylamide gel. After electroblotting on a nitrocellulose membrane, the fragments on the membrane were examined with regard to their reactivities with the mAb, as described in the footnote to Fig. 1. Panel A shows the SDS-PAGE patterns of fragments 1–4, corresponding to lanes 1–4. Panel C shows the electrophoresis patterns of fragments 5–8, corresponding to lanes 5–8. Panels B and D show the immunoblotting patterns of fragments 1–4 and fragments 5–8 respectively.

allergen purified from wheat flour. However, among the mAbs, only one, 1H11, strongly recognized the allergen (Fig. 1). Hence that mAb was used in subsequent experiments. It was to have κ light chains and γ_1 heavy chains.

To locate the epitope on the allergen to the mAb, the amino acid sequence of Tri a Bd 27K was divided into four fragments, sequences 1–86 (F1), 78–104 (F2), 98–154 (F3), and 147–203 (F4). cDNAs encoding F1, F2, F3, and F4 were amplified by PCR using forward primer 1 (ATATTAGGATCCGGGGATGTGGCAACGGGC) and reverse primer 1 (ATAATAGAATTCCTTAGACCACGGCGTTGCCATA), forward primer 2 (ATATATGGATCCCTCGTCCCCTATGGCAAC) and reverse primer 2 (GACTAAGAATTCCTTAGTTCTTCAACACCAGGTC), forward primer 3 (ATATATGGATCCGTGTGCGACCTGGTGTG) and reverse primer 3 (ATATAAGAATTCCTTAAACGAAGTGGTGAGGAGG), and forward primer 4 (ATATATGGATCCCTCGTGCCTCCTCACCAG) and reverse primer 4 (ATATAAGAATTCCTTAAAGTCGGCCGTACACATC) respectively. The single and double underlining in the above nucleotide sequences indicate *Bam*HI

and *Eco*RI cleavage sites respectively. The cDNAs were inserted into expression vector pGEX6p-1, and after transformation of the vector the fragment proteins were expressed in *E. coli* JM109, as described above. Of the four fragments, only one, corresponding to region 147–203 (F4) of Tri a Bd 27K, exhibited binding to the mAb (Fig. 2A). This suggests that the epitope to the mAb occurs on sequence 147–203 (F4). Subsequently, the fragment was divided into the four fragments, sequence 147–164 (F5), sequence 160–177 (F6), sequence 173–190 (F7), and sequence 186–203 (F8), and the cDNAs encoding the fragments F5, F6, F7, and F8 were amplified by PCR with forward primer 1 and reverse primer 5 (ATAATAGAATTCCTTAGAGCGGCTTGCCGTGCGAC), forward primer 6 (ATATATGGATCCGACGCAAGCCGCTCTAC) and reverse primer 6 (GACTAAGAATTCCTTACTTGCAGATGTAAGCTTT), forward primer 7 (ATATATGGATCCGCTTACATCTGCAAGGCG) and reverse primer 7 (ATATAAGAATTCCTTAGCTTCGACACGCCTCGAG), and forward primer 8 (ATATATGGATCCGAGGCGGTGTCGAAGCCTG) and reverse primer 4 respectively. The single and double underlining in the above nucleotide

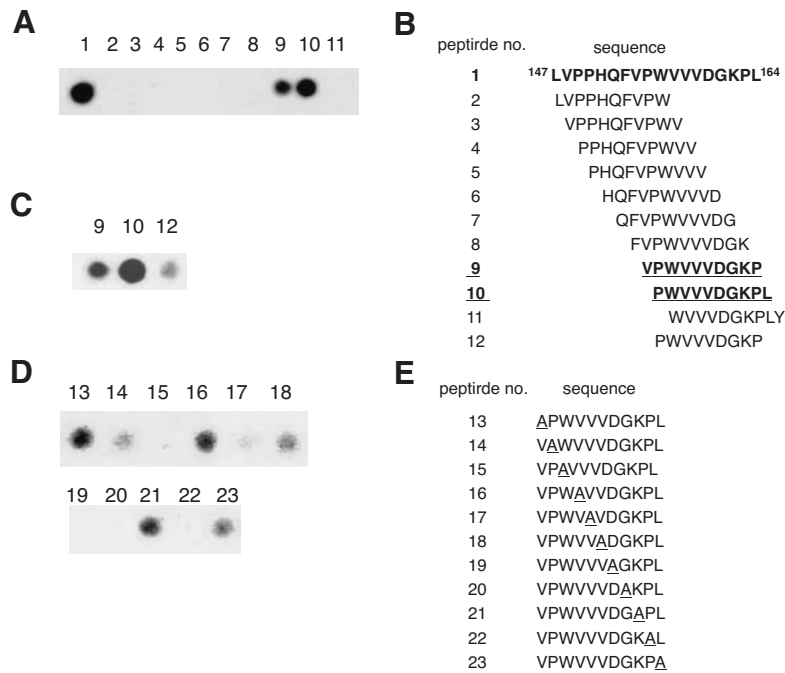


Fig. 3. Immunostaining Patterns of the Peptides with the mAb (A and C) and a Series of the Amino Acid Sequences of the Peptides Synthesized on SPOTs Membrane (B and E).

Panel A shows the immunostaining pattern of one 18-mer peptide (peptide 1) and 10 10-mer peptides (peptides 2–11) covering region F5, which were manually synthesized on a SPOTs membrane. Of the peptides, the C-terminal amino acid residue (Y) of peptide 11 corresponds to the N-terminal amino acid residue of fragment 6. Of the above 10-mer peptides, adjacent peptides had 9 amino acid overlaps with each other. In the experiment for the identification of amino acid residues of Tri a Bd 27K, involved in the binding to the mAb, 11 11-mer peptides, designated peptides 12–22, were manually synthesized on a SPOTs membrane, as described in the text. The peptides on the membrane, probed with the mAb and the mAb complexes, were detected by a chemiluminescence method with a rabbit anti-mouse IgG-horse radish peroxidase conjugate. In order to examine the amino acid residues responsible for the binding of the allergen to the mAb, each amino acid residue on sequence 154–164 (¹⁵⁴VPWVVVDGKPL¹⁶⁴) was replaced with an alanine residue. The 11 substituted peptides, designated on a SPOTs membrane, are shown in panel D. In panel D, alanine substitutions are underlined and indicated in bold. The peptides on the membrane were probed with the mAb, and the mAb bound on the membrane was detected as described above.

sequences indicate *Bam*HI and *Eco*RI cleavage site respectively. Construction of the expression vector with the cDNAs, transformation of the expression vector, and expression of the four peptide fragments were done as described above. The immunoblotting patterns of these peptide fragments with the mAb are shown in Fig. 2B. The mAb was observed to recognize only peptide fragment 5 (F5), corresponding to region 147–164 of Tri a Bd 27K. This suggests that the epitope to the mAb is on sequence 147–164.

We further attempted to elucidate the detailed structure of the epitope to the mAb using a SPOTs membrane with 10 10-mer peptides prepared by Sigma-Genosys, which overlapped with 9 amino acid residues. All of the peptides on the membrane covered perfectly sequence 147–164. The peptides on the membrane were examined with regard to their reaction with the mAb, 1H11, by immunostaining. The immunostaining pattern is shown in Fig. 3A. Only peptides 9 and 10, corresponding to sequences 154–163 and 155–164 of Tri a Bd 27K respectively, exhibited strong binding activity to the mAb, and the other peptides were not recognized by the mAb. These findings suggest that the common sequence on the two peptides, ¹⁵⁵PWVVVDGKP¹⁶³, corresponds to the minimum epitope to the mAb. The sequence ¹⁵⁵PWVVVDGKP¹⁶³ was found to bind to the mAb, although the peptide showed weak binding (Fig. 3C). This confirms that sequence 155–163 is the minimal epitope on the allergen to the mAb. Furthermore, the occurrence of V155 and L164 probably

made the affinity of the minimal epitope to the mAb stronger. Hence, sequence 154–164 was used in the identification of the amino acid residues on the epitope involved in the binding to mAb. A series of 11-mer peptides was synthesized on a SPOT membrane with alanine substitutions at each amino acid residue on ¹⁵⁴VPWVVVDGKPL¹⁶⁴, and their binding activities to the mAb were evaluated by immunostaining (Fig. 3D). Of the 11 amino acid residues on the epitope to the mAb, alanine substitutions at W156, D160, G161 and P163 remarkably inhibited the binding to the mAb (Fig. 3C). These findings indicate that amino acid residues W156, D160, G161, and P163 were responsible for binding of the epitope to the mAb.

It is very important to determine whether the mAb, 1H11, can be used to identify the allergen in wheat and in developing hypoallergenic wheat products. Hence, we carried out immunoblotting analyses of the allergen in an extract obtained from wheat. The immunoblotting pattern obtained with the mAb is shown in Fig. 1B. The mAb recognized only the protein corresponding to Tri a Bd 27K in the extract of wheat, and the protein was also found to react with IgE antibodies in the serum of a wheat-sensitive patient. This finding indicates that the mAb is a useful probe for detection of the allergen in wheat and its products. As described above, Tri a Bd 27K in wheat was found to bind to IgE antibody in the patient serum, whereas rTri a Bd 27K expressed in *E. coli* did not recognize it. Considering that rTri a Bd 27K has no glycan moiety, the difference between the

binding of the purified allergen and the recombinant protein to the IgE antibodies most have been due to the occurrence of the glycan moiety in the allergen. This means that the glycan moiety might be responsible for the binding to IgE antibodies in the serum of the wheat sensitive patient, as described in the previous paper.⁸⁾

At present, further investigation of the allergenicity of Tri a Bd 27K is in progress.

Acknowledgment

We are grateful to Ms. Yukari Yoshida (Nishiri Co., Kyoto) for excellent technical support.

References

- 1) Armentia A, Sanchez-Monge R, Gomez L, Barber D, and Salcedo CG, *Clin. Exp. Allergy*, **23**, 410–415 (1993).
- 2) Johansson A, Rasmussen SK, Harthill JE, and Welinder KG, *Plant Mol. Biol.*, **18**, 1151–1161 (1992).
- 3) Sander I, Flagge A, Merget R, Halder TM, Meyer HE, and Baur X, *J. Allergy Clin. Immunol.*, **107**, 907–913 (2001).
- 4) Pastorello EA, Farioli L, Conti A, Pravettoni V, Bonomi A, Lametti S, Fortunato D, Scibilia J, Bindslev-Jensen C, Ballmer-Weber B, Robino AM, and Ortolani C, *Int. Arch. Allergy Immunol.*, **144**, 10–22 (2007).
- 5) Kimoto M, Yoshikawa M, Takahashi K, Bando N, Okita M, and Tsuji H, *Ann. Report Interdiscipl. Res. Inst. Environ. Sci.*, **17**, 53–60 (1998).
- 6) Tsuji H, Kimoto M, and Natori Y, *Nutr. Res.*, **21**, 925–934 (2001).
- 7) Sanchez-Monge R, Gomez L, Barber D, Lopez-Otin C, Armentia A, and Salcedo G, *Biochem. J.*, **281**, 401–405 (1992).
- 8) Kimoto M, Suzuki M, Komiyama N, Kunimoto A, Yamashita H, Hiemori M, Takahashi K, and Tsuji H, *Biosci. Biotechnol. Biochem.*, **73**, 1–8 (2009).
- 9) Hiemori M, Bando N, Ogawa T, Shimada H, Tsuji H, Yamanishi R, and Terao J, *Int. Arch. Allergy Immunol.*, **122**, 238–245 (2000).
- 10) Ogawa T, Tsuji H, Kimoto M, and Sasaoka K, *Biochim. Biophys. Acta*, **1115**, 220–224 (1992).
- 11) Laemmli UK, *Nature*, **227**, 681–685 (1970).

Advance View Proofs