Identification of the major allergens in wheat flour responsible for baker's asthma

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Baker's asthma, a typical occupational allergic disease, is a serious problem in the food industries. In this study, purification and identification of major allergens recognized by IgEs in sera of allergic patients were performed. Major immunoreactive proteins were purified from the albumin fraction by gel filtration on a Toyopearl HW-50 column followed by reverse-phase HPLC. The N-terminal amino acid sequences and molecular masses measured by MS indicated that the major immunoreactive proteins are members of the α -amylase inhibitor family, 0.19 and 0.28. Significant leukotriene release by each purified protein was

observed in cell-associated stimulation tests, suggesting *in vivo* activity of these antigens. Carbohydrate analyses of major allergens indicated that they are monoglycosylated but not *N*-glycosylated in spite of the presence of a potential *N*-glycosylation site. Recombinant 0.19 expressed in *Escherichia coli* showed the same reactivity with IgE as native wheat 0.19 in Western blotting and ELISA using methyl vinyl ether maleic anhydride co-polymer as an immobilizing reagent, suggesting that the allergenic epitopes are located in the peptide portions.

INTRODUCTION

Inhalation of wheat flour often causes baker's asthma, a typical occupational allergic disease which has been known since ancient Roman times. It has been a most important occupational disease of workers in food industries. Baker's asthma is mainly a type-I allergy in which patients have a specific IgE for the allergen. Protein families, which include inhibitors of heterologous α -amylase and trypsin, have been identified as the major allergens associated with baker's asthma [1–5]. Extensive studies of bread and pasta wheat flours have separately identified a monomeric α -amylase inhibitor (AI), 0.28 [6], and an *N*-glycosylated tetrameric AI, CM16* [3], as prominent allergens. The possibility has been proposed that complex asparagine-linked glycans specifically found in plant glycoproteins may contribute to the allergenicity [7].

In recent years in Japan, the number of patients suffering from baker's asthma caused by bread wheat has been increasing, and includes not only people engaged in food industries, but also those who live near a factory using wheat flour products [8]. The major allergen of baker's asthma for these patients, however, has not yet been identified. In this study, major allergens recognized by IgEs in the sera of these patients were purified and identified. The differences between allergenic epitopes for these patients and those reported previously are discussed.

EXPERIMENTAL

Materials

Flour from hard spring wheat, *Triticum aestivum*, cv. Canada Western, Dark Northern Spring and Hard Winter grown in Canada and the U.S.A. and blended by the miller and com-

mercially available from Nisshin Seifun, Tokyo, Japan was used in this study. Another commercial flour from hard spring wheat, *Triticum aestivum*, cv. Western Red Spring grown in Canada was purchased from Showa Sangyo, Tokyo, Japan. Sera from four Japanese patients with allergic symptoms caused by inhalation of wheat flour were used for the analysis of major allergens. The scores were radioallergosorbent test (RAST) 6 in case 1, RAST 4 in case 2, RAST 2 in case 3, and RAST 2 in case 4. Horseradish peroxidase grade III (HRP) was purchased from Toyobo, Tokyo, Japan. *Clerodendron trichotomum* lectin was purified as described previously [9]. Alkaline phosphatase-labelled horse anti-human IgE antibodies were purchased from Biosource International, Camarillo, CA, U.S.A., and HRP-labelled goat anti-rabbit IgG was from KPL, Gaithersburg, MD, U.S.A. HRP-labelled lectins were purchased from Seikagaku Kogyo, Tokyo, Japan. Methyl vinyl ether maleic anhydride co-polymer (MMAC; molecular mass 41 kDa) was obtained from Showa Denko, Tokyo, Japan.

Preparation of crude allergen fraction from wheat flour

All the procedures were carried out at 4 °C unless otherwise described. Extraction was performed on 20 g of commercially available wheat flour with 100 ml of 150 mM NaCl for 1 h at room temperature, followed by centrifugation for 30 min at 19000 g (12000 rev./min) to separate the albumin and insoluble gluten fractions. Proteins were precipitated by 0–50 % (NH₄)₂SO₄ fractionation of the albumin fraction, separated by centrifugation for 30 min at 28000 g (15000 rev./min), dissolved, and dialysed against distilled water. The crude allergen fraction thus obtained was stored at –20 °C after lyophilization. The protein concentration was determined by using Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Abbreviations used: AI, α -amylase inhibitor; HRP, horseradish peroxidase; MMAC, methyl vinyl ether maleic anhydride co-polymer; SI, stimulation index; CAST, cellular antigen stimulation test; CBB, Coomassie Brilliant Blue; RAST, radioallergosorbent test; *Cry j* I, *Cryptomeria japonica* pollen allergen I.

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Purification of allergen fraction by gel filtration and reverse-phase HPLC

The crude allergen fraction was dissolved in 100 mM ammonium acetate buffer, pH 6.8, and applied to a Toyopearl HW-50 (Toso, Tokyo, Japan) column (1.6 cm × 90 cm) at 4 °C.

The fractions obtained by gel-filtration chromatography were tested for reactivity with pooled serum by Western blotting. The positive fractions were further purified by reverse-phase HPLC on a Vydak PROTEIN C-4 column (0.46 cm × 25 cm) with a linear gradient (0–70 %) of acetonitrile in 0.05 % trifluoroacetic acid for 30 min at a flow rate of 1.0 ml/min. Elution conditions were optimized for better separation of the components in a short time from the method previously reported for separation of albumin fractions [10]. The purified subfractions were stored at –20 °C.

SDS/PAGE and detection of allergens by Western blotting

SDS/PAGE was carried out as described by Laemmli [11] using a separation gel of 14 % acrylamide under reducing conditions with 5 % mercaptoethanol. After electrophoresis, the proteins were electrotransferred on to a PVDF membrane (Millipore Corp., Bedford, MA, U.S.A.) at 100 mA for 100 min at room temperature. The membrane was blocked with PBS containing 5 % BSA and 0.005 % Tween 20, and then incubated with serum from each patient or pooled serum diluted with blocking buffer (1:1) for 1 h at room temperature. After being washed three times with PBS containing 0.01 % Tween 20, the membrane was blocked and incubated with alkaline phosphatase-labelled goat anti-human IgE antibody (1:1000) for 1 h at room temperature and repeatedly washed. Development was carried out with a mixture of NitroBlue Tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt. Proteins on the membrane were detected by Coomassie Brilliant Blue (CBB). Reactivity with rabbit anti-(*Cryptomeria japonica* pollen allergen I) [anti-(Cry j I)] IgG antibodies, which recognize fucose- or xylose-containing *N*-linked oligosaccharides [12], was examined by Western blotting using peroxidase-labelled goat anti-rabbit IgG as a secondary antibody as described [13]. Development was carried out with 4-chloro-1-naphthol and H₂O₂.

N-Terminal amino acid sequence analysis

N-Terminal amino acid sequences of the purified proteins obtained by reverse-phase HPLC were determined by the auto Edman degradation method using an Applied Biosystems 476A sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Mass spectrometry

The molecular masses of the major allergens were determined by using a Kompact MALDI-IV mass spectrometer (Shimadzu Seisakusho, Kyoto, Japan). About 10 pmol of a sample was mixed with 10 µg of synaptic acid as a matrix and ionized by N₂ laser. Ions produced were detected in linear mode.

Carbohydrate analyses

Carbohydrate composition was analysed by procedures reported previously [14]. Purified allergens were hydrolysed with 2.5 M trifluoroacetic acid at 100 °C for 6 h and subjected to 2-amino-pyridylation of the reducing sugars. The fluorescent sugars were separated by anion-exchange HPLC, and total sugar content was calculated from the yield of fluorescent sugar. Lectin staining was performed on the proteins electrotransferred on to PVDF

membranes after SDS/PAGE using peroxidase-labelled concanavalin A as previously described [9].

Cellular antigen stimulation test (CAST)

CAST was performed by the method of de Weck et al. [15] to estimate the releasability of leukotrienes, a *de novo* formed allergic mediator, after *in vitro* stimulation of leucocytes. Briefly, leucocytes with IgEs were separated from a patient's peripheral blood by centrifugation of the upper layer of the dextran sedimentation, and incubated with interleukin 3, antigen and cell-dissolving solution at 37 °C for 40 min. After centrifugation, the supernatant was assayed for sulphidoleukotrienes by ELISA. Stimulation index (SI) was calculated by dividing the concentration of sulphidoleukotrienes formed under stimulation with antigen by the concentration of those formed in the absence of antigen. When the SI was more than 2, the antigen was judged significantly capable of releasing leukotrienes.

Preparation of recombinant 0.19 AI expressed in *E. coli* cells

Recombinant 0.19 AI was expressed in *E. coli* JM109 cells as described by Okuda et al. [16]. Briefly, expression vector pMKAI-II-(III) was also constructed from the chemically synthesized gene of 0.19 AI ligated into a pMK plasmid for *E. coli* JM109. In this study, the *E. coli* JM109 cells transformed with the pMKAI-II-(III) vector were grown at 37 °C overnight. After induction with isopropyl β-D-thiogalactopyranoside, the cells were cultured at 37 °C for 3 h and harvested by centrifugation; then the recombinant protein was extracted by sonication. As the expression product accumulated in an insoluble fraction as an inclusion body, the lysate was centrifuged, and the precipitate was solubilized by incubation with 8 M urea in 10 mM Tris/HCl, pH 8.0. The unfolded recombinant protein was refolded by two successive steps of dialysis in alkaline pH, and then purified by ion-exchange chromatography on a DEAE-Sephacel column (1.8 cm × 27 cm) with a linear elution gradient of 0–150 mM NaCl in 50 mM Tris/HCl, pH 9.0.

ELISA using MMAC

Aliquots (50 µl) of antigen solution (1–1000 µg/ml) were added to the wells of an Immulon 1 plate (Dynatech Laboratories, Chantilly, VA, U.S.A.) pre-activated with MMAC/DMSO (5 mg/ml) as reported previously [17]. Immobilization was performed at 4 °C overnight followed by blocking of the acid anhydride group with 1 M Tris/HCl, pH 7.4, and subsequently with 2 % BSA/PBS. Then the reactivity with human IgE was examined using pooled serum diluted with blocking buffer (1:1) and alkaline phosphatase-labelled rabbit anti-human IgE antibodies (1:1000) as a secondary antibody as described previously [13].

RESULTS

Reactivity of patients' sera with saline-soluble fraction and crude allergen fractions

The saline-soluble albumin fraction and the crude allergen fraction precipitated by (NH₄)₂SO₄ were examined for reactivity with the serum from individual patients after separation of proteins by SDS/PAGE and electroblotting on to a PVDF membrane. In control experiments, primary sera were omitted before application of the secondary antibody. Multiple bands corresponding to molecular masses of 12–17 kDa and a band of about 60 kDa were commonly stained in all patients' sera,

Figure 1 Separation of the crude allergen by gel-filtration chromatography

Top, the crude allergen fraction was separated by gel-filtration chromatography on a Toyopearl HW-50 column (3.0 cm × 90 cm), equilibrated with 100 mM ammonium acetate, pH 6.8. Bottom, reactivity of the subfractions with pooled serum. Western blotting was performed as described in the text. Immunostaining was performed with pooled serum and alkaline phosphatase-labelled goat anti-human IgE antibodies. A 0.8 µg portion of protein was applied to each lane. For CBB protein staining, 4 µg of protein was applied to each lane. N, Subfractions obtained from crude allergen from wheat flour of Nisshin Seifun; S, those of Showa Sangyo. Subfractions * and [D] were not apparently present in crude allergen from Showa Sangyo.

whereas salt-insoluble gluten fractions, including glutenin and gliadin, and supernatant obtained by (NH₄)₂SO₄ fractionation did not react with pooled serum by immunoblotting (results not shown). The pooled patient serum was used for detection of allergens in the following experiments.

Isolation of the major allergens

The crude allergen fraction was further separated by gel-filtration chromatography on a Toyopearl HW-50 column. As shown in the top panel of Figure 1, four major fractions, A–D, were obtained. The crude allergen fraction obtained from wheat flour from Showa Sangyo gave a similar elution profile on the column, with poor separation of C and D (results not shown). The reactivities of each fraction with pooled serum are shown in the bottom panel of Figure 1. From a comparison of staining intensity and protein content of fractions A–D, based on the A₂₈₀ value in the elution profiles in Figure 1 (top), the major allergens were considered to be present in fraction C.

Fraction C was further subjected to reverse-phase HPLC on a C-4 column and three fractions, C-a, C-b and C-c, were obtained as shown in Figure 2(a). Each of these fractions gave a single band on SDS/PAGE and showed high immunoreactivity with IgE in pooled serum, as shown in Figure 2(b). When fraction D was applied to a C-4 column, most of it was eluted at the same elution position as C-b (results not shown). Considering their

Figure 2 Purification of the subfraction C by reverse-phase HPLC

(a) Subfraction C prepared from Nisshin Seifun wheat flour was purified by reverse-phase HPLC on a Vydac PROTEIN C-4 column (0.46 cm × 25 cm). Elution was performed as described in the text. (b) Reactivity of the purified allergens with pooled serum. Western blotting was performed as described in the text. Immunostaining was performed with pooled serum and alkaline phosphatase-labelled goat anti-human IgE antibodies. C-a (2 µg of protein), C-b (1.6 µg of protein) and C-c (0.8 µg of protein) was applied to each lane. For CBB protein staining, C-a (10 µg of protein), C-b (8 µg of protein) and C-c (4 µg of protein) was applied to each lane.

Table 1

*, Identical amino acids; †, homologous substitutions; N, potential N-glycosylation sites. Sequence data for 0.19 AI, 0.28 AI and trypsin inhibitor CME precursor protein from barley are from references [18], [19] and [20] respectively.

Fraction	Sequence
C-a	SGP ⁵ WM CYPGQ AFQVP ALPAC RPLLR LQCNG ³⁰
0.19 AI	SGP ⁵ WM CYPGQ AFQVP ALPAC RPLLR LQCNGS ³⁰
C-b	SGP ⁵ WS WCNPA TGYKV SALTG CRAMV KLQCV ³⁰
0.28 AI	SGP ⁵ WS WCNPA TGYKV SALTG CRAMV KLQCV ³⁰NPS ¹⁰⁶
C-c	SFREQ CYPGR E I TYE CLNAC AEYAV RQ ²⁵
Trypsin inhibitor CME precursor protein from barley	SFGDS CAPGD ALPHN PLRAC RTYVV SQ ⁵⁰

relative amounts, C-a and C-b are considered to be major allergens and C-c to be a minor one.

Identification of the major allergens

The N-terminal sequences of the purified allergens were analysed and aligned with proteins of known sequence homologies (Table 1). The N-terminal 30 amino acid sequences of C-a and C-b were in perfect agreement with those of 0.19 and 0.28 AI respectively [18,19]. The molecular sizes estimated by SDS/PAGE are 14 and 13 kDa (Figure 2b) and those measured by MS are 13365 Da and 13336 Da for C-a and C-b respectively, as shown in Figure 3. The molecular sizes calculated from amino acid sequences of 0.19 and 0.28 AI are 13337 and 13326 Da respectively. On the

Figure 3 MS of fraction C-a, recombinant 0.19 AI expressed in *E. coli* and C-b

Procedure was as described in the text. Inset: magnification of a signal of C-a. Peak broadening observed corresponds to 162 Da.

basis of these findings, the major allergens for baker's asthma from wheat flour are identified as 0.19 and 0.28 AI. As to C-c, no known protein has a sequence identical with its N-terminal sequence, but trypsin inhibitor precursor protein from barley contains a 27-amino acid sequence having 41% similarity to it, and 22 of 27 amino acids are related including homologous substitutions [20].

CAST of 0.19 and 0.28 AI

As shown in Figure 4, significant release of sulphidoleukotrienes was observed with both C-a (0.19 AI) and C-b (0.28 AI) using serum of a RAST 6 patient. The SI values were 4.5 for C-a and C-b at a concentration of 10 µg/ml. The stimulation levels achieved were significantly higher than the SI obtained using normal serum with these proteins, that is less than 1.9 in the concentration range 15–0.04 µg/ml. Release of sulphidoleukotrienes was observed with wheat albumin fraction using serum from a RAST 6 patient, but the SI were relatively low compared

Figure 4 CAST of a patient's serum

Procedures were as described in the text. One patient's serum (RAST 6) and sera from five healthy individuals were used. The symbols used are: stimulation of patient's serum with fraction C-a (—□—), fraction C-b (—○—), wheat albumin fraction (—■—) and house dust mite *Dermatophagoides farinae* (—▲—), and stimulation of normal serum with fraction C-a (---□---) and fraction C-b (---○---).

with that of the purified proteins. It was probably due to the low concentration of these proteins in the albumin fraction. These results suggest that both 0.19 and 0.28 AI act as allergens *in vivo*. The SI values for C-a and C-b were less than 2 when serum from a RAST 2 patient was used (results not shown). The low SI in this case may be the result of the immunotherapy.

Carbohydrate analysis of 0.19 and 0.28 AI

Total sugar content was less than 1% (w/w) for both fractions C-a and C-b, and it consisted mainly of xylose and trace amounts of glucose and fucose. The mass spectrum of C-a in some batches showed a broadening of the signal corresponding to about 160 Da, as shown in Figure 3 (inset), with intensities varying by batch, suggesting that covalent modification with a sugar residue may partially occur. These inhibitors are therefore considered to contain sugar residues at less than 1 mol/mol of protein, but the glycosylation site and type of linkage are not known. As shown

Figure 5 Reactivities of patients' IgEs and anti-(*Cry j I*) IgGs with plant glycoproteins

SDS/PAGE and electroblotting were performed as described in the text. (I) Protein staining. Lane A, HRP (6 µg); lane B, *C. trichotomum* lectin (6 µg); lane C, fraction C-a (4 µg); lane D, fraction C-b (4 µg). (II) Immunostaining was performed with anti-(*Cry j I*) IgG and HRP-labelled goat anti-rabbit IgG antibodies. Lane A, HRP (1.2 µg); lane B, *C. trichotomum* lectin (1.2 µg); lane C, fraction C-a (0.8 µg); lane D, fraction C-b (0.8 µg). (III) Immunostaining was performed with pooled serum and alkaline phosphatase-labelled goat anti-human IgE antibodies. Amounts applied were the same as in (II).

Figure 6 Immunoreactivity of recombinant 0.19 AI with pooled serum

(a) Western blotting. For protein staining, 4 µg of protein was applied to each lane. Immunostaining was performed with pooled serum and alkaline phosphatase-labelled goat anti-human IgE antibodies. A 0.8 µg quantity of protein was applied to each lane. Lane A, fraction C-a; lane B, recombinant 0.19 AI expressed in *E. coli*. (b) ELISA with MMAC was performed as described in the text. □, C-a; ■, recombinant 0.19 AI.

in Figure 5(II), the binding assay of membranes indicated that neither C-a nor C-b was stained with the rabbit anti-(*Cry j* I) IgG antibody which recognizes xylose/fucose-containing *N*-linked oligosaccharides [12], suggesting that this type of *N*-linked oligosaccharide does not occur on these proteins in spite of the presence of a consensus *N*-glycosylation sequence, N-X-S, in the peptide backbone. These proteins did not react with HRP-labelled concanavalin A, lentil lectin, wheat germ lectin or phytohaemagglutinin E4 (results not shown). The pooled serum was tested for reactivity with plant glycoproteins possessing xylose/fucose-containing *N*-linked oligosaccharides, HRP and *C. trichotomum* lectin [9,21]. As shown in Figure 5(III), the pooled serum did not react with these glycoproteins, suggesting that the glycans are not an allergenic epitope for the patients tested.

Immunoreactivity of recombinant 0.19 AI

ELISA without MMAC failed to detect the interaction of both native and recombinant 0.19 AI with patients' IgE, suggesting that the direct immobilization of these proteins resulted in poor immobilization efficiency on to the wells without MMAC. Recombinant 0.19 AI expressed in *E. coli* showed a reactivity to patients' IgE similar to that of wheat 0.19 AI by Western blotting, as shown in Figure 6(a). ELISA using MMAC for immobilization of antigens revealed equal reactivities of native and recombinant 0.19 AI with IgE, as shown in Figure 6(b). These findings suggest that covalent glycosylation did not play an essential role in the reactivity of 0.19 AI with IgE.

DISCUSSION

In this study, the major allergens of baker's asthma patients were purified and identified as 0.19 and 0.28 AI. 0.19 AI has not been considered to be an important allergen for baker's asthma [1–5], although it was predicted that it might become a potent allergen if it coexisted with 0.53 AI [22]. In this work, 0.19 AI alone reacted strongly with IgE on ELISA and Western blotting, and induced sulphidoleukotriene release in the CAST analysis. Compared with Western blotting, ELISA and in particular CAST are

important methods of evaluating allergenicity *in vivo*, because the activity of allergens in native conformations can be measured by these methods, and the results of CAST do not always parallel RAST scores. However, ELISA and CAST had not been performed in the previous studies. In this work, 0.19 and 0.28 AI showed similar releasability of leukotrienes on CAST analysis. Major components of fractions C and D were identified with 0.19 and 0.28 AI respectively, and the relative content of 0.19 AI can be estimated to be twice that of 0.28 AI in the flour extract. Another immunoreactive protein, a homologue of a barley trypsin inhibitor precursor protein, was also found. The content of this unidentified protein was estimated from the elution pattern in Figure 2(a) to be a quarter of that of 0.19 AI, and thus its contribution as an allergen would be small compared with those of 0.19 and 0.28 AI.

0.28 AI has been reported to be a major allergen [22], and the N-terminal 9–26 residues have been proposed as a possible epitope sequence [6]. However, the epitopic structure of 0.19 AI remains unknown. There is 60% similarity throughout the amino acid sequence between 0.19 AI and 0.28 AI proteins and only 33% similarity in the N-terminal 9–26 residues.

Recently glycan moieties of barley and wheat glycosylated AIs, BMAI-1 and WTAI-CM16* respectively, were reported to play a critical role in the reactivity with IgEs of patients with baker's asthma [3,7]. These AIs were predicted to have *N*-linked oligosaccharides with β 1-2 xylose and α 1-3 fucose residues [7]. Interestingly, carbohydrate analysis, immunoblotting with anti-(*Cry j* I) IgGs and lectin-binding assays showed that neither 0.19 nor 0.28 AI possessed the *N*-linked oligosaccharides in spite of the fact that they both have one potential *N*-glycosylation site. The IgEs in allergic sera examined in this study did not cross-react with plant glycoproteins, HRP or *C. trichotomum* lectin, whereas both had *N*-linked oligosaccharides with β 1-2 xylose and α 1-3 fucose residues [9,21]. Furthermore, recombinant 0.19 AI showed the same IgE reactivity as wheat 0.19 AI. These results suggest that the allergenic epitopes of the patients examined here are in peptide portions and not in glycan moieties. When crude allergen subfractions were subjected to Western blotting using anti-(*Cry j* I) IgGs, multiple protein bands of 50–60 kDa in subfraction A1 reacted strongly, but low-molecular-mass bands in subfractions A–D showed very weak reactivities, which were indistinguishable from non-specific binding (results not shown). Therefore WTAI-CM16* could not be found in the subfractions in this study. The reason why the contributions of oligosaccharides to allergenicity are observed differently in the work of Sanchez-Monge et al. [3] and this study is not clear, but it may be that the allergens recognized by IgEs are not identical in Japanese and Caucasian patients, who were examined in the previous report, although the numbers of patients in the two studies are not sufficient to discuss the possibility of a racial difference.

The allergens for patients with atopic dermatitis with wheat allergy have been reported to be present in both saline-soluble and -insoluble fractions. The molecular masses of IgE-reactive bands in the saline-soluble fraction were around 15 kDa on SDS/PAGE [23], which is close to the molecular mass of the major allergens for baker's asthma found in this study. The epitopic sequences have been reported in gluten for patients with atopic dermatitis [24] and that in gliadin for patients with coeliac disease [25], but a major allergen in the saline-soluble fraction has not yet been elucidated. It would be interesting to know whether the major allergens for baker's asthma are commonly allergenic to atopic dermatitis. The reactivity with atopic dermatitis IgE and the epitopic structure of 0.19 AI are under investigation using the recombinant protein.

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