Primary structure and allergenic activity of trypsin inhibitors from the seeds of buckwheat (*Fagopyrum esculentum* Moench)

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Abstract The complete amino acid sequences of two trypsin inhibitors BWI-2a and BWI-2b from the seeds of buckwheat (*Fagopyrum esculentum* Moench) were determined. BWI-2b consists of 51 amino acid residues containing two disulfide bonds. BWI-2a shares all amino acids with BWI-2b except for the C-terminal tripeptide: BWI-2a lacks Glu-Gly-Asn and ends with the Asp residue, making a total of 48 residues in the chain. The two disulfide bonds connect Cys^{11} to Cys^{32} and Cys^{15} to Cys^{28}. BWI-2b shows no relatedness to the other buckwheat trypsin inhibitor reported [Belozersky et al. (1995) FEBS Lett. 371, 264–266]. Sequence comparison of BWI-2b with those of the other proteins included in PIR showed that BWI-2b is significantly homologous to the N-terminal region of storage proteins classified in the vicilin family. Furthermore, the allergenic activity of BWI-2b and the other buckwheat trypsin inhibitor BWI-1 was examined using the radioallergosorbent test. The result indicated that both inhibitors BWI-2b and BWI-1 have IgE binding activity, albeit to a low extent, suggesting that they might be minor allergenic proteins in buckwheat seeds.

Key words: Buckwheat trypsin inhibitor; *Fagopyrum esculentum* Moench; Amino acid sequence; Allergenic protein

1. Introduction

Proteinase inhibitors are of widespread occurrence in the plant kingdom and have been isolated from various sources. Although the biological function of proteinase inhibitors is not yet understood, the proteinase inhibitors in some nutritive seeds are known to be an anti-nutritional factor which would seem to affect the nutritive value [1,2].

Buckwheat is an important food for both humans and domestic animals [3], but its low digestibility, possibly due to the presence of proteinase inhibitors, has been reported[4]. Ikeda and Kusano [5,6] and Kiyohara and Iwasaki [7] have isolated three and seven, respectively, trypsin inhibitors from buckwheat seeds; Kiyohara and Iwasaki classified seven trypsin inhibitors, on the basis of the pH dependency of their inhibitory activities, into two groups: one group includes the permanent inhibitors with leucine as the N-terminal amino acid, the other containing the temporary inhibitors with serine as the N-terminal amino acid [8].

Apart from the nutritional interest, the proteinase inhibitors in buckwheat seeds have been suggested to be one of the allergenic proteins which cause highly explosive allergy [9]. Although buckwheat allergy is not very common, patients are affected with severe symptoms by very small amounts of allergens by either ingestion or inhalation.

In order to understand buckwheat trypsin inhibitors in detail, we have started to analyze their amino acid sequences. During the course of this study, Belozersky et al. [10] reported the complete amino acid sequence of buckwheat trypsin inhibitor BWI-1, which corresponds to one of the permanent inhibitors described by Kiyohara and Iwasaki, and showed that BWI-1 is a member of the proteinase inhibitor I family in plants.

In this study, we determined the primary structure of the buckwheat trypsin inhibitors, referred to as BWI-2a and BWI-2b. Furthermore, we examined the allergenic activity of the buckwheat trypsin inhibitors by the radioallergosorbent test (RAST).

2. Materials and methods

2.1. Materials

Seeds of buckwheat (*Fagopyrum esculentum* Moench) were purchased from Takii Seeding Co. (Japan). Bovine pancreatic trypsin was obtained from Sigma Chemical Co., (USA). Substrate for trypsin, Bz-L-Arg-pNA, was obtained from the Peptide Institute Co. (Japan). All other chemicals were of analytical grade.

2.2. Isolation of trypsin inhibitors

Defatted flour from seeds of buckwheat (200 g) was extracted by vigorous agitation with 10 mM sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl for 2 h at 4°C and the supernatant solution was isolated by centrifugation for 20 min at 13 000 rpm. The following operations were performed at 4°C. The protein solution was salted out with 100% ammonium sulfate and after 24 h, the precipitate was collected by centrifugation. After dialysis against 10 mM phosphate, pH 7.5, the concentrated protein was applied to a Sephadex G-50 column (3×118 cm) which was equilibrated with the same buffer. The fraction containing trypsin inhibitory activity was further chromatographed on a DEAE-cellulose column (2.2×40 cm) previously equilibrated with 10 mM Tris-HCl buffer, pH 8.0, and subsequently eluted by a linear gradient of NaCl from 0 to 0.3 M in the buffer. The fraction was finally purified by FPLC using a Mono-Q column and RP-HPLC using a Wakosil C18 column (4.6×250 mm).

2.3. Assay of inhibitory activity

All measurement were made in 96-well microtiter plates at 0.6 mM substrate in 50 mM Tris-HCl, pH 7.5, containing 0.15 M $CaCl_2$. The enzyme solution (15 μl) was incubated for 10 min at room temperature with inhibitor (10 μl). Thereafter, 150 ml of substrate solution was added and the residual enzyme activity was measured at 414 nm as described by Ikeda and Kusano [5].

2.4. Mass spectrometry

The molecular weight of the inhibitor was determined using a Voyager-RP (PerSeptive Biosystems, USA) matrix-assisted laser desorption and ionization time-of-flight mass spectrometer (MALDI-

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Abbreviations: MALDI-TOF-MS, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry; PE, pyridylethyl; RAST, radioallergosorbent test; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; SCB, sodium carbonate buffer; PBS, phosphate-buffered saline.

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TOF-MS). In this analysis, α-cyano-4-hydroxycinnamic acid was used as matrix.

2.5. Reduction and alkylation

The inhibitor was reduced and alkylated by 4-vinylpyridine as described by Thomson and Bayne [11]. The pyridylethylated (PE) protein was purified by RP-HPLC using a YMC gel C4 column (4.6×250 mm). The protein was eluted with a linear gradient from 0 to 56% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 30 min at a flow rate of 1.0 ml/min, and each peak was collected manually. The effluents were monitored by ultraviolet absorption at 220 nm.

2.6. Enzymatic digestion and separation of peptide

Limited proteolysis with trypsin was performed for 24 h at pH 4.0, using an enzyme/substrate weight ratio of 1:50. Lysylendopeptidase digestion was carried out as follows: BWI-2b was first reduced and alkylated, and then digested with the enzyme (enzyme/substrate, 1:50) in 0.2 M *N*-methylmorpholine acetate buffer, pH 8.1, at 37°C for 6 h. For placement of disulfides, BWI-2b was dissolved in 0.2 M *N*-methylmorpholine acetate buffer, pH 8.1, containing 2 M urea and digested with trypsin (enzyme/substrate, 1:50) at 37°C for 12 h. Separation of the peptides derived from enzymatic digestion was achieved by RP-HPLC on a Wakosil C18 column (4.6×250 mm) using an acetonitrile gradient in aqueous TFA, as described above.

2.7. Amino acid composition and sequence analysis

Proteins and peptides were hydrolyzed in the vapor of 5.7 N HCl containing 0.02% 2-mercaptoethanol at 110°C for 24 h in evacuated sealed tubes. The amino acids were analyzed in an LC6A system amino acid analyzer (Shimadzu, Japan) after derivatization with PITC, as described by Heinrikson and Meredith [12]. The amino acid sequence was determined on a PSQ-1 gas-phase protein sequencer (Shimadzu, Japan). The C-terminal amino acid sequence were determined by carboxypeptidase Y digestion [13] in pyridine acetate buffer, pH 5.0, using an enzyme/substrate ratio of 1:50 followed by MALDI-TOF-MS analysis.

2.8. Specific IgE antibodies for trypsin inhibitors

Specific IgE antibodies for BWI-1a and BWI-2b were measured by radioallergosorbent test (RAST) [14]. Briefly, a flexible polyvinylchloride microtiter plate (Sumitomo Bakelite, Japan) was used as the solid phase. 10 μg of antigen in 100 ml of 0.1 M sodium carbonate buffer (SCB), pH 9.8, was placed into each well and the plate was kept overnight at 4°C in a moist chamber. The wells were then blocked with SCB containing 2% human serum albumin (Sigma Chemical Co., USA) for 3 h at room temperature. After three washes with 0.1 M PBS containing 1% Tween 20 (PBS-Tween), 50 μl of 2-fold diluted serum from a buckwheat-sensitive patient was placed in the wells for 12 h at room temperature. After four washes with PBS-Tween, 50 μl of ^{125}I-labeled anti-human IgE (Dainbot, Japan), approx. 2200 Bq, was incubated in the wells for 16 h. After four additional washes, each well was measured for bound radioactivity of ^{125}I-labeled anti-human IgE in a gamma-counter (Packard, USA). RAST values were expressed as the percentage of total radioactivity added. Values exceeding the mean plus two standard deviations of the percentage binding of non-atopic controls were considered as RAST positive.

2.9. RAST inhibition

Five sera from subjects with high IgE antibodies against crude buckwheat antigen were used in RAST inhibition studies. A mixture of serum (25 μl) and solutions (25 μl) containing buckwheat, BWI-2b or BWI-1 as an inhibitor, was added to the well. The concentrations of buckwheat used were 10-fold serial dilutions from 10 to 1000 mg/ml. The procedure followed was the same as the RAST assay using crude buckwheat antigen as a solid phase. The percentage of RAST inhibition was expressed as [1 ± (cpm bound to an inhibited well/cpm obtained without an inhibitor)]×100%. A semilogarithmic plot enabled an estimate of the concentration of inhibitor required for 50% inhibition to be made.

3. Results and discussion

3.1. Isolation of trypsin inhibitors

Fig. 1 shows the pattern of elution of the buckwheat trypsin inhibitors on DEAE-cellulose column chromatography. Further chromatography of the trypsin inhibitors included in fractions 1–3 on a Mono Q column and RP-HPLC yielded four trypsin inhibitors with molecular masses of 6–7 kDa, as judged from Tricine-SDS-PAGE. Two of the trypsin inhibitors (referred to as BWI-1a and BWI-1b) isolated from fractions 1 and 2 have leucine as the N-terminal amino acid residue, whereas the other two (referred to as BWI-2a and BWI-2b) isolated from fractions 2 and 3 have serine as the N-terminal amino acid residue. Kiyohara and Iwasaki previously isolated seven trypsin inhibitors and classified them into two groups, viz. permanent inhibitors with leucine and temporary inhibitors with serine as the N-terminal amino acids. Hence, BWI-1a and BWI-1b, and BWI-2a and BWI-2b correspond to permanent and temporary inhibitors, respectively, as reported by Kiyohara and Iwasaki.

The amino acid compositions and molecular weights evaluated by MALDI-TOF-MS of four inhibitors are given in Table 1. The amino acid compositions of BWI-1a and BWI-1b agreed well with those of the permanent inhibitors, while the compositions of BWI-2a and BWI-2b were different from those of the temporary inhibitors. The number of residues in the temporary inhibitors reported by Kiyohara and Iwasaki appears to be slightly overestimated for all amino acids, as the authors assumed a molecular mass of 10–11 kDa instead of 6 kDa for BWI-2a and BWI-2b [8].

3.2. Amino acid sequence

Since Belozersky et al. very recently reported the complete amino acid sequence of buckwheat trypsin inhibitor BWI-1 which corresponds to BWI-1a in this study, the amino acid sequence determinations of BWI-2a and BWI-2b were carried out as described below.

Direct gas-phase sequencing of two inhibitors could identify the amino acid sequences up to 25 positions; the result indicated that two inhibitors had identical N-terminal amino acid sequences. Since BWI-2a and 2b showed very similar amino acid compositions and identical N-terminal sequences, BWI-2a was expected to be the C-terminal truncated form of BWI-

Table 1
Amino acid composition of inhibitors from buckwheat seeds

Amino acid	BWI-1a	BWI-1b	BWI-2a	BWI-2b
Asp/Asn	6.9	6.0	4.5	7.3
Glu/Gln	8.2	8.9	9.2	11.8
Cys	–	–	–	–
Ser	1.1	1.3	1.5	2.7
Gly	6.4	7.2	5.3	5.5
His	–	–	1.3	1.0
Arg	9.7	9.2	9.4	8.2
Thr	–	–	–	–
Ala	5.3	5.1	1.1	–
Pro	6.3	5.7	2.4	1.8
Tyr	–	0.5	0.7	0.6
Val	13.5	14.1	1.5	1.8
Met	0.7	–	0.5	0.2
Ile	3.0	2.9	1.5	2.7
Leu	4.0	3.6	4.2	3.6
Phe	1.4	1.5	1.7	1.0
Trp	–	–	–	–
Lys	3.7	4.1	2.8	2.7
M_r	7745.1	7708.6	5722.4	6029.5

Values denote the residues per molecule calculated on the basis of the molecular weights of the proteins. –, not detected.

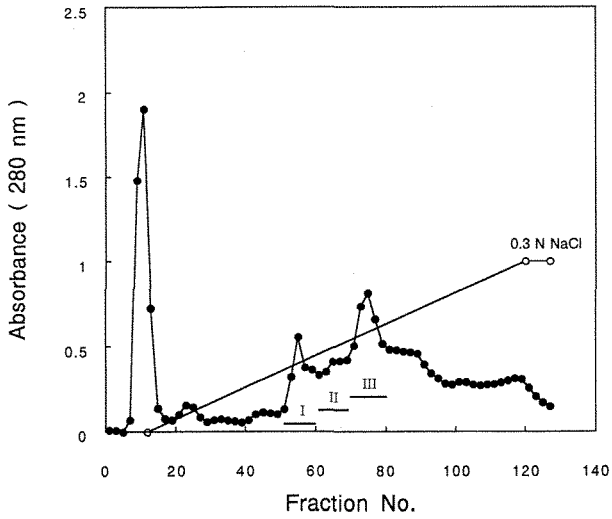

Fig. 1. Chromatography of buckwheat trypsin inhibitors on DEAE-cellulose. The buckwheat trypsin inhibitors were separated by Sephadex G-50 put on a DEAE-cellulose column (2.2×40 cm) previously equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The proteins were eluted with a linear gradient of NaCl from 0 to 0.3 M in the same buffer. The fraction containing trypsin inhibitory activity was pooled as indicated by the horizontal bars.

2b. Hence, the amino acid sequence of BWI-2b was first determined.

It is known that a trypsin inhibitor can be specifically cleaved at the scissile bond by trypsin under acidic conditions, i.e. pH 4.0 [15]. Thus, BWI-2b was first digested with trypsin at pH 4.0, and the resulting peptides were pyridylethylated and separated by RP-HPLC. Sequencing of two peptides (T-1 and T-2) thus obtained led to determination of 19 and 20 residues for peptides T-1 and T-2, respectively (Fig. 2). The result showed that the peptide bond between Arg^{19} and Trp^{20} was specifically cleaved with trypsin and therefore Arg^{19}-Trp^{20} was found to be the reactive site of BWI-2b for trypsin. This analysis could determine almost all amino acids except for those at the C-terminal part of the protein. To establish the C-terminal amino acid sequence of BWI-2b, pyridylethylated BWI-2b was further digested with lysylendopeptidase and the resulting peptides, which were separated by RP-HPLC, were sequenced. This analysis completed the amino acid sequence of BWI-2b as shown in Fig. 2.

The amino acid sequence of BWI-2a was determined using an identical procedure to those described for BWI-2b. The result revealed that BWI-2a had the same amino acid sequence as BWI-2b except for the three C-terminal amino acid residues: BWI-2a lacks the tripeptide Glu-Gly-Asn as compared with BWI-2b. This was further confirmed by C-terminal amino acid sequencing of BWI-2a and 2b using carboxypeptidase Y followed by MALDI-TOF-MS analysis. The analysis of BWI-2b showed the C-terminal sequence to be Glu-Gly-Asn (Fig. 3A), which is good agreement with that determined by the protein chemical method. As for BWI-2a, the C-terminal amino acid sequence: Ser-Ser-Asp, was deduced by MALDI-TOF-MS analysis, demonstrating that BWI-2a is the C-terminal truncated form of BWI-2b. The molecular masses of BWI-2a and BWI-2b calculated from sequences are 5730 and 6030 Da, respectively; these values agree well with those estimated by MALDI-TOF-MS, as described above.

3.3. Placement of disulfide bonds

Limited proteolysis of BWI-2b with trypsin did not yield the two peptides T-1 and T-2 without reduction and pyridylethylation (data not shown). It was therefore concluded that two cysteine residues, in peptides T1 (Cys^{11} and Cys^{15}) and T2 (Cys^{28} and Cys^{32}), were not connected by disulfide bonds. In order to locate the disulfide bonds in the chain, BWI-2b was dissolved in 0.2 M *N*-methylmorpholine buffer, pH 8.1, containing 2 M urea, digested with trypsin, and the resulting peptides were separated by RP-HPLC. This analysis gave two cystine-containing peptides TU-1 and TU-2: the amino acid compositions of TU-1 and TU-2 were in good agreement with those of the peptide from Ser^{1} to Arg^{12} and Cys^{32} to Arg^{38}, and of the peptide from Tyr^{13} to Arg^{16} and Cys^{28} to Lys^{31}, respectively. The direct sequencing of these peptides TU-1 and TU-2 identified PTH-cystine at the 11th and 3rd steps, respectively: PTH-cystine is eluted from the Wakosil PTH column (4.6×250 mm) around 8 min with an isocratic system using a PTH-amino acid mobile phase (Shimadzu, Japan). In this way, it was determined that two disulfide bonds connect Cys^{11} to Cys^{32} and Cys^{15} to Cys^{28}.

3.4. Sequence comparison

Sequence comparison of BWI-2b with that of the other buckwheat trypsin inhibitor BWI-1 determined by Belozersky et al. showed no relatedness with each other. A homology search in PIR using the computer program BLAST found that BWI-2b shows a considerable homology to storage proteins classified in the 7S vicilin-type globulin family [16]: they are a widely distributed group of storage proteins not only in dicotyledons but also in monocotyledons. In particular, BWI-2b is highly related to the N-terminal region derived from the first exon of the cotton (*Gossypium hirsutum*) vicilin gene [17], sharing 14 identical residues, i.e. 29% positions compared, and

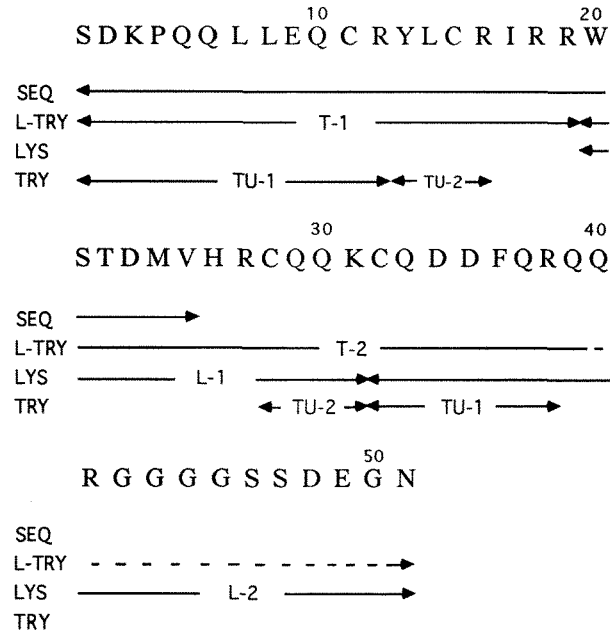

Fig. 2. Amino acid sequence of BWI-2b. SEQ indicates the amino acid residues identified by gas-phase sequencer. L-TRY, LYS, and TRY indicate the peptides obtained by limited tryptic, lysylendopeptidase, and tryptic digestions, respectively.

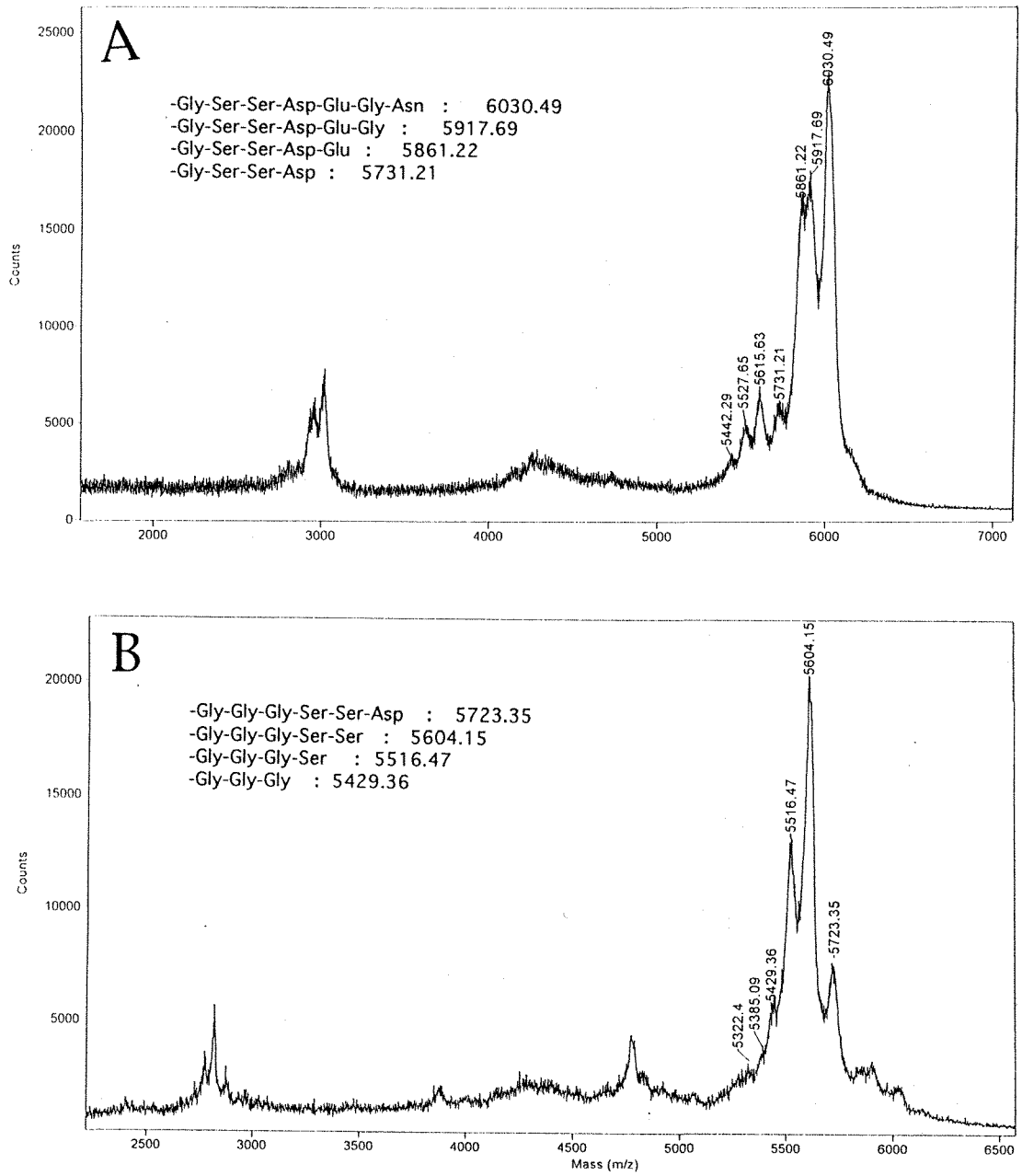

Fig. 3. C-terminal sequences of BWI-2a and 2b by carboxypeptidase Y digestion followed by MALDI-TOF MS analysis. MS spectrometry analysis of (A) BWI-2b and (B) BWI-2a.

at a considerable number of the other positions, there are chemically similar residues, as aligned in Fig. 4. This finding suggests that BWI-2b might be derived from the first exon of the ancestral vicilin gene or that the ancestral gene for BWI-2b might have given rise to the first exon of the present vicilin gene.

3.5. *RAST and RAST inhibition*

It was reported that three proteins with molecular masses of 7–8 kDa were allergen proteins and that one of these proteins exhibited inhibitory activity toward trypsin [9]. Since the molecular weights of four trypsin inhibitors isolated in this study were similar to that of the allergen protein reported, the IgE

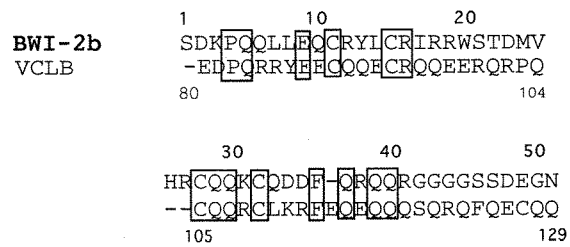

Fig. 4. Sequence comparison of BWI-2b with that of cotton vicilin. The amino acid sequence of BWI-2b was aligned with that of cotton vicilin [16] for maximum homology. Identical amino acid residues are boxed.

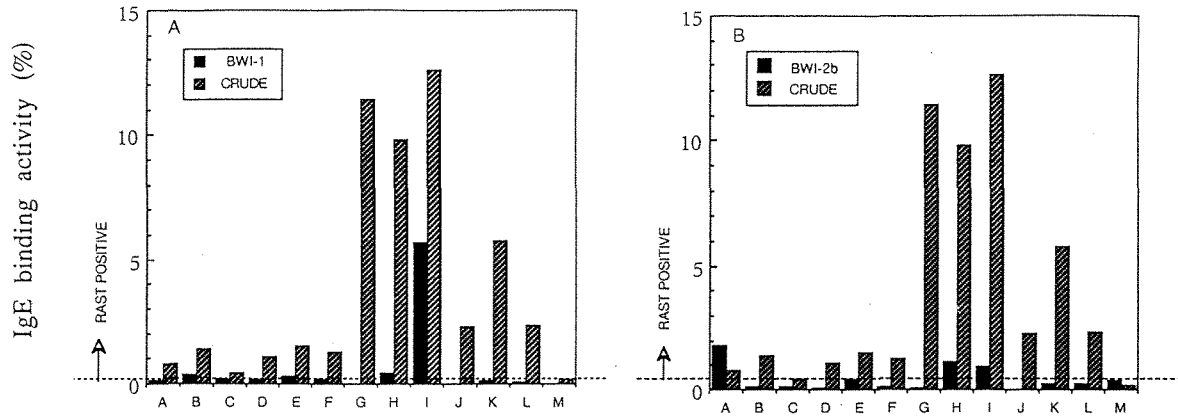

Fig. 5. Specific IgE binding activity of BWI-1 and BWI-2b evaluated by RAST. CRUDE indicates total proteins extracted from buckwheat seeds. The dotted lines denote the values of the mean plus two standard deviations of the percentage binding of non-atopic controls, as described in Section 2. Specific IgE binding activity of (A) BWI-1 and (B) BWI-2b.

binding activities of BWI-1, BWI-1a in this study, and BWI-2b were examined by RAST.

Seven (58%) and four (33%) out of 12 subjects (A–M) were RAST positive for BWI-1 and BWI-2b, respectively, as shown in Fig. 5. The percent binding for these antigens, however, was lower than those of crude buckwheat antigen. Significant RAST inhibition of more than 50% with BWI-1 and BWI-2b was observed in only one out of five sera tested (data not shown). These data indicate that BWI-1 and BWI-2b have IgE-binding activities but that the activities are not strong; they might be minor allergenic proteins in buckwheat seeds.

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