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Determination of the carbohydrate-binding site of *Bauhinia purpurea* lectin by affinity chromatography

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ABSTRACT

To determine the carbohydrate-binding site of *Bauhinia purpurea* lectin (BPA), a D-galactose- and lactose-binding lectin, a peptide which interacts with lactose was purified from endoproteinase Asp-N digests of BPA by chromatography on a lactose–Sepharose column. It consists of nine amino acids and its amino acid sequence is Asp–Thr–Trp–Pro–Asn–Thr–Glu–Trp–Ser. A tryptic fragment with the ability to interact with lactose was also purified and found to contain this sequence, consisting of nine amino acids. This nonapeptide was aligned in a part of the metal-binding region conserved in all legume lectins. The chemical synthesis of the nonapeptide was carried out by a solid-phase method and the synthetic peptide showed a lactose-specific binding activity in the presence of calcium. A chimeric lectin gene was constructed using a cDNA coding BPA in which the nonapeptide sequence was replaced by the corresponding region of the α-D-mannose binding *Lens culinaris* lectins. Although BPA is specific for β-D-galactose, the chimeric lectin expressed in *Escherichia coli* was found to bind α-D-mannosyl–bovine serum albumin and this binding was inhibited by D-mannose.

INTRODUCTION

Lectins, a class of proteins that bind sugars specifically, are widely distributed in nature [1]. Leguminous plant lectins resemble each other in their physico-chemical properties although they differ in their carbohydrate specificities. They usually consist of two or four subunits with a relative molecular mass of 25 000–30 000 and each subunit has one carbohydrate binding site. Interaction with carbohydrates requires tightly bound Ca^{2+} and Mn^{2+} [2]. The primary structures of *Lotus tetragonolobus* lectin [3], *Ulex europeus* agglutinin I and II [4], *Bauhinia purpurea* agglutinin (BPA) [5] and *Labrunum alpinum* lectin I [6] have already been determined by chemical or molecular genetic techniques. They show a remarkable homology with other legume lectins for which amino acid sequences have already been reported [7–16].

BPA consists of four subunits with a relative molecular mass of 30 000. This lectin is specific for the β-D-galactose (Gal) residue, especially for Galβ1-3-N-acetylgalactosamine [17]. cDNA coding BPA has already been cloned from the cDNA library constructed from the poly(A)$^+$ RNA from germinated *Bauhinia purpurea* seeds and the nucleotide sequence of BPA cDNA and its deduced amino acid sequence have been analysed [5]. The cloned cDNA coding BPA encodes a polypeptide of 290 amino acids, including a signal peptide composed of 28 amino acids.

This paper reports the purification and characterization of a nonapeptide consisting of the carbohydrate-binding site of BPA using affinity chromatography. In addition, it is shown that the synthetic nonapeptide specifically binds to lactose in the presence of calcium ions. To confirm the assumption that the nonapeptide is a part of the cabohydrate-binding site of BPA and defines the carbohydrate-binding specificity of this lectin, a chimeric lectin of BPA and *Lens culinaris* lectin (LCA) has been

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constructed and the alteration of the carbohydrate-binding specificity of BPA is shown.

EXPERIMENTAL

Materials

Bauhinia purpurea seeds were purchased from F. W. Schumacher Co. (Sandwitch, MA, USA). C_4 and C_{18} μBondasphere (100 Å) for reversed-phase chromatography were obtained from Waters (Burlington, MA, USA). Endoproteinase Asp-N from *Pseudomonas fragi* was purchased from Boehringer (Mannheim, Germany). Restriction enzymes were purchased from Boehringer or New England Biolabs (Beverly, MA, USA). Radioisotopes were obtained from Amersham (Buckingham, UK). Taq DNA polymerase was from Cetus (Norwalk, CT, USA). β-D-Galactosyl–bovine serum albumin (βGal–BSA), α-D-mannosyl–BSA (αMan–BSA), α-L-fucosyl–BSA (αFuc–BSA) and trypsin were obtained from Sigma (St. Louis, MO, USA) and Iodo-gen as an iodination reagent was from Pierce (Rockford, IL, USA). All other reagents used were of analytical-reagent grade.

Purification of BPA

Finely powdered *Bauhinia purpurea* seeds were extracted with 0.15 *M* NaCl. To the supernatant obtained by centrifugation, solid $(NH_4)_2SO_4$ was added to give 0.8 saturation. The precipitate formed was dialysed against phosphate-buffered saline (PBS; 10 m*M* phosphate buffer, pH 7.3 containing 0.15 *M* NaCl) and applied to a lactose–Sepharose 4B affinity column equilibrated with PBS. The column was washed with PBS and then eluted with 0.2 *M* lactose in PBS. The purity was checked with sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis by the method of Laemmli [18].

Affinity chromatography of peptides of BPA digested with endoproteinase Asp-N or trypsin on columns of lactose–Sepharose 4B

Purified lectins (0.2–0.5 mg in 200 μl of 50 m*M* Tris–HCl, pH 8.5) were digested with 2 μg of endoproteinase Asp-N or with 5 μg of trypsin for 18 h at 37°C. Endoproteinase Asp-N or trypsin digestion products obtained from BPA were applied to columns of lactose–Sepharose 4B (4.5 ml) without additional modification. After the column was washed with PBS at a flow-rate of 1.5 ml/h, the bound material was eluted with the same buffer containing 0.1 *M* lactose. Each 0.5-ml fraction was collected and analysed by reversed-phase high-performance liquid chromatography (HPLC) on a column of C_{18} using a linear gradient (0–60%) of propan-2-ol–acetonitrile (7:3) in distilled water containing 0.1% trifluoroacetic acid (TFA) in 30 min at a flow-rate of 1 ml/min. Amino acid sequence analyses of purified peptides were performed on a PSQ-1 gas-phase protein sequencer (Shimadzu, Kyoto, Japan).

Solid-phase peptide synthesis

Solid-phase peptide synthesis was carried out automatically on a 9020 peptide synthesizer (Milli-Gen/Bioserch, Burlington, MA, USA). Protected peptide resins were treated with 10% phenol in TFA to remove any protecting groups. The cleaved peptide products were precipitated with ether and collected by centrifugation. The crude peptides were immediately dissolved in water and purified by reversed-phase HPLC on a C_{18} column. The purified peptides were tritiated with [^{3}H]acetic anhydride (18.5 GBq/mmol) and purified by passage over a Sephadex G-25 column equilibrated in Tris-buffered saline (TBS; 10 m*M* Tris–HCl, pH 6.8, containing 0.15 *M* NaCl) containing 10 m*M* $CaCl_2$ and 10 m*M* $MnCl_2$.

Affinity chromatography of tritiated peptides on columns of lactose–, maltose–, L-fucose– and di-N-acetylchitobiose–Sepharose 4B

Amino-Sepharose 4B was prepared by the method of Matsumoto *et al.* [19]. Lactose, maltose, L-fucose and di-N-acetylchitobiose were coupled to amino-Sepharose 4B by reductive amination according to the method of Baues and Gray [20]. Tritiated synthetic peptides were applied to lactose–, maltose–, L-fucose– and di-N-acetylchitobiose–Sepharose 4B columns equilibrated with TBS containing 1 m*M* $CaCl_2$ and 1 m*M* $MnCl_2$ and eluted wtih the same buffer. Fractions of 0.4 ml were collected and their radioactivity was counted in a liquid scintillation counter.

Construction of the expression plasmid and expression of chimeric lectin of BPA–LCA in E. coli

The BPA coding region flanked by artificial sites

for *Nde*I and *Bam*HI was amplified by the polymerase chain reaction (PCR) using primers 5'GGCATATGACAATCTCAACCTTA3' (*Nde*I primer) and 5'GGATCCTGTTACATACTGGAATAAGAG3' (*Bam*HI primer) as described previously [5]. To construct a cDNA of the chimeric BPA–LCA lectin (which is a fusion protein of BPA, the metal-binding region of which is replaced by the corresponding region of LCA), the NH_2-terminal fragment of BPA cDNA, extending from nucleotides 1 to 405 of the BPA coding region and flanked by artificial sites for *Nde*I and *Fun4*HI, was first amplified by the PCR using primers 5'GGCATATGACAATCTCAACCTTA3' (*Nde*I primer) and 5'CGCTTGCTGCATTGTAGAAAGTGTCAAATTCATCATCAGC3' (*Fun*4HI primer-a). The COOH-terminal fragment of BPA cDNA from 429 to 786, flanked by artificial sites for *Fun*4HI and *Bam*HI, was also generated by the PCR using primers 5'CGCAGCATGGGACCTACGTTATCCACAT3' (*Fun*4HI primer-b) and 5'GGATCCTGTTACATACTGGAATAAGAG3' (*Bam*HI primer). These fragments were then digested with *Fun*4HI and ligated. The cDNA clone thus prepared was further amplified by the PCR using the same primers (*Nde*I and *Bam*HI primers) as just described. Finally, after digestion with *Nde*I and *Bam*HI, the PCR-generated DNA fragment was inserted between the *Nde*I and *Bam*HI sites of pET-3c to yield a plasmid pET-3c–BPA–LCA. The constructed plasmid was introduced into *E. coli* strain BL21(DE3) cells grown on an LB plate containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol. The BL21(DE3) cells containing the plasmid pET-3c–BPA–LCA were grown to mid log phase at 37°C in an LB medium containing 5 m*M* $CaCl_2$ and 5 m*M* $MnCl_2$ and then induced by adding isopropyl-β-D-thiogalactoside to a concentration of 1 m*M*. At the same time the temperature for the culture was shifted to 25°C. Six hours after the addition of isopropyl-β-D-thiogalactoside, *E. coli* cells were collected by centrifugation and the pellet was washed twice in PBS and then lysed with 1% SDS. The lysates were subjected to electrophoretic analysis using 10% polyacrylamide gels in the presence of 0.1% SDS. The chimeric and control lectins were purified from the cell lysates by reversed-phase HPLC on a column of C_4 under the same conditions as described previously [3]. The purified lectins were digested with an endoproteinase Asp-N at 37°C for 18 h. The peptide fragments thus obtained were separated by reversed-phase HPLC on a column of C_{18} using a linear gradient (0–70%) of propan-2-ol–acetonitrile (7:3) in distilled water containing 0.1% TFA in 60 min at a flow-rate of 1 ml/min [3]. The peptide fragments were collected manually. Sequence analyses of the intact protein and of the purified peptides were performed on a PSQ-1 gas-phase protein sequencer (Shimadzu, Kyoto, Japan).

Binding of ^{125}I-labelled β-D-galactosyl–BSA, α-D-mannosyl–BSA and α-L-fucosyl–BSA to the recombinant chimeric BPA–LCA lectin

βGal–BSA, αMan–BSA and αFuc–BSA were labelled wih ^{125}I by Iodo-gen according to the method of Fraker and Speck [21]. The specific activities of the ^{125}I-labelled βGal–BSA, αMan–BSA and αFuc–BSA were $1.55 \cdot 10^5$, $1.9 \cdot 10^5$ and $1.42 \cdot 10^5$ cpm/μg, respectively. *E. coli* expressing the recombinant chimeric BPA–LCA lectin was used for the binding experiments. The cells were lysed by freezing and thawing in 10 m*M* Tris–HCl, pH 6.8, containing 5 m*M* EDTA and 0.15 *M* NaCl, and then the lysate was treated with DNase I (50 μg/ml) at 37°C for 30 min. Suspensions of inclusion bodies containing recombinant chimeric lectins were prepared from the lysed *E. coli* cells by washing with 10 m*M* Tris–HCl, pH 6.8, containing 1% Triton X-100, 5 m*M* EDTA and 0.15 *M* NaCl and then suspended in 50 m*M* Tris–HCl, pH 6.8, containing 5 m*M* $CaCl_2$ and 5 m*M* $MnCl_2$. The washed inclusion bodies (100 μg) were incubated at 4°C for 1 h with ^{125}I-labelled βGal–BSA, αMan–BSA or αFuc–BSA in 0.25 ml of Tris–HCl, pH 6.8, containing 0.1% BSA, 1 m*M* $CaCl_2$ and 1 m*M* $MnCl_2$. After the incubation, the inclusion bodies were washed three times with the same buffer by centrifugation and then the radioactivity was counted.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of reversed-phase HPLC on a column of C_{18} of various fractions obtained by affinity chromatography of an Asp-N digest of BPA on a column of lactose–Sepharose 4B. The elution profile of the original Asp-N digest mixture is shown in the upper left-hand panel. The other panels show the elution profile of each fraction

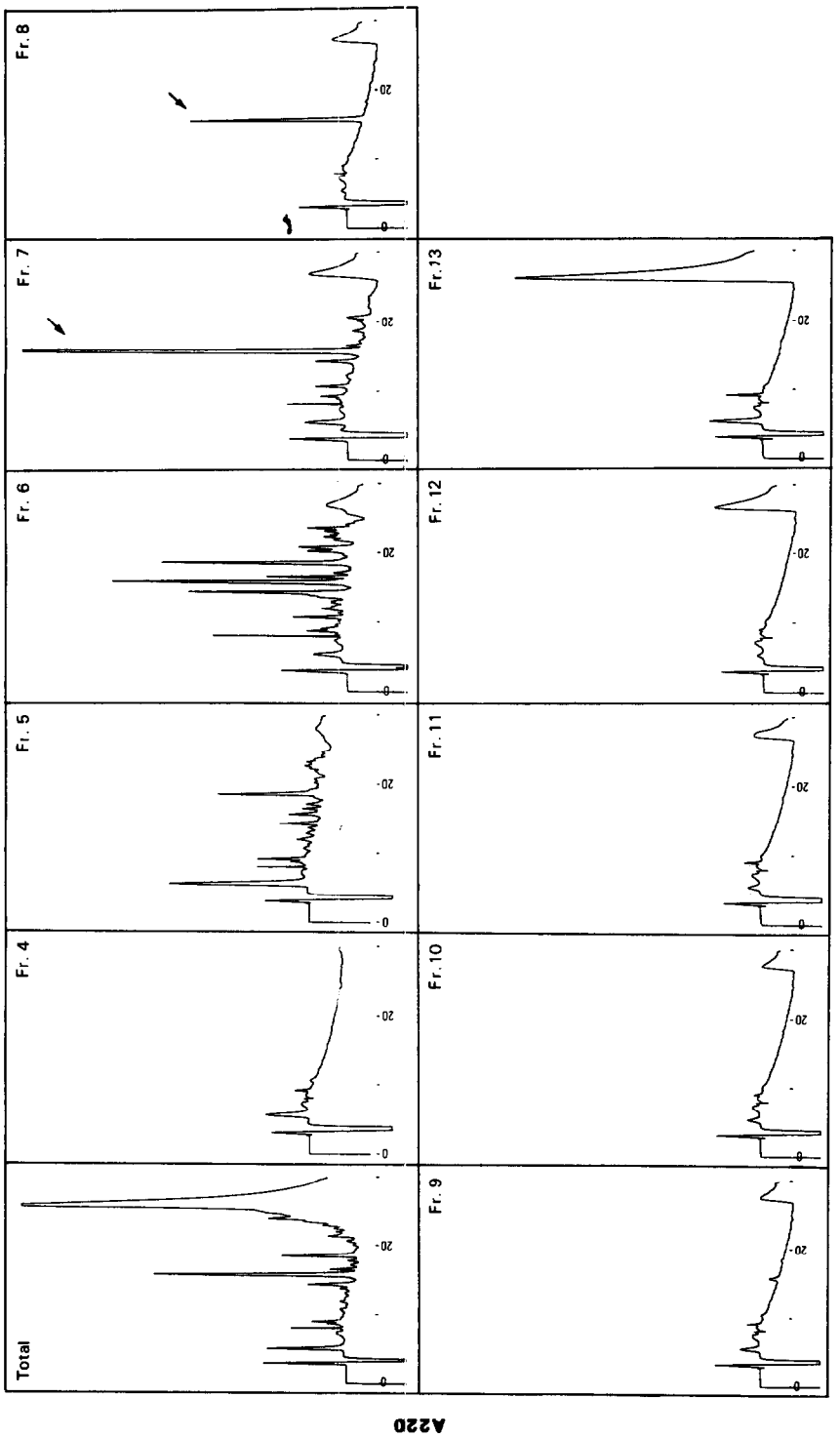

Fig. 1. Elution profiles of reversed-phase HPLC on a C_{18} column of the fractions obtained by affinity chromatography of an Asp-N digest of BPA on a column of lactose–Sepharose 4B. Purified BPA was digested with endoproteinase Asp-N at 37°C for 18 h. The reaction mixture was applied to a column of lactose–Sepharose as described under Experimental. After the column had been washed with PBS, the bound material (after fraction number 10) was eluted with the same buffer containing 0.1 *M* lactose. Each fraction was then analysed by reversed-phase HPLC on a C_{18} column as described under Experimental.

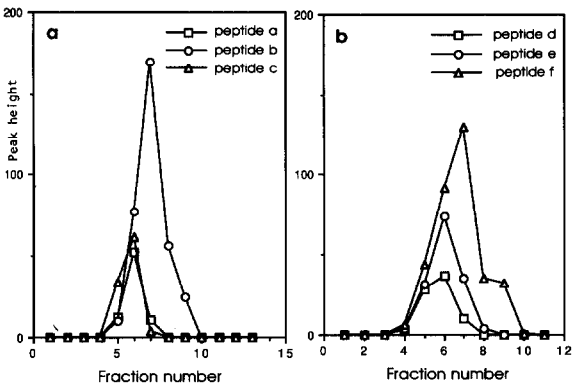

Fig. 2. Elution profiles of Asp-N and trypsin fragments of BPA on a lactose–Sepharose 4B column. Asp-N (a) or trypsin (b) fragments of BPA were determined by elution profiles on a C_{18} column shown in Fig. 1. Peptide a = DVPHITA; peptide b = DTWPNTEWS; peptide c = DPSKNQVVAVEF; peptide d = SFTSTLNSTK; peptide e = IGFSGGTGFNETQYILSW; peptide f = NQVVAVEFDTWPNTEWSDLR.

obtained by the affinity chromatography of an Asp-N digest of BPA on a column of lactose–Sepharose 4B. As shown in Fig. 2a, most of the fragments [for example, DVPHITA (peptide a) and DPSKNQVVAVEF (peptide c)] of BPA were recovered in fraction number 6. Undigested BPA bound to the column was eluted in fraction 13 by 0.2 *M* lactose. Interestingly, the peptide indicated by the arrow in Fig. 1 (peptide b in Fig. 2a) was retained on the column of lactose–Sepharose 4B and was eluted in fractions 7 and 8. This shows that this peptide specifically interacts with lactose. The peptide was purified again by reversed-phase HPLC on a C_{18} column and its amino acid sequence was determined by a gas-phase protein sequencer. The amino acid sequence of the peptide is shown in Fig. 3. It consists of nine amino acids and its amino acid sequence is DTWPNTEWS (BP-9). This peptide correspond to the fragment from aspartic acid-135 to serine-143

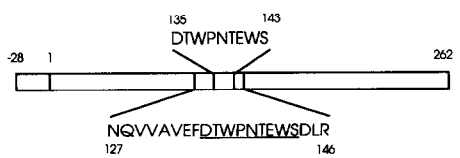

Fig. 3. Amino acid sequences of Asp-N and tryptic fragments of BPA retained on a column of lactose–Sepharose 4B.

based on the complete amino acid sequence of BPA reported elsewhere [5].

Tryptic fragments of BPA were then prepared and separated using these procedures. Most of the tryptic fragment [for example, SFTSTLNSTK (peptide d) and IGFSGGTGFNETQYILSW (peptide e)] of BPA was recovered in fraction 6, whereas a tryptic peptide (f in Fig. 2b) was retained on the column of lactose–Sepharose. The amino acid sequence of this tryptic peptide f is shown in Fig. 3. The tryptic fragment consists of 20 amino acids (NQVVAVEFDTWPNTEWSDLR) and it contains the same amino acid sequence as that of the Asp-N fragment described previously. When aligned with other legume lectins, these peptides were found to match to a relatively conserved region of legume lectins. Fig. 4 shows the local homology among legume lectins. This region seems to correspond to the metal-binding region, and for concanavalin A (Con A) X-ray crystallographic analysis showed that the metal ions bind to amino acid side-chains [Ca^{2+} to Asp-10, Tyr-12, Asn-14 and Asp-19 (bold letters in Fig. 4); Mn^{2+} to Glu-8, Asp-10, Asp-19, His-24, Val-32 and Ser-34 (outlined letters in Fig. 4)] [22]. This indicates that metal ions keep the conformation of this peptide and maintain its ability to bind lactose.

The crystal X-ray analyses of the complex of Con A with α-methyl-D-mannoside [23] and of the complex of *Griffonia simplicifolia* lectin IV (GSIV) with Le^b-OMe tetrasaccharide Fucα1-2Galβ1-3(Fucα1-4)-N-acetylglucasamine-OMe [7] were reported. In both instances, the carbonyl group of the Asn side-chain (Asn-14 for Con A, Asn-135 for GSIV) makes contact with the OH group of sugars via a hydrogen bond. In addition, for GSIV, Trp-133 and Trp-138 make three van der Waals contacts with Le^b-OMe oligosaccharides. These three amino acid residues (Asn-139, Trp-137, Trp-142 for BPA) are completely conserved in the BP-9 peptide of BPA. This indicates that the BP-9 peptide DTWPNTEWS may interact with lactose via the same hydrogen bonds and van der Waals forces. It is known that, of the six amino acids which are assumed to participate in the interaction of Con A with D-mannose, Asn-14 (which is involved in the binding with the positive calcium ion) and Asp-208 (which forms a hydrogen bond with an equatorial 4-OH group of D-mannose) are conserved in all

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BPA[a]      -VAVEFDTWP----NTEWSDLRYP---HIGINVNSTVSVAT-
                 *              *
GS4[b]      -VAVEFDTWI----NKDWNDPPYP---HIGIDVNSIVSVAT-
PHA-E[c]    -VAVEFDTLY----NKDW-DPT--ER-HIGIDVNSIRSIKT-
PHA-L[c]    -VAVEFDTLY----NVHW-DPK-P-R-HIGIDVNSIKSIKT-
ECorL[d]    -LGVEFDTFS----NP-W-DPPQVP--HIGIDVNSIRSIKT-
DBA[e]      -VAVEFDTLS----NSGW-DPSM-K--HIGIDVNSIKSIAT-
SBA[f]      -VAVEFDTFR----NS-W-DPPNP---HIGINVNSIRSIKT-
LTA[g]      -VAVEFDSYH----NI-W-DPKSLRSSHVGIDVNSIMSLKA-
LAA-I[h]    -IAVEFDTYFGKAYNP-W-DPD-FK--HIGVDVNSIKSIKT-
UEA-I[i]    -VAVEFDTIGSP-VNF-W-DPG-FP--HIGIDVNRVKSINA-
UEA-II[i]   -IAVEFDSYFGKTYNP-W-DPD-FK--HIGIDVNSIKSIKT-
PSA[j]      -VAVEFDTFY----NAAW-DPSNRDR-HIGIDVNSIKSVNT-
LCA[k]      -VAVEFDTFY----NAAW-DPSNKER-HIGIDVNSIKSVNT-
VFA[l]      -VAVEFDTFY----NAAW-DPSNGKR-HIGIDVNTIKSIST-
LOL[m]      -VAVEFDTFY----NTAW-DPSNGDR-HIGIDVNSIKSINT-
Con A[n]    -VAVELDTYP----NTDIGDPS-YP--HIGIDIKSVRSKKT-
              8  10 12        14      19          24        32 34
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Fig. 4. Amino acid homology of the BP-9 nonapeptide of BPA aligned with other legume lectins. Amino acid involved in calcium-binding (bold letters) and manganese-binding (outlined letters) are indicated. The bottom numbering is for the Con A sequence. Amino acid residues which bind both Ca^{2+} and Mn^{2+} are marked with asterisks. [a] Kusui *et al.* [5]; [b] Delbaere *et al.* [7]; [c] Hoffman and Donaldson [8]; [d] Arango *et al.* [9]; [e] Schnell *et al.* [10]; [f] Vodkin *et al.* [11]; [g] Konami *et al.* [3]; [h] Konami *et al.* [6]; [i] Komami *et al.* [4]; [j] Higgins *et al.* [12]; [k] Foriers *et al.* [13]; [l] Cunningham *et al.* [14]; [m] Yarwood *et al.* [15]; [n] Carrington *et al.* [16].

legume lectins sequenced so far. It is noteworthy that the asparagine residue is involved in this peptide.

To investigate further the role of this peptide, chemical synthesis of the nine-residue peptide was carried out in the solid-phase peptide synthesizer and the carbohydrate-binding activity of this peptide was tested. Synthetic peptide was purified by reversed-phase HPLC on a C_{18} column. The purified peptide was then tritiated with ^{3}H-acetic anhydride and labelled peptide was applied to lactose–, maltose–, L-fucose– and di-N-acetylchitobiose Sepharose 4B columns.

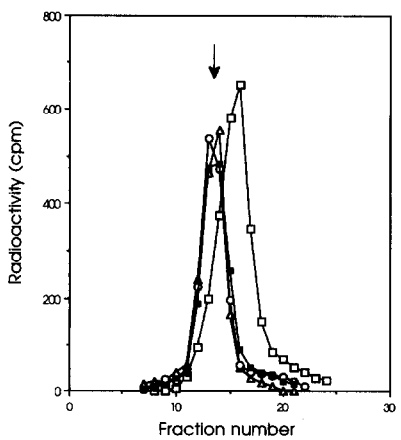

Fig. 5. Elution profiles of the synthetic BP-9 nonapeptide on columns of lactose– (□), maltose– (△), L-fucose– (○) and di-N-acetylchitobiose–Sepharose 4B (■). The tritiated synthetic peptide, DTWPNTEWS, was dissolved in 10 m*M* TBS containing 1 m*M* $CaCl_2$ and $MnCl_2$ and then applied to columns as described under Experimental.

Fig. 5 shows the respective elution profiles of the synthetic BP-9 peptide on lactose–, maltose–, L-fucose– and di-N-acetylchitobiose–Sepharose 4B columns. When the synthetic peptide was dissolved in TBS containing 1 m*M* $CaCl_2$ and 1m*M* $MnCl_2$ and was applied to the lactose column equilibrated with the same buffer, it was retained on the column and eluted at fractions 16 and 17 in the presence of Ca^{2+} and Mn^{2+}. In contrast, the BP-9 peptide was recovered without retention from the columns of L-fucose–, maltose– and di-N-acetylchitobiose–Sepharose 4B. In the absence of Ca^{2+}, retention of the BP-9 peptide on a column of lactose–Sepharose was not seen (data not shown). From these data, it is proposed that the BP-9 nonapeptide is a part of the carbohydrate-binding site and defines the sugar-binding specificity of BPA.

To assess whether the nonapeptide sequence of BPA is involved in the determination of carbohydrate-binding specificity, a chimeric lectin of BPA was produced. In this chimeric lectin, the BP-9

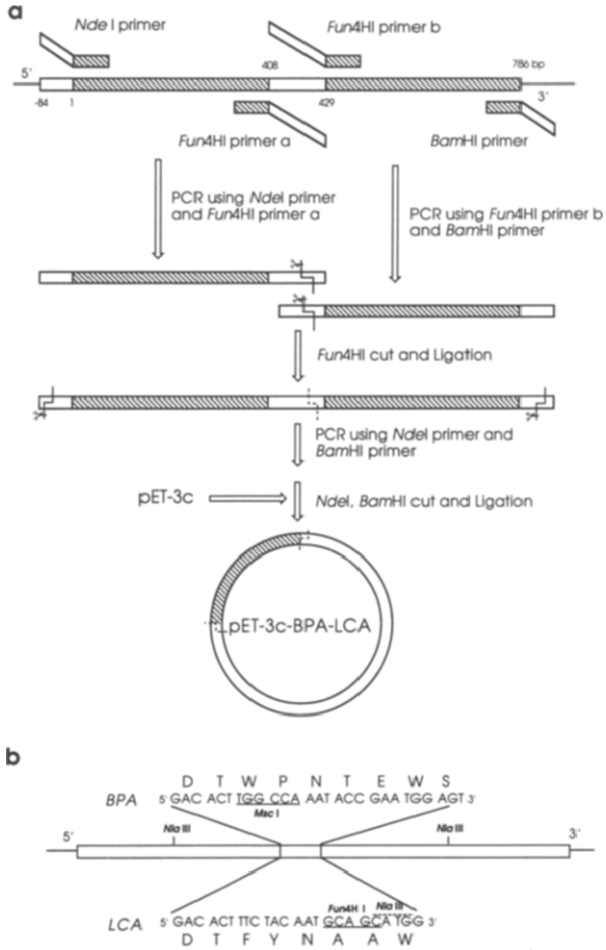

Fig. 6. (a) Schematic diagram for constructing the chimeric BPA–LCA lectin gene. (b) Schematic diagram of BPA and the chimeric BPA–LCA lectin and specific restriction sites for cDNAs coding BPA and BPA–LCA.

nonapeptide sequence was replaced by the corresponding region of LCA [13]. The amino acid sequence of LCA corresponding to BP-9 is also identical with that of the *Pisum sativum* lectin [12] or *Vicia faba* lectin [14]. These three lectins show almost the same α-D-mannose specific carbohydrate-binding specificity as described previously [24,25], whereas BPA is specific for β-D-galactosyl residues. A chimeric BPA–LCA lectin gene was constructed using the two-step PCR illustrated in Fig. 6a. A nucleotide sequence of LCA coding the corresponding portion of the BP-9 peptide was introduced using synthetic oligonucleotides as PCR primers. The prepared nucleotide sequence was designed to contain DTFYNAAW without additional framing sequences and contains a recognition sequence for a unique restriction enzyme, which is not found in BPA cDNA. For the production of the chimeric BPA–LCA lectin gene using a cDNA clone coding BPA, a cDNA coding the NH_2-terminal residues (BPA amino acid residues 1–136) was amplified by a PCR with *Nde*I primer and *Fun*4HI primer-a, which repositions a codon coding Phe–Asn–Ala–Ala–Trp after Thr-136. A cDNA coding the COOH-terminus (BPA residues 144–262) was then amplified with *Bam*HI primer and *Fun*4HI primer-b, which introduces a codon coding Ala–Ala–Trp before Asp-144. The products of the PCRs were digested with *Fun*4HI and ligated. After the ligation, a cDNA coding the full length of the chimeric BPA–LCA lectin was again amplified with *Nde*I and *Bam*HI primers using the ligated DNA as a template. The PCR product finally obtained was digested with *Nde*I and *Bam*HI and inserted into the expression plasmid pET-3c, which was digested with *Nde*I and *Bam*HI to yield a plasmid pET-3c–BPA–LCA.

To identify the difference of the nucleotide sequences between BPA and BPA–LCA cDNAs, the cleavage sites susceptible to restriction enzymes were detected (Figs. 6b and 7). The *Msc*I site was unique in BPA cDNA and the *Msc*I fragments of 416 and 390 base pairs (bp) were obtained from BPA cDNA

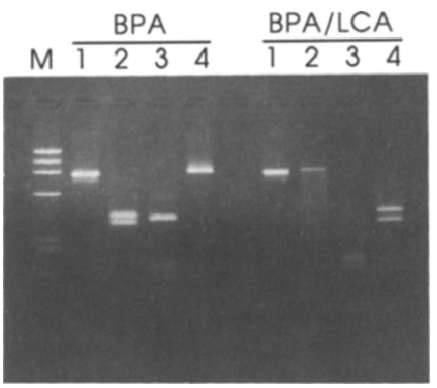

Fig. 7. Identification of the difference of the nucleotide sequences between cDNAs coding BPA and BPA–LCA. Lanes: 1 = cDNAs coding BPA and BPA–LCA generated by a PCR with *Nde*I and *Bam*HI primers; 2 = BPA and BPA–LCA cDNAs digested with *Msc*I; 3 = cDNAs digested with *Nla*III; 4 = cDNAs digested with *Fun*4HI; M = marker bands of φX174–*Hae*III digest (1353, 1078, 872, 603, 310, 281, 271, 234 bp).

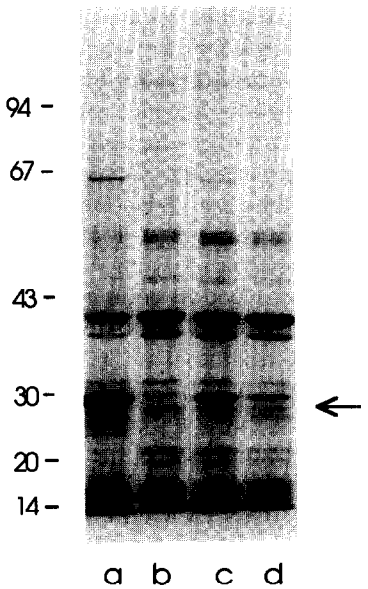

Fig. 8. Expression of the recombinant BPA and the chimeric BPA–LCA lectin in *E. coli*. Protein samples were analysed by electrophoresis on a 10% SDS-polyacrylamide gel, followed by staining with Coomassie blue. Lanes: a = *E. coli* containing the pET-BPA plasmid with the induction by isopropyl-β-D-thiogalactoside; b = *E. coli* containing the pET-BPA plasmid without induction by isopropyl-β-D-thiogalactoside; c = *E. coli* containing the pET-BPA–LCA plasmid with induction by isopropyl-β-D-thiogalactoside; d = *E. coli* containing the pET-BPA–LCA plasmid without induction by isopropyl-β-D-thiogalactoside. Relative molecular masses indicated on the left (· 10^3).

(Fig. 7, lane 2). The *Fun*4HI fragments of 425 and 378 bp were obtained from BPA–LCA cDNA (Fig. 7, lane 4). The 215, 214, 189 and 185 bp fragments were obtained after digestion of BPA–LCA cDNA with *Nla*III (Fig. 7, lane 3), whereas the 402 fragment remained for BPA cDNA (Fig. 7, lane 3). BPA cDNA is resistant to the digestion with *Fun*4HI and BPA–LCA cDNA with *Msc*I (Fig. 7, lane 2).

As shown in Fig. 8, the syntheses of the recombinant lectins in *E. coli* were measured by polyacrylamide gel electrophoresis of the cell lysates. The lectins expressed in *E. coli* after the induction gave a strong band to a relative molecular mass of 29 000. These recombinant lectins were purified by reversed-phase HPLC on a C_4 column and then digested with an endoproteinase Asp-N. The peptide fragments obtained were separated as reported previously [5]. Among these fragments, unique amino acid sequences of DTWPNTEWS from BPA and of DTFYNAAW from BPA–LCA were determined by the protein sequencer (data not shown).

The recombinant lectins expressed in *E. coli* formed insoluble aggregates in the *E. coli* cells, and the binding of ^{125}I-labelled βGal–BSA, αMan–BSA and αFuc–BSA to the aggregates of the recombinant lectins were assayed. The recombinant BPA bound ^{125}I-βGal–BSA as reported previously [5], and did not bind ^{125}I-αMan–BSA or ^{125}I-αFuc–BSA (Fig. 9). It was reported that cloned pea lectin cDNA expressed in *E. coli* possessed both the same haemagglutinating activity and carbohydrate-binding specificity as the native pea lectin [26]. The carbohydrate-binding specificities of the recombinant and native BPAs seem to be identical, as expected.

Interestingly, the chimeric BPA–LCA lectin in which the seven amino acid residues (WPNTEWS)

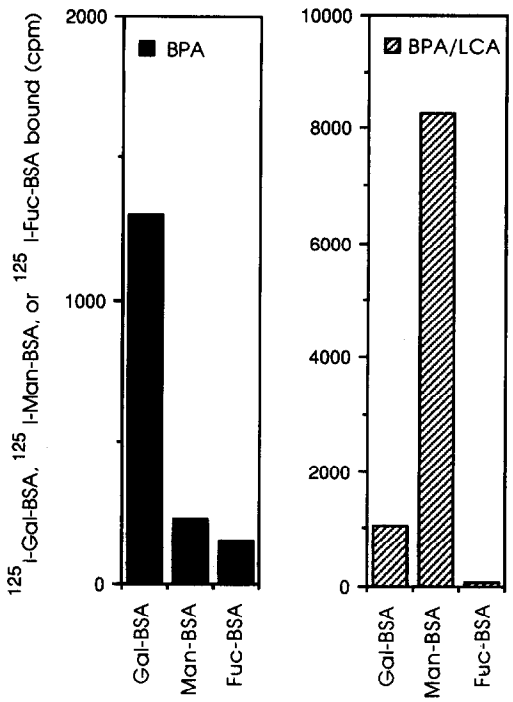

Fig. 9. Binding of ^{125}I-labelled βGal–BSA, αMan–BSA and αFuc–BSA to recombinant BPA or BPA–LCA. Recombinant BPA or BPA–LCA was incubated with ^{125}I-labelled βGal–BSA, αMan–BSA or αFuc–BSA at 4°C for 1 h. After incubation, the recombinant BPA or BPA–LCA was washed and then the radioactivity was counted. Each value is the mean of three experiments.

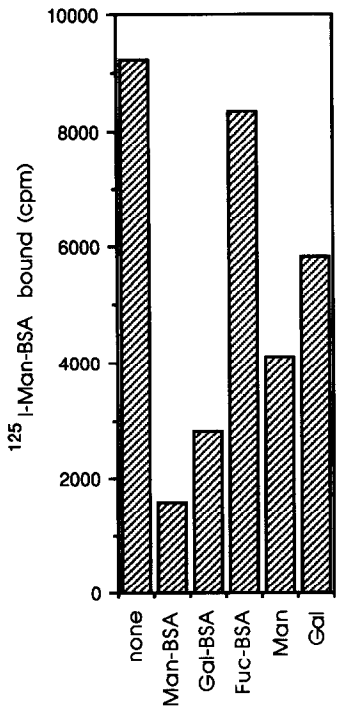

Fig. 10. Inhibition of ^{125}I-labelled αMan–BSA binding to the recombinant chimeric BPA–LCA lectin. The recombinant BPA–LCA was incubated with ^{125}I-labelled αMan–BSA in the presence of a twenty times excess of unlabelled αMan–BSA, βGal–BSA or αFuc–BSA, or 200 m*M* D-mannose or D-galactose. After incubation and washing, the radioactivity was counted. Each value is the mean of three experiments.

of BPA were replaced by the corresponding homologous residues of LCA (FYNAAW) bound αMan–BSA (Fig. 9). It also slightly bound βGal–BSA. Unlabelled αMan–BSA completely inhibited the binding of the chimeric BPA–LCA lectin with ^{125}I-labelled αMan–BSA, and unexpectedly unlabelled βGal–BSA also inhibited the binding of the BPA–LCA lectin with ^{125}I-labelled αMan–BSA (Fig. 10). D-Mannose (200 m*M*) and D-galactose (200 m*M*) also inhibited the binding of the chimeric BPA–LCA lectin with ^{125}I-αMan–BSA by up to 56 and 37%, respectively.

These facts suggest that the expression of the carbohydrate-binding specificity of BPA was altered by substituting the seven amino acid residues corresponding to a part of its metal-binding region. The chimeric BPA–LCA lectin was found to bind D-mannose, indicating that the amino acid sequence of a part of the metal-binding region is highly implicated in the carbohydrate-binding specificity of BPA. It is of interest to note that the chimeric lectin can bind both D-mannose and D-galactose. In the chimeric BPA–LCA lectin, a part of the metal-binding region is derived from that of the D-mannose-binding LCA lectin and other amino acids involved in the carbohydrate-binding are the same as those of BPA which binds D-galactose. It is, therefore, natural that the chimeric BPA–LCA lectin could bind both D-mannose and D-galactose. This may be explained by the fact that BP-9 peptide (DTWPNTEWS) does not represent the whole carbohydrate-binding site of BPA.

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