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Purification and characterization of a carbohydrate-binding peptide from *Bauhinia purpurea* lectin

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In order to examine the correlation between the amino acid sequence and sugar binding specificity of *Bauhinia purpurea* lectin (BPA), a galactose and lactose binding lectin, a peptide which interacts with lactose was purified from an Asp-N endoproteinase digest of BPA by means of affinity chromatography on a column of lactose-Sepharose. The amino acid sequence of this peptide is Asp-Thr-Trp-Pro-Asn-Thr-Glu-Trp-Ser. A tryptic fragment having the ability to interact with lactose was also purified and found to contain the above sequence, consisting of 9 amino acids. The chemical synthesis of this peptide was carried out by the solid-phase method and the synthetic peptide was found to exhibit lactose binding activity in the presence of calcium.

Amino acid sequence; Carbohydrate-binding peptide; *Bauhinia purpurea*

1. INTRODUCTION

Lectins, a class of proteins that specifically bind sugars, are widely distributed in nature [1]. Leguminous plant lectins resemble each other in their physiochemical properties although they differ in their carbohydrate specificities. They usually consist of two or four subunits having molecular masses of 25–30 kDa and each subunit has one carbohydrate binding site. Their interaction with carbohydrates requires tightly bound Ca^{2+} and Mn^{2+} [2]. We have already determined the primary structures of *Lotus tetragonolobus* lectin [3] and *Ulex europeus* lectins I and II [4] by use of a protein sequencer. They exhibit remarkable homologies with other legume lectins whose amino acid sequences have already been reported [5–12].

Bauhinia purpurea lectin (BPA) consists of four subunits each having a molecular mass of 30 kDa. This lectin is a galactose-binding lectin and is particularly specific for the Galβ1-3GalNac sugar sequence [13]. We cloned cDNA for BPA from a cDNA library constructed from poly(A)$^+$ RNA from *Bauhinia purpurea* seeds and the nucleotide sequence of the cDNA was determined (K. Kusui, Y. Konami, K. Yamamoto and T. Osawa, unpublished results). The open reading frame of the cDNA encodes a polypeptide of 280 amino acids. The signal peptide of this lectin is composed of 28 amino acids.

Here we report the purification of a peptide which is involved in the saccharide-binding site of BPA and the determination of its amino acid sequence. Furthermore, we demonstrate in this paper that a peptide synthesized on the basis of the amino acid sequence of the natural saccharide-binding peptide also specifically binds to lactose in the presence of calcium ions.

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Abbreviations: BPA, *Bauhinia purpurea* lectin; PBS, 10 mM sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl, HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; TBS, 10 mM Tris-HCl, pH 6.8, containing 0.15 M NaCl; Con A, concanavalin A; GS4, *Griffonia simplicifolia* lectin IV

2. MATERIALS AND METHODS

2.1. *Materials*

Bauhinia purpurea seeds were purchased from F.W. Schumacher Co. (Sandwich, MA, USA). A C18 μBondasphere (100 Å) column for reversed phase chromatography was obtained from Waters (Milford, MA). Endoproteinase Asp-N from *Pseudomonas fragi* was purchased from Boehringer Mannheim GmbH (Mannheim) and trypsin from Sigma (St. Louis, MO).

2.2. *Purification of* Bauhinia purpurea *lectin*

Finely powdered *Bauhinia purpurea* seeds were extracted with 0.15 M NaCl. To the supernatant obtained on centrifugation, solid $(NH_4)SO_4$ was added to 0.8 saturation. The precipitate formed was dialyzed against PBS (10 mM sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl) and then applied to a lactose-Sepharose 4B affinity column equilibrated with PBS. The column was washed with PBS and then eluted with 0.1 M lactose in PBS. The purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the method of Laemmli [14].

2.3. *Affinity chromatography of peptides obtained on digestion of BPA with Asp-N endoproteinase or trypsin on a column of lactose-Sepharose 4B*

The peptide fragments obtained on digestion of BPA with endoproteinase Asp-N or trypsin were applied to a column of lactose-Sepharose 4B (4.5 ml) without additional modification. After the column had been washed with PBS, the bound material was eluted out

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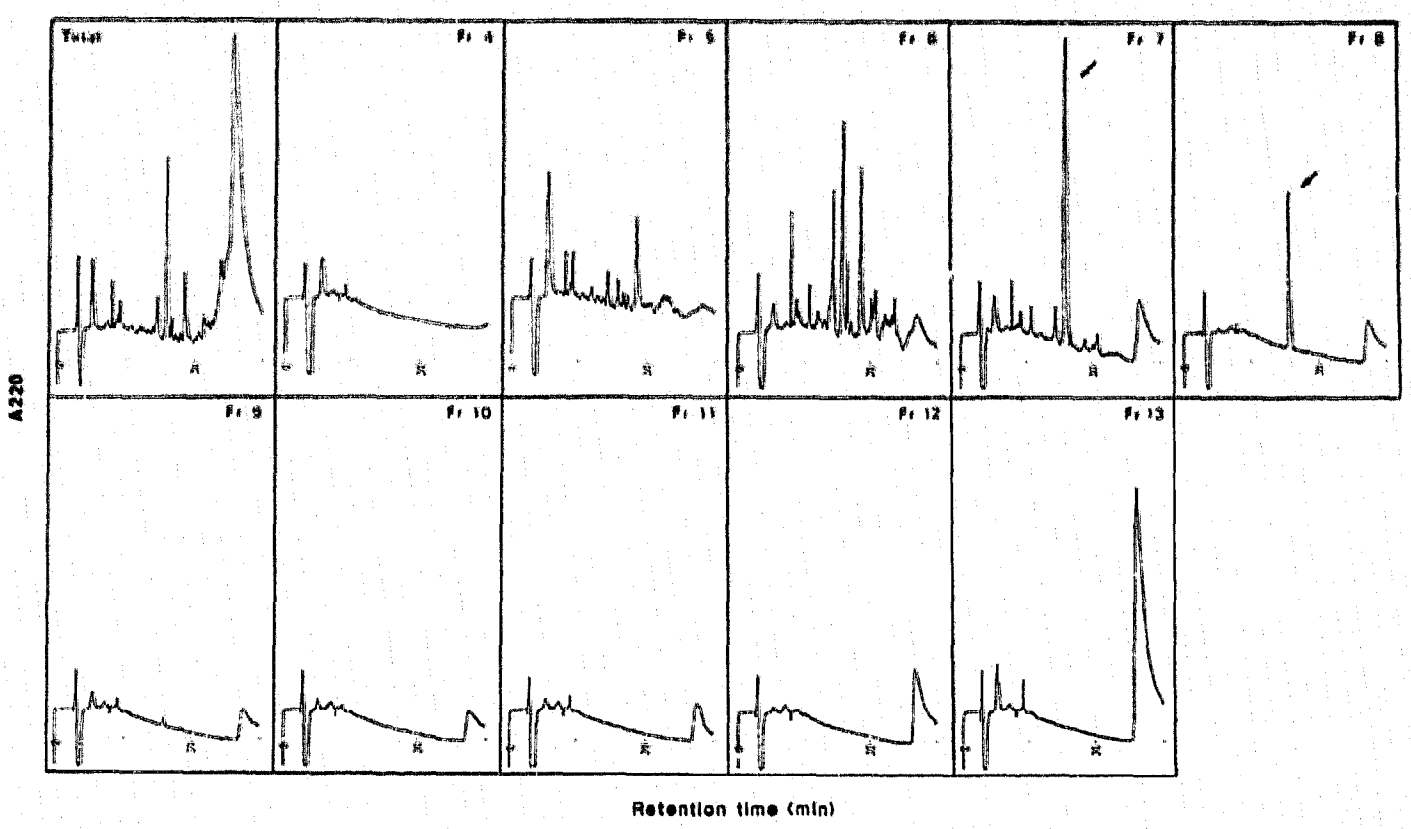

Fig. 1. Elution profiles of reversed phase HPLC on a column of C18 of the fractions obtained by affinity chromatography of an Asp-N digest of BPA on a column of lactose-Sepharose 4B. Purified BPA was digested with endoproteinase Asp-N at 37°C for 18 h. The reaction mixture was applied to a column of lactose-Sepharose without additional modification as described under Materials and Methods. After the column had been washed with PBS, bound material (after fraction number 10) was eluted with the same buffer containing 0.1 M lactose. Each fraction was then analyzed by reversed phase HPLC on a column of C18 as described under Materials and Methods.

with the same buffer containing 0.1 M lactose. Fractions of 0.5 ml were collected and then analyzed by reversed phase high performance liquid chromatography (HPLC) on a column of C18 with a linear gradient (0-60%) of 2-propanol/acetonitrile (7:3) in distilled water containing 0.1% trifluoroacetic acid (TFA) in 30 min at the flow rate of 1 ml/min. Amino acid sequence analysis of the purified peptides was performed on a PSQ-1 gas-phase protein sequencer (Shimadzu, Kyoto, Japan).

2.4. *Solid phase peptide synthesis*

Solid phase peptide synthesis was carried out in an automated manner with a 9020 peptide synthesizer (MilliGen/Biosearch, Burlington MA). Protected peptide resins were treated with 10% phenol in TFA to remove protecting groups. The cleaved peptide products were precipitated with ether and then collected by centrifugation. The crude water-soluble peptides were purified by reversed phase HPLC on a column of C18. The purified peptides were tritiated with [^{3}H]acetic anhydride (18.5 GBq/mmol; Amersham, Boston, MA) and then purified by passage through a Sephadex G25 column equilibrated with TBS (10 mM Tris-HCl, pH 6.8, containing 0.15 M NaCl) containing 10 mM $CaCl_2$ and 10 mM $MnCl_2$.

2.5. *Affinity chromatography of tritiated peptides on columns of lactose-, maltose-, fucose- and di-N-acetylchitobiose-Sepharose*

The tritiated synthetic peptides were applied to a lactose-, maltose-, fucose- or di-*N*-acetylchitobiose-Sepharose column equilibrated with TBS containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$, and then the column was eluted with the same buffer. Fractions of 0.4 ml were collected and their radioactivity was counted in a liquid scintillation counter.

3. RESULTS AND DISCUSSION

Fig. 1 shows the elution profiles of reversed phase HPLC on a column of C18 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 panel. Other panels show the elution profiles of fractions 4–13 obtained by the affinity chromatography on a column of lactose-Sepharose 4B. Most of the fragments of BPA were recovered in fraction number 6. Undigested BPA bound to the column was eluted with 0.2 M lactose in fraction 13. Interestingly, a peptide indicated by the arrow in Fig. 1, which was

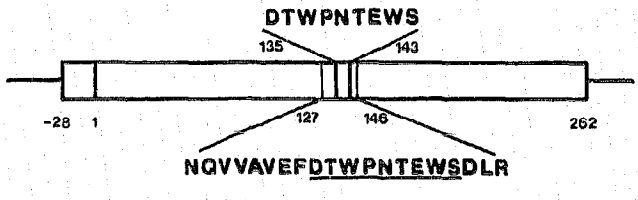

Fig. 2. Amino acid sequences of Asp-N and tryptic fragments of BPA retarded on a column of lactose-Sepharose 4B.

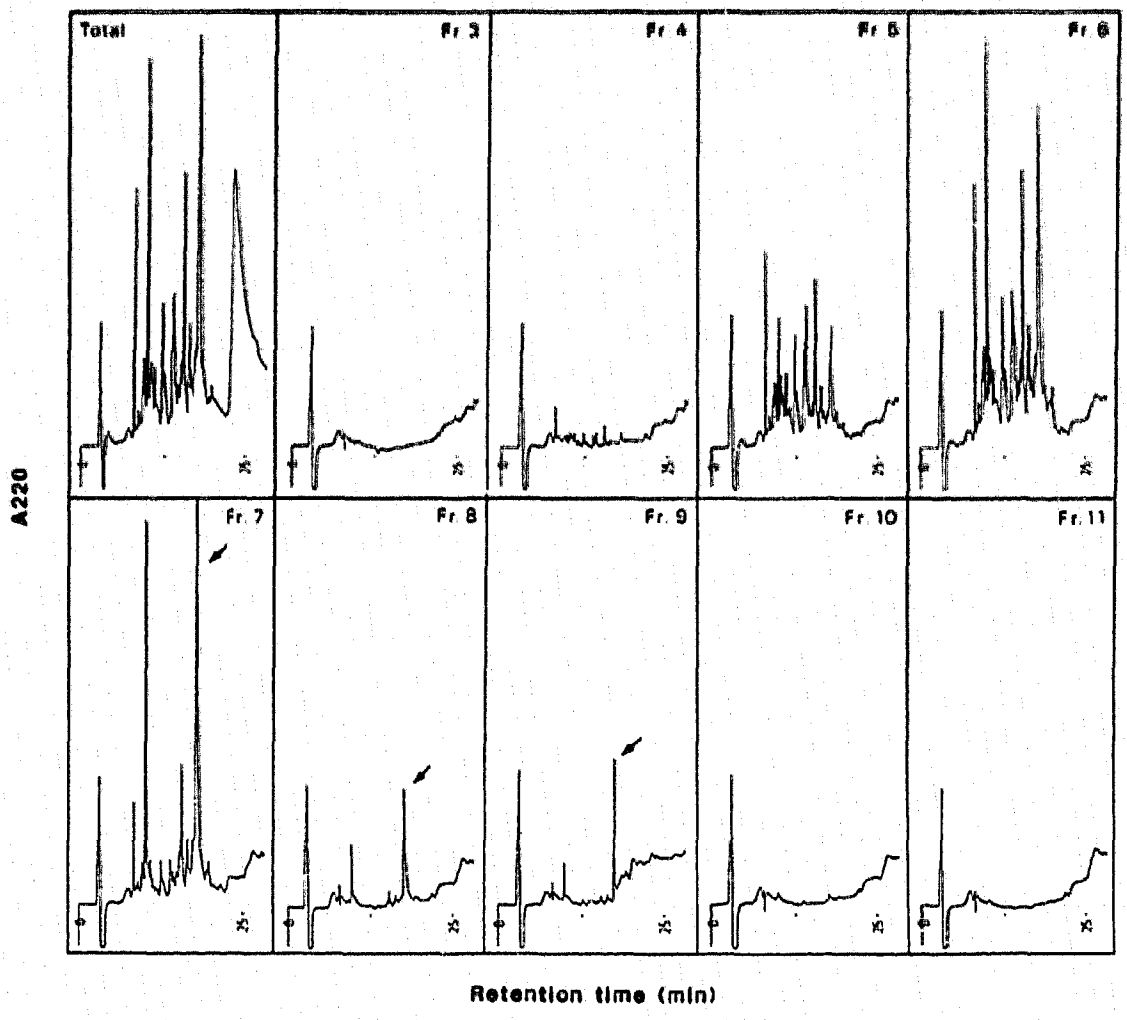

Fig. 3. Elution profiles of reversed phase HPLC on a column of C18 of the fractions obtained by affinity chromatography of a trypsin digest of BPA on a column of lactose-Sepharose 4B.

retarded on a column of lactose-Sepharose 4B, being eluted in fractions 7 and 8, was observed. These results show that this peptide specifically interacts with lactose. The peptide was purified by reversed phase HPLC on C18 again and its amino acid sequence determined with a gas-phase protein sequencer. Fig. 2 shows the amino acid sequence of this peptide. It consists of 9 amino acids, its amino acid sequence being DTWPNTEWS (BP-9). This peptide corresponds to the fragment from aspartic acid-135 to serine-143, based on the complete amino acid sequence of BPA to be reported elsewhere.

We, then, prepared tryptic fragments of BPA and separated them by the same procedures as described above. Fig. 3 shows the elution profiles on a column of C18 of fractions obtained from a column of lactose-Sepharose 4B. The recovery of the fragment indicated by the arrows was retarded on the lactose-Sepharose 4B column. The amino acid sequence of this tryptic peptide is also shown in Fig. 2. The tryptic fragment consist of 20 amino acids (NQVVAVEFDTWPNTEWSDLR) and contains the same amino acid sequence as that of the Asp-N fragment previously described. As for the homology of these peptides among legume lectins, it was found that the sequence of this peptide correspond to the relatively conserved region in legume lectins. Fig. 4 shows the local homology of the peptide from BPA among legume lectins. Highly-conserved residues are denoted by bold letters. All of these residues are known as amino acids involved in metal binding [15]. Therefore, these amino acids are highly conserved among these lectins. Three of the four amino acids are present in the Asp-N fragment, BP-9. This indicates that metal ions maintain the conformation of this peptide and maintain its ability to bind lactose.

Crystal X-ray analysis of the complex of Con A with α-methylmannoside [16] and of the complex of *Griffonia simplicifolia* lectin IV (GS4) with Le^b-OMe oligosaccharide, Fucα1-2Galβ1-3(Fucα1-4)GlcNAc-OMe [17], has been reported. In both cases, the positive calcium ion was found to cooperate with the negative

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               134         143
BPA        -VAVEFDTWPNTEWSDPKD-VPH-

CON A      -VAVELDTYPNTDIGDPS--VPH-
LTA        -VAVEFDSYHNI-W-DPKSLKSH-
DBA        -VAVEFDTLSNSGW-DPS-MK-H-
SBA        -VAVEFDTFRNS-W-DPPH--PH-
PHA-L      -VAVEFDTLYNKDW-DPTE--RH-
VFA        -VAVEFDTFYNAAW-DPSNGKRH-
LCA        -VAVEFDTFYNAAW-DPSNKERH-
PSA        -VAVEFDTFSNRAW-DPSNRDRH-
ECorL      -LGVEFDTFSNP-W-DPPQ-VPH-
GS4        -VAVEFDTWINKDWNDPP--YPH-
                   *  *   *
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Fig. 4. Comparison of BP-9 with other legume lectins. The alignment was maximized by leaving gaps (-). Bold letters denote the amino acids involved in metal binding. The amino acids of GS4 making contacts with Le^b-OMe oligosaccharides are marked (*). Concanavalin A (Con A) [6]; *Lotus tetragonolobus* lectin (LTA) [3]; *Dolichos biflorus* lectin (DBA) [7]; soybean lectin (SBA) [8]; *Phaseolus vulgaris* lectin-L4 (PHA-L) [9]; *Vicia faba* lectin (VFA) [10]; *Lens culinaris* lectin (LCA) [11]; *Pisum sativum* lectin (PSA) [12]; *Erythrina corallodendron* lectin (ECorL) [13]; Griffonia simplicifolia lectin IV (GS4) [14].

carboxylated group of aspartic acid (Asp-14 for Con A, Asp-135 for GS4) to enhance the charge delocalization for the strengthening of hydrogen bonds. In addition, in the case of GS4, Trp-133 and Trp-138 make three van der Waals contacts with Le^b-OMe oligosaccharides. The complete conservation of these three amino acid residues in the BP-9 peptide of BPA is of interest as to the sugar binding site of this lectin. These three amino acid residues in the BP-9 peptide, DTWPNTEWS, are presumed to play an important role in the interaction of the peptide with lactose. However, this interaction is weak compared with that of the native lectin, because the BP-9 peptide does not represent the whole carbohydrate binding site and, furthermore, the native lectin consists of four subunits [13], each having one carbohydrate binding site.

It is known that, among 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 of the positive calcium ion and Asp-208 which forms a hydrogen bond with an equatorial 4-OH group of D-mannose are found to be conserved in almost all legume lectins so far sequenced. However, in the case of BPA, the Asn residue is a part of the BP-9 peptide, but the Asp residue is not conserved (K. Kusui, Y. Konami, K. Yamamoto and T. Osawa, unpublished results).

In order to investigate further the role of this peptide, the chemical synthesis of several 9-residue peptides was carried out with a solid-phase peptide synthesizer and the carbohydrate binding activity of these peptides was examined.

The synthetic peptides thus obtained were purified by reversed phase HPLC on a C18 column. After the purified peptides had been tritiated with [^{3}H]acetic anhydride, the labeled peptides were applied to lactose-, maltose-, fucose and di-*N*-acetylchitobiose-Sepharose columns.

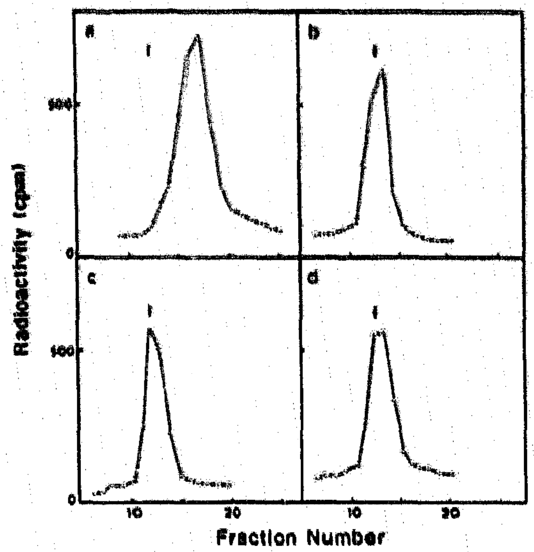

Fig. 5. Elution profiles of the synthetic BP-9 peptide on columns of lactose-, maltose-, fucose- and di-*N*-acetylchitobiose-Sepharose. The tritiated synthetic peptide, DTWPNTEWS, was dissolved in 10 mM Tris-buffered saline containing 1 mM $CaCl_2$ and $MnCl_2$ and then applied to a column (5 ml) equilibrated with the same buffer. Fractions of 0.4 ml were collected and their radioactivity was counted. (a) Lactose-Sepharose; (b) maltose-Sepharose; (c) fucose-Sepharose; (d) di-*N*-acetyl chitobiose-Sepharose.

Fig. 5 shows the respective elution profiles of the synthetic BP-9 peptide on lactose-, maltose, fucose- and di-*N*-acetylchitobiose-Sepharose 4B colums. When the synthetic BP-9 peptide was dissolved in TBS containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$, and then applied to a lactose column equilibrated with the same buffer, it was found to be retarded on the column, and to be eluted at fractions 16 and 17 in the presence of calcium and manganese ions. In the absence of calcium ions, retardation of the synthetic BP-9 peptide on the lactose-

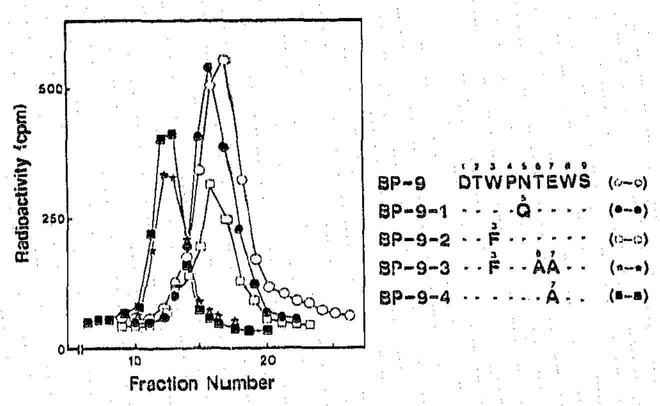

Fig. 6. Elution profiles of BP-9 and analogous peptides on a lactose-Sepharose column. The tritiated synthetic peptides were applied to a column of lactose-Sepharose 4B (5 ml) and fractions of 0.4 ml were collected. The experimental details are given in the legend to Fig. 5. (a) DTWPNTEWS (BP-9); (b) DTWPQTEWS; (c) DTFPNTEWS; (d) DTFPNAAWS; (e) DTWPNTAWS.

Sepharose column was not observed (data not shown). On the other hand, the synthetic BP-9 peptide was recovered without retardation from the fucose-, maltose- and di-*N*-acetylchitobiose-Sepharose columns.

For further clarification of the roles of the conserved amino acids, single and triple amino acid substitutions were introduced into the BP-9 peptide by means of chemical synthesis. Fig. 6 summarizes the interaction of these various peptides having amino sequences analogous to that of BP-9 with lactose. One mutant peptide (BP-9-2), in which Trp-3 was replaced by Phe, and another one (BP-9-1) having Gln instead of Asn-5 showed almost the same affinity for lactose as the BP-9 peptide, while a mutant peptide (BP-9-4) in which Glu-7 was replaced by Ala appeared to have little or no affinity for lactose. In the case of GS4, Asp is present at this position. These results suggest that the presence of a Glu residue at this position, 7, is important for the binding to lactose.

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