Volume 304, number 2,3, 129–135 FEBS 11132 June 1992

Correlation between carbohydrate-binding specificity and amino acid sequence of carbohydrate-binding regions of *Cytisus*-type anti-H(O) lectins

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Received 23 March 1992; revised version received 20 April 1992

A carbohydrate-binding peptide of the di-*N*-acetylchitobiose-binding *Cytisus sessilifolius* anti-H(O) lectin I (CSA-I) was isolated from the endoproteinase Asp-N digest of CSA-I by affinity chromatography on a column of *N*-acetyl-D-glucosamine oligomer-Sepharose (GlcNAc oligomer-Sepharose). The amino acid sequence of the carbohydrate-binding peptide of CSA-I was determined to be DTYFGKTYNPW using a gas-phase protein sequencer. This sequence corresponds to the sequence from Asp-129 to Trp-139 based on the primary structure of CSA-I, and shows a high degree of homology to those of the putative carbohydrate-binding peptide of the *Laburnum alpinum* lectin I (LAA-I) (DTYFGKAYNPW) and of the *Ulex europaeus* lectin II (UEA-II) (DSYFGKTYNPW). The binding of these three anti-H(O) lectins is known to be inhibited by di-*N*-acetylchitobiose but not by L-fucose. These results strongly suggest that there is a good correlation between the carbohydrate-binding specificity and the amino acid sequence of the carbohydrate-binding regions of di-*N*-acetylchitobiose-binding lectins.

Cytisus sessilifolius anti-H(O) lectin; Amino acid sequence; Carbohydrate-binding peptide

1. INTRODUCTION

Lectins are widely used as tools for the study of the carbohydrate constituents of cell surfaces and glycoproteins. They are also widely used for the isolation of glycoproteins by affinity chromatography. Leguminous lectins resemble each other in their physicochemical properties regardless of their carbohydrate-binding specificity. Several anti-H(O) lectins have already been isolated and characterized for their carbohydrate-binding specificity [1–19]. On the basis of the inhibition assays using simple sugars as inhibitors, these anti-H(O) lectins were known to be subdivided into two groups. The first group is the so-called eel serum type anti-H(O) lectins including *Ulex europaeus* lectin I (UEA-I) and *Lotus tetragonolobus* lectin (LTA). Their binding is inhibited by L-fucose. The second group is the so-called *Cytisus*-type anti-H(O) lectins including *Ulex europaeus* lectin II (UEA-II), *Laburnum alpinum* lectin I (LAA-I), and *Cytisus sessilifolius* lectin I (CSA-I). Their binding is inhibited by di-*N*-acetylchitobiose.

In previous papers [19–21] we have determined the primary structures of LTA [19], UEA-I [20], UEA-II [20] and LAA-I [21], and compared them with those of several other leguminous lectins. Extensive homology was found throughout the stretch of the peptides. Furthermore, carbohydrate-binding peptides have been isolated from LTA, UEA-I, UEA-II and LAA-I by affinity chromatography after treatment of the lectins with endoproteinase Asp-N or Lys-C [22]. The peptides having an affinity for the specific sugars were retarded on the affinity columns of GlcNAc oligomer-Sepharose for LAA-I and UEA-II, and Fuc-gel for LTA and UEA-I. The homology found in the amino acid sequences among these retarded peptides indicates the presence of a carbohydrate-binding region of homologous leguminous lectins. However, there was not enough available data to draw a conclusion on the relationship between the sequence of this region and the carbohydrate-binding specificity.

In this study, we determined the complete amino acid sequence of the di-*N*-acetylchitobiose-binding anti-H(O) CSA-I using a protein sequencer. After digestion of CSA-I with two kinds of endoproteinase, Lys-C or Asp-N, the resulting peptides were purified by reversed-

Abbreviations: CSA-I, *Cytisus sessilifolius* lectin I; GlcNAc oligomer-Sepharose, *N*-acetyl-D-glucosamine oligomer-Sepharose; LAA-I, *Laburnum alpinum* lectin I; UEA-II, *Ulex europaeus* lectin II; UEA-I, *Ulex europaeus* lectin I; LTA, *Lotus tetragonolobus* lectin; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

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phase high-performance liquid chromatography (HPLC) and then subjected to sequence analysis.

A carbohydrate-binding peptide was also isolated from the Asp-N digest of CSA-I by affinity chromatography on a column of GlcNAc oligomer-Sepharose and the amino acid sequence of this peptide was determined using a protein sequencer. More extensive homology was detected in the sequences of the carbohydrate-binding peptide of CSA-I (DTYFGKTYNPW), LAA-I (DTYFGKAYNPW), and UEA-II (DSYFGKTYNPW) than for the homology between isolectins having different carbohydrate-binding specificity. These results strongly suggest that there is a close relationship between the carbohydrate-binding specificity and the sequences of the carbohydrate-binding peptide of the di-*N*-acetylchitobiose-binding (*Cytisus*-type) lectins such as CSA-I, LAA-I and UEA-II.

2. MATERIALS AND METHODS

2.1. *Materials*

The seeds of *Cytisus sessilifolius* were obtained from Service plus S.A. (Venissieux cedex, France). Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). C_4 and C_{18} μ Bondaspheres (100 Å) for reversed-phase chromatography were obtained from Waters (Burlington, MA, USA). Endoproteinase Lys-C (*Lysobacter enzymogenes*) and Asp-N (*Pseudomonas fragi*) were purchased from Boehringer GmbH (Mannheim, Germany).

2.2. *Preparation of specific affinity adsorbent, GlcNAc oligomer-Sepharose*

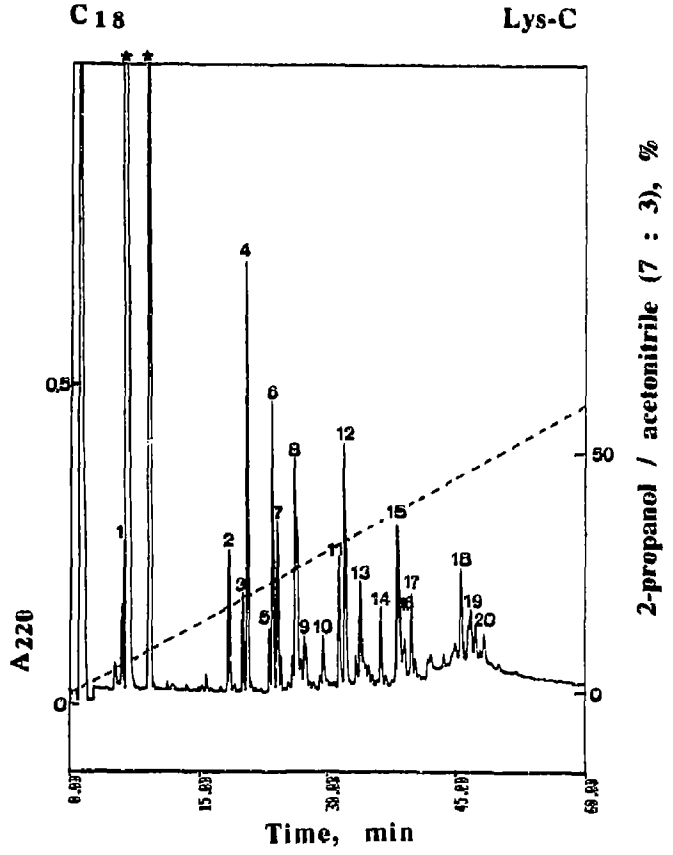
Chitin oligosaccharides were prepared by the limited hydrolysis of chitin according to the method of Rupley [23]. A mixture of tetra-*N*-acetylchitotetraose, penta-*N*-acetylchitopentaose, and hexa-*N*-acetylchitohexaose (1:1:1; w/w) prepared as already mentioned was coupled to amino-Sepharose 4B according to the method of Baues and Gray [24] (GlcNAc oligomer-Sepharose).

2.3. *Purification of CSA-I*

CSA-I was isolated and purified by affinity chromatography by the previously reported method [18]. The affinity-purified CSA-I was further purified by reversed-phase HPLC on a column of C_4 using a linear gradient (0–100%) of 2-propanol/acetonitrile (7:3) in distilled water containing 0.1% trifluoroacetic acid (TFA) in 60 min at a flow rate of 1 ml/min.

2.4. *Digestion of CSA-I with endoproteinase Lys-C or Asp-N*

The purified CSA-I (0.5 mg in 150 μl of 50 mM phosphate buffer, pH 8.0, 37°C) was digested with 5 μg of Lys-C or with 2 μg of Asp-N for 18 h. The peptide fragments obtained were separated by reversed-phase HPLC on a column of C_{18} using a linear gradient (0–60%) of 2-propanol/acetonitrile (7:3) in distilled water containing 0.1% TFA in 60 min at a flow rate of 1 ml/min. Elution profiles were monitored by the absorbance at 220 nm. The peptide fragments were manually collected.



Lys-C fragments

L1 Thr-Val-Lys
Δ Ser-Ile-Lys
L2 His-Ile-Gly-Val-Asp-Val-Asn-Ser-Ile-Lys
L3 Phe-Gly-Leu-Phe-Asn-Ser-Ser-Asp-Asn-Lys
Δ Trp-Asp-Trp-Arg
L4 Asn-Gly-Glu-Val-Ala-Asn-Val-Val-Ile-Thr-Tyr-Arg-Ala-Pro-Thr-Lys
L5 Thr-Tyr-Asn-Pro-Trp-Asp-Pro-Asp-Phe-Lys
L6 Thr-Gly-Arg-Val-Ala-Ser-Phe-Glu-Thr-Ser-*Phe-Ser-Phe*
L7 Leu-Asn-Asp-His-Leu-Ser-Phe-Asn-Phe-Asp-Lys
L8 Trp-Asp-Trp-Arg-Asn-Gly-Glu-Val-Ala-Asn-Val-Val-Ile-Thr-Tyr-Arg-Ala-Pro-Thr-Lys
L9 Ser-Trp-Tyr-Phe-Thr-Ser-Asn-Leu-Glu-Ala-Asn-Pro-Ala
L10 Leu-Ala-Pro-Ala-Asn-Ser-Gln-Ile-Pro-Ser-Gly-Ser-Ser
L11 Ser-Ser-Asn-Gln-Ile-Ile-Ala-Val-Glu-Phe-Asp-Thr-Tyr-Phe-Gly-Lys
L12 Phe-Val-Pro-Asn-Gln-Asn-Asn-Ile-Leu-Phe-Gln-Gly-Glu-Ala-Ser-Val-Ser
L13 Phe-Phe-Leu-Ala-Pro-Ala-Asn-Ser-Gln-Ile-Pro-Ser-Gly-Ser-Ser-Ala-Gly-Leu
L14 Ala-Ile-Leu-Pro-Glu-Trp-Val-Ser-Val-Gly-Phe
L15 Ser-Asn-Gly-Val-Asp-Gly-Leu-Thr-Phe-Phe-Leu-Ala-Pro-Ala-Asn-Ser-Gln-Ile-Pro-Ser-Gly-Ser-Ser-Ala-Gly-Leu-Phe-Gly-Leu-Phe-Asn-Ser-Ser-Asp-Asn-Lys
Δ Ser-Leu-Thr-Val-Ser-Leu-Ser-Tyr-Pro-Ser-Asp-Gln-Thr-Ser-Asn-Ile-Val-Thr-Ala-Ser-Val-Asp-Leu-Lys
L16 Ser-Ala-Gly-Val-Gly-Asn-Ala-Ala-Glu-Phe-Glu-Thr-His-Asp-Val-Leu-Ser-Trp-Tyr-Phe-Thr-*Ser-Asn*
Δ Val-Ala-Ser-Phe-Glu-Thr-Ser-Phe-Ser-Phe-Val-Val-Lys-Asp-Glu-Pro-Glu-Lys
L17 Phe-Val-Pro-Asn-Gln-Asn-Asn-Ile-Leu-Phe-Gln-Gly-Glu-Ala-Ser-Val-Ser-Thr-Thr-Gly-Val-Leu-Gln-Val-Thr-Lys
L18 Ala-Ile-Leu-Pro-Glu-Trp-Val-Ser-Val-Gly-Phe-Ser-Ala-Gly-Val-Gly-Asn-Ala-Ala-*Glu*-Phe-Glu-*Thr*
L19 Ala-Leu-Tyr-Ala-Ala-Pro-Val-His-Ile-Trp-Asp-Ser-Thr-Thr-Gly-Arg
L20 Pro-Ala-Thr-Arg-Ser-Ile-Gly-Arg-Ala-Leu-Tyr-Ala-Ala-Pro-Val-His-Ile-Trp-Asp-Ser-Thr-Thr-Gly-Arg

Fig. 1. Reversed-phase HPLC of Lys-C digest of purified CSA-I on a column of C_{18}. The residues, which were difficult to identify, are written in italics. Two peaks arising from dithiothreitol used for the reduction of CSA-I are marked with an asterisk. Minor peptides in the fragments are marked with a triangle.

2.5. *Isolation of a carbohydrate-binding peptide of CSA-I*

The purified CSA-I was digested with endoproteinase Asp-N and a mixture of all the obtained peptide fragments was applied to a column of GlcNAc oligomer-Sepharose (5 ml). The column was washed with 10 mM Tris/HCl, pH 6.8, containing 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ at a flow rate of 1.5 ml/h. Each 0.5 ml was collected and analyzed by reversed-phase HPLC on a column of C_{18} with a linear gradient (0–60%) of 2-propanol/acetonitrile (7:3) in distilled water containing 0.1% TFA in 30 min at a flow rate of 1 ml/min. The elution was monitored by the absorbance at 220 nm.

2.6. *Amino acid sequence analysis*

The amino acid sequence analyses of the peptide fragments fractionated by HPLC were performed with a 6600 ProSequencer solid-phase protein sequencer (MilliGen/Biosearch, Burlington, MA, USA) and a PSQ-1 gas-phase sequencer (Shimazu, Kyoto, Japan).

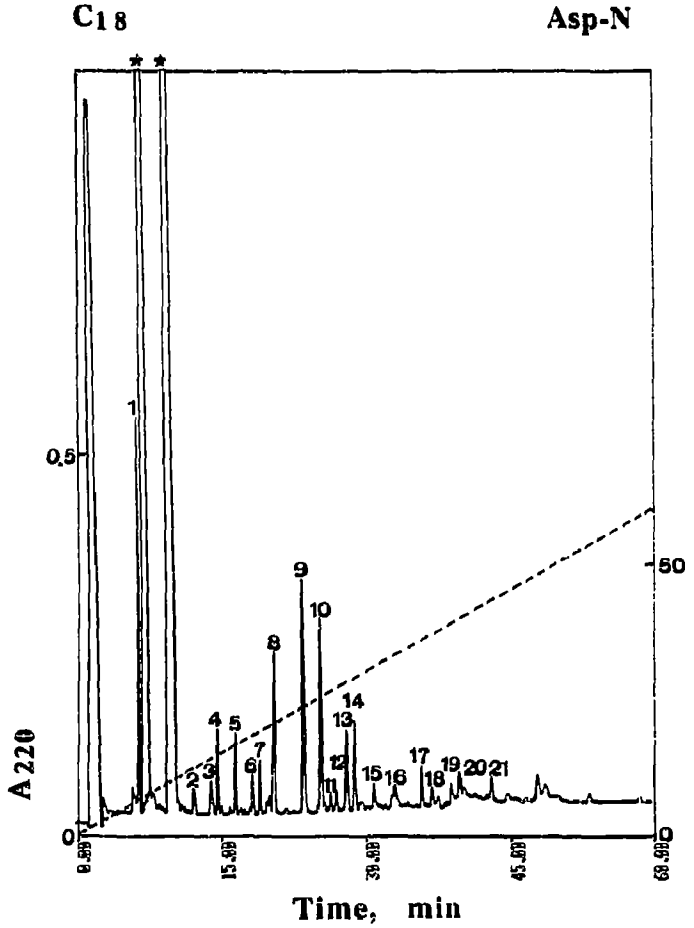
3. RESULTS AND DISCUSSION

3.1. *Determination of the primary structure of CSA-I*

Purification and sequencing of the peptides, obtained after digestion of CSA-I with endoproteinase Lys-C (Fig. 1) or Asp-N (Fig. 2), provided enough overlapping sequences to obtain the complete amino acid sequence of CSA-I (Fig. 3). Some sequenced peptide fragments (L8 and L15) were aligned by the use of the structural homology with several anti-H(O) leguminous lectins shown in Fig. 4. The structure of CSA-I contains 244 amino acid residues. The molecular weight of CSA-I calculated on the basis of the primary sequence is 26,575.02. This value is in good agreement with the apparent molecular weight previously determined by SDS-PAGE (27,000) [18]. As shown in Fig. 4, the complete amino acid sequence of CSA-I was compared with those of LAA-I [21], UEA-II [20] and UEA-I [20]. CSA-I, LAA-I and UEA-II are all di-*N*-acetylchitobiose-binding anti-H(O) lectins. The overall identity value between CSA-I and LAA-I is 86.1% and that between CSA-I and UEA-II is 87.7%. Although UEA-I and UEA-II are isolectins isolated from the seeds of the same species, *Ulex europaeus*, the overall positional identity value between UEA-I and UEA-II was 52.0% [20]. On the other hand, the overall identity values between CSA-I and LAA-I (86.1%), and between CSA-I and UEA-I (87.7%) are strikingly high.

3.2. *Affinity chromatography of the Asp-N digest of CSA-I*

The Asp-N digest of CSA-I was fractionated on a



Asp-fragments

A1 Glu-Ala-Asn-Pro-Ala
A2 Asp-Glu-Pro-Glu-Lys-Ser-Asn
A3 Thr-Lys-Val-Ser-Lys-Pro-Ala-Thr-Arg-Ser-Ile-Gly-Arg
A4 Asp-Thr-Tyr-Phe-Gly-Lys
A5 His-Ile-Gly-Val
A6 Asp-Phe-Lys-His
A7 Asp-Phe-Lys-His-Ile-Gly-Val
A8 Thr-Tyr-Asn-Pro-Trp
A9 Asp-Val-Asn-Ser-Ile-Lys-Ser-Ile-Lys-Thr-Val-Lys-Trp
A10 Asp-Thr-Tyr-Phe-Gly-Lys-Thr-Tyr-Asn-Pro-Trp
Δ Asp-Lys-Phe-Val-Pro-Asn-Gln-Asn-Asn-Ile-Leu-Phe-Gln-Gly
A11 Asp- X -Lys-Ser-Ser-Asn-Gln-Ile-Ile-Ala-Val
A12 Asp-Asn-Lys-Ser-Ser-Asn-Gln-Ile-Ile-Ala-Val-Glu-Phe
A13 Asp- X -Lys-Ser-Ser-Asn-Gln-Ile-Ile-Ala-Val
A14 Asp-Asn-Lys-Ser-Ser-Asn-Gln-Ile-Ile-Ala-Val-Glu-Phe
Δ Ala-Leu-Tyr-Ala-Ala-Pro-Val-His-Ile-Trp
A15 Asn-Gln-Ile-Ile-Ala-Val-Glu
A16 Asp-His-Leu-Ser-Phe-Asn-Phe
A17 Asp-Val-Leu-Ser-Trp-Tyr-Phe-Thr-Ser-Asn-Leu
A18 Asp-Leu-Lys-Ala-Ile-Leu-Pro-Glu-Trp-Val-Ser-Val-Gly-Phe-Ser-Ala-Gly-Val-Gly-Asn-Ala-Ala
Δ Asp-Trp-Arg-Asn-Gly-Glu-Val-Ala-Asn-Val-Val-Ile-Thr-Tyr-Arg-Ala-Pro
A19 Glu-Lys-Ser-Asn-Gly-Val-Asp-Gly-Leu-Thr-Phe-Phe-Leu-Ala-Pro-Ala-Asn-Ser-Gln-Ile-Pro-Ser-Gly-Ser-Ser-Ala-Gly-Leu
A20 Asp-Gly-Leu-Thr-Phe-Phe-Leu-Ala-Pro-Ala-Asn-Ser-Gln-Ile-Pro-Ser-Gly-Ser-Ser-Ala-Gly-Leu
A21 Asn-Gly-Val-Asp-Gly-Leu-Thr-Phe-Phe-Leu-Ala-Pro-Ala-Asn-Ser-Gln-Ile-Pro-Ser-Gly-Ser-Ser-Ala-Gly-Leu

Fig. 2. Reversed-phase HPLC of Asp-N digest of purified CSA-I on a column of C_{18}. The residues, which were difficult to identify, are written in italics. Two peaks arising from dithiothreitol used for the reduction of CSA-I are marked with an asterisk. Minor peptides in the fragments are marked with a triangle.

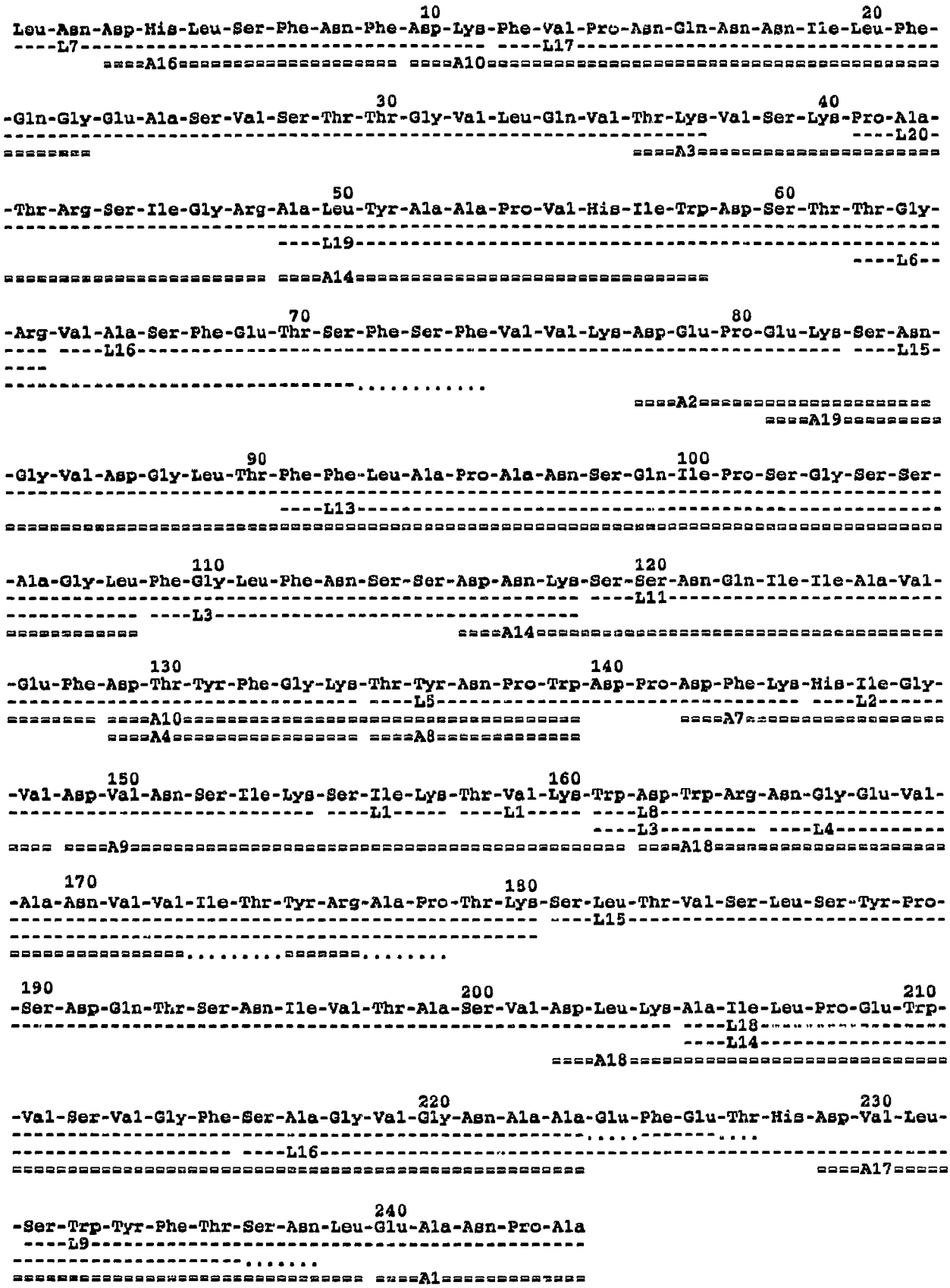


Fig. 3. Complete amino acid sequence of CSA-I. Primary structure in terms of the peptides obtained after digestion with Lys-C (L) and Asp-N (A). Residues were identified by automated sequence analysis of the Lys-C fragments (--L--) or the Asp-N fragments (==A==). The numbering starts from the N terminus. Dots show residues not clearly determined at this position.

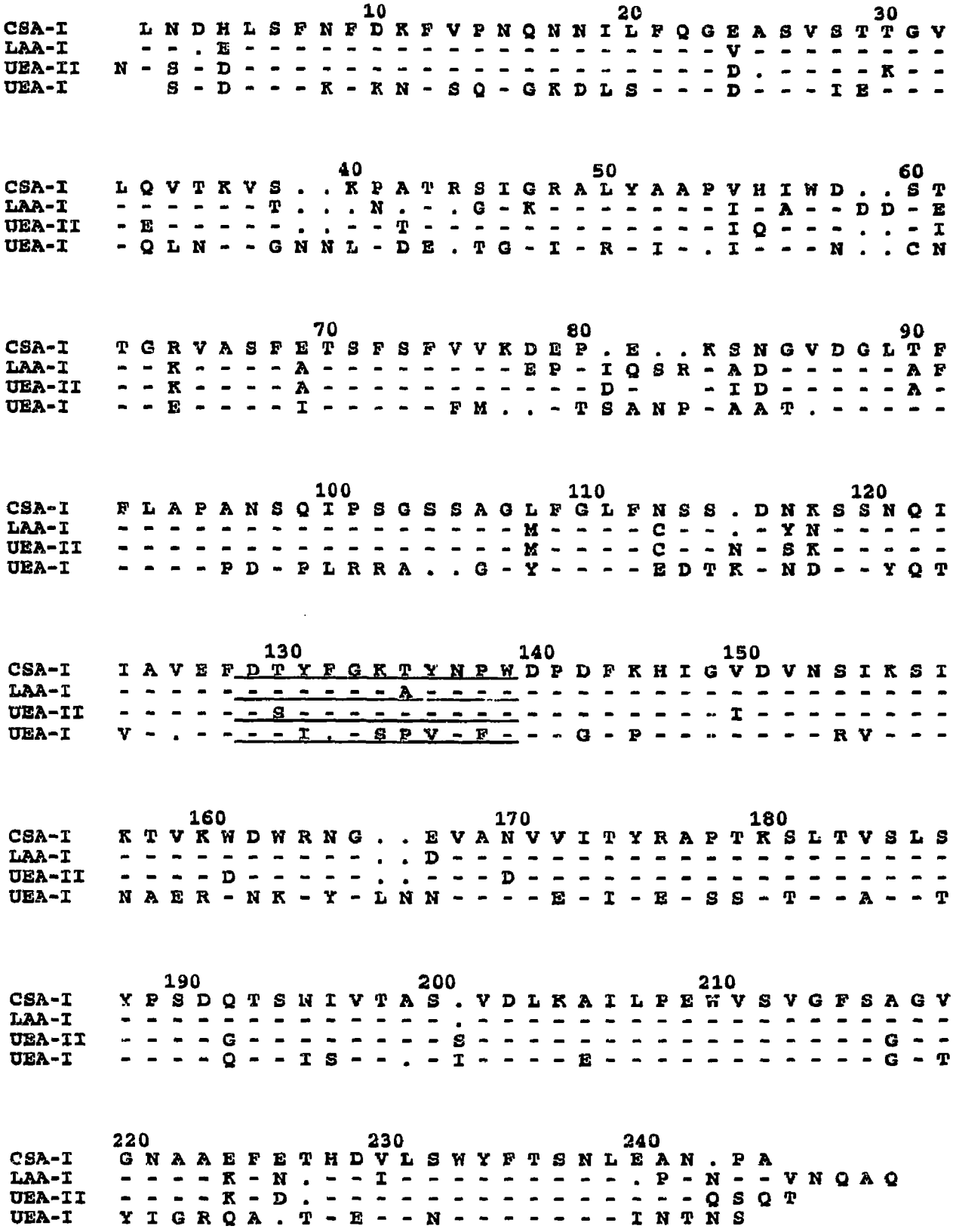


Fig. 4. Comparison of complete amino acid sequence of CSA-I with those of LAA-I, UEA-II and UEA-I and their carbohydrate-binding peptide sequences. The sequences were aligned to maximize homology. (–) denotes sequence identity with that of CSA-I. Dots represent gaps introduced for maximal alignment. The sequences of the putative carbohydrate-binding peptides of CSA-I, LAA-I, UEA-II and UEA-I are underlined.

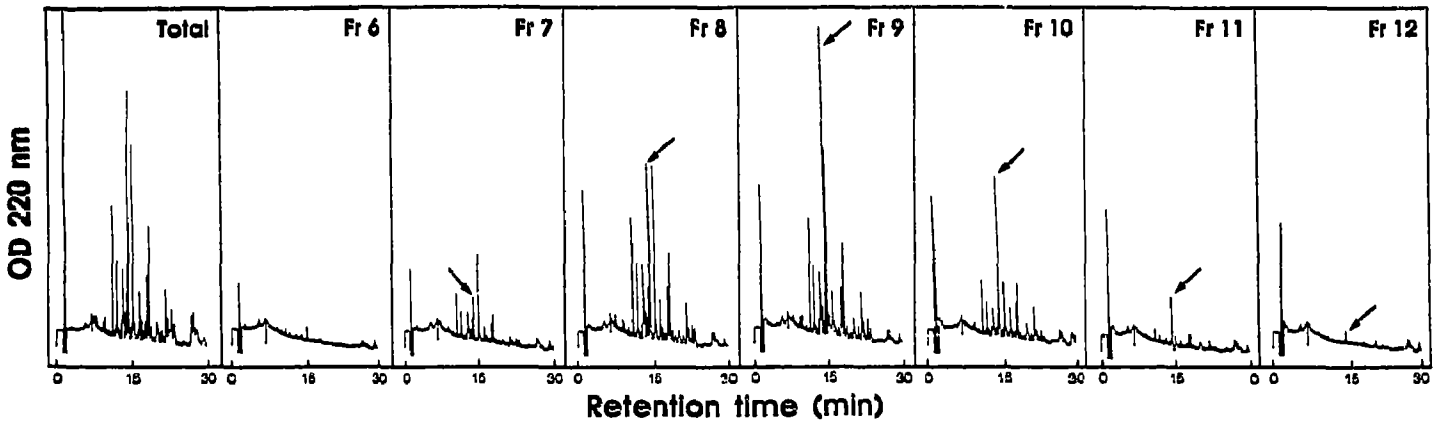


Fig. 5. Elution profiles on reversed-phase HPLC on a column of C_{18} of fractions obtained by affinity chromatography of Asp-N digest of CSA-I. The purified CSA-I was digested with endoproteinase Asp-N at 37°C for 18 h. The reaction mixtures were applied to a column of GlcNAc oligomer-Sepharose as described in section 2. After the column had been washed with Tris-HCl, pH 6.8, containing 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$, each fraction was analyzed by reversed-phase HPLC on a column of C_{18} as described in section 2. Arrows indicate the peptide of CSA-I retained on the affinity column.

column of GlcNAc oligomer-Sepharose. Each fraction was separately subjected to reversed-phase HPLC on a column of C_{18}. Fig. 5 shows the elution profiles on HPLC of the affinity fractions obtained from CSA-I. In this figure, the elution profile of the original mixture of the Asp-N digest of CSA-I is shown in the left panel (shown as Total). Comparison of the elution profiles of the affinity fractions with this original profile clearly shows that most of the Asp-N digest fragments of CSA-I were recovered in affinity fraction 8. The peptide fragment indicated by the arrows in Fig. 5 appeared to be retained on the GlcNAc oligomer-Sepharose column, because this fragment is a major component of fraction 9 and is also clearly observed in fraction 10, suggesting that it specifically interacts with the *N*-acetyl-D-glucosamine oligomer. The peptide retained on the column was purified by reversed-phase HPLC on C_{18} and the amino acid sequence of this peptide was determined using a gas-phase protein sequencer. On the basis of the complete amino acid sequence of CSA-I shown in Fig. 3, it appears that the carbohydrate-binding peptide, DTYFGKTYNPW, from CSA-I corresponds to the sequence from Asp-129 to Trp-139.

3.3. *Comparison of the sequence of the carbohydrate-binding peptide of CSA-I with those of LAA-I, UEA-II and UEA-I*

The sequence of the peptide of CSA-I (underlined in Fig. 4), which was retained on the GlcNAc oligomer-Sepharose column, was compared with those of LAA-I, UEA-II and UEA-I (also underlined in Fig. 4), and this sequence apparently corresponds to a relatively conserved region of the anti-H(O) leguminous lectins. This region has already been demonstrated to represent carbohydrate-binding sites of LTA, UEA-I, UEA-II and LAA-I [22].

The amino acid sequence of the putative carbohydrate-binding peptide of CSA-I was DTYFGKTYNPW and this amino acid sequence was compared with those of LAA-I (DTYFGKAYNPW) [22], UEA-II (DSYFGKTYNPW) [22] and UEA-I (DTIGSPVNFW) [22]. The sequence of the carbohydrate-binding peptide of CSA-I was almost identical with those of LAA-I and UEA-II. The sequence of the carbohydrate-binding peptide of UEA-I, an isolectin of UEA-II having L-fucose-binding specificity, was different from those of the three di-*N*-acetylchitobiose-binding lectins. In view of the differences in the species of the seeds from which CSA-I, LAA-I and UEA-II were isolated, the observed homology of these three lectins seems to be striking. The fact that these three kinds of anti-H(O) lectins have the same di-*N*-acetylchitobiose- binding specificity and no L-fucose-binding specificity is worthy of remark. The finding provides us with important clues as to the elucidation of the relationship between the carbohydrate-binding specificity and the structure of the carbohydrate-binding region of the leguminous plant seeds. Because the structural homology of these three di-*N*-acetylchitobiose-binding lectins is extremely high throughout the stretch of these polypeptides, these three lectins may consist of the products from a group of closely related genes.

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