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A putative carbohydrate-binding domain of the lactose-binding *Cytisus sessilifolius* anti-H(O) lectin has a similar amino acid sequence to that of the L-fucose-binding *Ulex europaeus* anti-H(O) lectin

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The complete amino acid sequence of a lactose-binding *Cytisus sessilifolius* anti-H(O) lectin II (CSA-II) was determined using a protein sequencer. After digestion of CSA-II with endoproteinase Lys-C or Asp-N, the resulting peptides were purified by reversed-phase high performance liquid chromatography (HPLC) and then subjected to sequence analysis. Comparison of the complete amino acid sequence of CSA-II with the sequences of other leguminous seed lectins revealed regions of extensive homology. The amino acid sequence of a putative carbohydrate-binding domain of CSA-II was found to be similar to those of several anti-H(O) leguminous lectins, especially to that of the L-fucose-binding *Ulex europaeus* lectin I (UEA-I).

Keywords: lactose-binding anti-H(O) lectin, lectin, primary structure

Abbreviations: BPA, *Bauhinia purpurea* lectin; Con A, concanavalin A; CMA-I, *Cytisus multiflorus* lectin I; CMA-II, *Cytisus multiflorus* lectin II; CSA-I, *Cytisus sessilifolius* lectin I; CSA-II, *Cytisus sessilifolius* lectin II; CSII, *Cytisus scoparius* lectin II; ECorL, *Erythrina corallodendron* lectin; GSIV, *Griffonia simplicifolia* lectin IV; HPLC, high performance liquid chromatography; LAA-I, *Laburnum alpinum* lectin I; LAA-II, *Laburnum alpinum* lectin II; LOL, *Lathyrus ochrus* lectin; LTA, *Lotus tetragonolobus* lectin; MAH, *Maackia amurensis* haemagglutinin; PSA, *Pisum sativum* lectin; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; UEA-I, *Ulex europaeus* lectin I; UEA-II, *Ulex europaeus* lectin II; VFA, *Vicia faba* lectin.

Introduction

Lectins are widely used as tools for the study of the carbohydrate constituents of cell surfaces and glycoproteins and 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 previously been isolated and characterized for their carbohydrate-binding specificity [1–11]. On the basis of assays using simple sugars as inhibitors, these anti-H(O) lectins are known to be subdivided into three groups. The first group is the so-called eel serum type anti-H(O) lectins including *Ulex europaeus* lectin I (UEA-I) [1] and *Lotus tetragonolobus* lectin (LTA) [1–4]. 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) [5–8], *Laburnum alpinum* lectin I (LAA-I) [9], *Cytisus multiflorus* lectin I (CMA-I) [10], and *Cytisus sessilifolius* lectin I(CSA-I) [11]. Their binding is inhibited by *N, N'*-diacetylchitobiose. The third group is a new type of anti-H(O) lectins inhibited most strongly by lactose and includes *Laburnum alpinum* lectin II (LAA-II) [9], and *Cytisus sessilifolius* lectin II (CSA-II) [11].

We have also purified and characterized *Cytisus multiflorus* lectin II (CMA-II) [10] specific for lactose. CMA-II has been found to be Le^b blood group-specific because it is strongly inhibited by lacto-*N*-difucohexaose I having a determinant structure for the Le^b blood group [12].

In previous papers we have determined the complete amino acid sequences of LTA [13], UEA-I[14], UEA-II[14], LAA-I[15], CSA-I[16], *Bauhinia purpurea* lectin (BPA) [17],

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Cytisus scoparius lectin (CSII) specific for 2-acetamido-2-deoxy-D-galactose [18], and *Maackia amurensis* haemagglutinin (MAH) [19], and compared them with those of several other leguminous lectins. BPA is a D-galactose or lactose-binding lectin and MAH is a lectin specific for sialylated carbohydrate chains. Extensive homology was found throughout the stretch of the peptides. Furthermore, carbohydrate-binding peptides have been isolated from LTA, UEA-I, UEA-II, LAA-I, and CSA-I by affinity chromatography after treatment of the lectins with endoproteinase Asp-N or Lys-C [16, 20].

In this study, we determined the complete amino acid sequence of the lactose-binding anti-H(O) CSA-II using a protein sequencer. After digestion of CSA-II with endoproteinase Lys-C or Asp-N, the resulting peptides were purified by reversed-phase HPLC and then subjected to sequence analysis. The complete amino acid sequence of CSA-II was compared with those of other leguminous anti-H(O) lectins.

Materials and methods

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 Bondasphere (100 Å) for reversed-phase chromatography were obtained from Waters (Burlington, MA, USA). Endoproteinases Lys-C (*Lysobacter enzymogenes*) and Asp-N (*Pseudomonas fragi*) were purchased from Boehringer GmbH (Mannheim, Germany).

Methods

Purification of CSA-II CSA-II was isolated and purified from *Cytisus sessilifolius* seeds by affinity chromatography according to a previously reported method [11]. The affinity-purified CSA-II 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) for 60 min at a flow rate of 1 ml min^{-1}. The homogeneity of CSA-II was confirmed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol [21].

Digestion of CSA-II with endoproteinase Lys-C or Asp-N The purified CSA-II (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 2 μg of Asp-N for 18 h. The obtained peptide fragments 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 for 60 min at a flow rate of 1 ml min^{-1}. Elution profiles were monitored by the absorbance at 220 nm. The peptide fragments were manually collected.

Amino acid sequence analysis The amino acid sequences of the peptides fractionated by HPLC and the NH_2-terminal sequence of the first 38 amino acid residues of CSA-II were determined using a 6600 ProSequencer solid-phase protein sequencer (MilliGen/Biosearch, Burlington, MA, USA) and a PSQ-1 gas-phase protein sequencer (Shimadzu, Kyoto, Japan).

Results

Determination of the primary structure of CSA-II The sequence of the NH_2-terminal 38 amino acid residues of CSA-II was determined. The digestion of CSA-II with endoproteinase Lys-C (Fig. 1, Table 1) or Asp-N (Fig. 2, Table 1) was performed and the amino acid sequences of the obtained peptides determined. The complete amino acid sequence of CSA-II was determined using several overlapping sequences among the recovered peptides and is shown in Fig. 3. The structure of CSA-II contains 243 amino acid residues. The molecular weight of CSA-II calculated on the basis of the primary sequence was 26 620.99. The apparent molecular weight previously determined by SDS-polyacrylamide gel electrophoresis was 34 000 [11]. The difference in these values is presumed to be derived from the presence of the carbohydrate

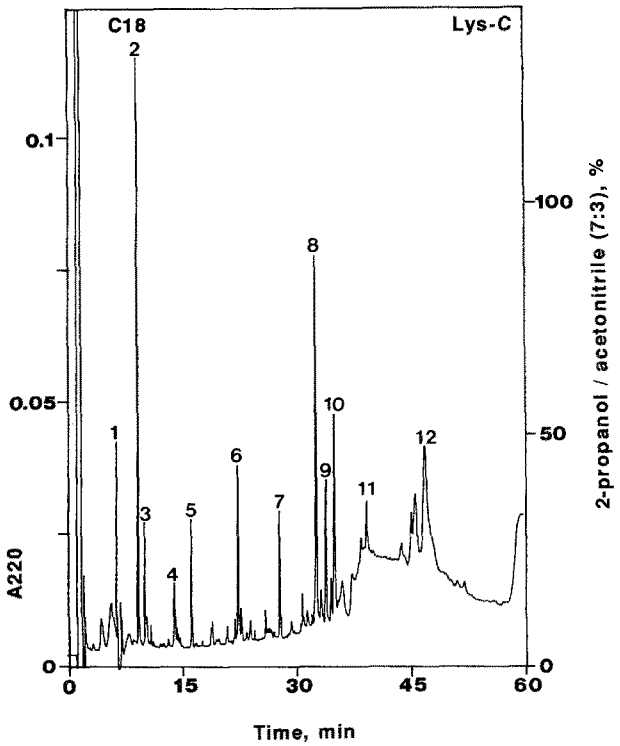

Figure 1. Reversed-phase HPLC of Lys-C digest of purified CSA-II on a column of C_{18}. The purified CSA-II (0.5 mg in 150 μl of 50 mM phosphate buffer, pH 8.0, 37°C) was digested with 5 μg of Lys-C for 18 h. The peptide fragments obtained were separated by reversed phase HPLC on a column of C_{18} using 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^{-1}. Elution profiles were monitored by the absorbance at 220 nm. The peptide fragments were manually collected.

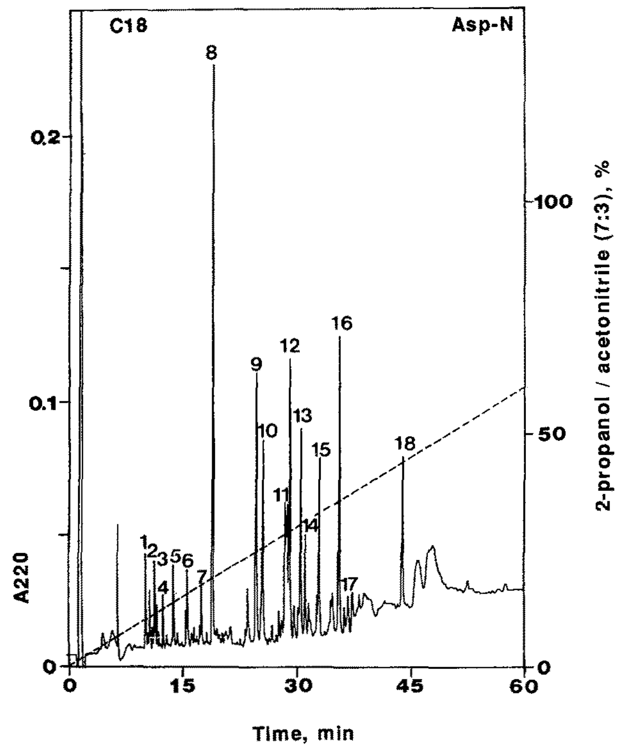

Figure 2. Reversed-phase HPLC of Asp-N digest of purified CSA-II on a column of C_{18}. The purified CSA-II (0.5 mg in 150 μl of 50 mM phosphate buffer, pH 8.0, 37°C) was digested with 2 μg of Asp-N for 18 h. The peptide fragments obtained were separated by reversed phase HPLC on a column of C_{18} using 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^{-1}. Elution profiles were monitored by the absorbance at 220 nm. The peptide fragments were manually collected.

```
                 10                  20                  30                  40                  50
S N D I S F K F D K F D P N G K Q L T F Q G Y A S V L D T G V L Q L N K V G T G L P K E I G G I A R-
=N=========================================================================
    =A11======= =A2== =A12======================     =A16=========================== =A15==========
                                                      =A17==========================================
=L5========== =L2== =L3========= =L10===================================== =L4========== =L8==========

                  60                  70                  80                  90                 100
-Y V A P F Q I W S K A T G E V A S F V T S F Q F F L E T S P N P A N G A S D G L T F F L A P P N S P-

========================== =A18====================== =A2===================== =A18=====================
======
==================== =L12==========================================================================

                  110                 120                 130                 140                 150
-L R R A G G Y L G L F E T S N K S D S S Y Q T V A V E F D T V G A P A N T W D P G Y P H I G V D V N-

====================== =A1======== =A10=================== =A8=================== =A9=============== =A7===

       =L6====================== =L10===================================================================
                                                         =L9==========================================

                  160                 170                 180                 190                 200
-R V T S I K T T K E K W N K R Y K R E V A N V W I T Y Q A S S K T L T A S L T Y P Q D Q T S D S V S-

================== =A4============== =A15=============================================== =A1==== =A3=====

======                 =L2== =L1== =L6============================ =L8=================================

                  210                 220                 230                 240
-V D F K A N L P E W V S V G F T G G T T V G G R E T T H E I L N W Y F S S T L E Y Q T

== =A13================================================= =A16=================
                                                                 =A13=================
======== =L11========================================================
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Figure 3. Complete amino acid sequence of CSA-II. Primary sequence in terms of the peptides obtained after digestion with endoproteinase Lys-C (L) and Asp-N (A). Residues were identified by automated sequence analysis of the Lys-C fragments (==L==), and the Asp-N fragments (==A==). The N-terminal 38 amino acid sequence (==N==) was also determined using a protein sequencer. The numbering starts from the N-terminus.

Table 1. Amino acid sequences of the Lys-C (a) and Asp-N (b) fragments of the purified CSA-II.

Fragments	Amino acid sequence
(a) Lys-C	
L 1	R-Y-K
L 2	W-N-K
	F-D-K
L 3	F-D-P-N-G-K
L 4	V-G-T-G-L-P-K
L 5	S-N-D-I-S-F-K
L 6	R-E-V-A-N-V-W-I-T-Y-Q-A-S-S-K
	A-G-G-Y-L-G-L-F-E-T-S-N-K
L 7	Y-V-A-P-F-Q-I-W-S-K
L 8	E-I-G-G-I-A-R-Y-V-A-P-F-Q-I-W-S-K
	T-L-T-A-S-L-T-Y-P-Q-D-Q-T-S-D-S-V-S-V-D-F-K
L 9	S-D-S-S-Y-Q-T-V-A-V-E-F-D-T-V-G-A-P-A-N-T-W-D-P-G-Y
	D-T-V-G-A-P-A-N-T-W-D-P-G-Y-P-H-I-G-V-D-V-N-R-V-T-S-I-K
L 10	S-D-S-S-Y-Q-T-V-A-V-E-F-D-T-V-G-A-P-A-N-T-W-D-P-G-Y-P-H-I-G-V-D-V-N-R-V-T
	Q-L-T-F-Q-G-Y-A-S-V-L-D-T-G-V-L-Q-L-N-K
L 11	A-N-L-P-E-W-V-S-V-G-F-T-G-G-T-T-V-G-G-R-E-T-T-H-E-I-L-N-W
L 12	A-T-G-E-V-A-S-F-V-T-S-F-Q-F-F-L-E-T-S-P-N-P-A-N-G-A-S-D-G-L-T-F-F-L-A-P-P
(b) Asp-N	
A 1	E-T-S-N-K-S
	D-Q-T-S
A 2	E-T-S-P-N-P-A-N-G-A-S
	D-K-F
A 3	D-S-V-S-V
A 4	E-K-W-N-K-R-Y-K-R
A 5	N-K-V-G-T
A 6	D-P-G-Y-P
A 7	D-V-N-R-V-T-S-I-K-T-T-K
A 8	D-T-V-G-A-P-A-N-T-W
A 9	D-P-G-Y-P-H-I-G-V
A10	D-S-S-Y-Q-T-V-A-V-E-F
A11	D-I-S-F-K-F
A12	D-P-N-G-K-Q-L-T-F-Q-G-Y-A-S
A13	D-F-K-A-N-L-P-E-W-V-S-V-G-F-T-G-G-T-T-V-G-G-R-E-T-T-H
	W-Y-F-S-S-T-L-E-Y-Q-T
A14	E-I-L-N-W-Y-F-S-S
A15	E-V-A-N-V-W-I-T-Y-Q-A-S-S-K-T-L-T-A-S-L-T-Y-P-Q
	E-I-G-G-I-A-R-Y-V-A-P-F-Q-I-W-S-K-A-T-G
	D-G-L-T-F-F-L-A-P-P-N-S-P-L-R-R-A-G-G-Y-L-G-L-F
A16	E-I-L-N-W-Y-F-S-S-T-L
	D-T-G-V-L-Q-L-N-K-V-G-T-G-L-P-K
A17	D-T-G-V-L-Q-L-N-K-V-G-T-G-L-P-K-E-I-G-G-I-A-R-Y-V-A
A18	D-G-L-T-F-F-L-A-P-P-N-S-P-L-R-R-A-G-G-Y-L-G-L-F
	E-V-A-S-F-V-T-S-F-Q-F-F-L

The fraction numbers for Lys-C and Asp-N fragments correspond to the number in Figs 1 and 2.

moiety (13%) of CSA-II [11]. As shown in Fig. 4, the complete amino acid sequence of CSA-II was compared with those of CSA-I [16], LAA-I [15], UEA-II [14], and UEA-I [14]. CSA-I, LAA-I, and UEA-II are all di-*N*-acetylchitobiose-binding anti-H(O) lectins. UEA-I is an L-fucose-binding anti-H(O) lectin. The overall identity values between CSA-II and UEA-I, CSA-I, LAA-I and UEA-II were respectively 70.2%, 53.1%, 50.8% and 52.0%.

Comparison of the amino acid sequence of a putative carbohydrate-binding domain of CSA-II with those of several other leguminous lectins The sequence of a putative carbohydrate-

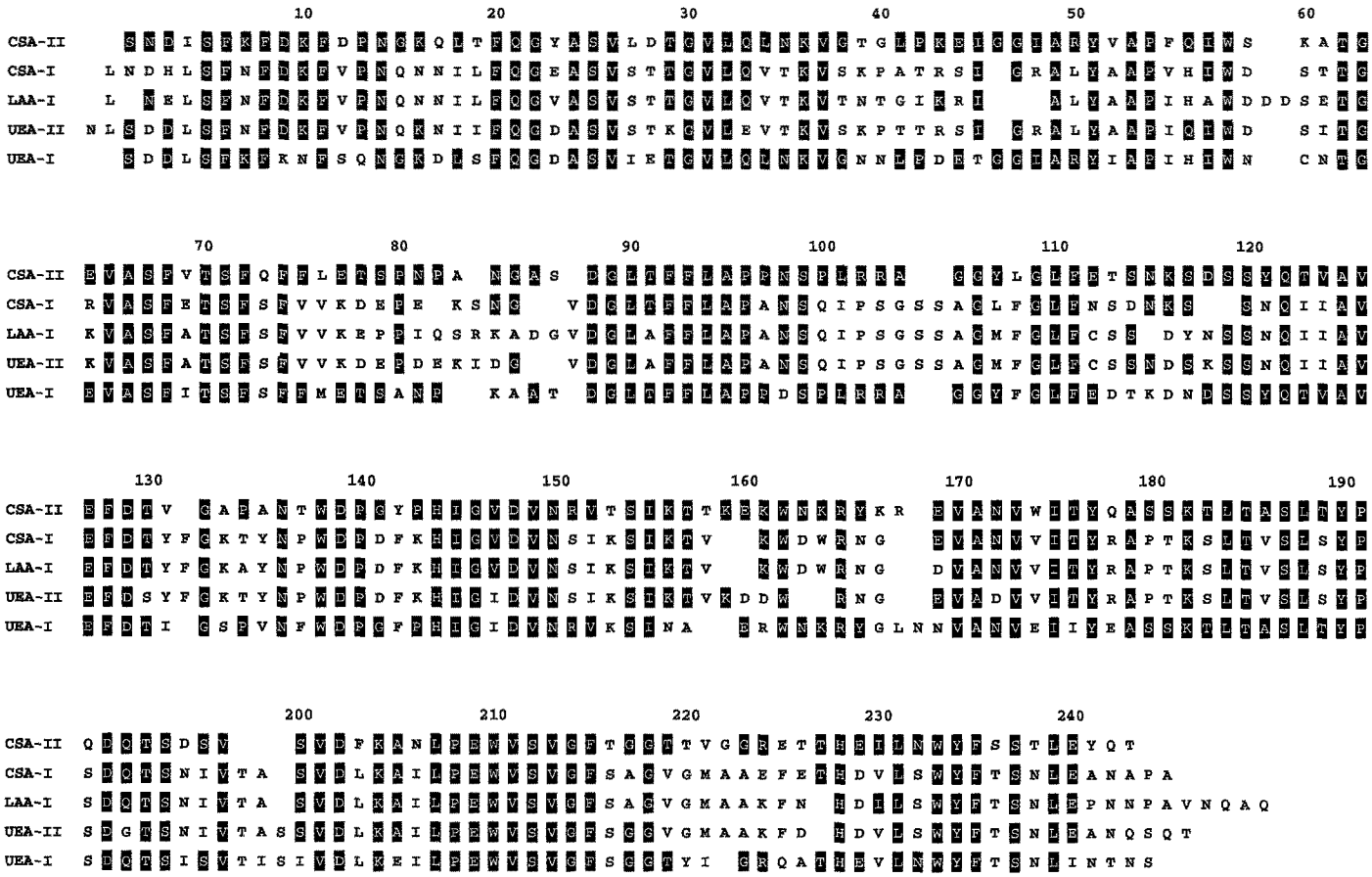

Figure 4. Comparison of complete amino acid sequence of CSA-II with those of CSA-I [16], LAA-I [15], UEA-II [14], and UEA-I [14]. The sequences were aligned to maximize homology. Residues identical to those of CSA-II are enclosed in black boxes.

binding domain of CSA-II was compared with all the available corresponding sequences of the lectins from the Leguminosae (Fig. 5). Amino acids involved in calcium-binding (bold letters) and manganese-binding (outlined letters) are also indicated in Fig. 5.

Discussion

The sequence analyses of the peptides obtained through the proteolytic cleavages mentioned above permit assignment of the amino acid sequence of CSA-II. The NH_2-terminal 38 amino acid residues were also identified by the use of the NH_2-terminal amino acid sequence data for the intact CSA-II. The COOH-terminal amino acid sequence of CSA-II may be incomplete because we did not carry out COOH-terminal sequence analysis. Therefore, the possibility of a longer sequence cannot be ruled out.

Figure 4 shows the comparison of the complete amino acid sequences of several leguminous lectins. UEA-I and UEA-II are lectins isolated from the seeds of the same species, *Ulex europaeus*, and the overall positional identity value between UEA-I and UEA-II is 52.0% [14]. CSA-II and CSA-I are both isolated from the seeds of *Cytisus sessilifolius* and the overall positional identity value between these two lectins is 52.7%. On the other hand, the overall identity values between CSA-I and LAA-I (86.1%) and between CSA-I and UEA-II (87.7%) is strikingly high [16]. These three lectins are di-*N*-acetylchitobiose-binding anti-H(O) lectins. The overall identity between CSA-II and UEA-I is 70.2%, although CSA-II and UEA-I were found to be respectively lactose- and L-fucose-binding anti-H(O) lectins.

Amino acid sequence comparisons carried out on several leguminous lectins [22–24] have suggested various conserved putative carbohydrate-binding domains, e.g. the conserved region shown in Fig. 5. The importance of the latter region, as well as of other conserved regions, in protein-carbohydrate interactions had been demonstrated by X-ray crystallographic studies on the three-dimensional structures of several complexes between leguminous lectins and their respective carbohydrate ligands [25–30]. X-ray crystallographic data is available on complexes between concanavalin A (Con A) and methyl α-D-mannoside [25], *Vicia faba* lectin (VFA) and glucose [26], *Pisum sativum* (pea) lectin (PSA) and glucose or mannose [27], *Lathyrus ochrus* lectin (LOL) and glucose or mannose [28], *Erythrina corallodendron* lectin (ECorL) and lactose [29], and *Griffonia simplicifolia* lectin IV (GSIV) with

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               130          140                150        160
CSA-II    -VAVEFDTV-GAPANT-W-DPG--YP---HIGVDVNRVTSIKT-

UEA-I     -VAVEFDTI-GSPVNF-W-DPG--FP---HIGIDVNRVKSINA-
LTA       -VAVEFDSY----HNI-W-DPKS-L-RSSHVGIDVNSIMSLKA-

MAH       -VAVEFDTYFGHSYDP-W-DP--NY-R--HIGIDVNGIESIKT-

CSA-I     -IAVEFDTYFGKTYNP-W-DP--DF--K-HIGVDVNSIKSIKT-
LAA-I     -IAVEFDTYFGKAYNP-W-DP--DF--K-HIGVDVNSIKSIKT-
UEA-II    -IAVEFDSYFGKTYNP-W-DP--DF--K-HIGIDVNSIKSIKT-

CSII      -VAVEFDTY----YNSAW-DPQTN-P---HIGIDVNTIKSKKV-
BPA       -VAVEFDTW---P-NTEWSDLR--YP---HIGINVNSTVSVAT-
GSIV      -VAVEFDTWI----NKDWNDPP--YP---HIGIDVNSIVSVAT-
ECorL     -LGVEFDTF---S-NP-W-DPPQ-VP---HIGIDVNSIRSIKT-

PSA       -VAVEFDTF----YNAAW-DPS-NRDR--HIGIDVNSIKSVNT-
VFA       -VAVEFDTF----YNAAW-DPS-NGKR--HIGIDVNTIKSIST-
LOL       -VAVEFDTF----YNTAW-DPS-NGDR--HIGIDVNSIKSINT-
Con A     -VAVELDTY---P-NTDIGDPS--YP---HIGIDIKSVRSKKT-
              10             20            30
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Figure 5. Amino acid sequence homology of a putative carbohydrate-binding domain of CSA-II aligned with other legume lectins, such as UEA-I [20], LTA [20], MAH [19], CSA-I [16], LAA-I [20], UEA-II [20], CSII [18], BPA [32], GSIV [31], ECorL [33], PSA [34], VFA [35], LOL [36], and Con A [37]. Amino acids involved in calcium-binding (bold letters) and manganese-binding (outlined letters) are indicated. Underlined residues represent carbohydrate-binding peptides demonstrated in the previous studies [16, 18, 20, 29]. The bottom numbering is for the Con A sequence. CSA-II is a lactose or D-galactose-binding anti-H(O) lectin. UEA-I and LTA are L-fucose-binding anti-H(O) lectins. MAH is a lectin binding to sialic acid-containing carbohydrate chains. CSA-I, LAA-I, and UEA-II are *N,N'*-diacetylchitobiose-binding anti-H(O) lectins. CSII, BPA, GSIV, and ECorL are a group D-galactose or *N*-acetyl-D-galactosamine-binding lectins, and the lower group (PSA, VFA, LOL and Con A) comprises D-mannose-binding lectins.

a blood-group specific tetrasaccharide [30]. Lectin-bound calcium and manganese ions were found to be located in the vicinity of the conserved region shown in Fig. 5 and the relative positions of these ions are identical to each other in all leguminous lectins examined to date. Residues corresponding to Glu-8, Asp-10, Asp-19 and His-24 of Con A, involved in manganese binding, and Asp-10, Asn-14 and Asp-19 of Con A, involved in calcium binding, are conserved in CSA-II (Fig. 5). Although these metal ions do not directly interact with carbohydrate ligands, they are required for maintaining the spatial structure necessary for carbohydrate binding.

High resolution X-ray crystallography of several leguminous lectins has shown that other residues not shown in Fig. 5 are important in carbohydrate ligand binding, e.g. Asp-208 and Gly-227 in Con A [25], Asp-81 and Gly-99 in LOL [28], Asp-89 and Gly-107 in ECorL [29] and Asp-89 in GSIV [30]. Figure 4 shows that Asp-88 and Gly-106 (corresponding to Asp-208 and Gly-227 in Con A) are conserved in CSA-II.

The isolation of carbohydrate-binding peptides from CSA-II was unsuccessful although we tried several times using several kinds of affinity columns including a column of Lactose-Gel (E. Y. Laboratory, San Mateo, CA, USA). The affinity of intact CSA-II for the Lactose-Gel column was weaker than that of other anti-H(O) lectins for their respective affinity columns with haptenic carbohydrates. The amino acid sequence of the putative carbohydrate-binding domain of CSA-II (Fig. 5) shows a high degree of homology to that of UEA-I. UEA-I is an L-fucose-binding anti-H(O) lectin and CSA-II is a lactose-binding anti-H(O) lectin. These facts indicate that CSA-II recognizes the carbohydrate structure, including the L-fucose residue, on the erythrocyte cell membranes similarly to lactose-binding LAA-II. Treatment of human O erythrocytes with a purified H-decomposing enzyme (α-L-fucosidase) from *Bacillus fulminans* destroyed the haemagglutinating activity of the cells by LAA-II, indicating the importance of the L-fucose residue for the recognition by LAA-II in the carbohydrate structure on the erythrocyte cell membranes [9]. Because of the lack of α-L-fucosidase, the possibility of the loss of activity by CSA-II after treatment of the erythrocytes with α-L-fucosidase could not be checked. However, CSA-II and LAA-II are both lactose-binding anti-H(O) lectins, and CSA-I and LAA-I isolated respectively from the same seeds, *Cytisus sessilifolius* and *Laburnum alpinum* were found to have very similar primary structures [16]. Therefore, an L-fucose residue might be required for the recognition of the carbohydrate structure on the erythrocyte cell membranes by CSA-II.

An *N*-glycosylation site in CSA-II is assumed to be Asn-115 as deduced from the complete amino acid sequence (Fig. 3). This site is located in the sequence -Ser-Asn-Lys-Ser-. Among the homologous lectins which contain carbohydrate, CSA-I is presumed to be glycosylated at Asn-117 [16], LAA-I at Asn-119 [15], UEA-I at-Asn-10 and 116 [14], UEA-II at Asn-118 and 245 [14], and LTA at Asn-4 [13]. The approximate molecular weight difference of 7000 between calculated and determined molecular weight values suggests the possibility of other *N*-glycosylation sites which could not be found in this determination, or of *O*-glycosylation.

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