

# Purification and Characterization of the $\alpha$ -1,3-Mannosylmannose-recognizing Lectin of *Crocus vernus* Bulbs\*

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**A unique mannose-binding lectin, highly specific for terminal Man( $\alpha$ 1,3)Man groups, was isolated from bulbs of crocus (*Crocus vernus* All.). The lectin failed to bind to a mannose affinity column and was purified by simple gel permeation chromatography (Sephacryl S200). The purified lectin, obtained in crystalline form, had a molecular mass of 44 kDa on gel filtration and showed a single peptide band with a molecular mass of 11 kDa on SDS-polyacrylamide gel electrophoresis, indicating it to be a tetrameric protein composed of four identical subunits. The N-terminal amino acid sequence analysis of the crocus lectin showed essentially no homology with that of other mannose-binding bulb lectins. The crocus lectin selectively interacted with the wild type *Saccharomyces cerevisiae* and other mannans carrying terminal Man( $\alpha$ 1,3)Man but not with those lacking this disaccharide unit. In hapten inhibition studies, methyl  $\alpha$ -mannopyranoside did not inhibit the mannan-lectin interaction. Of various  $\alpha$ -mannooligosaccharides, those having the Man( $\alpha$ 1,3)Man sequence showed the highest inhibitory potency, confirming the strict requirement of lectin for terminal  $\alpha$ 1,3-linked mannosylmannose units. An affinity column of immobilized lectin enabled the complete resolution of yeast mannan and glycogen. The immobilized lectin may provide a useful tool for purification and analysis of biologically important polysaccharides and glycoproteins.**

Since the first report on a yeast mannan-binding lectin from bulbs of tulip, *Tulipa generiana* (1), several kinds of  $\alpha$ -mannose-binding lectins have been studied in our laboratory, mostly from bulbs of the family *Amaryllidaceae*, such as *Galanthus nivalis* (snow drop; GNA)<sup>1</sup> (2, 3), *Hippeastrum hybrid* (amaryllis), *Narcissus pseudonarcissus* (daffodil) (4), *Sternbergia lutea* (5), and *Allium sativum* (garlic) (6), which belongs to the *Lilaceae* family. A similar lectin was also isolated from leaves of *Listera ovata* (twayblade) (7). These lectins are distinct from hitherto known mannose/glucose-binding lectins, such as concanavalin A and other legume lectins, in their strict requirement for the axial C-2 hydroxyl group of  $\alpha$ -D-mannopyranose. Our detailed studies of the carbohydrate binding specificity of these lectins have indicated some differences with

regard to the location of mannosidic linkages at the terminal and/or internal position in the carbohydrate chain. For instance, GNA recognizes terminal Man( $\alpha$ 1,3)Man (3) and also certain internal linkages (8). Similarly, *L. ovata* lectin can recognize the internal sequence of  $\alpha$ (1,3)-linked mannosidic linkages (7). In a survey of new plant lectins we found that the bulbs of *Crocus vernus* All., belonging to the family *Iridaceae*, accumulates a very unique mannose-binding lectin with a very strict requirement for terminal  $\alpha$ -1,3-mannosyl mannose units. This lectin, designated CVA, agglutinates rabbit but not human erythrocytes and does not appear to have homology with hitherto known mannose-binding lectins in its sequence of N-terminal amino acids.

This paper reports the purification, characterization, and detailed binding specificity of the crocus lectin, as revealed by interactions with a series of structurally defined yeast mannans, haptenic inhibition studies using a series of synthetic branched manno-trisaccharides, substituted at O-3 or O-6 of the  $\alpha$ -mannose units. The application of the immobilized lectin for the selective fractionation of mannans and plant glycoproteins is also described.

## EXPERIMENTAL PROCEDURES

**Isolation of CVA**—CVA was isolated from the crude extract of bulbs of spring flowering crocus. The peeled bulbs of *C. vernus* All. (160 g, water content 72%), cultivated in Niigata prefecture, Japan, in October 1994, were homogenized with 10 mM phosphate-buffered saline (PBS, pH 6.8) containing 0.1 M  $(\text{NH}_4)_2\text{SO}_4$  solution overnight at 10 °C, and the PBS extract was centrifuged. To the supernatant of the extract was added  $(\text{NH}_4)_2\text{SO}_4$  to 30% saturation, and the precipitate was collected, dialyzed against distilled water, and lyophilized (220 mg). Because CVA was not retained on a mannose-agarose (Sigma) column normally used for the isolation of most mannose-binding lectins, subsequent purification was carried out by gel filtration chromatography. The crude lectin (100 mg) obtained by  $(\text{NH}_4)_2\text{SO}_4$  precipitation was dissolved in a minimum volume of PBS and applied onto a Sephacryl S200 (Pharmacia Biotech Inc.) column (2.5 × 200 cm) equilibrated with PBS; elution was conducted with the same buffer. The protein peak(s) was monitored by absorption at 280 nm, and the lectin activity was tested by a precipitation reaction with *Saccharomyces cerevisiae* mannan. The lectin-containing fractions were collected and dialyzed against distilled water, concentrated, and lyophilized (yield, 65 mg).

**CVA Affinity Column**—To purified CVA (15 mg) dissolved in 100 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl (2 ml) was added 700 mg of AF-Tresyl Toyopearl 650 (Toso Co.) in 5 ml of the same buffer. The mixture was shaken gently for 6 h at 25 °C and kept for a further 4 h at 10 °C according to the technical specification. The reaction product was filtered and washed with 100 mM phosphate buffer (pH 6.8) in 0.5 M NaCl, and the free tresyl-groups were blocked with 100 mM Tris-HCl buffer (pH 8.0) for 1 h at 25 °C. Determination of protein in the washing solution indicated approximately 70% of the lectin was conjugated to the Toyopearl.

For affinity chromatographic resolution of mannans and other polysaccharides or glycoproteins, each mannan or glycoprotein (1 mg) was applied to the CVA-toyopearl column (0.5 × 5 cm) and eluted at 5 °C, first with PBS, and then with 20 mM diaminopropane (DAP). Elution was monitored by measuring absorption at 280 nm (protein) or determination of mannose at 490 nm by the phenol-sulfuric acid method (9)

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<sup>1</sup> The abbreviations used are: GNA, snowdrop *G. nivalis* lectin; CVA, *C. vernus* lectin; DAP, diaminopropane; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography(y).

in a microscale system consisting of 100  $\mu$ l of sample solution, 150  $\mu$ l of 5% phenol, and 750  $\mu$ l of concentrated  $H_2SO_4$ . When necessary, the glycan (glycoconjugate) eluted with DAP was collected, immediately dialyzed against water, and lyophilized.

**Polysaccharides and Glycoproteins**—The mannan of wild type *S. cerevisiae*, designated normal yeast mannan, was purified in our laboratory from the cell walls of bakers' yeast, as was sake yeast Kyoukai number 7. This mannan, isolated from the mechanically disintegrated cell walls, was further purified by digestion with glucoamylase to remove contaminating glycogen (10). Among various structurally different yeast mannans, the mannan of 4488 strain (mn 1 mutant) lacks  $\alpha$ -1,3-mannosyl linkages (11), the mannan of *Candida albicans* NIH B-792 strain contains both terminal and internal  $\alpha$ Man(1,3)Man linkages, and the *Candida parapsilosis* IFO 1396 strain lacks terminal but contains internal  $\alpha$ -1,3-linkages (12).  $\alpha$ -1,2-Mannosidase-treated mannan was a gift from Dr. T. Nakajima, Tohoku University.

Elsinan, an exocellular glucan of *Elsinoe leucospila*, was available from a previous study (13). Dextran B-1355-S was a gift of Dr. A. Jeans (Peoria, IL). Lima bean lectin and *Phaseolus vulgaris* lectin were available in our laboratory.

**Oligosaccharides**—All the monosaccharides and their methyl or *p*-nitrophenyl glycosides were purchased commercially or were available in our laboratories. Man( $\alpha$ 1,3)Man, Man( $\alpha$ 1,3)Man- $\alpha$ -OME, and Man( $\alpha$ 1,6)Man- $\alpha$ -OME were purchased from Sigma. Man( $\alpha$ 1,2)Man- $\alpha$ -OME, Man( $\alpha$ 1,6)[Man( $\alpha$ 1,3)]Man- $\alpha$ -OME, and Man( $\alpha$ 1,6)[Man( $\alpha$ 1,3)]Man( $\alpha$ 1,6)[Man( $\alpha$ 1,3)]Man- $\alpha$ -OME were purchased from Pfanstiel Laboratories Inc. Man( $\alpha$ 1,3)[Gal( $\alpha$ 1,6)]Man- $\alpha$ -OME, Man( $\alpha$ 1,3)[Glc( $\alpha$ 1,6)]Man- $\alpha$ -OME, and Man( $\alpha$ 1,6)[Glc( $\alpha$ 1,3)]Man- $\alpha$ -OME Man( $\alpha$ 1,6)[Gal( $\alpha$ 1,3)]Man- $\alpha$ -OME were available at our laboratory (University of Michigan). Gal( $\alpha$ 1,3)ManOME (14) and Man( $\alpha$ 1,3)Glc (15) were synthesized in our laboratory.

**Molecular Mass Determination**—The molecular mass of the purified CVA was estimated by gel filtration chromatography using Sephacryl S200 column (1.5  $\times$  120 cm) equilibrated with 100 mM PBS (pH 6.8). The column was calibrated with thyroglobulin (670 kDa), gamma globulin (158 kDa), bovine serum albumin (66.2 kDa), ovalbumin (44 kDa), myoglobin (17.5 kDa), and cobalamin (1.3 kDa); CVA (1.2 mg) was applied to the column and eluted with the same buffer, each 1.0-ml fraction being assayed for protein by determining absorbance at 280 nm.

**Polyacrylamide Gel Electrophoresis (PAGE)**—PAGE was conducted with an ATTO Mini-Slab electrophoresis, model AE-6000, using a precast 15% sulfate-polyacrylamide gel (SPU-15S) for molecular range, 1–6  $\times$  10<sup>6</sup>, or 12.5% sulfate-polyacrylamide gel (NPU-15L; 1.4–8  $\times$  10<sup>4</sup>), in the presence (reduced PAGE) or the absence of 2-mercaptoethanol (native PAGE). Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. Molecular mass markers were low range protein markers (Promega) containing carbonic anhydrase (31 kDa), soybean trypsin inhibitor (20.4 kDa), myoglobin (16.9 kDa), lysozyme (14.4 kDa), and CNBr-cleaved myoglobin (8.1, 6.2, and 2.5 kDa), and also a molecular standard protein kit (Pharmacia).

**Periodate Modification of Mannan and Oligosaccharide**—*S. cerevisiae* mannan (10 mg in 1 ml) was oxidized with 50 mM sodium periodate at 10 °C for 5 days. After decomposition of the excess periodate with ethylene glycol, the oxidized mannan was reduced with sodium borohydride at 25 °C for 3 h, the excess borohydride was decomposed by stirring with Amberlite IR 120 (H<sup>+</sup>-form), the product was dialyzed, and the oxidized-reduced mannan in the nondialyzable was lyophilized. An aliquot of Man( $\alpha$ 1-3)ManOME was also subjected to periodate oxidation and reduction, and the product was purified by passage through an Econo-pack 10 DG (Bio-Rad).

**Quantitative Precipitation and Hapten Inhibition**—Quantitative precipitation reactions were conducted essentially by the method of So and Goldstein (16). Varying amounts of polysaccharides or glycoproteins in microcentrifugal tubes were interacted with 20–25  $\mu$ g of CVA, each in a total volume of 150  $\mu$ l of 50 mM PBS (pH 6.8). After incubation at 35 °C for 1 h, the reaction mixture was kept at 5 °C for 48 h, centrifuged, and analyzed for protein in the precipitate by the micro Lowry method (17). For hapten inhibition studies, varying amounts of haptens were added to the reaction mixture containing 20  $\mu$ g of CVA and 20  $\mu$ g of *S. cerevisiae* native mannan in a total volume of 150  $\mu$ l. Protein in the precipitates of the reaction mixtures was determined using bovine serum albumin as standard, and the inhibition ratios were calculated.

**pH Profile of Precipitation Reaction**—A point on the yeast mannan (*S. cerevisiae*)-CVA precipitation curve was selected, and the amount of protein precipitated was determined at several pH values. Present in the reaction mixture were CVA (27  $\mu$ g) and mannan (22  $\mu$ g) in a total of 150  $\mu$ l of 50 mM buffer of the following composition: glycine-HCl (pH

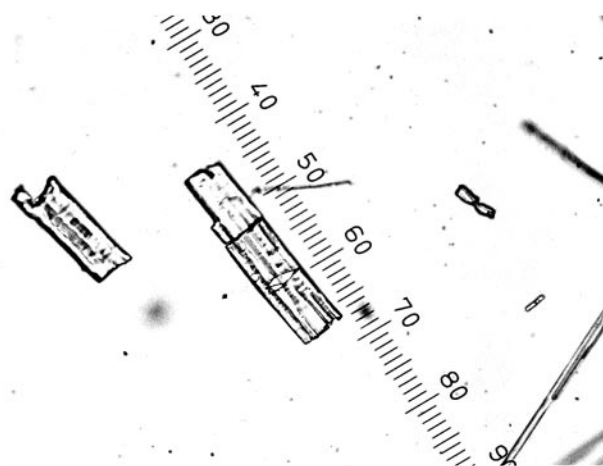


FIG. 1. Crystalline lectin from *C. vernus* bulbs.

2.0–3.5); acetate (pH 4.0–5.5); phosphate (pH 6.0–8.3), and glycine-NaOH (pH 8.5–10.4).

**Amino Acid Analysis**—The purified lectin (40  $\mu$ g) transferred onto polyvinylidene difluoride membrane was hydrolyzed with 6 M HCl for 21 h at 110 °C. Amino acid analysis was performed with a Shimadzu high performance liquid chromatograph (HPLC) LC-10A.

**N-terminal Amino Acid Sequence Analysis**—Amino acid sequencing analysis was carried out on an ABI 4473 (automated sequencer) at the Protein Structure Facility of the University of Michigan Medical School. The subunit (11 kDa) of CVA on SDS-PAGE was transferred onto a polyvinylidene difluoride membrane and was digested with Asp-N or Lys-C for 18 h at 37 °C. The endoproteinase digestion products were subjected to reverse phase HPLC, elution being monitored by  $A_{220\text{ nm}}$ . Amino acid sequence analysis of the peptide fractions was carried out as described above.

## RESULTS

**Purification of CVA**—A preliminary study showed that the PBS extract of *C. vernus* bulbs, which agglutinated rabbit erythrocytes, interacted with yeast mannan but not with  $\alpha$ -glucans, e.g. glycogen, dextran, etc., suggesting that it contained an  $\alpha$ -mannose-specific lectin. The lectin in the crude protein extract was precipitated with 30% saturated  $(NH_4)_2SO_4$ . Interestingly, however, the lectin was not retained on a mannose column that had been used for the isolation of most  $\alpha$ -mannose-binding lectins. Therefore, the crude lectin fraction obtained by  $(NH_4)_2SO_4$  precipitation was applied to a Sephacryl S200 column (2.5  $\times$  200 cm). Elution with 10 mM PBS indicated the presence of a single, symmetrical protein peak that interacted with *S. cerevisiae* mannan. It was rechromatographed on the same column, concentrated, dialyzed against distilled water, and lyophilized. Thus, this simple gel filtration procedure afforded the lectin (CVA) in a pure state; for example, 100 mg of the crude lectin extract yielded 67 mg of pure lectin.

The above purified CVA crystallized spontaneously in the form of needles or pillars when its concentrated aqueous solution (approximately 10 mg in 200  $\mu$ l of water) was stored at 5 °C for several weeks (Fig. 1).

Purified CVA gave a molecular mass of 44,000 Da as estimated by gel filtration on a Sephacryl S200 column, which was calibrated with standard proteins (Fig. 2). Upon SDS-PAGE, the purified lectin gave a single polypeptide band of 11,000–11,500 Da both in the absence and the presence of 2%  $\beta$ -mercaptoethanol. Figs. 2 and 3 show the gel filtration profile and the results of SDS-polyacrylamide gel electrophoresis. These results indicate CVA to consist of four identical subunits with no intersubunit disulfide bonds.

**Chemical Characteristics of CVA**—As noted above, the purified CVA, obtained as crystals, is a protein composed of four

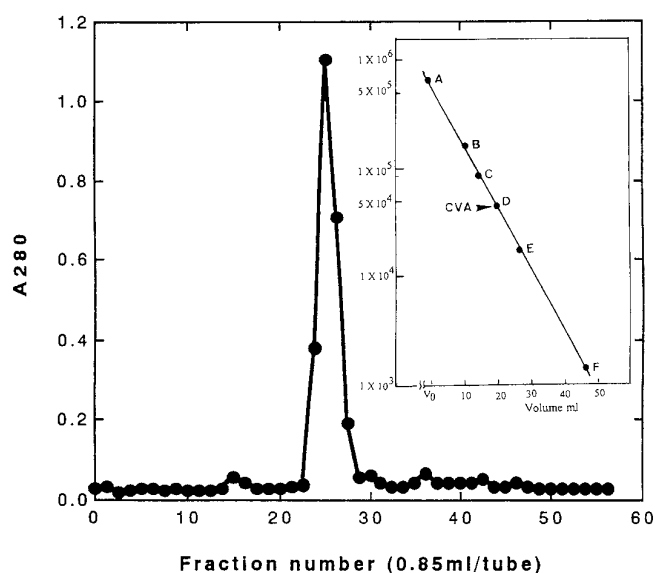


FIG. 2. Gel filtration of CVA on Sephacryl S200. The column ( $1.5 \times 120$  cm) was loaded with 2 mg of the purified lectin and eluted with 0.05 M PBS (pH 6.8). The molecular mass standards were thyroglobulin (670 kDa) (A); gamma globulin (158 kDa) (B); bovine serum albumin (66.2 kDa) (C); ovalbumin (45 kDa) (D); myoglobin (17 kDa) (E); and vitamin B<sub>12</sub> (F). Fractions of 0.85 ml were collected, and proteins were assayed by absorbance at 280 nm.

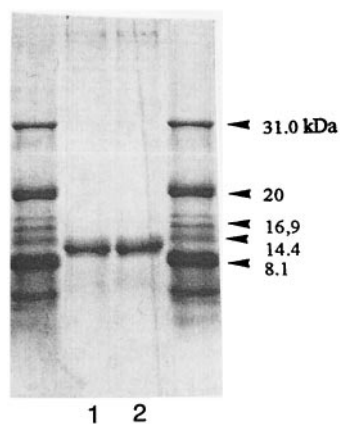


FIG. 3. SDS-polyacrylamide gel electrophoresis of purified CVA. Left and right lanes, standards: carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa); myoglobin (16.9 kDa); lysozyme (14.4 kDa); and CNBr-cleaved myoglobin (8.1–6.2 kDa). Lane 1, native CVA. Lane 2, CVA in the presence of  $\beta$ -mercaptoethanol.

subunits of a single polypeptide with a molecular mass of 11,000 Da; it is devoid of carbohydrate, similar to other mannose-binding lectins of *Amaryllidaceae* bulbs. CVA contains essentially no metals (metal analysis showed only 0.04% of metal cations, thought to be  $\text{Ca}^{+2}$ ).

The amino acid composition of CVA appeared somewhat similar to that of GNA, the mannose-binding lectins of the *Amaryllidaceae* family, as shown in Table I. The CVA molecule was estimated to consist of 410 amino acid residues. The lectin contains high proportions of asparagine/aspartic acid, glycine and leucine, like those found in GNA (2). It is also interesting that CVA contains a small proportion of methionine that is absent in GNA. The molecular mass (44,071–44,139 Da, calculated from the amino acid data) was consistent with the apparent molecular mass of 44,000 Da obtained by gel permeation chromatography. Therefore, assuming that the subunit mass is 11 kDa, the CVA molecule must contain four identical single peptide chains, each consisting of 102 amino acid residues.

The N-terminal amino acid sequencing analysis of CVA pro-

TABLE I  
Comparison in amino acid compositions of lectins of CVA and GNA

Amino acid	CVA		GNA
	mol %	Residues/subunit	mol %
Asx	22.0	22	15.4
Thr	5.9	6	7.5
Ser	7.3	7	10.5
Glx	8.5	9	7.2
Pro	3.5	4	4.1
Gly	9.0	9	12.0
Ala	6.2	6	3.5
Cys	2.0	3	1.7
Ile	3.2	3	5.4
Leu	7.6	8	8.9
Tyr	2.8	3	5.0
Phe	2.9	3	2.1
His	2.8	3	1.0
Lys	2.3	3	4.2
Arg	4.5	4	3.5
Val	7.1	7	4.8
Met	2.4	3	0.0
(Total 102)			
Molecular mass	44,000		50,000
Subunit mass	11 kDa		12 kDa

TABLE II  
Comparison of partial N-terminal amino acid sequence of CVA and two *Amaryllidaceae* lectins, GNA and SLA

CVA	1	5	10	15	20	25
	NIPQVRNVLFSSQVMYDNAQLATR					
GNA	1	5	10	15	20	25
	DNIYLSGETLSTGEFLNYGSFVVFVIM					
SLA <sup>a</sup>	1	5	10	15	20	25
	DNYLYSGETLFSQGLNYGNRYRIM					

<sup>a</sup> SLA, *S. lutea* lectin.

vided a partial N-terminal amino acid sequence. Table II indicates that the molecular structure of CVA is strikingly different from the hitherto characterized mannose-binding lectins of *Amaryllidaceae*; the N-terminal sequence of 24 amino acids of CVA indicated that its homology with GNA is only 8.3%, whereas the *S. lutea* lectin possesses 76% homology with GNA (7).

*Interactions of CVA with Polysaccharides and Glycoproteins*—Preliminary precipitation studies showed that CVA interacts to form a precipitate with the mannan of ordinary yeast (*S. cerevisiae*) and some plant glycoproteins, such as lima bean lectin (18) and *P. vulgaris* lectin (19), but not with  $\alpha$ -D-glucans (glycogen, dextran, etc.), ovalbumin, and ovomucoid. To ascertain the mannosyl linkage specificity of CVA, structurally different yeast mannans whose repeating unit structures have been well established (Fig. 4, I–V) were examined by quantitative precipitation. As shown in Fig. 5, CVA strongly precipitated normal types of yeast mannans, *i.e.*, bakers' yeast (*S. cerevisiae*) and *S. cerevisiae* Kyoukai number 7 mannan, both having essentially the same branched structure (Fig. 4, I), but dextran 1355S, having numerous  $\alpha$ 1,3-glucosidic linkages (20), did not react. Elsinan, a fungal linear  $\alpha$ -1,3/1,4 glucan (15) reacted only slightly, as in the case of GNA (3), indicating that CVA specifically recognizes the  $\alpha$ -mannosyl configuration. Interestingly, CVA does not interact with mannans that are devoid of Man( $\alpha$ 1,3)Man units at their nonreducing termini (Fig. 4). Fig. 6 shows precipitation curves of CVA with these structurally different mannans, *i.e.*, the normal yeast mannan of *S. cerevisiae* and other mannans, including enzymatically  $\alpha$ -1,2-mannose-deleted *S. cerevisiae* mannan (Fig. 4, IV), *C. albicans* B-792 mannan, which contains branched  $\alpha$ -1,3-mannosyl termini (Fig. 4, V), the  $\alpha$ -1,3-mannose-deficient mannan of *S. cerevisiae* 4484 mutant strain (Fig. 4, III), and *C. parapsilosis* 1396 mannan (Fig. 4, II) containing internal (1,3)-linked

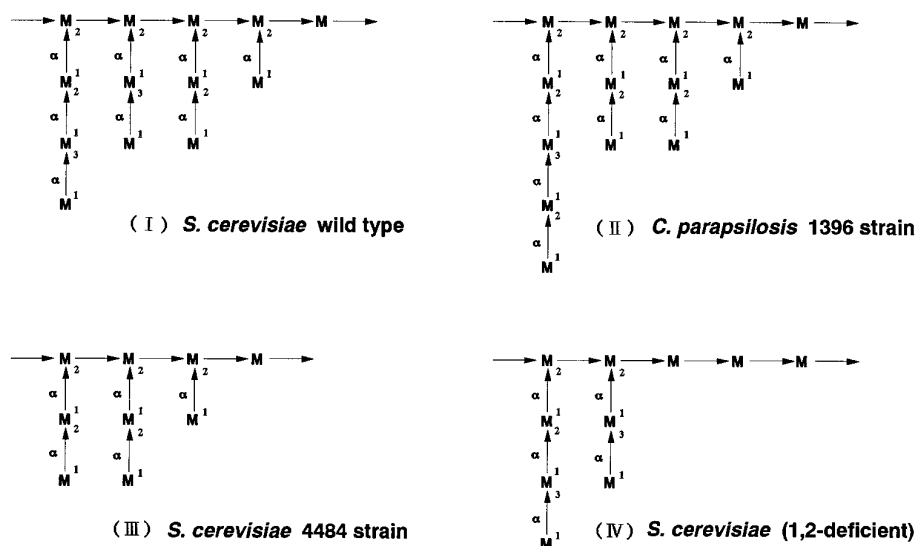


FIG. 4. Repeating unit structure of various yeast mannans used for interaction with CVA.

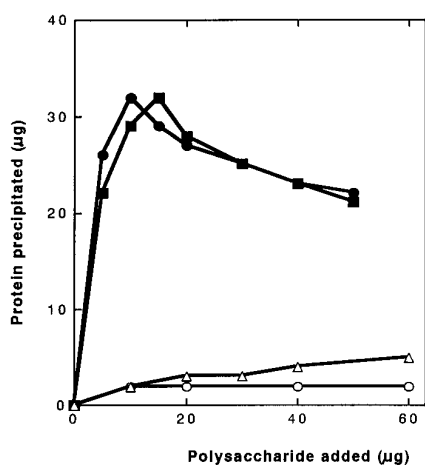
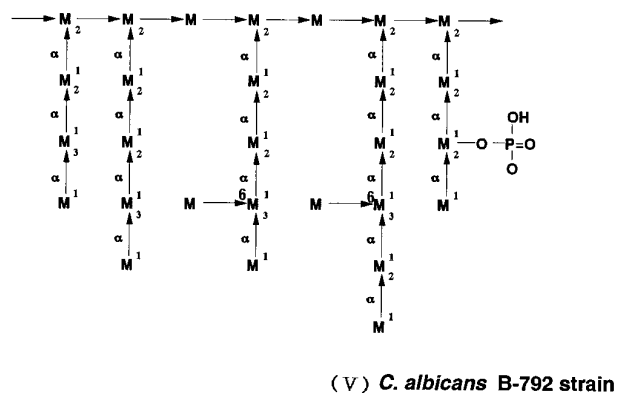


FIG. 5. Quantitative precipitation curves between CVA and "normal" yeast mannans (see Fig. 4) and  $\alpha$ -D-glucans. Shown are mannan of *S. cerevisiae* (bakers' yeast, ●); *S. cerevisiae sake* (Sake yeast, ■); *Leuconostoc mesenteroides* 1355S dextran (○); and Elsinan of *E. leucospila* (△). Varying amounts of polysaccharides were incubated with 35  $\mu$ g of CVA in a volume of 150  $\mu$ l, and the amount of protein precipitated in each tube was determined by the Lowry method (17).

FIG. 6. Quantitative precipitation curves of CVA and structurally different yeast mannans (see Fig. 4, I-V). ●, *S. cerevisiae* (I); ○,  $\text{NaIO}_4$  and  $\text{NaBH}_4$ -treated I; △, *C. parapsilosis* 1936 (II); ▲, *S. cerevisiae* 4484 mutant (III); ■, enzymically generated terminal ( $\alpha$ 1,2)Man-deleted *S. cerevisiae* mannan (IV); □, *C. albicans* B-792 mannan (V).

mannose residues not adjacent to their termini. Among these mannans it is striking that mannan III (Fig. 4), deficient in terminal  $\alpha$ -Man(1,3)Man, did not interact with CVA. On the contrary, under the same conditions, GNA and the *S. lutea* lectin gave appreciable precipitation with III (data not shown). The recognition of  $\alpha$ -Man(1,3)Man by CVA is confined to the

terminal position, because *C. parapsilosis* 1396 mannan (Fig. 4, II), which contains internal residues, did not react. The periodate-oxidized and borohydride-reduced *S. cerevisiae* mannan was no longer reactive with CVA, even though the intact  $\alpha$ -(1,3)-mannose units are present adjacent to the modified end groups (Fig. 6). These quantitative precipitation reactions con-

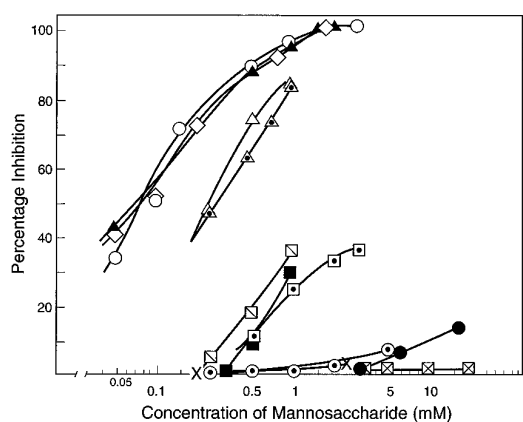


FIG. 7. Hapten inhibition of the CVA *S. cerevisiae* mannan precipitation system (see text and Table III).  $\circ$ , Man( $\alpha$ 1,3)Man- $\alpha$ -OME;  $\blacktriangle$ , Man[ $\alpha$ 1,6][Man( $\alpha$ 1,3)]Man- $\alpha$ -OME;  $\diamond$ , Man( $\alpha$ 1,6)[Man( $\alpha$ 1,3)]Man( $\alpha$ 1,6)[Man( $\alpha$ 1,3)]Man- $\alpha$ -OME;  $\triangle$ , Man( $\alpha$ 1,3)[Glc( $\alpha$ 1,6)]Man- $\alpha$ -OME;  $\Delta$ , Man( $\alpha$ 1,3)[Gal( $\alpha$ 1,6)]Man- $\alpha$ -OME;  $\square$ , Man( $\alpha$ 1,6)[Gal( $\alpha$ 1,3)]Man- $\alpha$ -OME;  $\blacksquare$ , Man( $\alpha$ 1,6)[Glc( $\alpha$ 1,3)]Man- $\alpha$ -OME;  $\square$ , Man( $\alpha$ 1,6)Man- $\alpha$ -OME;  $\odot$ , Man( $\alpha$ 1,3)Glc;  $\times$ , Gal( $\alpha$ 1,3)Man $\alpha$ OME;  $\bullet$ , Man( $\alpha$ 1,2)Man- $\alpha$ -OME;  $\boxtimes$ , Man $\alpha$ OME.

firmed that CVA possesses a unique, highly specific binding specificity with regard to its  $\alpha$ -1,3-mannosyl linkages and their localization. Due to its highly selective binding property, CVA was not retained on the mannose column, as described in the purification procedure.

**Inhibition of Precipitation Reaction by Haptenic Mannosaccharides**—To confirm the binding specificity of CVA, detailed inhibition studies were conducted using the precipitation system of CVA with the *S. cerevisiae* mannan under maximum precipitating conditions. Mannose, glucose, and other monosaccharides showed no inhibition. Methyl  $\alpha$ -mannopyranoside, known as one of the best inhibitors for most mannose-specific lectins, was found to be a very poor inhibitor; it does not inhibit up to 30 mM, and at 100 mM gave only 20% inhibition. This result is consistent with the fact that CVA does not bind to single  $\alpha$ -mannosyl end groups of any branched mannan. Inhibition of a series of linear and branched mannosaccharides were compared. The inhibition curves of these oligosaccharides are depicted in Fig. 7 and in Table II, in which the percentage of inhibition of the oligosaccharides at various concentrations are compared. Some oligosaccharides exhibited very poor inhibition and are not presented at 50% inhibition concentrations. According to the inhibition curves of mannooligosaccharides, they may be divided into several groups (Fig. 7). The best inhibitor group includes those having the Man( $\alpha$ 1,3)Man sequence, such as, Man( $\alpha$ 1,3)Man- $\alpha$ -OME, the branched trisaccharides, *i.e.*, Man( $\alpha$ 1,6)[Man( $\alpha$ 1,3)]Man- $\alpha$ -OME, and Man( $\alpha$ 1,6)[Man( $\alpha$ 1,3)]Man( $\alpha$ 1,6)[Man( $\alpha$ 1,3)]Man- $\alpha$ -OME. These all gave very similar inhibition curves with 50% inhibition at approximately 0.07 mM concentrations. The second best inhibitors are branched trisaccharides, *i.e.* Man( $\alpha$ 1,3)[Glc( $\alpha$ 1,6)]Man- $\alpha$ -OME and Man( $\alpha$ 1,3)[Gal( $\alpha$ 1,6)]Man- $\alpha$ -OME, both also containing the sequence of Man( $\alpha$ 1,3)Man. They gave 50% inhibition at 0.3 mM. The oligosaccharides belonging to the third group exhibited 30% inhibition at approximately 1 mM; they include Man( $\alpha$ 1,6)Man- $\alpha$ -OME and those having  $\alpha$ (1,3)glucosyl or  $\alpha$ (1,3)galactosyl branches, *i.e.* Man( $\alpha$ 1,6)[Glc( $\alpha$ 1,3)]Man- $\alpha$ -OME and Man( $\alpha$ 1,6)[Gal( $\alpha$ 1,3)]Man- $\alpha$ -OME. Man( $\alpha$ 1,2)Man- $\alpha$ -IME was much less active. It must be noted that both Man( $\alpha$ 1,3)Glc and Gal( $\alpha$ 1,3)Man $\alpha$ OME are not active inhibitors, indicating that binding to CVA must involve recognition of the terminal Man( $\alpha$ 1,3)Man disaccharide unit. In connection with these findings, when Man( $\alpha$ 1,3)Man- $\alpha$ -OME, a most potent inhibitor, was periodate-oxidized and reduced, the result-

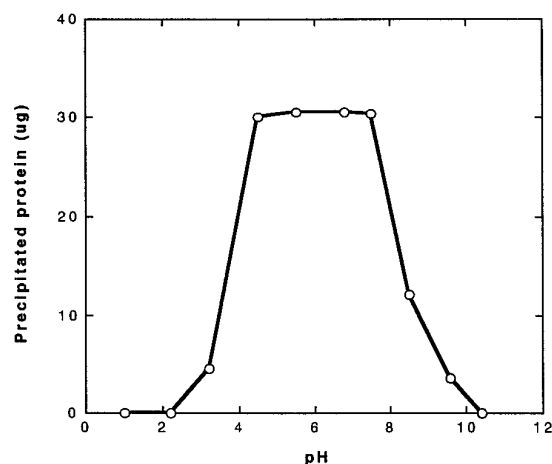


FIG. 8. Effect of pH on the precipitation reaction between CVA and *S. cerevisiae* mannan. Each microcentrifuge tube contained CVA (27  $\mu$ g) and mannan (22  $\mu$ g) in a total volume of 150  $\mu$ l of 50 mM buffer. See "Experimental Procedures" for buffer composition.

ing terminally modified  $\alpha$ -1,3-mannosaccharide was no longer active, supporting this unique binding specificity of CVA.

**The pH Profile of the Manan *S. cerevisiae* CVA Precipitation Reaction**—The pH profile of the precipitation reaction from pH 2 to 10 is depicted in Fig. 8. A plateau of constant protein precipitation was demonstrated over the pH range 4.5–7.5.

**Binding Characteristics of CVA Affinity Column**—An aliquot of CVA was conjugated to AF-tresyl Toyopearl 650 (binding efficiency, 70%), and its binding capacity for polysaccharides and plant glycoproteins carrying  $\alpha$ -1,3-mannosyl ends was investigated. As anticipated, normal yeast mannan of *S. cerevisiae* bound strongly to the CVA column and was eluted by weak alkali, such as 20 mM DAP, whereas the mannan of the 4484 mutant was not bound (Fig. 9, A and B). *C. parapsiosis* 1936 mannan also was not bound. As glycogen passed through the column unretarded, complete resolution of the mannan and glycogen in the yeast cell can be made (Fig. 9C), as previously reported for the *L. ovata* lectin affinity column (7). Some glycoproteins are known to contain high mannose-type carbohydrate chains. Among them are lima bean lectin (*P. lunatus*) and Tora bean lectin (*P. vulgaris*), both of which contain  $\alpha$ -1,3-mannosyl end group(s) in their glycan structures (18, 19). As shown in Fig. 10, they could also bind to the CVA affinity column and were readily eluted with dilute DAP.

## DISCUSSION

*C. vernus*, the spring flowering crocus, belongs to the family *Iridaceae*. It originated in middle Europe and was brought to Japan one hundred years ago. We found that its PBS extract precipitated bakers' yeast mannan but did not strongly agglutinate yeast cells, compared with concanavalin A and GNA. Furthermore, unlike ordinary glucose/mannose and other mannose-binding lectins, this lectin (CVA) did not bind to a mannose affinity column. Nevertheless, it was purified by successive  $(\text{NH}_4)_2\text{SO}_4$  precipitation and gel chromatography (yield, 145 mg/160 g bulbs).

The purified lectin, which crystallized spontaneously, is a carbohydrate-free protein (mass = 44,000 Da) composed of four identical peptide chains of about 100 amino acid residues; there was no evidence of disulfide interchain bonds. It was somewhat surprising that its N-terminal amino acid sequence of 24 residues showed essentially no homology with GNA and other *Amaryllidaceae* mannose-specific lectins (Table II), although its molecular size and amino acid composition are very similar to those of GNA and other bulb lectins (Table I). These results strongly suggest that the molecular structure of the CVA car-

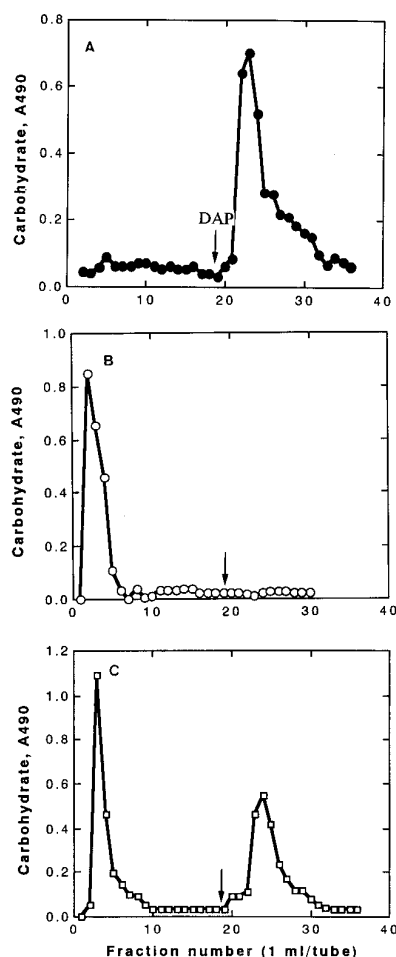


FIG. 9. Elution profile of *S. cerevisiae* mannan (A), *C. parapsilosis* 1396 mannan (B), and mannan and glycogen (1:1 mixture) of *S. cerevisiae* (C). Each polysaccharide (1 mg) was applied to CVA-Toyopearl column (5 ml). After elution with 0.05 M PBS, the polysaccharide retained on the column was eluted with 20 mM DAP, as indicated by the arrows. Carbohydrate was monitored by the phenol-sulfuric acid method at 490 nm.

bohydrate-binding sites must be different from *Amaryllidaceae* bulb lectins.

The precipitation reactions of CVA with various types of yeast cell wall mannans showed that this lectin possesses an unusual, very strict binding specificity. It reacted only with the mannans having terminal  $\alpha$ -1,3-mannosyl groups, such as normal yeast mannan (Fig. 4, I), the mannan lacking  $\alpha$ -1,2-linked terminal mannose units (Fig. 4, IV), and *C. albicans* B-792 mannan (Fig. 4, V). However, it did not react with other types of mannans, such as those lacking  $\alpha$ -1,3-linkage at its terminal ends or localized at internal positions, as in the case of the 4484 mutant mannan and of *C. parapsilosis* 1396 (Fig. 4, III and II); these mannans contain nonreducing, terminal  $\alpha$ -1,2-linked mannose residues.

The pH profile of the yeast mannan (*S. cerevisiae*) precipitation reaction indicates that the reaction is maximum over the pH range 4.5–7.5. It is possible that acidic residue(s) are involved in the carbohydrate-binding site of CVA inasmuch as the ascending portion of the pH profile ( $pK = \sim 3.5$ –4.0) is in the titration range of  $\beta$ - and  $\gamma$ -carboxyl groups of Asp and Glu. Acidic amino acid residues have been identified in many lectin-binding sites including the snowdrop lectin (22).

Detailed inhibition studies were conducted using the normal *S. cerevisiae* mannan-CVA precipitation system with various mannobioses and branched tri- and pentasaccharides contain-

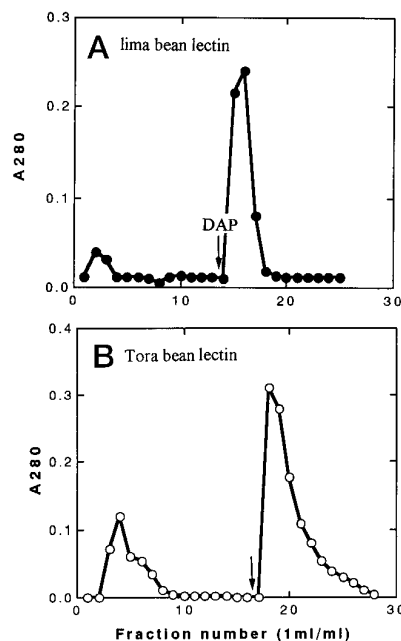


FIG. 10. Elution profile of some plant glycoproteins by CVA-Toyopearl column. A, lima bean lectin. B, Tora bean (*P. vulgaris*) lectin. Protein was assayed at 280 nm.

TABLE III  
Inhibition by mannosaccharides of *S. cerevisiae* mannan precipitation with CVA

Mannosaccharide	Concentration mM	Inhibition %
Man $\alpha$ OME	30	0
	40	7.4
	100	21.5
<i>p</i> -NP- $\alpha$ Man	6	2.5
	15	5.0
Man( $\alpha$ 1,3)Man $\alpha$ OME	0.05	32.7
	0.1	50.9
	0.2	72.2
	1.6	98.0
Oxidized, reduced-Man( $\alpha$ 1,3)Man $\alpha$ OME	1.0	10.0
Man( $\alpha$ 1,6)Man- $\alpha$ OME	2.0	33.0
	3.0	36.4
Man( $\alpha$ 1,2)Man $\alpha$ OME	6.0	8.5
	16.0	16.6
Man( $\alpha$ 1,3)Glc	2.0	2.0
	4.0	7.5
Gal( $\alpha$ 1,3)Man $\alpha$ OME	(No inhibition at 10 mM)	
$\alpha$ Man <sub>6</sub>		
↓		
Man( $\alpha$ 1,3)Man $\alpha$ OME	0.1	52.5
	0.5	79.9
	1.0	87.2
$\alpha$ Glc <sub>6</sub>		
↓		
Man( $\alpha$ 1,3)Man $\alpha$ OME	0.5	74.5
	1.0	83.5
$\alpha$ Gal <sub>6</sub>		
↓		
Man( $\alpha$ 1,3)Man $\alpha$ OME	0.5	63.6
	1.0	85.3
$\alpha$ Man <sub>6</sub> Man <sub>3</sub>		
↓            ↓		
Man( $\alpha$ 1,3)Man(1,6)Man $\alpha$ OME	0.1	49.0

ing  $\alpha$ -1,3- and/or  $\alpha$ -1,6-terminal mannosyl group(s). The results clearly indicate that only those having  $\alpha$ -1,3-mannosidic termini, either linear or branched, exhibited strong inhibitory activity. Thus, Man( $\alpha$ 1,3)Man- $\alpha$ OME, Man( $\alpha$ 1,3)[Man( $\alpha$ 1,6)]-Man- $\alpha$ OME, and Man( $\alpha$ 1,6)[Man( $\alpha$ 1,3)]Man( $\alpha$ 1,6) [Man( $\alpha$ 1,3)]-Man- $\alpha$ OME were the best inhibitors. When the nonreducing

terminal mannose group is periodate-modified, its original activity is destroyed. This group of oligosaccharides was 4.5 times more active than  $\alpha$ -1,3 mannobiose substituted at the O-6 position of the "reducing" mannosyl group with a glycosyl group other than an  $\alpha$ -mannosyl group, e.g. Man( $\alpha$ 1,3)[Glc( $\alpha$ 1,6)]-Man- $\alpha$ -OMe and Man( $\alpha$ 1,3)[Gal( $\alpha$ 1,6)]Man- $\alpha$ -OMe. These results suggests that a glycosyl substituent at the O-6 position of the reducing  $\alpha$ -mannosyl residue is tolerated by the CVA-binding sites. The  $\alpha$ -1,6-linked mannosylmannose unit, with or without an O-3-glucosyl or galactosyl branch were much less active. Man( $\alpha$ 1,3)Glc and Gal( $\alpha$ 1,3)Man $\alpha$ OMe were very poor inhibitors, slightly more active than methyl  $\alpha$ -mannoside, which showed no inhibition at a concentration of 30 mM (Table III). Thus, these hapten inhibition studies clearly confirm that terminal  $\alpha$ 1,3-linked mannosyl mannose units are required for binding to the lectin.

Our previous study indicated that GNA of the *Amaryllidaceae* family, also has a high affinity for terminal Man( $\alpha$ 1,3)-Man units (3), but the binding specificity of GNA appears more broad than CVA, because GNA is also able to recognize internal (1,3/1,6) mannose units (8, 21), and it precipitated periodate-modified *S. cerevisiae* mannan as well as the 4484 mutant mannan (data not shown).

Recently the three-dimensional structure of GNA was elucidated and its binding site for  $\alpha$ -methyl mannoside in each monomer was identified (22). With regard to molecular structure, CVA also is a tetrameric protein with similar molecular size and amino acid composition as that of GNA. CVA is rich in Asn, Leu, and Gly, like GNA, but the N-terminal amino acid sequence is completely different. Therefore, it will be of great interest to compare the three-dimensional structure of the two lectins. We have succeeded in crystallizing CVA, and its crystallographic x-ray structure is in progress.

The biological role of plant lectins is not fully understood and remains the subject of current debate, although their role as a host defender against animals, fungi, bacteria, or virus is an attractive hypothesis (23). Apart from such a biological role, the aforementioned unique binding specificity of CVA prompted us to prepare the corresponding immobilized lectin and to study its application to the affinity chromatographic analysis of polysaccharides and glycoproteins. The normal mannan of *S. cerevisiae* was strongly bound to the column, but  $\alpha$ -glucans, e.g. glycogen, starch, etc., and also certain yeast mannans genetically deficient in terminal  $\alpha$ -1,3-mannosyl groups readily passed through the column. Some plant glycoproteins, for in-

stance, lima bean lectin and *P. vulgaris* lectin, which contain 5% carbohydrate chains carrying two  $\alpha$ -1,3-mannosyl terminal groups, were retained on CVA column and were eluted with dilute DAP (Fig. 9); elution with methyl  $\alpha$ -mannoside was unsuccessful. From these examples, it may be anticipated that immobilized CVA may provide a useful probe for selective fractionation of biologically important glycans and glycoproteins containing Man( $\alpha$ 1,3)Man termini.

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