Purification and Characterization of a Mannan-Binding Lectin Specifically Expressed in Corms of Saffron Plant (*Crocus sativus* L.)

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Despite the economical interest of *Crocus sativus*, its biochemistry has been poorly studied. Herein, we have isolated a lectin present in saffron corm by gel-filtration, anion-exchange, and reversed-phase chromatography. One- and two-dimensional PAGE, MALDI-MS, and N-terminal amino acid sequence analyses indicated that the native protein forms noncovalently linked aggregates of about 80 kDa apparent molecular mass, mainly composed of two charged heterogeneous (p*I*'s, 6.69−6.93) basic subunits of approximately 12 kDa. Their N-terminal sequences shared 25% similarity and were homologous to the N- and C-terminal domains of monocotyledonous mannose-binding lectins, respectively. An additional polypeptide of around 28 kDa apparent molecular mass was also detected, probably corresponding to a precursor processed into two mature subunits. In addition, the N-terminal domain subunit exhibited 56% similarity with curculin, a sweet protein with taste-modifying activity. The native lectin specifically interacts with a yeast mannan and is a major corm protein specifically expressed in this organ.

Keywords: *Crocus sativus; corm; MALDI-MS; mannose-binding; lectins; yeast mannan*

INTRODUCTION

The saffron plant (*Crocus sativus* L.) has been cultivated with very little changes for at least 4300 years (Gadd, 1971). For centuries it was used both as spice and as medicinal plant, but nowadays its use has been restricted, although it still constitutes one of the most valuable old world spices. Despite its high price, the use of saffron in food applications has been steadily increasing, because of changes in consumer preference toward natural products (Knewstubb and Henry, 1988). Moreover, some studies have revealed the potential of saffron as a cardiovascular protective agent (Grisolia, 1974) and more recently as a source of antitumor compounds (Salomi, 1991; Tarantilis et al., 1994; Adbullaev, 1994; Nair et al., 1995; Escribano et al., 1996). Despite saffron's economical importance in Southern Europe and Asia, its molecular cell biology still remains largely unexplored.

Plant lectins are widely utilized and very useful proteins with both biological and industrial applications (Lis and Sharon, 1986). Several works have shown the existence of a group of monocotyledonous lectins with interesting properties regarding their carbohydrate-binding specificity. Some of them recognize exclusively mannose and have been useful tools in glycoconjugate research (Van Damme et al., 1991). Characterization and molecular cloning of several of these lectins have shown that they all belong to the so-called superfamily of mannose-binding proteins, which show considerable overall sequence homology within the mature protein and contain highly conserved domains (Van Damme et al., 1992a, 1995).

During the isolation of an acidic glycoconjugate from saffron corms (Escribano et al., 1999) we have purified a coeluting protein. In this paper we report that it is a multimeric mannan-binding lectin composed of two basic subunits of about 12 kDa, which shares homology with the superfamily of mannose-binding proteins. The lectin accounts for at least 30% of total protein content of corm tissues and could be one of the major storage protein in *C. sativus*.

MATERIALS AND METHODS

Materials. Corms of 'La Mancha' saffron (*C. sativus* L.) were acquired from farmers in Lezuza, Albacete (Spain), and stored at −80 °C until further use. HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Ultrapure water for HPLC, generated by a Milli-Q water-purification system (Millipore, Bedford, MA), was used in the preparation of buffers. All other chemicals used were reagent grade or of highest quality available.

Purification of Lectins. Saffron corms (50 g) were homogenized at 4 °C in 100 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and 5 mM 1,4-dithio-DL-threitol. The protein concentration in the initial extract, estimated by the Bradford assay, was 12 mg/mL. The extract was centrifuged at 12000*g* for 5 min. Then the supernatant was recovered and concentrated 50 times at 4 °C, using 30-kDa molecular weight cutoff centrifuge filters (Filtron, Northborough, MA). The initial volume was reconstituted with 100 mM ammonium acetate, pH 6.6. The process

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was repeated twice. Three to four milliliters of the fraction higher than 30 kDa were loaded on a Bio-Gel P-100 column (25 × 300 mm) and eluted with 100 mM ammonium acetate buffer, pH 6.6. Fractions of 5 mL were collected. Selected fractions obtained by size-exclusion chromatography were lyophilized, dissolved in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.4 M NaCl, and applied on a BioRad (Hercules, CA) Macro-Prep DEAE column (10 × 48 mm) equilibrated with the same buffer. The sample was stepwise eluted with 20 mL of each 0.4, 0.8, and 1.0 M NaCl in 0.02 M Tris-HCl buffer, pH 8.0. A Hewlett-Packard 1100 HPLC (Palo Alto, CA) connected on line with a equipped with a photodiode array detector, with a dynamic range from ultraviolet to visible region (190−800 nm), was employed. A Suplecosil LC-304 C-4 column (4.6 × 50 mm) was used for reversed-phase HPLC. The column was equilibrated with water, eluted with an acetonitrile gradient, and run at room temperature at a flow rate of 0.5 mL/min.

Affinity Chromatography. HPLC-purified protein was dissolved in 20 mM Tris-HCl buffer, pH 7.5, and applied to a mannan-agarose (Sigma, St. Louis, MO) column (5 × 5 mm) previously equilibrated with the same buffer. The column was eluted with 4.0 mL of equilibration buffer and then with 4.0 mL of the same buffer containing 1.0 M NaCl. The retained material was finally eluted with 0.1 M acetic acid. Fractions of 1 mL were collected and protein was detected by absorbance at 280 nm.

Electrophoretical Analysis. Analytical 12.5% polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed as reported by Laemmli (1970), using the Mini-PROTEAN II system from BioRad. Gels were stained with a 0.1% solution of Coomassie brilliant blue R250 in 40% (v/v) ethanol and 5% (v/v) acetic acid. Purified *C. sativus* corm lectin was analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) following O'Farrell's method (O'Farrell, 1975), in a mini-two-dimensional electrophoresis system (Bio-Rad). Samples were dissolved in 9.5 M urea, 2% Triton X-100, 5% mercaptoethanol, 3% ampholytes (pH 3 to 10), and electrophoresed at 1500 V for 3 h through 4% polyacrylamide capillary tube gels containing the same buffer. Gels were extruded from the capillaries, placed onto 12.5% polyacrylamide slab gels, and subjected to SDS−PAGE for the second dimension. Proteins in slab gels were stained with silver nitrate (Switzer et al., 1979).

Densitometry for protein band quantitation was performed by scanning photographs of SDS−PAGE gels (620 nm) using a Joyce and Loebl Chromoscan densitometer (Joyce-Loebl Ltd., Gateshead, U.K.).

Mass Spectrometry Analysis. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis was performed with a MALDI-II spectrometer (Kratos Analytical, Manchester, U.K.) and using synapinic acid (Sigma) as matrix. MALDI-MS calibration was carried out with trypsin (23 292 Da; Sigma) and barley LTP2 (8 774 Da; Molina et al., 1993).

Amino Acid Composition and N-Terminal Sequence Analysis. For amino acid analysis, samples were hydrolyzed at 110 °C for 20 h in 0.1 mL of 5.7 M HCl, containing 0.05% (v/v) 2-mercaptoethanol, in evacuated and sealed tubes. The quantitative analyses were performed using a Beckman system 6000 high-performance amino acid analyzer. Amino acid analysis were performed at the Protein Chemistry Facility of the Centro de Investigaciones Biológicas, CSIC, Madrid, Spain.

To determine both the N-terminal sequence and amino acid composition of the different polypeptide chains, the protein was incubated at 100 °C for 5 min, analyzed by SDS−PAGE, and blotted onto a Immobilon-P (Millipore) membrane, using a semidry transfer apparatus (Hoefer Scientific, San Francisco, CA). After Coomassie blue staining of the membrane, individual bands were cut from the blot and used for sequencing or amino acid analysis. The method of Edman and Begg (1967) was used for automated sequence analysis in an Applied Biosystems 470A sequencer (Applied Biosystems Inc., Foster City, CA), equipped with a phenylthiohydantoin (PTH) amino acid analyzer (model 120A). Searches for protein sequence homologies were made using the program Fasta in GenBank/EMBL and Swis-Prot databases at the World Wide Web Baylor College of Medicine Search Launcher.

Polyclonal Antiserum to *C. sativus* Lectin. Rabbit antiserum was raised against *C. sativus* lectin. One hundered micrograms of purified protein dissolved in 150 μL of phosphate-buffered saline (PBS) was mixed with 500 μL of complete Freund's adjuvant and used to immunize a rabbit for the first time, by subcutaneous injection. The rabbit was then administered an injection in incomplete adjuvant every 2 weeks for 45 days. Blood withdrawn after 2 months was pooled and used for immunobloting.

Western Blot Analysis. Different tissues of *C. sativus* were homogenized and protein concentrations were measured by Bradford assay (Bradford, 1976). Proteins were fractionated by SDS−PAGE (12.5% acrylamide) and gels were subsequently transferred in Tris-Gly buffer (25 mM Tris, 192 mM glycine, pH 8.3) for 15 min onto Immobilon-P (Millipore) using a semidry transfer apparatus (Hoefer Scientific). Filters were preincubated for 30 min at room temperature in a blocking buffer consisting of 5% (w/v) skim milk and 0.05% Tween 20 in PBS and then incubated for 1 h at room temperature with rabbit antiserum to the *C. sativus* lectin (1:1000 dilution) in the presence of blocking buffer. The blot was then washed twice in PBS, incubated for 30 min with alkaline phosphatase-conjugated antibodies against rabbit IgG (1:3000 dilution) in 5% (w/v) skim milk and 0.05% Tween 20 in PBS, and washed for 10 min in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl. The blot was washed in 10 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5, for 5 min and developed in the same buffer containing 0.12 mM *p*-nitroblue tetrazolium chloride and 13.5 mM 5-bromo-4-chloro-3-indolyl phosphate, until bands were visible.

Quantitative Mannan-Binding. Varying amounts of *Saccharomyces cerevisiae* mannan coupled to agarose (Sigma) were incubated for 30 min at room temperature with 15 μg of lectin, in a total volume of 50 μL of PBS, pH 7.4. After incubation, samples were kept at 5 °C for 60 min, centrifuged, and analyzed for protein in the supernatant by Bradford assay (Bradford, 1976). As control 15 μg of BSA were incubated with *S. cerevisiae* under the same conditions.

RESULTS

Purification of Lectin. While applying a purification scheme designed to isolate a complex rhamnose-rich proteoglycan from saffron corms (Escribano et al., 1999), we have found a protein further identified as a lectin. Saffron corm extracts were concentrated by ultrafiltration using 30-kDa molecular weight cutoff centrifuge filters. The fraction higher than 30 kDa was fractionated by size-exclusion chromatography. Two peaks absorbing at 280 nm were detected (Figure 1A). Material eluted in the first peak (P-1) was lyophilized and separated in a Macro-Prep DEAE column, stepwise eluted with 0.4, 0.8, and 1.0 M NaCl in 0.02 M Tris-HCl, pH 8.0, buffer (Figure 1B). Fraction 2, eluted with 0.8 M NaCl, was desalted and then fractionated by reversed-phase HPLC. Two major peaks absorbing at 280 nm were detected (Figure 1C). Peak P-2 showed an absorption spectrum with a band centered at 280 nm, suggesting the presence of aromatic amino acids (Figure 1C, insert). Peak eluted at 8 min corresponded to a previously described rhamnose-rich proteoglycan (Escribano et al., 1999).

Amino acid sequence analysis of peak P-2 showed two N-terminal sequences that are related to mannan-binding lectins. The total amount of lectin recovered was about 40 mg. To demonstrate the mannan-binding activity, the molecule purified by HPLC was loaded on a yeast mannan-agarose column. The protein was

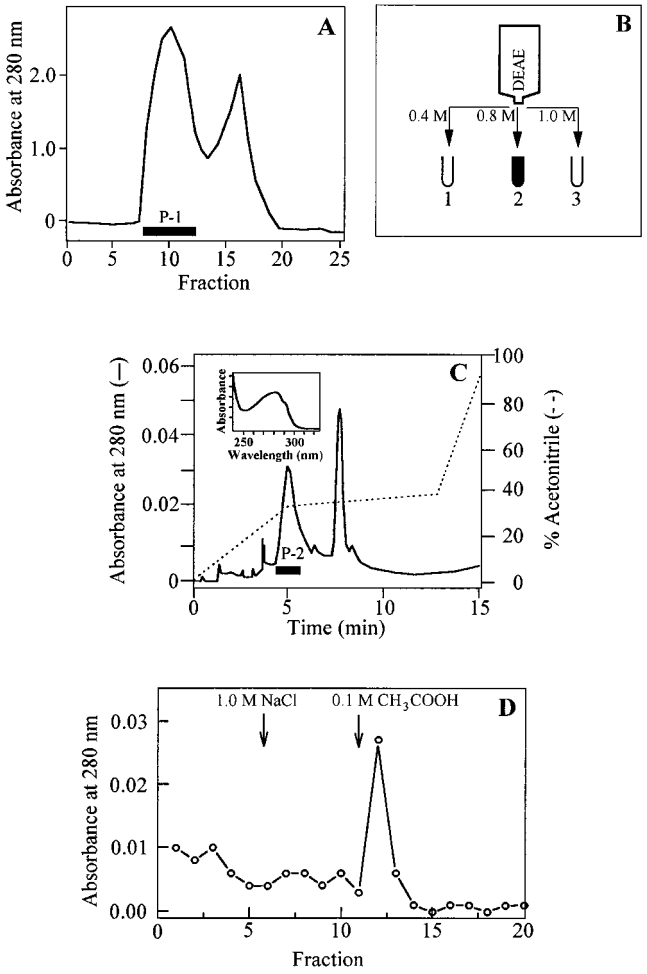

Figure 1. Purification of the lectin from *C. sativus* corm. A saffron aqueous extract was concentrated using 30-kDa molecular weight cutoff centrifuge filters. (A) The fraction higher than 30 kDa was loaded on a size-exclusion column (Bio-Gel P-100) and eluted with 100 mM ammonium acetate buffer, pH 6.6. Protein elution was monitored at 280 nm. (B) Fractions corresponding to peak P-1 were further chromatographed in an anion-exchange column (Macro-Prep DEAE) stepwise eluted with 0.02 M Tris-HCl buffer, pH 8.0, containing the indicated concentrations of NaCl. (C) Material eluted with 0.8 M NaCl (fraction 2, labeled in black) was desalted and fractionated by reversed-phase HPLC, using the acetonitrile gradient indicated on the right. The inset shows the absorption spectrum of peak P-2, recorded by a photodiode array detector. (D) An aliquot of 100 μg of fraction P-2 was loaded on a mannan-agarose column equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The column was eluted with the equilibration buffer (4.0 mL), then with the same buffer containing 1.0 M NaCl (4.0 mL), and finally with 0.1 M acetic acid. Fractions of 1.0 mL were collected.

strongly retained by the column and eluted with 0.1 M acetic acid, demonstrating its binding to mannan (Figure 1D).

Chemical Characterization. To elucidate the molecular structure of the isolated protein, unreduced samples incubated at different temperatures were analyzed by SDS−PAGE in the absence of reducing agents. The protein incubated at room temperature or at 40 °C migrated mainly as a single heterogeneous polypeptide of apparent M_r about 80 000. A comparable value for the molecular mass was obtained by gel-filtration chromatography of the native protein (data not shown). When aliquots of this molecule were incubated for 5 min at 60, 80, or 100 °C, four bands of 11 (B4), 15 (B5), 16 (B6), and 28 kDa (B7) apparent molecular mass were detected, suggesting that the native molecule is built of several subunits not covalently bound (Figure 2A). Densitometric analysis revealed relative proportions of 1:3:12 for bands B6, B5, and B4, respectively. Small amounts of band B4 were also detected when the protein was incubated at room temperature or at 40 °C, indicating that under these conditions some B4 molecules are dissociated from the aggregate.

Figure 2B shows the silver-stained two-dimensional pattern of this lectin. B4 is composed of at least five isoforms with isoelectric points ranging from 6.79 to 6.93. B5 apparently consists of at least two different isoforms which focused at pH's between 6.69 and 6.79. Finally, B6 showed at least two spots (p*I*'s, 6.69−6.79) and B7 three isoforms (p*I*'s, 6.79−6.93).

The isolated protein was subjected to MALDI-MS analysis that revealed the presence of two major groups of molecules, which had molecular masses between 11 800−12 100 and 12 600−12 850 Da, respectively (Figure 3).

The N-terminal amino acid sequences of these bands were determined. Bands B5, B6, and B7 shared the sequence D-N-N-V-L, while B4 showed major (N-I-P-R-V-R-N-V-L-F-S) and minor (D-N-N-V-L-L-T-G) amino acid sequences (Table 2). These two sequences shared 25% identity (Figure 4A). The N-terminal sequence alignment with homologous proteins is shown in Figure 4 (B and C). The N-terminal amino acid sequence present in bands B7, B6, and B5 (and with low yield in band B4) exhibited 56% similarity to curculin, a sweet protein with taste-modifying activity found in fruits of *Curculigo latifolia* (Yamashita et al., 1990), 44% homology to tarin G1d of taro (*Colocasia esculenta*) (de Castro et al., 1992) also called *Colocasia* type 2 polypeptide (Van Damme et al., 1995), and 38% to the *Arum maculatum* type 1 polypeptide (Van Damme et al., 1995). B5 amino acid composition also coincides with the amino acid proportions of taste-modifying proteins such as miraculin and curculin (Theerasilp et al., 1989; Yamashita et al., 1990). The major B4 N-terminal amino acid sequence showed 100% identity to the monomeric lectins called I, II, and IV of *C. sativus* (Oda and Tatsumi, 1993), 91% to the mannose-binding lectin of *C. vernus* (Misaki et al., 1997), and 64% to tarins G1a and G1c (*Col. esculenta*) (de Castro et al., 1992; Bezerra et al., 1995) also called *Colocasia* type 2 polypeptide (Van Damme et al., 1995), the type 2 polypeptide of tannia (*Xanthosoma saggitifolium*), and the *A. maculatum* type 2 polypeptide (Van Damme et al., 1995) (Figure 4C). All these proteins are lectins extracted form corms and tubers.

Amino acid composition analysis (Table 1) revealed that band B4 presents Asx (17.5%), Ser (12.4%), and Gly (12.2%) as predominant components. Although B4 is composed of two polypeptide chains, its profile is similar to the amino acid compositions reported by Oda and Tatsumi (1993) and Misaki et al. (1997) for *C. sativus* and *C. vernus* lectins, respectively. On the other hand, the amino acid composition of band B5 is also dominated by Asx (20.6%), Gly (12.7%), and Ala (12.2%). These amino acid composition data show that *C. sativus* lectins present proportions that are similar to those found in mannose-binding lectins of monocotyledons (Van Damme et al., 1991).

Lectin Expression Pattern. The expression pattern of this protein was determined in root, corm, leaf, flower, stigma, and callus corm of *C. sativus* by Western blot

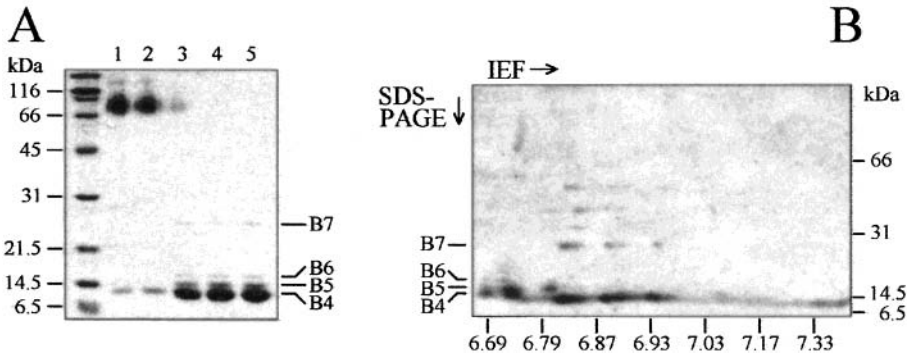

Figure 2. (A) SDS−PAGE analysis of *C. sativus* corm lectin. Aliquots of 10 μg of HPLC-purified lectin incubated in the absence of reducing agents at room temperature (lane 1), 40 °C (lane 2), 60 °C (lane 3), 80 °C (lane 4), or 100 °C (lane 5) were analyzed by electrophoresis on a 12.5% SDS−polyacrylamide gel. Proteins were stained with Coomassie blue. Molecular weight markers are shown on the left. (B) Two-dimensional gel electrophoresis of *C. sativus* corm lectin. Purified protein (15 μg) was resolved by 2D-SDS−PAGE and silver stained. The first (IEF) and second (12.5% SDS−polyacrylamide) electrophoretic dimensions are indicated by arrows at the top of the figure. The pH gradient along the first dimension is indicated beneath the gel. The positions of the molecular weight markers (in kDa) are shown at right.

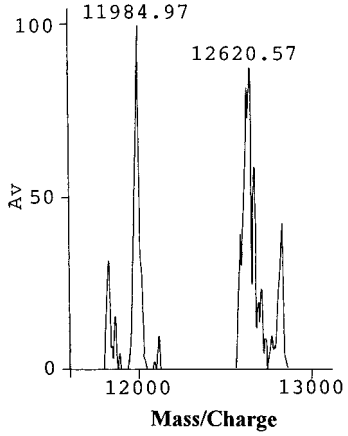

Figure 3. MALDI-MS determination of the purified lectin from saffron corms. The mass/charge values of the components between 11 500 and 13 000 Da of the lectin are shown. Av, arbitrary units.

Table 1. Mole Percent of Amino-Acyl Residues of the *C. sativus* Lectin Bands

amino acid	B4	B5	amino acid	B4	B5
Asx	17.5	20.6	Ile	1.8	1.0
Thr	5.4	4.7	Leu	4.7	6.1
Ser	12.4	9.0	Tyr	2.1	3.5
Glx	8.7	8.9	Phe	2.8	4.1
Pro	nd[a]	nd	His	2.6	4.0
Gly	12.2	12.7	Lys	1.4	0.2
Ala	9.5	12.2	Arg	7.3	3.3
Val	9.0	8.1	Cys	nd	nd
Met	2.6	1.6	Trp	nd	nd

[a] nd, not determined.

(Figure 5**)**. The lectin was immunodetected using a rabbit polyclonal antiserum raised against the purified protein. Two bands of apparent molecular masses of about 12 and 28 kDa were detected only in corm, showing a highly specific expression pattern (Figure 5B). The apparent molecular weights of these proteins coincided with those of bands B4 and B7. Under these conditions, signals from bands B4, B5, and B6 presumably overlapped. Two major bands of equivalent molecular weight and representing at least 30% of total protein, as determined by densitometry, were detected in corm (Figure 5A). This result indicated that the isolated lectin is one of the major protein components of saffron corm.

Interaction with *S. cerevisiae* Mannan. The interaction between the isolated protein and a yeast mannan coupled to agarose was quantitated as indicated in Figure 6A. A lineal fall of the percentage of free lectin was obtained after incubation with increasing concentrations of *S. cerevisiae* mannan. No binding of BSA to the mannan was detected, indicating the specificity of the interaction with this lectin.

DISCUSSION

Saffron, the world's most expensive spice, consists of the bright red stigmas of the saffron crocus, *C. sativus* L. This species is a sterile triploid (Dhar et al., 1988) that produces triannual replacement corms and is propagated solely from them (Mathew, 1983). During the isolation of a rhamnose-rich proteoglycan from saffron corms, by gel-filtration, anion-exchange, and reversed-phase chromatography (Escribano et al., 1999), we have also purified a lectin which represents at least 30% of total protein content as estimated by densitometry. Coelution of the lectin in anion-exchange chromatography with the proteoglycan could be due to their acid character, although an interaction between the lectin and the proteoglycan carbohydrate moiety cannot be completely discarded.

SDS−PAGE analysis at different temperatures and under nonreducing conditions showed that this lectin forms noncovalent 80-kDa aggregates, composed of polypeptides ranging from 11 to 28 kDa apparent molecular mass (bands B4−B7, Figure 2A). Oda and Tatsumi (1993) reported the isolation from saffron bulbs of lectins with an apparent molecular mass of 48 kDa and composed by six subunits of 8 kDa each (Oda and Tatsumi, 1993). In addition, a lectin purified from *C. vernus* in crystalline form has been reported to be a tetrameric protein (44 kDa) composed of four identical subunits of 11 kDa each (Misaki et al., 1997). Other mannose-binding lectins containing subunits of 11.5−14 kDa not linked by disulfide bonds and occurring as dimers, trimers, tetramers, or even octamers have been found in monocotyledonous plants (Peumans et al., 1986; Smeets et al., 1994; Van Damme et al., 1992b, 1993, 1995).

The lectin polypeptides we isolated in *C. sativus* corm are heterogeneous in charge (Figure 2B). Our two-dimensional electrophoresis studies show the existence of several isoforms (p*I*'s, 6.79−6.93). According to the

Table 2. Amino Acid Sequence of Blotted Polypeptides Obtained by SDS−PAGE of *C. sativus* Corm Lectin

polypeptide	sequence, PTH derivative (% mol)
B4	N(28.5)-I(51.3)-P(22.0)-R(17.5)-V(25.0)-R(17.7)-N(16.4)-V(21.9)-L(21.0)-F(18.8)-S(9.5) D(8.7)-N(6.7)-N(7.7)-V(7.7)-L(6.9)-L(8.6)-T(4.9)-G(5.2)
B5	D(30.0)-N(25.1)-N(29.5)-V(28.6)-L(27.9)-L(32.4)-T(17.4)-G(14.5)-D(12.4)-V(15.5)-L(13.3)-H(5.9)-T(7.9)-D(6.9)-N(5.2)-Q(5.2)
B6	D(0.9)-N(0.7)-N(0.7)-V(0.6)-L(1.0)
B7	D(3.2)-N(1.9)-N(2.7)-V(1.8)-L(1.9)-L(2.1)-T(0.9)-G(1.18)-D(0.8)-V(0.9)

(A)

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P1                D N N V L L T G D V L H T D N - Q
                  : : :   .     :     .   : : :   :
P2  N I P R V R N V L F S S Q V M - S D N A Q
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(B)

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P1              D N N V L L T G D V L H T D N Q
Curculin        D - N V L L S G Q T L H A D H S
Tarin-G1b     L G T N Y L L S G Q T L D T D G H
Tarin-G1d     L G T N Y L L S G Q T L N T D G H
Arum type 1   V G T N Y L L S G E T L N V D G H
Xan. type 1   L G T N Y L L S G Q T
Dieff 12 kDa  V N X N Y L L S G Q T I N T N G X
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(C)

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P2, Lectin-I  N I P R V R N V L F S S Q V M S D N A Q
Lectin-II     N I P R V R N V L F S S Q V M W D N A Q
Lectin IV     N I P R V R N V L F S S Q V M R D N A Q
CVA           N I P Q V R N V L F S S Q V M Y D N A Q
Tarin-G1a     N I P F T D N L L F S G Q V L Y G D G R
Tarin-G1c     N I P F T N N L L F S G Q V L Y G D G R
Arum type 2   N I P F T H N M L F S G Q I L Y G D D M
Xan. type 2   N I P T T N N L L F S
Dieff 14 kDa  N T P L T R N
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Figure 4. (A) N-Terminal amino acid sequence comparison of the two *C. sativus* lectin polypeptides. The amino acids have been aligned by introducing gaps (−) to maximize identity. Colons between lines indicate identical amino acid residues, whereas single dots indicate similar amino acids. (B) Alignment of N-terminal amino acid sequences of mannose-binding lectins (N-terminal domain) and related proteins: P-1 from *C. sativus* (N-terminal amino acid sequence from bands B5−B7 and minor sequence from B4); curculin from *Cur. latifolia* (Yamashita et al., 1990); tarins G1b and G1d from *Col. esculenta* (de Castro et al., 1992; Bezerra et al., 1995); *A. maculatum* type 1 lectin; *X. sagittifolium* type 1 lectin; and *D. sequina* 12-kDa lectin (Van Damme et al., 1995). (C) Alignment of N-terminal amino acid sequences of mannose-binding lectins (C-terminal domain): P-2 major sequence from band B4; lectins I, II, and IV from *C. sativus* (Oda and Tatsumi, 1993; this work); lectin CVA from *C. vernus* (Misaki et al., 1997); tarins G1a and G1c (de Castro et al., 1992; Bezerra et al., 1995); *A. maculatum* type 2 lectin; *X. sagittifolium* type 2 lectin; and *D. sequina* 14-kDa lectin (Van Damme et al., 1995).

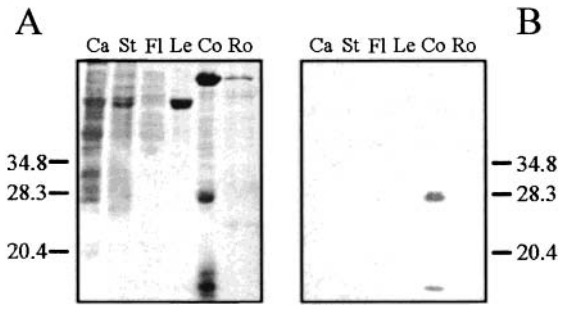

Figure 5. Expression analysis of *C. sativus* lectin by Western blot. (A) Total protein extracts (10−20 μg/lane) from different tissues were fractionated by eletrophoresis on a 12.5% SDS−polyacrylamide gel and stained with Coomassie blue. Samples were reduced with β-mercaptoethanol and incubated at 100 °C for 5 min. Samples were loaded as follows: Ca, callus from corm; St, stigma; Fl, flower without stigma; Le, leaf; Co, corm; Ro, root. (B) Samples fractionated by SDS−PAGE as indicated in (A) were transferred for 15 min to an Immobilon-P filter and probed with rabbit polyclonal antiserum (1:1000 dilution). Positions of standard molecular weight markers are shown on both sides.

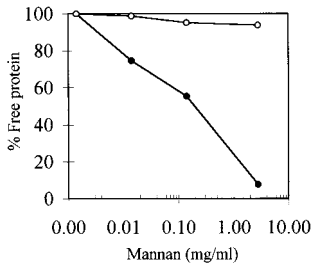

Figure 6. Binding of *C. sativus* lectin to *S. cerevisiae* mannan. Fifteen micrograms of lectin were incubated with different amounts of mannan coupled to agarose (●). As control, 15 μg of BSA were incubated with mannan under the same conditions (○).

MALDI-MS data, this lectin is composed of two basic subunits with multiple isoforms of 11800−12 100 and 12 600−12 850 Da, respectively (Figure 3). Oda and Tatsumi (1993) reported the existence of four isoforms of a *C. sativus* lectin, three of them with almost identical N-terminal sequences. Similarly, lectins with several isoforms have been found in corms of taro (*Col. esculenta*), an important edible crop in tropical regions, with p*I*'s from 5.5 to 7.6. These isoforms showed apparent molecular masses of about 14 kDa (de Castro et al., 1992). Since *C. sativus* is an autotriploid ($2n = 3x = 24$), one can expect three basic alleles per locus instead of two. Additional gene duplications in each haploid genome could explain the higher number of isoforms found in the present work. In this sense, high heterogeneity of the protein pattern and multiple isoforms have been confirmed at the molecular level (cDNA cloning and blot analysis) in the mannose-binding lectins (Van Damme et al., 1995).

From the N-terminal amino acid sequence alignments and the comparison of amino acid composition, we conclude that all the analyzed polypeptides are related to the group of monocot mannose-binding lectins. A

major polypeptide chain was detected in band B4 (N-I-P-R-V-R-N-V-L-F-S) which corresponds to the group of lectins previously identified by Oda and Tatsumi (1993) in *C. sativus* and more recently by Misaki et al. (1997) in *C. vernus*. Surprisingly, the latter authors did not find any homology between the *C. vernus* lectin and other mannose-binding lectins. However, the sequence they published was identical to that previously reported by Oda and Tatsumi (1993), sharing high homology with other previously well-known mannose-binding lectins (Oda and Tatsumi, 1993; Bezerra et al., 1995, Van Damme et al., 1995) (Figure 4). Actually, this polypeptide sequence has shown to be homologous to the C-terminal domain of monocot mannose-binding lectins (Van Damme et al., 1995).

A second N-terminal sequence identified in bands B7, B6, and B5 and as a minor component in band B4 (D-N-N-V-L-L-T-G) was a newly characterized *C. sativus* lectin subunit which shares homology to curculin, a sweet protein with taste-modifying activity (Yamashita et al., 1990), and with the N-terminal domain of monocot mannose-binding lectins (Van Damme et al., 1995). The existence of polypeptide bands with the same N-terminal amino acid sequence and different molecular masses (Figure 2) indicates the existence of a maturation process, as has been demonstrated in other related proteins such as garlic mannose-binding lectin ASAI, *A. maculatum* lectin, and tarins (Smeets et al., 1994; Bezerra et al., 1995; Van Damme et al., 1995). cDNA analysis of clones encoding tarins G1a and G1d indicated that they are encoded by the same gene, *tar 1*, which produces a precursor polypeptide chain of around 26−28 kDa, processed into the two mature subunits of around 12.5 kDa each (Bezerra et al., 1995). Likewise, *Arum* lectins are encoded by two separate domains of a single gene, and the lectin precursor (25.5 kDa) is also processed into two different polypeptides of 12.3 and 13.1 kDa. (Van Damme et al., 1995). Actually, polypeptides B5, B6, and B7 have a high sequence similarity to the N-terminal domain of Araceae lectins, whereas the B4 major sequence is highly similar to the C-terminal domain of these lectins (Bezerra et al., 1995; Van Damme et al., 1995) (Figure 4). According to that, polypeptide B7 (28 kDa) could be a precursor constituted by two different domains that, after processing, could give raise to two mature polypeptides of approximately 12 kDa, the N-terminal and C-terminal domains, respectively (Figure 7). In this scheme, polypeptides B6 and B5 might correspond to intermediates which, after cleavage of putative C-terminal sequences, could be finally converted into a mature N-terminal domain polypeptide, as has been described for mannose-binding lectins from garlic and ramsons (Smeets et al., 1994). Although band heterogeneity due to differences in glycosylation cannot completely be discarded, this possibility seems to be unlikely, because all these bands were not stained with Schiff's reagent, which allows detection of glycosylated proteins (data not shown). On the other hand, band B4 was mainly composed of the C-terminal domain subunit and minor amounts of the N-terminal domain subunit. The yield of Pth derivatives obtained from amino acid sequence of B4 indicated that the amino acid sequence N-I-P-R-V-R-N-V-L-F-S (C-terminal domain) was approximately 3 times more abundant in band B4 than sequence D-N-N-V-L-L-T-G (N-terminal domain). In addition, densitometric analysis showed that B4 polypeptides were 4 and 12 times more abundant than B5 and B6 polypeptides, respectively. We estimated that the C- and N-terminal domain subunits (major and minor components of band B4, respectively) including immature polypeptides (bands B5 and B6) were present in equal amounts.

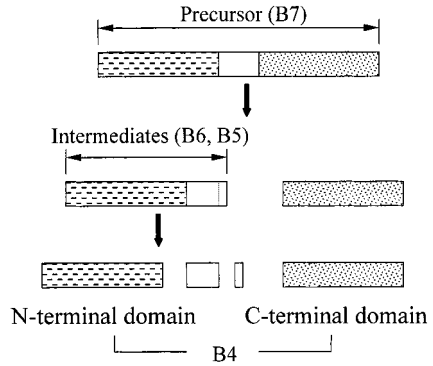

Figure 7. Model of *C. sativus* lectin maturation process. In this model a precursor (28 kDa) constituted of two domains give raise to the polypeptides of 12.6 and 11.9 kDa, which correspond to its N- and C-terminal domains, respectively. Polypeptide B6 (band B6 from Figure 2) could correspond to an intermediate which, after cleavage of a putative C-terminal peptide, releases the mature subunit corresponding to the N-terminal domain of the precursor.

Sequence alignment of these two saffron lectin domains showed 25% homology. This circumstance has been pointed out in other monocot lectins (Van Damme et al., 1992a, 1995) and suggests that both domains might appear evolutionary as result of a DNA segment duplication.

The *C. sativus* lectins I−IV isolated by Oda and Tatsumi (1993) were demonstrated to be mannose-binding proteins, which agglutinate the yeast *S. cerevisiae* but do not aggregate animal erythrocytes. On the other hand, the mannose-binding lectin from *C. vernus* corms was unable to bind to a mannose affinity column but showed a very strict binding specificity when assayed using a quantitative precipitation test with different polysaccharides and glycoproteins (Misaki et al., 1997). Herein, we have also shown the specific interaction of this lectin with yeast mannan.

Corms are perennial underground storage stems in which the deposition of storage products is under developmental control (Strauss et al., 1980; Wills et al., 1983). Corm development is associated with the temporal and spatial expression of corm-specific genes (de Castro et al., 1992). G1a, G1c, and G1d tarins are proteins specifically expressed in parenchyma cells of taro corms (Bezerra et al., 1995). They are major corm components, and it has been suggested that they can act as storage proteins, used for the growth and development of new corms and associated organ systems. Their nature as members of the mannose-binding lectins has also been established (Van Damme et al., 1995). Although the *Arum* lectin is undoubtedly the major tuber protein, it cannot be considered a tuber-specific protein, since it also occurs in all other tissues of the *Arum* plants (Van Damme et al., 1995). As tarins, the *C. sativus* lectins we report here are specifically expressed in corm and are one of the most abundant corm proteins. One can propose that these *C. sativus* lectins have molecular structures and posttranslational processing pathways similar to those of taro and *Arum* lectins. In addition, all these proteins show similar amino acid compositions and share high N-terminal

amino acid sequence homology and carbohydrate-binding characteristics. The expression pattern and the structural and functional similarity of the saffron lectin with respect to tarins and other lectins strongly suggest it could also be a spatially and temporally regulated protein. In addition, the numerous saffron lectin isoforms and the fact that tarins and other monocots' mannose-binding lectins are encoded by small gene families suggest the existence of such a gene family in *C. sativus*. To unravel all these questions, our laboratory is currently involved in the isolation and characterization of cDNAs encoding this corm lectin, as well as in the study of possible regulated expression patterns.

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