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Mannose-Binding Lectin from *Curcuma zedoaria* Rosc.

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A mannose-binding lectin was isolated from rhizomes of the medicinal plant *Curcuma zedoaria*. We used extraction with 20 mM phosphate buffer, ammonium sulfate precipitation, ion exchange chromatography on Q-Sepharose, gel filtration chromatography on Superdex 75, and reverse-phase HPLC. The purified lectin yielded a single band on SDS-PAGE that corresponded to a molecular mass of 13 kDa. This lectin exhibited hemagglutinating activity toward rabbit erythrocytes, which could be inhibited by mannose only. The lectin was digested with trypsin and its digests were analyzed using MALDI-TOF/TOF. Partial amino acid sequences were obtained from tandem mass spectra via automated *de novo* sequencing, and were then identified by MS-BLAST homology searches to enable recognition of related proteins in other species. Inferred peptide sequences exhibited similarity to a mannose-binding lectin from *Epipactis helleborine*, a member of the Orchidaceae.

Keywords: Curcuma zedoaria, de novo sequencing, hemagglutinating activity, MALDI-TOF/TOF, mannose-binding lectin

There are many active proteins from plants such as cytosolic small heat-shock protein from *Nicotiana tabacum* (Yoon et al., 2005) or cysteine-rich antifungal protein from *Capsicum annuum* (Lee et al., 2004). *Curcuma zedoaria* Rosc. (zedoary), a member of the family Zingiberaceae, is used as a medicinal plant in China and Japan (Cao et al., 2001). Crude zedoary serves as an antiseptic, aids in digestion, and relieves flatulence and colic. In addition, its polysaccharides have anti-tumor, genotoxic, and anti-clastogenic activities (Syu et al., 1998). Its rhizomes also possess hepatoprotective properties (Matsuda et al., 1998) as well as anti-microbial (Lai et al., 2004), cytotoxic (Jang et al., 2001), anti-inflammatory, and antioxidant effects (Kim et al., 2000). Hemagglutinating (lectin) activity has been found in crude protein extracts from various *Curcuma* species, including *C. zedoaria* (Sophon et al., 2007). Lectins or agglutinins are carbohydrate-binding proteins that are widely distributed in plants (van Damme et al., 1998a). Their functions in such tissues are defending against phytopathogenic microorganisms, phytophagous insects, and herbivorous animals (Vasconcelos and Oliveira, 2004). These roles have stimulated research into possible applications for lectins in crop protection. Their specific agglutination properties are based on defined recognition of and binding to carbohydrates (van Damme et al., 1998b). The mannose-binding lectins are commonly found in higher plants and are believed to play a role in the recognition of high-mannose-type glycans from foreign microorganisms or plant predators (Barre et al., 2001). Chen et al. (2005) have reported the first cloning of mannose-binding lectin cDNA from rhizomes of ginger (*Zingiber officinale* Roscoe), which belongs to the Zingiberaceae family. However, information is minimal for the extracted proteins from rhizomes of *C. zedoaria*. Therefore, the objective of our study was to purify and identify those proteins via tandem mass spectrometry. In particular, we combined similarity searching with MALDI-TOF/TOF (Suckau et al., 2003) to identify proteins from an organism that lacks genomic information.

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MATERIALS AND METHODS

Isolation of Protein

Protein was isolated from *C. zedoaria* by the method of Choi and Laursen (2000), using rhizomes (1.5 kg) obtained from a local market. These were cut into small pieces and homogenized in a blender with 20 mM phosphate buffer (pH 7.0) that contained 1 mM EDTA (3 mL g^{-1}). The mixture was stirred for 1 h and filtered through cheesecloth. NaCl was added to 2% (w/v) and stirred for 2 h before this suspension was centrifuged at 15000*g* for 30 min at 4°C. The supernatant was brought to 20% saturation with ammonium sulfate, stirred for 1 h, then centrifuged at 15000*g* for 30 min. The supernatant was retained and ammonium sulfate was added to 60% saturation, adjusted to pH 7.2 with NaOH, and stirred for 3 h at 4°C. This resulting suspension was centrifuged, the supernatant discarded, and the precipitate re-suspended in 20 mM phosphate buffer (pH 7.0). The solution was then dialyzed over 24 h (using tubing with a molecular weight cut-off of 3500 Da) against the same buffer containing 1 mM EDTA. This dialysate was centrifuged at 12000*g* for 30 min at 4°C to remove any insoluble material. The solution (≈ 300 mL) was applied to a column (1.6 × 15 cm) of fast-flow Q-Sepharose installed in an AKTA prime instrument (GE Healthcare, Sweden) equilibrated with Buffer A (20 mM phosphate buffer; pH 7.0). The column was washed with 5 volumes of Buffer A to remove unbound proteins. Bound protein was then eluted along a 0 to 100% linear gradient of Buffer B (20 mM phosphate buffer containing 0.35 M NaCl; pH 7.0), at a flow rate of 1.0 mL min^{-1} over 15 column volumes. Afterward, 8-mL fractions were collected and assayed for hemagglutinating activity. The activity-containing fraction was lyophilized, dis-

solved in water, and dialyzed against 0.1 M NH_4HCO_3 (pH 7.8). Insoluble proteins were removed by centrifugation at 13000g for 30 min at 4°C, before the supernatant was applied to a column (1.6 × 56 cm) of Superdex 75 (GE Healthcare) equilibrated in the same buffer at a flow rate of 0.5 mL min^{-1}. From there, 3-mL fractions were collected and assayed for hemagglutinating activity. The activity-containing fraction was lyophilized and finally purified by reverse-phase HPLC on a BDS C_8 column (4.6 × 250 mm; 5 μM particle size). This column was developed with a linear gradient of 10 to 60% mobile phase B (0 to 60 min) and 60 to 90% mobile phase B (60 to 90 min) [mobile phase A was milli Q water containing 0.1% (v/v) trifluoroacetic acid; mobile phase B was acetonitrile containing 0.1% (v/v) trifluoroacetic acid], at a flow rate of 1 mL min^{-1}. For our hemagglutinating assay and sequencing studies, the reverse-phase HPLC procedures were repeated ten times. All hemagglutinating-containing peaks were pooled and lyophilized.

Determination of Protein Concentration

Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as a standard and Coomassie Brilliant Blue G-250 (Amersham Bioscience, Sweden).

Assay for Hemagglutinating Activity

A serial two-fold dilution of the protein in micro-titer U-plates (50 μL) was mixed with 50 μL of a 2% (v/v) suspension of rabbit erythrocytes in phosphate-buffered saline (pH 7.2) at room temperature (RT). The results were read after 1 h, when the blank had become fully sedimented. This titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was considered equal to one hemagglutination unit. Specific activity was stated as the number of units per mg of lectin (Wang et al., 1998).

Inhibition of Lectin-Induced Hemagglutination by Various Carbohydrates

Hemagglutination inhibition tests were performed in a manner analogous to our hemagglutination test. Serial two-fold dilutions of sugar samples were prepared in phosphate-buffered saline and mixed with an equal volume (25 μL) of a protein solution having 8 hemagglutination units. This mixture was incubated for 30 min at RT, then mixed with 50 μL of a 2% (v/v) rabbit erythrocyte suspension. The minimum concentration of sugar in the final reaction mixture was calculated as the amount that could completely inhibit those 8 hemagglutination units from the lectin preparation (Wang et al., 1998).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

One-dimensional SDS-polyacrylamide gel electrophoresis was performed using standard methods on a Bio-Rad Mini-Protean II System. This discontinuous system comprised a 12% separating gel (pH 8.8) and a 4% stacking gel (pH 6.8), with a mini size (7 × 10 × 1 cm). Prior to electrophoresis, protein samples were re-dissolved in Laemmli (1970) buffer, and heated in the presence of dithiothreitol (DTT) for 5 min at 100°C. We used the SigmaMarker™ wide molecular-weight range (Sigma, USA). Electrophoresis was performed at 10 mA per gel. Our gels were stained with Brilliant Blue R concentrate (Sigma-Aldrich) for 30 min, then de-stained in solution of 50% methanol 10% acetic acid in water for 30 min and followed by solution of 5% methanol 5% acetic acid in water until bands appeared.

Mass Spectrometric Analysis

Mass spectrometric analysis was performed on a MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany). To determine its molecular mass, we spotted 0.5 to 1.0 μL of protein onto a target plate, followed by an equal volume of saturated sinapinic acid in 0.1% (v/v) TFA and 50% (v/v) ACN. Mass spectra were acquired with a Bruker MALDI-TOF Ultraflex II that operated in the positive reflectron mode. Ions were generated by a nitrogen laser emitting at 337 nm, then accelerated to 20 kV. All other parameters were set for an optimized mass resolution at about m/z 1000. Usually, 200 laser shots were combined for each mass spectrum.

Sequence Determination of Peptides

Protein samples were proteolyzed with trypsin (2% w/w) for 18 h at 37°C in 25 mM NH_4HCO_3. The resultant peptides were co-crystallized with a saturated solution of a-cyano-4-hydroxy cinnamic acid in 0.1% (v/v) TFA and 50% (v/v) ACN before being analyzed by MALDI-TOF/TOF. LIFT mass spectra were obtained on a Bruker Ultraflex II TOF/TOF mass spectrometer operated in the positive ion mode. Metastable fragmentation was induced by a nitrogen laser (337 nm) without the further use of collision gas. Precursor ions were accelerated to 8 kV and selected in a timed ion gate. In the LIFT-cell, the fragments were further accelerated to 19 kV. The reflector potential was 29 kV.

Database Searching

Protein database searches were performed via BioTools 2.2, using RapiDeNovo extension (Bruker). Homology searches based on the most likely *de novo* sequences were performed with MS BLAST (Shevchenko et al., 2001).

RESULTS

Proteins precipitating between 20 and 60% ammonium sulfate saturation were chromatographed using a Q-Sepharose column. Three peaks of UV intensity were eluted with a linear NaCl gradient (0.00 to 0.35 M) in 20 mM phosphate buffer (pH 7.0) over 20 column volumes. These were designated Q1, Q2, and Q3 (Fig. 1). A hemagglutinating assay was used to determine the functioning of active fractions at each purification step. The small peak -- Q1 -- showed activity at 249.61 titer mg^{-1} (Table 1). The fraction was then pooled, lyophilized, and loaded onto a Superdex 75 gel filtration column. Two major peaks were subsequently designated Q1S1 and Q1S2 (Fig. 2). The latter, containing the majority of the activity, was lyophilized and further purified by reverse-phase HPLC on a C_8 column.

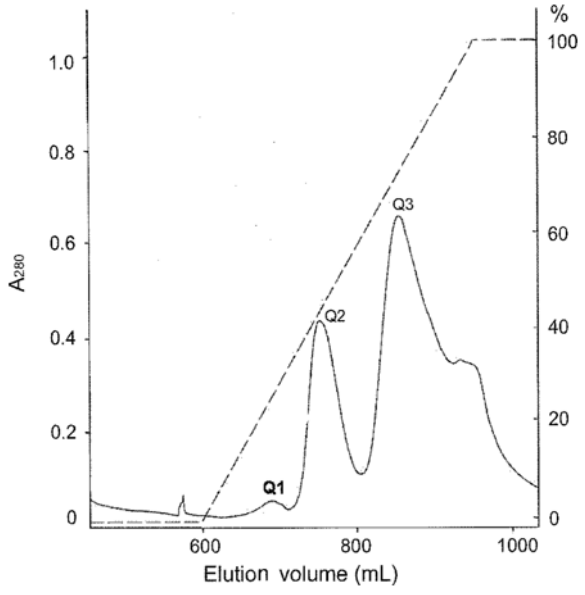

Figure 1. Ion exchange chromatography of crude *C. zedoaria* [20-60% saturated $(NH_4)_2SO_4$].

Table 1. Hemagglutinating activity during purification of *C. zedoaria* lectin from 1.5 kg of rhizomes.

Fraction	Protein yield	Specific hemagglutinating activity (titer[a]/mg)	Folds of Purification
20-60% $(NH_4)_2SO_4$	1100.00	75.58	1
Q1	5.80	249.61	3.3
Q1S2	2.15	656.60	8.69
Q1S2H7	0.23	5820.72	77.01

[a]Titer was defined as the reciprocal of the highest dilution for hemagglutination with 2%(w/v) rabbit erythrocyte in phosphate buffer saline (PBS).

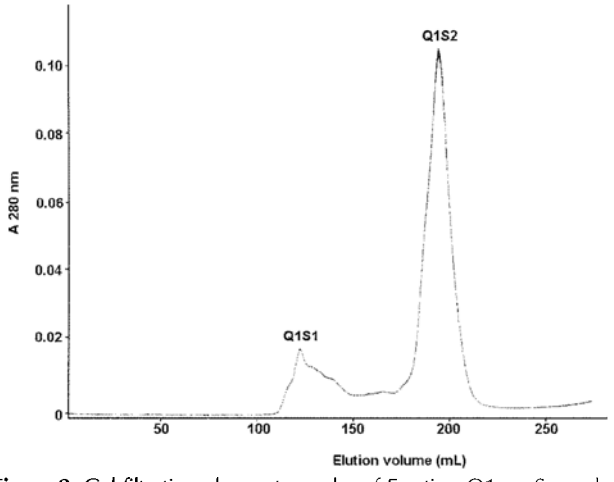

Figure 2. Gel filtration chromatography of Fraction Q1 on Superdex 75 in 0.1 M NH_4HCO_3 (pH 7.8).

Hemagglutinating activity located in the fraction at a retention time of 42.8 min was collected and named Q1S2H7 (Fig. 3). This pooled fraction was then lyophilized for our protein-identification step. Consequently, SDS-PAGE with Coomassie Blue staining revealed a single band of purified

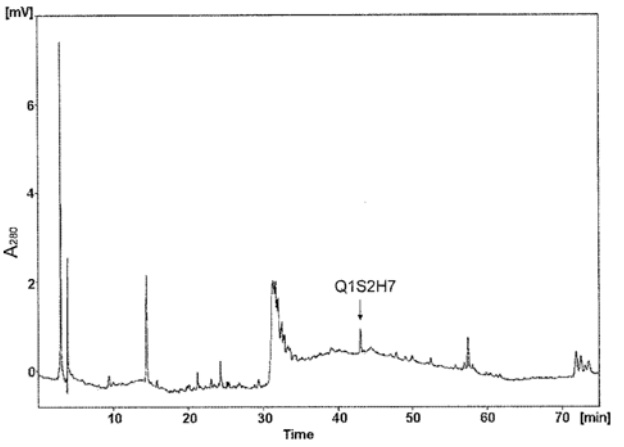

Figure 3. Reverse-phase HPLC of Fraction Q1S2 from gel filtration on C_8 column (4.6×250 mm), under conditions of linear gradient: 10 to 60% (v/v) mobile phase B (0-60 min), 60 to 90% (v/v) mobile phase B (60-90 min); flow rate of 1 mL min^{-1}. Mobile phase A is 0.1% (v/v) trifluoroacetic acid/water; mobile phase B is 0.1% (v/v) trifluoroacetic acid/acetronitrile.

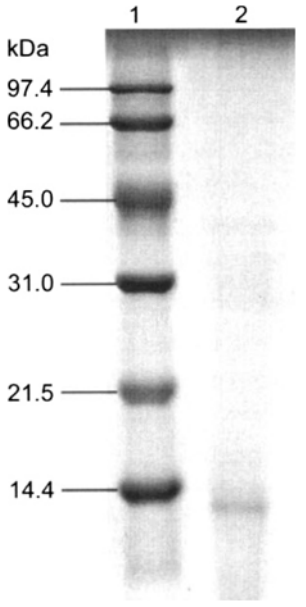

Figure 4. SDS-PAGE of purified protein. (**1**) Protein marker; phosphorylase b (97.4 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and α-macroglobulin (14.4 kDa). (**2**) Purified protein Q1S2H7.

protein with a molecular weight of 13 kDa (Fig. 4). An accurate molecular mass of 13448.7 Da was also recorded, using time-of-flight mass spectrometric analysis with sinapinic acid as the matrix (Fig. 5). This protein was then digested with trypsin. Afterward, the peptide mixtures were analyzed by MALDI-TOF/TOF. In all, 20 product ion spectra were obtained and peptide sequences were then determined according to a *de novo* sequencing algorithm to derive a short list of possible sequence candidates. These served as query sequences in our subsequent homology-based search against a non-redundant protein database. This search used MS BLAST (Shevchenko et al., 2001) to find likely candidates. As a result, we found a query sequence, LNTGDFLTEGEFLFLMK (m/z of 1973.98), which was similar to

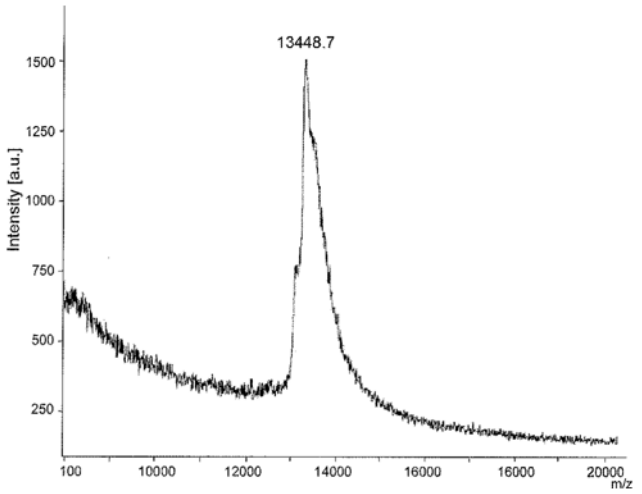

Figure 5. Molecular mass of purified protein, Q1S2H7, determined by MALDI-TOF.

those previously isolated for mannose-specific lectins (Q39728) from *Epipactis helleborine* (van Damme et al., 1994) at amino acid residues 34 to 50 (Fig. 6). The tandem mass spectrum for this precursor ion is shown in Figure 7. In addition, tandem mass spectra for precursor ion m/z of 1014.52 (Fig. 8) and 771.46 (Fig. 9) exhibited a peptide sequence similar to the same protein (Q39728). An amino acid alignment comparing Q1S2H7 from *C. zedoaria* and Q39728 is presented in Figure 10. Our hemagglutination activity assay suggested that an approx. 70-fold increase in specific activity was obtained when the crude extract was subjected to various purification steps (Table 1). Sugar specificity of this lectin was determined by comparing the ability of different carbohydrates to inhibit this hemagglutinating activity. A solution of the lectin containing 8 hemagglutinating units of activity was limited by a minimal inhibitory concentration at 31 mM of D(+)mannose, but was not

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^ = sptrembl|Q39728|Q39728 Lectin precursor.//:trembl|U02515|EH02515_1 product:
          "lectin";  Epipactis helleborine lectin mRNA, complete cds.
          //:pironly|S43462|S43462 mannose-binding lectin precursor -
          Epipactis helleborine//:gp|U02515|436825 lectin [Epipactis
          helleborine]
          Length = 172

Total Score: 77

                              0         40         80         120        160
                              |         |          |          |          |   |172
sptrembl|Q39728|Q3972   |  ____________________________________________________
Local hits (HSPs)       |  ____

 Score = 77 (41.2 bits)
 Identities = 11/17 (64%), Positives = 13/17 (76%)

Query:  6624 BLNTGDFLTEGEFLFLM 6640
             +L TG FLTEG F F+M
Sbjct:    34 RLTTGSFLTEGGFTFIM 50
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Figure 6. MS-BLAST result showing scored alignments of queried peptide sequence and corresponding homologous peptides from database.

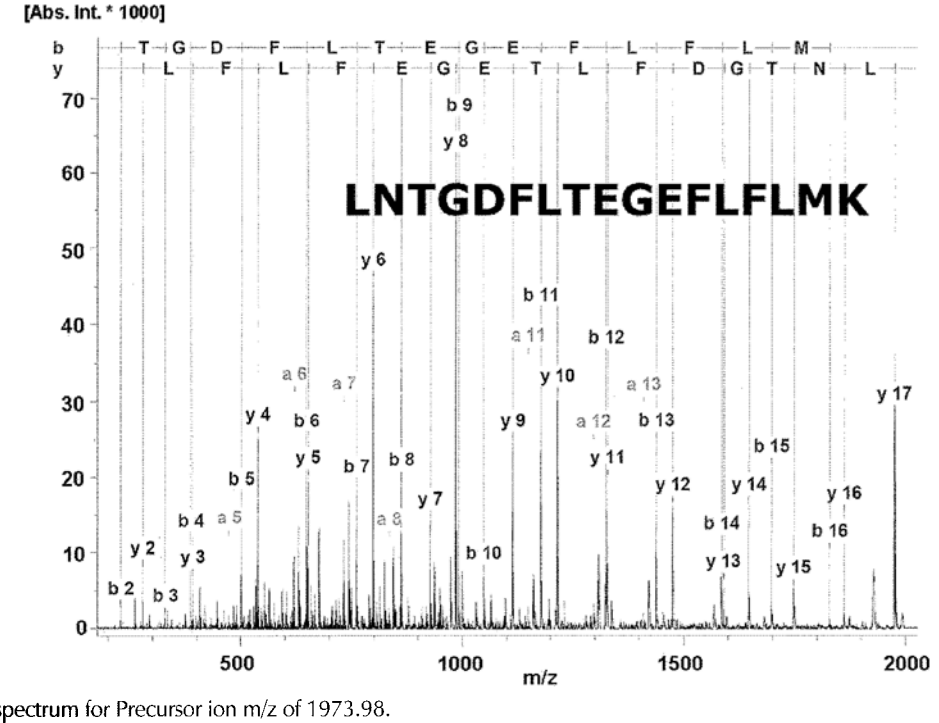

Figure 7. Tandem spectrum for Precursor ion m/z of 1973.98.

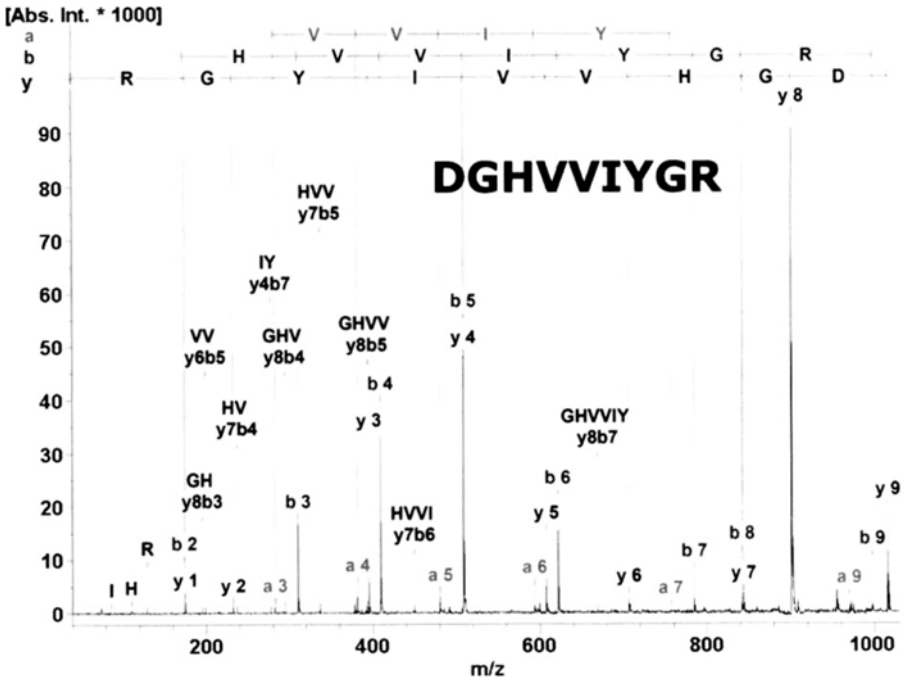

Figure 8. Tandem spectrum for Precursor ion m/z of 1014.52.

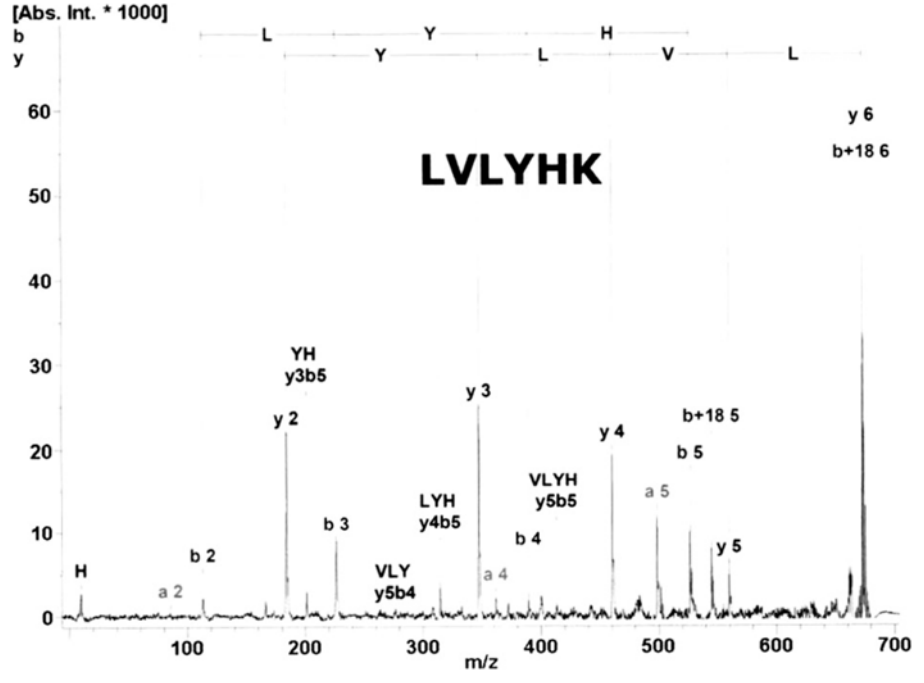

Figure 9. Tandem spectrum for Precursor ion m/z of 771.46.

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C. zedoaria                    RL   NTGDFLTEGE  FLFIMK      LVLYH
E. helleborine    1  DNHLLTGQRL   TTGSFLTEGG  FTFIMQSDCN  LVLYDLNRPI  WASGTYGKGT

C. zedoaria                                                          DGHVVIYGR
E. helleborine   51  GCFLSMQSDG   NLVVYDVRNI  AIWASNTARN  NGNYLLVLER  DRNVVIYSQP
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Figure 10. Amino acid sequence of purified protein, Q1S2H7, from *C. zedoaria*, aligned with known amino acid sequences of *E. helleborine*.

restricted by other carbohydrates (Table 2). These results indicate that carbohydrate-binding specificity of the lectin purified here from *C. zedoaria* is in the same group as the lectin from *E. helleborine* (van Damme et al., 1994).

Table 2. Test of inhibition of lectin-induced hemagglutination by various sugars.

Sugar	Minimal inhibitory concentration (mM)
D-mannose	>31
D-glucose	>125
D-galactose	>125
D-maltose	>125
D-lactose	>125
D-fructose	>125
D-xylose	>125
D-sorbital	>125

Concentration of lectin was 8 hemagglutination units; 2% (w/v) rabbit erythrocyte was used in this assay.

DISCUSSION

We purified protein from rhizomes of *C. zedoaria*, using three chromatography steps: anion exchange, gel filtration, and reverse-phase HPLC. Ion exchange chromatography was chosen as a first step because of its property by which a large sample volume can be loaded. After reverse-phase chromatography, we achieved a significantly mani-fold increase in this purification (Table 1). The molecular weight determination by SDS-PAGE revealed a single band at 13 kDa, whereas the accurate molecular mass produced by MALDI-ToF was recorded as 13448.7 Da. The minimum amount required for agglutination of rabbit erythrocytes was 0.17 μg mL^{-1} (5820.72 titer mg^{-1}), a level that is stronger than the one needed for lectins (Q39728) from *E. helleborine* (1.25 μg mL^{-1}; van Damme et al., 1994). Carbohydrate-binding specificity was studied through assays with various sugars, where we found that only mannose was inhibited. Because no genome and proteome information is yet available for *C. zedoaria*, we adopted an approach that allowed for the identification of its protein by determining partial or complete amino acid sequences via manual or automated *de novo* sequencing. An amino acid sequence was interpreted directly from spectra without referring to the database. Sequence candidates were then submitted to the protein database to compare them with proteins from other species. This was accomplished by using similarity searching and an MS-BLAST method. Our search utilized an alternative evaluation scheme, based on threshold scores that are set conditionally on the number of retrieved high-scoring segment pairs and the total number of fragmented precursors. Following this MS/MS experiment, sequence candidates were then generated from 20 product ion spectra by BioTools software version 2.2 (Bruker), and then submitted to the protein database. Our data revealed that *C. zedoaria* lectin shows sequence similarity (a positive hit) with a mannose-binding lectin from the orchid, *E. helleborine* (van Damme et al., 1994). Furthermore, a precursor ion m/z of 1973.98 indicated that this sequence is LNTGDFLTEGEFLFLMK (Fig. 7), as supported by 64% identities and 76% positives (Fig. 6). This sequence-producing high-scoring segment pair (HSP) equaled 77, which is higher than the threshold of statistical significance for a single matched HSP (used 20 peptides; threshold score of 1HSP=73). Moreover, the percentage of sequence coverage was increased from two product ion spectra m/z of 1014.52 with Sequence DGHVVIYGR (Fig. 8) and m/z of 771.46 with Sequence LVLYHK (Fig. 9), even though these fragments were not hit by MS BLAST-searching.

We have now combined the powerful tool of tandem mass spectrometry with sequence-similarity database-searching methods, which allowed us to identify a protein from *C. zedoaria*, a species with an as-yet unsequenced genome. This protein is assumed to be lectin because it possesses hemagglutinating activity to rabbit erythrocyte as well as specific binding to mannose. Based on the latter, it appears to share sequence similarity with a lectin from the orchid *E. helleborine*.

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