Complete Structural Characterization of a Chitin-Binding Lectin from Mistletoe Extracts

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Dedicated to Professor Dr. Horst Kunz, University of Mainz, on the Occasion of his 60th Birthday

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Abstract. From mistletoe extracts, a chitin-binding lectin (cbML) was isolated and its primary structure determined. The protein is composed of two identical protein chains, linked by am interchenary disulfide bond. Each chain is characterized by four intrachenary disulfide bridges. The structure shows high homology to hevein, one of the prominent allergens of natural rubber latex. cbML could also be detected in commercially available pharmaceutical mistletoe extract preparations. The described isolation procedure and characterization allows isolation of cbML in highly pure form and sufficient quantities, now ready for unequivocal determination for its pharmacological effects.

Mistletoe extracts are multicomponent mixtures, as it is usual for plant extracts. Low molecular weight plant constituents like alkaloids, plant acids, phenylpropane derivatives, terpenes, amino acids, amines as well as high molecular weight compounds like viscotoxins, pectins, arabinogalactans and lectins (ML-I, ML-II, ML-III) were identified [1]. About 10 years ago, *in vivo* studies on animals and humans gave the first indication that ML-I causes a significant increase and activation of natural killer cells and enhances phagocytic activity of granulocytes and monocytes [2, 3]. Recently, we could demonstrate that cultures of peripheral blood lymphocytes with ML-I led to the expansion and activation of T-cells [4]. Furthermore, we showed that the treatment of leukemic T- and B-cell lines with ML-I induces apoptosis which prior requires activation of proteases of the caspase family [5]. Such effects of ML-I on cells of the immune system are at present time intensively studied in *in vitro* test systems, animal experiments [6] and human trials [7] and their therapeutical values are under evaluation [8]. These results prompted us to put efforts into the detailed primary and conformational structure of ML-I, to rationalize the biological and medical effects on a molecular basis [9 – 13]. In the course of our work on the isolation of ML-I, II and III, we currently came across with a protein component of a molecular mass of approximately 12000 Da with chitin-binding properties [14]. This compound, according to our observations, is also present in pharmaceutical preparations. Mainly for this reason, we started studies on the isolation, structural identification and bioactivity of this biomolecule.

Experimental

Materials

All experiments were carried out with material from a single mistletoe plant, collected from an apple tree (*Malus domest.*) in the middle of September 1999. The plant was freed from the berries and stored at – 20 °C until use. All other chemicals used for the work were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) or Merck Eurolab GmbH (Darmstadt, Germany).

Isolation of the Chitin-binding Mistletoe Lectin (cbML)

Leaves and branches of mistletoe were cut into small pieces, frozen in liquid nitrogen and powdered in a Waring Commercial Blendor (Dynamics Corp. of America, New Hartford, Conneticut, USA). The fine ground tissue was extracted with 0.5 M NaCl solution (200 ml/100 g plant material) by stirring at 4 °C over night. The suspension was filtered through a nylon cloth and centrifuged at 6000x*g* for 30 min at 4 °C. Solid $CaCl_2$ (1.5 g/l) was added to the decanted supernatant and the pH was raised with 3 M NaOH to pH 9.0. The preparation was kept at 4 °C for 5 hours, and the precipitate was removed by centrifugation at 8000x*g* for 30 min at 4 °C. The pH of the supernatant was adjusted to 7.0 (with 3 M HCl), the extract clarified by centrifugation under the same conditions as described above and loaded onto a column (1.6 × 10 cm; 20 ml bed volume) of chitin beads (New England Biolabs, New York, USA), equilibrated with 0.5 M NaCl in 50 mM sodium phosphate buffer, pH 7.2. Unbound proteins were removed by washing the column with the same buffer until the absorbance at 280 nm decreased below 0.01. The chitin-binding mistletoe lectin was eluted from the column with 20 mM acetic acid, dialyzed against water and lyophilized [15].

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Reverse Phase High Performance Liquid Chromatography

The lyophilized chitin-binding mistletoe lectin, isolated by affinity chromatography as described above, was purified by reverse phase high performance liquid chromatography (RP-HPLC) on a Nucleosil 100 C18 column (7 µm, 250 × 10 mm; Macherey-Nagel, Düren, Germany) using a H_2O/TFA (trifluoroacetic acid)/CH_3CN gradient system. More detailed chromatographic conditions are given in the legends of the corresponding figures. After collection, the protein fractions were lyophilized and further analyzed.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

CbML preparations were analyzed by SDS-PAGE as described by Laemmli [16], using a 3.0% stacking and a 12% separating gel. Electrophoresis was carried out at a constant voltage of 60 V for 30 min and then over 2 hours at a constant voltage of 150 V.

Mass Spectrometric Analysis

Mass spectrometry (MS) was performed using a matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) instrument (Kratos Kompact MALDI II; Kratos Analytical, Manchester, United Kingdom) [17]. The samples were dissolved in 0.1% (v/v) TFA in water, aliquots of 0.5 µl were mixed with 0.5 µl matrix solution and applied on a stainless steel slide. The droplet was allowed to dry at atmospheric pressure and the sample slide was loaded for analysis into the mass spectrometer. As matrix, a mixture of α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybencoatic acid in 70% acetonitrile and 0.1% TFA was used [18]. MALDI-MS spectra were calibrated using several peptide ion peaks (*e.g.* substance P, *m/z* 1348.7; bovine ubiquitin, *m/z* 8565.9) as standards.

Amino Acid Analysis

For determination of the amino acid composition of the protein, a sample of 299.7 µg was hydrolyzed in 6 M HCl at 110 °C for 24 h and analyzed by a LC 3000 amino acid analyzer (Eppendorf-Biotronik, Hamburg, Germany). Amino acids were quantified with post-column ninhydrin detection [19]. The results were edited using our developed calculation program, based on the software Excel 97 (Microsoft Cooperation, Redmond, WA, USA).

Reduction and Alkylation of Cysteine Residues

Reduction of lyophilized chitin-binding mistletoe lectin (2.8 mg) with 2-mercaptoethanol (7 µl) was carried out in a final volume of 1 ml 0.25 M Tris/HCl, 6 M guanidine-hydrochloride, 1 mM EDTA, pH 8.5, at RT for 16 h in the dark. Then, 10 µl of vinylpyridine was added and the reaction mixture was stirred in the dark for 4 h [20]. The alkylated cbML was desalted by RP-HPLC on an Aquapore RP300 C8 column (7 µm, 30 × 2.1 mm; Perkin Elmer AB, Weiterstadt, Germany). The elution was performed using the following conditions: eluent A, 0.1% TFA in water; eluent B, 0.085% TFA, 80% acetonitrile and 20% water; gradient program: 0 – 80% B in 60 min at a flow rate of 0.2 ml/min. The absorbance of the eluate was recorded at 214 nm. The fraction containing the modified protein was collected and recovered by lyophylization.

Enzymatic Digestion and HPLC Fractionation of the Peptides

Digestion of the reduced and alkylated chitin-binding lectin with endoproteinase Lys-C (enzyme:substrate = 1:40) was carried out in 25 mM Tris-HCl containing 1mM EDTA and 5% acetonitrile, pH 8.5, at 37 °C for 16 h [21]. The resulting peptides were fractionated by RPHPLC on a Grom-Sil 100 C18 column (5 µm, 250 × 4.6 mm; Grom, Herrenberg Kayh, Germany) with: eluent A, 0.1% TFA in water; eluent B, 80% acetonitrile in A; gradient program: 0% B for 5 min, then 0% to 60% B in 45 min at a flow rate of 1.0 ml/min. Eluted peptides were detected by UV absorbance at 214 nm, collected manually and recovered by lyophylization.

Amino Acid Sequence Analysis

Automated Edman degradation was performed by using an Applied Biosystems pulsed liquid sequencer model 473A (Weiterstadt, Germany) with on-line analysis of the phenylthiohydantoin (PTH) derivatives [22]. The sequence of the peptides, dissolved in 0.1% (v/v) TFA and spotted onto polybrene-coated filters, was evaluated by comparing the HPLC chromatograms of standard PTH-derivatized amino acids with the profiles produced by sequential Edman degradation of the protein fragments.

Haemagglutination Assays

Agglutination assays were carried out in 96 well microtiter plates in a final volume of 0.1 ml containing 50 µl of a 2% suspension of washed red blood cells in PBS (phosphate buffered saline) and 50 µl of a twofold serial dilution of the lectin sample solution (initial concentration 1 mg/ml) with PBS. Agglutination was controlled visually after 1 h at room temperature. Haemagglutination units were defined as maximum dilution factor of sample solution in which clear agglutination was detectable. Papaine treatment of erythrocytes was performed as follows: 1.5 ml of a 2% suspension of washed red blood cells in PBS (pH 7.3) was mixed with 2 mg papaine in a volume of 500 µl PBS. After incubation for 30 min at 37 °C in a thermomixer, the cells were centrifuged for 2 min at 2000xg and the supernatant was discarded. The cells were resuspended in 2 ml PBS and the washing procedure repeated four more times. Finally, the cell suspension was reconstituted with 1.5 ml PBS and stored at 4 °C until use.

Protein Similarity Search and Sequence Alignments

The sequence, derived from *N*-terminal amino acid sequencing, was compared against published protein sequences with the program PSI-BLAST [23] at NCBI (National Center for Biotechnology Information). Multiple sequence alignments were performed and presented using the software ClustalX [24] and GeneDoc [25].

Results

To avoid isolation of isoform mixtures from different

plant species, a single mistletoe plant, growing on an apple tree, was used as starting material for the isolation of the chitin-binding protein. Leaves and branches were cut into small pieces , frozen in liquid nitrogen, powdered and extracted with a sodium chloride solution. After filtration of the suspension through a nylon cloth, centrifugation, adding $CaCl_2$, adjusting the pH to 9.0 with NaOH, followed by another centrifugation step, the supernatant was adjusted to pH 7.0. After the final centrifugation procedure the extract was loaded onto a chitin beads column. The loaded column was then washed with 0.5 M NaCl in 50 mM sodium phosphate buffer, pH 7.2, for several hours to remove unbound material of the plant extract. At absorption of the eluate below 0.05, the chitin-binding mistletoe lectin was eluted from the column using 20 mM acetic acid as eluent (Fig. 1). Protein-containing fractions were pooled, dialyzed against distilled water and lyophilized.

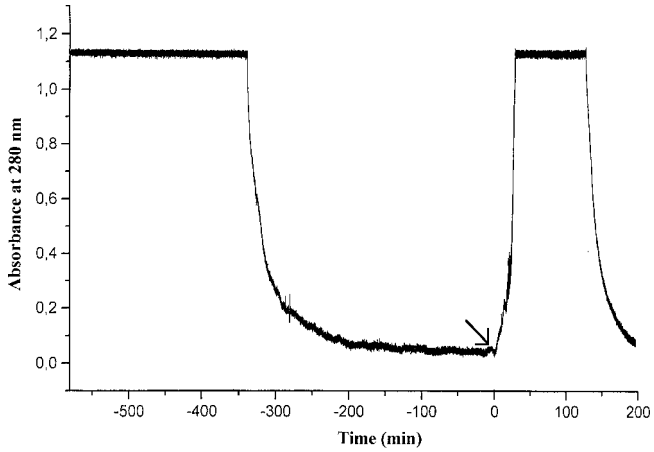

Fig. 1 Affinity chromatogram of the mistletoe plant extract. Column: Chitin beads (10 × 1,6 cm; New England Biolabs, New York, USA). Washing buffer: 0.5 M NaCl in 50 mM sodium phosphate, pH 7.2; elution buffer: 20 mM acetic acid. Washing flow rate: 0.5 ml/min; elution flow rate: 0.7 ml/min. Detection: UV at λ = 280 nm. The arrow indicates start of the elution.

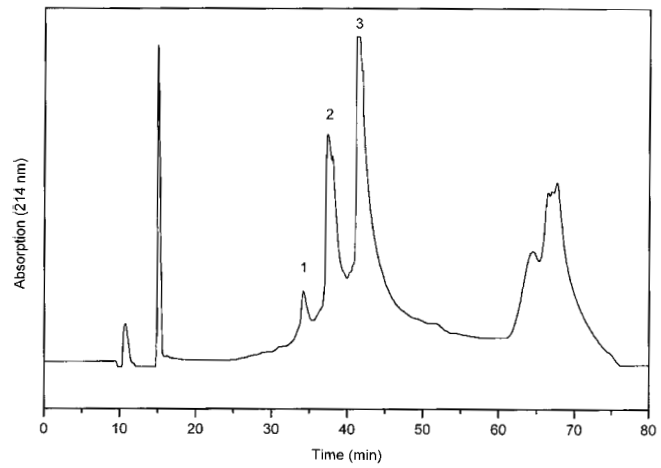

Fig. 2 HPLC profile of the chitin-binding mistletoe lectin purified by affinity chromatography (see Fig. 1). Column: Nucleosil 100 C18 (7 µm, 250 × 10 mm; Macherey-Nagel, Düren, Germany). Gradient: 27% B for 10 min, 27–40% B within 39 min, 40–100% B within 20 min; eluent A: 0.1% TFA in H_2O; eluent B: 80% CH_3CN, 0.1% TFA in H_2O. Flow rate: 1.2 ml/min. Detection: UV at λ = 214 nm.

Further purification of the isolated chitin-binding mistletoe lectin by RP-HPLC resulted in three fractions (Fig. 2, peaks 1, 2, 3) with retention times between 30 and 45 minutes. The *N*-terminal amino acid sequences of the three fractions were identical for the first five amino acids. The peak fractions with retention times larger than 60 minutes consisted of a mixture of different components as indicated by Edman degradation. In this communication we concentrate on detailed structure information of fraction 3 only.

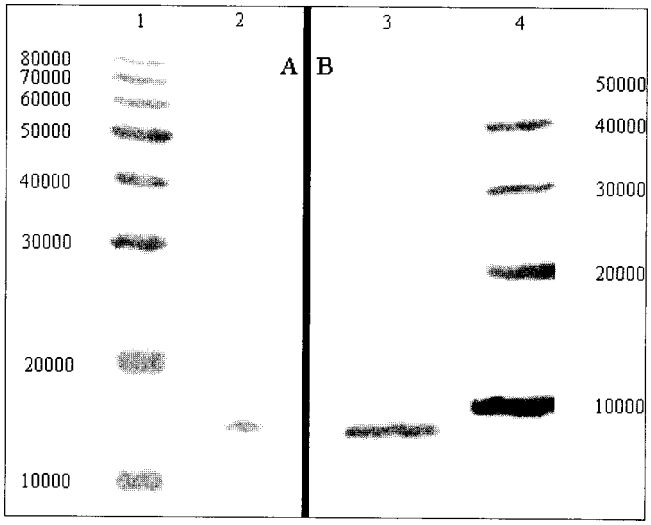

Fig. 3 SDS-PAGE (3.0% stacking and 12% separating gel) of reduced (B) and unreduced (A) chitin-binding mistletoe lectin. Standard: 10 kDa protein ladder (Life Technologies, Karslruhe, Germany) . Detection: Coomassie Blue G dye. Lanes 1: 10 kDa protein ladder; 2: unreduced cbML; 3: reduced cbML; 4:10 kDa protein ladder.

For protein purity control and molecular mass determination of fraction 3, SDS polyacrylamide gel electrophoresis was carried out (Fig. 3). The unreduced protein showed a molecular weight of about 14 kDa, whereas the reduced form migrated lower than 10 kDa. No further bands were detectable with the Coomassie staining [26]. More precise information was obtained with MALDI-MS analysis of fraction 3 (Fig. 4). A molecular mass of 10847 Da was determined, which is in coincidence with the results obtained by acrylamide gel electrophoresis. Furthermore, *m/z* values of 5410, 3604 and 2705 were detectable in the lower mass range, corresponding to double-, three- and fourfold loaded species.

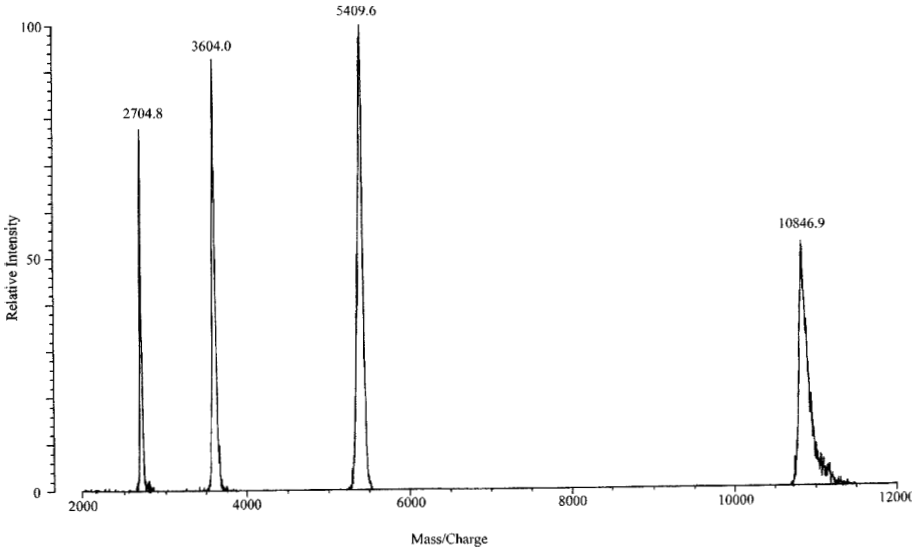

Fig. 4 MALDI-MS spectrum of purified chitin-binding mistletoe lectin. Matrix: α-cyano-4-hydroxycinnamic acid. Number of shots: 50. Laser power: 94. Wavelength: 337 nm

Table 1 Amino acid analysis of cbML after 24 h hydrolysis

Amino acid (aa)	Concentration [nmol/ml]	Amount aa [nmol]	Calculated number aa	Theoretical number aa	Weight contribution of aa [µg]
Asx	880,12	264,04	6,05	6	30,39
Thr	415,27	124,58	2,85	3	12,60
Ser	368,82	110,65	2,53	3	9,64
Glx	718,39	215,52	4,94	5	27,82
Gly	883,61	265,08	6,07	6	15,14
Ala	312,69	93,81	2,15	2	6,67
Cys	1298,00	389,40	8,92	9	40,15
Val	5,29	1,59	0,04	0	0,16
Met	6,42	1,93	0,04	0	0,25
Ile	141,46	42,44	0,97	1	4,80
Leu	264,62	79,39	1,82	2	8,99
Tyr	144,51	43,35	0,99	1	7,08
Phe	4,92	1,48	0,03	0	0,22
His	161,82	48,55	1,11	1	6,66
Lys	323,29	96,99	2,22	2	12,43
Arg	616,24	184,87	4,23	4	28,88
Pro	293,92	88,18	2,02	2	8,56
Trp [a])			2,00	2	16,26
Sum		**2051,82**	**49,00**	**49**	[b]) **237,46**
Average		**43,66**			
Sample weight:		**299,7 µg**			
Protein content of sample:		**79,23%**			

[a]) The extrapolated values for Trp (2 residues) are based on the sequencing data; [b]) Sum of all aa middle-unit contributions including one average molar amount of water; aa: amino acid (residue)

For determination of the amino acid composition of cbML, the highly purified protein was hydrolyzed with 6N HCl. The detection and quantification of the separated amino acids was achieved by post-column ninhydrin derivatization. The obtained results are presented in Table 1, characterizing cbML as rich of cysteine, glycine and Asx residues. The determined amino acid ratios are in close relation to the subsequently established sequence data (see below).

For primary structure elucidation of the chitin-binding mistletoe lectin, the protein was reduced, alkylated and digested with endoproteinase Lys-C. The resulting fragments were isolated *via* HPLC separation on a C18 RP column (Fig. 5). Besides the three main fractions (LC 1-3), a considerable number of further minor ones were detected. *N*-Terminal sequencing and MALDI-MS

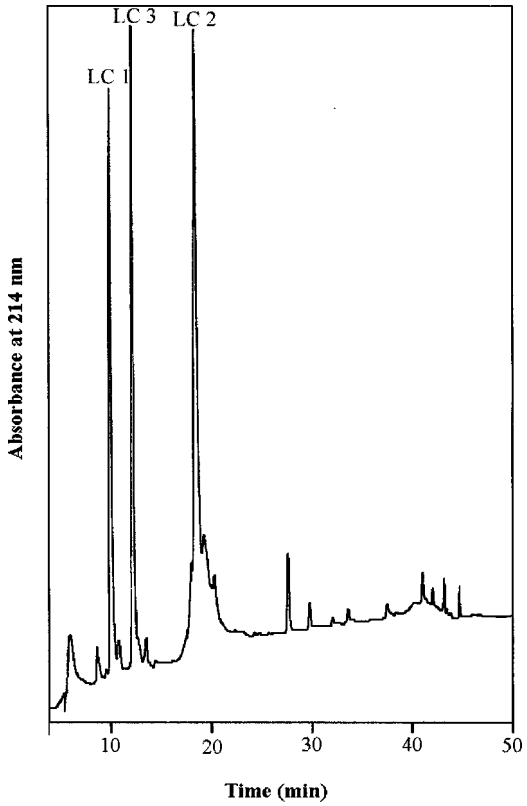

Fig. 5 HPLC fractionation of the fragment mixture obtained after endoproteinase Lys-C cleavage of the purified chitin-binding mistletoe lectin (Fraction 3, Fig. 2). Column: Grom-Sil 100 C18 (5 µm, 250 × 4,6 mm; Grom, Herrenberg-Kayh, Germany). Gradient: 0% B for 5 min, 0–60% B within 45 min; eluent A: 0.1% TFA in H_2O; eluent B: 80% CH_3CN, 0.1% TFA in H_2O. Flow rate: 1.0 ml/min. Detection: UV at λ = 214 nm.

analysis demonstrated that they resulted from incomplete alkylation and/or digestion of cbML (data not shown). Therefore, only fractions 1–3 were further processed. Their primary structures were identified by Edman degradation and confirmed by MALDI-MS measurements (Fig. 6, Table 2). The amino acid sequence of pyridylethylated intact cbML could unequivocally be identified up to residue 40. The information of the three Lys-C peptides, together with the *N*-terminus, allowed the complete alignment of the cbML sequence, furthermore confirmed by MALDI-MS.

The highly purified cbML showed no agglutination activity at an initial concentration of 1 mg/ml cbML, neither against type A, B nor AB human erythrocytes. However, a suspension of papaine-treated human red blood cells of type B were agglutinated in a concentration of 1 mg/ml cbML. These results indicate a relatively weak haemagglutination activity of cbML in contrast to ML-I. For ML-I (see introduction), one of the major bioactive components of mistletoe extracts, a minimal agglutination concentration against papaine-treated human B erythrocytes of less then 1 µg/ml is observed [27].

Discussion

Recently, Peumans *et al.* [15] reported on the isolation and partial characterization of a small chitin-binding lectin. The published 26 *N*-terminal amino acid residues are identical with the sequence of cbML, isolated *via* affinity and subsequent HPL chromatography (Fig. 2, fraction 3). However, there is some doubt, if the par-

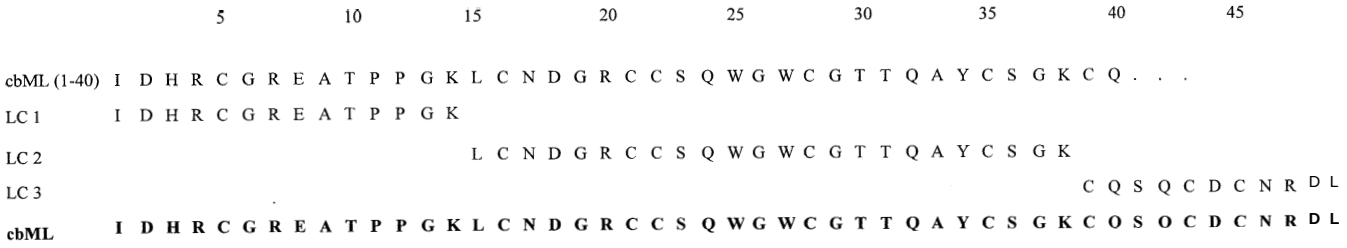

Fig. 6 Complete amino acid sequence of the chitin-binding mistletoe lectin (cbML) as determined from overlapping sequenced Lys-C fragments and its *N*-terminus

Table 2 Comparision of experimentally (MALDI MS) and theoretically determined molecular masses of Lys-C (LC) fragments of cbML

Fraction (Fig. 5)	Alkylated chitin-binding mistletoe lectin fragments	Theoretical mass (Da)	Experimental Mass (Da)
LC 1	1–14	1642	1643 (MH^+)
LC 2	15–38	3154	3172 (MNa^+)
LC 3	39–49	1600	1600 (MH^+)

tially characterized protein, reported in reference [15], is identical with the biopolymer, described in this communication, for several reasons:

1. Based on MALDI-TOF measurements, SDS-PAGE and gel filtration chromatography, the authors of reference [15] determined a molecular mass of 21.6 kDa for their lectin. We found a molecular size of 10.8 kDa only for the biopolymer, characterized in this report.
2. For our lectin, we could not determine any agglutination activity for A, B and AB type human erythrocytes up to initial concentrations of 1 mg/ml cbML. The chitin-binding lectin, described in reference [15] agglutinated untreated and trypsin-treated human type A erythrocytes at concentrations as low as 5 and 30 µg/ml, respectively. One reason for this discrepancy might be that minor contamination of ML-I caused errors in the determination of the haemagglutination units. *E.g.* we observed for papain-treated human type B erythrocytes a minimal agglutination concentration of less then 1 µg/ml for ML-I [14].
3. Preliminary *N*-terminal sequencing data of different HPLC fractions of chitin-binding mistletoe lectins, isolated by affinity chromatography on chitin beads (see Figs 1, 2), demonstrate that the plant produces several chitin-binding protein isoforms with identical *N*-termini.

Based on the sequencing data, the cbML chain consists of 49 amino acid residues, including nine cysteines, for which a theoretical molecular mass of 5413.1 Da is calculated. However, by MALDI-MS an exactly twofold higher mass is determined for the molecular ion. This result is in coincidence with the value determined by SDS-PAGE. Therefore, cbML is a homodimer, composed of two identical units, linked by a disulfide bridge.

By sequence homology search [23], a high identity of cbML to hevein, ragweed allergen Ra5G and the domains of wheat germ agglutin [28, 29] was found (Fig.7). In these proteins, especially the cysteine bridges are highly conserved, and with high probability an analogous disulfide bridging for cbML, can be assumed. Therefore, in the two identical chains of cbML 16 cysteine residues form 8 intrachenary disulfide bridges (residues 5(5')→22(22'), 16(16')→28(28'), 21(21')→35(35'), 39(39')→43(43')). The cysteine residues at position 45 and 45' link the two identical protein chains by an interchenary disulfide bond (Fig. 8).

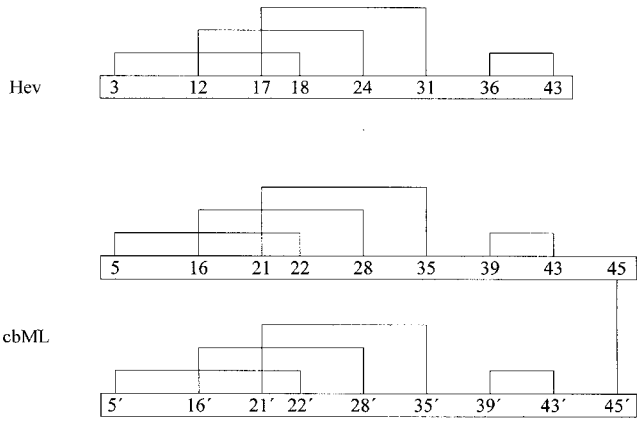

Fig. 8 Hevein and cbML chains with identified, respectively postulated disulfide bridges

The complete primary structure elucidation of cbML allowed to discover its close relationship to hevein. It is well known that hevein is a major IgE-binding polypeptide in *Hevea latex* and is responsible for development of latex allergies [30]. Therefore, similar effects can be expected from the structurally closely related cbML. As we could determine cbML in different commercially available pharmaceutical preparations in quantities up to 5 µg/ampoule, it is essential to screen different pharmacological parameters for the lectin, like *e.g.* toxicity, allergic effects, stimulation of lymphocyts *etc.* One prerequisite for these studies is, however, that highly pure and characterized material becomes available in sufficient quantities. This is one of the main aims of this communication.

Position	1				5					10					15					20					25
cbML	I	D	H	R	C	G	R	E	A	T	P	P	G	K	L	C	N	D	G	R	C	C	S	Q	W
Hev	-	-	E	Q	C	G	R	Q	A	G	-	-	G	K	L	C	P	N	N	L	C	C	S	Q	W

	26				30					35					40					45				49
cbML	G	W	C	G	T	T	Q	A	Y	C	S	G	K	-	-	C	Q	S	Q	C	D	C	N	R	D	L
Hev	G	W	C	G	S	T	D	E	Y	C	S	P	D	H	N	C	Q	S	N	C	K	D				

Fig. 7 Sequence homology between cbML and hevein (Hev). Cysteine residues are black shaded

References

[1] P. Luther, H. Becker, Die Mistel, Springer-Verlag Berlin Heidelberg 1987
[2] T. Hajto, K. Hostanska, H.-J. Gabius, Cancer Res. **1989**, *49*, 4803
[3] T. Hajto, K. Hostanska, H.-J. Gabius, Therapeutikon **1990**, *4*, 136
[4] C. N. Baxevanis, I. F. Voutsas, M. H. Soler, A. D. Gritzapis, O. E. Tsitsilonis, S. Stoeva,W. Voelter, P. Arsenis, M. Papamichail, Immunopharmacol. Immunotoxicol. **1998**, *20*, 355
[5] H. Bantel, I. H. Engels, W. Voelter, K. Schulze-Osthoff, S.Wesselborg, Cancer Res. **1999**, *59*, 2083
[6] J. Beuth, B. Stoffel, H. L. Ko, J. Jeljaszewicz, G. Pulverer, Arzneimittel-Forschung/Drug Res. **1995**, *45*, 1240
[7] J. Beuth, H.L. Ko, H.-J. Gabius, H. Burrichter, K. Oette, G. Pulverer, Clin. Investig. **1992**, *70*, 658
[8] H. Mayer, R. Steppkes, R. Roth, C.-P. Richter, Pharm. Zeitung **1996**, *27*, 11
[9] M. H. Soler, S. Stoeva, C. Schwamborn, S. Wilhelm, T. Stiefel, W. Voelter, FEBS Lett. **1996**, *399*, 153
[10] M. H. Soler, S. Stoeva, W. Voelter, Biochem. Biophys. Res. Comm. **1998**, *246*, 596
[11] S. Stoeva, T. Maier, M. H. Soler, W. Voelter, Polish J. Chem. **1999**, *73*, 125
[12] S. Eschenburg, R. Krauspenhaar, A. Mikhailov, S. Stoeva, C. Betzel, W. Voelter, Biochem. Biophys. Res. Comm. **1998**, *247*, 367
[13] R. Krauspenhaar, S. Eschenburg, M. Perbandt, V. Kornilov, N. Konareva, I. Mikailova, S. Stoeva, R. Wacker, T. Maier, T. Singh, A. Mikhailov, W. Voelter, C. Betzel, Biochem. Biophys. Res. Comm. **1999**, *257*, 418
[14] R. Wacker, Ph. D. Thesis, University of Tübingen, **2000** (in preparation)
[15] W. J. Peumans, P. Verhaert, U. Pfüller, E. J. M. Van Damme, FEBS Lett. **1996**, *396*, 261
[16] U. K. Laemmli, Nature **1970**, *227*, 680
[17] M. Karas, F, Hillenkamp, Anal. Chem. **1988**, *60*, 2299
[18] K. Gevaert, H. Demol, M. Puype, D. Brokaert, S. De Boeck, T. Houthaeve, J. Vandekerckhove, Electrophoresis **1997**, *18*, 2950
[19] S. Moore, W. H. Stein, Methods Enzymol. **1963**, *6*, 819
[20] M. Friedman, L. H. Krull, J. F. Cavins, J. Biol. Chem. **1970**, *245* (15), 3868
[21] S. Tsunasawa, T. Masaki, M. Hirose, M. Soejima, F. Sakiyama, J. Biol. Chem. **1989**, *264* (7), 3832
[22] P. Edman, Eur. J. Biochem. **1967**, *1*, 80
[23] S. F. Altschul, T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, Nucleic Acids Res. **1997**, *25* (17), 3389
[24] J. D. Thompson, T. J. Gibson, F. Plewniak, F. Jeanmougin, D. G. Higgins, Nucleic Acids Res. **1997**, *25* (24), 4876
[25] K. B. Nicholas, H. B. jr. Nicholas, GeneDoc: a tool for editing and annotating multiple sequence alignments, distributed by the author, **1997**
[26] W. Diezel, D. Koppenschlager, E. Hofmann, Anal. Biochem. **1972**, *48(2)*, 617
[27] R. Wacker, unpublished results
[28] N. H. Andersen, B. Cao, A. Rodriguez-Romero, B. Arreguin, Biochemistry **1993**, *32*, 1407
[29] J. J. Beintema, FEBS Letters **1994**, *350*, 159
[30] Z. Chen, A. Posch, C. Lohaus, M. Raulf-Heimsoth, H. E. Meyer, X. Baur, J. Allergy Clin. Immunol. **1997**, *99*, 402

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