Isolation and partial characterization of a small chitin-binding lectin from mistletoe (*Viscum album*)

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Abstract A novel lectin, called VisalbCBA, was isolated from European mistletoe (*Viscum album*). This lectin differs completely from the classical galactose/*N*-acetylgalactosamine-binding mistletoe lectins MLI, MLII and MLIII. Biochemical analyses indicated that VisalbCBA is a dimeric protein composed of two identical subunits of approx. 10 kDa. VisalbCBA exhibits specificity towards oligomers of *N*-acetylglucosamine and shows sequence homology to the previously isolated chitin-binding plant proteins. Although VisalbCBA is less toxic than the other mistletoe lectins, it definitely exhibits cytotoxic properties. The possible involvement of VisalbCBA in the biological and therapeutic effects of mistletoe is discussed.

Key words: *Viscum album*; Mistletoe; Lectin; Cytotoxicity; Chitin binding

1. Introduction

European mistletoe (*Viscum album*) has become an important issue in lectinology because there are indications that the therapeutic applications of this medicinal plant are linked to the biological activities of its lectins. Detailed studies have demonstrated that leaves and other tissues of this half parasite contain three different lectins [1,2]. Although these lectins differ from each other with respect to their molecular structure and carbohydrate-binding specificity, they all clearly belong to the so-called type 2 ribosome-inactivating proteins (RIP) [3]. Type 2 RIP are bifunctional proteins composed of two different disulphide-bridge-linked polypeptide chains. The RNA *N*-glycosidase activity resides in the so-called A chain whereas the sugar-binding activity is associated with the B chain. Both chains are derived from a single precursor by the post-translational excision of a linker sequence between the (N-terminal) A-domain and the (C-terminal) B-domain and are held together by a disulphide bridge. Mistletoe lectin I (MLI) is a dimer of two [A-s-s-B] pairs and exhibits a preferential specificity towards galactose [4–7]. In contrast, lectin II (MLII) and lectin III (MLIII) are monomeric type 2 RIP exhibiting specificity towards galactose/*N*-acetylgalactosamine and *N*-acetylgalactosamine, respectively [1,2]. As could be expected from the differences in carbohydrate-binding specificity the three mistletoe lectins exhibit quite different biological activities. Most likely, the therapeutic effects of mistletoe preparations are based on the immunomodulatory activity of MLI [1,2,8].

In this report we present evidence that mistletoe contains besides the three above-mentioned type 2 RIP/lectins also a small agglutinin, which on the basis of its structure and specificity can be classified in the group of chitin-binding plant lectins composed of hevein domains [9]. The discovery of the novel agglutinin not only demonstrates that mistletoe contains two groups of totally different lectins, but also raises the question of whether this new lectin possibly contributes either positively or negatively to the therapeutic effects of mistletoe preparations. Since a similar chitin-binding lectin from the rhizomes of stinging nettle (*Urtica dioica*) is a potent superantigen in mice [10], the latter question is certainly relevant.

2. Materials and methods

2.1. Materials

All experiments were carried out with materials from a single mistletoe (*V. album* L.) plant grown on a local poplar (*Populus* hybr.) tree. The plant was collected at the end of September and processed immediately.

A mixture of *N*-acetylglucosamine oligomers was prepared from crude crab shell chitin (type C-7170 from Sigma) as described by Kilpatrick and Yeoman [11].

2.2. Isolation of the chitin-binding mistletoe lectin

1 kg of leaves and young branches of mistletoe were homogenized in 10 l of 20 mM acetic acid with a Waring blender. The homogenate was filtered through cheese cloth, centrifuged (8000×*g* for 10 min) and the resulting supernatant decanted and filtered through glass wool (to remove the floating particles). After the addition of 1.5 g/l $CaCl_2$, the extract was brought to pH 9.0 (with 1 N NaOH) and kept in the cold (2°C) for about 3 h. The precipitate was removed by centrifugation (3000×*g* for 10 min) and the cleared extract adjusted to pH 3.0 (with 1 N acetic acid). After standing overnight in the cold room, the extract was recentrifuged (3000×*g* for 10 min) and the supernatant filtered through filter paper (Whatman 3MM). The filtrate was diluted with an equal volume of distilled water and applied onto a cation exchanger column (10 cm×5 cm; 200 ml bed volume) of S Fast Flow (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM acetic acid. After loading the proteins, the column was washed with 2 l of 20 mM Na-formate (pH 3.8) and the bound proteins eluted with 500 ml of 0.5 M NaCl in the same buffer.

To remove the type 2 RIP MLI, MLII and MLIII, the protein mixture desorbed from the S Fast Flow column was subjected to consecutive affinity chromatography on galactose-Sepharose 4B and fetuin-Sepharose 4B. Therefore, the partially purified protein fraction was adjusted to pH 7.4 (with 1 N NaOH) and loaded onto a column (10 cm×2.6 cm; about 50 ml bed volume) of galactose-Sepharose 4B on which MLI is retained. After passing the protein solution, the column was washed with 200 ml of PBS. The MLI-depleted protein fraction and the washing solution were combined and applied onto a column (10 cm×2.6 cm; about 50 ml bed volume) of fetuin-Sepharose 4B to remove MLII and MLIII. After loading the MLI-depleted fraction, the column was washed with 200 ml of PBS. The unbound

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Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time of flight; ML, mistletoe lectin; MS, mass spectrometry; PBS, phosphate-buffered saline (1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 3 mM KCl, 140 mM NaCl, pH 7.4); RIP, ribosome-inactivating protein; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VisalbCBA, *Viscum album* chitin-binding agglutinin

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proteins (which were free of MLI, MLII and MLIII) were combined with the first 200 ml of PBS of the washing solution and applied onto a column (20 cm×2.6 cm; about 100 ml bed volume) of chitin (type C-7170 from Sigma). Unbound proteins were removed by washing the column with phosphate-buffered saline (PBS) until the A_{280} fell below 0.01. Finally, the lectin was desorbed with 20 mM acetic acid, dialyzed against PBS and stored at −20°C until use. Alternatively, the lectin was dialyzed against water and lyophilized.

2.3. Gel filtration

Analytical gel filtration of the purified lectin was performed on a Pharmacia Superose 12 column using PBS containing 10 mg/ml of a mixture of *N*-acetylglucosamine oligomers (to avoid binding of the lectin to the column) as running buffer. Since the chitin-binding lectins composed of hevein domains behave somewhat anomalously upon gel filtration, the molecular mass of the novel mistletoe lectin was estimated using wheat germ agglutinin (34 kDa) and nettle lectin (8.5 kDa) as marker proteins.

2.4. Analytical methods

Total neutral sugar was determined by the phenol/H_2SO_4 method [12], with D-glucose as standard.

Lectin preparations were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5–25% (w/v) acrylamide gradient gels as described by Laemmli [13].

2.5. Agglutination assays

Agglutination assays were carried out in small glass tubes in a final volume of 0.1 ml containing 90 μl of a 1% suspension of red blood cells and 10 μl of crude extracts or lectin solutions (each serially diluted with 2-fold increments). Agglutination was controlled visually after 1 h at room temperature.

The carbohydrate-binding specificity of the lectin was determined using hapten inhibition assays with some glycoproteins (thyroglobulin, fetuin, asialofetuin and ovomucoid) and a series of simple sugars (cf. Table 1).

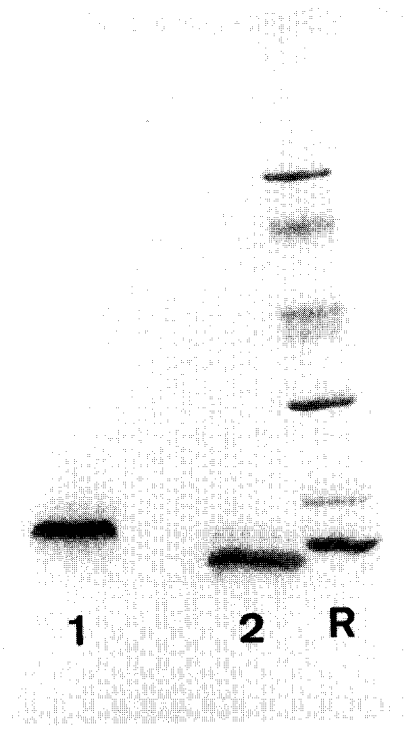

Fig. 1. SDS-PAGE of purified VisalbCBA. Samples (25 μg each) of unreduced and reduced VisalbCBA were run in lanes 1 and 2, respectively. Molecular mass reference proteins (lane R) were lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase *b* (94 kDa).

2.6. Amino acid sequence analysis

The N-terminal amino acid sequence of the mistletoe lectin was determined by automated Edman degradation using a gas-phase sequenator (Beckman LF 3600 TC) coupled to a high-performance liquid chromatograph (Beckman Gold) for PTH amino acid identification.

2.7. Mass spectrometry

Mass spectrometry (MS) was performed using a MALDI-TOF (matrix-assisted laser desorption ionization time of flight) instrument; a VG TofSpec by Micromass Ltd. (Manchester, UK). Calibration was performed externally using horse heart cytochrome *c* singly and doubly charged ions ($[M+H]^+$ 12361.10 and $[M+2H]^{2+}$ 6181.05, respectively). The matrix employed was α-cyano-4-hydroxycinnamic acid (Aldrich). The instrument was operated in the linear mode with an accelerating voltage of 24 000 V. The energy of the pulsed UV laser was adjusted to near threshold values for desorption/ionization in order to obtain optimal resolution at the observed masses. Approx. 30 shots were averaged to obtain a spectrum.

2.8. Cytotoxicity assays

The cytotoxicity of VisalbCBA was determined on Molt4 cells (a cell line derived from human T cell leukemia) kindly provided by Prof. A.G. Tonevitsky (Institute of Immunology, Moscow). Cells were cultured as described by Ribereau-Gayon et al. [14]. For the cytotoxicity assays, cells were growth-arrested for 48 h in serum-free medium, plated in 96-well microtiter plates in culture medium containing increasing concentrations of lectins and incubated for 72 h. Cell viability was determined by the cytotoxicity assay of Mossman [15]. To each well 100 μl of nitroblue tetrazolium chloride was added. After incubation for 4 h the supernatant was removed and 200 μl solubilizer (20% SDS (w/v) dissolved in dimethylformamide/water 1:1 (v/v)) added before reading the absorption at 580 nm in a microtiter plate reader.

3. Results

3.1. Nomenclature of the mistletoe lectins/RIP

According to the currently used nomenclature, the type 2 RIP/lectins from mistletoe are referred to by the abbreviations MLI, MLII and MLIII. Since these abbreviations are trivial and, in addition, there is a possibility that mistletoe also contains other as yet unidentified type 2 RIP or related proteins, the novel lectin is not called MLIV. To distinguish the new chitin-binding agglutinin from the previously described galactose/*N*-acetylgalactosamine-binding type 2 RIP, it will be referred to as *V. album* chitin-binding agglutinin or VisalbCBA.

3.2. Isolation and characterization of the V. album chitin-binding agglutinin (VisalbCBA)

VisalbCBA was isolated by a combination of classical protein purification techniques and affinity chromatography. After partial purification by ion-exchange chromatography on a S Fast Flow column, the type 2 RIP MLI, MLII and MLIII were removed from the total lectin fraction by two consecutive affinity chromatography steps on immobilized galactose and fetuin, respectively. The final purification of VisalbCBA was achieved by affinity chromatography on a chitin column. Using the procedure described above the total yield was about 10 mg affinity purified VisalbCBA per kg of starting material. Since VisalbCBA is very well retained on the S Fast Flow column, and all the agglutinating activity of the MLI, MLII and MLIII-free fraction was quantitatively retained on the chitin column, we presume that the overall recovery is reasonably high. Accordingly, the total content of VisalbCBA in the mistletoe leaves and stems is rather low.

To determine the molecular structure of VisalbCBA, the affinity-purified lectin was analyzed by SDS-PAGE, mass

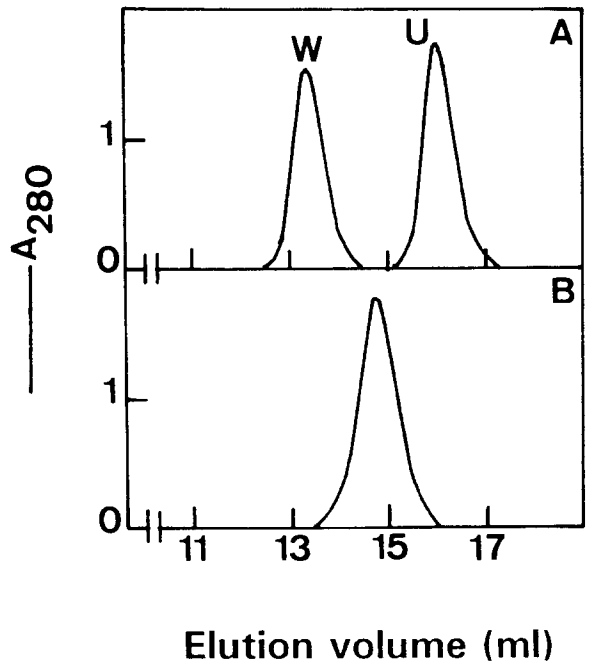

Fig. 2. Gel filtration of native VisalbCBA on a Superose 12 column. (A) Elution of a mixture of the chitin-binding lectins wheat germ agglutinin (34 kDa) (W) and *Urtica dioica* (stinging nettle) agglutinin (8 kDa) (U). (B) Elution pattern of VisalbCBA.

spectrometry and gel filtration. Reduced (with 2-mercaptoethanol) VisalbCBA yielded a single polypeptide band of about 9 kDa upon SDS-PAGE whereas the unreduced protein migrated as a diffuse band with an apparent molecular mass of about 18 kDa (Fig. 1). Mass spectrometry yielded a molecular ion $[M+H]^+$ at 10847 mass units and a doubly charged species $[ML+2H]^{2+}$ at 5425 mass units (results not shown). Native VisalbCBA eluted as a single symmetrical peak with an apparent molecular mass of about 18 kDa upon gel filtration chromatography on a Superose 12 column using the chitin-binding lectins from wheat germ and stinging nettle rhizomes as standards (Fig. 2). On the basis of the gel filtration, MS and SDS-PAGE data, it is concluded that VisalbCBA is a dimer of two identical subunits of 10.8 kDa.

No carbohydrate could be detected in purified VisalbCBA by the phenol-sulphuric acid method indicating that the lectin is not glycosylated.

VisalbCBA is readily soluble in water (up to 5 mg/ml) and in 20 mM acetic acid (up to 20 mg/ml). A solution of 1 mg/ml gives an A_{280} of 1.83.

VisalbCBA was challenged with antibodies against MLI, MLII and MLIII. Since neither polyclonal (goat and human) nor monoclonal antibodies reacted with VisalbCBA (results not shown), it is evident that the chitin-binding lectin is serologically unrelated to the formerly described mistletoe type 2 RIP/lectins.

3.3. *Agglutination properties and carbohydrate-binding specificity of VisalbCBA*

The specific agglutination activity (defined as the lowest lectin concentration which still yields a visible agglutination) was determined with untreated and trypsin-treated rabbit and human type A erythrocytes. VisalbCBA agglutinated untreated and trypsin-treated rabbit red blood cells at concentrations as low as 2.5 ml and 10 μg/ml, respectively. When human erythrocytes were used, the values were 2–3-times higher (5 and 30 μg/ml, respectively).

The carbohydrate-binding specificity of VisalbCBA was determined in some detail by hapten inhibition assays of the agglutination of rabbit erythrocytes. As shown in Table 1, only glcNAc and glcNAc oligomers were inhibitory. Since the dimers, trimers and tetramers of glcNAc were 19-, 300- and 2400-times, respectively, more potent inhibitors than the monomer, it is evident that the carbohydrate-binding site of VisalbCBA is most complementary to the trimer and tetramer of GlcNAc. It is also noteworthy that none of the glycoproteins tested (ovomucoid, fetuin, asialofetuin, thyroglobulin, mucin and asialomucin) had any inhibitory effect at concentrations below 2 mg/ml.

3.4. *Stability of VisalbCBA*

Most of the chitin-binding lectins composed of hevein domains are very stable proteins. To assess the stability of VisalbCBA, the effects of adverse conditions and some proteolytic enzymes were investigated in some detail. Commonly used proteases such as trypsin and chymotrypsin did not affect the activity of the lectin. VisalbCBA was fully stable in the pH range between 1 and 12. In addition, VisalbCBA is

Table 1
Carbohydrate-binding specificity of VisalbCBA

Sugar[a]	IC_{50} (μM)[b]	Relative inhibitory potency
GlcNAc	37500	1
GlcNAc dimer	2000	19
GlcNAc trimer	125	300
GlcNAc tetramer	15	2400

[a]Sugars and glycoproteins which were not inhibitory at concentrations below 200 mM and 2 mg/ml, respectively, are not listed. They were: saccharose, glucose, 6-deoxyglucose, glucosamine, GlcNAc, cellobiose, glycogen, amylose, glucuronic acid, methyl-α-D-mannopyranoside, methyl-α-D-glycopyranoside, maltose, trehalose, fructose, 2-deoxyglucose, methyl-α-D-galactopyranoside, gal, GalNAc, melibiose raffinose, gentobiose, galacturonic acid, arabinose, L-fucose, D-fucose, xylose, rhamnose, sorbose, ribose, deoxyribose, NAN, erythreitol, arabitol, xylitol, sorbitol, laminarin, mucin, asialomucin ovomucoid, fetuin, asiolofetuin, mucin, asialomucin, and thyroglobulin.
[b]IC_{50}: concentration required to give a 50% inhibition of the agglutination of trypsin-treated rabbit erythrocytes at a lectin concentration of 20 μg/ml.

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IDH RCGRE ATPPG KLCND GRCCS QWG: VisalbCBA
  E QCGRQ A  GG KLCPN NLCCS QWG: hevein
  Q RCGSQ G  GG GTCPA LWCCS IWG: nettle lectin Domain 1
  E QCGRQ A  GG ALCPG GNCCS QFG: bean chitinase
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Fig. 3. Comparison of the N-terminal amino acid sequences of VisalbCBA and some chitin-binding plant proteins composed of hevein domains. Cysteine residues (indicated in italics) were not identified positively in the sequencer due to the instability of the underivatized PTH-cys (undetectable in the HPLC chromatogram). However, dehydro-alanine, a typical β-elimination product of cysteine was detected in all relevant cycles. The sequence data for hevein, nettle lectin and bean chitinase were taken from Broekaert et al. [23], Beintema and Peumans [24] and Broglie et al. [25], respectively. On the bottom line asterisks indicate identical amino acids at that position of the sequence whereas dots represent similar amino acids.

Table 2
Comparison of the cytotoxicity of the different mistletoe lectins

Lectin	Concentration (ng/ml)				
	0	10	50	100	500
VisalbCBA	100 ± 0.2	101 ± 1.1	91.1 ± 2.6	82.5 ± 1.4	38.4 ± 3.8
MLI	100 ± 0.2	4 ± 1.8	0.5 ± 1.3	0	0
MLII	100 ± 0.2	10 ± 2.7	0.2 ± 0.9	0	0
MLIII	100 ± 0.2	24 ± 1.8	0.8 ± 1.2	0	0

Cell viability is expressed as % of the control (without lectin). Experiments were performed in triplicate.

also fairly heat-resistant. Over 50% of the agglutination activity was retained after boiling the lectin solution in PBS for 5 min.

3.5. *Amino acid sequencing of VisalbCBA*

N-terminal amino acid sequencing of affinity-purified VisalbCBA yielded a single sequence. A comparison of the N-terminus of VisalbCBA to those of some other chitin-binding plant proteins and lectins revealed a striking homology (Fig. 3). Although a few gaps have to be created to align the sequences of VisalbCBA and the N-termini of hevein, nettle lectin and bean chitinase, the conserved positions of especially the cysteine residues strongly suggest that VisalbCBA belongs to the family of chitin-binding plant lectins composed of hevein domains.

3.6. *Cytotoxicity of VisalbCBA*

The possible cytotoxic properties of VisalbCBA were assayed using Molt4 cells. As shown in Table 2, VisalbCBA definitely has a toxic effect on these cells at concentrations above 100 ng/ml. It is evident, however, that the toxicity of VisalbCBA is much lower than that of MLI, MLII and MLIII.

4. Discussion

The present paper describes the isolation and partial characterization of a novel lectin from mistletoe, which based on its molecular structure, biochemical properties and sugar-specificity studies belongs to the superfamily of chitin-binding plant proteins composed of so-called hevein domains [9]. It is evident, therefore, that VisalbCBA has nothing in common with the previously described *V. album* lectins MLI, MLII and MLIII, all three of which are genuine type 2 RIP. To the best of our knowledge, mistletoe is the first plant species in which the simultaneous occurrence of both a typical chitin-binding lectin and several type 2 RIP has been demonstrated. In addition, besides mistletoe there are only a few other plants in which two different types of lectins have been found. Several legume species are also known to contain two or more lectins. However, in all cases the different lectins clearly belong to the family of legume lectins [16]. Similarly, the different lectins found in elderberry (*Sambucus* sp.) species have all been identified as type 2 RIP or proteins derived thereof [17,18]. Detailed studies of the different lectins present in a number of monocot species such as garlic (*Allium sativum*), ramsons (*Allium ursinum*) and tulip (*Tulipa* sp.), have demonstrated that they are all closely related at the molecular level [19–21].

The discovery of a chitin-binding agglutinin in mistletoe raises some intriguing questions about the possible contribution of this previously unknown lectin to the presumed biological and therapeutic activity of mistletoe preparations. Since VisalbCBA is a very stable protein, one can reasonably expect that it survives the processing of the raw materials into medicinal preparations much better than the type 2 RIP. Therefore, the question must be addressed whether the presence of biologically active VisalbCBA contributes to or might be responsible for the observed activity of mistletoe preparations. It is worth mentioning in this context that the lectin from stinging nettle, which closely resembles VisalbCBA with respect to its molecular structure and specificity [22], is a potent superantigen in mice [10]. On the analogy of the effects of the latter lectin, it is quite possible that VisalbCBA influences the immune system. Besides a direct effect on the immune system, VisalbCBA may also indirectly influence the immune system through an interaction (either synergistic, additive or antagonistic) with the putative immunomodulatory MLI. At present one can only speculate about the possible involvement of VisalbCBA in the therapeutic effects of mistletoe preparations. However, it is of primary importance that the presence of this previously unknown chitin-binding lectin is considered whenever the biological activities of mistletoe preparations or proteins are questioned.

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