Glycoconjugate Journal (1997) **14:** 259–265

Isolation of a novel plant lectin with an unusual specificity from *Calystegia sepium*

Willy J. Peumans[1]*, Harry C. Winter[2], Veronique Bemer[3‡], Fred Van Leuven[4], Irwin J. Goldstein[2], Paolo Truffa-Bachi[3] and Els J.M. Van Damme[1]

[1] *Laboratory for Phytopathology and Plant Protection, Katholieke Universiteit Leuven, Willem de Croylaan 42, 3001 Leuven, Belgium*
[2] *Department of Biological Chemistry, The University of Michigan, 1301 Catherine Road, Ann Arbor, MI 48109-0624, USA*
[3] *Unité d'Immunophysiologie Moléculaire, CNRS LA 1961, Département d'Immunologie, Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France*
[4] *Centre for Human Genetics, Katholieke Universiteit Leuven, Herestraat 49, 3001 Leuven, Belgium*

A novel plant lectin has been isolated from the rhizomes of *Calystegia sepium* (hedge bindweed) and partially characterized. The lectin is a dimeric protein composed of two identical non-covalently linked subunits of 16 kDa. Hapten inhibition studies indicate that the novel lectin is best inhibited by maltose and mannose and hence exhibits a sugar binding specificity that differs in some respects from that of all previously isolated plant lectins. Mitogenicity tests have shown that the *Calystegia* lectin is a powerful T-cell mitogen. Affinity purification of human, plant and fungal glycoproteins on immobilized *C. sepium* lectin demonstrates that this novel lectin can be used for the isolation of glycoconjugates from various sources. Moreover, it can be expected that by virtue of its distinct specificity, the new lectin will become an important tool in glycobiology.

Keywords: ***Calystegia*, lectin, maltose, mannose**

Abbreviations: **Calsepa, lectin isolated from *Calystegia sepium;* ConA, concanavalin A; LPS, lipopolysaccharide; PBS, phosphate buffered saline (1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 3 mM KCl, 140 mM NaCl, pH 7.4)**

Introduction

Many plant species contain carbohydrate-binding proteins which are commonly referred to as lectins [1]. In the past, several hundred of these proteins have been purified and characterized in some detail with respect to their biochemical properties, sugar-binding specificity and biological activities. At first sight, plant lectins are a rather heterogeneous group of proteins which have little in common except their ability to recognize and bind specific carbohydrates. However, a closer examination of the available data reveals that the majority of all currently known lectins can be classified into four groups of structurally and evolutionary related proteins. These four groups are the legume lectins [2], the chitin-binding lectins composed of hevein domains [3], the so-called type 2 ribosome-inactivating proteins (RIP) [4] and the monocot mannose-binding lectins [5]. Whereas the first group is strictly confined to the legume family, the latter three groups comprise lectins from species belonging to several plant families. Evidently not all plant lectins can be classified into one of these four major groups. Molecular cloning of the lectins from *Amaranthus hypochondriacus* [6] and jack fruit (*Artocarpus integrifolia*) seeds [7], for instance, clearly indicated that they have no homology with any other lectin.

In this report we describe the isolation of a novel type of plant lectin from *Calystegia sepium.* It is shown that the carbohydrate binding specificity of this lectin differs in some respects from that of all previously described plant lectins.

Materials and methods

Plant material

Plant material of the hedge bindweed (*C. sepium* (L.) R.Br.) was collected in Leuven in December, and stored at −20 °C. For the isolation of the lectin whole rhizomes were used.

Isolation of the *C. sepium* agglutinin (Calsepa)

Rhizomes were homogenized in 10 volumes (v/w) of phosphate buffered saline (PBS, 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 3 mM KCl, 140 mM NaCl, pH 7.4). The homogenate was filtered through cheese cloth and

* To whom correspondence should be addressed.
‡ This paper is dedicated to the memory of Dr Véronique Bemer whose untimely death deprived us of an excellent colleague.

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centrifuged at 8000 g for 10 min. The supernatant (about 1 l starting from 100 g of rhizomes) was decanted, filtered through filter paper and applied onto a column (5 cm × 2.6 cm; 25 ml bed volume) of mannose-Sepharose 4B equilibrated with PBS. After passing the extract, the column was washed with PBS until the A_{280} fell below 0.01 and the lectin desorbed with PBS containing 0.1 M mannose. The affinity-purified lectin was dialysed against water and lyophilized. Virtually all the agglutinating activity was retained on the mannose-Sepharose 4B column. The total yield was about 0.2 mg lectin per gram rhizomes (on a fresh weight basis).

Agglutination assays

Agglutination assays were carried out either 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 in twofold increments), or in V-well microtitre plates in a final volume of 60 µl containing 30 µl of lectin solutions and 30 µl of a 3% suspension of red blood cells. Agglutination was assessed visually after 1 h at room temperature.

The carbohydrate-binding specificity of the lectin was determined by inhibition of agglutination of formaldehyde-stabilized trypsinized rabbit erythrocytes. Stock solutions of the inhibitors (glycoproteins, mono- and oligosaccharides, and methyl monoglycosides) were serially diluted in V-well microtitre plates, followed by addition of 0.25 µg lectin (final concentration, $3.8\ \mu g\ ml^{-1}$). After 1 h incubation, the erythrocyte suspension was added, and agglutination evaluated as above. All sugars tested are of the D-configuration.

Analytical methods

Lectin preparations were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5–25% (w/v) acrylamide gradient gels as described by Laemmli [8]. Total neutral sugar was determined by the phenol/H_2SO_4 method [9], with D-glucose as standard. Analytical gel filtration of the purified proteins was performed on a Pharmacia Superose 12 column using PBS containing 0.2 M mannose (to avoid possible binding to the column) as running buffer. Molecular mass reference markers were concanavalin A (ConA, 104 kDa), pea lectin (60 kDa), the snowdrop lectin (48 kDa) and garlic lectin (24 kDa).

Amino acid analysis

Samples of the *Calystegia* lectin were hydrolysed *in vacuo* in 6 N HCl at 110 °C for 20, 40 and 64 h. Amino acid analysis on hydrolysates was performed using an Beckman model 120C amino acid analyser [10] modified for single column operation. Values for serine and threonine were estimated by extrapolation to 0 time of hydrolysis.

Amino acid sequence analysis

Protein sequencing was conducted on an Applied Biosystems (Foster City, CA, USA) Model 477A protein sequencer interfaced with an Applied Biosystems model 120A on-line analyser.

Preparation of immobilized *C. sepium* agglutinin

Affinity-purified Calsepa was coupled to divinylsulphone activated Sepharose 4B. Briefly, 10 ml (packed matrix) of washed Sepharose 4B was suspended in 20 ml of 0.5 M carbonate-buffer (pH 11) containing 0.5 ml divinylsulphone and gently shaken for 2 h. The activated matrix was extensively washed with water (on a Büchner funnel), transferred into a small Erlenmeyer flask containing 20 mg Calsepa in 10 ml of 0.5 M carbonate-buffer (pH 9.0) and incubated overnight in a shaker at 37 °C. After coupling, the gel was extensively washed with PBS and gently shaken for 3 h in 0.1 M Tris-HCl (pH 8.5) to block the remaining activated groups. Then, the gel was washed again, resuspended in PBS containing 0.01% sodium azide and stored in the cold room until use.

Affinity chromatography on immobilized *C. sepium* agglutinin

Twenty ml of human serum was loaded on a column (1.6 cm × 5 cm; 10 ml bed volume) of Calsepa-Sepharose 4B equilibrated with PBS. After passing the serum, the column was washed with PBS until the A_{280} fell below 0.01 and the bound glycoproteins desorbed with PBS containing 0.1 M mannose. About 3 mg protein was retained per ml packed gel. After elution of the glycoproteins, the column was washed with 50 ml of a 0.5 M solution of NaCl in 50 mM Tris-HCl (pH 10) followed by 200 ml of PBS containing 0.01% sodium azide.

Plant and fungal glycoproteins were isolated similar to the human serum proteins. For the fungal proteins, 250 ml of the medium of a 8-day-old *Botrytis cinerea* culture (grown in Czapek-Dox medium) was lyophilized, redissolved in 25 ml of PBS and dialysed against PBS for 15 h. After dialysis, the solution was clarified by centrifugation (20 000 g for 20 min), filtered through paper (Whatmann 3MM) and applied onto the column of Calsepa-Sepharose 4B. For the isolation of plant glycoproteins an extract was prepared from garlic bulbs. Five grams of bulbs were homogenized with mortar and pestle in 50 ml of PBS. The homogenate was clarified by centrifugation (3000 × **g** for 10 min) and the supernatant dialysed against PBS for 15 h. After dialysis, the solution was clarified by centrifugation (20 000 × **g** for 20 min), filtered through paper (Whatmann 3MM) and applied onto the column of Calsepa-Sepharose 4B. Affinity chromatography of the fungal and plant glycoproteins on Calsepa-Sepharose 4B was essentially as described for the serum proteins.

Cyanogen bromide cleavage

Lyophilized Calsepa (2 mg) was dissolved in 0.1 ml of 70% formic acid. Ten mg of solid cyanogen bromide was added and the mixture incubated overnight at 37 °C (in the dark). Peptides were recovered by evaporation under vacuum, separated by SDS-PAGE and sequenced as described above.

Mitogenicity tests

Cell proliferation assays were carried out as described previously [11]. Briefly, 2×10^5 spleen cells or 2×10^5 enriched B-cells from 5–8–week-old BALB/c mice (IFFA-CREDO, L'Arbresle, France) were cultured in 100 µl of RPMI 1640 (Life Technologies, Inc., Grand Island NY) supplemented with 2 mM L-glutamine, 50 µg ml^{-1} streptomycin, 50 U ml^{-1} penicillin, 10% heat-inactivated foetal calf serum, 5×10^{-5} M 2-mercaptoethanol in a humidified atmosphere of 5% CO_2 in air. For the experiments 96-wells flat-bottom tissue culture cluster plates (Costar) were used. Various amounts of Calsepa were added at the onset of the culture. Concanavalin A (ConA) was used at 2 µg ml^{-1}; *Escherichia coli* O55 : B5 lipopolysaccharide (LPS), was used at 10 µg ml^{-1}. Cultures were pulsed with 0.25 µCi tritiated thymidine (^{3}H-Tdr, 2 Ci mM^{-1} = 74GBq mmol^{-1}, Amersham, UK) for the last 4 h of culture and the radioactivity incorporated was measured in a Betaplate (LKB) scintillation counter.

Depletion of T-lymphocytes was carried out by a single-step method as previously described [12]. Briefly, 4×10^7 cells ml^{-1} were incubated for 60 min at 37 °C in the presence of 1 : 10 dilution of anti-Thy 1.2, and a 1 : 2 dilution of anti-CD4 monoclonal antibody and Low-Tox-M rabbit complement (Cedarlane, Horny, Ontario, Canada).

Results

Isolation and characterization of the *C. sepium* agglutinin (Calsepa)

Since no universal classification system has been elaborated yet for plant lectins, they are usually referred to by three letter abbreviations. By analogy to other lectins the *C. sepium* agglutinin should be indicated by the abbreviation CSA. However, the latter abbreviation is already used for a lectin from *Cytisus sessilifolius.* To avoid further confusion, the novel *C. sepium* lectin will be referred to as Calsepa.

Calsepa was purified by affinity chromatography on immobilized mannose and analysed by SDS-PAGE and gel filtration. Unreduced as well as reduced (with 2-mercaptoethanol) Calsepa yielded a major polypeptide band of 16 kDa and a minor band of 17 kDa upon SDS-PAGE (Figure 1). The native lectin eluted as a single symmetrical peak with an apparent Mr of about 35 kDa upon gel filtration chromatography on a Superose 12 column (results not shown). It can be concluded, therefore, that Calsepa is a dimeric protein composed of two identical subunits of about 16 kDa.

As shown in Table 1 the amino acid composition of the purified lectin is typified by high concentrations of Asx, Glx, Gly, Thr, Ile, Ser and Val.

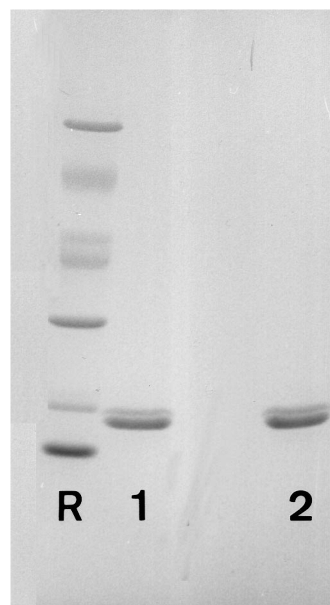

Figure 1. SDS-PAGE of the *C. sepium* lectin. Unreduced and reduced Calsepa (25 µg each) were run in lanes 1 and 2, respectively. Molecular mass reference proteins (lane R) were lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase b (94 kDa).

Table 1. Amino acid composition of the *C. sepium* lectin.

Amino acid	*Number of residues*[a]	*Mol (%)*
Ala	6	3.9
Arg	5	2.7
Asp/Asn	21	14.0
Cys	0	0
Glu/Gln	5	3.4
Gly	26	17.0
His	2	1.6
Ile	12	7.9
Leu	6	4.2
Lys	6	4.2
Met	2	1.1
Phe	7	4.6
Pro	7	4.6
Ser	12	7.8
Thr	15	9.6
Trp	ND[b]	ND
Tyr	7	4.2
Val	12	8.2

[a]The number of residues was the nearest integer calculated from the estimated molecular mass of 16 kDa.
[b]ND, not determined.

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

Calsepa is readily soluble in water (up to 25 mg ml^{-1}). A solution of 1 mg ml^{-1} gives an A_{280} of 1.25.

Agglutination properties and carbohydrate-binding specificity

The minimum agglutinating concentration of Calsepa was determined with untreated and trypsin-treated rabbit and human type A erythrocytes. The values observed for untreated and trypsin-treated rabbit red blood cells (0.3 and 0.6 μg ml^{-1}, respectively) indicate that the treatment of the cells has little effect. Trypsin-treated human erythrocytes agglutinated only at higher concentrations of Calsepa (5 μg ml^{-1}) whereas no clear agglutination was observed with untreated human red blood cells. We also observed that rabbit erythrocytes (trypsin-treated or untreated) stabilized with formaldehyde or glutaraldehyde [13, 14] required a somewhat higher concentration of lectin to be agglutinated (~ 2 μg ml^{-1}) than did fresh erythrocytes.

The carbohydrate-binding specificity of Calsepa was determined in some detail by hapten inhibition assays of the agglutination of rabbit erythrocytes. Because of the convenience of their stability, formaldehyde-stabilized erythrocytes were used in most of the studies. As shown in Table 2, the best inhibitor was methyl α-mannopyranoside, followed by methyl α-glucoside, which was about 1/3 as effective, as were several other α-glucosides (maltose, isomaltose, panose, palatinose) or α-glucosides modified at the 2-position (methyl 2-deoxy-α-arabinoglucopyranoside, and methyl 2-acetamido-2-deoxy α-glucopyranoside). In the case of the corresponding free monosaccharides (which were generally less inhibitory), glucose was at least 20-fold less reactive than mannose. This distinguishes Calsepa from the legume mannose/glucose binding lectins. None of the glycoproteins tested were strongly inhibitory, although all exhibited some reactivity, the best being asialothyroglobulin and ovomucoid. We also noted that when fresh rabbit cells were used, some quantitative differences were observed in the relative inhibitory potency of some sugars (data not shown). For example glucose was not inhibitory up to 200 mM, whereas mannose, fructose and maltose exhibited nearly the same inhibitory effect as observed with formaldehyde-stabilized cells.

Table 2. Specificity of the *C. sepium* lectin.

Sugar/glycoprotein	IC_{50}[a] *mM*	*Relative potency*	*μg ml^{-1}*
Mannose	4.2	1.0	
Glucose	77	0.05	
Galactose	> 200	< 0.02	
Talose	> 33	< 0.13	
Xylose	> 200	< 0.05	
Lyxose	> 200	< 0.02	
Fructose	25	0.17	
2-deoxy-Glucose	> 200	< 0.02	
ManNAc	> 200	< 0.02	
GlcNAc	42	0.1	
Maltose	2.1	2.0	
Isomaltose	2.1	2.0	
Isomaltitol	> 200	< 0.05	
Melezitose	30	0.14	
Palatinose	1.8	2.3	
Panose	1.7	2.3	
Sucrose	> 200	< 0.02	
Trehalose	> 200	< 0.02	
Turanose	> 200	< 0.02	
Me*α*Man	0.67	6.3	
Me*α*Glc	2.1	2	
Me*α*-d-Glc	1.8	2	
Me*α*Gal	> 200	< 0.05	
Me*α*GlcNAc	2.1	2	
Me*β*Man	> 200	< 0.05	
Me*β*Glc	> 200	< 0.05	
Me*α*Xyl*p*	> 200	< 0.05	
Fetuin			333
Asialofetuin			177
Thyroglobulin			167
Asialothyroglobulin			82.5
*α*1 AGP			418
Ovomucoid			100

[a] IC_{50}: concentration required to give a 50% inhibition of the agglutination of formaldehyde-stabilized trypsin-treated rabbit erythrocytes at a lectin concentration of 3.8 μg ml^{-1}.

Amino acid sequencing of Calsepa

N-terminal sequencing of intact Calsepa yielded no signal indicating that the protein is blocked. Cleavage of the protein with cyanogen bromide yielded a weak band of about 16 kDa (at almost the same position as the intact lectin polypeptide) and a broad band of polypeptides with an estimated Mr of about 7–8 kDa (data not shown). The 16 kDa polypeptide as well as the upper and lower part of the major polypeptide band were sequenced. In contrast to the fragment of 16 kDa, which yielded a single sequence, the upper and lower part of the broad band of 7–8 kDa fragments gave a double sequence. However, since the intensity of the signals of the two amino acids in each run differed sufficiently, the double sequence could easily be resolved (Figure 2).

A search in the database indicated that the sequences shown in Figure 2 show no homology to any other known protein.

Splenic lymphocytes response to Calsepa

Preliminary experiments were performed on BALB/c spleen cells using different doses of Calsepa and different times of

DTISG PWGNN GGNFW SFRPV: 17 kDa polypeptide

VNIGT DEYLT GISGT YLFGI: main sequence of the upper part of the 7-8 kDa band

DTISG PWGNN GGNFW SFRPV: second sequence of the upper part of the 7-8 kDa band

DTISG PWGNN GGNFW SFRPV: main sequence of the lower part of the 7-8 kDa band

VNIGT DEYLT GISGT YLFGI: second sequenceof the lower part of the 7-8 kDa band

Figure 2. N-terminal amino acid sequences of the cyanogen bromide cleavage fragments of the *C. sepium* lectin.

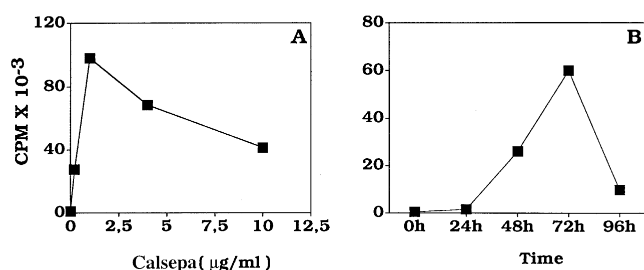

Figure 3. Dose-response titration of the mitogenic activity of Calsepa. (A) Spleen cells (2×10^6 ml^{-1}) were cultured for 72 h in complete RPMI supplemented with various amounts of Calsepa. Cells were pulsed with 0.25 µCi of (^{3}H)Tdr for the last 4 h. (B) Spleen cells (2×10^6 ml^{-1}) were cultured with 1 µg ml^{-1} of Calsepa for the indicated time, and pulsed as above. The results are presented as mean cpm (10^{-3}) of triplicate cultures; SD did not exceed 10% of the mean. The data are representative of three independent experiments.

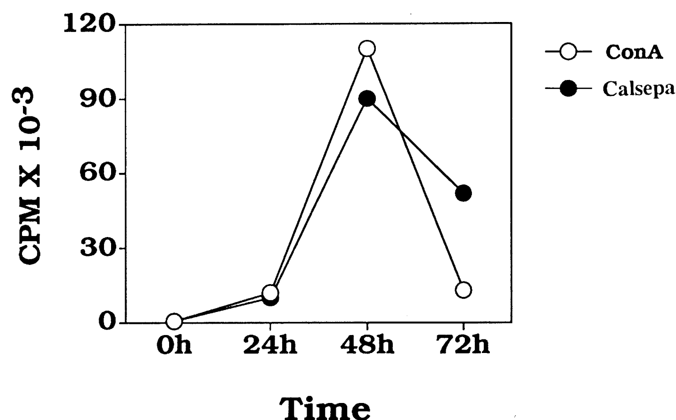

Figure 4. Comparison of the response of spleen cells to Calsepa and to ConA. Spleen cells (2×10^6 ml^{-1}) were cultured in complete RPMI supplemented either with ConA (2 µg ml^{-1}) or Calsepa (1 µg ml^{-1}). Thymidine incorporation was performed as described in Figure 3.

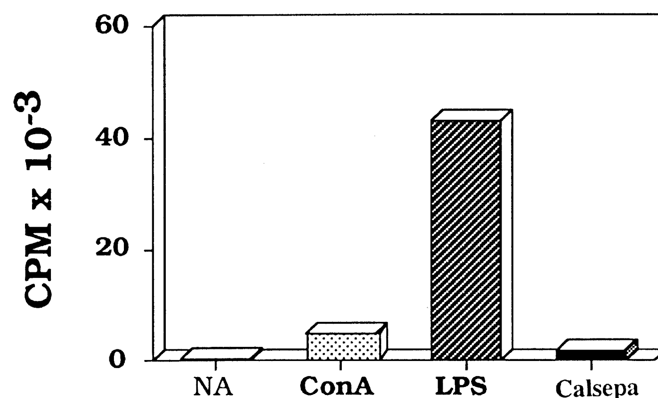

Figure 5. Calsepa stimulates only murine T-lymphocytes. Spleen cells, depleted of T-lymphocytes as described in Materials and Methods, were stimulated with ConA (2 µg ml^{-1}) or Calsepa (1 µg ml^{-1}) or LPS (10 µg ml^{-1}). Thymidine incorporation was measured as described in the legend of Figure 3.

thymidine pulse as previously reported [15]. A thymidine uptake was recorded after 48–72 h of stimulation with Calsepa doses ranging from 0.5 to 10 µg ml^{-1}. The optimal conditions for spleen cell stimulation, *ie* dose-response and kinetics, were then determined. As shown in Figure 3 the concentration of Calsepa giving the optimal thymidine uptake was of 1 µg ml^{-1}, and the peak of the response was observed at days 2–3 (Figures 3B, 4). Figure 4 shows a comparison of the kinetics of the responses to Calsepa and to ConA. The proliferation kinetics and the magnitude of the response caused by the two lectins were similar.

In order to determine the lymphoid subset responsible for the thymidine incorporation, T-cell depletion was performed prior to activation. Stimulation was carried out with the optimal dose of Calsepa (1 µg ml^{-1}) and thymidine incorporation was measured at 48 h. As shown in Figure 5, the T-cell depleted population responded only to LPS, a B-cell mitogen, demonstrating that Calsepa is a T-cell mitogen.

Affinity chromatography of glycoproteins on immobilized Calsepa

To evaluate the potential use of Calsepa as a tool for the isolation of glycoconjugates some experiments were performed to check the binding of glycoproteins from different

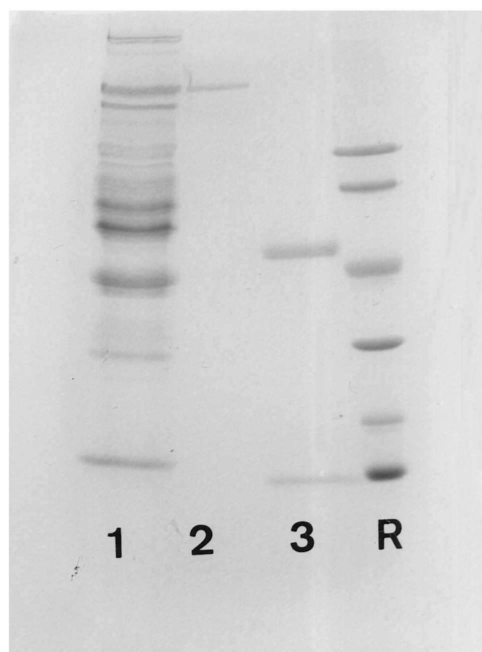

Figure 6. SDS-PAGE of glycoproteins isolated on immobilized *C. sepium* lectin. Affinity-purified glycoproteins from human serum, *B. cinerea* culture medium and an extract from garlic bulbs were loaded in lanes 1, 2 and 3, respectively. Molecular mass reference proteins (lane R) were the same as in Figure 1.

sources on the immobilized lectin. Human serum, a crude extract from garlic bulbs and a mixture of the extracellular proteins from a liquid culture of *B. cinerea* were applied onto a column of Calsepa-Sepharose 4B, and the bound proteins analysed by SDS-PAGE. As shown in Figure 6 many serum proteins were specifically retained by the immobilized lectin. On the contrary, in the case of the culture filtrate of *B. cinerea* and the garlic bulb extract only one or two proteins were recognized by Calsepa. Although these results do not give any information about the proteins, which are bound on the immobilized lectin, they clearly demonstrate that Calsepa can be used for the isolation of human as well as fungal and plant glycoproteins.

Discussion

The present paper describes the isolation and partial characterization of a novel lectin from *C. sepium*, a typical representative of the plant family Convolvulaceae (bindweed family). To our knowledge, this is the first lectin from this plant family that has been characterized. The known sequence of Calsepa, which comprises about one quarter of the total sequence, has no apparent sequence homology to any other lectin or to other plant proteins, suggesting a new type of plant lectin. Moreover, Calsepa also exhibits somewhat unusual carbohydrate-binding properties that conform neither to the glucose/mannose binding lectins (concanavalin A, pea, lentil, *Vicia faba* lectins) nor to monocot mannose-binding lectins which bind solely to mannose-containing glycans (snowdrop, daffodil, amaryllis lectins) [16, 17]. At first sight, there is some resemblance with the legume glucose/mannose lectins, which are also inhibited by mannose and maltose. However, unlike concanavalin A, the prototype Glc/Man-binding lectin which shows a 4–5-fold preference for mannose over glucose, Calsepa binds mannose more than ten-fold more avidly than glucose. Fructose and *N*-acetylglucosamine and sucrose, which are potent inhibitors of the legume glucose/mannose lectins [2] are either weakly or non-inhibitory to Calsepa. Calsepa also differs with respect to its specificity from the monocot mannose-binding lectins, which exhibit an exclusive specificity towards mannose and are not inhibited by maltose or methyl α-glucopyranoside [16]. It appears that this lectin reacts with α-glucosides of mannose or glucose, but is quite sensitive to the anomeric configuration and to the nature of the aglycone or reducing sugar. For example, methyl-α-glucopyranoside, maltose, isomaltose, etc. are good inhibitors, but isomaltitol, sucrose, trehalose, etc. are not inhibitory, even though they are also α-D-glucosides of similar size and functionality. It can be concluded, therefore, that Calsepa exhibits a distinct sugar-binding specificity that differs in some respects from that of all previously-isolated plant lectins.

Some plant lectins mimic the effect of the physiological interaction of antigen with the T-cell receptor and activate all T-lymphocytes. These polyclonal activators, called mitogens, have been important tools in the analysis of lymphocyte activation [for review, see 18, 19]. The data reported here show that Calsepa is a powerful T-cell mitogen, giving similar proliferation to ConA, the prototypic T-cell mitogen in the mouse. Whether these two mitogens bind to the same or to different proteins of the TCR/CD3 complex is under investigation.

The distinct sugar specificity of the *Calystegia* lectin offers interesting perspectives for the isolation and characterization of glycoconjugates from different sources. In addition, Calsepa can be used as an alternative to ConA for the mitogenic stimulation of lymphocytes. Thereby the high solubility of the lyophilized lectin in water or aqeous buffers – even in the absence of the complementary sugar – is certainly an important advantage.

Acknowledgements

This work was supported in part by grants from the Catholic University of Leuven (OT/94/17) and the National Fund for Scientific Research (Belgium, Fonds voor Geneeskundig Wetenschappelijk Onderzoek grant 2.0046.93). W.P. is Research Director and E.V.D. is Postdoctoral Fellow of this fund. Furthermore we want to acknowledge grant 7.0047.90 from the National Fonds voor Wetenschappelijk Onderzoek-Levenslijn fund.

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Received 15 May 1996, revised 1 August 1996, accepted 6 August 1996