Chemical and physicochemical characterization of the sialic acid-specific lectin from *Cepaea hortensis*

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Abstract

A sialic acid-specific lectin was isolated from the albumin glands of the garden snail *Cepaea hortensis* by affinity chromatography on fetuin-Sepharose following gel filtration on Superdex 200. The purified native lectin showed a molecular mass of about 95 kDa by gel filtration and 100 kDa by SDS electrophoresis. It was cleaved by boiling in buffer containing SDS in three serologically identical bands corresponding to molecular masses of about 24, 20 and 16 kDa, respectively. From these three fragments, only the 24- and the 20-kDa bands were found to be glycosylated. Only the three sugars mannose, galactose and N-acetylglucosamine could be detected in a molar ratio of 3:8.6:2. The oligosaccharide moieties seem to be N- and partially O-glycosidic bound. Isoelectric focusing (IEF) of the purified lectin revealed a heterogeneous pattern with bands in the pH range of 4.3–5.0. Isolated bands of different isoelectric points showed in SDS electrophoresis the same three fragments with molecular masses of 24, 20 or 16 kDa. The heterogeneity of the lectin was revealed either by IEF or amino acid sequencing of internal tryptic peptides. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Lectin; Sialic acid; Snail; *Cepaea hortensis*; *Streptococcus agalactiae*

1. Introduction

Lectins were originally defined as agglutinins which could discriminate among types of red blood cells. They have been found in plants, viruses, micro-organisms and animals, but despite their ubiquity, their function in nature is unclear. They represent a heterogeneous group of oligomeric proteins that vary widely in size, structure, molecular organization, as well as in the constitution of their combining sites. Nonetheless, many of them belong to distinct protein families with similar sequences and structural features. Prokop et al. [13–15] found in the albumin glands of snails high concentrations of lectins specifically reacting with human blood cells. It has been assumed that these agglutinins play some role in the defence mechanism against bacterial infections and consequently, these lectins were called first protectines [15]. In the albumin gland of the garden snail *Cepaea hortensis* two lectins with different binding specificities were identified, a sialic acid-binding agglutinin (CHA-I) and an N-acetyl-D-galactosamine-specific lectin (CHA-II) [20]. CHA-I reacts specifically with all encapsulated group B streptococci (*Streptococcus agalactiae*) [11,22] and promotes also the phagocytosis of these bacteria (lectinophagocytosis) [6]. The specificity of this lectin has been reported to the submolecular level [2]. Binding of the sialic acid to CHA-I requires an intact N-acetyl group, the trihydroxypropyl side chain and an axially orientated carboxyl group. In contrast to some other sialic acid-specific lectins, CHA-I does not distinguish between the α(2-3)- and α(2-6)-glycosidic linkage of sialic acid. Histochemical investigations on formalin-fixed human tissues confirmed the high specificity of the lectin for O-glycosidically linked sialic acids [5]. Up to now only preliminary studies on its biochemical nature have been reported [21]. The aim of the present paper was to
give a more comprehensive chemical and physicochemical characterization of this lectin.

2. Materials and methods

2.1. Extraction and purification of the lectins

The crude lectins were isolated as described previously for the sialic acid-binding lectin [2], with some slight modifications. In short, 2.9 g of isolated frozen albumin glands from the snail C. hortensis were ground and extracted two times with buffer A (0.15 M NaCl, 0.025 M CaCl₂, 0.05 M Tris–HCl, pH 7.5) for each 30 min. The cleared extract was loaded directly on a fetuin-Sepharose column (2 cm × 5 cm). After washing with buffer A (the N-acetyl-D-galactosamine-specific lectin was not retained) the CHA-I was eluted with 0.1 M NaCl, 0.05 M Tris–HCl buffer (pH 7.5) containing 0.04 M Na₂EDTA. A final purification was done by gel chromatography on a Superdex 200 column (16/60, Amersham Pharmacia Biotech).

2.2. Protein determination

The bicinchoninic acid protein assay (Sigma Chemicals Co.) with bovine serum albumin as standard was used according to the instructions of the manufacturer.

2.3. Hemagglutination assay

For determining the hemagglutination activity serial dilutions of 100 µl lectin were prepared on microtiter plates using Tris–HCl buffer (0.10 M NaCl, pH 7.5) containing 0.01 M CaCl₂. To each well 50 µl 5% sheep red blood cell suspension in the same buffer was added and the titer was read after 30 min. Alternatively, 25 µl of the lectin dilution was mixed with the same volume red cell suspension on a white foil. Hemagglutination titers were calculated from the highest dilution giving complete agglutination.

2.4. Production of antiserum

Purified sialic acid-specific lectin (CHA-I) as well as the fragments with molecular masses of 20 and 16 kDa were suspended in Freund’s adjuvant to raise antibodies in rabbits. The both fragments were prepared after SDS-PAGE and cut out from the stained gel.

2.5. SDS–PAGE and Western blotting

The purity of samples was monitored by SDS-PAGE on 12.5% running gels [9]. After blotting to nitrocellulose membrane and blocking the blots were incubated with specific antisera raised against either the whole lectin or its fragments and developed with peroxidase-labeled pig anti-rabbit IgG (Dako, Denmark) and detected after reaction with 3,3′-diaminobenzidine tetrahydrochloride and H₂O₂. For detection of glycosylated bands the periodic acid Schiff’s reagent (PAS) staining was performed according to Carlsson [3].

2.6. Isoelectric focusing (IEF)

The purified lectin was applied to Ampholine–PAGE plates, pH 3.5–9.5 (Amersham Pharmacia Biotech) and run according to the instructions of the manufacturer. For measuring the lectin activity or the pH values gel portions were cut out and used for elution.

2.7. Determination of amino acid sequence

N-terminal amino acid sequencing was performed with lectin fragments blotted on PVDF membranes. Internal sequences of the lectin were obtained after cleavage with sequencing grade trypsin (Promega, Madison, WI, USA) following separation of tryptic peptides on an RP18 (Sephasil® C18 SC 2.1/10; Amersham Biosciences, Freiburg, Germany) column. Micro-sequence analysis was done by using an amino acid sequencer model 494A (Applied Biosystems, Foster City, CA, USA).

2.8. Carbohydrate analysis

For analysis gas liquid chromatography (GC) using a Varian 3700 GC or a Hewlett-Packard (HP 5890 series II) GC on a capillary column (fused-silica SPB-5, 30 m, Supelco) was used. Combined GC/mass spectrometry was performed using a mass spectrometer (HP model 5989) equipped with a HP-1 capillary column (30 m). The neutral sugars were determined as alditol acetates.

2.9. Chemical and enzymatic deglycosylation

0.5 mg lectin was treated with 100 µl trifluoromethane-sulfonic acid at 0°C for 2 and 72 h, respectively, according to Sojar and Bahl [18]. Subsequently the reaction mixture was neutralized by addition of 260 µl of 60% (w/v) pyridine, dialyzed against 0.02 M Tris–HCl buffer pH 8.0 and analyzed by SDS–PAGE. For testing of O-glycosidically bound carbohydrates the chemical release of O-glycans by β-elimination was performed on Western blots of the lectin on PVDF membranes according to Duk et al. [4]. Enzymatic deglycosylation was done by use of N-glycosidase A and N-glycosidase F according to the instructions of the manufacturer (Roche, Mannheim, Germany).

3. Results

3.1. Purification of the lectins

The effect of the purification steps of both lectins is
demonstrated in Table 1. The crude and cleared extract from the albumin glands was applied directly on a fetuin-Sepharose column for the removal of the N-acetyl-D-galactosamine-specific lectin. The CHA-I lectin could be specifically eluted from this column by addition of Na₂EDTA to the neutral washing buffer. The final purification by chromatography on Superdex 200 removed the last impurities of a N-acetylgalactosamine-specific lectin which is strongly bound to this gel [8]. The CHA-I lectin eluted from the Superdex 200 column at a position corresponding to a molecular mass of about 95 kDa (Fig. 1). The absorbance spectrum of the purified lectin was found typically for proteins having a specific absorbance at 1 mg ml⁻¹ of 1.0 at pH 7.6 (λ_max 277.5 nm, λ_min 251 nm, E_{λ_max}/E_{λ_min} = 1.87). The purified lectin agglutinated specifically only group B streptococci and not group C streptococci as described by Wagner [11,20,22]. Inhibition of the hemagglutination was only possible by N-acetylneuraminic acid but not by D-galactose, D-lactose, N-acetyl-D-mannosamine, N-L-acetyl-D-glucosamine, D-mannosamine, D-galacturonic acid, dextran sulfate, D-sorbitol or N-acetyl-D-galactosamine at 2 mg ml⁻¹.

### 3.2. Analysis of the purified lectin (CHA-I)

In the gel filtration on Superdex 200 the CHA-I lectin

<table>
<thead>
<tr>
<th>Amounts Puriﬁcation step</th>
<th>Abs. 280 nm</th>
<th>Abs. total</th>
<th>HA titer</th>
<th>Agg. str. group B</th>
<th>Agg. str. group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.59 g albumin glands</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62.5 ml crude extract</td>
<td>68</td>
<td>425</td>
<td>≥4096</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>120 ml unretained fractions fetuin-Sepharose column</td>
<td>3.78</td>
<td>454</td>
<td>≥4096</td>
<td>☐</td>
<td>++++</td>
</tr>
<tr>
<td>35 ml eluted fractions from fetuin-Sepharose column</td>
<td>517</td>
<td>181</td>
<td>1:128</td>
<td>++++</td>
<td>☐</td>
</tr>
</tbody>
</table>

Table 1
Puriﬁcation protocol for the sialic acid-speciﬁc lectin (CHA-I) from the garden snail *C. hortensis*

Ammonium sulfate precipitation (0.7 g ml⁻¹) and gel ﬁltration on Superdex 200 (1/2 of lectin) 17 ml V_e (66-81 ml) 0.476 0.5 mg ml⁻¹ HA 1:128

The high agglutination titers of the crude extract resulted from the much higher speciﬁc agglutination of the N-acetylgalactosamine-binding lectin (CHA-II). Abs, absorbance at 280 nm; HA, hemagglutination titer; Agg. str., ability to agglutinate group B or group C streptococci; V_e, elution volume.

Fig. 1. Analysis of puriﬁed sialic acid-speciﬁc lectin (CHA-I) from the snail *C. hortensis* by gel ﬁltration on a Superdex 200 column. The lectin eluted as a homogeneous protein peak at a position corresponding to an apparent molecular mass of 95 kDa. Inserted pictures: left, SDS-PAGE separation: CHA-Ir, reduced and boiled lectin; MW, molecular mass standard proteins; CHA-I, non-boiled native lectin. Right: CHA-Id, lectin after chemical deglycosylation with trifluoromethanesulfonic acid; CHA-Ir, reduced boiled lectin before deglycosylation; MW, molecular mass standard proteins (97, 66, 39, 27, 21, and 14.4 kDa).
eluted as a homogeneous protein peak corresponding to a molecular mass of about 95 kDa. The SDS–PAGE of the non-boiled lectin resulted in one band corresponding to a molecular mass of about 100 kDa (Fig. 1). After boiling with or without β-mercaptoethanol in SDS containing buffers, the lectin was split into three fragments with molecular masses of about 24, 20 and 16 kDa, respectively, with a quantitative relation of about 1:1:0.3 (Table 3). Western blotting of the lectin on nitrocellulose membrane and development using polyclonal antisera produced against the whole lectin or the fragments of 20 and 16 kDa resulted in reactions to all three bands, demonstrating their serological identity (Fig. 2). By treatment of the SDS–PAGE with PAS only the two larger fragments (24 and 20 kDa) but not the 16-kDa band were stained. This indicates that only the both higher molecular mass fragments (molecular masses of 20 or 24 kDa) are glycosylated (Fig. 2). As suggested from the different staining intensity by PAS, the fragments may differ in the content of oligosaccharide moiety, i.e. the 24-kDa fragment seems to have the double amount of oligosaccharide residues per molecule in contrast to the 20-kDa fragment. Accordingly, the deglycosylation with trifluoromethanesulfonic acid resulted in only one band at a molecular mass of 16 kDa and some low molecular mass smear (Fig. 1). By IEF on polyacrylamide gel the lectin showed about four to six bands with isoelectric points between pH 4.3 and 5.0. All bands of the IEF with different isoelectric points showed, if separately analyzed by SDS–PAGE, the same pattern of the three bands with molecular masses of 24, 20 and 16 kDa (Fig. 3). Attempts to get the N-terminal sequence of these fragments after SDS and blotting on PVDF membranes were unsuccessful. Only internal sequences could be obtained after cleavage with trypsin and high-performance liquid chromatography (HPLC) separation on an RP18 column (Fig. 4).

MALDI-TOF analysis (matrix-associated laser desorption ionization spectrometry-time of flight analysis) of the lectin determined the exact mass of the ‘16-kDa fragment’ to 15,504.69 Da and that of the ‘20-kDa fragment’ to 18,899.31 Da (Fig. 3). The highly glycosylated ‘24-kDa fragment’ gave no signal, which is a known phenomenon of high glycosylated structures. The difference of the molecular mass of the two fragments amounts 3394 Da, which could correspond to the calculated molecular mass of the oligosaccharide moiety.
The sugar analysis of the lectin revealed only the presence of mannose, galactose and N-acetylglucosamine. No fucose, sialic acid or uronic acids could be detected. A relation of 3:8.6:2 of mannose:galactose:N-acetylgalactosamine was calculated (Table 2). Reductive cleaving with NaBH₄ did not result in a significant release of O-glycosidically linked sugars. Also an incubation for 1 h at 80°C with 0.025 M H₂SO₄ following incubation for 16 h with 0.055 M NaOH according to Duk et al. [4] did not significantly change the PAS staining pattern of the blots (Fig. 2). Therefore we assume that most of oligosaccharides are N-glycosidically linked to asparagine although no quantitative deglycosylation could be observed in SDS-PAGE after treatment with N-glycosidases A or F. Analysis of lectin bands on the stained gels resulted in a reduction of the 24- and the 20-kDa fragments after N-glycosidase A treatment for 24 h only to 74% and 47%, respectively (Table 3).

Amino-terminal sequence analysis of tryptic peptide numbers 33 and 40 revealed blanks on the first four sequencing cycles (Fig. 4). Because peptides 25 and 26 showed on these positions DKSS or SS we suggest O-glycosylation there and that could be responsible for the blanks in sequencing.

4. Discussion

The results of Table 1 show that the albumin gland of the C. hortensis contains two lectins with different specificities and different specific activities to agglutinate sheep erythrocytes. The sialic acid-specific lectin (CHA-I) binds to the glycoprotein molecule on the red cells while the N-acetyl-D-galactosamine-specific lectin (CHA-II) is directed to the blood group A substance. Essential for the binding of CHA-I to sialic acid residues is the intact N-acetyl group, the presence of a trihydroxypropyl side chain, and an axial-oriented carboxyl group [2].

The molecular mass of the native lectin was detected to be about 100 kDa by gel filtration or SDS electrophoresis. It could be cleaved by heating in SDS containing buffers in three subunits having molecular masses of about 24, 20 and 16 kDa. All of these subunits were found to be serologically identical. The molecular mass differences between the subunits resulted from an individual's different glycosylation grade. In contrast to the glycosylated 24- and the 20-kDa subunits the 16-kDa fragment did not contain sugars. After chemical deglycosylation with trifluoromethanesulfonic acid only the 16-kDa fragment was observed by SDS electrophoresis. Deglycosylation by β-elimination...
on Immobilon membranes was unsuccessful, therefore we assume a N-glycosidic binding of the most part of the bound polysaccharide. MALDI-TOF analysis revealed fragments at molecular masses of 15 504.69 and 18 899.31 Da. The higher molecular fragment of 24 kDa could not be detected here. The difference between these fragments of 3394 Da seems to be caused by a different glycosylation grade. From the analysis of the sugar components a possible structure of the polysaccharide could be:

\[
[(\text{Gal})_6 - \text{GlcN} \rightarrow \text{Man}]_2 \rightarrow \text{Man} - \text{GlcN} \rightarrow \text{GlcN} \rightarrow \text{GlcN} \rightarrow \text{Asn}
\]

The native lectin resulted in three to six stronger bands by IEF in polyacrylamide gel with isoelectric points between 4.3 and 5.0. SDS electrophoresis of four isolated bands from the IEF gel revealed identical patterns showing the same three subunits of molecular masses 24, 19 and 16 kDa. N-terminal sequencing of the pI 4.6 band revealed about three or more residues at each step. A 16-kDa subunit isolated after SDS electrophoresis and blotting on PVDF membrane produced by N-terminal sequencing only weak and diffuse signals [GAW][AL][IP][PFK][NTH]-[NA][ID][YLV][NAL][QY]GHND. Internal amino acid

![HPLC separation of tryptic cleavage of the CHA-I lectin](image)

**Table 2**
Quantitative analysis of the sugar components from the sialic acid-specific lectin CHA-I from the garden snail *Cepaea hortensis*

<table>
<thead>
<tr>
<th>Components</th>
<th>First analysis</th>
<th>Second analysis</th>
<th>Mean value</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol mg⁻¹</td>
<td>wt%</td>
<td>nmol mg⁻¹</td>
<td>wt%</td>
</tr>
<tr>
<td>Mannose</td>
<td>48.6</td>
<td>0.9</td>
<td>49.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Galactose</td>
<td>131.7</td>
<td>2.4</td>
<td>141.7</td>
<td>2.5</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>28.2</td>
<td>0.6</td>
<td>35.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Total weight</td>
<td>1.3</td>
<td></td>
<td>4.2</td>
<td></td>
</tr>
</tbody>
</table>

No sialic acid or uronic acids could be detected by mild methanolysis of the lectin followed by peracetylation as well as strong methanolysis. Hydrolysis with 2 M TFA (100°C, 4 h) followed by reduction with NaBH₄ and peracetylation detected only mannose, galactose and N-acetylgalactosamine.
sequences were obtained after tryptic fragmentation of the purified lectin. Interestingly, we found there peptides on different positions of HPLC separation (no. 25, 26, 33 and 40) with very similar N-terminal sequences. The first four amino acid residues on peptides 33 and 40 could not be verified. The reason for this result could be the presence of glycosylated amino acid residues. Such residues cause zones indistinguishable from the background and will be appearing as blanks at the appropriate cycles. With peptides no. 25 and 26 we found on these first positions the sequences DSSK or SS, so we assume that O-glycidic residues on the serine could be responsible for the blanks on the first cycles on peptides 33 and 40. Furthermore, the eighth and 13th amino acid residues on peptide 33 were different to peptides 25, 26 and 40. These differences in the amino acid sequences and variable glycosylation site occupancy could be responsible for the observed heterogeneity in IEF. Micro-heterogeneity could be the result by multiple N- and O-glycosylation along a polypeptide and a widespread among lectins [7]. Furthermore, some lectins are expressed as an inactive precursor and were converted to the mature form through a complex of steps of deglycosylation, proteolytic cleavage and transpeptidation or ligation resulting in N-terminal amino acid heterogeneity of the mature lectin [17].

From the quantitative relation of the three subunits of about 1:1:0.5 and an estimated molecular mass of about 100 kDa for the native C. hortensis sialic acid-specific lectin we prefer a oligomeric structure like (AB)2C, whereby A and B correspond to the 20- and the 16-kDa fragments and C to the 24-kDa subunit. Their combination results in a molecular mass of 96 kDa in good correspondence to the value estimated for the native lectin by gel filtration or SDS electrophoresis. A same similar structure (AB)2 was verified. The reason for this result could be the presence of glycosylated amino acid residues on peptides 33 and 40 could not be verified. The reason for this result could be the presence of glycosylated amino acid residues. Such residues cause zones indistinguishable from the background and will be appearing as blanks at the appropriate cycles. With peptides no. 25 and 26 we found on these first positions the sequences DSSK or SS, so we assume that O-glycidic residues on the serine could be responsible for the blanks on the first cycles on peptides 33 and 40. Furthermore, the eighth and 13th amino acid residues on peptide 33 were different to peptides 25, 26 and 40. These differences in the amino acid sequences and variable glycosylation site occupancy could be responsible for the observed heterogeneity in IEF. Micro-heterogeneity could be the result by multiple N- and O-glycosylation along a polypeptide and a widespread among lectins [7]. Furthermore, some lectins are expressed as an inactive precursor and were converted to the mature form through a complex of steps of deglycosylation, proteolytic cleavage and transpeptidation or ligation resulting in N-terminal amino acid heterogeneity of the mature lectin [17].

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Furthermore, we could not find homologues to known sialic acid-specific lectins on the basis of amino acid sequences or biochemical properties. Sialic acid-specific lectins are mostly characterized as oligomeric glycoproteins requiring calcium, manganese or magnesium ions. For example, the lectin from the African giant snail Achatina fulica (achatinin H) consists of 16 identical subunits of 15 kDa, whereas the molecular mass of the native lectin was found to be 242 kDa. Its carbohydrate moiety was quite different form the CHA-I lectin consisting of xylose and fucose [1,16]. The active form of sialic acid-specific lectin of the freshwater prawn Macrobrachium rosenbergii was a dimeric glycoprotein with 9.5 kDa per subunit [23], or the lectin of the Southeast Asian horseshoe crab Tachypleus gigas which had a molecular mass of 396 kDa and was composed of 13 identical subunits with a molecular mass of 31 kDa [19].

Acknowledgements

We would like to thank Mrs. Anita Willitzer from the Institute of Molecular Biotechnology Jena for N-terminal sequencing of peptide probes.

References


Table 3
Quantitative analysis of the Coomassie blue-stained bands after SDS electrophoresis of native lectin or lectin treated by N-glycosidase A

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Native lectin CHAI</th>
<th>CHAI after N-glycosidase A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abs. 592 nm %</td>
<td>Abs. 592 nm %</td>
</tr>
<tr>
<td>16 kDa</td>
<td>0.511 100</td>
<td>0.196 100</td>
</tr>
<tr>
<td>20 kDa</td>
<td>0.566 111</td>
<td>0.102 52</td>
</tr>
<tr>
<td>24 kDa</td>
<td>0.158 31</td>
<td>0.045 23</td>
</tr>
</tbody>
</table>

Bands were cut out, eluted by 0.2 ml 1% SDS solution and the absorbances at 592 nm were measured.


