Purification and characterization of a hemagglutinin isolated from the leaves of Chenopodium (Chenopodium amaranticolor)

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A hemagglutinin was isolated and purified from the leaves of Chenopodium (Chenopodium amaranticolor) using ion-exchange chromatography and affinity chromatography on fetuin-agarose matrix. It agglutinated rabbit erythrocytes. The hemagglutinin had a native molecular mass of 58 kDa, as estimated by gel filtration and showed a single band of molecular mass of 33 kDa on SDS-PAGE. It showed hemagglutination activity over the pH range 3-12 and was found to be stable up to 70°C. On isoelectric focussing, the pI of this hemagglutinin was estimated to be 5.25. However, it was found to contain seven charge variants when isoelectric focussing was performed in presence of 6M urea.

Plant lectins are a unique heterogeneous group of proteins or glycoproteins classified on the basis of their ability to recognize and specifically bind carbohydrate ligands. Besides lectins with specific sugar binding properties, there are many proteins which possess a unique property of agglutinating erythrocytes and other cell types. Widely distributed in nature, they are present in seeds, roots, leaves and stems of most plants. Plant agglutinins are commonly known as phytohemagglutinins, phytoagglutinins or hemagglutinins. These proteins usually have non-specific carbohydrate binding specificities.

While investigating the nature of pathogenesis-related (PR) proteins in Chenopodium (Chenopodium amaranticolor) in response to tobacco mosaic virus (TMV) infection, we detected the presence of hemagglutination activity in the leaf extract as well as in the intercellular fluid in both virus-free and virus-infected leaves. In the present paper, we report the isolation and partial characterization of Chenopodium leaf hemagglutinin (CLH).

Materials and Methods

Leaves were collected from 45-60 day old Chenopodium (Chenopodium amaranticolor) plants and used immediately or stored at -20°C.

All the chemicals and reagents used were of analytical grade. Fine chemicals and molecular weight markers for gel filtration were procured from Sigma Chemical Company, St Louis, Mo, USA. DEAE-Sephacel and molecular weight markers for gel electrophoresis were obtained from Pharmacia AB, Uppsala, Sweden.

Extraction and purification of Chenopodium leaf hemagglutinin (CLH)

Chenopodium leaves (100 g) were cut into small pieces and were homogenised in 1 litre of 20 mM Tris-HCl buffer, pH 8.0 containing 2% insoluble PVP in a Sorvall Omnimixer. The slurry was stirred for 2 hr at room temperature and allowed to stand overnight at 6°C. It was then centrifuged at 17,000 g for 20 min.

The protein in the supernatant was precipitated by 70% saturation with (NH₄)₂SO₄. This was again centrifuged at 17,000 g for 20 min. The precipitate was dissolved in and dialysed against 20 mM Tris-HCl, pH 8.0.

Ion-exchange chromatography

The dialysed sample of 70% (NH₄)₂SO₄-saturated fraction was purified using pre-equilibrated DEAE-Sephacel chromatography column of 20 ml capacity. The protein was loaded on the column and the column was washed with 20 mM Tris-HCl, pH 8.0 containing 100 mM NaCl until the absorbance of the eluate at 280 nm became zero. The bound protein was eluted by a linear gradient of 100-500 mM NaCl prepared in the equilibration buffer at a flow rate of 12 ml/hr. Fractions (3 ml) containing the agglutination activity were pooled. The protein was precipitated with 70%
(NH₄)₂SO₄ saturation. The precipitated protein was dissolved in 20 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl and was dialysed against the same buffer.

**Affinity chromatography**

The dialysed sample after ion-exchange chromatography was further purified on Fetuin-agarose column. The regenerated Fetuin-agarose column was equilibrated with 20 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl. The sample was loaded on the column and the column was washed with the equilibration buffer until the absorbance of the washings at 280 nm was zero. The bound protein was desorbed with 6 M urea prepared in equilibration buffer. The eluted protein was precipitated with 70% saturation of (NH₄)₂SO₄ at 0°C. The precipitate was dissolved in 20 mM Tris-HCl, pH 8.0 and dialysed against the same buffer.

**Gel filtration chromatography**

The native molecular weight of CLH was determined by gel filtration on FPLC (Pharmacia) using Superose-12 column, pre-equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl. The protein was loaded and elution was done with the equilibration buffer. The flow rate was adjusted to 0.5 ml/hr.

**Hemagglutination assay**

Hemagglutination assay was carried out in a microtitre plate following 2-fold serial dilution method. Fifty microlitre of protein solution was incubated with 50 µl of 2% suspension of trypsin-treated rabbit erythrocytes for 1 hr at room temperature. The agglutination was observed under the microscope and titre was calculated from the last dilution which showed detectable agglutination. Specific hemagglutination activity was determined from the agglutination activity units (AU) per mg of protein.

**Protein estimation**

Protein was estimated by biuret method⁹, using lipid-free BSA as the standard protein.

**Production of antibody**

Antibodies against purified CLH were raised in six months old New Zealand white rabbit. The CLH solution (1 ml) containing 1 mg protein was emulsified with 1 ml of Freund’s adjuvant (complete) and was injected subcutaneously at four sites. After 30 days, a booster dose was injected in the same manner using Freund’s incomplete adjuvant. The blood was collected 10 days after the booster.

**Immunodiffusion assay**

Immunodiffusion assay was done on 0.75% agarose prepared according to Jensen¹⁰. The precipitin line was observed after 16 hr of diffusion at room temperature (25°C).

**pH Stability**

The pH dependence of CLH was examined using different buffers of pH ranging from 1-12. A volume of 50 µl CLH solution (50 µg) was incubated with 50 µl of buffer for 1 hr at room temperature. After incubation with respective buffers, an aliquot was checked for agglutination activity.

**Effect of temperature**

In a screw-cap tube 200 µl of CLH (200 µg) in 20 mM Tris-HCl buffer pH 8.0 was taken and 200 µl of the same buffer was added to it. The sample was heated at 85°C in a multi-block heater up to 90 min. Our initial studies have shown that CLH remains active upto 70°C. An aliquot of 25 µl was withdrawn at a regular interval of 20 min for checking the agglutination activity.

**Electrophoresis**

Electrophoretic analysis of the purified CLH was performed under non-dissociating conditions¹¹ using 10% polyacrylamide gel in a discontinuous system and also under dissociating conditions using 12% SDS-polyacrylamide gels (SDS-PAGE)¹². For dissociating system, the protein sample was treated with diluent (0.1 M Tris-HCl pH 7.0, 10% glycerol, 1% SDS, 5% 2-mercaptoethanol (βME), 0.05% Coomassie brilliant blue) for 3 min at 100°C. The sample was centrifuged for 5 min in an Eppendorf centrifuge before loading.

**Isolelectrofocussing**

Isolelectrofocussing of native CLH was performed on polyacrylamide gel in tubes (14x4 mm) using ampholine of pH range 3-10, for 6000 Volt-hours. A solution of 20 µl (60 µg) CLH was treated with 20 µl of diluent (10 mg methyl red, 0.3 ml ampholine pH 3-10, 0.75 g sucrose in a final volume of 5 ml). Ethylenediamine (0.01 M) and iminodiacetic acid (0.01 M) were used as cathode and anode buffers,
respectively. The gels were pre-focussed for 30 min at 450 volts (constant).

Isoelectrofocusing was also performed in the presence of 6 M urea using an ampholine of narrow range of pH 4-6.5. About 150 µl (150 µg) of CLH solution was treated with 6 M urea (solid), 5% βME and 10 µl of methyl red (saturated solution) to a final volume of 100 µl. Sodium hydroxide (0.1 M) and 

H₃PO₄ (0.1 M) were used as cathode and anode buffers, respectively. The gels were pre-focussed for 30 min at 450 volts (constant) and focussing were done for 6000 volt-hours. After focussing the gel was incubated in 30% methanol containing 10% acetic acid for several hours to remove ampholine. The gel was then stained with Coomassie brilliant blue and destained in 30% methanol containing 10% acetic acid.

Assay for inhibition of agglutination

Inhibition of agglutination was studied by using various monosaccharides, disaccharides, oligosaccharides, polysaccharides and glycoproteins using the method described by Kurokawa et al. Two-fold serial dilution of various carbohydrate solutions (25 µl) in 25 µl of 20 mM Tris-HCl, pH 8.0 were incubated with 25 µl (25 µg) of CLH solution for 30 min at room temperature. The agglutination assay was performed as described above. The inhibitory concentration was determined as the concentration that failed to agglutinate erythrocytes.

Blood group specificity

Agglutination assays were carried out as mentioned earlier, using human erythrocytes of groups A, B and O, collected from three donors. The assay was carried out against the untreated as well as trypsin treated erythrocytes.

Results

Purification of Chenopodium leaf hemagglutinin

Ion-exchange chromatography

After initial precipitation from crude extract using 70% saturation of (NH₄)₂SO₄, the protein was subjected

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Fig. 1—Ion-exchange chromatography of CLH on DEAE-sephadex. (---), NaCl gradient of 100-500 mM; (●), protein measured at 280 nm; (○), agglutination activity. Bar (—[]) indicates the number of active fractions pooled for CLH]
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Table 1—Purification of Chenopodium leaf hemagglutinin (100 g leaf tissue)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (AU)</th>
<th>Sp. activity</th>
<th>Purification fold</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2697.0</td>
<td>4,807,849</td>
<td>1783</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>70% (NH₄)₂SO₄ fraction</td>
<td>485.9</td>
<td>4,533,467</td>
<td>9330</td>
<td>5.2</td>
<td>94.3</td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>142.7</td>
<td>3,293,266</td>
<td>23078</td>
<td>12.9</td>
<td>68.4</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>14.3</td>
<td>2,315,578</td>
<td>1,619,288</td>
<td>90.8</td>
<td>48.2</td>
</tr>
</tbody>
</table>

Fig. 2—Gel filtration chromatography on FPLC using Superose-12 column. [●], Log molecular weight vs elution volume plotted. The points indicate the molecular weights in decreasing order as the elution volume increases, 1, Catalase (232000); 2, γ-globulin (160000); 3, Bovine serum albumin (66000); 4, Cytochrome c (124000). The peak represents the CLH fractions and the bar (I—I) represents the fractions pooled which showed agglutination activity.

Fig. 3—Electrophoresis of CLH on 10% polyacrylamide gel (non-denaturing). [50 μg protein was loaded and the protein was visualized by Coomassie brilliant blue staining]

Criterion of purity

Gel filtration

Affinity purified CLH when subjected to gel filtration chromatography on Superose-12 column using FPLC produced a single peak showing a native molecular weight of 58 kDa (Fig. 2).

Electrophoresis

The CLH preparation resolved into a single band when subjected to electrophoresis on native polyacrylamide gel (Fig. 3).

Subunit structure of CLH

The isolated CLH was analysed by SDS-PAGE both in presence and absence of βME. In the absence
of βME, CLH yielded a single band of 58 kDa, while in presence of βME the CLH molecule dissociated into its subunits and produced a single band of 33 kDa, leading to the conclusion that CLH is homodimeric in nature and its two subunits are linked by S-S bonds (Fig. A).

**Immunodiffusion**

The immunodiffusion experiment with anti-CLH antiserum showed a single band for CLH. The pre-immune serum did not give any precipitin line (Fig. 4).

The CLH antibodies did not produce any precipitin line with other legume lectins like pea lectin, conA, peanut lectin, blackgram lectin (BGL), and Abrus lectin and also soluble proteins isolated from Chenopodium seeds.

**Properties of purified Chenopodium leaf hemagglutinin**

**Blood group specificity**

The purified CLH agglutinated native and trypsin-treated rabbit erythrocytes. No agglutination was observed with either native or trypsin-treated human erythrocytes of blood groups A, B or O. This showed that CLH was not blood group specific.

**Hemagglutination inhibition assay of CLH**

The results of inhibition of hemagglutination are summarised in Table 2. D-glucose, L-glucose, D(+)-galactose, D(+)-mannose, D(+)-xylose, D(+)-fucose, stachyose, raffinose, lactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine did not inhibit agglutination up to 100 mM. Some glycoproteins which were tested, inhibited the agglutination activity of CLH. Asialofetuin was found to be a relatively better inhibitor for CLH followed by thyroglobulin and fetuin. Mucin inhibited agglutination activity at a concentration of 50 mg/ml but ovalbumin and BSA did not inhibit agglutination activity up to 100 mg/ml.

**pH profile of CLH**

The pH stability profile of CLH is shown in Fig. 6a. CLH was found to have a broad pH range for its agglutination activity. The lectin activity remained stable from pH 3 to 12. However, at pH below 3, the activity was found to be decreased.

<table>
<thead>
<tr>
<th>Sugar/glycoprotein</th>
<th>Minimum concentration* (mg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucin</td>
<td>50.00</td>
</tr>
<tr>
<td>Fetuin</td>
<td>12.50</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>6.25</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>0.78</td>
</tr>
</tbody>
</table>

*Minimum concentration required for complete inhibition of agglutination activity.

D(+)-glucose, L-glucose, D(+)-galactose, D(+)-mannose, D(+)-xylose, D(+)-fucose, stachyose, lactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine did not inhibit agglutination activity up to 100 mM. The glycoproteins like ovalbumin and BSA also did not inhibit agglutination up to a concentration of 100 mg/ml.
Kinetics of heat inactivation of \( \text{CLH} \)

On heating \( \text{CLH} \) from room temperature (25°C) to 100°C at an interval of 10°C, the agglutination activity was found to be retained up to 80°C (data not shown). The kinetics of heat inactivation were studied at 85°C. At this temperature, \( \text{CLH} \) lost 50% of its agglutination activity in 5 min and 97% activity was lost in 10 min (Fig. 6b).

Isoelectric point (\( \text{pI} \))

In the native isoelectrofocussing, \( \text{CLH} \) showed a single band of \( \text{pI} \) 5.25, when the experiment was performed using ampholine of \( \text{pH} \) 3-10. In presence of 6M urea and ampholine of a narrow \( \text{pH} \) range 4-6.5, \( \text{CLH} \) produced six bands of \( \text{pI} \) ranging from 5.1-5.6 with varied intensities (Fig. 7).

Discussion

A hemagglutinin (CLH) from \textit{Chenopodium} leaves was purified about 91-fold and had 160 agglutination activity units per \( \mu \text{g} \) of protein. The CLH was found to be a homodimer. It agglutinated native as well as trypsin-treated rabbit erythrocytes. The purified CLH on SDS-PAGE demonstrated a single band of 33 kDa under reducing conditions, which in absence of \( \beta \text{ME} \) showed a single band of 58 kDa showing the presence of disulfide linkage between the subunits (Fig. 4). Such an agglutinating activity was neither detected in the \textit{Chenopodium} seeds, nor was CLH immunologically cross-reactive to the putative seed lectin in the same plant (data not shown). The differences between hemagglutinin in seeds and hemagglutinin in other vegetative parts have been reported in many plants\(^\text{2,5}\). On isoelectrofocussing with 6M urea CLH showed the presence of six charged species, which could not be isolated by the present methodologies employed. CLH is the first hemagglutinin to be isolated from the leaves of \textit{Chenopodium} species belonging to the family Chenopodiaceae and it differs from other leaf
agglutinins so far described with respect to its carbohydrate specificity and structure.

CLH was found to be a thermostable protein which could retain its agglutination activity at a broad range of pH (3-12) (Fig. 6a), similar to certain other seed lectins\(^\text{14,15}\) and also the bark lectins of rubber tree, *Hevea brasiliensis*\(^\text{16}\).

The hemagglutinin from *Chenopodium* leaves did not show any immunological cross-reactivity with pea lectin, Con A, PHA, peanut lectin, BGL and *Abrus* lectin isolated from seeds, as no precipitin lines were formed by these lectins in the immunodiffusion test done with CLH-antibodies.

CLH does not qualify to be a lectin as it did not show inhibition of hemagglutination activity with specific sugars, although it could bind to a glycoprotein like fetuin—the affinity matrix used for its purification. Several glycoproteins interfered with its binding to erythrocytes in hemagglutination assays. There are several lectins which do not show specific carbohydrate binding properties\(^\text{7,8}\). For example, the well known lectin, *Phaseolus vulgaris* agglutinin (PHA) showed that the hemagglutinin activity could not be effectively inhibited by simple sugars, but only by complex oligosaccharide structures\(^\text{17}\).

**Acknowledgement**

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**References**

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