Purification and Characterization of a Lectin from *Amaranthus hypochondriacus* var. Mexico Seeds

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A lectin from *Amaranthus hypochondriacus* var. Mexico (AHML) was purified by affinity chromatography using asialofetuin-Sepharose 4B. AHML is specific for N-acetyl-D-galactosamine as are the other *Amaranthus* lectins. AHML has no carbohydrate moiety and requires no metal ion for the hemagglutination activity. The pI of AHML is 6.8. AHML has a native molecular mass of 45.0 kDa and is composed of homo-subunits having molecular masses of 36.8 kDa.

**Key words: Amaranthus; lectin; hemagglutinin; purification**

Lectins are carbohydrate-binding proteins (or glycoproteins) of nonimmune origin that agglutinate cells or precipitate glycoconjugates. They are widely distributed in plants, animals, and microorganisms and a large number of them have been purified from various sources, especially legume seeds.

Recently, some lectins were purified from *Amaranthus* seeds. Zenteno and Ochoa purified a lectin from *A. leucocarpus* (syn. *A. hypochondriacus*) using a blood group 'A' stroma affinity column. The lectin was shown to be a glycoprotein (10% w/w carbohydrate) and specific to T-antigen (Galβ1→3GalNAc-O-R). Singh et al. also purified a lectin from *A. hypochondriacus* using an asialofetuin affinity column. They showed that the lectin is a glycoprotein (1.13–1.17% neutral carbohydrate). The hemagglutination of the lectin was inhibited by N-acetyl-D-galactosamine, fetuin, and asialofetuin. Moreover, both of these two lectins were composed of homo-subunits and their subunit molecular masses were 45.0 and 29.0 kDa, respectively. Accordingly, these two *A. hypochondriacus* lectins have similar properties except for their molecular masses and sugar contents.

In this paper, we report the characterization of a novel lectin having no carbohydrate moiety from *A. hypochondriacus* var. Mexico seeds (AHML).

**Materials and Methods**

**Materials.** *A. hypochondriacus* var. Mexico seeds were obtained from the Foundation of Agricultural Industry Development Assistance, Tokyo, Japan. Fetuin, asialofetuin, mucin, bovine serum albumin, and ovalbumin were purchased from Sigma (St. Louis, MO, U.S.A.). Ovomucoid was from Miles (Kankakee, IL, U.S.A.). Chymotrypsinogen A was obtained from Wako (Osaka, Japan). Polyvinylidene difluoride membrane (Immobilon, pore size 0.45 μm) was a product of Millipore (Bedford, MA, U.S.A.). Other reagents were the highest grade available.

**Purification of AHML.** The seeds were homogenized by an Ultraturrax apparatus in 10 volumes of 150 mM NaCl. The homogenate was kept at 4°C for 4h and then squeezed through a cheese cloth. The resulting solution was centrifuged at 9000 rpm for 15 min to remove the insoluble materials. The crude extract was incubated with asialofetuin-Sepharose 4B prepared by the method of the manufacturer at 4°C overnight. The suspension was packed into the column (3 × 14 cm). After the column was washed with 1 M NaCl, the adsorbed proteins were eluted with 200 mM glycine–HCl buffer (pH 2.5). The eluates were dialyzed against deionized water and lyophilized.

**Hemagglutination and hapten inhibition assays.** Hemagglutination activity was assayed by a 2-fold serial dilution of the lectin in a micro U-plate using 3% (v/v) rabbit erythrocyte suspension. Titer was defined as the reciprocal of the highest dilution showing detectable agglutination. A hapten was diluted serially and mixed with the lectin solution of titer 4. After incubation at room temperature for 1 h, 3% erythrocyte suspension was added. The inhibitory activity was defined as the concentration of hapten required for the complete inhibition of the hemagglutination.

**Polyacrylamide gel electrophoresis.** Acidic-polyacrylamide gel electrophoresis (PAGE) was done in a 15% gel at pH 4.3 by the method of Resfeld et al. SDS-PAGE was done by the method of Laemmli. The subunit molecular mass was estimated by electrophoresis on 10% polyacrylamide gel in 0.1% SDS with or without the addition of 2% (v/v) 2-mercaptoethanol. The molecular mass standards (Bio-Rad, Richmond, VA, U.S.A.) used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.3 kDa), and trypsin inhibitor (21.5 kDa). Gels were stained with Coomassie brilliant blue.

**Molecular mass measurement.** The native molecular mass of AHML was measured by gel filtration by the method of Andrews on a column (2 × 110 cm) packed by Toyopearl HW-50 F equilibrated with 50 mM Tris–HCl (pH 8.0) containing 100 mM NaCl. Standard proteins used were bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), and chymotrypsinogen A (25.0 kDa). The void volume of the column was measured with Blue Dextran 2000 (Pharmacia).

**Isoelectric focusing.** Isoelectric focusing was done in a 110-M column using a carrier ampholyte (Ampholine, pH range of 4-6, Pharmacia) at a final concentration of 1.0% (v/v) and a stepwise sucrose gradient (0–50% (w/v)) by the method of Vesterberg and Svensson. The electrophoresis was done for 6 h (at 300 V for the first 0.5 h and 900 V for the remaining 5.5 h) in a column kept around 10°C by cooling with tap water. After electrofocusing, the column was drained at a flow rate of 0.7 ml/min and the eluate was collected in 2-ml fractions. Each fraction was assayed for pH, absorbance at 280 nm for protein, and hemagglutination activity.

**Amino acid analysis.** AHML was electrophoresed by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. The amino acid composition of AHML was analyzed using a Waters Pico Tag system (Millipore). AHML was hydrolyzed by 6 M HCl in the presence of 1% HCl for 24 h at 110°C; 1 M NaCl was added to dissolve the proteins before analysis.
phenol at 150°C for 1 h using a Waters Workstation, and the amino acids were analyzed after derivatization with phenylisothiocyanate. Cyst(e)ine was measured as cysteic acid by the method of Schram et al.13

Results

Purification of a lectin from *A. hypochondriacus* var. Mexico seeds

A summary of the purification scheme for a lectin from *A. hypochondriacus* var. Mexico seeds is shown in Table I. In the preliminary study, it had been shown that the crude extract had a hemagglutination activity and its activity was inhibited by asialofetuin, which was a good ligand for affinity chromatography. Non-adsorbed proteins had no hemagglutinating activity. Bound proteins were desorbed by the change of pH. The purity of desorbed proteins was analyzed by acidic-PAGE (Fig. 1). This showed a single band, so the lectin could be purified by one step from crude extract using affinity chromatography.

Molecular mass estimation of native AHML and subunits

AHML yielded a single band at the position of 36.8 kDa on SDS-PAGE in the presence of 2-mercaptopethanol (Fig. 2, lane 2). In the absence of 2-mercaptopethanol, some smaller bands were observed, but no bands in the higher range (Fig. 2, lane 3). Accordingly, AHML was shown to be composed of homo-subunits and the subunits seemed to be held together by non-covalent forces.

The molecular mass of native AHML was estimated to be 45.0 kDa on the finding of the identical migration with ovalbumin (45.0 kDa) on the gel filtration experiments (data not shown).

Amino acid composition

The amino acid composition of AHML is presented in Table I. AHML had a high content of Asx or Glx and was rich in hydrophobic amino acids. Moreover, this lectin contained a few cyst(e)ine residues. The N-terminal amino acid of AHML was shown to be blocked (data not shown).

Physicochemical properties of AHML

AHML was found to have no carbohydrate moiety from periodic acid/Smith staining18 and phenol-sulfuric acid methods15 (data not shown). The pI of AHML was 6.8. The hemagglutination activity of AHML was not affected by demetalization with EDTA. On the other hand, AHML lost the activity completely upon heat treatment at 80°C for 20 min.

Carbohydrate specificity of AHML

The results of the hemagglutination inhibition assay of AHML are summarized in Table III. Only N-acetyl-D-galactosamine was shown to be an inhibitor among the monosaccharides tested. Hemagglutination of AHML

![Fig. 1. Acidic-PAGE of AHML.](image)

Electrophoresis was done on 15% gel in acidic buffer system as described in Materials and Methods. Protein bands were detected with Coomassie brilliant blue.

![Table 1. Purification of AHML (from 25 g)](image)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (Titer × volume)</th>
<th>Specific activity (Titer/mg proteins)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extracts</td>
<td>2696.8</td>
<td>56633</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td>Asialofetuin-Sepharose 4B</td>
<td>32.7</td>
<td>41169</td>
<td>125</td>
<td>72.7</td>
</tr>
</tbody>
</table>

* Titer defined as the reciprocal of the end-point dilution with the hemagglutination with 0.85% rabbit erythrocytes in phosphate-buffered saline.
was inhibited by Galβ1→3GalNAc but not by Galβ1→3GlcNAc. N-Acetyl-d-galactosamine and Galβ1→3GalNAc inhibited at the minimum concentrations of 7.8 mM and 0.039 mM, respectively. Accordingly, Galβ1→3GalNAc is about a two hundred times stronger inhibitor than N-acetyl-d-galactosamine.

Hemagglutination of AHML was also inhibited by some glycoproteins that have GalNAc-containing oligosaccharide chains such as mucin, fetuin, and asialofetuin. On the other hand, glycoproteins having no GalNAc-containing oligosaccharide chain such as ovomucoid and ovalbumin could not inhibit the hemagglutination of AHML. These results suggest that AHML is specific for GalNAc-containing oligosaccharide chains.

**Discussion**

In this study, AHML, an *A. hypochondriacus* lectin, was purified and characterized. There were no other lectins in the saline extract. AHML was shown to consist of homo-subunits. The other *Amaranthus* lectins have been reported to consist of homo-subunits.2,8) On this point, AHML was similar to the other lectins from *Amaranthus* species. Zenteno and Ochoa reported that the lectin from *A. leucocarpus* is a glycoprotein composed of homo-subunits of 45.0 kDa.31 Singh et al. also showed that the *A. hypochondriacus* lectin is a glycoprotein consisting of homo-subunits of 29.0 kDa.8) AHML has no carbohydrate moieties and its subunit molecular mass is 36.8 kDa on SDS-PAGE. The pl of AHML was 6.8, but the other two *A. hypochondriacus* lectins focus in multiple bands in the range of 4-6 on isoelectric focusing. These results show that AHML has a molecular structure distinct from those of the other *A. hypochondriacus* lectins. However, their properties, especially carbohydrate specificities, closely resemble each other. These three lectins have been purified from the same species but possibly different varieties. For example, PHA, a typical *Phaseolus vulgaris* lectin, is composed of two kinds of subunits (E and L).16) On the other hand, GNL-1 and 2, novel lectins from Great Northern bean, a cultivar of *P. vulgaris*, were shown to have a different subunit structure from PHA.17) GNLs are composed of three kinds of subunit (α, β, and γ, or α′, β′, and γ′, respectively). These findings show that there are lectins having structurally different subunits in the same species. We are interested in the relationship between subunit structures and biological roles of lectins in plants.

On SDS-PAGE, AHML gave a single band in the presence of 2-mercaptoethanol, although a few bands were observed in the absence of 2-mercaptoethanol. In Fig. 2, lane 3, these bands might be due to the heterogeneity of disulfide bonds. We propose that AHML may be in a heterogeneous form in the seed, or this result might be an experimental artifact. Koeppe and Runow obtained a similar SDS-PAGE profile with *A. cruentus* lectin2) to that we observed in this study (Fig. 2, lane 3). Maeshima et al. showed that sporamin A and B migrated to the same position corresponding to the molecular mass of 25.0 kDa in reducing conditions on SDS-PAGE, but these two sporamin bands appeared at the positions corresponding to molecular mass of 31.0 kDa (A) and 22.0 kDa (B) in the non-reducing condition.18) They proposed that sporamin A and B might have the same molecular mass but different surface charge and stereo-structure when bound with SDS in the presence of 2-mercaptoethanol. These show that a polypeptide having an intramolecular disulfide bond(s) may form a compact three-dimensional structure, hence the molecular mass seems smaller and the polypeptide possibly migrates to a smaller mass position on SDS-PAGE. AHML has a few cyst(e)ine residues by amino acid analysis. Therefore, AHML may have intramolecular disulfide bond(s).

It should be noted that there is a possibility of the occurrence of isoelectins. It was reported that two *A. hypochondriacus* lectins were electrophoretically homogeneous on native- or SDS-PAGE, but each showed multiple bands on isoelectric focusing.3,8) Zenteno and Ochoa proposed this might be due to microheterogeneity in carbohydrate composition. AHML was focused as a single peak on isoelectric focusing in our experiments, so we concluded that there might be no isoelectin.

AHML is a *N*-acetyl-d-galactosamine-specific lectin. It has been reported that T-antigen interacts specifically with *Amaranthin* or *A. leucocarpus* lectin.4,5) In order to evaluate whether the sugar specificity of AHML resembles *Amaranthin* or *A. leucocarpus* lectin, two kinds of disac-
charide were tested. The hemagglutination of AHML was inhibited by Galβ1→3GalNAc but not Galβ1→3GlcNAc. Thus the sugar specificity of AHML is similar to A. caudatus and A. leucocarpus lectins. Among the glycoproteins tested mucin, fetuin, and asialofetuin inhibited hemagglutination of AHML. Asialofetuin was a more potent inhibitor than fetuin. This may be due to interference with binding of the oligosaccharide chain by the terminal sialic acids. AHML seems to recognize the structure of the carbohydrate moiety of fetuin and asialofetuin. Moreover, these three inhibitory glycoproteins are known to have GalNAc-containing oligosaccharide chains. Accordingly, AHML might bind to the carbohydrate moiety of these inhibitory glycoproteins.

In this paper, we report a structurally novel Amaranthus lectin that has a similar carbohydrate specificity to the other Amaranthus lectins.

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References