High Molecular Weight Lectin Isolated from the Mucus of the Giant African Snail Achatina fulica

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To understand better the host defense mechanisms of mollusks against pathogens, we examined the antimicrobial activity of mucus from the giant African snail Achatina fulica. Hemagglutination activity of the mucus secreted by the integument of snails inoculated with Escherichia coli was observed to increase and to cause hemagglutination of rabbit red blood cells. Purification of the mucus lectin by column chromatography revealed that the relative molecular mass of the lectin was 350 kDa. The hemagglutination activity of the lectin was Ca\(^{2+}\)-dependent and was inhibited by galactose. Growth arrest tests showed that the lectin did not inhibit bacterial growth, but did induce agglutination of gram-positive and gram-negative bacteria. Tissue distribution analyses using a polyclonal antibody revealed that the lectin was expressed in the tissues of the mantle collar. The lectin isolated from the mucus of the snail appeared to contribute to its innate immunity.

Key words: Achatina fulica; host defense; innate immunity; lectin; mucus

The immune response in organisms can be categorized into two systems, innate and adaptive immunity. Innate immunity an important component of host defense mechanisms in all plant and animal species. In vertebrates, defenses against pathogens are mediated by both innate and adaptive systems. However, since invertebrates do not exhibit adaptive immunity, they rely on innate immune mechanisms to respond to pathogens. Mollusks have two general defense reactions: cellular defense reactions and reactions mediated by humoral defense factors. Cellular defense reactions involve a phagocytic system against foreign pathogens and to encapsulate foreign materials. Humoral defense factors involve lysozyme, lecithins, antibacterial or anti-viral factors, and opsonization. Lectins are widely distributed among bacteria, plants, and animals, and are prominent factors in innate immunity. They are classified into several types based on structural characteristics. Of these, C-type lectins exhibit Ca\(^{2+}\)-dependent activity and form a diverse group of proteins including collectins and selectins. A number of C-type lectins have been isolated and characterized, mainly on the basis of whether they exhibit hemagglutinating activity. In addition, numerous primary sequences have been submitted to databases such as GenBank. Among invertebrates, C-type lectins have been isolated and sequenced from tunicates, flies, barnacles, and sea urchins, all of which show an affinity for galactose. The monosaccharide-binding specificity of a lectin is an important determinant of its function, and many animal lectins have been observed to recognize monosaccharides. Skin mucus lectins exhibiting lactose-binding ability have been identified in the Japanese eel. While this lectin is structurally a C-type lectin, it was observed to exhibit Ca\(^{2+}\)-independent activity. Although the physiological role of the other invertebrate lectins is not well understood, it is thought that they play roles in host defense mechanisms.

In general, the monomeric size of lectins ranges between 30 and 40 kDa, and few high molecular mass lectins have been reported. Nonetheless, high molecular weight lectins, such as d-Hml, identified in hemocyte-derived cells of Drosophila, have been reported. This C-type lectin has a molecular mass of 400 kDa, and has been reported to play a role in the hemostasis of drosophila through its involvement in blood clotting. Similarly, a lectin with a molecular mass of 280 kDa was also purified and sequenced from the hemolymph of the silkworm, Bombyx mori. Another high molecular weight lectin (350 kDa) localizes in the vitelline layer of sea urchin eggs and binds to an acrosomal protein of sea urchin sperm during fertilization.

In addition to the lectin-complement system, action in combination with an antimicrobial system is a very important aspect of innate immunity. We have reported that an antibiotic protein, achacin, is present in mucus of the giant African snail. Achacin is a most abundant mucus protein and is secreted by the collar of the body. Primary sequence analysis and biochemical evidence showed that achacin exhibits l-amino acid oxidase (LAO) activity and that it binds to bacteria, which it kills with H\(_2\)O\(_2\) generated by enzymatic reaction in vitro. The present investigation sought to purify and characterize a high molecular weight lectin from the mucus of the giant African snail. Purified lectins specific to a sialic acid have been reported in the snail mucus.
and hemolymph. Both lectins agglutinating erythrocytes from different animals in a Ca$^{2+}$-dependent manner. Native forms of the lectins from the mucus and the hemolymph were 78 kDa and 242 kDa respectively, the latter consisting of identical 15 kDa subunits. In this study we characterized a lectin isolated from the giant African snail and found that it is both novel and distinct from previously reported lectins.

Materials and Methods

**Bacterial strains and media.** *Staphylococcus aureus* (IAM1011) and *Escherichia coli* (K12 W3110) grown in Luria-Bertani (LB) broth were used.

**Purification of lectin.** Giant African snails, *Achatina fulica* Ferussac, were captured in Okinawa, Japan, and transported by air to the laboratory, where they were maintained. Lectin was purified from mucus of the snails by the procedure described below. Mucus collected from the collar of a snail not inoculated with *E. coli* was diluted twice with 50 mM Tris–HCl buffer (pH 7.5). To reduce the viscosity of the mucus, polysaccharides were precipitated by adding polyvinyl poly pyrrolidone (PVPP) to a final concentration of 4% w/v. After centrifugation at 15,000 × g for 30 min, the supernatant was further fractionated by ammonium sulfate precipitation. The 40–60% w/v ammonium sulfate precipitates were then dissolved in 50 mM Tris–HCl buffer (pH 7.5) containing 50 mM NaCl (buffer A) and dialyzed against the same buffer. The clarified supernatant was applied to a TSK-gel DEAE-Toyopearl 650M column (Tosho, Tokyo, 2.6 × 23 cm) pre-equilibrated with buffer A. After washing with 600 mL of buffer A at a flow rate of 2 mL/min, the crude lectin was eluted with a 400 mL linear gradient of NaCl (50–300 mM) in 50 mL Tris–HCl (pH 7.5). The eluates were dialyzed against a 10 mM potassium phosphate-buffered solution (pH 6.8), and adsorption chromatography was applied using hydroxyapatite gels (Nacalai Tesque, Kyoto; 10×10 cm) pre-equilibrated with 10 mM potassium phosphate-buffered solution (pH 6.8). The flow-through fractions were collected and dialyzed against buffer A. The supernatant after clarification was concentrated with Centriprep 30 (Millipore, Bedford, MA) and further subjected to high performance liquid chromatography (HPLC) using a TSK-gel G 4000 SW (Tosho, 0.75 × 60 cm) pre-equilibrated with buffer A.

The protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA), using bovine serum albumin (BSA) as standard.

**Hemagglutinating activity.** Hemagglutinating activity was tested by making a serial dilution of samples in physiological saline (150 mM NaCl, 5 mM CaCl$_2$, and 10 mM Tris–HCl, pH 7.5) in a 96-well plate. To each well a 2% erythrocyte suspension prepared from rabbit blood was added. The hemagglutination unit (HAU) was expressed as the reciprocal of the highest dilution showing detectable hemagglutination. For the pathogen inoculation test, the integument was excised using a scalpel (2 mm deep and 1 cm long), and 100 µL of *E. coli* (approximately 10$^8$ colony forming units or CFU/mL) suspended in potassium-buffered saline (PBS) or PBS as control was inoculated into the wound. After the appropriate period, mucus was collected from the body surface and hemagglutinating activity was examined. Agglutination activity relative to the protein concentration of the mucus was presented as the mean value with a standard deviation for the experiment, which was performed in triplicate. Statistical significance was assessed by Student’s t-test.

**Sugar specificity.** The sugar specificity of the high molecular weight lectin was tested by inhibiting the hemagglutinating activity using simple sugars. The results were expressed as the minimal concentration of carbohydrates required to inhibit four hemagglutinating dose units (HDU) of the lectin effectively.

**Agglutination tests against bacteria.** The agglutination activity of the lectin was tested against *S. aureus* and *E. coli*. The bacteria were washed with PBS, and the precipitated cells were suspended in 0.5% formaldehyde and incubated overnight to kill the bacteria. The bacterial samples were incubated in the presence of 5 mM CaCl$_2$ with lectin added to a final concentration of 2 µg/mL (experiment) or PBS (control) at room temperature for 1 h, and agglutination activity was examined under a light microscope.

**Measurement of bactericidal activity.** The bactericidal effects of the lectin were examined by metabolic inhibition assay (Kubota et al., 1985). *Staphylococcus aureus* and *E. coli* (approximately 10$^7$ CFU/mL) were cultured in plastic cuvettes with 1 mL of LB-broth containing 2 µg of lectin for 16 h at 37°C. Antibacterial activity was determined by monitoring the turbidity of the culture medium at OD 600 nm. Two-factor analysis of variance (ANOVA) was used to compare the results of the bacterial growth tests.

**Gel electrophoresis and Western blot analysis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) of the protein samples was performed by the method of Laemmli. Protein samples were mixed with a SDS sample buffer to final concentrations of 2.5% SDS, 5% 2-mercaptoethanol, 65 mM Tris–HCl (pH 6.8), and 5% glycerol. The proteins, separated on 7.5% polyacrylamide gels under reducing conditions, were stained with Coomassie Brilliant Blue R-250 (CBB), and the molecular mass of the lectin was measured by 4% PAGE. The migration distances of the endogenous protein projectin (1,200 kDa), kettin (500 kDa), and myosin heavy chain (220 kDa) from crayfish skeletal muscle, a connectin/triton fragment (1,200 kDa), and myosin heavy chain (220 kDa) from rabbit skeletal muscle were measured and used as size markers for electrophoresis.

To generate a polyclonal antibody against the purified lectin, the purified lectin sample were dialyzed against PBS and used as the antigen. Rabbits were immunized with the antigen emulsified in Freund’s complete adjuvant, and were boosted 3 times with the same antigen emulsified in Freund’s incomplete adjuvant. Serum containing anti-lectin antibody was purified by incubating the serum with nitrocellulose-bound antigen and then eluting it at low pH, as described previously.

For western blot analysis, proteins were separated on 10% SDS–PAGE gels and transferred to nitrocellulose membranes. The membranes were incubated for 60 min at room temperature in 10 mM PBS (pH 8.0) and 0.05% Tween 20 (PBST), containing 5% skim milk (Yukijirushi, Tokyo). They were then incubated with anti-lectin antibody (diluted 1:2,000 with PBST) for 2 h at room temperature, and then rinsed 5 times for 7 min each time in PBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (DAKO, Kyoto, Japan) diluted 1:2,000 with PBS for 1 h at room temperature. Each membrane was then washed 5 times for 7 min each time in PBS, and then bound antibody was visualized using enhanced chemiluminescence (ECL) detection reagents (GE Healthcare Bio-Sciences, Piscataway, NJ) following the manufacturer’s instructions.

**Immunofluorescence analysis.** For immunofluorescence analysis, snail integument and collar tissues measuring 4–7 mm were excised with a scalpel, fixed overnight in 4% paraformaldehyde, and then frozen in Tissue-Tek OCT Compound (Miles, Elkhart, IN) and cut into 10-µm sections using a cryostat (model 505E; Leica Microsystems, Wetzlar, Germany). The sections were mounted on glass microscope slides, air dried, fixed again in methanol at −20°C for 5 min, and permeabilized at room temperature in 0.1% Triton X-100 for 5 min. After washing with PBS, nonspecific binding was blocked with 10% normal goat serum for 30 min and 5% BSA in PBS for 30 min. Affinity-purified anti-lectin antibody and polyclonal antibody against achatin were used as the primary antibodies (diluted 1:400 and 1:1,000 respectively), with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (diluted 1:150) (Sigma-Aldrich) as the secondary antibody. The specimens were examined under a confocal microscope (LSM410; Carl Zeiss, Oberkochen).

**Results**

**Pathogen inoculation tests**

To test changes in the hemagglutinating activity of the mucus in response to pathogen inoculation, an incision, made in the integument of the snail with a scalpel, was inoculated with *E. coli*. The hemagglutinating activity...
of the mucus collected from the inoculated snails increased 6 h after inoculation and decreased slightly thereafter. Conversely, the hemagglutinating activity of the mucus collected from the control snails remained constant for the duration of the test (Fig. 1A). The observed increase in the hemagglutinating activity thus appeared to be due to the presence of pathogen and not to disruption of the integument.

**Lectin purification**

Lectin from the mucus of the giant African snail was purified by monitoring hemagglutinating activity (Fig. 1B). A high (350 kDa) molecular weight protein band was one of the major protein bands in the mucus. Fractions exhibiting hemagglutinating activity after purification with the anion exchange column contained this high molecular protein band and a smaller band, of approximately 76 kDa (Supplemental Fig. 1; see **Biosci. Biotechnol. Biosci.** Web site). Hydroxylapatite gels were used to remove all other minor protein bands (Supplemental Fig. 2). Flow-through from the gels was concentrated and applied to a gel filtration column (Supplemental Fig. 3). Eluted fractions exhibiting high hemagglutinating activity were found to contain the high molecular weight protein band, which had specific hemagglutinating activity approximately 2,000 times that of the crude mucus (Table 1). Hence we designated the purified lectin from the mucus of *Achacin fulica* AfHML.

**Biochemical characterization of the lectin**

The purified lectin was detected between the myosin heavy chain and kettin and had an estimated molecular mass of 350 kDa (Fig. 2). Since the lectin was eluted near the void volume of the HPLC column, its native size could not be estimated (Supplemental Fig. 3).

We examined the effects of various sugars and calcium ions on the hemagglutinating activity of AfHML. As
shown in Table 2, hemagglutinating activity was observed in the presence of Ca\(^{2+}\), and was removed by the addition of Ca\(^{2+}\) chelating agent. In the subsequent hemagglutination experiments, CaCl\(_2\) was added to the saline to a final concentration of 5 mM or 10 mM. EDTA was added to a final concentration of 10 mM to chelate Ca\(^{2+}\). Agglutination not observed.

Table 2. Effects of Ca\(^{2+}\) on the Hemagglutinating Activity of AfHML

<table>
<thead>
<tr>
<th>Conditions</th>
<th>HAU (dilution ratio)</th>
</tr>
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<tbody>
<tr>
<td>Physiological saline w/o CaCl(_2)</td>
<td>(2^1)</td>
</tr>
<tr>
<td>+5 mM CaCl(_2)</td>
<td>(2^6)</td>
</tr>
<tr>
<td>+10 mM CaCl(_2)</td>
<td>(2^6)</td>
</tr>
<tr>
<td>+5 mM CaCl(_2), +10 mM EDTA</td>
<td>—</td>
</tr>
</tbody>
</table>

AfHML was diluted so that HAU might become 2\(^1\) with a physiological saline without (w/o) CaCl\(_2\) (150 mM NaCl and 10 mM Tris-HCl, pH 7.5), and was used in experiments. CaCl\(_2\) was added to the saline to a final concentration of 5 mM or 10 mM. EDTA was added to a final concentration of 10 mM to chelate Ca\(^{2+}\). —, agglutination not observed.

Table 3. Saccharide Inhibition of AfHML Hemagglutinating Activity against Rabbit Erythrocytes

<table>
<thead>
<tr>
<th>Saccharides</th>
<th>Minimum inhibitory concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Galactose</td>
<td>10</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>25</td>
</tr>
<tr>
<td>L-Galactose</td>
<td>25</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>25</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>50</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>50</td>
</tr>
<tr>
<td>Neuraminic acid</td>
<td>50</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>100</td>
</tr>
<tr>
<td>N-Acetyl-D-galactosamine</td>
<td>100</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>&gt;250</td>
</tr>
</tbody>
</table>

Fig. 3. Agglutination Activity of AfHML against Gram-Positive and Gram-Negative Bacteria. 
(A and B) Formaldehyde-treated S. aureus and (C and D) E. coli were incubated with (B and D) purified lectin and with (A and C) PBS at room temperature for 1 h.

*Effects of lectin on bacterial agglutination and growth*

The lectin agglutinated gram-positive (S. aureus) and -negative (E. coli) bacteria (Fig. 3). The effect of the lectin on bacterial growth was assessed by monitoring absorbance of a buffer containing bacteria at 600 nm. No marked inhibitory effects were observed on the bacterial agglutination or bacterial growth test (Fig. 4).

Fig. 5. Specificity Tests of the Generated Antibody. 
Affinity-purified polyclonal antibody raised against AfHML was used in western blotting. High-molecular weight protein bands were detected in both the mucus (Mucus) and the purified fractions (Lectin). CBB, Coomassie Brilliant Blue staining; WB, western blotting; MWM, molecular weight marker.
Immunofluorescence analysis using the membrane-purified antibody against lectin showed that signals were present in tissue of the mantle collar but not in the foot (Fig. 6b and e). The localization pattern was similar to that of the antibacterial protein achacin (Fig. 6c and f). No obvious signals in tissues such as the intestinal organ were observed (data not shown).

**Discussion**

In the present study, we detected the presence of a high molecular weight lectin, designated AfHML, in the mucus of the giant African snail. Most lectins are involved in internal physiological activities, in tissues or the blood plasma. But less is known about how lectins function outside the body. Lectins specific to sialic acid and GalNAc/GlcNAc have been purified from tissues of the garden snail *Cepaea hortensis*.28,29) The sialic acid-binding lectin of *C. hortensis*, CHAI, is a 80-kDa protein composed of two subunits (23 and 16 kDa). Similarly, the GalNAc/GlcNAc-specific lectin of *C. hortensis*, CHAII, is also composed of two subunits (17 and 15.5 kDa). Lectins purified from the giant African snail *Achatina fulica* have also been reported in the literature, including lectins specific to a sialic acid in the mucus23) and hemolymph,24) both of which agglutinated erythrocytes from various animals in a Ca\(^{2+}\)-dependent manner. In addition, the molecular masses of the native forms of the lectins purified from the mucus and the hemolymph were 78 kDa and 242 kDa respectively, consisting of identical 15 kDa-subunits. Low molecular weight lectins in the mucus appear to have been separated by DEAE column and/or by hydroxyapatite column (Supplemental Figs. 1 and 2); thus, any hemagglutinating activity was not detected in the low molecular weight protein fractions separated by gel filtration column (Supplemental Fig. 3).

The hemagglutinating activity of AfHML was inhibited by d-galactose, suggesting some affinity to the sugar. Similarly, lectins exhibiting an affinity for galactose have been isolated in many invertebrates, and the galactose-specific C-type lectins from *Drosophila* are well characterized. The lectins in *drosophila* form a gene cluster with other C-type lectin genes and have roles in host defense against pathogens.30,31) Of these lectins, the DL1 protein bound to some gram-negative bacteria (*E. coli* and *Erwinia chrysanthemi*) but not to others or gram-positive bacteria. Conversely, AfHML bound both gram-negative and gram-positive bacteria. To clarify the bacterial recognition mechanism of AfHML, the inhibitory effect of lipopolysaccharide (LPS) on hemaglutination activity was tested. The binding of AfHML to *E. coli* was not affected by the addition of LPS (data not shown). Additionally, AfHML showed no affinity to GalNAc, the major component of bacterial peptidoglycans. Taken together, these findings indicate that AfHML does not bind to bacterial cells by LPS, but rather by binding to other polysaccharide chains on the bacterial cell surface.

Generally, lectins have a molecular mass of less than 30 kDa. High molecular weight lectins, such as d-Hml of *Drosophila* and lectins of sea urchins have unique domain structures and functions.17,19) Because of its size, AfHML may also have unique structural features, but the N-termini of the purified lectin could not be determined by Edman degradation on an automated
sequences. In order to elucidate the innate function of the lectin isolated in this study, AFHML, the primary sequence of AFHML must be determined in future research. The drosophila lectin, δ-Hml, is known to be involved in hemostasis, and the upregulation of this lectin has been reported in instances where the larval integument was pierced with a thin needle. However, our results indicate an increase in the hemagglutinating activity of the mucus in response to inoculation of E. coli, not just to injury. It is thus possible that AFHML functions in host defense after pathogen attack. However, although agglutination of bacteria occurs in the presence of AFHML and Ca\(^{2+}\) ions, no bactericidal activity of AfHML was observed after exposure to this lectin, and the contribution of another antibacterial substance is considered necessary to kill bacteria.

We have reported the presence of an antibacterial protein, achacin, in the mucus of the giant African snail. Achacin is a member of the l-αmino acid oxidase family and generates hydrogen peroxide to kill bacteria. Gene expression and the secretion of achacin from mantle collar tissue have also been reported. Tissue localization analysis revealed that AFHML is also secreted from the collar tissue, where achacin is present. Since achacin is known to bind gram-positive and gram-negative bacteria, it is possible that the agglutination activity of AFHML accelerates the antibacterial activity of achacin by increasing the local concentration of hydrogen oxides in the mucus.

Acknowledgment

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References