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Purification of a secreted lectin from *Andrias davidianus* skin and its antibacterial activity

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ABSTRACT

A lectin secreted from *Andrias davidianus* skin (ADL) was purified by affinity chromatography on porcine stomach mucin (type III) (PSM)-crosslinked albumin, followed by gel filtration on Sephadex G-100 and HPLC on TSK gel G3000PW$_{XL}$. The purified lectin was found to be a dimeric protein, as revealed by SDS-PAGE and MALDI-TOF analysis. SDS-PAGE showed that the ADL protein had a molecular mass of 17 kDa. ADL produced an 8.5 kDa band when examined using SDS-PAGE under reducing conditions. ADL agglutinated native and trypsinized human B erythrocytes. The hemagglutination activity was inhibited by glycoproteins, such as PSM and asialo-PSM, but not by any of the monosaccharides tested. The activity was stable between 4 °C and 50 °C. Significant ADL activity was observed between pH 4–5. The lectin reaction did not depend on the presence of the divalent cation Ca^{2+} or Mg^{2+}. The N-terminal ADL sequence was determined to be VGYTVGATPM. The lectin exhibited antibacterial activity, involving growth and respiration inhibition in *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Bacillus subtilis* and *Shewanella* sp. Furthermore, ADL showed inhibition activity against the yeast *Saccharomyces cerevisiae*. These findings suggest that ADL plays an important role in the innate immunity of *A. davidianus* on the body surface.

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1. Introduction

The giant salamander, *Andrias davidianus*, is a rare amphibian in China. It lives in clear water in mountain rivulets without environmental pollution (Lan et al., 1990). Since the 1980s, artificial *A. davidianus* cultures have been developing in many provinces of China (Jiang et al., 2011). Many *A. davidianus* farms have been established and developed in artificial streams. In recent years, with the rapid growth of *A. davidianus* aquaculture, *A. davidianus* diseases have been reported frequently, and these diseases do serious damage to the cultures. To address this issue, numerous studies have focused on *A. davidianus* skin.

Amphibian skin is a defense organ that fulfills the functions of antimicrobial defense, anti-infection and anti-oxidation (Clarke, 1997; Li et al., 2007; Yang et al., 2009). The skin gland secretion of *A. davidianus* is a source of defense molecules with biological activities. In particular, numerous antimicrobial peptides, phospholipase A_2 and proteolytic enzymes are secreted from *A. davidianus* skin (Wang et al., 2011; Guo et al., 2012). Recently, the skin secretions of *A. davidianus* have been investigated in our laboratory, and glycopeptides have been prepared and characterized (Jin et al., 2011; Feng et al., 2012). However, *A. davidianus* skin components have not been well studied.

Lectins are widely distributed among plants, bacteria and animals, including amphibians. Lectins are one of the important pattern-recognition proteins that have been described as playing a role in processes as diverse as self-defense, parasitism and symbiosis (Jing et al., 2011). They can bind or immobilize microorganisms through agglutination or limit pathogen infection (Holmskov et al., 2003). Unfortunately, there are limited data and literature concerning lectins from amphibian skin secretions compared to that concerning their other organs. For instance, an egg lectin (13.5 kDa) from *Rana japonica* was isolated by gel filtration and successive ion-exchange chromatography on diethylaminoethyl cellulose and carboxymethylcellulose columns (Sakakibara et al., 1979). A β-galactoside binding lectin (30 kDa) from *Bufo arenarum* oocytes was isolated by salt extraction and affinity chromatography, and it was partially characterized with data on

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its amino acid content and physico-chemical characteristics (Fink de Cabutti et al., 1987). A β-galactoside-binding lectin was isolated and characterized from *Rana catesbeiana* frog eggs (Ozeki et al., 1991). Two β-galactoside-binding lectins (50 and 56 kDa) from the *B. arenarum* skin were isolated and characterized; they showed strong bacteriostatic activity against Gram-negative bacteria (*Escherichia coli* K124100 and wild strains of *E. coli* and *Proteus morganii*) and Gram-positive bacteria (*Enterococcus faecalis*) (Riera et al., 2003). Odorranalectin, a small peptide lectin that recognizes L-fucose, was purified and characterized from *Odorrana grahami* frog skin secretions. This lectin is composed of only 17 amino acid residues (YASPKCFRYPNGVLACT) and contains a single disulfide bridge (Li et al., 2008). Although lectins are found in various organs, the search for new lectins from amphibian skin secretions remains a problem. Studying the properties and functions of lectins from *A. davidianus* skin secretions is a promising endeavor for understanding innate immune defense and for curing related diseases.

In this report, we describe the purification, characterization, carbohydrate specificity and antibacterial activity of a new Ca^{2+}-independent mucin-binding lectin from the giant salamander *A. davidianus*.

2. Materials and methods

2.1. Materials

Monosaccharides were obtained from Merck (Darmstadt, Germany). PSM, porcine stomach mucin (type III), albumin (egg white), thyroglobulin and trypsin were purchased from Sigma Chemical (USA). Human erythrocytes were obtained as outdate red cell concentrates from the Center of Blood Utilization (Dalian). Sephadex G-100 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The TSK gel G3000PW$_{XL}$ column was purchased from TOSOH (Japan). The standard proteins used for apparent molecular mass estimation by SDS-PAGE and HPLC were purchased from Beijing Solarbio Science and Technology Company (Beijing, China).

Gram-positive bacterial strains (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923) and Gram-negative bacterial strains (*E. coli* ATCC 35218, *Clostridium perfringens* ATCC 13124 and *Shewanella* sp.) were used. All strains were kindly provided by the Liaoning Entry–Exit Inspection and Quarantine Bureau. The yeast *Saccharomyces cerevisiae* was obtained from a commercial store (Angel Yeast Co., Ltd., China). The affinity sorbent for crosslinking albumin with PSM was prepared according to the method described previously (Kowal and Parsons, 1980; Belogortseva et al., 1994).

2.2. A. davidianus skin secretion collection

Five 4-year-old, sexually mature giant salamanders (*A. davidianus*) were obtained from Zhangjiajie Jinchi Giant Salamander Biological Technology Company Ltd. (Zhangjiajie city, Hunan province of China). Their use in this research was approved by the Aquatic Wild Animal Researching License of Hunan province (2011-021). Both male and female ($n = 5$, 2 males, 3 females) animals (2-3 kg in mass) were selected randomly for skin secretions. Animals were washed with distilled water to remove contaminants from their skin. Then, the surface was stimulated by pulse durations of 1–2 min with a maximum stimulus strength of 20 V (Tyler et al., 1992). A milky skin secretion was collected and centrifuged at 1200 *g* for 20 min. The supernatant was lyophilized to yield a powder. The powder was stored at 4 °C.

2.3. ADL isolation and purification

100 mg of the powdered sample was suspended in 2 mL of 0.01 M Tris–HCl buffer (TB), pH 7.4, and then centrifuged at 1200 *g* for 20 min. The clear supernatant was applied to a PSM-crosslinked albumin column (3 × 11 cm), which was previously equilibrated and eluted with TB. After the elution of unbound proteins in TB, adsorbed proteins were eluted with 1.5 M NaCl in TB. The protein concentration and hemagglutination titer of each fraction were measured. Fractions containing hemagglutination activity were pooled and further purified by gel filtration on Sephadex G-100 (2.5 × 96 cm) equilibrated with 0.1 M TB, pH 7.4, containing 0.15 M NaCl (TBS). The column was eluted with the same buffer at 10 mL/h, and fractions showing hemagglutination activity were dialyzed against water and lyophilized to yield a powder (6 mg). 50 μg of the purified sample was suspended in 20 μL of 0.01 M TB, pH 7.4, and then centrifuged at 8000 rpm for 20 min. The clear supernatant was additionally purified by high performance liquid chromatography (HPLC) on a G3000PW$_{XL}$ column (7.8 mm × 30 cm). The column was washed with TB.

2.4. Molecular mass measurement of the purified ADL and its subunits

The molecular mass of the purified ADL subunit was measured by SDS-PAGE using a 15% polyacrylamide separation gel and a 4% polyacrylamide stacking gel (Laemmli, 1970). The ADL molecular mass was determined by SDS-PAGE in the presence and absence of dithiothreitol. ADL reduction was performed by heating at 100 °C for 5 min in sample buffer containing 2% SDS and 2.5% dithiothreitol. Gels were calibrated using the following standard proteins: phosphorylase B (94,000), BSA (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and alpha-lactalbumin (14,400). Proteins were stained with Coomassie brilliant blue.

The molecular mass of the purified native lectin was measured by passing it through a G4000PW$_{XL}$ column (7.8 mm × 30 cm) in 0.01 M TBS, pH 7.4. The standard proteins used were rabbit muscle phosphorylase B (97,000), albumin bovine V (68,000), albumin egg (45,000) and trypsin (23,300).

The molecular masses were further investigated by MALDI-TOF mass spectrometry. The measurements were performed on a 4800 Proteomics Analyzer time-of-flight mass spectrometer (Applied Biosystems, USA) at the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.5. Hemagglutination assay

To assay the hemagglutination activity, ADL was serially diluted 2-fold with 0.01 M TBS (25 μL) into microtiter U-plates. To each well, an equal volume of 2% human B type erythrocyte suspension was added, and the mixture was agitated. The hemagglutination was visually evaluated after 30 min (Belogortseva et al., 1998b).

For the hemagglutination inhibition assay, aqueous solutions of various substances were serially diluted 2-fold with TBS. ADL (25 μL, 4 doses of agglutinated material) and a 2% erythrocyte suspension (25 μL) were added to each sample (25 μL) successively. The obtained mixture was gently stirred by pipette and incubated for 1 h. The minimal concentration of each substance required for complete inhibition was determined.

2.6. Effect of divalent cations and pH

ADL was dialyzed for 24 h against 0.1 M TBS, pH 7.4, containing 50 mM $EDTANa_2$ or 50 mM $CaCl_2$. Human B type erythrocytes were used as indicator cells.

The ADL pH dependence was determined by preincubating the samples with different pH buffers for 1 h at 25 °C as follows: 0.02 M sodium acetate/acetic acid, pH 3–5; 0.02 M sodium phosphate/HCl, pH 6–7; 0.02 M Tris/HCl, pH 8–8.5; and 0.02 M glycine/NaOH, pH 9–10. The samples were subsequently dialyzed against 0.1 M PBS, pH 7.8, and the agglutination activity was assessed using B type erythrocytes (Belogortseva et al., 1998b).

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