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Target-directed isolation and identification of a serum lectin from lamprey (*Lampetra japonica*) by chromatographys and MALDI-TOF/TOF

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Abstract

A 105-kDa polymer lectin was purified from lamprey (*Lampetra japonica*) serum by chromatography methods including cation ion-exchange chromatography with a SP-Sepharose^{TM} XL column and size exclusion chromatography with a Superdex 200 column. The target fractions were collected according to the direction of hemagglutinating activity. The results revealed that the active fractions could adsorb on SP-Sepharose column and showed a 280 nm UV absorbance peak corresponding to molecular weights of 105 kDa in the following size exclusion chromatography. The target fractions with hemagglutinating activity were further checked by Native-PAGE and SDS-PAGE. Two single bands at around 105 kDa and 35 kDa were displayed by two electrophoresis methods respectively, indicating that the protein exists as a trimer in solution. After Native-PAGE and SDS-PAGE, two bands were excised from the gels respectively and further identified by MALDI-TOF/TOF as serum lectin (gi: 13094239). The lectin was able to agglutinate rabbit red blood cells (RRBCs) and sheep red blood cells (SRBCs) *in vitro*. The lectin isolated from lamprey serum in the current study might be helpful for deeply understanding the innate immune molecules dependent immune defence in jawless vertebrates which have been proved recently that they possess a lymphocyte-based system of anticipatory immunity with variable lymphocyte receptors as mediators.

Key words: *Lampetra japonica*, lectin, purification, identification, MALDI-TOF/TOF

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1 Introduction

Lectins are carbohydrate-binding proteins which have been investigated extensively in recent years for application in medical and immunological research (Ogawa et al., 2011). They have various biological functions from the glycoprotein synthesis to regulation of cell adhesion and the control of protein levels in the animal bloods (Rutishauser and Sachs, 1975). In addition, lectins can serve many important roles in innate immune defense by recognizing carbohydrates (Ourth et al., 2008) and agglutinating red blood cells *in vitro* (Guo et al., 2013). Agnathans, represented by lamprey and hagfish, are agreed to be the oldest vertebrates currently possessing the adaptive and innate immune defenses. The study of jawless vertebrate provides a theoretical basis for the origin of immune system. Though there are no T cell receptor (TCR) and B cell receptor (BCR) singling pathway in these jawless vertebrates, recent findings in the Agnathans have revealed that they possess an alternative adaptive immune system which could specifically recognize and respond to external pathogens (Cooper and Alder, 2006). This system undergoes germline genomic rearrangements of insertion of diverse leucine-rich repeat modules (LRRs) to generate a large number of different variable lymphatic receptors (VLRs) for the resistance to the pathogen invasion. Three types of receptors, VLRA, VLRB and VLRC have been identified in lampreys (Kasamatsu et al., 2010). Recent evidence indicates that VLRA and VLRB are expressed in different cell types that resemble T cells and B cells in jawed vertebrates, respectively. After being infected by specific a pathogen, VLRB-like lymphocytes expressing specific VLRB molecules undergo amplified expression, and begin to secrete VLRB in a manner analogous to the secretion of immunoglobulins by B cells (Guo et al., 2009). Comparing to the most advances achieved in the adaptive immune system of lamprey, little is known about the effective components that exist in lamprey blood and their roles played in innate immune system. The current study reports that a compon-

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ent with hemagglutinating activity was purified from lamprey (*Lampetra japonica*) serum by several chromatography steps, and it was identified as lectin by MALDI-TOF/TOF.

2 Materials and methods

2.1 *Animals and reagents*

The handling of lamprey (*Lampetra japonica*) and all experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University (Permit Number: SYXK2004—0029). Adult lampreys were purchased from Tongjiang section of the Heilongjiang River (Tongjiang City, Heilongjiang Province, China) in December. Rabbit red blood cells (RRBCs) and sheep red blood cells (SRBCs) were obtained from rabbit (*Oryctolagus cuniculus*) and sheep (*Oreamnos americanus*), respectively. $NaHCO_3$, C_2H_3N, IAA, and TFA were purchased from the Sigma Company (Sigma-Aldrich, St. Louis, MO). NaCl, coomassie brilliant blue G250, CH_3COOH and the phosphate buffer were of analytical grade from Sangon Biotech (Sangon Biotech (Shanghai) Co., Ltd.). AKTA avant 25 was purchased from GE Company (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Autofelx MALDI-TOF spectrometer was purchased from Bruker Corporation (Bruker Daltonics, Bremen, Germany).

2.2 *Lampreys serum*

About 100 healthy adult lampreys (200–220 g in weight) were tail-severed for collecting blood. The blood (about 530 mL) was aliquoted into 10 mL plastic centrifuge tubes and allowed to clot at 4°C overnight. Serum was separated by centrifugation (4 000 r/min) for 10 min at 4°C and kept in 1.5 mL centrifuge tube at –20°C before use.

2.3 *Cation exchange chromatography with SP-Sepharose column*

Twenty milliliters of lamprey serum were dialyzed against three changes of 6 L starting buffer consisting of 0.005 mol/L EDTA in 0.01 mol/L NaPB (pH 6.5) for 24 h. The pellets were removed by centrifugation (12 000 r/min) for 10 min at 4°C and the cleared supernatant was applied to a SP-Sepharose column (300 mm×45 mm, i.d., GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) which was equilibrated with two column volumes of starting buffer followed by a linear salt gradient elution. The concentration of NaCl raised from 0.2 to 0.6 mol/L in 0.025 mol/L Tris-HCl buffer (pH 7.5) in one column volume. Five milliliter fractions were collected at the flow rate of 1 mL/min. The eluted protein fractions were then conducted the hemagglutinating activity examination.

2.4 *Size exclusion chromatography*

The eluted protein fractions with hemagglutinating activity were pooled together and dialyzed against three changes of 6 L elution buffer (0.005 mol/L EDTA and 0.15 mol/L NaCl in 0.01 mol/L NaPB (pH 6.5)) for 24 h. The dialyzed sample (5 mL) was applied to size exclusion chromatography on a Sephadex 200 column (700 mm×20 mm i.d., GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) equilibrated with the elution buffer. The elution was carried out at a flow rate of 0.3 mL/min, and 1 mL-fractions were collected for hemagglutinating activity examination. Finally, the protein fractions with hemagglutinating activity were collected, dialyzed, lyophilized and stored at -20°C for future assay. The protein concentrations of the samples were determined by Bradford assay (Sigma-Aldrich, St. Louis, MO) using bovine serum albumin as standard.

2.5 *Mass spectrometry*

The protein fractions with hemagglutinating activity were electrophoresed by native polyacrylamide gel electrophoresis (Native-PAGE) and SDS polyacrylamide gelelectrophoresis (SDS-PAGE). Albumin from bovine serum 66 kDa (monomer) and 132 kDa (dimer) were used as Native-PAGE gel molecular-weight standards (Sigma-Aldrich, St. Louis, MO). The SDS–PAGE was carried out using 12% (w/v) separation gel. The molecular-weight standards (TaKaRa Biotechnology, Dalian, China) including phosphorylase b (97.2 kDa), albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa) and trypsin inhibitor (20.1 kDa) were used for SDS–PAGE. The protein bands were visualized with Coomassie Brilliant Blue R-250. Specific protein bands were excised from the gel matrix and subjected to in-gel tryptic digestion. A total of 0.5 μL of matrix solution (10 mg of a-cyano-4-hydroxycinnamic acid dissolved in 1 mL of 30% ethanol) and 0.5 μL of the diluted analyte solution were spotted on the MALDI target plate (Bruker Daltonics). The MALDI-TOF mass spectrometry was operated in the positive ion mode and measured in reflectron modes on an Autoflex MALDI-TOF spectrometer within a mass range of 700–3 500 Da. The positive-ion mass spectra were calibrated externally using the Bruker Protein Calibration Standard I. Each spectrum corresponded to an accumulation of 3 000 laser shots (6×500 laser shots from different positions of the sample spot). The generated spectra were visualized and compared with FlexAnalysis 3.3 software (Bruker Daltonics, Bremen, Germany). All MS raw data were analyzed by biotools 3.0 (Bruker Daltonics, Bremen, Germany) and searched against all lamprey sequences available in NCBI and ensemble database using Mascot searching engine. Data were run assuming trypsin digestion with a parent ion tolerance of 0.5 Da and fragment ion mass tolerance of 0.8 Da. Oxidation was specified as global modifications and carboxymethyl as variable modifications. Database analysis was performed using the same parameters plus the possibility of up to one missed enzyme cleavages sequence coverage greater than 10%.

2.6 *Hemagglutination assay*

To assay the hemagglutinating activity, a serial two-fold dilution of the sample fractions (100 μL) was mixed with equal volume of 2% suspension of RRBCs and SRBCs in phosphate-buffered saline (PBS, pH 7.2) in a 96-well flat-bottom plate at room temperature for 1 h, respectively. After incubation, red blood cells agglutination was observed by light microscopy *in vitro*. All statistical analyses were performed with the SAS proprietary software release 8.02 and student's two-sample *t*-test.

3 Results and discussion

The target protein with hemagglutinating activity was first isolated from lamprey serum using cation exchange chromatography with a SP-Sepharose column. Twenty milliliters of the lamprey serum was applied to the column and eluted with a linear salt gradient elution as described in materials and methods. Each of the 5 mL-fractions of the effluent were collected and assayed for protein at 280 nm (Fig. 1). Forty-two fractions showed absorbance at 280 nm were assayed hemagglutinating activity and the Hemagglutinating activity was only found in Fraction 25. The dialyzed sample (Fraction 25) was applied on a Sephadex 200 column for conducting size exclusion chromatography. Under UV 280 nm, there were a major peak (Fractions 27 to 34) and a minute peak (Fractions 21 and 24) (Fig. 2). The hemagglutinating activity was only detected in Fraction 26. The elution volume of Fraction 26 was approximately 26 mL, and the molecular

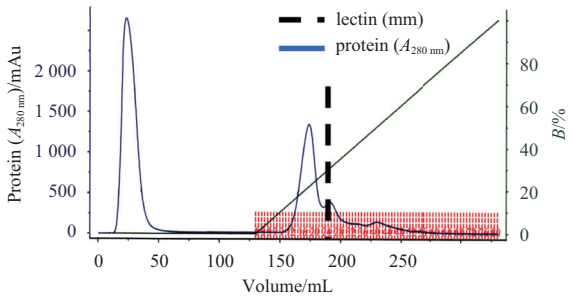

Fig. 1. Ion exchange chromatography profile of lamprey serum crude extract on SP-Sepharose column. Flow rate was 50 mL/h. Hemagglutinating activity was detected in Fraction 25. The dotted line indicates the location of hemagglutinating activity. *B* indicates concentration of elution buffer (PBS+1 mol/L NaCl).

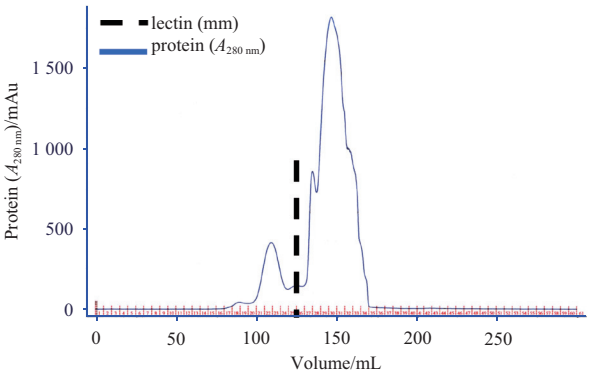

Fig. 2. A Gel filtration chromatography profile of Fraction 25 on Superdex 200 column. Flow rate was 20 mL/h. Hemagglutinating activity was detected in Fraction 26. The dotted line indicates the location of hemagglutinating activity.

weight of Fraction 26 is deduced to be approximately 105 kDa according to the calculation equation given in the manufacturer's instruction manual. The concentration of the target protein is 1.25 mg/mL in the lamprey serum by BCA assay. The result of Native PAGE analysis showed a single protein band whose molecular weight was calculated to be about 105 kDa (Fig. 3a). A single protein band was also found on the gel of SDS-PAGE analysis (Fig. 3a). It can be deduced that its molecular weight is around 35 kDa from the molecular weight calibration graph. From the results of Native PAGE and SDS-PAGE analysis, it can be concluded that the protein with hemagglutinating activity exists as a trimer. On MALDI-TOF MS/MS analysis, the peptide fragments of the protein showed significant high scores (p>0.05) from Mascot searches of peptide mass fingerprints of proteins (Fig. 3b). The complete sequence of a peptide fragment with m/z 1 671 was identified as R.WSSQLGSNP.A which showed a typical character of the product of trypsin (Fig. 3b). The identified sequence of the protein with hemagglutinating activity possesses 100% identity with the serum lectin (GenBank: BAB32787) from lamprey *Lethenteron camtschaticum*. The hemagglutinating activity of purified lamprey serum lectin was further examined by a 10-fold dilution series of sample. The high hemagglutinating ability of the lamprey serum lectin can be observed to agglutinate RRBCs and SRBCs even diluted to 100 fold (12.5 μg/mL) and 200 fold (6.25 μg/mL), respectively (Fig. 4). The ability of the lamprey serum lectin to agglutinate SRBCs was slightly stronger than RRBCs. This result suggests that the hemagglutinating activities of RRBCs and SRBCs depend on the dose of lectin.

In the current study, serum lectin was purified from lamprey *L. japonica*, a representative of jawless vertebrate. The lectin was eluted as a single peak with a molecular weight of around 105 kDa in size exclusion chromatography step. Its molecular weight was verified as 105 kDa by Native-PAGE analysis under native condition, while it was revealed a 35-kDa protein band on SDS-PAGE gel in reducing conditions. These data suggest that the lectin is a 105-kDa trimerical protein and possesses hemagglutinating activity. Agnathans, represented by lamprey and hagfish, are agreed to be the oldest vertebrates currently possessing the

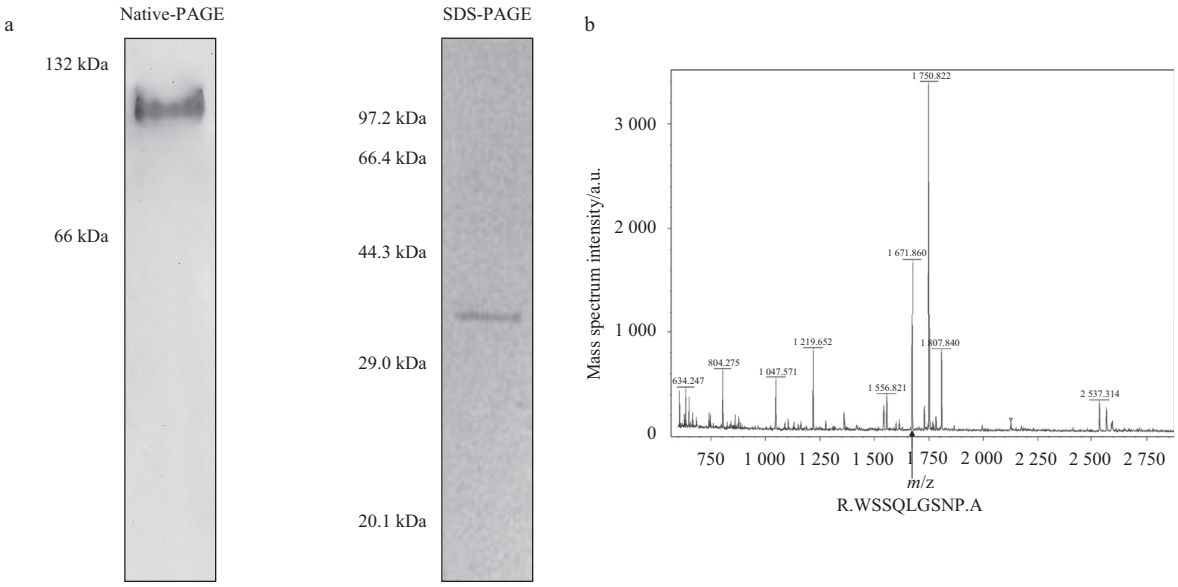

Fig. 3. Gel analyses and Peptide mass fingerprints. a. Native-PAGE and SDS–PAGE. Left lane: Native-PAGE. Right lane: SDS-PAGE. b. The peptide with m/z 1 671 corresponds to a tryptic fragment of the complete protein was identified as R.WSSQLGSNP.A, an identical homologue of serum lectin (GenBank: BAB32787), by MALDI-TOF MS/MS analysis.

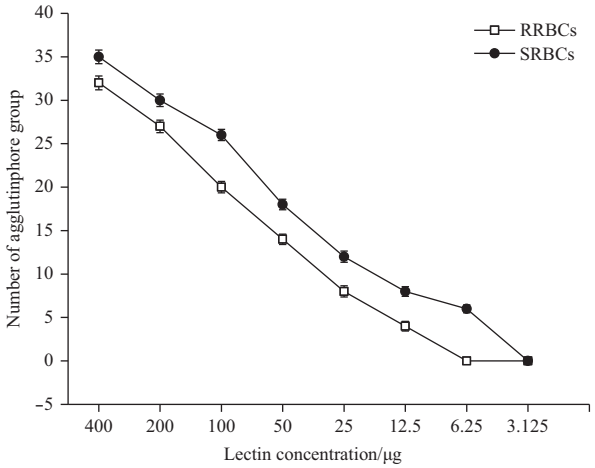

Fig. 4. Agglutination assay of lamprey lectin against erythrocytes. The antigens for agglutination assay were RRBCs and SRBCs. Data represent mean numbers±SE. Error bars, n=3.

adaptive and innate immunity. Variable lymphatic receptors are the important components for adaptive immune response in jawless vertebrates (Guo et al., 2009). In jawed vertebrates, lectin serves many important roles in the innate immunity by recognizing exogenous antigen (Guo et al., 2013). Here, we found lamprey lectin also possesses hemagglutinating activity to allogeneic red blood cells. These findings provided basic information for deeply understanding the components of innate immune system of jawless vertebrate.

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