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Purification and characterization of a novel C-type hemolytic lectin for clot lysis from the fresh water clam *Villorita cyprinoides*: A possible natural thrombolytic agent against myocardial infarction

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ABSTRACT

Villorita cyprinoides (black clam) is a fresh water clam that belongs as a bivalve to the group of mollusc. The saline extracts from the muscle reveal high titers of agglutination potency on trypsin-treated rabbit erythrocytes. With the help of affinity chromatography a hemolytic protein with lectin activity which could all be inhibited by D-galactose were isolated. The lectins were separated on DEAE-cellulose and the main component was purified after an additional step of gel filtration on sephadex G-75. The main component is a non-glycosylated protein with a molecular weight of 96,560 Da determined by MALDI-ToF, consisting of a single protein chain and characterized by the lack of polymers and intermediate disulfide bonds. The pure main lectin with clot lytic feature shows two bands at molecular weights 36,360 and 26, 520 Da. Optimal inhibition of the pure lectin is achieved by D-galactose containing oligo- and polysaccharides. The lectin activity decreased above 40 °C and was lost at 62 °C, the stability over the pH range between 7.0 and 8.0 and requires divalent cations for their activity. The novel C-type hemolytic lectin for clot lysis from the clam *Villorita cyprinoides* was identified and evaluated, the purified hemolytic lectin (0.35 mg/ml and 0.175 mg/ml) enhanced clot lysis activity when compared to the different concentration (5 mg/ml and 1 mg/ml) of commercial streptokinase. In the present study identified hemolytic lectin was a rapid and effective clot lytic molecule and could be developed as new drug molecule in future.

1. Introduction

In Invertebrates, there are many natural substances which have toxic effects on the blood coagulation and fibrinolytic systems of mammals [1]. Some of these substances have been well characterized and studied as alternative drugs in the treatment of pathological processes that affect the hemostatic system, such as bee venom serine protease [2], venom of *Lonomia obliqua* caterpillar extract [3], venom of *Lonomia achelous* caterpillars [4], Hirudin [5], tick anticoagulant peptide [6], snake venom [7], both experimentally and clinically as possible antithrombotic drugs because of their anticoagulant activity *in vivo*. However, there are no specific reports on lectins especially the hemolytic lectin which have involved in clot lysis processes and probably nil. Among lectins, hemolytic lectin is a lytic factors, generally protein in nature, had been demonstrated in the body fluids or tissue extract of sipunculids, annelids, arthropods, mollusks and echinoderms using vertebrate erythrocytes as experimental targets, and consequently named as hemolysins [8]. Hemolytic lectin was reported in different invertebrates, such as echinoderm *Cucumaria echinata* (CEL-III) [9], mushroom *Laetiporus sulfureus* [10] and the seeds of *Croton tiglium* [11].

However, except hemolysin, reports on the presence of hemolytic lectin in molluscan species are lacking. Consequently, from the beginning the significance of hemolysins to mollusks is unclear. Recently, the hemolytic lectin properties, mode of action, interaction studies and functional properties were well studied [12]. Thrombolytic drugs have been used in the clinical arena to treat wide varieties of venous and arterial thromboembolic disorders which are a main cause of death. In thrombolytic therapy, ability to produce rapid clot lysis an effective alternative is needed [13]. Now, the hemolytic lectins have emerged as significant molecules in all aspect of research especially in the cancer research. Considering its

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importance and functionality, the present study and the first report, attempted to utilize effectively hemolytic lectin as a clot lytic molecule and characterized the clot lytic bioprocess.

2. Materials and methods

2.1. Materials

The clams, *Villorita cyprinoides* were collected from the Anchalikadavu, a tributary of Thamirabharany River near Athencode, Vilavancode taluk, Kanyakumari district, Tamil Nadu South India. CNBr-activated fetuin Sepharose 4 Fast Flow was purchased from Sigma Chemicals (USA), Sephadex™ G-75 (Amersham Biosciences, Sweden), DEAE-Cellulose (HiMedia), Sugars, glycoprotein and all basic chemicals from Sigma chemicals (USA), and Streptokinase.

2.2. Preparation of sample

The shell of the clam was cleaned in fresh water and dried using absorbent paper. The muscle was dissected out by gently opening the shell and cleaned with distilled water. 100 mg of dissected muscle in 1 ml of ice cold TBS buffer (pH 7.6) and homogenized in a tissue homogenizer, centrifuged at 4000 × g for 10 min at 4 °C. The clear supernatant was dialyzed against TBS buffer and the dialyzed extract was stored at −20 °C for further study.

2.3. Purification of hemolytic lectin

i) **Anion exchange chromatography**: The clam muscle extract was dialyzed overnight against TBS containing 10 mM $CaCl_2$ and by centrifugation at 10,000 g (10 min, 4 °C). Two milliliter of sample was applied to a column (1.5 × 30 cm) of DEAE-cellulose (HiMedia) previously stabilized with TBS + $CaCl_2$. The column was washed with the same buffer until optical density attains zero. The retained material was eluted with different concentration of NaCl. Two milliliter of fraction were collected and analyzed by absorption at 280 nm and test by hemolytic activity against rabbit erythrocyte.

ii) **Affinity chromatography** − Two ml of DEAE-cellulose purified active fraction was applied for affinity chromatography purification and applied to CNBr-activated fetuin sepharose 4 fast flow column was equilibrated with TBS (pH 7.6) at 4 °C. After column packing, it was washed with TBS until it attains an optical density of 0.002 at 280 nm. The elution was done with elution buffer containing 0.1 M galactose and the fractions were collected at the rate of 0.4 ml/min. The emergence of the lectin was monitored by measuring absorbance at 280 nm. The fraction having high protein content was identified using hemolytic assay and was estimated using Lowry method [14].

iii) **Gel filtration chromatography:** Affinity purified protein obtained was further fractionated on a column Sephadex™ G-75 (Amersham Biosciences, Sweden) (0.6 × 100 cm). The column was equilibrated with TBS-Ca and separation was carried out at a flow rate 0.3 ml/min and fractions were collected. The active hemolytic fractions were collected and the combined material was dialyzed against deionized water for 24 h and concentrated.

2.4. Lectin purification by RP-HPLC

The gel filtration purified lectin derived from *V. cyprinoides* was applied to the HPLC C_{18} column (250 × 4.6 mm) Varian (Lake forest, CA, USA) Cyberlab, USA. The column was equilibrated with required percentage of acetonitrile and water. Fractions were carried out at the flow rate of 1 ml/min. The active peak was collected and concentrated using speed vac. The concentrated sample was subjected to native PAGE and SDS-PAGE.

2.5. Hemolytic assay

The hemolytic assays were performed in V-bottomed microtiter plates by serial two − fold dilution of a 25 μl serum sample with an equal volume of TBS-Ca, addition of 25 μl red blood cells suspension and incubated for 1 h at room temperature under the same condition as for the hemagglutination assay. The hemolytic titer was recorded as the reciprocal of the highest dilution of the sample causing complete lysis of red blood cells. Each experiment was performed in duplicate, and the hemolytic activities were expressed as the median hemolytic titer. Finally the purified lectin was assayed with the hemolytic assay.

The hemolytic activity (HL = HLU mL^{-1}) was defined as the Hemolytic Units per volume used. Specific hemolytic activity was expressed as the activity per mg of protein.

2.6. Determination of hemolytic activity

According to the method described by [9], hemolytic activity was determined either by visual examination of lysis of the erythrocytes under the same condition as for the hemagglutination assay or by measurement of the absorbance of 550 nm due to hemoglobin released from the erythrocytes.

2.7. Erythrocyte agarose diffusion test

Hemolytic assay was carried out as described by [15]. The hemolytic activity was detected using a 1 mm thick agarose layer containing 1.5% erythrocytes in 0.9% NaCl. Wells of 4 mm diameter were cutout from the agarose and filled with 5 μl of sample. The agarose wells were incubated for 16 h at room temperature. After the period of incubation, the clear zones of hemolysis if any around the wells were noted. The diameter of plaques were measured and used as a degree of hemolytic activity.

2.8. Effect of pH and thermal stability

The pH stability of the lectin was determined by extensive dialysis of the lectin (1 mg/mL) against buffers of different pH values ranging from pH 3.5–10.0 using acetate buffer, Tris–HCl and glycine NaOH. The pH of the lectin solution was adjusted to pH 7.6 by the addition of 0.1 N HCl or 0.1 N NaOH before hemolytic activity was determined. The dialysates were centrifuged and the supernatant was tested for hemolytic activity.

The thermal stability of hemolytic lectin was examined by holding 100 μl of purified hemolytic lectin for 30 min at the temperatures ranging from 10 to 80 °C. All the samples were centrifuged at 400 × g for 10 min at R.T and the clear supernatant was used to determine the hemolytic activity using rabbit erythrocytes.

2.9. Divalent cation dependency and EDTA sensitivity

The initial hemolytic activity of the purified lectin was determined in Tris buffered saline (TBS) containing 10 mM $CaCl_2$. The purified lectin was dialyzed extensively against TBS (to test divalent cation dependency) or in TBS − EDTA (to examine EDTA sensitivity) at 15 °C .The samples were dialyzed against TBS - EDTA and were re-equilibrated by dialysis in TBS. After centrifugation at 400 × g for 5 min at room temperature, the supernatant was used to determine the hemolytic activity using human and animal RBC in the presence