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Purification and characterization of lectin from humoral fluids of *Charybdis feriatus**

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Abstract To search new sources of lectin, an experiment on lectin distribution in humoral serum of crab *Charybdis feriatus* (in short, CFL) was conducted March, 2002. When adding solid ammonium sulfate into the fluids up to 50% saturation at 4℃, most CFL activity showed precipitates who were then continually extracted by ammonium sulfate of different concentrations. The supernatant, which was called primary CFL fluids, was given a 17.60-fold purification and 45.70% recovery of total activity. Finally, by using Sephadex G-100 column chromatography, the CFL in the primary CFL fluid was highly purified. Compared to the original humoral fluids, the last purified CFL got a 203.90-fold purification and 30.48% recovery of total activity, and demonstrated a single band on SDS-PAGE. In the same time, the purified CFL was detected for agglutination activity with 7 kinds of animal erythrocytes. Other characterization, such as sugar inhibition, and the effect of temperature on the agglutination activity of CFL were also studied. Our results indicate that agglutination activity of CFL was influenced by sugar and temperature.

Key words: ammonium sulfate; purification; CFL (lectin from *Charybdis feriatus*); characterization

1 INTRODUCTION

Lectins have been regarded as having a putative role in non-self recognition in vertebrate and invertebrate immunities (Arason, 1996; Matsushita, 1996; Olafsen, 1996; Vasta et al., 1999; Wilson, et al., 1999; Marques and Barracco, 2000). They are ubiquitous proteins that can promote cell agglutination by specific binding to various carbohydrates expressed on cell surfaces.

The first case of lectins in invertebrates was from the humeral fluid of *Limulus polyphemus* in 1903(Yeaton, 1981). Being lack of adaptive immune systems, invertebrates have developed various defense systems that can recognize antigens on the surface of potential pathogens (Vasta et al., 1994; Olafsen,1996). These systems include hemolymph coagulation, melanization, cell agglutination, antimicrobial action, active oxygen formation and phagocytic activity (Cooper et al., 1992; Vazquez et al., 1998). Among them, hemolymph coagulation and phenoloxidase-mediated melanization, as well as cell agglutination, are directly triggered by foreign invaders with phagocytosis. The invaders would be immobilized and finally killed by antimicrobial substances released from many kinds of hemocytes (Olafsen, 1996; Kawabata and Iwanaga, 1999). Lectins in invertebrates play such a role in defense mechanism, recognizing foreign invaders, attacking their cells and mediating the biological activity themselves (Vasta et al., 1994).

As for crustacean lectins, a number of reports on purification and molecular characterization are now available (Marques and Barracco, 2000). In the present study, we detected the distribution of lectins in *Charybdis feriatus*, and furthermore, we isolated and purified them from the animal's humoral fluids.

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2 MATERIALS AND METHODS

2.1 Animals

Adult native crabs, *Charybdis feriatus*, males and females, were collected from the Dabai Is. (118º27'12"E, 24°33'57"N) of Fujian Province, China, in March 2002. After the collection, they were soon transferred to laboratory, and kept in large tanks with constant aeration under controlled temperature (26–28℃) and salinity (33–35). Only apparently healthy animals in inter-molt stage were used.

2.2 Preparation of humoral fluid and erythrocyte

Hemolymph was collected by shearing crab's feet. Before bleeding, the crab's feet were cleaned with 70% alcohol. Each crab was bled for only once. For humoral fluids preparation, the extracted hemolymph (pools of 5–10 animals) was coagulated for 16–24 h at 4℃. The clot was then centrifuged (2000×g) for 10 min at 4℃ and the supernatant was used as source of humoral fluids, and kept in refrigerator ready for use.

Animal erythrocytes (from sheep, rabbit, quail, chicken, snake, frog and fish, respectively) were collected in a sterile Alsever's solution (100 mmol/L glucose, 20 mmol/L NaCl and 30 mmol/L sodium citrate, pH 7.2) and kept at 4℃ for at most 1 week. Prior to their use in conventional agglutination assays (96-well microtitration plates), the red blood cells (RBC) were washed for three times by centrifuging (800×g for 10 min) with TBS (TBS: 50 mmol /L Tris–HCl, 0.15 mol /L NaCl, pH 7.4), and then suspended at 2% (V/V) in the same buffer.

2.3 Purification by ammonium sulfate and Sephadex G-100 column chromatography

Solid ammonium sulfate was slowly added into the humoral fluids up to 50% saturation at 4℃, and then centrifuged (12 000×g for 30 min). The precipitates were collected and continually extracted by ammonium sulfate with 50%, 45%, 40%, 35%, 30% saturation, respectively. The agglutination activity of each supernatant was detected after dialyzation.

The supernatant with strongest agglutination activity was called primary CFL (lectin from *Charybdis feriatus*), and it was collected and freeze-dried for use. Further purification of the primary CFL was performed on Sephadex G-100 (Pharmacia FineChem, Uppsala, Sweden) column (1.6×80 cm) chromatography as follows: 500 mg of the primary CFL was applied onto the column before dialyzation against TBS at 4℃ over-night. The column was previously equilibrated with TBS, at a flow rate of 1 ml/min with a maximal pressure of 40 bars. The protein was then eluted with TBS, and 1 ml fraction was collected. The optical density was monitored at 280 nm and the eluted peak was auto-recorded by the Nucleic Acid Protein Detector (HD-2000). Agglutinating activity was determined by the 2-fold dilution procedure in the presence of a 2% solution of quail erythrocytes in TBS. Each eluted peak was exhaustively dialyzed against distilled water and freeze-dried for further analysis. The fraction with the highest eluted peak was called purified CFL, and it was collected and detected for its proteins concentration, and then placed in refrigerator ready for use.

Polyacrylamide gel electrophoresis (PAGE/SDS) of humoral fluids, primary CFL and purified CFL fractions were carried out according to the method of Laemmli (1970), in a polyacryla-mide running gel (10%), under reduction and non-reduction conditions. The separated proteins were stained with silver nitrate according to the method of Blum et al. (1987).

2.4 The characterization of CFL

Agglutination assays were performed with erythrocytes from sheep, rabbit, quail, chicken, snake, frog and fish, respectively. Agglutination tests using serial dilution procedure were done in 96-well microtitration plates. Equal volumes of erythrocytes suspension were added to each well containing test solution. The plates was gently shaken and kept at room temperature for 2 hours. The resulting agglutinations were observed macroscopically and their activity was expressed as the minimum active concentration. All assays were carried out in duplicate. Agglutination inhibitions by sugars were assayed only with quail erythrocytes, using the same assay method as described above, but before adding 2% erythrocyte suspension, a solution (0.2 mol/L, 25 μl) of the following sugars were added: Lactose, D-Mannose, D-Fructose, D-Galactose, D-Arabinose, D-Glucose and Sucrose. The effect of temperature on agglutinating activity was tested by heating a 1 ml of test solution of each humoral fluids for 10 min, at intervals of 10℃, from

40℃ to 90℃in a small air-tight centrifuge tube. Each of the cooled solutions was tested in the same procedure for agglutinating activity using quail erythrocytes.

3 RESULTS

Substances with agglutinating activity towards vertebrate erythrocytes were detected in humoral fluids of crab *Charybdis feriatus*. Precipitated CFL in the crab's humoral fluids was collected with high saturation of ammonium sulfate. The experiment showed that after adding solid ammonium sulfate up to 50% saturation into the humoral fluids at 4℃, most CFL precipitated and deactivated. Some precipitate was dissolved inTBS-Ca^{2+} solution (TBS 0.01mol/L $CaCl_2$, pH7.4), and regained its activity by dialysis against distilled water at 4℃ over-night.The agglutination assay was performed with 2% (V/V) quail erythrocyte solution. Compared with the humoral fluids, the precipitates had 7-fold purification and 91.40% recovery of total activity.

The precipitates were continually extracted by ammonium sulfate in different saturations, and 40% $(NH_4)_2SO_4$ was the best concentration. The supernatant or called primary CFL fluid was given a 17.60-fold purification and 45.70% recovery of total activity and compared with the humoral fluids. By using Sephadex G-100 column chromatography, the curve of elution was built up (Fig.1). The agglutination titer of purified CFL reached 2^8. Compared to the humoral fluids, the final purified CFL reached 203.90-fold purification and 30.48% recovery of total activity (Table 1 and Fig. 2).

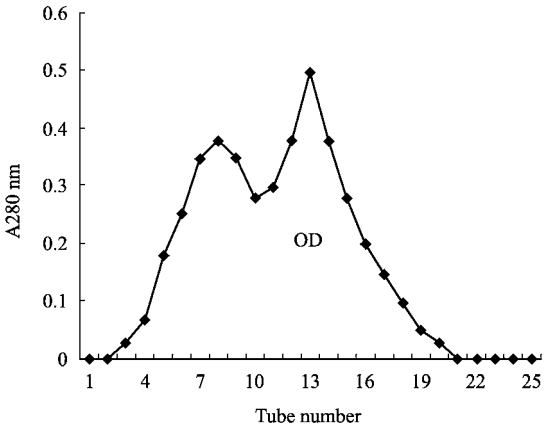

Fig.1 SephadexG-100 column chromatography

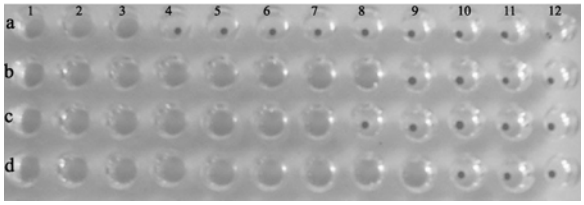

Fig.2 Agglutinating titer

a: Humoral serum; b: Precipitate of 50% $(NH_4)_2SO_4$; c: Supernatant of extracting by 40%$(NH_4)_2SO_4$; d: Activity apex eluted from Sephadex G-100 column chromatography

Table 1 The purification of CFL

Component	Total volume (ml)	Total Protein (mg)	Agglutinating titer (2^n)	Recovery of total Activities (%)	Specific activity (total activity/mg)	Purification
Humoral serum	420	974.4	2^2	100	1.72	1
Precipitate in 50% $(NH_4)_2SO_4$ saturation	12	127.2	2^7	91.4	12.08	7
Supernatant extracted by 40% $(NH_4)_2SO_4$ saturation	12	25.32	2^6	45.7	30.33	17.60
Purification measured Sephadex G-100	2	1.46	2^8	30.48	350.68	203.90

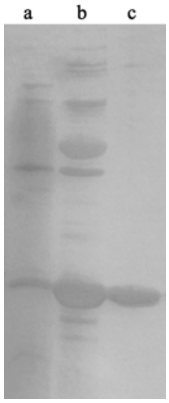

Fig.3 The result of different samples on SDS-PAGE

a: Humoral serum

b: Supernatant extracted in 40% $(NH_4)_2SO_4$ saturation

c: Activity apex eluted from Sephadex G-100 column chromatography

The final purified CFL showed a single band on SDS-PAGE, indicating that the purified CFL has reached high purity (Fig.3).

Agglutination assay results (Table 2.) show that the CFL could agglutinate all the erythrocytes tested. The minimum concentration of CFL protein for producing agglutination varied remarkably, ranging from 0.36 to 11.4 μg/ml. The maximum activity in the agglutination was to fish erythrocytes, and the minimum one was to that of sheep.

Results of anti-agglutination assays (Table 3) show that CFL could retain agglutination against quail erythrocyte when D-glucose was added. Therefore, CFL was not D-glucose sensitive. However, other 6 sugars could de-agglutinate the CFL against quail erythrocyte.

In terms of temperature, CFL could retain agglutination against quail erythrocyte until the test solutions were heated at 40℃ for 10 minutes. When heated at 50℃ for 10 minutes, the agglutination disappeared (Table 4).

Table 2 Agglutination activity of CFL using animal erythrocytes

Animal	Sheep	Rabbit	Quail	Chicken	Snake	Frog	Fish
Agglutination titer (2^n)	2^6	2^7	2^8	2^9	2^6	2^6	2^{11}
Total Protein* (μg)	11.4	5.70	2.85	1.43	11.4	11.4	0.36

*Contains the minimum concentration (μg/ml) that caused agglutination.

Table 3 Agglutination inhibition with quail erythrocyte with seven sugars

	Lac	D-man	D-fru	D-gal	D-glc	D-ara	Sur
CFL	+	+	+	+	-	+	+

+: Inhibition; -: no inhibition. The minimum concentration of sugar that showed inhibition was 100 mmol /L; Lac: lactose; D-man: D-mannose; D-fru: D-fructose; D-gal: D-galactose; D-glc: D-glucose; D-ara: D-arabinose; Sur: sucrose

Table 4 Influence of temperature on the agglutination activity of CFL against quail erythrocyte

Temperature	4℃	40℃	50℃	60℃	70℃	80℃	90℃
Charybdis feriatus	+	+	-	-	-	-	-

+: Agglutination; -: no agglutination

4 DISCUSSION

Insect and horseshoe crab often have coexisting multiple lectins having different sugar-binding abilities among species. However, this is quite limited in crustaceans. The first case of multiple lectins in crustacean was reported more than three decades ago in lobster *Homarus americanus* by Hall and Rowlands (1974). Two different plasma lectins, LAg-1 and LAg-2 were purified, but the occurrence of other lectins could not be excluded. These two lectins, which could be obtained in hemocyte extract or in plasma, reacted with human (LAg-1) and mouse erythrocytes (LAg-2). In *C. feriatus*, we found some substances with agglutination to vertebrate erythrocyte in muscle extraction and humoral fluids. However, due to technical limitation, we could be able to purified only one lectin from humoral fluids, namely CFL.

As far as lectin purification is concerned, more than 9 kinds of protein and enzyme have been purified from acid extract of cattle pancreas, using am-

monium sulfate in different concentrations (Department of Biology, Sun Yat-sen University, 1978). Zhao et al. (1992) first purified lectin from sea clam by precipitating with solid ammonium sulfate, and the precipitate was continually extracted by cold ammonium sulfate in different saturations, and at last a lectin with very high agglutination was obtained (Zhao et al., 1992). They considered this method as being facile and effective in purification of lectins from sea clam. However, we found the method not so satisfactory in purifying lectins from crabs and shrimps, since only one lectin was obtained from the crab *C. feriatus*, when the method was used to purify lectins from the humoral fluids of 12 species of crabs and shrimps.

In the characterization of CFL, results of agglutination assay showed that CFL could agglutinate against many kinds of animal erythrocytes. The agglutination of CFL against quail erythrocyte could not work by adding many types of sugars in 40℃ or higher for 10 min. in test solutions.

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