

Isolation and Partial Characterization of a Lectin from the Internal Organs of the Sea Cucumber (*Holothuria scabra* Jaeger)

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Abstract: The internal organs of the sea cucumber (*Holothuria scabra* Jaeger) were used as source of lectin. Lectin was isolated by extraction with 0.01 M Tris buffer (0.15 M NaCl at pH 7.5), ammonium sulfate fractionation and gel chromatography using Sephadex G-200. The isolated lectin was found to be non-blood type specific and non-blood group specific since it agglutinated all types of human blood as well as animal erythrocytes. Trypsin and calcium ions increase the isolated lectin's agglutinating activity. Optimum activity of the lectin was achieved between 20-50°C and at pH 6-8. Hapten inhibition studies did not show any specificity towards the nineteen sugar standards tested. The lectin is a glycoprotein containing 1.33% total sugars as determined by the phenol-sulfuric acid method. The molecular weight of the native lectin was estimated to be 355 kD using Sephadex G-200. SDS-PAGE revealed five sub-units with estimated molecular weights of 120, 98, 80, 65 and 51 kD.

Key words: Lectin, *Holothuria scabra*, glycoprotein, hemagglutination

INTRODUCTION

Lectins are carbohydrate-binding proteins or glycoproteins of non-immune origin, which are able to agglutinate cells and precipitate polysaccharides and glycoconjugates^[1]. Lectins are present in a wide range of organisms from bacteria to animal, being present in all class and families, although not in all the kinds and species^[2]. In invertebrates, lectins have been detected in hemolymph and coelomic plasma^[3-5]. Lectins have been implicated in several diverse physiological processes in invertebrates including host immune responses like non-self recognition, phagocytosis, encapsulation and hemocoelic clearance of foreign cells^[6].

They are of great interest because of various applications. Their carbohydrate-binding specificity enables their use in cell separation techniques, in isolation and purification procedures in biochemistry, cell biology, pharmacology, immunology and other related areas. It has been used in the isolation, purification and structural studies of carbohydrate-containing polymers^[2]. They are also used in human blood typing and in the characterization of the oligosaccharide structures of glycoconjugates present in many cell types^[7].

The numerous and diverse application of lectins in the different fields show the economic importance of

uncovering new source of lectins that are cheap and readily available. Sea cucumbers are echinoderms that live in relatively shallow areas of the ocean in areas with little or no current. Lectins have been found in several species of sea cucumbers like *Stichopus japonicus*^[3,8], *Cucumaria echinata*^[9], *Cucumaria japonica*^[10] and *Holothuria* sp.^[11]. The Philippines, being an archipelago, is a rich source of sea cucumbers. This study was done to isolate and partially characterize the lectin from the internal organs of sea cucumbers particularly *Holothuria scabra* Jaeger.

MATERIALS AND METHODS

Extraction: Composite samples of *Holothuria scabra* were collected from shallow waters of Bauan, Batangas, Philippines in the summer of 2002 and identified by the Bureau of Fisheries and Aquatic Resources (BFAR). The internal organs of the collected samples were separated and homogenized in an Osterizer blender with 0.1 M Tris Buffer Saline (TBS) containing 0.15 M NaCl at 1:5 (w/v) ratio. The homogenate was stirred for 6 h at 10°C and then filtered through a cheesecloth. The supernatant collected was then centrifuged at 10,000 rpm for 10 min at 10°C. Clarified extract was collected and stored in a freezer for subsequent analysis.

Isolation and purification: The crude extract was precipitated at 90% saturation with ammonium sulfate and allowed to stand overnight to separate the protein from other cellular components. The resulting precipitate was collected through centrifugation at 10,000 rpm at 10°C for 10 min and dissolved in minimum amount of buffer. The ammonium sulfate was removed by dialysis against a dilute solution of TBS using a Sigma dialysis tubing with a molecular weight cut-off of 12,000 at 10°C. The lectin was isolated from the protein by fractional precipitation using different ammonium sulfate saturation followed by gel chromatography using Sephadex G-200.

Agglutination assay: Agglutination assay using the crude extract, ammonium sulfate fractions and gel filtration eluates was carried out in multiwell microtiter plates using human erythrocytes (types A, B, AB and O) following the method of Goldstein *et al.*^[12]. Blood samples of carabao, chicken, calf and goat were also used. A 50 µL portion of the lectin solution was serially diluted two fold in TBS. The solution was added with 50 µL 2% (v/v) erythrocyte suspension in the same buffer. The plates were incubated for 1 h at room temperature and were examined visually for agglutination. A positive test was indicated by the formation of a uniform layer over the surface of the well. A negative test was indicated by the formation of a discrete button at the bottom of the wall.

Protein content determination: The protein content of the crude extract, the different ammonium sulfate fractions (supernatant and precipitate) and the fractions obtained from gel filtration was determined using the Bradford method^[13] with Bovine Serum Albumin (BSA) as standard.

Polyacrylamide Gel Electrophoresis (PAGE): The purified lectin was electrophoresed under non-denaturing conditions in a discontinuous polyacrylamide slab gel system following the method of Laemmli^[14]. The gel was stained with 0.1% Coomassie Blue R-250 in (4:1:5 v/v) methanol: acetic acid: water for 8 h at room temperature followed by destaining the gel for one hour in destaining solution I (50% methanol and 10% acetic acid) and in destaining solution II (5% methanol and 10% acetic acid) until proteins bands are clearly defined.

Characterization of the lectin: The effect of calcium on lectin activity was determined by adding 20 mM CaCl₂ on the lectin extract prior to agglutination assay. For the effect of trypsin, 1% trypsin was added to a 2% blood suspension at a ratio of 1:10 (v/v). The mixture was incubated for 1 h at 37°C and was used in the agglutination assay.

The optimum temperature for lectin activity was evaluated by incubating the purified lectin samples for 30 min in a water bath at different temperatures (0-90°C) prior to hemagglutination assay. For temperature below 20°C, the hemagglutination assay was done inside the cold cabinet.

The effect of pH on lectin activity was also determined by agglutination assay using 0.01M glycine-HCl buffer (pH 2.0, 3.0), 0.01 M acetate-acetic buffer (pH 4.0, 5.0), 0.01M KH₂PO₄-K₂HPO₄ buffer (pH 6.0, 7.0), 0.01 M Tris-HCl buffer (pH 8.0, 9.0), 0.01 M Na₂CO₃-NaHCO₃ buffer (pH 10.0, 11.0) and 0.01 M glycine-NaOH (pH 12.0). All buffers were made up in 0.15 M NaCl.

The effect of prolonged exposure to UV radiation was also investigated by subjecting the lectin to different time of exposure to UV light.

Hapten inhibition assay: The effect of soluble sugars on the agglutination of blood types AB, A, B and O was determined by the inclusion of different sugars and glycosides at 125, 250, 500 and 1000 mM in the agglutination assay. The following sugars were used: xylose, inositol, lactose, mannose, glucuronic acid, mucic acid, dulcitol, fucose, cellobiose, glucose, fructose, glucosamine, fucose, rhamnose, galactose, arabinol, arabinose, methyl mannopyranoside and benzoyl-DL-arginine-paramitrilide (BAPNA).

Carbohydrate analysis and chemical tests: Total carbohydrate content of the purified lectins was determined using the phenol-sulfuric acid method developed by Dubois *et al.*^[15]. Chemical tests like Molisch, Biuret, Benedict, lead acetate and Schiff's tests were performed to determine the chemical nature of the isolated lectin^[16].

Molecular weight determination: The molecular weight of the purified lectin was determined by gel permeation chromatography on Sephadex G-200. The molecular weights (kD) of the standard proteins used were: thyroglobulin, 669; β-Amylase, 200; Concanavalin A, 102; albumin, 45. A calibration curve was constructed by plotting elution volume (V_e) over void volume (V₀) against the logarithm of the Molecular Weight (MW) of the standard proteins.

SDS-PAGE following the method of Laemmli^[14] was used to determine the molecular weights of the lectin sub-units. The following proteins with their respective molecular weight were used as standards: myosin, 220 kD; α₂-macroglobulin, 170 kD; β-galactosidase, 116 kD; transferrin, 76 kD and glutamate dehydrogenase, 53 kD.

RESULTS AND DISCUSSION

Isolation and purification: Hemagglutination assay at different stages of purification was done using all human blood types. After using different ammonium sulfate concentration for precipitation, it was found out that agglutination was strongest at 0-60% ammonium sulfate concentration. Because of this, the 0-60% saturation point was considered and used for partial purification. At this level of saturation, much of the other proteins are eliminated while retaining most of the lectin in the precipitate.

Gel filtration on Sephadex G-200 of the 0-60% ammonium sulfate precipitate gave three peaks with the first one as the major peak (Fig. 1). The different fractions were freeze dried and then assayed for agglutination. Fractions found in the first two peaks exhibited agglutinating activity. Homogeneity of each fraction was determined by Polyacrylamide Gel Electrophoresis (PAGE) under denaturing condition and fractions showing identical single band were then pooled together. The agglutinating activity decreases as purification was achieved but the titer values decreased possibly because of a decrease in the protein content (Table 1). An increase in the specific activity was also noted which indicates increased purity of the lectin isolated.

Characterization

Sugar specificity: Sugar specificity of a lectin was determined by the hapten inhibition assay. The sugars that inhibit agglutination of lectin are the sugar specific for the lectin. This inhibitory effect of sugar can be attributed to their ability to compete for binding sites on the lectin molecule, which can interfere with the attachment of the lectin to sugar units on the surface of the erythrocytes.

Results of the hapten inhibition assay showed no inhibition of agglutination of the different sugars added at concentrations ranging from as low as 125 mM to as high as 1 M. This could only mean that the sugar determinants of blood types A, B and O which are N-acetyl-D-galactosamine, D-galactose and L-fucose respectively fit better on the binding sites of the lectin such that the test sugars were not able to displace them. Another possibility is the presence of multiple binding sites in the lectin such that it can interact with a wide variety of sugars or it is possible that the binding site is flexible on the size and shape of sugar residue^[17]. In addition, the results can be explained by the absence of structural features needed for sugar inhibition. Osawa^[18] gave the following structural features needed to be present in lectins for sugar inhibition to take place: (1) a

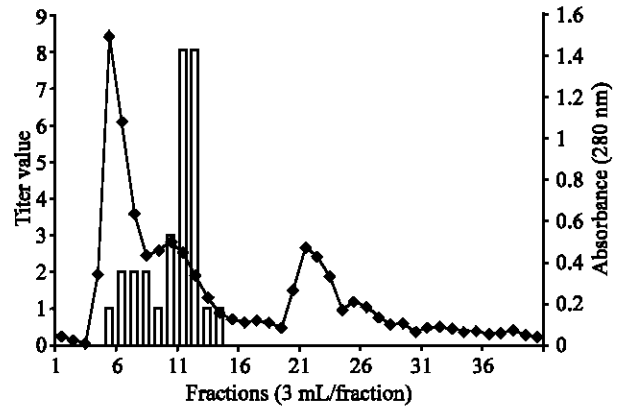


Fig. 1: Elution profile and titer value of fractions of 0-60% ammonium sulfate eluted by gel filtration using Sephadex G-200

non-reducing end sugar residue that is linked to the remainder of the molecule by a β -glycosidic linkage; (2) a glucosidic linkage of the non-reducing end sugar residue that is linked to a ring carbon atom of the next residue or a benzene ring without interposition of a methylene group and (3) a non-reducing sugar residue that have an unsubstituted hydroxymethyl group. It is therefore possible that the above features are not present resulting to non-inhibition of agglutination.

Blood specificity: The purified lectin was found to be non-blood type specific since it agglutinated all types of human erythrocytes (A, B, AB and O). Table 1 showed the agglutination assay at each stage of purification. It has been observed that agglutination assay showed a lower titer value for type B. This could mean the preference of lectin to N-acetyl-D-galactosamine and L-fucose, the sugar moieties present in type A and type O over D-galactose, the sugar moiety present in type B. Type AB both have the sugar moieties of both type A and type B.

The purified lectin is also non-blood group specific since it agglutinated animal blood (Table 2). It agglutinated calf, carabao, goat and chicken blood with the chicken blood having the lowest titer value. The titer values for animal blood are quite low in comparison with that of the human blood. The reason as cited by Merca and Reyes^[19] maybe due to the lesser number of receptor sites in animal red blood cells or incompatibility of these receptor sites with the lectin binding sites.

The non blood type and non blood group specificity of the isolated lectin suggests the presence of multiple binding sites such that it can interact with a wide variety of sugars or it is possible that the binding site is flexible on the size and shape of sugar residue^[17]. It could also be

Table 1: Purification of lectin present in the internal organs of black sea cucumber (*Holothuria scabra*)

| Stage | Blood type | Titer value | Protein content ($\mu\text{g mL}^{-1}$) | Total protein (mg) | Agglutination activity ($\mu\text{g mL}^{-1}$) | Specific activity | Purification fold |
|------------------------------------------------|------------|-------------|-------------------------------------------|--------------------|--------------------------------------------------|-------------------|-------------------|
| Crude extract | A | 16 | | | 17.36 | 57.62 | |
| | B | 8 | 277.7 | 27.77 | 34.72 | 28.84 | - |
| | AB | 16 | | | 17.36 | 57.62 | |
| | O | 16 | | | 17.36 | 57.62 | |
| 0-90% $(\text{NH}_4)_2\text{SO}_4$ precipitate | A | 64 | | | 17.92 | 55.79 | 1 |
| | B | 64 | 1147.1 | 11.47 | 17.92 | 55.79 | 2 |
| | AB | 64 | | | 17.92 | 55.79 | 1 |
| | O | 64 | | | 17.92 | 55.79 | 1 |
| 0-60% $(\text{NH}_4)_2\text{SO}_4$ precipitate | A | 64 | | | 16.50 | 60.61 | 1 |
| | B | 32 | 1056 | 8.45 | 33.00 | 30.30 | 1 |
| | AB | 64 | | | 16.50 | 60.61 | 1 |
| | O | 64 | | | 16.50 | 60.61 | 1 |
| G-200 eluate | A | 4 | | | 10.20 | 98.04 | 1.7 |
| | B | 2 | 40.8 | 0.0816 | 20.40 | 49.02 | 1.7 |
| | AB | 4 | | | 10.20 | 98.04 | 1.7 |
| | O | 4 | | | 10.20 | 98.04 | 1.7 |

Table 2: Titer value of *Holothuria scabra* lectin using animal erythrocytes

| | Chicken | Carabao | Cattle | Goat |
|-----------------------|---------|---------|--------|------|
| No. of positive wells | 3 | 4 | 4 | 4 |
| Titer value | 4 | 8 | 8 | 8 |

due to the flexibility of the sugar binding requirements that permits the binding of structurally related carbohydrates^[20].

The difference in agglutination activity could be attributed to several factors. Lis and Sharon^[2] mentioned the following factors that can affect agglutination: nature, number, distribution, exposure and mobility of receptors and fluidity and surface charges of the membrane. Aside from these, Nicolson^[21] cited the number and structure of surface receptors as other factors that could affect agglutination.

Effect of trypsin and calcium: Addition of trypsin is done to increase the susceptibility of erythrocytes to agglutination without affecting the total number of lectin binding sites^[2]. The increase in agglutinability maybe due to the exposure of the cryptic receptor sites on the surface of the cell membrane^[22] and the removal of close interfering structures by possible rearrangement of receptor sites on the erythrocytes^[21].

Results of the trypsin treatment on agglutination (Table 3) showed a slight increase in titer value. The isolated lectin from *H. scabra* Jaeger can therefore be classified as a complete lectin since it can agglutinate untreated erythrocytes, although agglutination increased with typosination.

Addition of Ca^{2+} on the other hand, resulted to an increase in the agglutination. On this basis, the *H. scabra* lectin maybe classified as a C-type lectin. C-type lectins are calcium-dependent animal lectins. Carbohydrate-

binding activity of C-type lectins is based on the function of the Carbohydrate Recognition Domain (CRD) whose structure is highly conserved among this family^[23]. Calcium is not only directly involved in the carbohydrate binding itself at the binding site^[24] but contributes to the structural maintenance of the lectin domain that is essential for the lectin activity^[25]. The C-type CRDs are incorporated in a variety of contexts of molecular organization. This fact may reflect the importance of carbohydrate recognition in diverse biological functions.

Most of the isolated lectins coming from marine invertebrates are C-type. Most of the invertebrate C-type lectins consist of only single C-type CRDs^[26,4], except for horseshoe crab (*Tachypleus tridentatus* Leach) factor C, a serine protease zymogen in the hemolymph coagulation system. This protein comprises several domains, including a serine protease domain and a C-type CRD whose carbohydrate-binding capacity is unclear^[27]. Muta *et al.*^[27] suggested that there might still be other C-type lectins with multiple domains in marine invertebrates. C-type CRDs in marine invertebrates show rather low homology with each other; sequence identities are generally below 40% even between closely related species. Each CRD has one carbohydrate-binding site, many of which have specificity toward galactose or N-acetylgalactosamine.

Lectins isolated from sea cucumbers, which are dependent on calcium, includes the two lectins from the coelomic plasma of *Stichopus japonicus* Selenka^[3], one from *Cucumaria echinata*^[5], another from the coelomic fluid of *Cucumaria japonica*^[10] and lastly one from the body wall of brown sea cucumber *Holothuria* sp.^[11].

Effect of temperature, pH and UV treatment: The heat stability of the purified lectin was studied by subjecting

Table 3: Effect of trypsin and Ca²⁺ on agglutination reaction of *Holothuria scabra* lectin

| Treatments | No. of positive wells | | | | Titer value | | | |
|--------------------------------|-----------------------|----|----|----|-------------|------|------|------|
| | A | B | AB | O | A | B | AB | O |
| Control | 5 | 4 | 6 | 5 | 16 | 8 | 32 | 16 |
| + Trypsin | 6 | 6 | 7 | 6 | 32 | 32 | 64 | 32 |
| + Ca ²⁺ | 10 | 11 | 12 | 9 | 512 | 1024 | 2048 | 256 |
| + Trypsin and Ca ²⁺ | 12 | 12 | 13 | 11 | 2048 | 2048 | 4096 | 1024 |

Table 4: Chemical tests on the isolated lectin

| Chemical tests | Observation | Conclusion |
|----------------|----------------------------------|-------------------------------------------|
| Molisch | Formation of purple interface | Presence of carbohydrates |
| Biuret | Formation of violet solution | Presence of protein |
| Schiff's | Formation of pink spot | Presence of aldehyde |
| Benedict's | Absence of brick red precipitate | Absence of reducing sugars |
| Lead acetate | Presence of black precipitate | Presence of sulfur-containing amino acids |

to different temperatures. The lectin is most stable at 20-50°C and it can therefore be isolated from *H. scabra* Jaeger at room temperature without denaturation of the lectin.

The lectin was found stable at a pH range of 3-11. The optimum pH is from 6-8 with blood type O exhibiting the maximum agglutinating activity. The lower activity at extreme pH could be due to the denaturation of lectin. Like the *Holothuria* sp. lectin isolated by Gana and Merca^[11], the isolated lectin had a marked stability over a wide range of pH. It had a different behavior compared to the lectin from *Tridacna derasa* Roding, which is unstable at pH below 6.2^[8].

Exposure to UV light showed that there was only a slight decrease in the activity as time exposure to UV light increases. Reduction of activity was only observed after exposing the sample at a wavelength of 254 nm after 12 h. Further reduction was observed after 24 h of UV exposure.

Carbohydrate analysis and chemical tests: The purified lectin was found to be a glycoprotein containing 1.33% total sugar as determined by the phenol-sulfuric acid method. Several chemical tests were conducted to further characterize the nature of the isolated lectin (Table 4). Positive results using Molisch test confirm the presence of carbohydrates in the isolated sample which further support the glycoprotein nature of the lectin. The presence of protein was confirmed based on the positive result of the Biuret test. Negative results were obtained in the Benedict's test which would indicate the absence of reducing sugars. However, hydrolyzate of the lectin gave positive result in the Benedict's test. This confirmed that glucose and galactose are the possible sugar components of the lectin as both are reducing sugars. Sulfur-containing amino acids are also present in the isolated sample since black precipitate was formed upon addition of lead acetate.

Molecular weight: The molecular weight of the native lectin was estimated using gel permeation



Fig. 2: SDS-PAGE of lectin from internal organs of *Holothuria scabra* Jaeger purified by gel chromatography. (A-MW markers, B-isolated lectin, 5 sub-units namely A=120, B=98, C=80, D=65 and E= 51 kD)

chromatography to be 355 kD. SDS-PAGE of the isolated lectin yielded five bands (Fig. 2) with molecular weights of 120, 98, 80, 65 and 51 kD. The calculated total molecular weight is 414 kD which is higher in comparison to the molecular weight of the native lectin.

The molecular weight obtained was comparable to one of the lectins that was isolated from the coelomic plasma of *Stichopus japonicus* Selenka. The two lectins isolated from this species have molecular weights of about 400 (*Stichopus japonicus* lectin 1 or SPL-1) and 60 kDa (SPL-2)^[14]. The brown sea cucumber lectin on the other hand, has a molecular weight of 305 kDa using

Sephadex G-200 and contain sub-units with molecular weights of 145, 87, 69 and 44 kDa on SDS-PAGE^[11].

CONCLUSIONS

A lectin was isolated from the internal organs of black sea cucumber, *Holothuria scabra* Jaeger by extraction with 0.01M TBS pH 7.5, ammonium sulfate fractionation and gel chromatography using Sephadex G-200. The isolated lectin is a non-blood type and non-blood group specific lectin since it agglutinated all human blood types and animal erythrocytes. Addition of calcium and trypsin increased the agglutinability of the lectin. Optimum activity of the lectin was achieved between 20-50°C and at pH 6-8. The native molecular weight was estimated to be 355 kD using Sephadex G-200. SDS-PAGE of the lectin yielded five sub-units of molecular masses of 120, 98, 80, 65 and 51 kD. Chemical tests like Biuret, Molisch and Schiff's tests indicated that the isolated lectin is glycoprotein with 1.33% total sugar.

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