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Lectin from the Body Walls of Black Sea Cucumber (*Holothuria atra* Jaeger)

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A survey of lectin activity was made on the crude extracts of 15 species of locally available marine invertebrates. Thirteen species were found to show lectin activity. The body wall of Holothuria atra Jaeger, which had the highest lectin activity, was chosen for further examination.

Lectin was extracted from the body walls of black sea cucumber (*Holothuria atra* Jaeger) using Tris buffer saline (TBS) pH 7.5 containing 0.15 M NaCl and purified by ammonium sulfate fractionation and gel chromatography using Sephadex G-200. The isolated lectin was non-blood type specific as it agglutinated erythrocytes of all human blood types (A, B, AB, O) and animal erythrocytes (calf, carabao, chicken and goat). Addition of trypsin and calcium ions increased the agglutinating activity of both lectins.

The isolated lectin was found to be glycoprotein containing 0.50% total sugar. Based on gel chromatography, the estimated molecular weight of lectin from the body wall is 439 kD. SDS-PAGE gave two protein bands for the isolated lectin with molecular weights estimated to be 113 kD and 77 kD.

INTRODUCTION

Lectins are carbohydrate-binding proteins or glycoproteins of non-immune origin, which are able to agglutinate cells and precipitate polysachaccharides and glycoconjugates (Goldstein et al. 1980). 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 (Lis and Sharon 1986). They are also used in human blood typing and in the characterization of the oligosaccharide structures of glycoconjugates present in many cell types (Sharon and Lis 1987).

Lectins have been described in plant, microorganisms, vertebrates and invertebrates (Lis and Sharon 1986). A variety of hemaglutinnins have been isolated in the coelomic plasma and coelomocytes of invertebrates (Brown et al. 1968; Giga et al. 1987). One of the roles of marine invertebrate lectins is to act as humoral factors in the defense mechanism, as do immunoglobulins in vertebrates. This is suggested from some observations such as the activation of phagocytes by the binding of lectin to foreign cell (opsonin activity) or the enhancement of lectin production in body fluids after injection of foreign substances (Hatakeyama et al. 1995). Lectins are also involved in processes in marine invertebrates such as non-self recognition, inflammation, cell-cell or cell-extracellular matrix interactions, fertilization, development and regeneration.

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Sea cucumbers are small marine animals belonging to the phylum Echinodermata. They 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. They have been isolated from *Stichopus japonicus* (Hatakeyama et al. 1993; Matsui et al. 1994), *Cucumaria echinata*, (Hatakeyama et al. 1994) and *Cucumaria japonica* (Bulgakov et al. 2000). Locally, a sialicacid-specific lectin exhibiting mitogenic and cytotoxic activity was isolated from the body wall of brown sea cucumber (Gana and Merca 2002). This study was done to isolate, purify and partially characterize lectin from the body walls of *Holothuria atra* Jaeger.

MATERIALS AND METHODS

Materials

Fifteen (15) species of marine invertebrates were collected from Oriental Mindoro, Batangas, Quezon and Cavite, Philippines, and were used in the preliminary survey (Table 1). Voucher specimens of the samples were kept in the laboratory.

Proximate Analysis

Proximate analysis of the body walls of sea cucumbers was done using the standard procedures of the Association of Official Analytical Chemists (AOAC 1993).

Isolation and Purification

The body walls from fresh composites samples of *Holothuria atra* were homogenized in an Osteorizer blender with 0.1M Tris buffer saline (TBS) at 1:5 (w/v) ratio. Extraction was continued by stirring both homogenate at 10°C for 6 h. The homogenate was then filtered through a cheesecloth. The collected supernatant 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.

The crude extract was precipitated at 90% saturation with ammonium sulfate using Cooper's nomogram (1971). The resulting precipitate was collected through centrifugation at 10,000 rpm at 10°C for 10 min. The collected precipitate was dissolved in a minimum amount of buffer and dialyzed against a dilute solution of TBS using a Sigma dialysis tubing with a molecular weight cut-off of 12,000 at 10°C. Polyethylene glycol was added to concentrate the dialyzed sample.

The concentrated sample was applied to Sephadex G-200 column and eluted with 0.01M TBS pH 7.5 Eluted fractions of 2.5 mL were collected, monitored for absorbance at 280 nm and assayed. The homogeneity of fractions showing agglutinating activity was determined by performing native polyacrylamide gel electrophoresis (PAGE) under non-denaturing condition. Fractions with same PAGE profiles were pooled, lyophilized and used for further characterization.

Table 1. Marine invertebrates collected in summer of 2003 and used for preliminary screening for the presence of lectin

Scientific Name	Common Name	Place of Collection
Diadema sp.	sea urchin	Puerto Galera, Oriental Mindoro
Holothuria scabra Jaeger (body wall)	black sea cucumber	Bauan, Batangas
Tripneustes sp.	sea urchin (spineless)	Puerto Galera, Oriental Mindoro
Murex sp.	murex	Tanza, Cavite
Strombus sp.	strombus	Tanza, Cavite
Peneaus sp	shrimp	Infanta, Quezon
Nerita planospira Anton	nerite snail/sihi	Infanta, Quezon
Telecopium telesopium	telescopium	Tanza, Cavite
Paphia undulata Born	small paros	Infanta, Quezon
Placuna placenta	kapiz	Tanza, Cavite
Spondylus princeps Broderip	thorny oyster	Tanza, Cavite
Tellina sp.	amatilis	Tanza, Cavite
Venus mercenaria L.	round clam	Tanza, Cavite
Soletellina cumingiana	kabibe	Tanza, Cavite
Loligo pealli	squid/pusit	Infanta, Quezon

Agglutination Assay

Agglutination assay was carried out in multiwell microtiter plates using whole human blood (types A, B, AB and O). A 50-μL portion of the lectin solution was serially extracted two-fold in Tris buffer solution. 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. A positive test was indicated by the formation of a uniform layer over the surface of the well. On the other hand, a negative test was indicated by the formation of a discrete button at the bottom of the wall. The blood samples of carabao, chicken, calf and goat were also tested for agglutination.

Protein Content Determination

The Bradford method (1976) was used in determining the protein content of the crude extract, ammonium sulfate precipitate and the fractions obtained from gel filtration with bovine serum albumin (BSA) as standard. Different concentrations of protein standard (BSA) were plotted against their corresponding absorbance to obtain a standard curve. The protein content of the sample was calculated based from the standard curve.

Polyacrylamide Gel Electrophoresis (PAGE)

The collected gel filtration fractions or purified lectins were subjected to electrophoresis under non-denaturing conditions in a discontinuous polyacrylamide slab gel system following the method of Laemmli (1970). The gel was stained with 0.1% Coomasie 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 1 h 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 Lectins

Effect of Ca^2+ and Trypsin on Lectin Activity

The effect of calcium on lectin activity was determined by adding 20 mM $CaCl_2$ 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.

Hapten Inhibition Assay

The effect of soluble sugars on the agglutination of blood types A, B, AB and O or hapten inhibition assay was done to determine the sugar that would inhibit agglutination. The following sugars, having an initial concentration of 1000, 500 and 250 mM, were used: D(+) xylose, inositol, α-lactose, D(+) mannose, D-glucuronic acid, mucic acid, dulcitol, L(-) fucose, D(+)cellobiose, D(+)glucose, β-D(-) fructose, glucosamine, L(-)fucose, α-L-rhamnose, D(+) galactose, D(+) arabitol, L(+) arabinose, methyl α-D-mannopyranoside and BAPMA.

The inhibition assay was carried out in multiwell microtiter plates. To each 50 μL of a two-fold serial dilution of the sugar solution, an equal volume of the lectin solution was added. This was followed by the addition of 50 μL of 2% (v/v) erythrocyte suspension. The solution was examined after incubation for 1 h at room temperature.

Determination of Total Carbohydrate Content

Total carbohydrate content of the purified lectin was determined using the phenol-sulfuric acid method developed by Dubois et al. (1956). Molisch and Schiff's tests were also performed to determine if the fractions containing lectin were glycoprotein.

Molecular Weight Determination

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

SDS-PAGE following the method of Laemmli (1970) was employed using 10% polyacrylamide separating gel. The molecular weights (in kilodalton) of the protein standards used to determine the approximate molecular weights of the lectin sub-units were as given: myosin, 220; α2-macroglobulin, 170; β-galactosidase, 116; transferrin, 76 and glutamate dehydrogenase, 53.

RESULTS AND DISCUSSION

Fifteen (15) species of marine invertebrates were assayed for lectin activity. From the results (Table 2), thirteen species were found to contain lectin, of which seven agglutinated all human blood types. The body wall of black sea cucumber (*Holothuria atra*) had the highest titer value and was used for further isolation and purification of the lectin.

Table 2. Preliminary screening for lectin activity of some marine invertebrates

Scientific Name	English/Common Name	Crude Extract				Crude Extract + Ca^{2+}			
		A	B	AB	O	A	B	AB	O
Diadema sp.	sea urchin	0	0	0	0	8	0	0	0
Holothuria atra Jaeger (body wall)	black sea cucumber	32	32	32	32	256	256	256	256
Tripneustes sp.	sea urchin	0	0	0	0	2	16	4	16
Peneaus sp	shrimp	0	0	0	0	0	0	0	0
Nerita planospira Anton	nerite snail/sihi	0	0	0	1	8	16	8	16
Telecopium telesopium	telescopium	8	8	8	8	32	32	32	32
Paphia undulata Born	small paros	0	0	0	0	0	0	0	0
Placenta sella	kapiz	1	1	8	4	16	16	32	16
Placuna placenta	kapiz	2	1	8	8	32	16	32	32
Spondylus princeps Broderip	thorny oyster	1	2	1	1	8	4	1	16
Strombus sp.	strombus	2	1	0	2	2	1	0	4
Murex sp.	murex	0	0	0	0	0	0	0	0
Tellina sp.	amatilis	0	0	2	0	0	0	4	0
Venus mercenaria L.	round clam	0	0	0	1	2	1	0	1
Soletellina cumingiana	kabibe	0	0	0	0	0	0	0	1
Loligo pealli	squid	0	1	0	0	2	1	0	0

Proximate Analysis

Proximate analysis of the body wall of *Holothuria atra* showed very high moisture content (Table 3). Defatting was not performed because of the low crude fat content of the samples. In addition, the presence of fats can prevent hemagglutination by destabilization of intermolecular forces (e.g. H-bond and van der Waals interaction), which is believed to be formed between protein and saccharide residues (Naismith and Field 1996) leading to inhibition of agglutination. On the other hand, Tsivion and Sharon (1981) observed false agglutination introduced by certain lipids using rabbit and rat erythrocytes.

Table 3. Proximate analysis of the body wall and internal organs of *Holothuria atra*

Proximate Analysis	Body wall
Moisture content (%)	94.00
Crude fat (%)	0.04
Crude protein (%)	4.79
Crude fiber (%)	0.11
Ash content (%)	0.73

Purification

The crude extract was first subjected to 0-90% saturation with ammonium sulfate to eliminate all the undesired proteins and to keep the lectin in stable form to prevent possible denaturation.

Gel filtration of the crude extract that passed through a Sephadex G-200 column yielded only a single peak (Figure 1). The fractions under the peak were freeze-dried and then assayed for agglutination. All fractions under the peak showed agglutination activity. Single band with the same PAGE profiles or Rf values were obtained in the positive fractions.

Result of the purification step by gel chromatography of *Holothuria atra* body wall lectin is presented in Table 4. The agglutinating activity decreases as purification was achieved but the titer values decreased because of a decrease in the protein content. An increase in the specific activity was also noted which indicates increased purity of the lectin isolated.

Characterization of the Lectins

The purified lectin was found to be non-blood type-specific since it agglutinated all types of human erythrocytes (A, B, AB and O). The lack of blood type specificity could be caused by the inability of the lectin to distinguish between α-and β-linked saccharide determinants, since the sugar determinants in blood group substances are α-linked (Sharon and Lis 1974). The sugar determinants for the different blood types are the following: α-linked N-acetylgalactosamine for Type A, α-linked D-galactose for Type B and α-L-fucose for Type O. It is also possible that the isolated lectin has independent binding sites other than for the saccharides specific for blood types, which can be

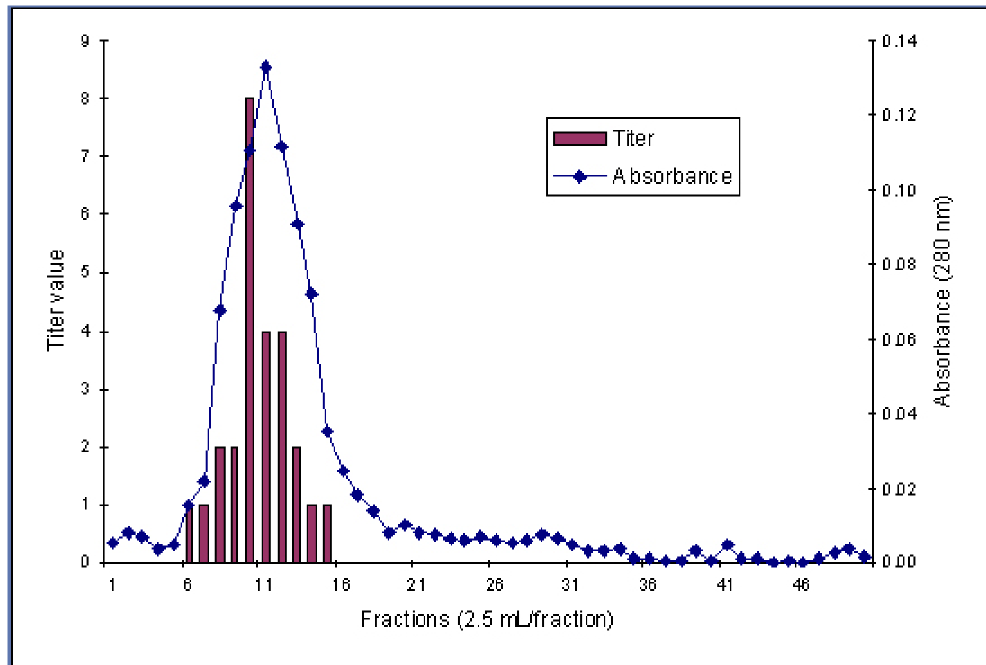

Figure 1. Elution profile and titer value of fractions of body wall extracts using Sephadex G-200

Table 4. Agglutination activity of *Holothuria atra* lectin at different stages of purification

Sample	Protein content (mg mL^{-1})	Total protein (mg)	Blood type	Titer[1]	Agglutination Activity[2] (µg ml^{-1})
Crude Extract	0.76	378	A	32	23.6
			B	32	23.6
			AB	32	23.6
			O	32	23.6
0-90%	1.68	16.8	A	128	13.1
			B	128	13.1
			AB	128	13.1
			O	128	13.1
Gel chromatography	0.03	0.06	A	4	7.8
			B	4	7.8
			AB	4	7.8
			O	4	7.8

[1]Titer – reciprocal of maximal dilution of the lectin extract that causes agglutination
[2]Agglutination activity – least concentration of lectin needed to cause detectable agglutination

used for attachment to the erythrocyte surface (Merca and Reyes 1989). In addition, the isolated lectin was also non human blood group specific as they also agglutinated animal erythrocytes (Table 5).

An increased in titer values in all blood groups was observed upon addition of calcium ions (Table 5 and 6). It may be possible that the isolated lectin is a C-type lectin. C-type lectins are usually calcium dependent (Drickamer 1988). Calcium is not only directly involved in the carbohydrate binding itself at the binding site (Weis et al. 1992) but contributes to the structural maintenance of the lectin domain that is essential for the lectin activity (Kimura et al. 1995). 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 lectins isolated from sea cucumbers are C-type (Matsui et al. 1994; Bulgakov et al. 2000) and consist of only single C-type carbohydrate recognition

Table 5. Titer value[1] of *H. atra* body walls lectin using animal erythrocytes

Treatment	Chicken	Carabao	Cattle	Goat
Control	32	32	16	32
+ Trypsin	64	64	64	64
+ Ca^{2+}	128	128	128	128
+ Trypsin and Ca^{2+}	512	512	512	512

[1] Titer – reciprocal of maximal dilution of maximal dilution of the lectin extract that caused agglutination

domains (CRDs) (Hatakeyama et al. 1995; Takagi et al. 1994), except for horseshoe crab (*Tachypleus tridentatus*), which is composed of several domains (Muta et al. 1991).

Trypsinization also showed a slight increase in titer value of the isolated lectin (Table 6). Trypsin is one of the proteolytic enzymes that acts specifically on the peptide bonds of basic amino acid (Murray et al. 1990). This is added to increase the susceptibility of erythrocytes to agglutination without affecting the total number of lectin binding sites (Lis and Sharon 1986). The increase in agglutinability of human erythrocytes with trypsin treatment has been attributed to exposure of additional agglutination receptor sites which are believed to be in a "cryptic" form on untreated cells or rearrangement of pre-existing receptor sites that interfering structures close to the binding sites are removed giving rise to a topological distribution which is more favorable for agglutination (Gordon et al. 1972) and the removal of close interfering structures by possible rearrangement of receptor sites on the erythrocytes (Nicolson 1971). The isolated lectin can therefore be classified as a complete lectin since it can agglutinate untreated erythrocytes, although agglutination increases with trypsinization.

Hapten Inhibition Assay

No inhibition was observed in any of the sugars used in the hapten inhibition assay. 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 residues 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 (Lacsamana and Merca 1994). It can also be due to the flexibility of the sugar binding requirements that permits the binding of structurally related carbohydrates (Singh et al. 1993).

Table 6. Effect of Ca^{2+} and trypsin on agglutination reaction of *Holothuria atra* lectin

Treatment	Human blood type			
	A	B	AB	O
Control	32	32	32	32
+ Trypsin	64	64	64	64
+ Ca^{2+}	256	256	256	256
+ Trypsin and Ca^{2+}	1024	1024	1024	1024

Determination of Total Carbohydrate Content

Using D-glucose as standard, the *H. atra* body wall lectin was found to be a glycoprotein containing 0.50% total sugar. This was confirmed by the positive results in Molisch test and Schiff's test.

Molecular Weight Determination

Gel filtration using Sephadex G-200 gave an approximate molecular weight of 439 kD for the isolated lectin (Figure 2). SDS-PAGE for the lectin gave two protein bands with molecular weights estimated to be 113 kD and 77 kD (Figure 3). It is possible that the lectin from the body wall is a tetramer.

The molecular weight of the isolated lectin was comparable to the lectin that was isolated from the coelomic plasma of *Stichopus japonicus* Selenka. The two lectins isolated from this species have a molecular weight of about 400 kD (SPL-1) and 60 kD (SPL-2) on gel filtration. However, on SDS-PAGE, both lectins yield a single band with a molecular weight of 17 kD (Matsui et al. 1994). This is higher than the lectin isolated from the body wall of brown sea cucumber which has a molecular weight of 305 kD using Sephadex G-200 and contain sub-units with molecular weights of 145 kD, 87 kD, 69 kD and 44 kD on SDS-PAGE (Gana and Merca 2002). In addition, this is also higher than the lectins isolated from *Stichopus japonicus* Selenka (Hatakeyama et al. 1993), *Cucumaria echinata* (Hatakeyama et al. 1994) and *Cucumaria japonica* (Bulgakov 2000).

The differences in the molecular weight obtained from the two methods can be attributed to the nature of glycoproteins. A study explained that some glycoproteins containing substantial amounts of carbohydrate are known to act in an anomalous manner during the said separation techniques (Wittenbach 1983). Gel

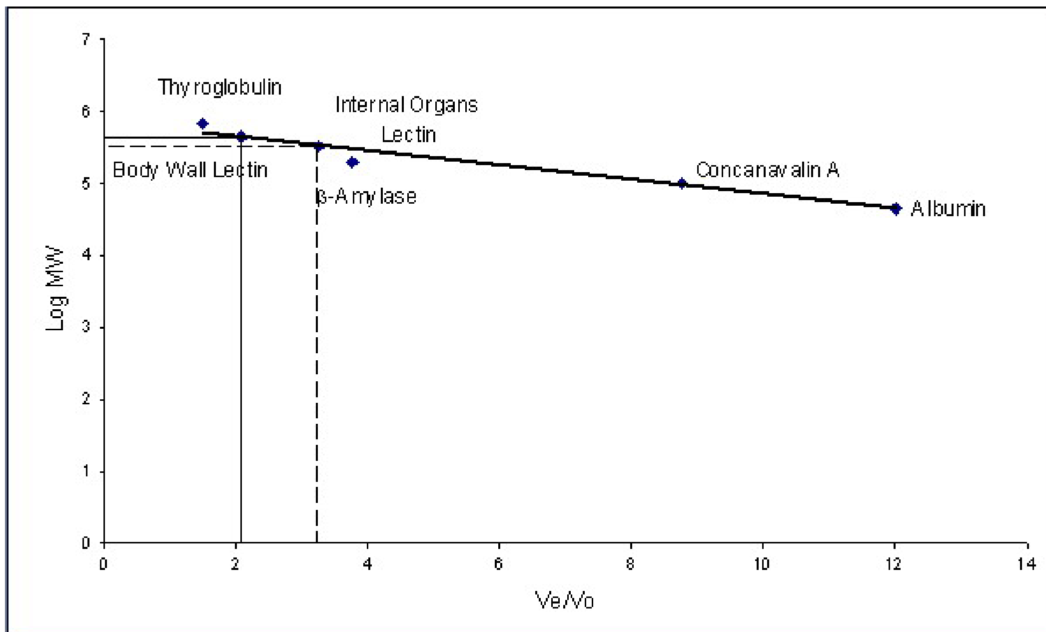

Figure 2. Calibration curve for the estimation of the molecular weight of the *Holothuria atra* lectin by gel filtration using Sephadex G-200

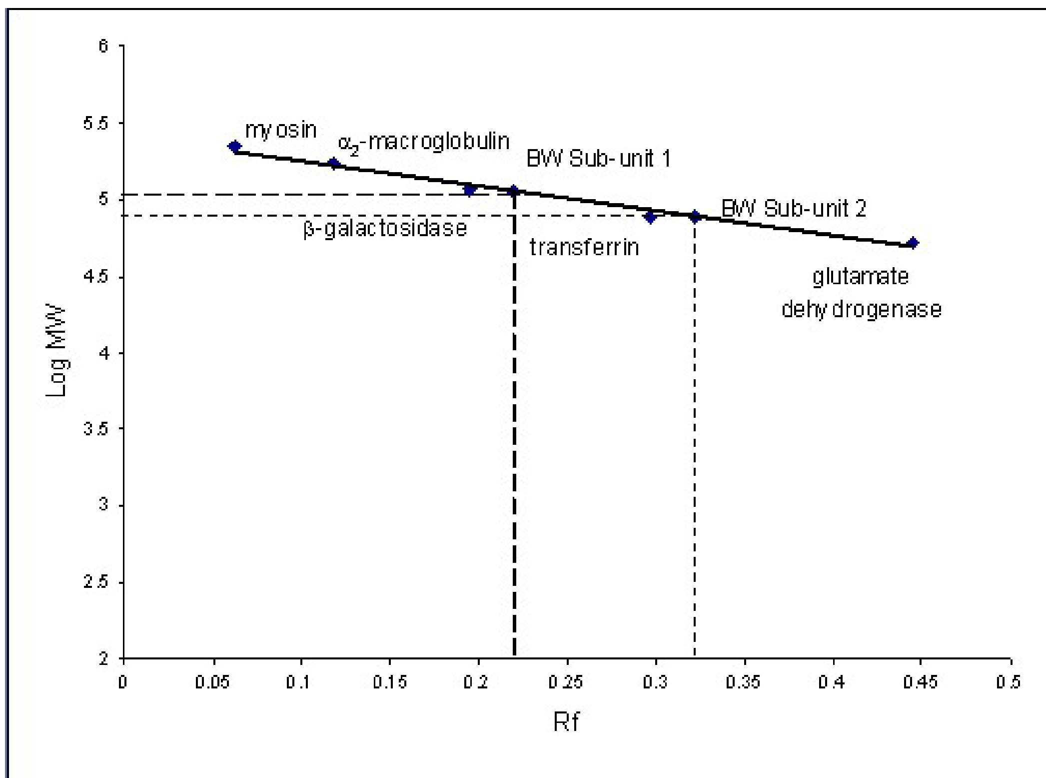

Figure 3. Calibration curve for the estimation of the molecular weight of lectin from the body wall of *Holothuria atra* by SDS-PAGE

filtration is based on the relative period native proteins of varying molecular sizes stay within the interstices of sephadex or dextran, which compose the gel (Hagel 1989) while SDS-PAGE, on the other hand, separates denatured proteins based on their capacity to traverse the gel network.

In addition, molecular weight determination using SDS-PAGE is not directly applicable for glycoproteins since these contain relatively large amounts of carbohydrates and behave anomalously using SDS-PAGE when compared to standard proteins. This is because of a decreased charge to mass ratio for glycoproteins, which results from lower SDS binding. As a consequence, there is decreased mobility during SDS-PAGE and thus a higher apparent molecular weight (Segrest and Jackson 1972).

CONCLUSION

Lectin was isolated from the body walls of the sea cucumber, *Holothuria atra*. Isolation was done by extraction with 0.01 M TBS and subsequent purification by ammonium sulfate precipitation and gel chromatography. The lectin was found to be non blood type specific and non human blood group specific since it agglutinated all types of human erythrocytes (A, B, AB and O) as well as animal erythrocytes. Addition of both trypsin and calcium increased the agglutinating activity of the isolated lectin.

The isolated lectin is a glycoprotein containing 0.50% total sugars. The specificity of this lectin, however, was not directed towards any of the sugars used in this study. The estimated molecular weight of lectin from the body wall is 439 kD by gel filtration and SDS-PAGE gave two protein bands with molecular weights estimated to be 113 kD and 77 kD.

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