Fish & Shellfish Immunology 27 (2009) 1–8

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Purification and characterization of an *N*-acetylglucosamine specific lectin from marine bivalve *Macoma birmanica*

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A R T I C L E I N F O

Article history:
Received 5 October 2007
Received in revised form
17 October 2008
Accepted 6 November 2008
Available online 17 November 2008

Keywords:
Macoma birmanica
Lectin
Antibacterial effect
Marine bivalve
ELLSA

A B S T R A C T

A calcium independent lectin of molecular mass 47 kDa was isolated from the foot muscle of marine bivalve *Macoma birmanica* by ammonium sulphate precipitation followed by affinity chromatography on immobilized GlcNAc column and designated as *M. birmanica* agglutinin (MBA). The lectin agglutinated rabbit erythrocytes strongly compared to human erythrocytes over a wide pH range from 5 to 9 and up to 50 °C. MBA is a glycoprotein and consists of 7.63% sugar. Among the tested sugars for analysis of carbohydrate recognition properties, Me-βGlcNAc was the most potent inhibitor followed by Me-αMan. Enzyme linked solid phase assay revealed that MBA interacted well with complex type N-linked glycans and moderately to high mannose type N-linked glycans. Fluorescence study of MBA indicated that tryptophan was present in a non-hydrophobic region and its binding to GlcNAc was neither quenched nor altered λ_{max} position. The denaturation of MBA induced by urea was a reversible process and urea could not significantly change the Trp environment. MBA interacted with both Gram-positive and Gram-negative bacteria by recognizing their surface exposed GlcNAc containing antigens.

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1. Introduction

Host defence of invertebrates against microbial infection solely depends on the innate immune system that differs from vertebrates [1]. Due to the lack of acquired immune response, invertebrates respond through pattern-recognition proteins (PRPs) that bind conserved pathogen associated molecular pattern molecules (PAMPs) expressed as the array of carbohydrate components on the surface of microorganisms. Lectins are one of the important PRPs that trigger a series of protective immune responses by interacting with PAMPs [1]. Lectins are sugar-specific proteins or glycoproteins of non-immune origin capable of agglutinating cells or precipitating glycoconjugates in solution [2]. Animal lectins are involved in multifarious functions like cell–cell interactions, protein trafficking, and defence system against foreign invaders by specifically recognizing a glycoside sequence or a cell surface exposed glycoconjugate, and in glycoproteins, glycolipids, bacterial lipopolysaccharides or peptidoglycans [3].

The role of several invertebrate lectins as mediators of nonself recognition in the innate immune response against invading microbes and foreign substances has been well documented [4]. The endogenous lectins in the cockroach hemolymph are capable of acting as nonself recognition molecules for a wide range of microorganisms [5]. Immunolectin-2 from *Manduca sexta* induced its function as a pattern-recognition protein specific for Gram-negative bacteria through its interaction with LPS [6]. Lectins derived from hemocytes and hemolymph of the Japanese horseshoe crab, *Tachypleus tridentatus* showed on host defence against invading microbes and foreign substances [7]. However, very few lectins from molluscs were isolated and classified with their defensive roles. Tridacnins, a family of C-type lectin, found in the hemolymph of giant clam, *Hippopus hippopus*, primarily removed and digested the clam symbiotic algae [8]. Heparin binding lectin, Anadarin MS from the plasma of *Anadara granosa* demonstrated the agglutinating activity towards the infective promastigotes of *Leishmania donovani* [9]. It has been reported that Manila clam (*Ruditapes philippinarum*) after infected with the protozoan parasite

Abbreviations: MBA, *Macoma birmanica* agglutinin; TBS, tris-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; ESI-MS Q TOF, electron spray ionization mass spectrometry with quadruple time of flight; TBS-T, TBS containing 0.05% Tween 20; HRP, horseradish peroxidase; PTG, porcine thyroglobulin; BTG, bovine thyroglobulin; BSM, bovine submandibular mucin; SBA, soybean agglutinin; PRP, pattern-recognition proteins; CRD, carbohydrate recognition domain; A and AA GP, asialo and asialoagalacto glycoproteins.

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doi:10.1016/j.fsi.2008.11.001

Perkinsus olseni expressed different lectins in hemocytes as immune response [10].

In the present study, we have reported isolation of a new lectin from the foot muscle of a marine bivalve *Macoma birmanica* and it was characterized including sugar specificity. The binding ability and growth inhibition activity of this lectin with pathogenic bacteria *Escherichia coli* O142, *Bacillus subtilis*, *Staphylococcus aureus* and *Vibrio parahaemolyticus* KX-V 138 (O3:K6) are also described herein.

2. Materials and methods

2.1. Materials

Methyl-α-glucose, methyl-β-glucose, 3-*O*-methyl-glucose, methyl-α-*N*-acetylglucosamine and methyl-β-*N*-acetylglucosamine were kindly donated by Prof. N. Roy, Indian Association for the Cultivation of Science, Kolkata, India. Other sugars, sugar derivatives, glycoproteins and enzymes were purchased from Sigma, USA. *B. subtilis*, *E. coli* O142 and *S. aureus* were obtained from Prof. A.K. Guha of Indian Association for the Cultivation of Science, Kolkata, India. *V. parahaemolyticus* KX-V 138 (O3:K6) was supplied by Dr. A. Sen Jr., Indian Institute of Chemical Biology, Kolkata, India. All other chemicals used were of highest purity available.

Asialo and asialoagalacto glycoproteins were prepared from bovine submandibular mucin (BSM), porcine and bovine thyroglobulin (PTG and BTG). The glycoproteins (10 mg) in 50 mM sodium acetate buffer, pH 5.5 were incubated with neuraminidase (0.1 units) at 37 °C for 24 h [11]. The enzyme was deactivated by heating at 100 °C for 5 min and centrifuged. The supernatants were dialyzed against water and lyophilized. Asialo glycoproteins were separated by gel filtration. Five milligrams asialo glycoproteins of bovine and porcine thyroglobulin were dissolved in 0.1 M phosphate buffer saline (PBS), pH 7.3 containing 1.0 mM $MgCl_2$ and 100 mM 2-mercaptoethanol and incubated with β-galactosidase (2 units) at 37 °C for 24 h [12]. The enzyme was deactivated and asialoagalacto glycoproteins were isolated as before.

2.2. Purification of M. birmanica lectin (MBA)

For the purification of the lectin, *N*-acetylglucosamine was immobilized on Sepharose 4B following the procedure of Porath and Ersson [13] by divinyl sulphone activation method.

M. birmanica bivalves were collected from the intertidal mud flat of the Chemagari Creek of Sagar Island, Sundarban, West Bengal, India. The foot muscle was separated by a scissor and preserved at −20 °C.

The foot muscles of *M. birmanica* (20 g) were homogenized in presence of 1 L TBS (20 mM Tris–HCl, 150 mM NaCl, pH 7.2). The extract was clarified by centrifugation at 40,000 g for 30 min. The supernatant was subjected to 0–50% ammonium sulphate precipitation and centrifuged. The protein pellet was dissolved in TBS and dialyzed extensively against the same buffer. The dialyzate was filtered through 0.2 μm filter and percolated slowly through GlcNAc-Sepharose 4B column (20 cm × 1 cm) pre-equilibrated with TBS. The column was washed extensively with the same buffer until the OD at 280 nm was below 0.005. The bound protein was specifically eluted with 0.4 M glucose in TBS. The affinity-purified lectin was dialyzed against TBS and concentrated by ultrafiltration (YM 10). Protein concentration was measured following the method of Lowry [14] using BSA as the standard.

2.3. Physico-chemical properties, composition and structure

To understand the stability of MBA, the effect of heat and pH of the agglutinin was studied as described earlier [15]. To examine the requirement of metal ion for the activity of the agglutinin, it was dialyzed in TBS containing 10 mM EDTA for 6 h at 4 °C followed by dialysis against TBS. Hemagglutination was performed with rabbit erythrocytes. All the above experiments were carried out with 40 μg ml^{-1} agglutinin.

The molecular mass of the agglutinin was determined by 10% SDS-PAGE according to the method of Laemmli [16] by comparing the relative mobility of the agglutinin with the Precision Plus Protein Standards (Bio-Rad). The gel was stained with Coomassie Brilliant Blue G-250. Gel filtration by FPLC was carried out on Superose 12 HR 10/30 column (Amersham Biosciences, Sweden) at 4 °C in TBS at a flow rate 24 ml h^{-1}. Protein elution was monitored by absorbance at 280 nm. The molecular mass was determined from relative elution of different protein markers (gel filtration kit, Sigma): sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa). Absolute mass was measured on ESI-MS Q TOF Micro (Waters) by dissolving MBA in $CH_3CN:H_2O$ (1:1) mixture.

MBA (50 μg) was hydrolyzed in vapour phase with 6 N HCl containing 1% phenol (v/v) in PICO-TAG workstation at 110 °C for 24 h. The amino acid was derivatized by phenyl isothiocyanate at ~25 °C for 20 min. The derivatives were then analyzed in reverse phase HPLC using PICO-TAG C18 column (3.9 × 150 mm). Tryptophan was determined spectrophotometrically by the procedure of Edelhoch [17]. For sugar analysis, MBA (300 μg) was hydrolyzed using 2 N trifluoroacetic acid (TFA) in a sealed tube at 120 °C for 2 h [18]. The alditol acetates of the released sugars in chloroform were run on fused silica HP-5 column (30 m × 0.25 μm × 0.32 mm) in Agilent (model 6890 series GC system) gas chromatograph equipped with a HP 3898 A Chemstation. The neutral and amino sugar standards used were Glc, Gal, Man, Xyl, L-Fuc, GlcNAc and GalNAc.

The conformational stability and reversible denaturation of MBA against urea were investigated by steady state fluorescence measurement in TBS at 20 °C to ensure maximum hemagglutinating activity. Denaturation of MBA (0.55 μmol) was done by incubating with different concentrations of urea (1–7 M) for overnight at 4 °C to attain the equilibrium. The change in the tertiary structure of MBA as a function of urea was monitored by hemagglutinating activity and shift in fluorescence spectra. Renaturation was carried out with slow removing of urea by dialyzing against TBS at 4 °C. Fluorescence measurements were monitored by exciting the sample at 280 nm in order to assess the contribution of both tyrosine and tryptophan residues and the emission spectra were recorded from 290 nm to 400 nm in FluoroMax-3 spectrofluorometer (Jobin Yvon, Horiba) at 20 °C by using a 1 cm path length cell and 1 nm emission slit width and corrected for background signals. To account for the number of binding sites of MBA, fluorescence quenching study was performed with MBA and its inhibitory sugar GlcNAc. MBA (0.55 μmol) was incubated with different concentrations of GlcNAc (10–40 mM) at 20 °C for 2 h. Fluorescence spectra of the complexes were recorded as above.

2.4. Biological activities

The hemagglutinating activity of the lectin was determined by incubating a 2-fold serially diluted lectin solution (25 μl) in TBS with an equal volume of 2% (v/v) erythrocyte suspension solution in TBS for 30 min in room temperature. Normal, pronase- and neuraminidase-treated human as well as rabbit erythrocytes were used for hemagglutination study. The normal and enzyme-treated erythrocytes were prepared as described earlier [18]. Hemagglutination unit (HU) is defined as the minimum amount of protein (μg ml^{-1}) showing hemagglutination after 30 min at room temperature [18,19]. The preliminary sugar specificity of MBA was carried out by

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