Isolation and characterization of a new hemagglutinin from the red alga *Gracilaria bursa-pastoris*

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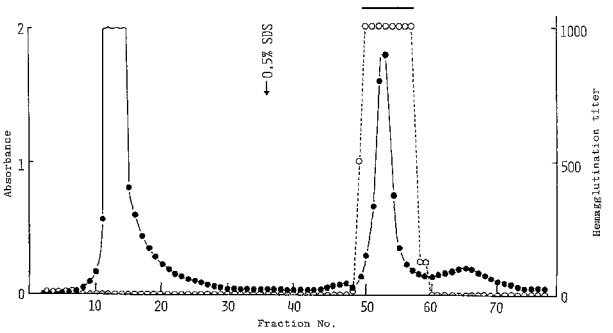
Summary. A new agglutinin has been isolated from the red alga *Gracilaria bursa-pastoris* by affinity chromatography on a yeast mannan-Cellulofine column. This agglutinin was isolated as a monomeric glycoprotein with a relatively low molecular weight. It had an isoelectric point of 4.7 and contained large amounts of Gly, Asx and Glx. It agglutinated trypsin-treated rabbit erythrocytes at the low concentration of 30 ng/ml. The activity was inhibited only by glycoproteins bearing N-glycans. This agglutinin also showed mitogenic activity for mouse splenic lymphocytes.
Key words. Marine alga; agglutinin; mitogen; glycoprotein; sugar-binding specificity; biological activity.

It has now been demonstrated that agglutinins are widely distributed in marine algae[2-4], and has been suggested that agglutinins may be involved in common but as yet unknown physiological functions in the algae[4]. So far, however, agglutinins have been isolated and characterized from only eleven algal species[5-15]. Our systematic investigation of marine algal agglutinins has shown that algal agglutinins are of small molecular size, are monomeric and have no affinity for monosaccharides[12-16], unlike most terrestrial plant agglutinins which are composed of subunits and have an affinity for monosaccharides[17,18]. These features of marine algal agglutinins led us to characterize other algal agglutinins. Recently, we have isolated a new agglutinin of a relatively low molecular weight, named Granin-BP, with mitogenic activity, from the red alga *Gracilaria bursa-pastoris* (Gmelin)-Silva. This paper describes the isolation and some of the properties of this agglutinin.

Specimens of *G. bursa-pastoris* were collected from the coast of Hiroshima, Japan, in June 1987, freeze-dried and ground to powder. The powdered alga was extracted with 10 volumes of 50 % ethanol. The agglutinin was recovered as a precipitate with 50–83 % cold ethanol (−20 °C) from the 50 % ethanolic extract. The precipitate was dissolved in a small volume of distilled water and dialyzed against distilled water. The non-dialysate was subjected to affinity chromatography on a yeast mannan-Cellulofine column (1 × 10 cm) equilibrated with 0.02 M phosphate buffer, pH 7 containing 0.85 % NaCl (PBS). The affinity gel was prepared by coupling yeast mannan to Formyl-Cellulofine according to the Seikagaku Kogyo manual[19]. It contained 7.5 mg of yeast mannan per 1 ml of gel. The column was washed with PBS until the washings showed no absorbance at 280 nm, then eluted with 1 M NaCl, 0.5 M lactose, 50 % ethylene glycol and 0.5 % SDS in PBS in this order.

The eluate was monitored by absorbance at 280 nm and hemagglutinating activity. Hemagglutinating activity was determined with trypsin-treated rabbit erythrocytes as described previously[16]. The absorbed agglutinin could be eluted with 0.5 % SDS. Therefore, 0.5 % SDS was used as a sole eluant in subsequent affinity chromatography as shown in the figure. The active fractions were



Affinity chromatography on a yeast mannan-Cellulofine column of the crude agglutinin. ●–●: Absorbance at 280 nm; o–o: hemagglutinating activity. Ten ml of each fraction was collected. Active fractions denoted by bar were pooled.

pooled and precipitated with 20 volumes of cold acetone (−30 °C) in order to remove SDS. The precipitate was washed three times with a small volume of cold acetone and dialyzed against distilled water. The agglutinin thus obtained was homogeneous in SDS-polyacrylamide gel electrophoresis (PAGE). The agglutinin was designated Granin-BP after the generic name of the alga.

The yield of Granin-BP was 1.7 mg of protein from 230 g of dry material. The molecular weight of Granin-BP was estimated to be 15,500 from gel filtration on a Asahipak GS-520 column with 0.05 M phosphate buffer, pH 7 containing 0.3 M NaCl, and 30,000 from SDS-PAGE. SDS-PAGE was carried out using a 12.5 % polyacrylamide gel, by the method of Laemmli[20]. The sample was treated with 2 % SDS and 5 % 2-mercaptoethanol at 100 °C for 3 min before running. Bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), chymotrypsinogen A (MW 25,000), β-lactoglobulin (MW 18,000) and lysozyme (MW 14,300) were used as reference proteins in both gel filtration and SDS-PAGE. Granin-BP was positive to PAS staining[21] after SDS-PAGE and contained approximately 20 % neutral sugars as determined by the phenol-sulfate method[22]. Granin-BP is thus a small monomeric glycoprotein, similar to other agglutinins[5,12-15] from marine algae, although it remains to be determined how such a monomeric form causes the agglutination of cells. The larger molecular weight ob-

Table 1. Amino acid composition of Granin-BP

Amino acid	Mol %	Amino acid	Mol %
Asx	10.3	Ile	4.0
Thr	7.8	Leu	5.7
Ser	9.0	Tyr	2.8
Glx	12.5	Phe	4.0
Gly	14.8	Lys	4.8
Ala	8.5	Arg	3.9
Cys	2.7	Pro	5.2
Met	1.9	Trp	1.6

Amino acid composition was determined on a Hitachi 835 amino acid analyzer after hydrolysis of the sample in an evacuated tube with 6 N HCl containing 3 % thioglycolic acid at 110 °C for 24 h. Cysteine was determined by the performic acid method[24].

tained using SDS-PAGE may be due to the acidic or glycoprotein nature of the protein, because in SDS-PAGE, acidic proteins or glycoproteins tend to migrate slowly and therefore to appear larger than they really are. Granin-BP gave a single band with a pI of 4.7 in isoelectric focusing, which was carried out as described previously[15]. Analysis of the amino acid composition of Granin-BP (table 1) showed that it contained large amounts of Gly, Asx and Glx. Cysteine or cystine was also present.

Granin-BP strongly agglutinated trypsin-treated rabbit erythrocytes. The minimum agglutination concentration was 30 ng protein/ml.

In the hemagglutination-inhibition test, the activity of Granin-BP was not inhibited by any of the monosaccharides and oligosaccharides tested; D-glucose, D-galactose, D-mannose, D-fructose, L-rhamnose, L-fucose, lactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-melibiose, maltose, D-raffinose, N,N'-diacetylchitobiose. This phenomenon has been seen in many other algal agglutinins[5,7,9,10,12–15]. However, Granin-BP was inhibited by glycoproteins such as fetuin, asialofetuin, yeast mannan and ovalbumin (table 2). Transferrin and α_1-acid glycoprotein were not inhibitory, whereas their desialylated glycoproteins[15] were inhibitory. The glycopeptide-fraction[16] from fetuin was also inhibitory, suggesting that the sugar moiety was responsible for inhibition. Among the inhibitory glycoproteins tested, ovalbumin was the best inhibitor against Granin-BP. Thus, Granin-BP seems to recognize glycoproteins bearing N-glycans irrespective of type whether complex, high mannose or hybrid N-glycans. Among the glycoproteins with complex N-glycans, Granin-BP was preferentially inhibited by fetuin which contains a relatively high proportion of tri-antennary complex N-glycans.

Yeast mannan has a high mannose N-glycan. Granin-BP was not inhibited by N,N'-diacetylchitobiose, which is the common constituent of the N-glycans, or by mannobiose, mannotriose, and mannotetraose prepared by acetolysis of the N-glycopeptide of the mannan[12]. Since acetolysis cleaves only the α-(1-6)-linkage in the backbone of the mannan, the mannosaccharides tested contained α-(1-2)- and α-(1-3)-linkages in the side chains[23].

Table 2. Inhibition of hemagglutinating activity of Granin-BP by sugars and related compounds

Sugars and related compounds	Minimum inhibitory concentration (μg/ml)
Monosaccharides *1	–
Oligosaccharides *1	–
Glycoprotein	
Transferrin	–
Fetuin	125.0
α_1-Acid glycoprotein	–
Asialotransferrin *2	125.0
Asialofetuin *2	15.6
Asialo-α_1-acid glycoprotein *2	2000.0
Yeast mannan	15.6
Ovalbumin	1.0
Glycopeptide-fraction from fetuin *2	+
Mannosaccharides from yeast mannan *2	
Mannobiose	–
Mannotriose	–
Mannotetraose	–

Trypsin-treated rabbit erythrocytes were used. The minimum inhibitory concentration is that required to inhibit completely the hemagglutinating activity with a titer of 4. + and – indicate presence and absence of inhibition, respectively, at a concentration of 100 mM in the case of mono- and oligosaccharides, and at 2 mg/ml in the case of glycoproteins and related compounds. *1 Mono- and oligosaccharides are given in the text. *2 These compounds were prepared as described previously[15,16].

Table 3. Mitogenic activity of Granin-BP for mouse splenic lymphocytes

Concentration of Granin-BP in medium (μg protein/ml)	Uptake of [3H]-thymidine in lymphocytes *1 (cpm)	Stimulation index *2
0	816,225	1.00
1.6	941,000	1.15
3.1	1601,333	1.96
6.3	2414,667	2.96
12.5	2112,667	2.59
25.0	1791,667	2.20

*1 Assay was performed in triplicate. The values indicate the means.
*2 Stimulation index expresses the uptake values as a ratio of the control.

Therefore, it is suggested that Granin-BP recognizes the moiety of α-(1-6)-linked polymannose and/or mannotriose at the core of the high mannose N-glycan. This indicates that Granin-BP could be a useful reagent for the elucidation of complex carbohydrate structures.

Granin-BP showed weak mitogenic activity for splenic lymphocytes of BALB/c mice (table 3). The activity was dose-dependent and the maximal activity was observed at 6–12 μg protein/ml. The mitogenic activity was assayed as described previously[15].

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Presence of anti-*Trypanosoma cruzi* antibodies in the sera of mice with experimental autoimmune myocarditis

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Summary. The existence of antigens shared in common by *T. cruzi* and heart muscle cells is suggested by the presence of antibodies binding to the parasite surface in the serum of mice with autoimmune myocarditis induced by immunization with syngeneic heart antigens.
Key words. Trypanosoma cruzi; Chagas' disease; myocarditis; autoimmunity.

South American trypanosomiasis (Chagas' disease) affects several million people in Latin America. About 10–20% of humans infected with *Trypanosoma cruzi* develop a severe chronic myocarditis 10 to 30 years after infection. On the basis of this long asymptomatic interval, and the fact that usually no parasites are seen at the site of the lesions, indirect mechanisms, mainly immunological, have been postulated for explaining the tissue damage [1]. Among them autoimmunity has been claimed to be a possible pathogenetic mechanism, but the conditions which may lead to the appearance of autoreactivity have not been clearly defined. Persistence of autoreactive clones after the polyclonal lymphocyte activation present during the acute phase of infection, or the existence of epitopes shared in common by the parasite and the mammalian host tissues, have been suggested as putative mechanisms of autoreactivity [2].

In the last few years most of the search for epitopes shared by the parasite and the host tissues has been carried out by developing monoclonal antibodies against nerve tissue, *T. cruzi* or closely related trypanosomatids, and determining their cross-reactivity with parasite and mammalian tissues. An alternative approach for investigating antigenic determinants common to the parasite and the host is to search for *T. cruzi* antibodies in patients with autoimmune disease, or in animals with experimentally-induced organ-specific autoimmune diseases. In the present report we investigate whether mice with experimental autoimmune myocarditis develop antibodies that recognize *T. cruzi* epitopes.

Materials and methods

Experimental autoimmune myocarditis (EAM) was induced in 3-month-old female BALB/c mice. Animals received, in multiple intradermal sites, 8 weekly injections (0.2 ml each) of a BALB/c heart homogenate incorporated in Freund's adjuvant (FA). For the first injection complete FA was employed; incomplete FA was used for the remainder. Fifteen days after the last injection a booster injection of 0.2 ml of the heart homogenate in phosphate buffered saline (PBS) was given intraperitoneally, and 7 days later the mice were bled to death by retroorbital puncture. Controls were mice receiving, according to a similar schedule, a BALB/c kidney homogenate prepared in the same way as the heart homogenate, or mice injected with FA alone. Eight animals were employed for each group.

For the preparation of the organ homogenates the organs were carefully dissected, sectioned into small pieces, washed repeatedly in Hanks' balanced salt solution, suspended 1/10 (w/v) in Hanks', and homogenized in a Potter-Elvehjem tissue homogenizer with a tightly-fitting Teflon pestle. The homogenate was centrifuged for 15 min at 100 × g to remove tissue clumps, divided into small aliquots and stored at −70 °C. Its protein concen-