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Purification and Characterization of Two Lectins from a Toxic Moray, *Gymnothrax javanicus*

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Two lectins, *Gymnothrax javanicus* lectin-I (GJL-I) and *Gymnothrax javanicus* lectin-II (GJL-II) were isolated from the stomach and intestine, and the liver, respectively, of a toxic moray eel, *Gymnothrax javanicus*,. GJL-I is a polymer of two heterogeneous subunits of 67 and 51 kDa. In a hemagglutination inhibition assay, it had sugar-binding specificity toward lactose and lactulose among the mono- or oligo-saccharides and bovine submaxillary mucin (BSM) among the glycoproteins tested. The lectin stimulated nerve growth factor (NGF) synthesis by astroglial cells. GJL-II was a polymer of subunit of 41 kDa. This lectin had *N*-acetyllactosamine binding specificity.

Key words: lectin; *Gymnothrax javanicus*; lactose-specific; *N*-acetyllactosamine-specific; nerve growth factor-synthesis

Lectins are carbohydrate-binding proteins present in a wide variety of animals, plants, and microorganisms. In the course of our continuing screening for new lectins from various organisms,[1-13] we found lectin activity with unique sugar-binding specificity in the extract of a toxic moray, *Gymnothrax javanicus*. On the other hand, during our continuing screening for new stimulators of NGF-synthesis from various organisms,[14-20] we found that the crude lectin-containing fraction strongly stimulated NGF synthesis by astroglial cells. This paper reports on the isolation and characterization of two lectins from the moray and stimulation by one of the lectins of NGF synthesis.

Materials and Methods

Materials. Gymnothrax javanicus were collected in Okinawa Prefecture, Japan, frozen upon collection, and stored at −30°C. Sepharose 4B, Superose 6 HR10/30 column, and an FPLC system were products of Pharmacia (Sweden). Galβ1→4GalNAc, Galβ1→4GlcNAcβ1→Me, Galβ1→4GlcNAcβ1→*p*-nitrophenyl, and Galβ1→6GlcNAc were synthesized by enzymatic methods.[21-22] All the other sugars for the hemagglutinating inhibition tests were products of Nacalai Tesque (Japan), Wako Pure Chemicals (Japan), or Sigma (U. S. A.). All glycoproteins for the test were purchased from Sigma (U. S. A.).

Isolation of GJL-I and GJL-II. All the procedures were done at 4°C except for defrosting the morays. After defrosting, the stomach and intestine were homogenized with acetone in a blender. The resulting suspension was filtered. The residue obtained was extracted in saline with stirring overnight. The suspension was filtered with gauze and the filtrate was centrifuged (7500 *g*, 25 min). Solid ammonium sulfate was added to the supernatant to give 80% saturation. The resulting precipitate was collected by centrifugation (10,000 *g*, 20 min), resuspended in distilled water, dialyzed against distilled water and lyophilized. The liver of the moray was treated in a similar manner.

The lyophilized extract of stomach and intestine was redissolved in 10 mM phosphate buffered saline, pH 7.4, (PBS) and put on a column of Sepharose 4B equilibrated with the buffer. After unbound materials were washed with the buffer, the bound fraction was desorbed with 0.3 M lactose in the buffer. The eluates were concentrated by ultrafiltration, dialyzed against PBS, and further purified by gel filtration on a Superose 6 HR10/30 column equilibrated with the buffer using a FPLC system. The hemagglutinating

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Abbreviations: GJL, *Gymnothrax javanicus* lectin; PBS, 10 mM phosphate-buffered saline (pH 7.4); BSM, bovine submaxillary mucin; PAGE, polyacrylamide gel electrophoresis; NGF, nerve growth factor. All sugars are of D-configuration unless otherwise stated

fraction was dialyzed against distilled water and lyophilized, giving a purified lectin, GJL-I.

The lyophilized extract from the liver was redissolved in PBS and put on a column of Sepharose 4B equilibrated with the buffer. After unbound materials were washed with the buffer, the bound fraction was desorbed with 0.3 M lactose in the buffer. The eluates were dialyzed against distilled water and lyophilized, giving a purified lectin, GJL-II.

Hemagglutination test Human blood was collected in 3% sodium citrate. The erythrocytes were washed three times with PBS and suspended at concentration of 3% in the buffer.

A 10% suspension of erythrocytes in PBS (10.0 ml) was treated with Pronase P (7.0 mg) for 30 min at 47°C, then the erythrocytes were washed three times with the buffer and suspended at a concentration of 3% in the buffer.

Agglutination of 3% erythrocytes and inhibition of the agglutination by sugars and glycoproteins were done in 96-well microtiter U-plates. The titer was defined as the reciprocal of the end-point dilution causing hemagglutination. Inhibition was expressed as the minimum concentration of each sugar or glycoprotein required for inhibition of hemagglutination of titer 4 of the lectin.

SDS-PAGE SDS-PAGE was done by the method of Laemmli.[23] Samples were heated in the presence or absence of 2-mercaptoethanol for 10 min at 100°C. Gels were stained with Coomassie Brilliant Blue. The molecular mass standards (Pharmacia, Sweden) used were phosphorylase b (*M*r 94,000), albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000).

Isoelectric focusing Isoelectric focusing on a gel (LKB Ampholine Pagplate, pH 3.5–9.5) was done in an LKB 2117 Multiphor apparatus with an LKB 2197 constant power supply. The pI standards (Pharmacia, Sweden) used were trypsinogen (pI 9.30), lentil lectin basic band (8.65), lentil lectin middle band (8.45), lentil lectin acidic band (8.15), myoglobin basic band (7.35), myoglobin acidic band (6.85), human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), β-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55), and amyloglucosidase (3.50).

Gel filtration for molecular weight estimation Gel filtration for measuring the molecular weight of native lectins was done on a Superose 6 HR10/30 column with a FPLC system. The molecular weight standards (Pharmacia, Sweden) used were thyroglobulin (*M*r 669,000), ferritin (440,000), catalase (232,000), and aldolase (158,000).

Sugar analysis Sugar content was measured by the phenol-sulfuric acid method with reference to glucose.[24]

Amino acid analysis The lectins were hydrolyzed with 6 M HCl at 110°C for 24 h in a sealed evacuated tube and analyzed on a Hitachi L-8500A amino acid analyzer. The content of cysteine and methionine was measured by oxidation of the lectin with performic acid followed by hydrolysis under the same conditions as those for the intact protein.

The *N*-terminal amino acids of the protein were analyzed on a PPSQ-10 Protein Peptide Sequencer (Shimadzu, Japan).

Thermostability Samples in PBS were heated for 30 min at the temperatures indicated, cooled on ice, and titrated.

pH stability The pH stability of the lectins was measured by incubating the samples in the following buffers for 24 h at room temperature, dialyzing against PBS, and titrating in PBS: 0.02 M sodium acetate buffer (pH 3.5–5.5), 0.02 M sodium phosphate buffer (pH 6.0–7.5), 0.02 M Tris-HCl Buffer (pH 8.0–9.0), and 0.02 M glycine-NaOH buffer (9.5–11.0).

Effects of metal cations on the lectin activity To examine metal cation requirements of the hemagglutination by the lectin, a sample (100 μg/ml) was incubated in 2 mM EDTA for 1 h at room temperature and titrated. $CaCl_2$, $MgCl_2$, or $MnCl_2$ was added to the treated lectins, and titrated.

NGF stimulating assay Quiescent astroglial cells from rats were used for the assay.[14–20,25,26] To the cells maintained in wells of a 96-well microplate, GJLs and the positive control, epinephrine, at various concentrations were added. The culture was kept for 24 h and then the NGF content secreted into the culture medium was measured by an enzyme immunoassay.

Results and discussion

The isolation procedure is summarized in Table 1. The saline solution of stomach and intestine extract of *Gymnothrax javanicus* was adjusted to 80% saturation with ammonium sulfate. Since the extract showed lactose-binding specificity in the hemagglutination inhibition assay, Sepharose 4B was used as an affinity adsorbent. After centrifugation, the precipitate obtained by the ammonium sulfate precipitation was put on a Sepharose 4B column. All the lectin activity was adsorbed to the column and eluted with 0.3 M lactose. The lectin-containing fraction was further purified by FPLC using a Superose 6 column, giving a purified lectin, GJL-1. On the other hand, the saline extract of the liver was also adjusted

Table 1. Purification of GJL-I (from 16.0 g Defatted Stomach and Intestine) and GJL-II (from 11.0 g Defatted Liver)

Lectin	Step	Total Protein (mg)	Total agglutination activity (titer)*	Specific agglutination activity (titer/mg protein)	Recovery (%)
GJL-I	80% $(NH_4)_2SO_4$ precipitate	200.0	8192	40.9	100
	Sepharose 4B	2.5	2048	820	25
	Superose 6	1.0	900	900	11
GJL-II	80% $(NH_4)_2SO_4$ precipitate	510.0	6093	12.0	100
	Sepharose 4B	1.0	853	853	14

* Titer was defined as the reciprocal of the end-point dilution causing the hemagglutination with Pronase-treated type A erythrocytes.

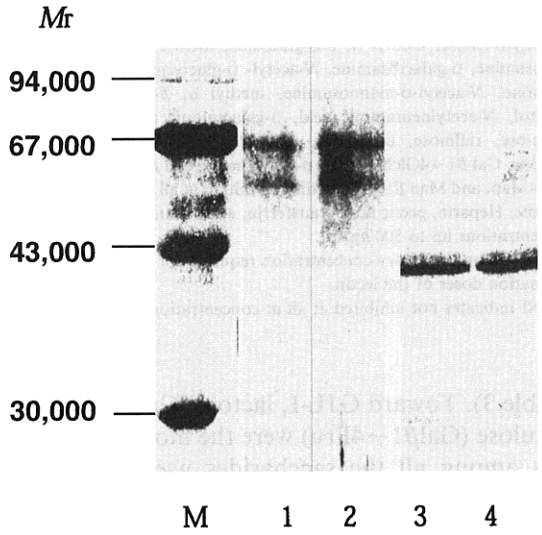

Fig. 1. SDS-PAGE of GJL-I and GJL-II.
Lane M, marker, lane 1, GJL-I (reduced with 2-mercaptoethanol); lane 2, GJL-I (non-reduced); lane 3, GJL-II (reduced with 2-mercaptoethanol); lane 4, GJL-II (non-reduced),.

Table 2. Amino Acid Compositions of GJL-I and GJL-II

Amino acid	mol% GJL-I	GJL-II
Asx	4.5	4.4
Glx	8.8	10.1
Ser	9.4	10.2
Gly	14.8	17.5
His	2.5	1.5
Arg	5.3	3.6
Thr	4.9	5.2
Ala	8.7	5.2
Pro	5.6	3.8
Tyr	0.0	4.7
Val	5.4	3.7
Met	0.0	0.0
Cys	4.3	9.0
Ile	4.2	3.0
Leu	8.1	8.6
Phe	5.1	4.9
Lys	8.4	4.6
Trp	ND*	ND

* ND indicates not determined.

to 80% saturation with ammonium sulfate and the resulting precipitate was used for affinity chromatography using Sepharose 4B. All the lectin activity was adsorbed to the column and desorbed with 0.3 M lactose, giving another purified lectin, GJL-II.

GJL-I gave two bands corresponding to the molecular masses of 67 and 51 kDa on SDS-PAGE, regardless of the presence (lane 1) or absence (lane 2) of 2-mercaptoethanol (Fig. 1). Gel filtration of the lectin on Superose 6 gave a peak at its void volume. The void volume of the column is more than *Mr* 5,000,000. These results allowed us to conclude that the lectin was a multisubunit protein containing two kinds of subunits of 67 and 51 kDa without S-S linkage, or a mixture of various kinds of polymers having a 51-kDa subunit and those having a 67-kDa subunit. SDS-PAGE of GJL-II indicated a single band of 41 kDa both in the presence (lane 3) and the absence (lane 4) of 2-mercaptoethanol (Fig. 1). Gel filtration of the lectin also gave a peak at the void volume of the Superose 6 column, suggesting that GJL-II was also a polymer of an identical subunit of 41 kDa. A sea urchin, *Anthocidaris crassispina*, also produces a multimeric lectin.[25] The molecular mass of the native lectin was 300,000 and the lectin is a polymer of two polypeptides of *Mr* 13,000 that are linked by a disulfide bond to each other. So far as we are aware, there is no other lectin having such a very high molecular mass like GJLs.

Amino acid analysis of GJL-I found a high content of Gly, Ser, Glx, Ala, Lys and Leu. Tyr and Met were not detected by the analysis. The analysis of GJL-II showed a high content of Glx, Ser, Gly, Cys and Leu. Met was also not detected (Table 2). Although 3 nmol of the proteins was put into a protein sequencer, the *N*-terminal amino acid could not be detected. It was suggested that *N*-termini of the proteins were blocked. The isoelectric focusing of both lectins gave similar bands at pH 6.7 (data not shown) and the carbohydrate contents of the proteins was 15.0% and 20.0%, respectively.

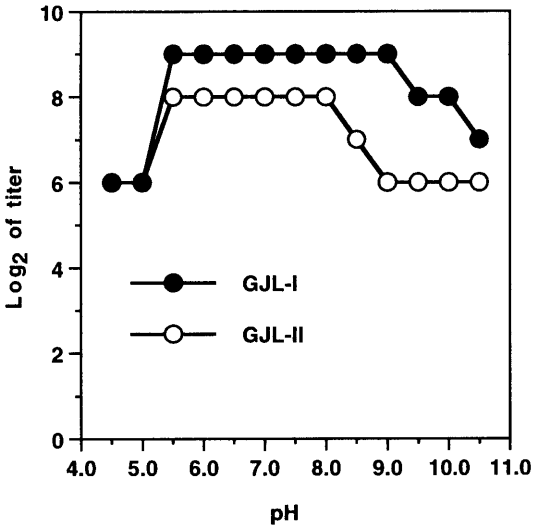

Fig. 2. pH Stability of GJL-I and GJL-II.

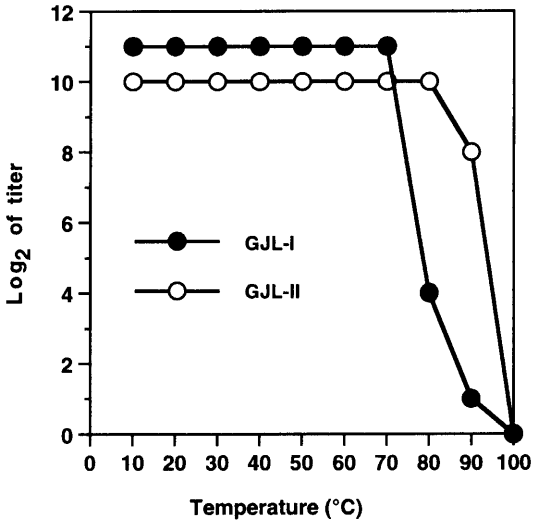

Fig. 3. Thermostability of GJL-I and GJL-II.

GJL-I and GJL-II were stable between pH 5.5 and 9.0, and 5.5 and 8.0, respectively (Fig. 2). The activity of GJL-I rapidly decreased over 70°C, but GJL-II was stable below 80°C (Fig. 3).

EDTA treatment or addition of metal cations such as $CaCl_2$, $MgCl_2$, and $MnCl_2$ had no effect on the activity of either lectin, suggesting that these lectins were not concerned with metal cations or the lectins bound to metal cations very tightly.

Both lectins did not agglutinate any type of intact human erythrocytes, but bound to Pronase-treated erythrocytes and showed a slight preference to Pronase-treated type O erythrocytes: the titer of GJL-I (1.0 mg/ml) was 256 to types A and B of the enzyme-treated erythrocytes and 512 to type O; GJL-II (1.0 mg/ml), 64 to types A and B, 128 to type O.

The sugar-binding specificity of both lectins was assayed by the hemagglutination inhibition method

Table 3. Inhibition of Hemagglutination Activity of GJL-I and GJL-II

Inhibitors*	Minimum inhibitory Concentration†	
	GJL-I	GJL-II
	(mM)	
Lactose (Galβ1→4Glc)	100	100
Lactulose (Galβ1→4Fru)	100	100
N-Acetyllactosamine (Galβ1→4GlcNAc)	NI‡	50
	(mg/ml)	
BSM	0.97	0.48
Asialo-BSM	15	0.48
α_1-Acid glycoprotein	250	1.92
Asialo-fetuin	60	15.6

* D-Glucose, D-galactose, D-mannose, D, L-fucose, D,L-arabinose, D-fructose, D-xylose, D-ribose, L-rhamnose, L-sorbose, 2-deoxy-D-glucose, 2-deoxy-D-ribose, methyl α, β-D-glucoside, methyl α, β-D-galactoside, D-glucosamine, D-galactosamine, *N*-acetyl- D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-D-mannosamine, methyl α, β-D-galactosaminide, D-manitol, *N*-acetylneuraminic acid, D-galactulonic acid, lactobionic acid, melibiose, raffinose, cellobiose, Gal β1→4GalNAc, Gal β1→4GlcNAc β1→Me, Gal β1→4GlcNAc β1→p-nitrophenyl, Gal β1→6GlcNAc, GlcNAc β1→4Man, and Man β1→4Man did not inhibit at all at concentrations up to 200 mM. Heparin, pectic acid, transferrin, and fetuin did not inhibit at all at concentrations up to 500 μg/ml.

† Minimum inhibitory concentration required for inhibition of 4 hemagglutination doses of the lectin.

‡ NI indicates not inhibited at all at concentrations up to 200 mM.

(Table 3). Toward GJL-I, lactose (Galβ1→4Glc) and lactulose (Galβ1→4Fru) were the most potent inhibitors among all the saccharides used in the assay. However the minimum inhibitory concentration of the sugars was very high. Bovine submaxillary mucin (BSM) was the strongest inhibitor among the glycoproteins tested. Asialo-BSM and asialo-fetuin were also inhibitory. *N*-acetyllactosamine was the most potent inhibitor toward GJL-II-mediated hemagglutination. Lactose and lactulose also inhibited hemagglutination by the lectin. Among glycoproteins, BSM and asialo-BSM were the best inhibitors and α_1-acid glycoprotein was next. Probably both lectins recognize specific sugar sequences including the β-galactoside moiety in the inhibitory glycoproteins.

GJL-I stimulated NGF synthesis by rat astroglial cells (Fig. 4) but GLJ-II had no activity;[14-20,25,26] the amount of NGF secreted into the culture medium in the presence of the lectin at 1.2 μg/ml (10 nM, calculated taking its molecular mass to be 117,000, which is the sum of the two subunits' molecular masses) was 101 ± 15 pg/ml. This optimum concentration was much lower than that (169 ± 17 pg/ml at 183 μM, 33.3 μg/ml) of a positive control compound, epinephrine. Stimulators of NGF-synthesis are the candidates for medicines for degenerative neuronal disorders and peripheral nerve regeneration. We have isolated some low-molecular-weight NGF inducers from mushrooms.[14-20] The mechanism of inducing NGF by the stimulators remains unknown. Probably

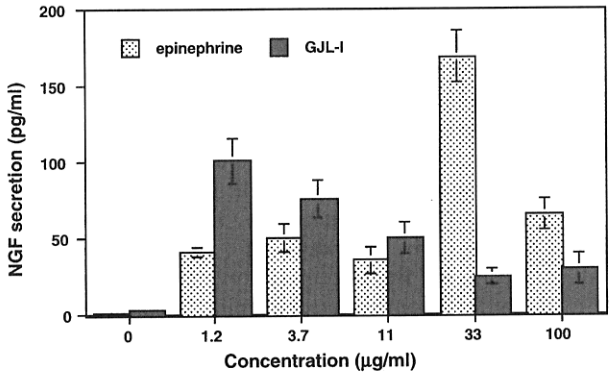

Fig. 4. Effects of GJL-I and Epinephrine on NGF Secretion of Astroglial Cells.

Values are means ±SEM obtained from 5 wells at the same concentraion.

the mechanism by low-molecular-weight NGF inducers is different from that by GJL-1. This lectin may become a useful tool for studying the mechanism. This issue remains under investigation. To our knowledge, this is the first lectin that stimulates NGF synthesis.

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