Molecular and biological characterization of a mannan-binding lectin from the holothurian *Apostichopus japonicus*

Aleksandr A Bulgakov¹,², Marina G Eliseikina³, Irina Yu Petrova⁴, Evgeny L Nazarenko², Svetlana N Kovalchuk², Valery B Kozhemyako², and Valery A Rasskazov²

¹Pacific Institute of Bioorganic Chemistry and ²Institute of Marine Biology, Far Eastern Branch, Russian Academy of Science, Vladivostok 690041, Russia

Received on January 15, 2007; revised on August 30, 2007; accepted on August 31, 2007

To elucidate the origin and evolution of mannann-binding lectins (MBLs), a new C-type lectin (CTL) specific for high-mannose glycans (MBL-AJ) was isolated from the coelomic plasma of the holothurian *Apostichopus japonicus*. MBL-AJ has oligomeric forms with identical 17-kDa subunits on SDS-PAGE. Among natural ligands, lectin hemagglutination activity was competitively inhibited by extracellular α-D-mannans isolated from marine halophilic bacteria and composed of α-1,2 and α-1,6 linked D-mannose residues. This suggests that the lectin interacts with backbone or inner side chain mannose residues, but not with terminal ones. The activity of the lectin was Ca²⁺, pH-, and temperature-dependent. MBL-AJ cDNA was cloned from a holothurian coelomocyte cDNA library. The subunit of the mature protein has 159 amino acids and a single carbohydrate-recognition domain (CRD) of CTL. CRD contains a Glu-Pro-Asp amino acid sequence (EPN-motif) conserved for all known MBLs. A monospecific polyclonal antibody against MBL-AJ was obtained using the 34-kDa lectin dimer as an immunogen. The MBL-AJ has demonstrated immunochemical identity to the earlier isolated mannann-binding CTL from another holothurian, *Cucumaria japonica*. But a more interesting finding was cross-reactivity of MBL-AJ and human serum lectin detected by the antibody against MBL-AJ. Taking into consideration such MBL-AJ peculiarities as its carbohydrate specificity, the presence of a conserved region forming the mannose-binding site, common antigenic determinants with human MBL, and participation in defense reactions, it is possible that MBL-AJ belongs to the family of evolutionary conserved mannann-binding proteins.

**Keywords:** *Apostichopus japonicus*/bacterial mannans/echinoderms immunity/mannann-binding lectins

Introduction

Mannann-binding lectins (MBLs) belong to the C-type animal lectin superfamily. Detailed descriptions of MBLs from a number of vertebrate species have been published (Ezekowitz and Stahl 1988; Kurata et al. 1994; Mogues et al. 1996; Storgaard et al. 1996; Laursen and Nielsen 2000). Structurally, they are homooligomers specific to mannose, fucose, and N-acetylgalactosamine. However, natural high-affinity MBL ligands are high mannose and hybrid type oligosaccharides (Childs et al. 1990).

MBLs are an important component of innate immunity, responsible for the first line of host defense (Holmskov et al. 1994; Epstein et al. 1996; Kawasaki 1999). They participate in elimination of altered self-structures (Gadjeva et al. 2004). MBLs mediate recognition and elimination of the pathogens expressing mannan-rich glycoconjugates on their surface (Kuhlman et al. 1989; Neth et al. 2000). After binding to mannan-rich carbohydrate structures expressed on the surface of yeasts, bacteria, and viruses, MBLs can activate the complement system by the C1q-independent mechanism (Anders et al. 1994; Ohta and Kawasaki 1994). This defense strategy was named lectin-dependent pathway of complement activation (Sato et al. 1994; Petersen et al. 2001.) MBLs trigger activation of the complement system by promoting MBL-associated serine proteases (MASPs) attachment to the target surface. Upon its binding to the pathogen surface mannans, the resultant MBL-MASP complex exhibits C4 and C2 activating capacities (Matsushita 1996; Thiel et al. 1997; Fugita et al. 2004). Soluble MBLs constantly circulate in body fluids and can therefore, immediately interact with pathogens like C1q complement component (Thiel et al. 1997).

The question is still open about when the precursors of mammalian MBLs have first arisen in the course of evolution. Recent advances in comparative immunology strongly suggest that the immune system of all extant animals appeared early in the evolution (Gamulin et al. 1994). Tunicates are a deuterostome group occupying a pivotal intermediary position between invertebrates and vertebrates (Ji et al. 1997; Nonaka and Azumi 1999; Nonaka et al. 1999). An MBL-like protein (GBL), structurally and functionally analogous to mammalian MBLs, was identified using a variety of methods in the solitary ascidian *Halocynthia roretzi*. The GBL was associated with two ascidian MASP. It is capable for activation of the primitive complement system in this ascidian. The GBL was isolated with a yeast mannan-Sepharose 4B column using mannann for elution of bound lectins. The carboxy-terminal half of the ascidian lectin contains a carbohydrate recognition domain that is homologous to other C-type lectins, but lacks the collagen-like region present in mammalian MBLs. The purified lectin bound specifically to glucose, but not to mannose.
or N-acetylg glucosamine, was considered as the glucose-specific lectin (Sekine et al. 2001). The variants of MASP/C1r/C1s family were also identified in other ascidians, like, for example, Ciona intestinalis (Azumi et al. 2003; Nonaka and Yoshizaki 2004).

Echinoderms are among the most primitive deuterostomes. They contain molecules homologous to the components of vertebrate complement system. Initially, three homologues of the vertebrate complement components C3 and Bf (named SpC3, SpC3-2, and SpBf, respectively) were cloned and sequenced from a coelomocyte cDNA library of the sea urchin Strongylocentrotus purpuratus (Smith et al. 1996; Al-Sharif et al. 1998; Smith et al. 1998; Zarkadis et al. 2001). According to the results of phylogenetic analysis, they are the most ancient members of the complement protein group. Smith et al. (1998) suggested a hypothetical model of sea urchin complement function, which accelerates opsonization and phagocytosis of microorganisms via positive feedback loop based on autocatalytic activation of C3b/Bf complexes. They also suggested that the echinoderm complement system may be activated by collectins (C1q and MBL) binding specifically to microbial surface oligosaccharides. This is consistent with the fact that vertebrate serum MBLs can interact with the C3 component and launch a lytic attack of the complement system. A search through sea urchin genome provided a number of homologues for vertebrate genes that mediate the alternative and lectin pathways (Hibino et al. 2006; Rast et al. 2006; Smith 2002). These genes encode C3-like Sp-TCP1 (thioester containing protein) and C4-like Sp-TCP2 belonging to the complement C3/4/5 family, additional two Sp-Bf paralogues, proteins containing single domain MACPF (membrane attack complex/Perforin) characteristic of terminal pathway proteins (C6, C7, C8, and C9), and five members of collectin family, among the latter four, members are similar to C1q and one to MBP.

Proceeding from the literature data on invertebrate and vertebrate lectins, the family of vertebrate MBLs must have an ancient precursor, as is the case in another lectin family, S-type lectins (Gamulin et al. 1994). To find evidence for this, we performed a screening of mannan-binding type lectin activity in the echinoderm species from the Peter the Great Bay (Sea of Japan). CTL with specificity for branched D-mannans was isolated from the coelomic fluid of the holothurian Cucumaria japonica (Bulgakov et al. 2000). Based on its properties, we suggested that the holothurian lectin is possibly related to mammalian MBLs. Its agglutinating activity is not inhibited by monosaccharides including N-acetyl-D-glucosamine, D-glucuronic and D-galacturonic acids, L-fucose, D-galactose, and D-mannose. The lectin is a homooligomer with identical subunits, each about 22 kDa. Its activity is Ca^{2+}-, temperature-, and pH-dependent and is reversibly inhibited by EDTA. The highest activity is observed at 4°C, 10 mM of Ca^{2+}, and within the pH range of 7–9.

We proposed that lectins of the same type activity may occur in other echinoderms, in particular, in the holothurian Apostichopus japonicus. Earlier, Matsui et al. (1994) isolated two lectins, SPL-I and SPL-II, from the coelomic plasma of the Far Eastern holothurian Stichopus (Apostichopus) japonicus. SPL-I is a CTL with a molecular mass of about 400 kDa on gel filtration. It is specific for D-glucuronic and D-galacturonic acids. SPL-II is also a CTL with a molecular mass of about 68 kDa on gel filtration. It is specific for N-acetyl-D-galactosamine and D-galactose. Both lectins have apparent molecular masses of about 17 kDa under reducing conditions (Matsui et al. 1994). SPL-I and SPL-II participate in coelomocyte clotting. This function is strongly inhibited by synthetic GRGDSP peptide or EGTA suggesting key role of its integrin region in clotting events.

Contrary to these findings, we found a new lectin in the coelomic plasma of A. japonicus (Holothuroidea, Aspidochirota). In this project, we have elucidated the primary structure and properties of MBL-AJ, a Ca^{2+}-dependent lectin from the holothurian A. japonicus. We found that MBL-AJ demonstrates preferential affinity for bacterial branched α-D-mannans, but not for monasaccharides. MBL-AJ is a homooligomer consisting of 17-kDa subunits. The subunit of the mature protein consists of 159 amino acid residues and includes a single carbohydrate-recognition domain (CRD) of CTL. CRD contains a Glu-Pro-Asp amino acid sequence conserved in all known MBLs. A database search showed a sequence homology between the MBL-AJ and CTLs from echinoderms and vertebrate MBLs. Moreover, we have found a partial immunochemical identity between MBL-AJ and the human serum MBL using an antibody against MBL-AJ, this fact suggests the presence of common antigenic determinants in these lectins.

Results

Hemagglutination and inhibition assays

Agglutinating activity of A. japonicus coelomic plasma was analyzed using tryspinized and gluteraldehyde fixed human erythrocytes (Table 1). O-type erythrocytes were agglutinated by holothurian plasma with high efficiency, corresponding to 256 agglutination titer. Native and trypsin-treated erythrocytes of type A, B, AB were agglutinated significantly weaker. Based on these data, hemagglutination of O blood group human erythrocytes was used for lectin activity monitoring during lectin isolation. Hemagglutinating activity was inhibited by EDTA, so it means that lectin activity was Ca^{2+}-dependent. Hemagglutination was most effectively inhibited by a number of low-branched bacterial mannan.

Isolation of MBL-AJ

An analysis of fractions following anion-exchange chromatography showed that the main lectin activity was eluted with 0.2 M NaCl (Figure 1). This fraction was used for further purification. Based on the results of inhibition experiment, mannan from Vibrio fluvialis strain 0010 was selected as a ligand for affinity sorbent synthesis. About 90% of Ca^{2+}-dependent lectin activity was bound on mannan-Sepharose column and eluted with EDTA (Figure 2). The EDTA-eluted fraction contained multiple components with apparent molecular masses on SDS-PAGE 17, 34, 68 kDa and high molecular weight complex in nonreducing condition (Figure 3). After β-mercaptoethanol treatment of this sample, only one band with molecular weight 17 kDa was detected.

The components were separated into three fractions by gel filtration on Sephacryl S-200 column (Figure 4). SDS-PAGE, used under nonreducing conditions, showed that the first peak contained a high molecular weight complex and a 68-kDa component; the second peak contained a 34-kDa component; and the third one comprised a 17-kDa component. Our data
Fig. 1. Anion-exchange chromatography of MBL-AJ on a DEAE TSK 650M column. Coelomic plasma was applied to the column equilibrated with TB containing 10 mM CaCl₂. Adsorbed proteins were eluted with stepwise gradient of NaCl in TB-Ca (0.1 M, 0.2 M, 0.3 M, 0.5 M). Hemagglutination activity was measured with human O-type trypsinized erythrocytes. The fractions 9, 10, 11 were pooled as active fraction. Elution profile, solid line, was formed by absorbance of protein solution measured at 280 nm. Hemagglutination titer (squares) was defined as the reciprocal value of the end point dilution causing hemagglutination.

Fig. 2. Affinity chromatography of MBL-AJ on a mannan-Sepharose CL-4B column. Fraction obtained previously from anion-exchange chromatography experiment was applied to the column equilibrated with TBS-Ca. The adsorbed proteins were eluted with TBS containing 20 mM EDTA. Hemagglutination activity was measured with human O-type trypsinized erythrocytes. The fractions 5, 6, 7, 8 were pooled as the active fraction. Elution profile, solid line, was formed by absorbance of protein solution measured at 280 nm. Hemagglutination titer (squares) was defined as the reciprocal value of the end point dilution causing hemagglutination.

suggested high cross-reactivity of antibody against 34 kDa dimeric MBL-AJ with 68 kDa polypeptide under nonreducing condition (Figure 3, lane E). After β-mercaptoethanol treatment of the sample, only one band with molecular weight 17 kDa was detected and western blot analysis revealed high cross-reactivity of the antibody with this component. However, we

Fig. 3. SDS-PAGE and corresponding immunoblot analysis of affinity purified and homogeneous MBL-AJ preparations. Coomassie-stained SDS-PAGE gels of nonreduced (A, B) and reduced (C) MBL-AJ. Lane A, MBL-AJ-enriched fraction isolated from coelomic plasma subsequently by anion-exchange chromatography on a DEAE TSK 650M and affinity chromatography on mannan-Sepharose. Lanes B and C, homogeneous MBL-AJ after last purification step using gel-permeation chromatography on a Sephacryl S-200 column. Lane D, molecular weight markers. Positions of molecular mass standards are indicated by arrows. Standard proteins used for molecular markers were phosphorylase B from rabbit muscle (97 kDa), bovine serum albumin (68 kDa), ovalbumin from chicken egg white (43 kDa), carbonic anhydrase from bovine erythrocyte (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-10 lactalbumin from bovine milk (14.4 kDa). Lanes E, F, G, H, western immunoblot analysis of of nonreduced (E, F, H) and reduced (G) MBL-AJ using anti-MBL-AJ rabbit polyclonal antibody. Lane E, affinity purified MBL-AJ. Lanes F and G, homogeneous MBL-AJ after gel-permeation chromatography on a Sephacryl S-200 column (nonreduced and reduced, respectively). Lane H, the absence of antibody cross-reactivity with 17-kDa component of third peak eluted from the Sephacryl S-200 column. Protein bands were transferred to nitrocellulose membrane and overlaid with anti-MBL-AJ antibody.
Isolation and properties of the mannans

The glycopolymers were isolated from cultural fluids of several marine proteobacteria (V. fluvialis strain AQ-0002B and AQ-00010A, Marinomonas communis strain ATCC 27118T, Pseudoalteromonas atlantica strain IAM 14175, Pseudoalteromonas flavigulchra strain NCIMB 2033T) by successive concentration, dialysis, and precipitation with ice-cold ethanol. Further purification of mannans was carried out by ion-exchange chromatography on DEAE TSK 50(M) and gel-permeation chromatography on TSK 50(S) gels. All preparations were analyzed using chemical methods and 13C NMR spectroscopy.

The glycosyl composition, obtained by analysis of polymers by paper chromatography and GLC as alditol acetates, indicated the only monosaccharide – mannose. GLC analysis of the acetylated glycosides obtained with optically active 2-octanol, using authentic compounds as references, showed that mannose had the D-configuration. Thus, the polysaccharides represented D-mannans. The results of the methylation analysis showed the presence of differently linked mannose units suggesting a mannan structure. Briefly, analysis of methylated mannans by GLCMS as partially methylated alditol acetates showed the presence of terminal Manp, 2-substituted Manp, 6-substituted Manp, and 2,6-di-substituted Manp residues in different proportions. In the case of mannans from the both V. fluvialis stains the respective ratio was 1.3:1:0.6:0.8, whereas for all other mannans it was 3:1:0.12:2. The 13C NMR spectra of the polysaccharides (Figure 5 and Table II) exhibited four broad anomeric signals at 103.5, 101.6, 100.6, and 99.5 ppm, all attributable to mannose units. Coupling constant value (175 Hz) for the anomeric carbons, determined from the gated-decoupling spectra, demonstrated the pyranoid form (Cyr and Perlin 1979) and the α-configuration of all mannose residues (Bock and Pedersen 1974). Besides, attach-proton test (APT)-experiment (Patt and Shoolely 1982) showed the presence 6-substituted mannose residues. From these combined data, the mannans obtained from culture fluid of some marine proteobacteria represented the glycopolymers similar to yeast cell-wall mannans (Shibata et al. 2003).

However, several structural differences between the mannans are apparent (Figure 5 and Table II). For the mannans from both strains of V. fluvialis, the integration of carbon anomeric signals of 6-linked and 2,6-linked mannopyranose units (∼3:4) demonstrated a low-branched structure (∼60%) confirmed by methylation analysis. The assignment of chemical shifts in 13C NMR spectra showed that these mannans had a α-1,6-linked mannoxylose backbone with few branching points and long α-1,2-linked mannoxylose side-chains. Similar structure was shown for mannans isolated from the bacteria P. atlantica (the strain IAM 14165) and M. communis (the strain ATCC 27118T) also showing strong inhibitory effect.

Table 1. Agglutinating activity of Apostichopus japonicus coelomic plasma with glutaraldehyde fixed native and trypsinized erythrocytes

<table>
<thead>
<tr>
<th>Blood group of human erythrocytes</th>
<th>Hemagglutination titer with native human erythrocytes</th>
<th>Hemagglutination titer with trypsin treated human erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>O</td>
<td>32</td>
<td>256</td>
</tr>
</tbody>
</table>

Fig. 4. Gel-permeation chromatography of MBL-AJ on a Sephacryl S-200 column. Fraction obtained previously from affinity chromatography experiment was applied to the column equilibrated with TBS-Ca2++. Proteins were eluted with TBS-Ca2++. Hemagglutination activity was measured with human O-type trypsinized erythrocytes. The fractions 14, 15, 16 were pooled as the active fraction. Elution profile, solid line, was formed by absorbance of protein solution measured at 280 nm. Hemagglutination titer (squares) was defined as the reciprocal value of the end point dilution causing hemagglutination.
permethylation data (see above) between branched and 6-linked units. Thus, these mannans have a highly branched α-1,6-linked manno pyranosyl backbone with many more numerous branching points and short α-1,2-linked manno pyranosyl side-chains.

Carbohydrate specificity of MBL-AJ

Carbohydrate-binding specificity of MBL-AJ was examined by inhibition assay. Effects of carbohydrates on the hemagglutination are listed in Table III. Activity of MBL-AJ was not inhibited by most of the mono- and oligosaccharides including mannose and fucose. Galactose, melibiose, and N-acetyl-D-galactosamine had weak inhibitory effect. Monosaccharide D-man did not inhibit lectin activity, disaccharide man-α-(1→2)-man and trisaccharide man-α-(1→2)-man-α-(1→2)-man showed weak inhibitory effect.

MBL-AJ showed a much more affinity to low branched bacterial α-d-mannans from V. fluvialis strains AQ-0002B and AQ-00010A, M. communis strain ATCC 27118T and P. atlantica strain IAM 14175.

High-branched bacterial mannan isolated from P. flavipulchra strain NCIMB 2033T showed no inhibitory effect. A structurally different mannan isolated from Pseudoalteromonas syringae pv. ciccaronei (Corsaro et al. 2001) that contains additional terminal D-glucose residues also showed no inhibitory effect.

Effect of Ca$^{2+}$ concentration, pH, and temperature on the lectin activity

Since the activity of MBL-AJ requires the presence of Ca$^{2+}$, the effects of Ca$^{2+}$ concentration on the lectin activity was examined. As shown in Figure 6, the concentration of Ca$^{2+}$ necessary for exhibiting of maximum hemagglutination was 10 mM.

As shown in Figure 7, lectin activity reached maximum value at pH 7 and was stable in the range from 7 to 9. Significant decrease hemagglutination activity was observed in the acidic region. The activity was lost completely at pH value below 5.

To study the influence of temperature, the lectin samples were incubated at various temperatures under optimal Ca$^{2+}$ concentration and pH (10 mM of Ca$^{2+}$ and pH 7.5). The activity remained stable between 4 and 30°C and declined at higher temperatures (Figure 8). The lectin solution heated at 90°C and then incubated at 4°C for 12 h did not agglutinate erythrocytes, thus indicating irreversible heat denaturation at this temperature.

Oligomeric structure of MBL-AJ

Western-blot analysis of affinity purified lectin fraction revealed two main bands corresponding to 34 and 68 kDa and high molecular weight complex with IgG against 34 kDa MBL-AJ (Figure 3). The results of this experiment and SDS-PAGE suggest that in coelomic plasma, MBL-AJ has several oligomeric forms. The immunochemical relationships of these forms were investigated by immunelectrophoresis. Immunelectrophoresis is the combination of electrophoretical separation of antigens in agarose gel with immunodiffusion. After electrophoretic separation of antigens, solution of anti-MBL-AJ antibody was added

### Table II. Data of the $^{13}$C NMR spectra of the mannans (δ, ppm)

<table>
<thead>
<tr>
<th>Sugar residue</th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
<th>C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>→2)-α-D-Manp(1→</td>
<td>101.2</td>
<td>78.7</td>
<td>70.1</td>
<td>67.5</td>
<td>74.5</td>
<td>74.5</td>
</tr>
<tr>
<td>→6)-α-D-Manp(1→</td>
<td>99.9</td>
<td>71.1</td>
<td>70.7</td>
<td>67.4</td>
<td>72.1</td>
<td>66.0</td>
</tr>
<tr>
<td>→2,6)-α-D-Manp(1→</td>
<td>98.8</td>
<td>79.2</td>
<td>71.0</td>
<td>67.0</td>
<td>72.7</td>
<td>66.7</td>
</tr>
<tr>
<td>α-D-Manp(1→</td>
<td>102.8</td>
<td>71.2</td>
<td>70.5</td>
<td>67.4</td>
<td>74.5</td>
<td>61.6</td>
</tr>
</tbody>
</table>

Pseudoalteromonas flavipulchra NCIMB 2033T

<table>
<thead>
<tr>
<th>Sugar residue</th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
<th>C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>→2)-α-D-Manp(1→</td>
<td>101.1</td>
<td>78.9</td>
<td>70.5</td>
<td>67.1</td>
<td>73.8</td>
<td>61.6</td>
</tr>
<tr>
<td>→6)-α-D-Manp(1→</td>
<td>99.9</td>
<td>71.2</td>
<td>70.6</td>
<td>68.0</td>
<td>71.7</td>
<td>66.1</td>
</tr>
<tr>
<td>→2,6)-α-D-Manp(1→</td>
<td>98.7</td>
<td>79.2</td>
<td>71.7</td>
<td>67.2</td>
<td>73.8</td>
<td>66.9</td>
</tr>
<tr>
<td>α-D-Manp(1→</td>
<td>102.7</td>
<td>71.4</td>
<td>71.5</td>
<td>67.2</td>
<td>73.8</td>
<td>61.6</td>
</tr>
</tbody>
</table>
Table III. Inhibition of MBL-AJ hemagglutination activity

<table>
<thead>
<tr>
<th>No.</th>
<th>Carbohydrates</th>
<th>Inhibition of hemagglutination, (initial concentration of carbohydrates 2.5 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L-fucose</td>
<td>NI</td>
</tr>
<tr>
<td>2</td>
<td>D+galactose</td>
<td>NI</td>
</tr>
<tr>
<td>3</td>
<td>2-deoxy-D-galactose</td>
<td>NI</td>
</tr>
<tr>
<td>4</td>
<td>D+galacturonic acid</td>
<td>NI</td>
</tr>
<tr>
<td>5</td>
<td>α- methyl-galactose</td>
<td>NI</td>
</tr>
<tr>
<td>6</td>
<td>N-acetyl-D-galactosamine</td>
<td>0.3125 mg/ml (1.21 mM)</td>
</tr>
<tr>
<td>7</td>
<td>D-ribose</td>
<td>NI</td>
</tr>
<tr>
<td>8</td>
<td>2-deoxy-D-ribose</td>
<td>NI</td>
</tr>
<tr>
<td>9</td>
<td>D+glucose</td>
<td>NI</td>
</tr>
<tr>
<td>10</td>
<td>D+glucuronic acid</td>
<td>NI</td>
</tr>
<tr>
<td>11</td>
<td>N-acetyl-D-glucosamine</td>
<td>NI</td>
</tr>
<tr>
<td>12</td>
<td>D-mannose</td>
<td>NI</td>
</tr>
<tr>
<td>13</td>
<td>α- methyl-mannose</td>
<td>NI</td>
</tr>
<tr>
<td>14</td>
<td>dimannoside (manα1→2 man)</td>
<td>1.25 mg/ml (3.65 mM)</td>
</tr>
<tr>
<td>15</td>
<td>trimannoside (manα1→2 manα1→2 man)</td>
<td>0.3125 mg/ml (0.61 mM)</td>
</tr>
<tr>
<td>16</td>
<td>α-L-Lactose (Galβ1→4Glc)</td>
<td>NI</td>
</tr>
<tr>
<td>17</td>
<td>melibiose (Galβ1→6Glc)</td>
<td>1.25 mg/ml (3.65 mM)</td>
</tr>
<tr>
<td>18</td>
<td>raffinose (Galα1→6Glcα1→2βFru)</td>
<td>1.25 mg/ml (2.48 mM)</td>
</tr>
<tr>
<td>19</td>
<td>α-1,4 Glucan Vibrio fisheri</td>
<td>1.25 mg/ml</td>
</tr>
<tr>
<td>20</td>
<td>α-1,4 Glucan Pseudoalteromonas infernis</td>
<td>NI</td>
</tr>
<tr>
<td>21</td>
<td>α- D-mannan Vibrio alginolyticus (strain 845–87)</td>
<td>0.078 mg/ml</td>
</tr>
<tr>
<td>22</td>
<td>α- D-mannan Vibrio alginolyticus (strain 945–80)</td>
<td>0.078 mg/ml</td>
</tr>
<tr>
<td>23</td>
<td>α- D-mannan Vibrio alginolyticus (strain 710)</td>
<td>0.0195 mg/ml</td>
</tr>
<tr>
<td>24</td>
<td>α- D-mannan Pseudoalteromonas atlantica-4</td>
<td>0.0195 mg/ml</td>
</tr>
<tr>
<td>25</td>
<td>α- D-mannan Pseudoalteromonas atlantica (strain IAM 14165)</td>
<td>0.00244 mg/ml</td>
</tr>
<tr>
<td>26</td>
<td>α- D-mannan V. fluvialis (strain AQ-0002B)</td>
<td>0.0012 mg/ml</td>
</tr>
<tr>
<td>27</td>
<td>α- D-mannan V. fluvialis (strain AQ-00010A)</td>
<td>0.0006 mg/ml</td>
</tr>
<tr>
<td>28</td>
<td>α- D-mannan Marinomonas communis (strain ATCC 27118T)</td>
<td>0.0006 mg/ml</td>
</tr>
<tr>
<td>29</td>
<td>α- D-mannan Pseudoalteromonas flavipulchra (strain NCIMB 2033T)</td>
<td>NI</td>
</tr>
<tr>
<td>30</td>
<td>α- D-mannan Pseudomonas syringae pv. ciccaronei</td>
<td>NI</td>
</tr>
<tr>
<td>31</td>
<td>α- D-mannan Candida albicans I</td>
<td>NI</td>
</tr>
<tr>
<td>32</td>
<td>α- D-mannan Candida albicans II</td>
<td>NI</td>
</tr>
</tbody>
</table>

NI: No inhibition at 2.5 mg/mL.

*aDetails are described in Materials and methods.

Cross-reactivity of MBL-AJ and human MBL-C, and mannan-binding lectin from coelomic plasma of C. japonica

Immunological cross-reactivity of MBL-AJ and human MBL was shown by “sandwich” ELISA with antibody against MBL-AJ. The percentage of cross reactivity of MBL-AJ with human MBL was estimated as approximately 10% (Figure 10). This result suggested that MBL-AJ and human MBL have common antigenic determinants. The absence of interactions between MBL-AJ and other components of the assay (rabbit IgG, bovine serum albumin, and horseradish peroxidase) was shown in our experiments carried out earlier on. According to the result of double immunodiffusion, MBL-AJ demonstrates complete immunochemical identity with the lectin from coelomic plasma of C. japonica (Figure 11).

MBL-AJ cDNA cloning and sequence analysis

Full-length cDNA sequence of MBL-AJ was determined by RT-PCR in conjunction with the rapid amplification of cDNA ends (RACE) methods. The MBL-AJ cDNA sequence and deduced
Ca\textsuperscript{2+} -dependence of MBL-AJ activity. To determine Ca\textsuperscript{2+} dependence of hemagglutinating activity, lectin solution with a known titer was dialyzed overnight against TBS containing 20 mM EDTA and then against TBS. Aliquots of lectin solution were diluted in TBS with 1.25, 2.5, 5, 10, 20, and 40 mM of Ca\textsuperscript{2+}, respectively. Hemagglutination was performed with human O-type trypsinized erythrocytes washed preliminarily with the same buffer. After 2 h-incubation at room temperature, hemagglutination titer was determined.

Amino acid sequence (GenBank accession no. AAT42221) are shown in Figure 12. The predicted MBL-AJ subunit consists of 159 amino acid residues. Its deduced N-terminal amino acid sequence coincides completely with the peptide N-terminal sequence of the isolated MBL-AJ. Calculated Mr of 17.5 kDa and pl of 4.82 agree well with experimental data. The analysis of the MBL-AJ deduced amino acid sequence by SMART (Schultz et al. 2000) has revealed that MBL-AJ subunit contains a CTL domain started at position 24 aa and ended at position 169 aa and includes the mannose residues binding site, Glu115-Pro116-Asn117 ("EPN-motif") (numbering of the polypeptide without signal sequence), conserved for all known mannan-binding proteins (MBP) (Figure 12, 13).

A database search performed with the BLAST2 software showed that the MBL-AJ had a significant sequence homology with the CTLs from echinoderms. MBL-AJ revealed the closest homology with CEL-IV (CTL) from *Cucumaria echinata* (GenBank accession no. AAB35250) – 65% identity and 79% similarity. A somewhat lower degree of similarity was observed with lectin SJL-1 from *Stichopus japonicus* (GenBank accession no. Q7M3Y0) (36% identity and 47% similarity), with CTL from sea urchin *S. purpuratus* (GenBank accession no. AAM70488) (35% identities and 48% similarity), with echinoind from sea urchin *Anthocidaris crassispina* (GenBank accession no. P06027) and SpEchinoid from *S. purpuratus* (GenBank accession no. AAR02404) (36% identity and 48% similarity). Shown in Figure 13 is multiple alignment of CTL domains of MBL-AJ, CEL-IV and vertebrates MBP.

**Discussion**

Characterization of the echinoderm immune system is important for understanding the ancestral deuterostome immune defense.

It is also important for understanding of evolutionary changes in the immune system of higher vertebrates that took part from the time, when precursor of the mammalian MBLs have appeared in the course of evolution.

Echinoderms are one of the most ancient and primitive extant groups of deuterostomes. They possess many defense systems.
Molecular and biological characterization of a mannan-binding lectin

Fig. 9. Immunochemical identity of MBL-AJ oligomeric forms by immunoelectrophoresis. A fraction of MBL-AJ eluted with 20 mM EDTA from the mannan-Sepharose column was used as an antigen. After electrophoretic separation of the proteins in agarose gel, the antibody anti-MBL-AJ was added to the well 1 and the plate incubated at 4 °C overnight. Agarose gel was stained with Coomassie brilliant blue R-250. 1, anti-MBL-AJ antibody in TBS, IgG fraction, 2, 3, antigen solution (MBL-AJ in TBS).

Fig. 10. Cross-reactivity of MBL-AJ and human serum MBL in ELISA using antibody against MBL-AJ. Purified preparations of MBL-AJ and human MBL were used as antigens. Curve was formed by absorbance of the resultant solution measured at 492 nm. MBL-AJ curve, (squares), was compared to curve obtained for human MBL (triangles).

undergoing further evolution in higher Deuterostomata. They lack antibody based humoral immune system, but both vertebrates and invertebrates have defensive molecules with the similar functions (lectins, agglutinins, hemolysins, components of the complement system, antimicrobial factors) (Arason 1996; Magor and Magor 2001). A survey of the S. purpuratus genome sequence identified more than 1000 gene models with relevance to immunity. They can be classified to several groups: scavenger receptor cysteine rich proteins (SRCR); toll-like receptors, interleukin-1 and -17-like protein families; two Recombinant Activating Genes (RAG) which encode an enzyme that is the primary mediator of Ig and T-cell receptor rearrangement in vertebrates; MACP-containing genes (membrane attack complex/Perforin) domain, characteristic for vertebrate terminal pathway proteins (C6, C7, C8, and C9); and five members of collectin family, four of which are similar to C1q and one to MBP (Hibino et al. 2006).

Some authors believe that the lectin-mediated defense system preceded integral cell and humoral immunity in vertebrates (Canciitti and Ancona 1989). Most of the lectins with known amino acid sequences belong to the CTL family. In echinoderms, a CTL with specificity to branched mannans was first isolated from the coelomic plasma of the holothurian C. japonica (Bulgakov et al. 2000). The screening of local echinoderms for mannan-specific lectins revealed their presence in some species.
in particular, in the holothurian *Apostichopus* (Stichopus) *japonicus* (Holothuroidea, Aspidochirota). In the present study, we reported on isolation, characterization, and cDNA cloning of a Ca\(^{2+}\)-dependent lectin (MBL-AJ) from the coelomic fluid of the holothurian *A. japonicus*. Lectin purification was performed under conditions similar to those described previously for a lectin from *C. japonica*. Given that *A. japonicus* defense system is most likely to interact with marine microorganisms, to choose a ligand for affinity sorbent, several bacterial strains were screened for inhibitory capacities of their polysaccharides. The lectin activity, in the presence of Ca\(^{2+}\), was efficiently inhibited by D-mannans isolated from a number of marine halophilic bacterial strains. This turned out to be a successful approach as α-D-mannans from *Halobacterium halobium* showed no inhibitory effect and had a highly efficient hemagglutination inhibitors.

The efficiency of lectin purification was to a large degree determined using the mannose-Sepharose affinity sorbent. MBL-AJ is an oligomeric lectin with 17-kDa subunits linked by disulfide bonds. MBL-AJ preparation selected for property analysis was judged to be a dimeric protein with identical subunits.

MBL-AJ is a Ca\(^{2+}\)-dependent lectin, with maximum activity in the range 10–40 mM of Ca\(^{2+}\). Concentration of Ca\(^{2+}\) in holothurian coelomic fluid varies between 10.7 and 11.8 mM (Matsui et al. 1994). Therefore, the maximum of MBL-AJ activity is determined using the mannan-Sepharose affinity sorbent. MBL-AJ is a thermolabile protein with an optimum activity at 4–30°C. Incubation of the lectin solution at 90°C resulted in irreversible heat denaturation. Since carbohydrate-recognition domains of CTLs are formed on the level of spatial structure (Weis et al. 1992), the observed temperature-dependent changes in MBL-AJ activity may be caused by changes in the secondary and tertiary structure of native lectin molecules.

### Carbohydrate specificity of the lectin

The specificity and avidity of lectin–polysaccharide interaction depend on the structure of the terminal monosaccharide residue, configuration of the glycoside bond between monomers, and branching degree of a glycane (Ezekowitz and Stahl 1988; Sharon and Lis 1989; Holmskow et al. 1994; Neth et al. 2000).

The mannans used to study MBL-AJ carbohydrate specificity comprised two different groups. The mannans of the first group inhibited the activity of lectin and had an α-1,6-linked manno-oligosaccharide backbone with few branching points and long α-1,2-linked manno-oligosaccharide side-chains, while those of the second group showed no inhibitory effect and had a highly branched α-1,6-linked manno-oligosaccharide backbone with considerably more branching points and short α-1,2-linked manno-oligosaccharide side-chains. This suggests that MBL-AJ interacts with the backbone core or inner side chain mannose residues, but not with terminal ones. It means that exists a correlation between structural peculiarities of polysaccharides and their inhibitory capacities. High specificity of MBL-AJ to branched mannans allows one to refer it to MBLs (Ikeda et al. 1987; Anders et al. 1994; Kurata et al. 1994; Sato et al. 1994). Multitissue binding between lectins and carbohydrate structures, so-called cooperative effect, is known to increase binding constant (Jobst et al. 1994). For example, vertebrate MBLs display much higher affinity for branched mannans than for mannoside monomers and oligomers (Jobst et al. 1994; Sato et al. 1994). Hoffman et al. 1994) MBL-AJ bound weakly, if at all to D-galactose, N-acetylgalactosamine, N-acetylgalactosamine, and α-lactose and did not bind to uronic acids. Noteworthy, it is these carbohydrates that determine the carbohydrate specificity of lectins SPL-1 and SPL-2 isolated from the *A. japonicus* coelomic plasma by Matsui et al. (1994). This suggests that MBL-AJ and lectins SPL-1 and SPL-2 are different proteins.
Oligomeric structure is typical for animal lectins, particularly for mammalian MBLs. They are presented in various oligomeric forms (dimers, trimers, tetramers, hexamers) in circulation (Holmskov et al. 1994; Leslie et al. 1996; Kawasaki 1999). Oligomerization of subunits is responsible for multisite binding of lectins with glycoconjugates, and, in turn, this is necessary for effective binding with microbial surfaces and complement activation (Neth et al. 2000; Storgaard et al. 2001). Fact of the oligomerization was found also for MBL-AJ. The immunoblotting analysis of the affinity purified MBL-AJ with antibody against MBL-AJ confirmed oligomeric structure of the lectin and revealed a high molecular complex and two components with molecular weights of 34 and 68 kDa, with the 68-kDa component predominating. The immunoelectrophoresis revealed complete immunochemical identity of the components detected by immunoblotting.

Earlier, we reported complete immunochemical identity of MBL-AJ and the MBL from C. japonica as revealed by their interaction with polyclonal antibodies against MBL from C. japonica raised in our laboratory (Bulgakov et al. 2000). Interaction of the lectin from C. japonica with polyclonal antibodies against MBL-AJ also revealed their complete immunochemical identity. But more interesting finding was cross-reactivity of MBL-AJ and human serum MBL detected by the antibody against MBL-AJ. The percentage of cross reactivity MBL-AJ of MBL-AJ and human serum MBL was approximately 10%. This means that they share common antigenic determinants and are partially structurally homologous.

cDNA cloning of MBL-AJ

The analysis of the MBL-AJ deduced amino acid sequence showed that MBL-AJ is a single domain protein containing a characteristic domain of CTL, but lacking a collagen-like domain present in mammalian MBLs as in the case of the ascidian lectin GBL (Sekine et al. 2001).

MBL-AJ as vertebrate MBPs contains EPN-motif which is essential for binding to mannose residues and Ca$^{2+}$ ions (Zelensky and Gready 2005). This site is typical of, and conservative for, vertebrate MBLs (Zelensky and Gready 2005). This suggests partial structural homology between these lectins and provides additional evidence that the lectin from A. japonicus is a MBL.

MBL-AJ amino acid sequence is mostly identical to that of the galactose-specific lectin CEL-I (65%), whose CRD contains EPN, but not QPN sequence. This reflects the close phylogenetic relationship between the two holothurian species. Other MBLs showed lower, yet significant identity with MBL-AJ (18–28%). The analysis of the multiple alignments (Figure 13) showed that MBL-AJ and CEL-I have two extended amino acid residue inserts. One of them, comprising 11 amino acid residues (from Ser40- to His50), is located after the first alpha helix of the rat mannan-binding protein (PDB ID: 1MSB) (Weis et al. 1991), while the other, comprising 13 amino acid residues common with for MBL-AJ (from Pro72 to Glu84) (11 amino acids residues common with for CEL-I) is located after the second alpha helix. Another dissimilarity between the holothurian lectins MBL-AJ and CEL-I, on one hand, and vertebrate MBLs, on the other hand, is the absence of two conserved cysteine residues (at positions 125 and 141) suggesting that MBL-AJ and CEL-I molecules are stabilized with disulfide bonds in a different manner. The analysis of amino acid sequences of MBL-AJ and CEL-I revealed that these proteins contain the conserved cysteine residue Cys41 in the first insert, Cys5, and Cys16 (Figure 13). It was shown that CEL-I contains two intrachain disulfide bonds, Cys5-Cys16 and Cys41–Cys147, and one interchain disulfide bond between subunits formed by Cys1 of polypeptide chains (Hatakeyama et al. 1995). Probably, these disulfide bridges are also present in MBL-AJ. It is also mentioned that holothurian MBLs MBL-AJ and CEL-I have an amino acid residue replacement in Ca-binding site 2 conserved for vertebrate MBLs (Zelensky and Gready 2005). In canonical WND motif (position 204–206 in 1MSB) in MBL-AJ and CEL-I, molecule asparagine is replaced by Ala137. It was shown that Asn205 and Asp206 of rat MBP-A are involved in Ca-coordination forming three Ca-coordination bonds (two from the side chains and one from the backbone carboxyl of Asp) and hydrogen bonds with the mannose residue. The carbonyl side chain of Glu193 of 1MSB is also involved in site 2 formation by forming one coordination bond with the Ca$^{2+}$ ion (Giga et al. 1987; Himeshima et al. 1994; Hatakeyama et al. 1995). In MBL-AJ and CEL-I, the amino acid residue Arg123 is located in this position. There seems to be a difference in Ca$^{2+}$-binding site 2 which indicates that holothurian lectins bind Ca$^{2+}$ ions and mannose residue by a different bond, which is presently unknown.

Partial structural similarity of invertebrate and vertebrate CTLs was confirmed by comparing their sequence. The carbohydrate-recognizing domains of some echinoderm lectins, such as echinoinid and lectin CEL-I from C. echinata are structurally homologous to vertebrate CTLs, in particular, to mammalian MBLs (Giga et al. 1987; Himeshima et al. 1994; Hatakeyama et al. 1995). A number of highly conserved structural elements typical of CTLs were revealed in all major vertebrate radiations. This is especially the case regarding amino acid residues of the carbohydrate-recognizing domains that form carbohydrate- and Ca$^{2+}$-binding sites (Gamulin et al. 1994; Hatakeyama et al. 1995).

As regards the functions of MBL-AJ in the organism, they are the part of the defense system of A. japonicus (Eliseikina et al. 2003; 2004). Their participation in the agglutination and opsonization of the foreign particles was demonstrated in the experiments with inoculation of the gram-negative bacteria Yersinia pseudotuberculosis into the coelomic cavity of A. japonicus. The results show functional similarity of MBL-AJ in mammals and echinoderms. But neither in the experiments with bacterial cells, nor in the test system with the O-group human erythrocytes, the coelomic liquid of A. japonicus did produce a lysis of these model cells. Consequently, in spite of the presence of MBL in the coelomic plasma of A. japonicus, the defense reactions did not result in the formation of membrane attack complex. On the basis of our data and the data of other researches concerning the presence of the complement components in the purple sea urchin (Smith et al. 1996; Hibino et al. 2006; Rast et al. 2006), we can conclude about the absence of arranged and complicated complement system in echinoderms. The functional differences between echinoderm and mammalian MBL can point out the structural differences of the lectin molecules. Actually, according to our data, MBL-AJ contains the conservative carbohydrate-recognizing domain, but does not have the collagen-like domain, which is responsible for the complement activation.
To summarize the above, we showed that MBL-AJ is structurally and functionally similar to vertebrate MBLs. The major evidence for this conclusion is its carbohydrate specificity, the presence in its structure of common antigen determinants and conserved region forming the manno-binding site, and its participation in defense reactions. Taken together, these properties suggest that the MBL from the holothurian *A. japonicus* is phylogenetically related to an evolutionary precursor of vertebrate MBLs.

**Materials and methods**

**Materials**

Sepharose CL-6B, Sephacryl S-200, Amicon UM-10 ultrafiltration system, and low molecular weight markers were purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden). ELISA microtiter plates and U-bottomed microtitre plates (Greiner, Germany). Horseradish peroxidase, Freund’s complete adjuvant, nitrocellulose membrane (0.45 µm), secondary antibody (goat anti-rabbit IgG conjugate with horseradish peroxidase), o-phenylendiamine, 3,3’-diaminobenzidine, divinylsulfone, phenylmethylsulfonlfonylfluoride, sodium azide, mono- and oligosaccharides were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). DEAE-Toyopearl TSK 650M were purchased from Toyoda Soda (Tokyo, Japan). Polysaccharides from marine bacteria were kindly provided by the Laboratory of carbohydrate chemistry (PIBOC, Russia). Mannan from *Pseudomonas syringae pv. ciceraronei* was kindly provided by Prof. Antonio Molinaro (University of Napoli, Italy)

**Preparation of coelomic plasma**

The holothurians *A. japonicus* were collected in the Bay of Peter the Great (the Sea of Japan) at a depth of 2–10 m. Coelomic plasma was prepared by prolonged gentle centrifugation of whole coelomic fluid (5000 × g for 60 min). The cell-free supernatant was dialyzed against tris buffer, containing 10mM CaCl₂ (0.01 M Tris-HCL, pH 7.5, 10 mM CaCl₂) (TB-Ca²⁺), clarified at 10,000 × g for 10 min, and used for lectin isolation. Sodium azide was added to prevent bacterial growth and phenylmethylsulfonlfonfluoride, to inhibit protease activity.

**Lectin activity assays**

Standard hemagglutination assay and carbohydrate inhibition of hemagglutinating activity were performed using trypsin treated glutaraldehyde fixed O blood group human erythrocytes. The assays were performed in U-bottomed microtitre plates by serial two-fold dilution of a 100-µL lectin solution with an equal volume of tris buffered saline, containing 10 mM CaCl₂ (0.01 M Tris-HCL, pH 7.5, 10 mM CaCl₂) (TB-Ca²⁺), clarified at 10,000 × g for 10 min, and used for lectin isolation. Sodium azide was added to prevent bacterial growth and phenylmethylsulfonlfonfluoride, to inhibit protease activity.

**Purification of MBL-AJ**

Anion-exchange liquid chromatography was performed on a TSK 650M column (40 × 100 mm) equilibrated with TB-Ca²⁺. Two liters of the coelomic fluid with titer 256 was applied to the column and the sorbent was washed with starting buffer. Bound proteins were eluted with a step gradient of NaCl (0.1, 0.2, 0.3, and 0.5 M respectively) in TB-Ca²⁺. The elution profile was monitored at 280 nm and lectin-containing fractions were identified by hemagglutination assay. The active fraction eluted at 0.2 M NaCl in TB-Ca²⁺ was subjected to affinity chromatography.

An affinity sorbent was synthesized by immobilization of the extracellular mannan isolated from *V. fluvialis* (strain AQ-00010A) on a Sepharose CL-6B. For this purpose, 40 mL of Sepharose CL-6B was washed with water and equilibrated with 0.5 M Na₂CO₃ (pH 11.0). The gel was resuspended in 80 mL of 0.5 M Na₂CO₃ (pH 11.0); 4 mL of divinylsulfone was added to the suspension and the mixture was incubated for 70 min at room temperature with constant shaking. The activated Sepharose was washed with water, 400 mg of mannan in 80 mL of 0.5 M Na₂CO₃ (pH 10.0) was added, and the suspension was incubated for 48 h at 4°C with constant shaking. The mannan-Sepharose was washed with water, resuspended in 80 mL 0.5 M Na₂CO₃, (pH 8.5), containing 1.6 mL β-mercaptoethanol, shaken for 3 h at room temperature, washed with water, equilibrated with TBS-Ca²⁺, and transferred to the column. Sixty milliliters of lectin-containing fraction obtained after ion-exchange chromatography was applied to the mannan-Sepharose column. Lectin binding was monitored by the hemagglutination activity assay. The sorbent was washed successively with TBS-Ca²⁺. The active fraction was eluted with 0.01 M EDTA in TBS and concentrated by ultrafiltration on an Amicon UM-10 system to the volume 4 mL.

Gel permeation chromatography was performed on a Sephacryl S-200 column (12 × 1200 mm) equilibrated with TBS-Ca²⁺. Fractions were identified as stated above and protein composition was analyzed by SDS-PAGE.

The protein concentration was estimated by the method of Lowry (Lowry et al. 1951) using bovine serum albumin as the standard.

**SDS-PAGE**

Electrophoresis was performed in 12.5% polyacrylamide gel by the method of Laemmli (Laemmli 1970) under reducing and nonreducing conditions. Protein bands were visualized by Coomassie brilliant blue R-250 staining. Molecular masses of the proteins were determined using low molecular weight markers.

**Peptide sequencing**

Peptide sequencing of the N-terminal end (10 amino acids: Cys-Leu-Thr-Ala-Cys-Pro-Glu-Phe-Trp-Thr) was performed on an Edman Automated Sequencing Apparatus Beckman 890C.

**Ca²⁺, pH, and temperature dependence of lectin activity**

To determine pH dependence of lectin activity, lectin solutions were dialyzed against 0.15 M NaCl, containing 10 mM Ca²⁺ and aliquoted. The pH in samples was adjusted with
the following buffers: 0.025 M CH₃COONa, pH 4.0 and 5.0; 0.025 M 2-[N-Morpholino]ethanesulfonic acid (MES), pH 6.0; 0.025 M N-2-Hydroxyethylpipеразине-N’-2-ethanesulfonic acid (HEPES), pH 7.0; 0.025 M Tris-HCl, pH 8.0 and 9.0; 0.025 M Na₂CO₃, pH 10 and 11. All buffers contained 10 mM Ca²⁺ and 0.15 M NaCl. Each lectin sample was incubated with the corresponding buffer overnight at 4°C. Hemagglutination was performed with human erythrocytes washed preliminarily with the same buffer.

To determine Ca²⁺ dependence of hemagglutinating activity, lectin solution with a known titer was dialyzed overnight against TBS containing 20 mM EDTA and then against TBS. Two-fold dilutions of lectin aliquots were prepared in a 96-well microplate containing TBS with 40, 20, 10, 5, 2.5, and 1.25 mM of Ca²⁺ in each row, respectively. After 2 h incubation at room temperature, hemagglutination titer was determined.

The effect of temperature denaturation on lectin activity was also examined using aliquots of lectin solution preincubated in TBS-Ca²⁺ at 20, 30, 40, 50, 60, 70, 80, and 90°C for 1 h. The agglutinating activity of lectin was measured at room temperature immediately after incubation. To determine lectin activity at low temperature, the lectin solution was incubated at 4°C for 1 h, mixed with erythrocyte suspension, and the hemagglutination titer was estimated at 4°C.

**Producing of the polyclonal monospecific antiserum against MBL-AJ**

A polyclonal monospecific antiserum against purified 34 kDa MBL-AJ homodimer was raised in rabbits by repeated injection of purified lectin with or without Freund’s complete adjuvant. Preimmune and anti-MBL-AJ sera were stored with sodium azide at −20°C until used. Specificity of antiserum was confirmed using double immunodiffusion and immunoelectrophoresis. IgG was isolated by ammonium sulfate precipitation and ion exchange chromatography on DEAE-Sephadex A-50 (Axelsen et al. 1977). MBL-AJ-specific antibodies concentration in IgG fraction was 110 μg/mg IgG. The specificity of antibody was determined by double immunodiffusion using both lectin-enriched fractions obtained from anion-exchange chromatography and purified lectin preparation. Immunochromatography revealed one precipitin band for both lectin preparations. This suggests that the obtained antiserum was monospecific.

**Conjugation of IgG anti-MBL-AJ with horseradish peroxidase**

Conjugated IgG anti-MBL-AJ with horseradish peroxidase was obtained by a modified method of Nakane and Kawaoi 1974. In this method, 0.5 mL of horseradish peroxidase solution (2 mg/mL in H₂O) was added to 0.1 mL freshly prepared 0.1 M NaIO₄. Mixture was incubated for 30 min in darkness at room temperature. The obtained solution of oxidized peroxidase was dialyzed against 0.001 M Na-acetate buffer, pH 4.4, overnight at 4°C and supplemented with 4 μL of IgG anti-MBL-AJ dissolved in 1 mL 0.1 M Na-carbonate buffer, pH 9.5. The mixture was incubated for 2 h at room temperature, supplemented with 50 μL of 0.4% fresh solution NaBH₄, and incubated for 2 h at room temperature. BSA 5 mg and equal volume of glycerol were added to the conjugate solution, and the resulting mixture was stored at −20°C.

**Isolation of human MBL fraction**

The human serum MBL-enriched fraction was obtained from serum of healthy donors using affinity chromatography on mannan-Sepharose as described above for MBL-AJ.

**ELISA**

In this method, 100 μL of IgG against MBL-AJ (10 μg/mL) dissolved in 0.1 M carbonate buffer, pH 9.5, was added to each well of a polystyrene 96-well ELISA microtiter plate and incubated at 4°C overnight. After incubation, the plate was washed three times with tris buffered saline, containing 0.05% Triton X-100 (TBS-T) and then three times with water. After that, 150 μL of 1% bovine serum albumin in TBS-T was added to each well to block nonspecific binding sites. The plate was then incubated for 1 h at room temperature and washed as described above. Samples containing human MBL (initial concentration 200 ng/mL) were two-fold serially diluted in TBS-T and added in 100 μL aliquots to each well. TBS-T was used as negative control, and interaction of MBL-AJ with adsorbed antibody under the same conditions, as positive control. After that, the plate was kept for 1 h at room temperature and washed again. Rabbit IgG against MBL-SJ conjugate with horseradish peroxidase was added in 100 μL aliquots to each well. The plate was incubated for 1 h at room temperature, washed according to standard procedure. Then 100 μL of substrate (o-phenylenediamine in 0.1M phosphate-citrate buffer, pH 5.0) was added to each well. After 15-min incubation in darkness at room temperature, 50 μL of 5% sulfuric acid was added to stop the reaction. Absorbance of the resultant solution was measured at 492 nm with a plate spectrophotometer (Bio-Tek Instruments, Winooski, VT.) The calibration curve obtained for human MBL was compared to MBL-AJ curve.

**Immunodiffusion and immunoelectrophoresis**

Double immunodiffusion was performed according to modified Ouchterlony procedure (Khramkova and Abelev 1961).

Immunoelectrophoresis is the combination of electrophoretical separation of antigens in agarose gel with immunodiffusion. Antigen solutions (MBL-AJ fraction eluted with 20 mM EDTA from the mannan-Sepharose column) was added in 25 μL to wells 2 and 3. Immunoelectrophoresis was performed in Tris/Barbital buffer, (pH 8.6) at 14 V/cm for 1.5 h in Mini Sub Cell GT unit (Bio-Rad, Hercules, CA) as described in (Grabar and Williams 1953; Axelsen et al. 1977). As the standard of protein electrophoretic mobility, Evans blue in PBS (BSA stained with bromphenol blue) was used. After electrophoretic separation of antigens (lectin isoforms), antibody solution (IgG anti-MBL-AJ in TBS) was added to the trough 1, then gel was incubated at room temperature overnight. Immunodiffusion and immunoelectrophoresis gels were stained with Coomassie blue R-250.

**Western blotting**

After electrophoresis in 12.5% polyacrylamide gel, proteins were transferred onto a nitrocellulose membrane. The transfer was carried out at 100 V, 400 mA for 1 h in Towbin buffer (Towbin et al. 1979.) To verify transfer, the gel was stained with Coomassie brilliant blue R-250. The membrane was incubated with TBS-T containing 1% bovine serum albumin as blocking solution for 1 h at room temperature. The membrane
was then washed three times for 10 min each in TBS-T and incubated with an antibody against MBL-AJ diluted in TBS-T with 0.1% bovine serum albumin for 2 h at room temperature. The membrane was then washed three times and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase solution in TBS-T. The membrane was washed again, and the antigen-antibody complexes were detected using 3,3′-diaminobenzidine as a substrate. Once proper staining intensity was achieved, the membrane was washed several times with water to stop the reaction.

Growth of bacteria
All bacterial strains were grown on Youschimizu–Kimura medium (Youschimizu and Kimura 1976) at 25°C for 2 days. Bacterial cells were harvested by centrifugation and collected by centrifugation at 8000×g for 10 min at 4°C. The pellets were washed twice with cold PBS and then with 50 mM Tris-HCl–0.15 M NaCl at pH 7.5. The bacterial cells were resuspended in 50 mM Tris–HCl–0.15 M NaCl at pH 7.5, and 1 ml of bacterial cell suspension was added to 10 ml of 50 mM Tris–HCl–0.15 M NaCl at pH 7.5. The suspension was incubated at 37°C for 2 h, and the reaction mixture was then centrifuged at 12000×g for 10 min at 4°C. The supernatant was used for the subsequent experiments.

Isolation of mannans
Paper chromatography was performed on Whatman 1 paper using mixture n-butanol–pyridine–water (6:4:3 v/v) as eluent. Gas-liquid chromatography was carried out using an Agilent 6890N gas chromatograph equipped with a GC-5MS capillary column (30 m × 0.25 mm i.d.). Helium as a carrier gas within the temperature range 150–230°C. GLC-mass-spectra were recorded on Finnigan MAT ITD-700 instrument at the same conditions. Anion-exchange chromatography was performed on a DEAE TSK 650M column (50 × 500 mm) eluted with 50 mM Tris-HCl buffer (pH 7.0) and then with 0.5 M NaCl in the same buffer. Gel permeation chromatography was carried out on a column (1.5 × 90 cm) with TSK 50(F) gel in water.

Sugar analysis
Polysaccharide samples were hydrolyzed with 2 M trifluoroacetic acid at 120°C for 3 h and analyzed by paper and gas-liquid chromatography.

Methylation analysis
Mannan samples were methylated as described in (Hakomori 1968). The partially methylated products were reduced with NaBH₄, acetylated, and analyzed by gas-liquid chromatography and mass-spectroscopy. Molar ratios were obtained using effective response factors, and peak areas were normalized with respect to that of inositol hexa-acetate, used as the internal standard.

NMR spectroscopy
The 13C NMR spectra were recorded on a Bruker WM-250 spectrometer for solutions in D₂O at 30°C (internal methanol, δC 50.15 ppm).

Determination of absolute configuration
Absolute configuration was determined as described in (Leontine et al. 1978). A 1-mg mannan sample was hydrolyzed with a of 2 M CF₃COOH for 1 h at 120°C. After neutralization, the crude reaction was treated with (−)-2-octanol (0.5 mL) with one drop of CF₃COOH overnight at 130°C with constant stirring. After usual work-up, the crude reaction was acetylated with Ac₂O and C₅H₅N and analyzed by gas-liquid chromatography.

RNA isolation and cDNA synthesis
Total RNA was isolated from the coelomocytes by guanidine thiocyanate–phenol–chloroform method (Chomczynski and Sacchi 1987). A cDNA library was synthesized from total RNA and amplified by a SMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA) using the provided protocol. cDNA amplification PCR was carried out for 23 cycles at 95°C (8 s), at 63°C (30 s), and at 72°C (1.5 min).

cDNA cloning and sequencing
Degenerate primers were designed to the determinate N-terminal amino acid sequence: TACPEFW and the sequence GLHASIH conserved for holothurians’ CTLs CEL-1V (GenBank accession no. AAB35250) and SJL-1 (GenBank accession no. Q7M3Y0). They were respectively 5′-ACNGCNTG(T/C) CCNGA(G/A)TT(T/C)TGG-3′ and 5′-TG(A/G)ATNGANGC- NAA(A/G)TGNCC-3′.

The amplified cDNA was diluted 50-fold in water, and 1 μL of this dilution was used for PCR with these primers. PCR reactions were carried out for 32 cycles of 10 s at 95°C, 30 s at 58°C, and 30 s at 72°C. Approximately 160-bp PCR fragment was obtained. The PCR product was cloned by InstAclone™ PCR Product Cloning Kit (Fermentas, Glen Burnie, MD) and sequenced using ABI 310 automated sequencer with Big Dye™ (ABI Prism) dye oxidetinators. The 3′- and 5′-terminal regions of the cDNA encoding MBL-AJ were amplified by the method of 3′- and 5′-RACE with specific primers designed on the determined sequence adapter-specific primer as described (Matz et al. 1999). Gene specific primers for 5′- and 3′-RACEs were AJ3 -5′−CCATGATCATCTGACCGTAC−3′; AJ5: 5′-GTTAACGTCAGTAGCATAGGG-3′. The PCR profile was 95°C, 10 s; 60°C, 20 s; 72°C, 1 min. Two obtained fragments with lengths 270 bp and 550 bp were cloned and sequenced as described above.

Sequence analysis and comparisons
Sequences were analyzed using the NCBI-BLAST2 website (http://www.ncbi.nlm.nih.gov/blast2/). Domain architectures were identified by SMART tool (http://smart.embl-heidelberg.de/). The presence and location of signal peptide cleavage sites in amino acid sequences was predicted by program SignalP (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignment was performed with the Clustal W version 1.8 software (http://www.ebi.ac.uk/clustalw/index.html).

Funding
Russian Foundation for Basic Research (RFBR) (07-04-01247) and (07-08-00638); Far Eastern Branch Russian Academy of Sciences- RFBR (FEBRAS-RFBR) (06-04-96039).

Acknowledgements
We are thankful to Prof. Antonio Molinaro (University of Napoli, Italy) for providing the sample of mannan from
Pseudomonas syringae pv. ciccaronei. We are thankful to Mr. Eric F. Howard (Department of Biochemistry, Vanderbilt University) for peptide sequencing of the MBL-AJ N-terminal end.

Conflict of interest statement
None declared.

Abbreviations
CRD, carbohydrate-recognition domain; CTL, C-type lectin; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MBL, mannan-binding lectin; MES, 2-[N-Morpholinol] ethanesulfonic acid; TB-Ca<sup>2+</sup>, tris buffer, containing 10 mM CaCl<sub>2</sub> (0.01 M Tris-HCL, pH 7.5, 10 mM CaCl<sub>2</sub>); TBS-Ca<sup>2+</sup>, tris buffered saline, containing 10mM CaCl<sub>2</sub> (0.01 M Tris-HCI, pH 7.5, 0.15 M NaCl and 10 mM CaCl<sub>2</sub>); TBS-T, tris buffered saline, containing 0.05% Triton X-100.

References


