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Characterization and bacterial-binding activity of a novel C-type lectin from the red-spotted grouper, *Epinephelus akaara*

Z.W. Zhang[1,2], X.F. Shi[1], M. Qu[1] and S.X. Ding[1]

[1]State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, Fujian, China
[2]Fishery Research Institute of Shanghai, Shanghai, China

Corresponding author: S.X. Ding
E-mail: sxding@xmu.edu.cn

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ABSTRACT. Because of their specific binding to carbohydrates, lectins play a crucial role in pathogen recognition and clearance in vertebrate animals. Previously, only two types of collectins had been isolated from bony fish: mannan-binding lectin (MBL) and galactose-binding lectin (GalBL). We sequenced a novel collectin (designated *EALec1*) from the red-spotted grouper, *Epinephelus akaara*. The gene structure of *EALec1* and the alignment of the carbohydrate recognition domain of the three collectins demonstrated that EALec1 is a new type of collectin derived from MBL. We examined the expression pattern of the *EALec1* transcripts in 12 tissues of the red-spotted grouper. The *EALec1* gene was found to have multiple copies; their transcripts were detected in all 12 tissues. *EALec1* was also recombined and expressed in *Escherichia coli* to investigate its immune functions and carbohydrate-binding characterization. We concluded that EALec1 belongs to the mannan-binding lectin group, despite its different Ca^{2+}-dependent sites in the carbohydrate recognition domain, and that it is involved in the recognition and

clearance of invaders in the red-spotted grouper.

Key words: *Epinephelus akaara*; C-type Lectin; Genomic structure; mRNA expression; Recombinant expression; Bacterial agglutination

INTRODUCTION

The vertebrate immune system comprises two mechanisms: the innate and the adaptive defense mechanisms (Schluter et al., 1997; Ellis, 2001; Pancer and Cooper, 2006). As primitive vertebrates, fish likely obtained their adaptive immunity in ancient evolutionary history (Carroll and Prodeus, 1998). The adaptive immune defense in fish has more constraints than in endothermic vertebrates because of its temperature-dependence, limited antibody repertories, affinity maturation and relatively slow response (Janeway and Travers, 1997; Magnadottir, 2006). Thus, innate immunity is considered to be an important defense system against infection in fish (Medzhitov and Janeway, 2000; Whyte, 2007).

The complement system is a crucial component in the innate immunity of teleost fish (Walport, 2000; Boshra et al., 2006). This system can be activated through three separate pathways: the classical pathway (i.e., the C1q pathway), the alternative pathway, and the lectin pathway (Fujita et al., 2004a; Boshra et al., 2006). The latter pathway functions by binding pathogens to two distinct groups of lectins, mannan-binding lectin (MBL) and ficolins, with which MBL-associated serine proteases (MASPs) associate to activate C4, C2 and C3 (Endo et al., 2003; Dodds and Matsushita, 2007; Gaboriaud et al., 2007). The MBL and ficolins have three similar domains: a cysteine-rich N-terminal region, a collagenous region characterized by Gly-X-Y repeats (X and Y stand for any amino acids) (Dodds and Matsushita, 2007), and a neck region. The major differences between them include fewer repeats of Gly-X-Y within ficolin and a fibrinogen-like domain within the C-terminal region of ficolin (Matsushita and Fujita, 2002; Atkinson et al., 2004; Nakao et al., 2006; Runza et al., 2008).

As a component of innate immunity capable of activating complement reactions by the lectin pathway (Zapata et al., 1997; Nonaka and Smith, 2000), the MBL was first discovered in human liver (Miller et al., 1968). Recently, an MBL-like lectin, the glucose-binding lectin (GBL), was isolated from ascidians (genus *Urochordata*) (Sekine et al., 2001; Endo et al., 2006), which have been identified as the most primitive species with a lectin pathway (Fujita et al., 2004b; Endo et al., 2006). In comparison to the vertebrate MBL, the GBL lacks a collagen region but has an identical tripeptide-binding motif, Glu-Pro-Asn (EPN type), in its carbohydrate recognition domain (CRD). The GBL can bind to MASPs and then activate C3/C4-like TEP. Thus, the GBL is considered to be a prototype of the MBL in an early evolutionary stage (Fujita et al., 2004b; Dodds and Matsushita, 2007). The lamprey is the most primitive animal found to possess the MBL (Takahashi et al., 2006; Dodds and Matsushita, 2007). Two types of the MBL group have been characterized in the bony fish: one has a QPD-type galactose-binding CRD (with Gln-Pro-Asp motif) and is designated GalBL, and the other has an EPN-type mannose-binding CRD and is designated MBL (Vitved et al., 2000; Nakao et al., 2006; Jackson et al., 2007). Previous studies have shown that GalBL was probably mutated from an MBL extra copy and has since acquired galactose-binding capability (Nonaka and Smith, 2000).

In a previous study, we isolated a complete lectin cDNA sequence from the red-spotted grouper, *Epinephelus akaara*, a valuable commercial fish in southern China (Heemstra and Ran-

dall, 1993). This locus possesses all of the distinct characteristics of a collectin, yet has an EPD-type CRD (with Glu-Pro-Asp motif) (manuscript submitted). Here, we designated it *EALec1*. Although a lectin with the same carbohydrate recognition sites has been isolated from the Japanese flounder (Kondo et al., 2007), an analysis of the protein domain features showed that the protein does not have a collagenous region characterized by Gly-X-Y repeats, and thus does not belong to the collectin group (Thiel, 2007). EALec1 can be regarded as a novel collectin that was first identified in the bony fish. To study the evolution of EALec1, we sequenced its genome structure and analyzed its expression pattern in different tissues using single-strand conformation polymorphism (SSCP) analysis and the qRT-PCR technique. The bacterial agglutination activity and carbohydrate-binding specificity of the recombinant EALec1 protein were also investigated to understand the roles of EALec1 in the immune responses of the red-spotted grouper.

MATERIAL AND METHODS

Material

One-year-old specimens of *E. akaara*, with a fresh weight of about 400 g, were collected from a local market in Xiamen, China, and were maintained in filtered seawater for 1 week before processing.

Three bacterial strains were used in this study. *Staphylococcus aureus* Rosenbach and *Bacillus subtilis* were supplied by the Institute of Microbiology at the Chinese Academy of Sciences, and *Vibrio harveyi* was isolated in our laboratory (Xu et al., 2009).

All primers used in this study (Table 1) were synthesized at Invitrogen (Shanghai, China).

Cloning of the full-length genomic sequence of the *EALec1* gene

Based on the complete cDNA sequence of *EALec1* (NCBI accession No. FG 392023), isolated from *E. akaara* in our laboratory, the specific PCR primers LectinD1F and LectinD1R (Table 1) were designed to amplify the genomic sequence of the *EALec1* gene. Extraction of genomic DNA from the muscle of *E. akaara* was performed using the Invisorb Spin Tissue Mini kit (Invitek, Germany). PCR was carried out with one denaturation step of 5 min at 94°C, 32 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 3 min, and a final extension at 72°C for 5 min. The PCR products were gel-purified using the QIAEXII Gel Extraction kit (Qiagen, USA) and cloned into the pMD18-T simple vector (TaKaRa, Japan). They were then transformed into competent cells of *Escherichia coli* JM109. The positive clones were sequenced at Invitrogen.

Expression detection of EALec1 in different tissues

The mRNA expression pattern of EALec1 was determined in the different tissues using the qRT-PCR and SSCP techniques. Total RNA of the tissues, including blood, ovary, liver, spleen, muscle, gill, head kidney, intestine, stomach, fin, heart, and brain, was extracted using Trizol (Invitrogen, USA) in accordance with manufacturer instructions. After treatment with RNase-free DNase (Qiagen), 1 μg RNA from each tissue was used for cDNA synthesis following the manual for Promega M-MLV use. RT-PCR and real-time RT-PCR were then performed using the two specific primers (LectinF197 and LectinR599) designed from the EALec1 cod-

ing sequence (Table 1). A 400-bp fragment of the EALec1 sequence was amplified. In parallel, a 300-bp fragment of the b-actin sequence was also amplified to serve as an internal control of RT-PCR with the primers actinF and actinR (Table 1). The PCR procedure involved one cycle of 94°C for 2 min and 30 cycles (or 28 cycles for b-actin) of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension of 72°C for 5 min.

Table 1. The oligonucleotide primers used in these experiments.

Primer name	Sequence
LectinF	5'-ACTTCAGCCATATGCCTGCAGGTCGACG-3'
LectinR	5'-CTCGAATTCACTT GAATGGGGT-3'
LectinD1F	5'-ATGAACCTCATCAAAAAGGAA-3'
LectinD1R	5'-TCACTTGAATGGGGTAGGCGCC-3'
LECTINF197	5'-TGAGACTAAACTTGTTGGAGGAT-3'
LECTINR599	5'-GCCTGGCT GTTGTCTGGCTCTGT-3'
actinF	5'-AAGCCAACAGGGAGAAGATGAC-3'
actinR	5'-GCCAGGTCTTCACCGCCGTCATGGT-3'
Oligo-dT-RA	5'-AAGCAGTGGTATCAACGCAGAGTGTAC$(T)_{25}$VN-3'

Before SSCP analysis, 2 µL RT-PCR product were mixed with 2 µL denaturing buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanole FF, 1 mM EDTA, and 10 mM NaOH). The samples were denatured at 95°C for 5 min and immediately cooled on ice for 2 min before they were separated by 1-mm 8% native polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed on 1X TBE buffer under optimized conditions (200 V for 5 h at 4°C). Prior to silver staining, the gels were fixed with 10% acetic acid for 20 min, rinsed twice in deionized water and soaked in 0.01 M silver nitrate for 30 min. The gels were then rinsed twice in deionized water, and reduction was carried out in a solution of 0.75 M sodium hydroxide and 0.085 M formaldehyde until the DNA bands were clearly visible (maximum 10-15 min). The reaction was stopped by transferring the gels to 0.07 M sodium carbonate for 30 min. The different DNA bands were purified and cloned into the pMD18-T simple vector (Takara), and then transformed into the competent cells of *E. coli* JM109. Three positive clones of each band were sequenced at Invitrogen.

Real-time RT-PCR was performed on a Rotor-Gene RG3000 (Corbett Research, Australia) using a SYBR Green 2-Supermix kit (Applied Biosystems, USA). PCR was carried out with one cycle of 94°C for 2 min and 40 cycles of 94°C for 15 s, 55°C for 20 s and 72°C for 30 s. The fluorescence output for each cycle was measured and recorded at the end of the entire run. Cycle threshold values were calculated using the Rotor-Gene-6171 software and were converted into an equivalent target amount (ETA) using the established statistical standard curve. The expression levels of EALec1 were with respect to b-actin as previously described.

Protein expression and purification

The protein-coding region of the *EALec1* gene was amplified using the specific PCR primers LectinF and LectinR from the cDNA of the liver. To enable cloning, a *Nod*eI site was added to the 5'-end of LectinF and an *Eco*RI site to the 5'-end of LectinR. The RT reaction began with a 5 min denaturation step at 95°C, followed by 32 cycles of 1 min at 95°C, 1 min at 52°C, and 1 min at 72°C, with a final extension at 72°C for 5 min. After running on a 1% agarose gel, the PCR fragment was gel-purified and completely digested with the restriction enzymes *Nod*eI and *Eco*RI

(NEB, USA) and then cloned into the *Nod*eI / *Eco*RI sites of the expression vector pET28(A). The recombinant plasmid (pET-28(A)-EALec-1) was transformed into *E. coli* BL21(DE3)-pLysS.32a. The positive clones were screened using PCR with the specific primers LectinF and LectinR. The recombinant plasmid was extracted from the positive clones and digested with *Nod*eI and *Eco*RI, then sequenced to confirm the exact insert. The positive clone was named EALec-12.

EALec-12 was added to 100 mL Luria broth solution with 50 µg/mL kanamycin. The solution was shaken overnight at 37°C. Next, 10 mL starter culture were added, and the solution was incubated for about 2 h until the density approached OD_{600} = 0.2-0.3. The expression of the protein was induced by adding 0.2 mM isopropyl-D-thiogalactopyranoside (IPTG). After 4 h of induction at 37°C, the cells were harvested by centrifugation at 3500 *g* for 2 min at 4°C. Subsequently, all of the cells were stored in liquid nitrogen overnight. After thawing, the bacterial cells were resuspended in a 40 mL column buffer and sonicated in an ice-water bath. After centrifugation at 12,000 *g* for 30 min at 4°C, the supernatant was purified with a Ni-NTA Agarose column (Qiagen), and the isolated protein was used for the activity assay. The purified recombinant EALec was subjected to 12% SDS-PAGE at 200 V for 3 h. The protein separation was visualized by staining with Coomassie brilliant blue R-250 (Sigma, USA). Molecular weight protein standards were used to determine the target protein size.

Bacterial agglutination and bacterial agglutination inhibition assay

The Gram-negative pathogenic *V. harveyi* and Gram-positive non-pathogenic *S. aureus* Rosenbach and *B. subtilis* were incubated in 100 mL LB separately. Subsequently, they were harvested by centrifugation at 3500 *g* for 2 min at 4°C, suspended in a Tris-buffered saline (TBS) (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) at OD = 0.5, and then stored at 4°C before further processing. The purified protein was desalted via centrifugation in a Millipore Amicon Ultra (Millipore, USA) at 7000 *g* for 40 min. The concentration was then adjusted to 100 µg/mL using TBS1 (100 mM Tris, 1 mM NaCl, pH 7.5). The protein concentration was measured using the Bradford method (Thiel, 2007). An aliquot of 8 µL bacterial suspension was added to 1 µL recombinant EALec. TBS2 (100 mM Tris, 1 mM NaCl, 100 mM $CaCl_2$, pH 7.5) was used to adjust the concentration of Ca^{2+} to 20 mM in the mixtures. TSB1 was used as the negative control. The mixtures were incubated overnight at 4°C. To examine whether the agglutination was calcium dependent, the cells were observed using light microscopy and photographss were taken.

To test the carbohydrate-binding specificity of EALec, 4 µL serial dilutions (80, 60, 50, 40, and 20 mM) of D-glucose, D-galactose, D-mannose, and mannose-6-P (Sigma) were premixed with 1 µL 100 mg/mL EALec at room temperature for 1 h. Next, 14 mL *V. harveyi* suspension (20 mM Ca^{2+}) were added to each of the carbohydrate - CLHd mixtures. The mixtures were stored overnight at 4°C before observation.

RESULTS

Genome organization of the *EALec1* gene from *E. akaara*

The genome structure of the *EALec1* gene of *E. akaara* was elucidated by comparing the full-length cDNA sequence of EALec1 to the corresponding genomic fragments sequenced. The coding region of this gene consists of 2714 bp and is grouped into 5 exons with

lengths of 22, 183, 152, 122, and 169 bp, and 4 introns with lengths of 85, 162, 96, and 1723 bp (Table 2). All exon-intron junctions follow the consensus rule of the splice donor [GT and acceptor sites (AG) for splicing (Figure 1)]. The CRD domain of the EALec1 of *E. akaara* is encoded by exons 3, 4 and 5 (Figure 2).

Table 2. The introns and exons in the *Epinephelus akaara EALec* genomic sequence.

EXON	Genomic coordinates	mRNA coordinates	Length	Encoding domain
EXON1	1-22	1-22	22	M
EXON1	108-290	23-205	183	Col+N
EXON3	453-604	206-357	152	N+CRD
EXON4	701-822	358-479	122	CRD
EXON5	2546-2714	480-648	169	CRD

Figure 1. The *Epinephelus akaara* EALec genomic sequence. The sequences with frames are the specific primers, and the shaded ones represent the mRNA sequence.

The constitutive expression of the *EALec1* transcript in different tissues

To identify where the *EALec1* gene was expressed, RNA analyses were performed with qRT-PCR for 12 tissues including blood, ovary, liver, spleen, muscle, gill, head kidney, intestine, stomach, fin, heart, and brain. The results suggest a broad distribution of constitutive expression. Nevertheless, the *EALec1* mRNA was best expressed in the head kidney and spleen, and least expressed in the liver (Figure 3). Based on the SSCP analysis, four genotypes were identified in all the tissues (Figure 4). The four Genbank accession numbers are FJ426399, FJ426400, FJ426401, and FJ426402.

FJ426399 and FJ426400 have a same-sense mutation. FJ426401 has a mutation that leads to an amino acid substitution, and the mutation in FJ426402 results in an unexpected translation stop (Figure 5). The result showed that there were at least 2 *EALec1* paralogs in the *E. akaara* genome.

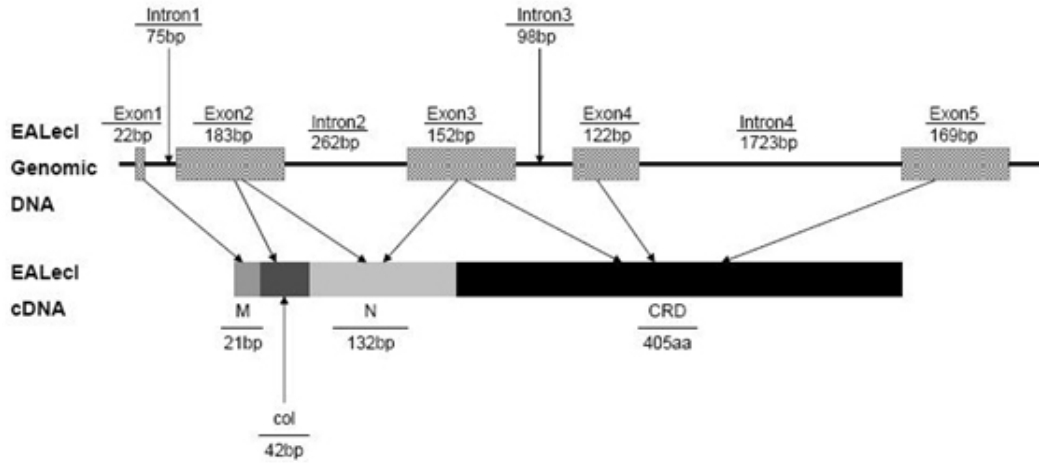


Figure 2. The genomic and cDNA structure of *EALec1* of *Epinephelus akaara*. The exons coding for the proteins are shown as boxes, and the introns are shown as straight lines. The sizes are shown above. M = initiation methionine; Col = collagenous region; N = neck region; CRD = carbohydrate recognition domain.

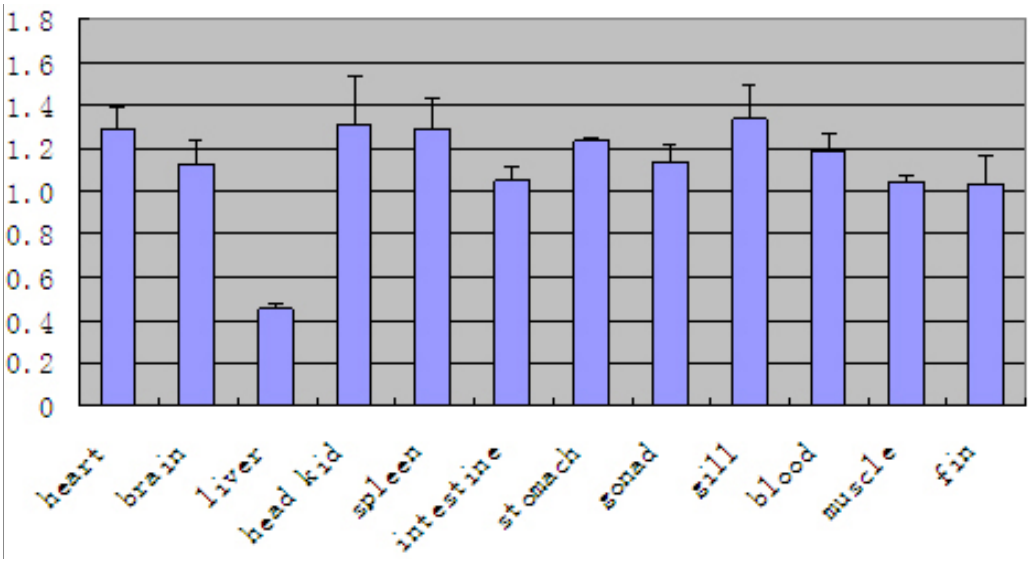


Figure 3. The distribution of the tissues of the EALec1 transcripts that were measured by real-time PCR.

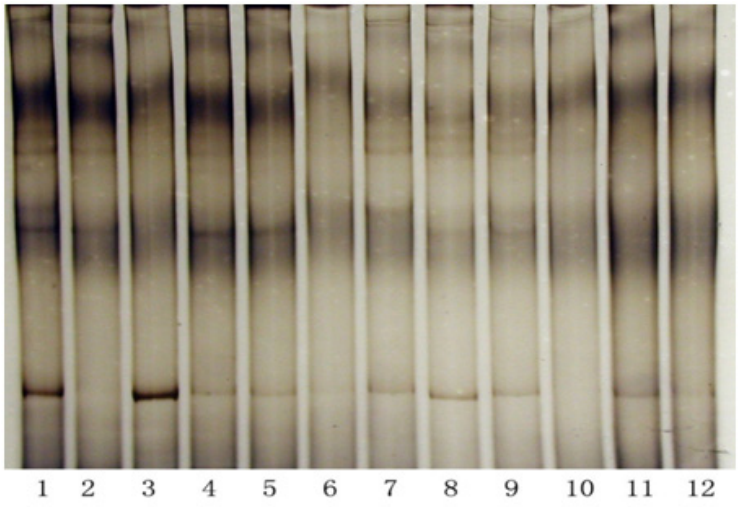


Figure 4. The EALec1 homologues detected by RT-PCR-SSCP. *Lane 1* = fin, *lane 2* = muscle, *lane 3* = blood, *lane 4* = gill, *lane 5* = gonad, *lane 6* = stomach, *lane 7* = intestine, *lane 8* = spleen, *lane 9* = head kidney, *lane 10* = liver, *lane 11* = brain, and *lane 12* = heart.

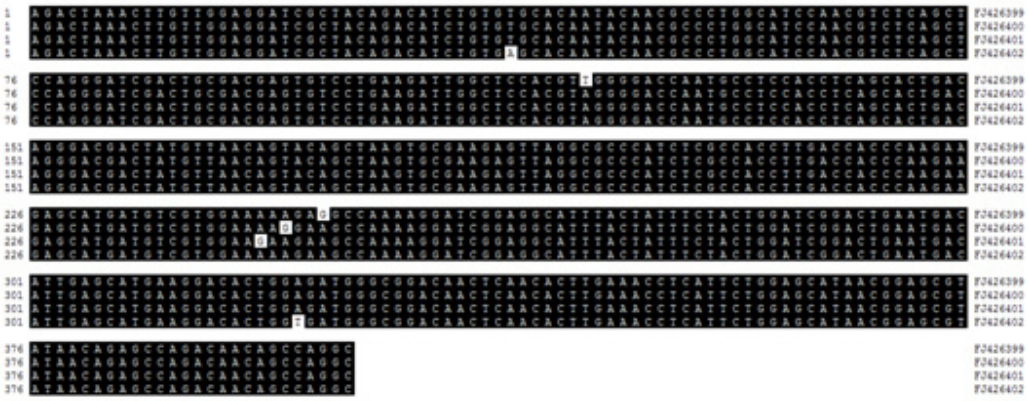


Figure 5. The alignment of 4 EALec1 homologues identified by SSCP.

Recombinant expression of EALec1 in *E. coli*

The cDNA sequences encoding the complete EALec1 of *E. akaara* were fused to the His-Tag coding sequence at the 5'-end. This allows the recombinant protein to be expressed with an additional 6-His-Tag at the C-terminal and to be induced with IPTG. As shown in Figure 6, the recombinant proteins were well induced. In contrast to the cells before induction, a strong band (Figure 6A) was found around 26 kDa in the lysate and the supernatant of the induced cells. The molecular weight was also consistent with the theoretical value of the entire fusion protein of complete EALec1. Each recombinant protein with the His-Tag was purified via Ni-NTA agarose affinity chromatography. The result showed that only one band was distinguishable (Figure 6B).

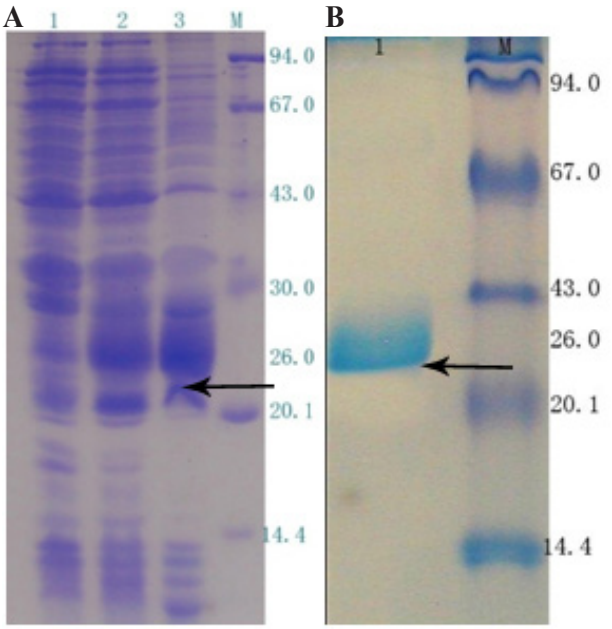


Figure 6. **A.** SDS-PAGE analysis of recombinant CLHd expressed in *Escherichia coli*. *Lane 1* = lysate of *E. coli*. BL21(DE3) without induction; *lane 2* = recombinant EALec with expression induced by 0.5 mM IPTG in *E. coli* BL21(DE3) incubated 3 h; *lane 3* = recombinant EALec with expression induced by 0.5 mM IPTG in *E. coli* BL21(DE3) incubated 5 h; *lane 4* = protein marker. The specific band of recombinant CLHd is indicated by an arrow. **B.** SDS-PAGE analysis of purified recombinant EALec. *Lane 1* = purified recombinant CLHd; *lane 2* = protein marker. The molecular mass of each protein marker band is indicated on the right side of the acrylamide gel.

Bacterial agglutination activity of EALec1

The Gram-negative pathogenic *V. harveyi* and the Gram-positive non-pathogenic *S. aureus* and *B. subtilis* were used to test the bacterial agglutination activity of EALec1. In 20 mM Ca^{2+}, agglutination was evident when *V. harveyi* (Figure 7B), *S. aureus* (Figure 7D) and *B. subtilis* (Figure 7F) were incubated with EALec1 at a concentration >10 μg/mL. The agglutination of *V. harveyi* (Figure 7A), *S. aureus* (Figure 7C) and *B. subtilis* (Figure 7E) was not, however, observed in the absence of Ca^{2+}. This result indicated that EALec1 is a Ca^{2+}-dependent type lectin.

The carbohydrate-binding specificity of EALec1

The carbohydrate-binding specificity of EALec1 was examined using competitive inhibition of various sugars on bacterial agglutination. Table 3 shows the minimum inhibitory concentration of each sugar. When the D-mannose concentration was greater than 50 mM, the bacterial agglutination activity of EALec1 was inhibited. In contrast, glucose, galactose and mannose-6-P did not show any inhibitory activity. These results suggested that EALec1 is a mannose-binding type lectin.

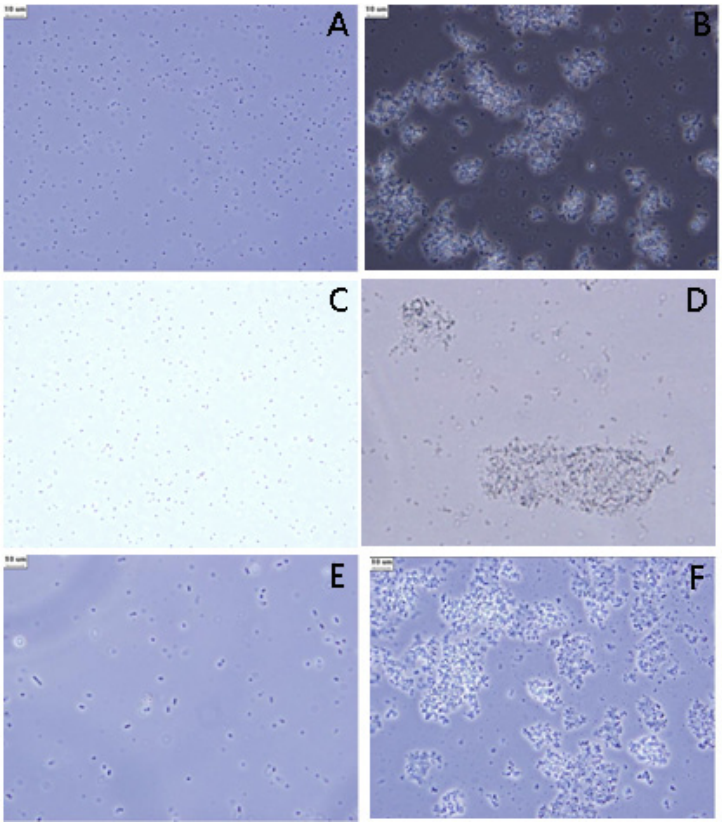


Figure 7. Bacterial agglutination induced by EALec1. **A. C. E.** control, *Vibrio harveyi*, *Staphylococcus aureus* and *Bacillus subtilis* were incubated in Ca^{2+}-free TBS buffer. **B. D. F.** *V. harveyi*, *S. aureus* and *B. subtilis* were incubated in 20 mM Ca^{2+}.

Table 3. The inhibition of the bacterial agglutination activity of EALec1 by carbohydrates.

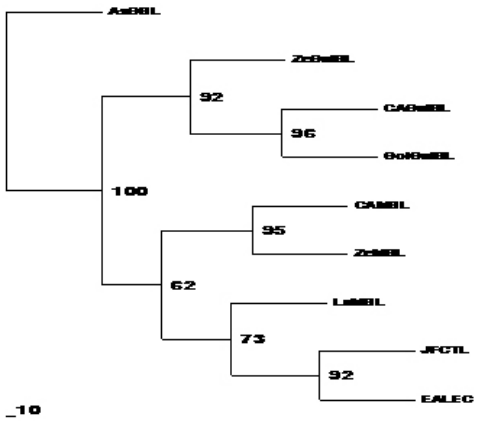
Saccharide	Minimum inhibitory concentration
Mannose	50 mM
Galactose	NI
Glucose	NI
Mannose-6-P	NI

NI = no inhibitory activity.

DISCUSSION

In the ascidian genome, nine collectin-like genes have been identified. In comparison to all of the C-type lectin domains of the non-collectin type genes, these C-type lectin domains with collagen form a monophyletic cluster and become a sister group to the vertebrate collectin cluster (Dodds and Matsushita, 2007). Three collectin-like isomers that possess EPN, EPS or EPT-type CRDs have also been identified in *Ciona intestinalis*. This evidence suggests that the *C. intestinalis* collectin-like genes may share an ancestor with the vertebrate MBL genes

(Dodds and Matsushita, 2007). Different types of CRD further evolved through gene duplication in fish (Nakao et al., 2006). The EPN-type collectin in lamprey is the earliest vertebrate MBL, whereas GalBL, which has a QPD carbohydrate recognition domain in bony fish, is believed to be a mutated copy from MBL duplication at a certain stage (Vitved et al., 2000; Nakao et al., 2006). In this study, a new C-type collectin with EPD-type CRDs, designated EALec1, was isolated from *E. akaara*. In the carbohydrate recognition domain, this new collectin seems to be an interim type between MBL and GalBL. Nevertheless, the alignment analyses of the amino acid sequences of MBL (or MBL-like) partial CRD from ascidians to human beings show that EALec1 has more evident insert residues than the MBL or GalBL of primitive vertebrates (Figure 8). This suggests that EALec1 likely evolved early as a prototype of the bony fish MBL.

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C-TYPE          W         P       G       C             WND   C
                |         |       |       |             |||   |
LaMBL       NWNAG----EPNNA--GGD--EDCAVIVANGGK-WNDVRCS
ZeMBL       NWKEK----EPNDYN-GA---EDCTAVYKTGV--WNDINCN
CAMBL       NWKEK----EPNDYN-GA---EDCTVMYKSGV--WNDVNCN
CAGalBL     NWGPG----QPDDYK-GL---QDCGYIEDSGL--WDDGSCD
GolGalBL    NWGPG----QPDDYK-GL---QDCGVREDTGL--WDDGGCG
ZeGalBL     NWGPN----QPDNYK-GA---QDCGAIADSGL--WDDVSCD
AsGBL       AWGPN----EPNDS--G--N-ERCGQLNRDSNYLINDKSCL
EALEC       FWSITERITEPDNSQAGGQEGEDCVVVDSTHV--WSDVPCN
JFCTL        SWNILKS--EPDNNLSDGPKGEDCAVVDSRSQS-WYDVPCS
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Figure 8. Alignment of the partial CRD amino acid sequences of C-type lectins.

Three copies of the GalBL gene in the *Danio rerio* genome have been reported (Jackson et al., 2007). All of them were found to comprise 3 exons and to have the same QPD motif in the CRD domain. However, the MBL gene that possesses the EPN motif has only one copy in the *D. rerio* genome and is composed of 4 exons (Nakao et al., 2006; Jackson et al., 2007). In the present study, the *EALec1* gene was found to have multiple copies in the *E. akaara* genome and is composed of 5 exons (Figure 9). The most remarkable difference between EALec1 and MBL/GalBL in the genomic structure occurs in the first exon. Compared to MBL and GalBL, the first exon in EALec1 lacks a partial collagenous sequence (Gly-X-Y repeats).

The whole EALec1 protein has only 4 Gly-X-Y repeats. In contrast, MBL and GalBL have more than 10 repeats in the collagenous region (Thiel, 2007). This apparent lack of the partial collagenous region-encoding sequence in EALec1 could be due to an incomplete assembly of contigs and scaffolds such as with MBL in *Takifugu* (Nakao et al., 2006). It also implies that EALec1 might have originated from a common ancestor of vertebrate collectin genes, including MBL.

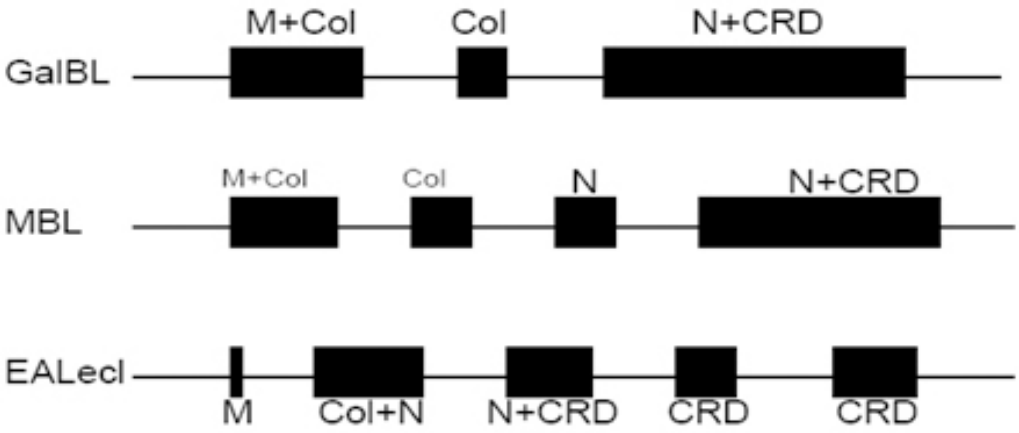

Figure 9. The genomic structure of the three type of collectins. The abbreviations for domain names: M, the initiation methionine; Col, collagenous region; N, neck region; CRD, carbohydrate recognition domain.

MBLs seem to have different expression pattern in different tissues. Two MBL homologues from rainbow trout (*Oncorhynchus mykiss*) are expressed exclusively in the liver or the spleen (Nikolakopoulou and Zarkadis, 2006). GalBL can be detected in the spleen, liver, gill and intestine in the cyprinidae, gold fish and zebra fish (Vitved et al., 2000). In this study, EALec1 was detected in all 12 tissues examined, which means that expression of this novel C-type lectin was not specific to any tissues of *E. akaara*, thereby resulting in a broader defensive area than that of MBL.

As a C-type lectin, EALec1 also has a calcium-binding site. It can agglutinate Gram-negative pathogenic *V. harveyi* and Gram-positive non-pathogenic *S. aureus* and *B. subtilis* in the presence of Ca^{2+}. This study shows that EALec1 has a broad-spectrum bacterial agglutination activity in vitro when compared to some other lectins (Ewart et al., 2001; Zheng et al., 2008; Russell et al., 2008). To our knowledge, this is the first time that a lectin gene from a fish was isolated, recombinantly expressed in *E. coli* and processed into a bacterial agglutination activity test *in vitro*. The carbohydrate-binding specificity test demonstrated that EALec1 is a mannose-binding lectin, despite the presence of the EPD-type CRDs. The results also imply that the sequences of the carbohydrate-recognizing sites in the lectin could be flexible in some way to recognize specific carbohydrates.

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