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A unique epidermal mucus lectin identified from catfish (*Silurus asotus*): first evidence of intelectin in fish skin slime

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Shigeyuki Tsutsui[1,*†], Yukie Komatsu[1], Takaya Sugiura[1], Kyosuke Araki[2] and Osamu Nakamura[1]

[1]School of Marine Biosciences, Kitasato University, 160-4, Uto, Okirai, Sanriku, Ofunato, Iwate 022-0101 and [2]Faculty of Fisheries, Kagoshima University, Shimoarata, Kagoshima 890-0056, Japan

*Shigeyuki Tsutsui, School of Marine Biosciences, Kitasato University, 160-4, Uto, Okirai, Sanriku, Ofunato, Iwate 022-0101, Japan. Tel: +81-42-778-8111, Fax: +81-42-778-8111, email: tsutsui@kitasato-u.ac.jp

†Present address: Shigeyuki Tsutsui, 1-15-1, Kitasato, Sagamihara, Kanagawa 252-0373, Japan.

The nucleotide and deduced amino acid sequences reported in this article are available in the DDBJ/EMBL/GenBank databases under accession numbers AB598141 and AB598142.

The present study reports a new type of skin mucus lectin found in catfish *Silurus asotus*. The lectin exhibited calcium-dependent mannose-binding activity. When mannose eluate from chromatography with mannose-conjugated agarose was analysed by SDS–PAGE, the lectin appeared as a single 35-kDa band. Gel filtration showed that the lectin forms monomers and dimers. A 1216-bp cDNA sequence obtained by RACE–PCR from the skin encoded a 308 amino acid secretory protein with homology to mammalian and fish intelectins. RT–PCR demonstrated that the lectin gene was expressed in the gill, kidney and skin. Subsequent sequencing revealed the presence of an isoform in the gills. Antiserum detected the intelectin protein in club cells in the skin and gill, renal tubules and blood plasma. Although intelectin gene expression was not induced by *in vivo* bacterial stimulation, the intelectin showed agglutination activity against the pathogenic bacterium *Aeromonas salmonicida*, suggesting that the lectin plays an important role in self-defence against bacteria in the skin surface of the catfish. These findings represent one of the few examples of characterization and functional analysis of a fish intelectin protein.

Keywords: catfish/intelectin/lectin/self-defence/skin mucus.

Abbreviations: Chr, chromosome; IntL, intelectin; PBL, peripheral blood leucocyte; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends.

Compared to air, water generally contains greater numbers of microorganisms such as bacteria, fungi and protozoa. The skin of fish is continuously exposed to the dangerous environment, but does not have a stratum corneum and is composed of live cells. The epidermis is therefore a high-risk site of constant attack from microorganisms. It is likely that fish possess a unique defence system in their skin surface, which is different from that of land vertebrates.

Fish skin is covered with mucus, in which various defence molecules such as IgM, muramidase and anti-microbial peptides are present (*1*). In addition to these molecules, lectins are often present in fish skin secretions (*2*). Lectins are defined as proteins with one or more carbohydrate-binding domains, excepting enzymes and immunoglobulins (*3*, *4*), and are believed to mediate innate immunity by specifically binding to saccharides on the surface of pathogenic microorganisms. In fact, some fish skin mucus lectins agglutinate bacteria (*5*–*8*) and enhance phagocytic uptake of microbeads by macrophages (*9*).

Since Muramoto and Kamiya (*10*) reported the amino acid sequence of a skin mucus lectin from the conger eel *Conger myriaster* in 1992, the primary structures of several fish mucus lectins have been revealed. Notably, fish skin mucus lectins exhibit marked structural diversity and are classified into five distinct families, *i.e.* galectin (*6*, *10*, *11*), C-type (*5*, *12*), B-type (*13*, *14*), rhamnose-binding lectin (RBL) (*15*) and pentraxin (*8*). Because fish have a long evolutionary history and thus display huge species diversity, it may not be surprising to find other types of lectins in their skin secretions.

During our comprehensive investigation of fish integument mucus lectins, we identified a unique lectin in the catfish *Silurus asotus*. The lectin is similar to intelectins, also called X-lectins, which is a newly discovered lectin family that plays an important role in innate immunity (*16*). Only 5 years have passed since the first identification of fish intelectin by Chang and Nie (*17*), so information about fish intelectins is still far from sufficient. To date, descriptions of fish intelectins are limited to those of grass carp (*Ctenopharyngodon idella*) (*17*), rainbow trout (*Oncorhynchus mykiss*) (*18*, *19*), channel and blue catfishes (*Ictalurus punctatus* and *Ictalurus furcatus*) (*20*) and zebrafish (*Danio rerio*) (*21*). All these studies are based on cDNA cloning and expression or immunohistological analyses, except for the study by Russell *et al.* (*18*), who purified intelectin from rainbow trout blood plasma and showed its binding activity with several bacteria. Because nobody had yet found intelectin in fish cutaneous secretions, our investigation of the catfish skin mucus intelectin will contribute to further understanding of fish intelectins and the significance of molecular diversity in fish skin mucus lectins. The goal of this study was to characterize

the catfish intelectin protein. We also report the primary structure, gene expression and histological localization of the lectin.

Materials and Methods

Fish

Catfish *S. asotus*, averaging ~185 g in weight and 33 cm in length, were purchased from a commercial dealer in Akita prefecture, Japan, and kept in tanks at Kitasato University with running freshwater. The fish were used in all experiments except for the *in vivo* bacterial immersion and injection tests. For the bacterial test, 24 catfish weighing ~65 g were purchased from a fish farm in Ibaraki prefecture, Japan.

Preparation of skin mucus extract

Skin mucus was collected by scraping the skin surface with a spatula and was homogenized in an equal volume of phosphate-buffered saline containing 0.9 mM $CaCl_2$ and 0.33 mM $MgCl_2$ [PBS (+)]. The homogenate was centrifuged at 15,000*g* for 15 min at 4°C and the supernatant was used as the crude mucus extract.

Haemagglutination assay and inhibition assay

Crude mucus extract was serially diluted with 30 µl PBS (+) in microtitre plates and mixed with 30 µl of 2% rabbit red blood cell suspension. After 1 h at room temperature, samples were checked for haemagglutination. Lectin titre was expressed as the maximum dilution at which agglutination was observed.

The inhibitory effects of various sugars on haemagglutination were assayed as follows: 15 µl of crude mucus extract, pre-diluted 1 : 1000 with PBS (+), was mixed with the same volume of PBS (+) containing 200 mM of D-glucose, D-galactose, D-mannose, D-fucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine or lactose in microtitre plates. After 1 h at room temperature, 30 µl of 2% rabbit erythrocyte suspension was added to each well and lectin titre was determined after an additional 1 h.

Purification of lectins by mannose-affinity chromatography

Twenty millilitres of the crude mucus extract was incubated with 2 ml mannose-agarose (Sigma) suspension at 4°C for 16 h. The agarose was packed into an empty column and washed with 20 ml PBS (+). After overnight incubation at 4°C with 2 ml PBS containing 200 mM D-mannose, the eluted fraction was concentrated and subjected to SDS–PAGE.

SDS–PAGE

SDS–PAGE was performed on a 12.5% separating gel. In brief, 10 µl of the affinity-purified fractions were added to 10 µl SDS sample buffer with or without 10% 2-mercaptoethanol. Electrophoresis was performed using a constant current of 20 mA for 1.5 h and the gel was visualized by staining with Coomassie brilliant blue (CBB) R-250.

N-terminal amino acid sequencing

Purified lectin was subjected to SDS–PAGE under reducing conditions and blotted onto a PolyVinylidene DiFluoride (PVDF) membrane (ATTO) at 100 mA for 1 h. The membrane was stained with Ponceau S and protein bands were carefully excised. Protein sequencing was performed on a PPSQ-21 A protein sequencer (Shimadzu).

Isolation and sequencing of the lectin gene

Catfish were anaesthetized with 2-phenoxyethanol and the skin was dissected. Total RNA was extracted with Isogen RNA extraction solution (Nippon gene).

First-strand cDNA for 3′- and 5′-RACE was synthesized from the skin total RNA using the SMARTer™ RACE cDNA amplification kit with Superscript II reverse transcriptase (Clontech). Each PCR amplification described below was carried out using a thermal cycler (i-Cycler, BIO-RAD) with Advantage2 DNA polymerase (Clontech).

Initially, four forward primers corresponding to the N-terminal amino acid sequence of the lectin (Leu–Phe–Thr–Phe–Asn–Glu–Gln) were separately mixed with the template for 3′-RACE. After 10 cycles PCR (95°C for 30 s, 60°C for 30 s and 72°C for 45 s), Nested Universal Primer-A (NUP-A; Clontech) was added to each reaction as a reverse primer and further PCR was performed for 33 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 45 s. Agarose gel electrophoresis showed that a PCR product derived from forward primer 5′-CTCTTCACNTTYAATGAACA-3′ gave smear bands. To obtain specific amplified DNA fragments, nested PCR was performed in the 25 µl reaction mixture containing one of the primers, corresponding to Phe–Thr–Phe–Asn–Glu–Gln–Glu–Tyr in the lectin N-terminus, and 1 µl of 100-fold diluted first PCR product for 10 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 45 s. One microlitre of NUP-A was added to each sample and further amplification was performed for 33 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 45 s. After agarose gel electrophoresis, a major band at ~900 bp derived from forward primer (5′-CACCTTCAA YGARCAGGAATA-3′) was excised and the DNA extracted with the QIAEX® II gel extraction kit (QIAGEN).

Five-prime RACE was carried out with NUP-A and a reverse primer (5′-GAGATCGTACACCAGAGGAACAGA-3′), which was designed for the 3′-sequence. Denaturation was performed at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 30 s.

Finally, the nucleotide sequences obtained by 3′- and 5′-RACE over the coding region were reconfirmed by PCR amplification using a primer set (ORF-F: 5′-AAGCTCAGGACCTTCTGAGAAG-3′ and ORF-R: 5′-GAAAATGATTCTGGAATGTTCTCC-3′). The primers were also used for the tissue expression analysis described below.

The DNA fragment was subcloned into the pGEM®-T Easy vector (Promega). *Escherichia coli* Hit-DH5α cells (RBC Bioscience) were transformed and grown overnight on Luria Bertani (LB) agar under ampicillin selection. Colonies containing the recombinant plasmid were isolated and sequenced with a 3100-Avant Genetic Analyzer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems).

Bioinformatic analyses

Homology of the deduced amino acid sequence was searched against the DNA Data Bank of Japan homology search system tblastn program (http://www.ddbj.nig.ac.jp/search/blast-j.html). Motifs were searched using the SMART program (http://www.smart.emble-heidelberg.de/). Multiple sequence alignments were performed by ClustalW. A neighbour-joining phylogenetic tree was constructed using ClustalW and MEGA4.

Tissue expression of lectin mRNA

Expression of the lectin mRNA was investigated by RT–PCR of nine tissues (skin, gill, stomach, intestine, liver, kidney, spleen, ovary and brain) from a healthy fish. Peripheral blood leucocytes (PBL) were also prepared as described by Evans *et al.* (*22*) with minor modification. In brief, peripheral blood was collected from the caudal blood vessel using a syringe with a needle, and red cells were removed by centrifugation at 500*g* for 40 min on a cushion of 45% Percoll (Amersham Biosciences). Cells at the top of the cushion were collected and used as PBL. One microgram of total RNA from each tissue was treated with Superscript II reverse transcriptase (Invitrogen) using the SMARTer™ RACE cDNA amplification kit, as described above. Genomic DNA was extracted from the muscle according to the methods of Asahida *et al.* (*23*).

PCR amplification with primers ORF-F and ORF-R, which were designed to cover the full-length open reading frame described above, was performed for 30 cycles. Each cycle was as follows: 94°C for 30 s, 62°C for 30 s and 72°C for 45 s. The PCR products were analysed by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The corresponding β-actin bands for these samples are shown as controls.

Calcium–ion requirement test

Skin mucus was homogenized with the same volumes of 20 mM Tris–HCl (pH 7.2) containing 150 mM NaCl and 10 mM $CaCl_2$ (TBS-Ca) and centrifuged at 15,000*g* for 15 min at 4°C. The supernatant (~20 ml) was incubated with 2 ml mannose-agarose for 16 h at 4°C, and the agarose was packed into an empty column. After the agarose was washed with 20 ml TBS-Ca, 2 ml 20 mM Tris–HCl (pH 7.2) containing 150 mM NaCl and 100 mM EDTA was added to the column. After overnight incubation at 4°C, the eluted fraction was concentrated and analysed by SDS–PAGE.

Determination of intact molecular mass

In order to determine the molecular mass of the lectin in its intact form, gel filtration was carried out by BioLogic Duo Flow system (Bio-Rad) on a column of Superose 6 (Amersham) with 50 mM Tris–HCl (pH 8.0) containing 150 mM NaCl and 100 mM lactose at a flow rate of 0.3 ml/min; absorbance was monitored at 280 nm. Thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B-12 (1.35 kDa) were used as standards.

Production of recombinant lectin

The full-length cDNA-coding ORF with appropriate sites at both ends was synthesized by PCR amplification with primer-Nco-F, 5′-CCATGGACTGGATCATTCTGCTAG-3′ (NcoI site underlined) and primer-Xho-R, 5′-CTCGAGGCGGTAGAAGATGAGCAC-3′ (XhoI site underlined). The PCR product was digested with NcoI and XhoI, and ligated into pre-digested pET-28 a (Novagen).

The expression plasmid was transformed into *E. coli* BL21 (DE3) pLysS Singles Competent Cells (Novagen). Transformants were cultured in 500 ml (LB) containing 20 μg/ml kanamycin at 37°C, and isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM; incubation continued for an additional 3 h at 37°C.

The bacterial cells were suspended in 50 mM Tris–HCl (pH 7.5) including 150 mM NaCl (TBS). The suspension was sonicated and centrifuged at 10,000*g* for 10 min at 4°C. Inclusion bodies were solubilized with TBS containing 8 M urea (TBS-U) and centrifuged at 10,000*g* for 10 min at 20°C. The supernatant was applied to a column in which 1 ml Ni Sepharose™ 6 Fast Flow (GE Healthcare) was packed. After 30 min incubation at room temperature, the resin was washed with TBS-U containing 40 mM imidazole; the column was refilled with 5 ml TBS-U containing 500 mM imidazole and bound protein was eluted. The expression and purification of the recombinant lectin was verified by SDS–PAGE and CBB staining. The N-terminal amino acid sequence was verified as described above.

Preparation of antiserum

A rabbit antiserum to the recombinant epidermal mucus lectin was prepared by multiple injections of purified recombinant protein emulsified in equal volumes of TiterMax Gold (Pharmaceutical Services). Two New Zealand white rabbits were given an initial subcutaneous injection followed by two injections at 2-week intervals. One week after the last immunization, exsanguinations were conducted from blood vessel of the ear, and sera were obtained by centrifugation.

Specificity of the antiserum against the catfish lectin was checked by western blotting as described later, in which skin mucus extract was subjected to SDS–PAGE.

Distribution of the lectin protein

In order to examine the distribution of the catfish lectin, skin, gill and kidney were homogenized with PBS and the extracts were prepared by centrifugation. Blood plasma was also prepared by centrifugation of catfish blood collected from the caudal blood vessel. For western blotting, proteins separated by SDS–PAGE were electroblotted onto a PVDF membrane, blocked for 1 h with 1% skim milk in PBS containing 1% Tween-20 (PBS-Tw) for 30 min at room temperature, and reacted with the antiserum diluted 1 : 2,000 in Solution 1 of Can Get Signal (Toyobo) overnight at 4°C. The membrane was washed three times with PBS-Tw and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG goat antibody (Sigma-Aldrich) diluted 1 : 1,000 in Solution 2 of Can Get Signal (Toyobo). Hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris–HCl (pH 7.5) was used as the HRP substrate.

Immunohistochemistry

The skin, gill and kidney were collected from healthy catfish and fixed with Bouin's solution. These samples were embedded in paraffin blocks and sliced at 5 μm. After removing the paraffin and rehydration, endogenous peroxidase activity was blocked by incubation in MeOH containing 0.3% H_2O_2 for 30 min, and washed with PBS containing 0.5% Triton-X (PBS-Tx). For blocking, the slides were incubated with normal goat serum at room temperature for 30 min. After washing three times with PBS-Tx, the rabbit antiserum against the catfish lectin (1 : 2,000 diluted in PBS-Tx) was added and incubated for 18 h at 4°C. Following three washes with PBS-Tx, sections were incubated with a HRP-conjugated anti-rabbit IgG goat antibody (Sigma-Aldrich) diluted 1 : 1,000 for 1 h at room temperature. Finally, the slides were incubated with 50 mM Tris–HCl (pH 7.5) containing DAB and H_2O_2 for 20 min followed by counterstaining with Mayer's haematoxylin.

Bacteria

Three pathogenic bacteria, *Aeromonas salmonicida* (OTH0234), *Vibrio anguillarum* (NUF113) and *Edwardsiella tarda* (FPC498), were inactivated by formalin as described elsewhere (*24*). In brief, cells were cultured in Bacto™ Tryptic Soy Broth (TSB, BD Difco) and cultured at 20 or 25°C for 48 h with shaking at 100 rpm. Formalin was added at a final concentration of 1%. After incubation at 4°C for 16 h, the cells were washed with PBS and re-suspended in the same buffer.

In vivo bacterial immersion and injection

For the bacterial immersion experiment, 12 catfish were selected, randomly divided into two groups (six individuals each) and kept in tanks with 50 l freshwater at 20°C. One group of catfish was immersed in freshwater in which formalin-killed *A. salmonicida* was present (50 μg/ml) for 1 h, and the other group was immersed in freshwater for 1 h. All catfish were transferred into other tanks, and three individuals from each group were randomly collected at 1 and 3 days post-challenge. The expression of the lectin transcripts in the skin was investigated by RT–PCR as described above.

Bacterial injection was also carried out. Six catfish were injected intraperitoneally with 100 μl of the bacterial suspension (10 mg/ml). Control fish ($n = 6$) received the same volume of PBS alone. At the 1st and 3rd day post-injection, fish were sacrificed and the expression of the lectin transcripts in the skin and kidney were investigated by RT–PCR.

Bacterial agglutination

Agglutination ability of the epidermal mucus lectin was examined. One microlitres of the *A. salmonicida*, *V. anguillarum* and *E. tarda* suspensions (100 mg/ml) were incubated with 10 μl of either purified skin mucus lectin (250 μg/ml TBS-Ca) or the lectin in 0.2 M mannose containing TBS-Ca at room temperature for 1 h. The mixture was placed on a slide glass and agglutination was observed under a light microscope.

Results

Haemagglutination activity of catfish skin mucus

Haemagglutination activity against rabbit red blood cells was detected in catfish skin mucus extract at a titre of more than 2^{15} (Fig. 1A), which indicated that the skin mucus had strong lectin activity. Haemagglutination was inhibited in the presence of mannose, but not other tested sugars (Fig. 1B).

Lectin purification

Mannose-affinity chromatography was performed to purify the skin mucus lectin. On both reduced and unreduced SDS–PAGE, the affinity-purified fraction yielded a single band at ~35 kDa (Fig. 2). We thus conclude that the lectin is a 35-kDa monomer or a non-covalently associated multimer.

Primary structure of the lectin

An N-terminal amino acid sequence, His–Leu–Phe–Thr–Phe–Asn–Glu–Gln–Glu–Tyr–Ser–Xaa–Thr, was obtained by protein sequencing. We then succeeded in isolating full-length cDNA encoding the lectin using the RACE technique and primers designed from the N-terminal amino acid sequence. The nucleotide and deduced amino acid sequences of the catfish

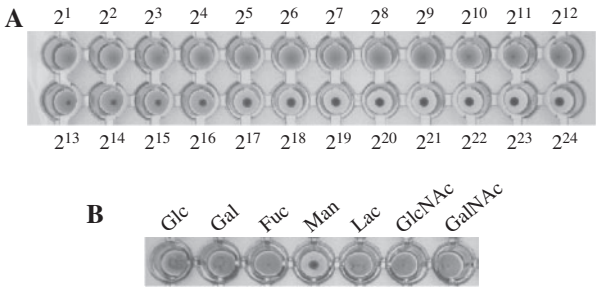

Fig. 1 Haemagglutination activity of catfish skin mucus extract (A) and saccharide inhibition (B). (A) Serially diluted skin mucus extract was mixed with 2% rabbit red blood cell suspension. (B) The 1/1,000 diluted extract was mixed with one of seven saccharides.

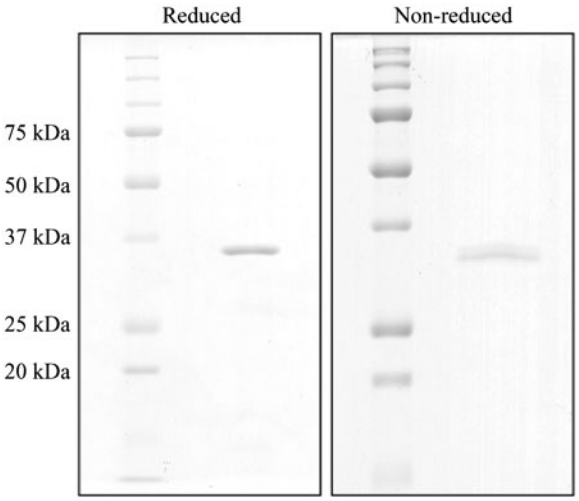

Fig. 2 SDS–PAGE of purified skin lectin under reducing and non-reducing conditions. Affinity-purified lectin was subjected to SDS–PAGE on a 12.5% gel. Molecular markers are shown on the left.

skin mucus lectin are shown in Fig. 3. The full-length 1216-bp cDNA includes a 927-bp open reading frame encoding a protein of 308 amino acid residues. An 80-bp 5′-untranslated region (UTR) and 209-bp 3′-UTR were found in the cDNA sequence. The amino acid sequence obtained by N-terminal protein sequencing was found between the 21st and 33rd residues, indicating that the amino acid region from Met^1 to Gln^{20} is a signal peptide. SignalP program predicted the same signal peptide. The mature protein has a putative molecular mass of 31,413.7 Da with an isoelectric point of 5.3.

Homolog search with tblastn demonstrated that the lectin protein sequence was significantly similar to intelectins of other vertebrates such as blue catfish *I. furcatus* IntL1 (accession no. EU030380, *E*-value: e-141), channel catfish *I. punctatus* IntL1 (EU030378, e-137), zebrafish *D. rerio* intelectin 2 (EU583682, e-110), *Xenopus tropicalis* intelectin 1 (BC061445, 5 e-96), mouse *Mus musculus* intelectin 1 (AB016496, 2 e-94) and human *homo sapiens* intelectin 2 (BC143341, 7 e-94) (Fig. 4). When full-length protein sequences including signal peptides were compared, the catfish *S. asotus* skin mucus lectin shared the most identity with blue catfish *I. furcatus* IntL1 (77.7%), followed by channel catfish *I. punctatus* IntL1 (71.8%) (Fig. 4). The fibrinogen-related domain (FreD), which commonly exists in the vertebrate intelectins, was also present in the amino acid sequence of the catfish *S. asotus* lectin (Fig. 4, underlined). We concluded that the skin mucus lectin of catfish *S. asotus* is a member of the intelectin family, and named the lectin 'saIntL' (short for '*Silurus asotus* intelectin').

The catfish intelectin requires Ca^{2+}

Intelectin is generally known to be a Ca^{2+}-dependent enteric lectin (*16*, *25*), so we determined whether the saIntL needs Ca^{2+} for its mannose-binding activity. Skin mucus extract resolved in Ca^{2+} ion-containing buffer was subjected to a mannose-agarose column, and the EDTA-eluted fraction was analysed by SDS–PAGE. As shown in Fig. 5, the eluate gave a single band at 35 kDa, indicating that saIntL binds mannose in a Ca^{2+}-dependent manner.

Quaternary structure of the catfish intelectin

While mammalian and amphibian intelectins are known to have several variations in their quatemary structures, there has been no such report in fish intelectins. We thus investigated the quaternary structure of the saIntL by gel filtration.

Two peaks were obtained when purified saIntL was subjected to gel filtration (Fig. 6), indicating that intact saIntL forms two distinct quaternary structures. Molecular masses of the intact saIntL were estimated at 76.4 and 40.2 kDa. This result suggests that saIntL exists in dimeric and monomeric forms.

Tissue expression of the catfish intelectin gene

Expression of saIntL mRNA in the skin, gill, stomach, intestine, liver, kidney, spleen, ovary, brain and PBL was examined by RT–PCR. Surprisingly, a single band was obtained from the kidney in addition to external mucosal tissues, *i.e.* skin and gill (Fig. 7). We then checked the sequence of the PCR products from the gill and kidney and found that the nucleotide sequence derived from the kidney was 100% identical to that of saIntL, while the sequence of the gill sample contained nine variant nucleotides, which induced six amino acid changes (Fig. 8). Thus, we renamed the lectin obtained from the skin and kidney as 'saIntL-sk' and from gill as 'saIntL-g'.

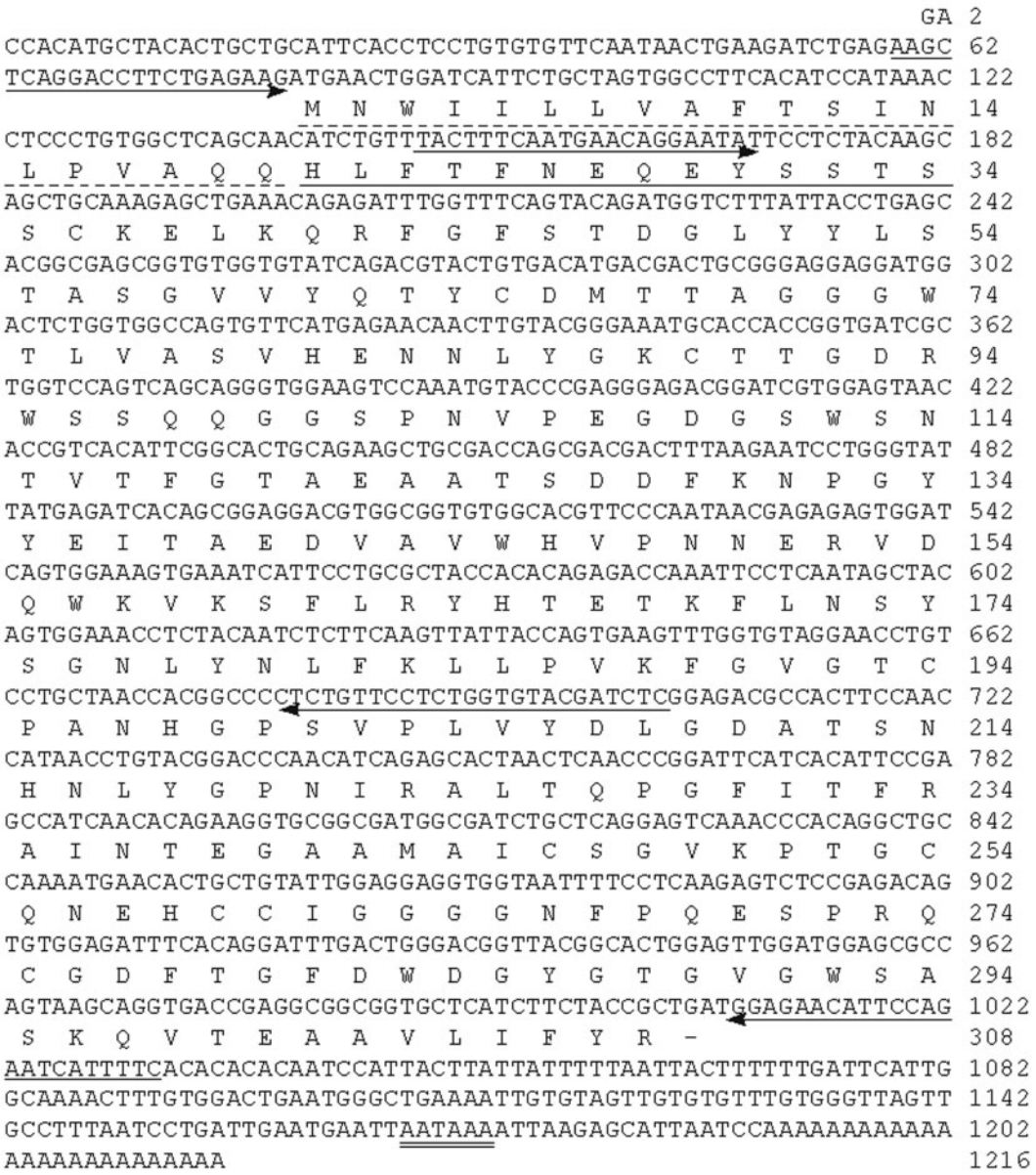

Fig. 3 Nucleotide sequence of cDNA and predicted amino acid sequence encoding the catfish skin mucus lectin protein. The signal sequence is indicated by a dotted underline, and the N-terminal amino acid sequence, obtained by protein sequencing, is underlined. The mRNA destabilization motif (AATAAA) is double-underlined. Arrows indicate primer locations.

More than two bands of the genomic PCR products were visualized in the analysis. This result may suggest the presence of multiple genes of catfish intelectins in addition to saIntL-sk and saIntL-g.

Phylogenetic analysis

To gain insight into the evolution of animal intelectins, the amino acid sequences of vertebrate intelectins including skin- and gill-type saIntLs were subjected to phylogenetic analysis by the neighbour-joining method (Fig. 9). The analysis grouped both saIntLs with IntL1s of blue and channel catfishes *I. punctatus* and *I. furcatus*. As described by Takano *et al.* (*20*), clear orthologies were difficult to establish between fish intelectins and their mammalian counterparts.

Production of recombinant protein and polyclonal antiserum

We attempted to produce a bacterially expressed His-tagged protein corresponding to the full-length form of saIntL-sk. Expression of recombinant proteins was monitored in the soluble fraction and in inclusion body by SDS–PAGE, and the protein was included in the latter (data not shown). We thus purified the denatured protein with urea-containing buffer, and tried to refold the protein. But refolding was unsuccessful, so we used the denatured protein as an antigen to generate an antiserum.

A rabbit polyclonal antiserum was generated against the purified recombinant saIntL-sk. The specificity of the antiserum was checked by western blotting. As shown in Fig. 10A, a single signal at ~35 kDa was

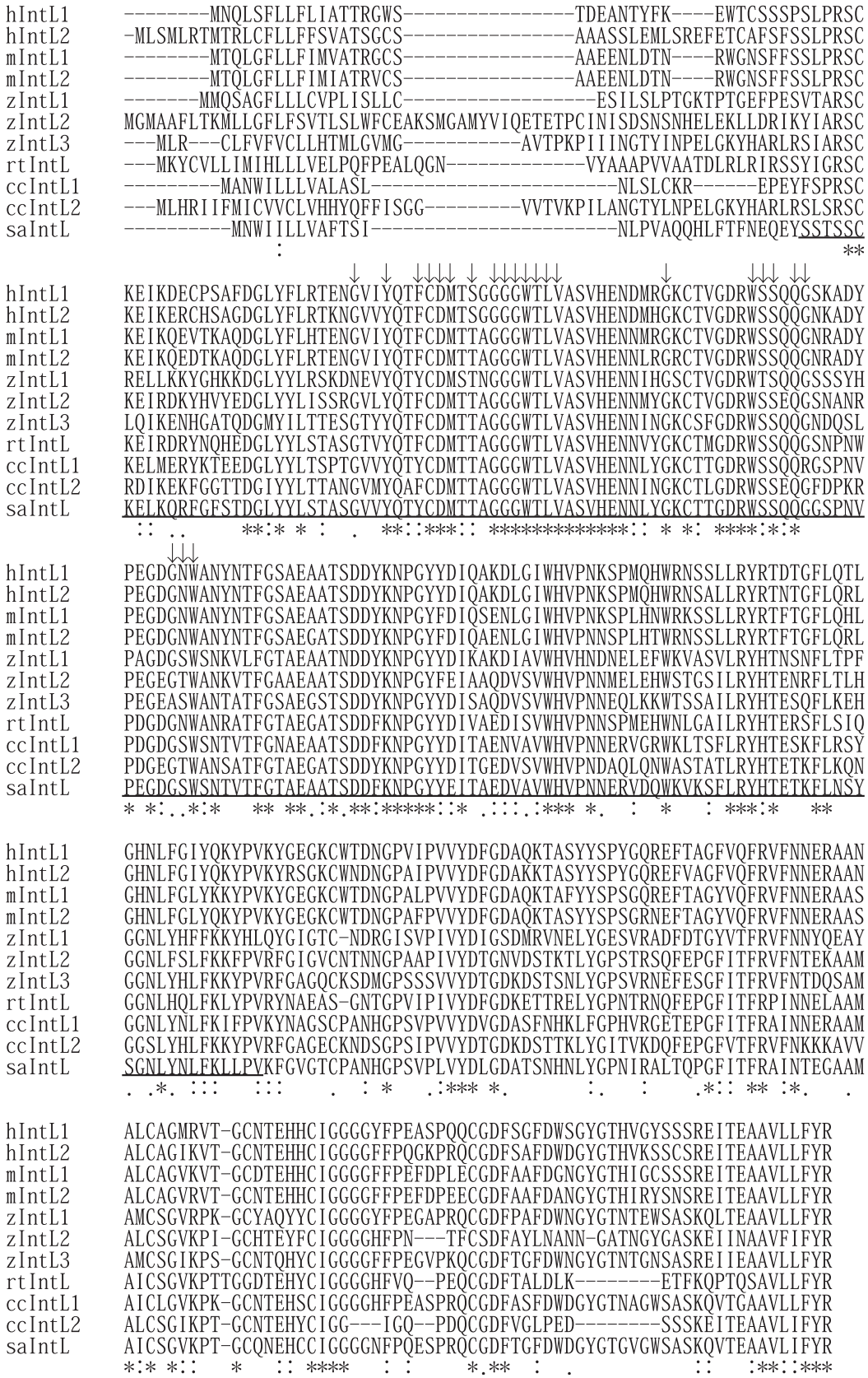

Fig. 4 Amino acid sequence alignment of catfish skin lectin with vertebrate intelectins. hIntL, mIntL, zIntL, rtIntL and ccIntL represent human, mouse, zebrafish, rainbow trout and channel catfish intelectins. The symbols (asterisks, colons and dots) indicate amino acid identity, conserved substitutions and semi-conserved substitutions. The FreD that was predicted by the SMART program (www.smart.emble-heidelberg.de) is underlined. Arrows indicate the amino acid residues resembling the fibrinogen-like motif of members of the ficolin/opsonin/p35 lectin family. Accession numbers for the sequences in this figure are shown in Fig. 9.

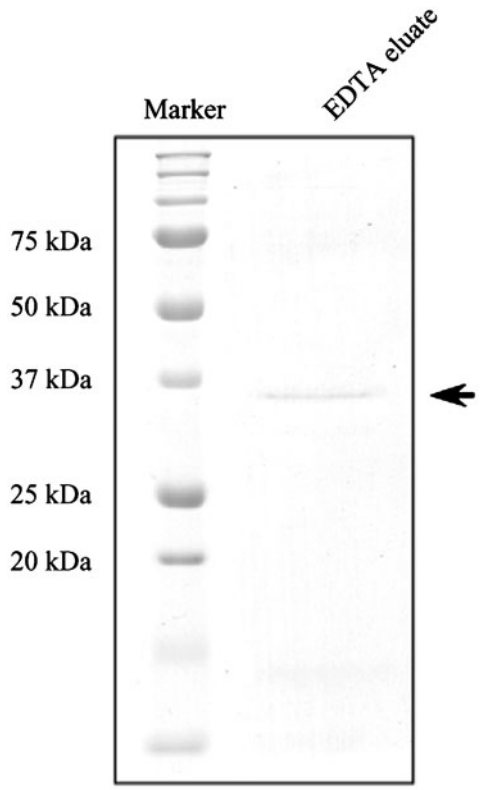

Fig. 5 Ca^{2+}-ion requirement of the skin mucus lectin. After incubation of skin mucus extract with mannose-agarose in the presence of Ca^{2+}, the EDTA-eluted fraction was subjected to reduced-SDS–PAGE. Protein markers are shown on the left.

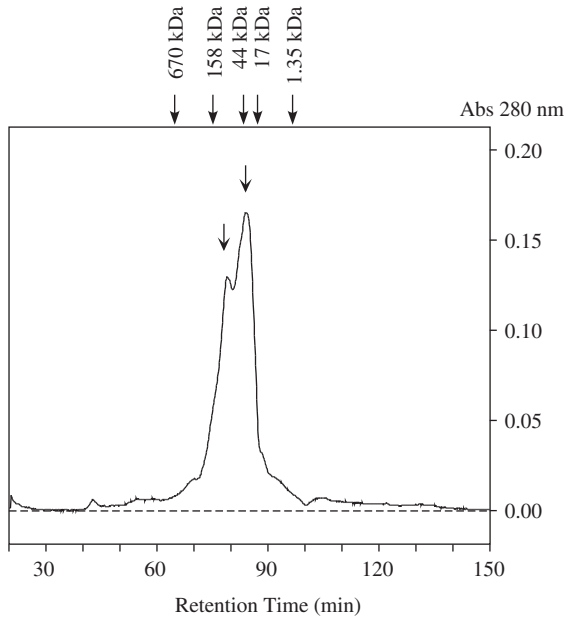

Fig. 6 Gel filtration of the skin mucus lectin. Affinity-purified lectin was subjected to gel filtration with a BioLogic Duo Flow system on Superose 6 at a flow rate of 0.3 ml/min. Elution positions of standard proteins are indicated by arrows (top).

detected in the skin mucus sample, indicating high specificity of the antiserum against the catfish intelectin.

Tissue distribution of the catfish intelectin protein

Western blotting with the antiserum yielded a strong signal at 35 kDa from the gill sample (Fig. 10B). Weak signals at 35 kDa were also observed in samples of skin, kidney and blood plasma (Fig. 10B). These results showed that the antiserum recognizes both isotypes of saIntL. The result of western blotting also indicated that the intelectin is present in the blood plasma of catfish. Because no expression of the intelectin gene was observed in the PBL (Fig. 7), the intelectin detected in the blood plasma may be produced in the kidney.

Immunohistochemical localization of saIntLs

To detect saIntLs-containing cells in the skin, gill and kidney, immunohistochemistry was performed with the anti-saIntLs rabbit serum. Fish epidermis contains various types of cells in addition to normal epidermal cells. In the Siluriformes, two different types of glandular cells, mucous cells and club cells, are present in the epidermis (*26*, *27*). As shown in Fig. 11A, positive immunohistochemical reactions were clearly detected in club cells, which possessed round-shaped heads. Epithelial and mucous cells were completely negative in the immunostaining.

Club cells are deposited in the gill epithelium of catfishes (*28*). The cells were exclusively stained by the anti-saIntL rabbit serum (Fig. 11C), indicating that saIntL-g is contained in the club cells in the catfish gill.

In the kidney, an immunopositive reaction was observed in the renal tubules (Fig. 11E). No other components in the kidney were stained by the antiserum.

In these tissues, no staining was produced when antiserum was pre-incubated with excess recombinant saIntL as a primary antibody (Fig. 11B, D and F).

Low sensitivity of catfish intelectin to bacterial challenge

In order to investigate whether gene expression of saIntL-sk in the skin is altered by bacterial immersion, we performed PCR analysis of the skin lectin of individuals that had been immersed for 1 or 3 days with *A. salmonicida*. However, PCR revealed no difference between these and untreated individuals (data not shown).

Additionally, expression of saIntL-sk mRNA in the kidney and skin was assessed in individuals intraperitoneally injected with *A. salmonicida* and PBS. In this experiment, it was also difficult to detect obvious differences in lectin expression in both tissues (data not shown).

Bacterial agglutination

SaIntL-sk agglutinated *A. salmonicida* (Fig. 12A), while did not *V. anguillarum* and *E. tarda* (Fig. 12C and D). Agglutination of the *A. salmonicida* was not observed when the cells were incubated with the lectin in the presence of mannose (Fig. 12B).

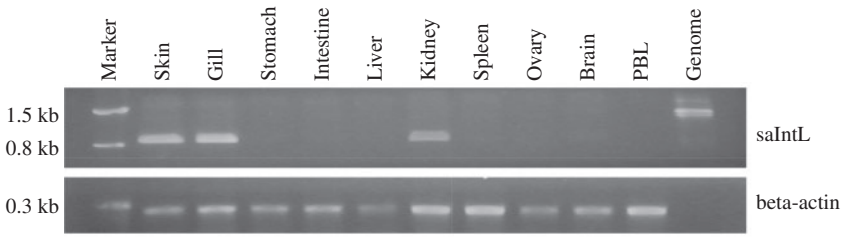

Fig. 7 RT–PCR detection of intelectin mRNA in various tissues of healthy catfish. Total RNA from nine tissues and PBL was isolated and reverse-transcribed. The intelectin gene was PCR amplified and electrophoresed on a 1.8% agarose gel. β-Actin was amplified as a control. DNA markers are shown on the left.

```
A Skin-type     ATGAACTGGATCATTCTGCTAGTGGCCTTCACATCCATAAACCTCCCTGTGGCTCAGCAACA
  Gill-type     ............................................................

  Skin-type     TCTGTTTACTTTCAATGAACAGGAATATTCCTCTACAAGCAGCTGCAAAGAGCTGAAACAGA
  Gill-type     ............................................................

  Skin-type     GATTTGGTTTCAGTACAGATGGTCTTTATTACCTGAGCACGGCGAGCGGTGTGGTGTATCAG
  Gill-type     ............................................................

  Skin-type     ACGTACTGTGACATGACGACTGCGGGAGGAGGATGGACTCTGGTGGCCAGTGTTCATGAGAA
  Gill-type     ......................A.....................................

  Skin-type     CAACTTGTACGGGAAATGCACCACCGGTGATCGCTGGTCCAGTCAGCAGGGTGGAAGTCCAA
  Gill-type     ............................................................

  Skin-type     ATGTACCCGAGGGAGACGGATCGTGGAGTAACACCGTCACATTCGGCACTGCAGAAGCTGCG
  Gill-type     ............................................................

  Skin-type     ACCAGCGACGACTTTAAGAATCCTGGGTATTATGAGATCACAGCGGAGGACGTGGCGGTGTG
  Gill-type     ..............C.............................................

  Skin-type     GCACGTTCCCAATAACGAGAGAGTGGATCAGTGGAAAGTGAAATCATTCCTGCGCTACCACA
  Gill-type     ............................................................

  Skin-type     CAGAGACCAAATTCCTCAATAGCTACAGTGGAAACCTCTACAATCTCTTCAAGTTATTACCA
  Gill-type     ............................................................

  Skin-type     GTGAAGTTTGGTGTAGGAACCTGTCCTGCTAACCACGGCCCCTCTGTTCCTCTGGTGTACGA
  Gill-type     ............................................................

  Skin-type     TCTCGGAGACGCCACTTCCAACCATAACCTGTACGGACCAACATCAGAGCACTAACTCAACC
  Gill-type     ...................................................A........

  Skin-type     CCGGATTCATCACATTCCGAGCCATCAACACAGAAGGTGCGGCGATGGCGATCTGCTCAGGA
  Gill-type     ...........................................C................

  Skin-type     GTCAAACCCACAGGCTGCCAAAATGAACACTGCTGTATTGGAGGAGGTGGTAATTTTCCTCA
  Gill-type     ..................C........T.....................C........G.

  Skin-type     AGAGTCTCCGAGACAGTGTGGAGATTTCACAGGATTTGACTGGGACGGTTACGGCACTGGAG
  Gill-type     ..C.........................................................

  Skin-type     TTGGATGGAGCGCCAGTAAGCAGGTGACCGAGGCGGCGGTGCTCATCTTCTACCGCTGAT
  Gill-type     ...........................................................

B Skin-type     MNWIILLVAFTSINLPVAQQHLFTFNEQEYSSTSSCKELKQRFGFSTDGLYYLSTASGVVYQ
  Gill-type     ............................................................

  Skin-type     TYCDMTTAGGGWTLVASVHENNLYGKCTTGDRWSSQQGGSPNVPEGDGSWSNTVTFGTAEAA
  Gill-type     ............................................................

  Skin-type     TSDDFKNPGYYEITAEDVAVWHVPNNERVDQWKVKSFLRYHTETKFLNSYSGNLYNLFKLLP
  Gill-type     ............................................................

  Skin-type     VKFGVGTCPANHGPSVPLVYDLGDATSNHNLYGPNIRALTQPGFITFRAINTEGAAMAICSG
  Gill-type     ....................................I.................L.....

  Skin-type     VKPTGCQNEHCCIGGGGNFPQESPRQCGDFTGFDWDGYGTGVGWSASKQVTEAAVLIFYR
  Gill-type     ......H..........H..EA.....................................
```

Fig. 8 Nucleotide (A) and amino acid (B) sequence alignments of the skin- and gill-type intelectins in catfish. The alignment was constructed by CLUSTAL W. Dots indicate identity between the skin- and gill-type intelectin sequences.

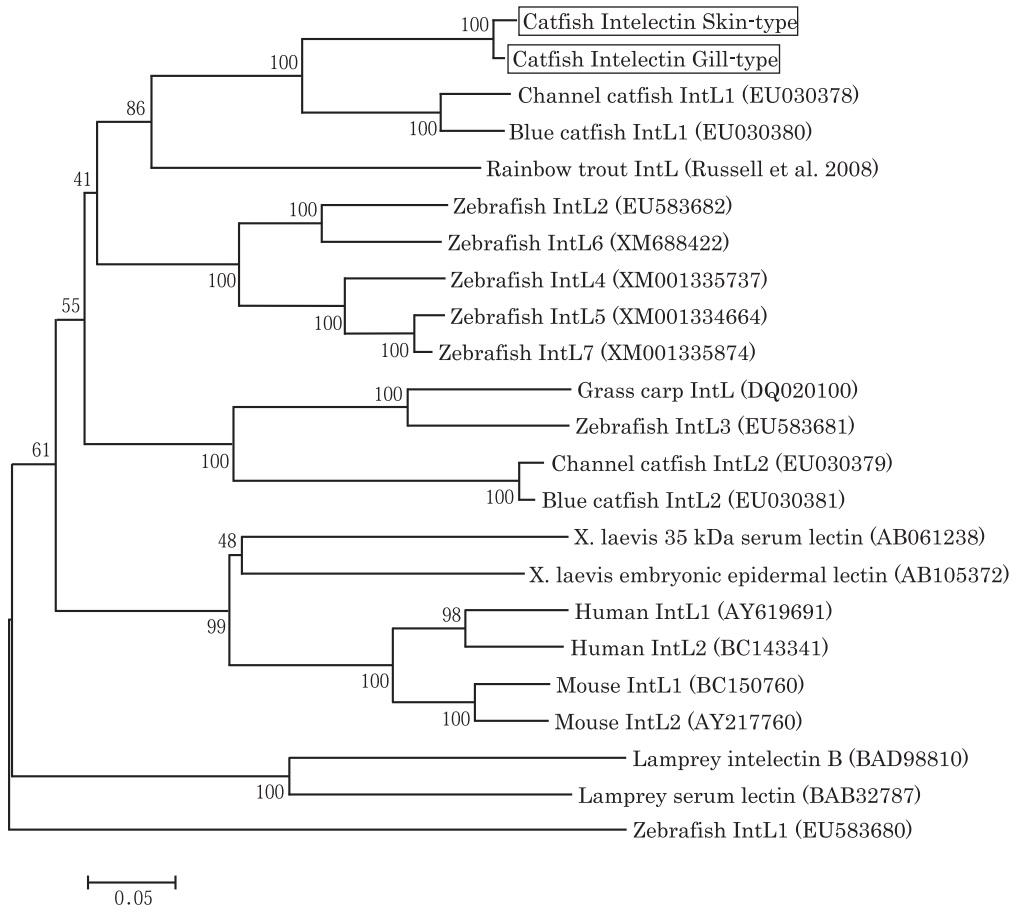

Fig. 9 A neighbour-joining phylogenetic tree of vertebrate intelectin protein sequences. The bootstrapping values are shown at the nodes. The bar (0.05) indicates genetic distance. Accession numbers are provided.

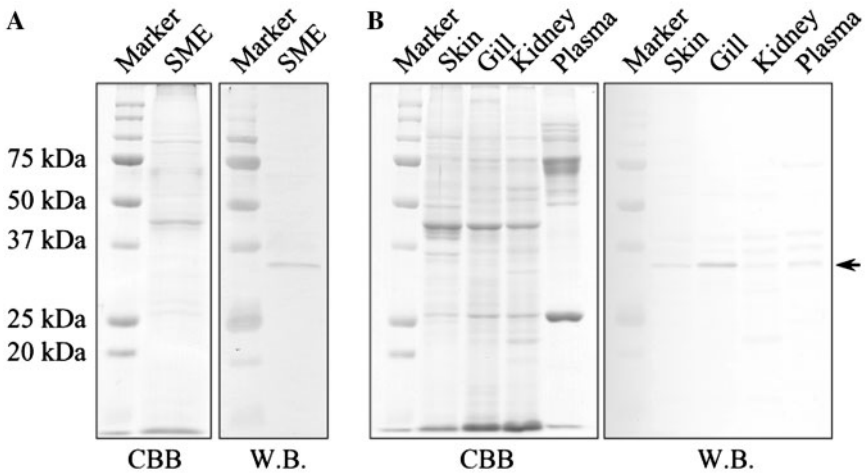

Fig. 10 Specificity of antiserum against catfish intelectin (A) and detection of intelectin protein in the skin, gill, kidney and blood plasma with the antiserum by western blotting (B). Extracts of skin mucus (SME), skin, gill, kidney and blood plasma were subjected to SDS–PAGE. The samples were stained with CBB (left panels) or antiserum against recombinant catfish intelectin (right panels). Pre-stained molecular weight markers were used in this analysis.

Discussion

We identified two types of intelectin in catfish *S. asotus* from important defence tissues, *i.e.* extra-body surface including the skin and gill and an important immune-organ in fish, namely the kidney. The saIntL-sk showed agglutination activity against *A. salmonicida*, suggesting the participation of the lectin in the catfish self-defence mechanism.

Intelectins are recently discovered animal lectins that possess a fibrinogen-like motif similar to that of members of the ficolin/opsonin/p35 lectin family (*29*, *30*).

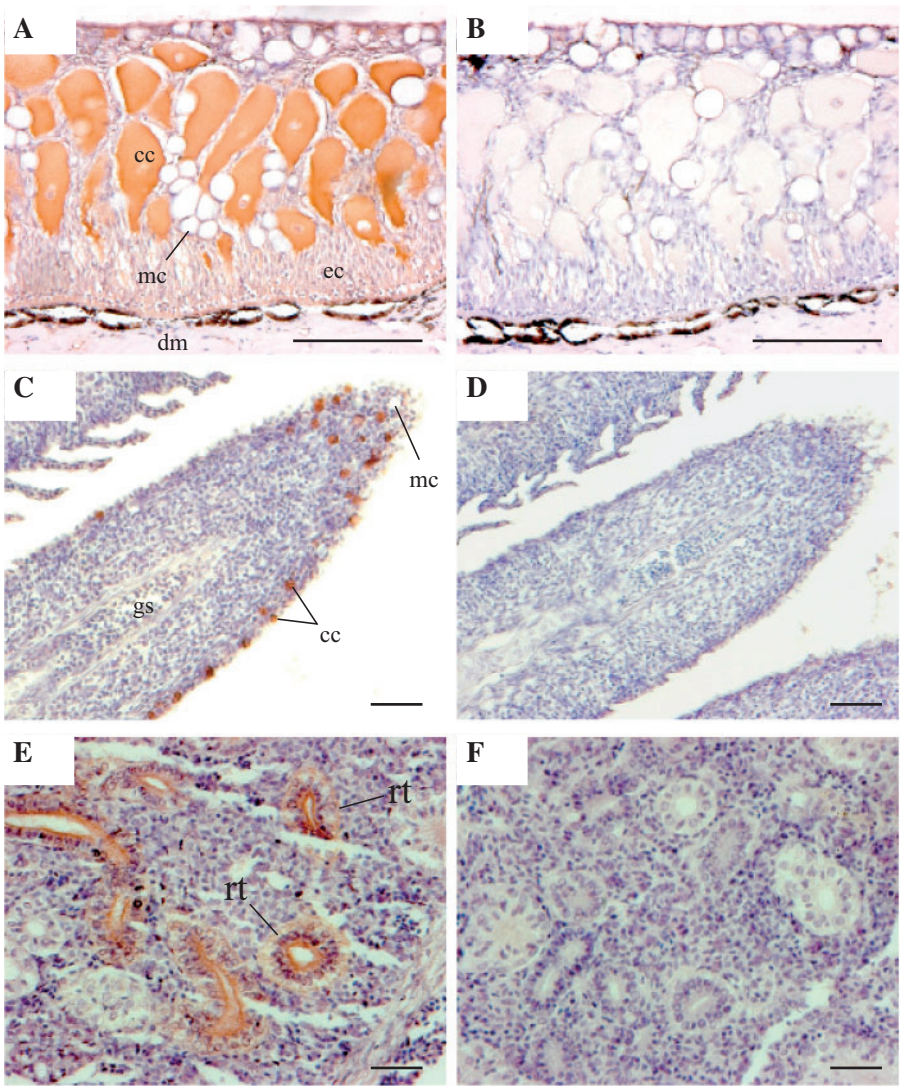

Fig. 11 Immunohistochemical localization of catfish intelectins in the skin (A and B), gill (C and D) and kidney (E and F). Controls (B, D and F) used identical tissue sections with pre-adsorbed antiserum with recombinant antigen. cc, club cell; mc, mucous cell; ec, epithelial cell; dm, dermis; gs, gill support and rt, renal tubule. Scale bars indicate 50 μm.

This lectin was originally identified in mouse as a homolog of *Xenopus laevis* oocyte lectin (*31*) and is therefore called 'X-lectin' (*16*). Most intelectins appear to bind to mannose, although there may be some exceptions; *e.g.* rainbow trout intelectin has binding ability to GlcNAc as well as mannose (*18*). Human intelectin 1 (HL-1) shows 100% amino acid identity with human lactoferrin receptor (LfR) (*32*) and mammalian intelectins actually act as LfR with milk lactoferrin binding activity during in iron metabolism (*33*). Intelectins also play an important role in development: XL35, representing ~70% of *X. laevis* oocyte cortical granules, contributes to the prevention of polyspermy (*34*). In addition to these functions, intelectins are believed to act in the immune response. For example, mouse intelectin that was specifically localized to Paneth cells of intestinal crypts (*31*) was induced by infection with the nematode *Trichinella spiralis* (*35*). Up-regulation of the mouse intelectin gene has also been reported in the lung after helminth parasite *Nippostrongylus brasiliensis* infection (*36*). HL-1 can bind to D-pentose and D-galactofuranosyl residues of arabinogalactan cell wall components from the *Nocardia rubra* bacterium (*25*). In fish, some evidence of intelectins taking part in self-defence has been also reported. For instance, intelectins of channel catfish *I. punctatus* and blue catfish *I. furcatus* were originally identified as being among the most highly up-regulated hepatic genes after bacterial infection with *Edwardsiella ictaluri* (*37*, *38*). Previous studies in rainbow trout also identified intelectin as one of the acute phase response genes in the liver following infection with *V. anguillarum* (*39*, *40*). In addition, a direct binding assay demonstrated that plasma intelectin in rainbow trout bound several Gram-negative bacteria including *A. salmonicida* (*18*).

Intelectins appear to exhibit highly differential patterns of tissue expression among species as well as isotypes within a species. For example, HL-1 is widely distributed in several tissues, while the second human

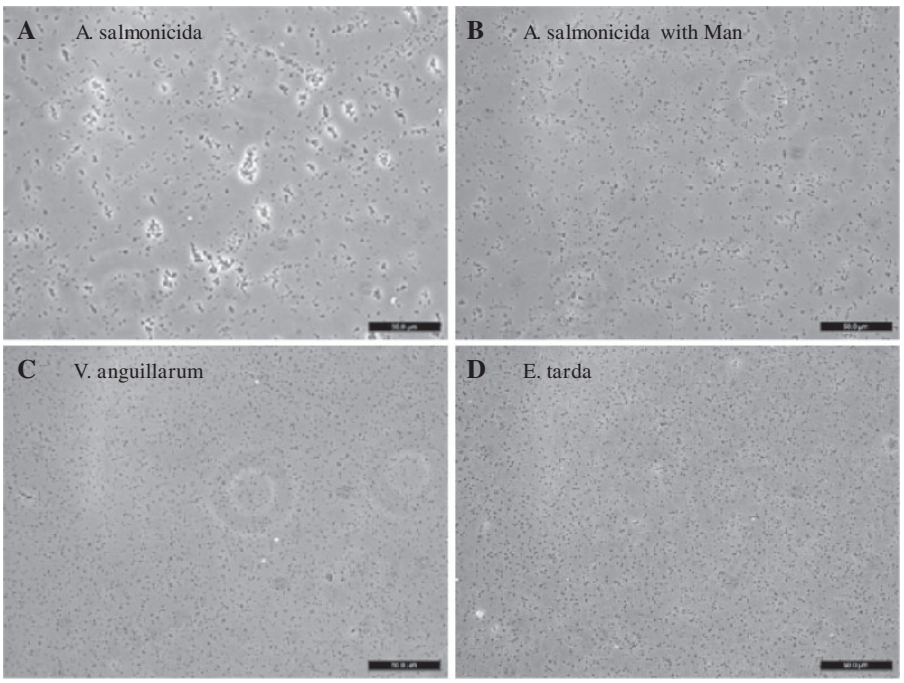

Fig. 12 Agglutination of pathogenic bacteria by catfish intelectin. Pathogenic bacteria *A. salmonicida* (A and B), *V. anguillarum* (C) and *E. tarda* (D) were incubated with purified intelectin in the presence (B) or absence (A, C and D) of mannose. Scale bars indicate 50 μm.

intelectin variant, HL-2, is specifically expressed in the small intestine (*41*). In fish species, rainbow trout intelectin gene is strongly expressed in the spleen, liver, intestine, kidney and swim bladder, weakly in the gill, and most weakly in the skin (*18*). Although expression in the skin was not examined, grass carp intelectin mRNA was detected in the brain, intestine, heart, kidney and gill (*17*). In channel catfish *I. punctatus*, IntL1 was widely expressed in various tissues including head kidney and trunk kidney, while IntL2 was predominantly expressed in the liver, with very low expression in the trunk kidney and intestine (*20*). Interestingly, even in blue catfish *I. furcatus*, a closely related species in the same genus with channel catfish, expression patterns were slightly different; IntL1 is strongly expressed in the muscle and IntL2 expression is high in the heart and head kidney in addition to the liver (*20*). It is notable that gene expression of the *Ictalurus* catfish intelectins is completely or nearly undetectable in the skin (*20*). Lin *et al.* (*21*) investigated the expression pattern of seven intelectins in several tissues in zebrafish, and showed that expression in the skin is negative or very weak in all intelectins. Thus, it is unlikely that large amounts of intelectin are present in the skin mucus of these fishes. As shown in Fig. 7, we clearly demonstrated that catfish *S. asotus* intelectins are strongly transcribed in the skin, gill and kidney. Furthermore, PBL was negative in the catfish *S. asotus* while it was one of main sources of intelectin mRNA in channel catfish *I. punctatus* (*20*). This expression pattern of intelectin has thus far been limited to catfish *S. asotus*.

SaIntL-sk is constitutively expressed in the skin and its expression was not affected by *A. salmonicida* injection and immersion. Nor was induction of intelectin expression apparent after intraperitoneal injection with the bacterium in the kidney of catfish *S. asotus*. In channel catfish *I. punctatus*, IntL1 is not induced while IntL2 is drastically up-regulated in the liver by intraperitoneal injection with *E. ictaluri* (*20*). Phylogenetic analysis indicated that both saIntLs are grouped with IntL1 of channel catfish *I. punctatus* and blue catfish *I. furcatus*, but not with IntL2 (Fig. 9). Therefore, it is likely that a counterpart of *Ictalurus* catfish IntL2 is present in catfish *S. asotus*, which may be sensitive to bacterial challenge.

Aside from the diversity in tissue expression patterns, great variations of quaternary structures have also been reported in intelectins. Human intelectins exist in disulphide-linked trimeric form, while mouse intelectins are monomers (*42*). Multiple structures of intelectins have been also reported; XL35 from *X. laevis* can form 9–12 multimers (*43*, *44*) and ascidian intelectin exists in multimers including dimers, tetramers and hexamers (*45*, *46*). We used gel filtration to demonstrate that intact saIntL-sk is present as monomers and dimers. Although recombinant zebrafish IntL3 protein was produced by mammalian cells (293 T cell) as a monomer (*21*), the quaternary structure of intact fish intelectin has not yet been reported. Our study is therefore the first description of quaternary structure in fish intelectins.

In the phylogenetic tree, it was difficult to establish clear orthologies between fish and mammalian intelectins (Fig. 9). Mammalian intelectins from the same species clustered together. Furthermore, human and mouse IntL1 and IntL2 genes are located in a single chromosome (chr. 1 in each species). These results suggest, as discussed by Takano *et al.* (*20*) and Lin *et al.* (*21*), that mammalian intelectins have been

independently expanded by partial gene duplication after separation to each species lineage. On the other hand, fish intelectins formed three distinct clades: (i) catfishes IntL1s, rainbow trout IntL and zebrafish IntLs except for IntL1 and IntL3; (ii) catfishes IntL2s, grass carp IntL and zebrafish IntL3; and (iii) zebrafish IntL1 and lamprey lectins. It is surprising that zebrafish IntL1 was located at the distal position in phylogenetic tree from other zebrafish intelectins. The zebrafish IntL1, however, showed lower sequence identity among seven zebrafish intelectins (*21*). In addition, the IntL1 was not grouped with other zebrafish in the phylogenetic tree constructed by Lin *et al.* (*21*). These facts may explain why the zebrafish IntL1 was clustered into distal position. Furthermore, of the seven zebrafish intelectins, IntL1 is located on chr. 1, IntL3 is on chr. 14 and IntL7 is on chr. 13, while IntL2, IntL4, IntL5 and IntL6 are on chr. 7 (*21*). Notably, zebrafish chromosomes 7 and 13 are tightly linked, *i.e.* these chromosomes are suggested to be generated from a common ancestral chromosome by whole gene duplication in the teleost lineage. It is thus likely that zebrafish intelectin genes on chr. 7 and 13 were generated by recent species-specific gene duplication. These facts also suggest the presence of at least three prototypes of intelectin genes in teleosts. Interestingly, in contrast to the case of zebrafish, intelectin genes are not found in the genome databases of two pufferfish species, *Takifugu rubripes* and *Tetraodon nigroviridis* (*20*). Further studies are warranted to determine why numbers of fish intelectin genes vary so significantly between species.

SaIntLs were located in club cells in the skin and gill of catfish (Fig. 11), suggesting that the lectins are produced by the cells and secreted into skin and gill mucus. Club cells have been found in many fish species of Anguilliformes, Cypriniformes, Cyprinodontiformes and Gonorynchiformes, as well as Siluriformes (*47*). The cells include various bioactive substances. For example, in a freshwater catfish *Heteropneustes fossilis* and a pearlfish *Carapus acus*, serotonin-like immunoreactivity was detected in the club cells (*48*). Alarm substances that mediate the fright reaction are also found in the cells of Ostariophysi (*49*). In the club cells of Siluriformes, a protein toxin was found in *Plotosus lineatus* (*50*). In addition to these molecules, lectins are often found in the club cells. Nakamura *et al.* (*51*) demonstrated that galectin, named congerin, is present in the club cells in the external surfaces, including skin and gill, in the conger eel *C. myriaster*. C-type lectin (AJL-2) is also immunohistochemically detected in the epidermal cells of the Japanese eel *Anguilla japonica* (*5*). It should be noted that lectin-containing club cells have been limited in Anguilliformes. This is the first report demonstrating the presence of a lectin in the club cells in Ostariophysi. These lectins are also detected in the skin mucus. It is thus feasible that club cells act as lectin-secreting cells in several fish species, not only in Anguilliformes.

So far, the only fish intelectin for which cellular localization has been analysed is that of rainbow trout, belonging to the Salmoniformes, which lack club cells. Russell *et al.* (*18*, *19*) examined immunohistochemical localization of intelectin protein in various tissues including the skin, gill and kidney in the trout. They demonstrated that trafficking leucocytes in the epidermis and dermis are positive in the trout skin, and epithelial cells in the gill are the only cells stained by the antibody. In the kidney of rainbow trout, intelectins are detected in the cytoplasm of leucocytes within the renal interstitium (*19*). We clearly demonstrated that catfish intelectin is contained in the renal tubules, but not in leucocytes in the catfish kidney (Fig. 11). These facts indicate that intelectin-producing cells are markedly diverse among not only species but also tissues. The role of catfish intelectin in the renal tubule is not clear. Intelectins have multiple functions in addition to their immunological function, *e.g.* some of them are also associated with iron metabolism (*33*) and fertilization (*34*). Catfish intelectin in the renal tubule may be involved in transfer or reabsorption of sugar during urine production.

Although the saIntL-sk agglutinated *A. salmonicida*, it did not show agglutination activity against other pathogenic bacteria, *V. anguillarum* and *E. tarda* (Fig. 12). Like this, skin mucus galectin of Japanese eel agglutinates a Gram-positive bacterium, *Streptococcus difficile*, but not four tested Gram-negative bacteria (*6*). Fugu skin mucus lectin, furthermore, agglutinates only 11 bacterial isolates among tested 120 isolates (*7*). It is likely that agglutination activity of fish skin mucus lectin is limited to certain bacterial species.

In the last decade, structural studies on fish skin mucus lectins have been intensive. As a result, the striking diversity of fish skin mucus lectins has been revealed. This study describes the sixth type of fish skin mucus lectin following galectin, C-type, B-type, RBL and pentraxin. In addition, some other fish mucus lectins that have not been fully identified may be classified into other families because of differences in their molecular properties. For example, the N-terminal amino acid sequence (21 residues) of the mucus lectin in kingklip *Genypterus capensis* (*52*) did not have any homologs in a BLAST search. Loach *Misgurnus anguillicaudatus* mucus lectin may also belong to another lectin family because of its large molecular mass (40 kDa) and absence of Cys residues (*53*). Additionally, the mucus lectin in windowpane flounder *Lophopsetta maculate* specifically recognizes *N*-acetylneuraminic acid (*54*), which is different from any other reported mucus lectins. More fish species should be analysed to understand the means of diversity in skin mucus lectin.

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Conflict of interest
None declared.

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