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Common Skate (*Raja kenojei*) Secretes Pentraxin into the Cutaneous Secretion: The First Skin Mucus Lectin in Cartilaginous Fish*

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A lactose-specific lectin with a molecular mass of about 25 kDa was purified from the skin mucus of a cartilaginous fish—the common skate (*Raja kenojei*). The complementary DNA sequence of the lectin was 1540 bp long and contained a reading frame encoding 226 amino acids, which showed ~38% identity to pentraxins of mammals and teleosts. Gene expression was observed in the skin, gill, stomach and intestine in the healthy skate. We also identified an isotype gene from the liver whose deduced amino-acid sequence shared 69.0% identity with the skin type gene. The antiserum detected protein in the skin, where the lectin is localized in the epidermal cells, and in the blood plasma. The lectin genes are multicopied in the common skate genome. Although pentraxins are acute phase proteins, mRNAs of both the isotypes were not upregulated after the *in vivo* challenge with formalin-killed *Escherichia coli*, which suggests that they are constantly present in the skin mucus and blood plasma to protect against pathogenic invasion. This lectin is the fifth type of lectin found in the cutaneous secretions of fish, demonstrating that skin mucus lectins have evolved with marked molecular diversity in fish.

Key words: cartilaginous fish, diversity, lectin, pentraxin, skin mucus.

Abbreviations: CRP, C-reactive protein; PBS, phosphate-buffered saline; PTX, pentraxin; RACE, rapid amplification of cDNA ends; SAP, serum amyloid P-component; RT, reverse transcription.

Lectins, which are non-enzymatic saccharide–binding proteins, are ubiquitous in nature and are present in micro-organisms, plants, invertebrates and vertebrates (*1*). Animals produce a diverse array of lectins, which are classified into several families, e.g. galectins and C-type lectins. Pentraxins (PTXs) are able to bind to various ligands, such as microbial polysaccharides, membrane phospholipids and carbohydrate moieties, in a calcium-dependent manner; hence, PTXs exhibiting saccharide–binding activity can be defined as lectins (*2*, *3*). PTXs comprise a family of multifunctional proteins including the C-reactive protein (CRP) and serum amyloid P-component (SAP), both of which are mainly produced in the liver (*4*). Sudden increases in the serum levels of some PTXs are observed during acute phase responses against infections or injuries (*5*, *6*). Moreover, these molecules are believed to play an important role in inflammation (*7*); they can activate the complement cascade via the classical pathway and can act as opsonins by interacting with the Fcγ receptor present in neutrophils and macrophages (*8*, *9*).

The fish skin is coated with mucus secreted by the mucous cells present in the epidermis. Lectins are often found in the mucus (*10*, *11*). The mucus on the fish skin constitutes a critical interface between the body surface and the surrounding environmental water, which harbours a considerable number of microorganisms. The lectins present in the fish skin mucus play an important role in the self-defence system by acting as pattern recognition proteins (PRPs) that bind to pathogen-associated molecular patterns (PAMPs) and trigger cellular and humoral responses. Although skin mucus lectins have been purified from many fish species, the structural information on several molecules of the lectins is limited. Nevertheless, skin mucus lectins can be classified into four different families on the basis of shape, i.e. galectins (*12–14*), C-type (*15*, *16*), Lily-type (*17*) and L-rhamnose-binding lectin (*18*). The aquatic environment offers markedly diverse natural conditions, resulting in considerable diversity among pathogenic micro-organisms. It is therefore likely that greater numbers of different types of lectins act as PRPs against various pathogens in the skin mucus of different fish species, which is one of the most diverse taxons in animals. We believe that surveying mucous lectin in more fish species will lead us to a better understanding of how such a marked molecular diversity of fish lectin has arisen.

Another problem is that no skin mucus lectins have been studied in cartilaginous fish, which are represented by sharks, rays and skates. Because they are the most

*The nucleotide and deduced amino-acid sequences reported in this article are available in the DDBJ/EMBL/GenBank databases under accession numbers AB295474 and AB371645.

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primitive living vertebrates having the complex immune system typical of all jawed vertebrates, cartilaginous fish are attractive organisms for studying comparative immunology; hence, investigation into cartilaginous fish skin mucus lectins will give significant insight into the evolution of mucosal immunity in vertebrates. Thus, we studied an elasmobranches species—the common skate (*Raja kenojei*)—and succeeded in identifying the skin mucus lectin. The lectin exhibited homology with the PTX, indicating that it was a new type of fish skin mucus lectin. This is the first report on lectin present in the skin mucus of cartilaginous fish.

MATERIALS AND METHODS

Fish Sampling—Common skates, which had been captured in the Okirai Bay, Iwate Prefecture, Japan, were kept in FRP tanks with running seawater at the Kitasato University. The fish were anaesthetized with 2-phenoxyethanol (Wako), and the skin mucus was collected by scraping the body surface with a spatula and then stored at –80°C until use. The skin, gill, oesophagus, stomach, intestine, liver, heart, kidney, spleen, ovary, uterine tube and muscle were dissected and immediately stored at –80°C for RNA, DNA and protein extractions. A blood sample was collected from the caudal blood vessel using a syringe with a needle and the plasma was immediately frozen. Small portions of skin were also fixed with Bouin's solution for immunohistochemical analysis.

Preparation of Skin Mucus Extract—The collected skin mucus was homogenized with an equal volume of 10 mM phosphate buffered saline (pH 7.5) containing 0.9 mM $CaCl_2$ and 0.33 mM $MgCl_2$ (PBS (+)). The homogenate was then centrifuged at $15{,}000 \times g$ for 30 min at 4°C and the supernatant was used as the skin mucus extract.

Detection of Lectin Activity Using Yeast Cells—Yeast cell agglutination caused by the skin mucus extract was examined using the method described by Tsutsui *et al*. (*16*). In brief, 5 µl of the skin mucus extract was mixed with 5 µl of yeast cell suspension (1×10^8 cells/ml in final concentration). After 1 h of incubation at room temperature, the sample was checked for yeast cells agglutination with a microscope.

The inhibitory effects of lactose and D-mannose on yeast agglutination were assayed as follows. Of each skin mucus extract, 5 µl was incubated at room temperature for 1 h with 5 µl of PBS containing either 200 mM lactose or 200 mM D-mannose. Thereafter, 10 µl of the yeast cell suspension was added to each sample and the agglutination was checked under the microscope.

Purification of Lectins by Lactose-Affinity Chromatography—Lactose was coupled to epoxy-activated Sepharose 6B (GE Healthcare) according to the manufacturer's protocol. Of the skin mucus extract, 20 µl was incubated with ~2 ml of lactose-bound sepharose suspension at 4°C for 16 h. Sepharose was packed into an empty column and washed with ~20 ml of PBS (+). Following overnight incubation at 4°C with the sepharose and 2 ml of PBS containing 200 mM lactose, the elute fraction was concentrated and subjected to SDS–PAGE.

Table 1. **Primers used in this study.**

Primers	Nucleotide sequences
primer-FA	5′-GCAGGATTGATGCAAAAA-3′
primer-FB	5′-GCAGGATTGATGCAAAAG-3′
primer-FC	5′-GCAGGATTGATGCAGAAA-3′
primer-FD	5′-GCAGGATTGATGCAGAAG-3′
primer-F1	5′-TTGGTCAGGAGCAAGACTCA-3′
primer-R1	5′-AAAAAGATGGCACCAAATGC-3′
primer-R2	5′-GGTTCTCTTCAGAGGCTGCT-3′
Liver-F1	5′-GCAATAAGGATTCCACACTTTGAATA-3′
Liver-R1	5′-GAGACAACCCTGACTGACCTTC-3′
Liver-F2	5′-AGCAGTGCAGAGCGGTGT-3′
Liver-R2	5′-TCACTGGAAGCACAAAACACA-3′
NUP-A	5′-AAGCAGTGGTATCAACGCAGAGT-3′

SDS–PAGE—SDS–PAGE was performed using a 12.5% separating gel. In brief, 10 µl of the affinity-purified fractions was added to 10 µl of the 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. Periodic acid Schiff (PAS) staining was also carried out to examine if the lectin was a *glycoprotein*.

N-Terminal Amino-Acid Sequencing—Purified lectin was subjected to SDS–PAGE under reducing conditions and blotted onto a polyvinylidene difluoride membrane (ATTO) at 100 mA for 1 h. The membrane was stained with CBB and protein bands were carefully cut out. The N-terminal amino-acid sequence was analysed by a PPSQ-21A protein sequencer (Shimadzu).

Primers—Primers used in this study were purchased from Sigma–Aldrich Japan. The sequences are listed in Table 1.

Molecular Cloning—Total RNA was extracted from the skate skin using the RNA extraction solution (ISOGEN, Nippon gene). First-strand cDNA was synthesized from 1 µg of the total RNA with the SMART™ RACE cDNA amplification kit (Clontech) for 5′ and 3′ rapid amplification of cDNA ends (RACE). The PCR reactions described below were carried out using i-Cycler (BIO-RAD) in a total volume of 20 µl with *Taq* DNA polymerase (Takara) and 0.5 µM each of the antisense and sense primers.

First, 3′ RACE was performed with the Nested Universal Primer-A (NUP-A; Clontech) and antisense primer-FA, -FB, -FC or -FD, corresponding to Ala-Gly-Leu-Met-Gln-Lys in the N-terminal amino-acid sequence of the lectin. The PCR protocol was 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s. Thereafter, a partial sequence was amplified with the antisense primer-F1 and sense primer-R1 for 30 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 1 min. Finally, 5′ RACE was carried out with NUP-A and sense primer-R2, which was designed on the basis of the 3′ sequence. Denaturation was performed at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s.

Each product was subcloned into the pGEM®-T Easy vector (Promega), and nucleotide sequencing was carried out using a 3100-Avant Genetic Analyzer (Applied Biosystems). Analyses were repeated with at least

three independent PCR amplifications to avoid any PCR errors.

Bioinformatic Analyses—Homologue search was performed with the BLASTX programme in the DNA Data Bank of Japan homology search system (http://www.ddbj.nig.ac.jp/search/blast-j.html). Motifs were searched using the MOTIF software (http://motif.genome.ad.jp/). *N*-glycosylation sites were predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). A multiple-sequence alignment was made using the ClustalW Multiple Sequence Alignment Program (version 1.8; http://www.ddbj.nig.ac.jp/search/clustalw-j.html). A phylogenetic tree was constructed using the neighbour-joining method with ClustalW and MEGA4.

Gene Expression Analysis by Reverse Transcription PCR—Isolation of total RNA and synthesis of cDNA from the skin, gill, oesophagus, stomach, intestine, liver, heart, kidney, spleen, ovary, uterine tube and muscle were carried out as described previously. For reverse transcription (RT)–PCR analysis, specific primers-F1 and -R1 (Table 1) were used with a protocol of 30 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 45 s, yielding a product size of 735 bp. The PCR product from the skin was also used as a probe in Southern and northern blot analyses, described below. The cDNA encoding for β-actin was amplified as a positive control. The PCR products were visualized by electrophoresis on a 1.8% agarose gel and stained with ethidium bromide.

Isolation of Liver-Specific Isoform cDNA—Liver-derived PCR-templates for 3′ RACE and 5′ RACE were obtained as described above. 3′ RACE was carried out with a primer pair of Liver-F1 and NUP-A for 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. Thereafter, the partial sequence was amplified with Liver-F2 and Liver-R2 primers for 30 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s. Finally, 5′ RACE was carried out with two primers, Liver-R1 and NUP-A, using the same amplification protocol as for 3′ RACE. Each product was ligated into the vector and sequenced as described previously.

Purification of Lactose-Binding Lectin from Serum—Approximately 10 ml of the skate blood plasma was subjected to lactose-affinity chromatography, as described above. The fraction eluted with 1 ml of PBS (+) containing 0.2 M lactose was electrophoresed on a polyacrylamide gel of 12.5%.

Southern Blot Analysis—Two PCR products, amplified from the skin-derived (described above) and the liver-derived templates (with primer pair of liver-F1 and liver-R2), were used as probes in the Southern blot analysis. These probes were labelled with an AlkPhos Direct Labelling kit (GE Healthcare).

Common skate genomic DNA was isolated from the muscle with standard methodologies, after which it was digested with either *Eco*RI, *Hind*III, *Pst*I or *Xho*I. The digested DNA was electrophoresed (10 μg/lane) on a 0.8% agarose gel, and the gel was treated with 0.25 M HCl for 30 min, followed by 1.5 M NaOH containing 0.5 M NaCl for 30 min. The DNA was then transferred onto a nylon membrane (Hybond-N^+, GE Healthcare) using 0.4 M NaOH as a transfer buffer at room temperature for 16 h. The membrane was hybridized with the probes at 55°C for 16 h. After stringency washing, the signals were detected using CDP-star Chemiluminescent detection reagent (GE Healthcare) and X-ray film.

Peptide Synthesis and Antibody Production—The antigenic domain in the amino-acid sequence of the skin mucus lectin was predicted using PREDICTED ANTIGENIC PEPTIDES software (http://bio.dfci.harvard.edu/Tools/antigenic.pl). A putative antigenic sequence, NH_2-DFSDLTAFTVCLR, corresponding to the internal amino-acid sequence of the lectin (from 45th to 57th), was used to generate 13 residues of a synthetic peptide (>80% purity, ARK Resource). The peptide was conjugated with keyhole limpet haemocyanin (KLH) as a carrier protein.

The rabbit antiserum of the peptide was raised by intracutaneous injection with the KLH-conjugated antigen with complete adjuvant. Three booster injections were performed after 2, 4 and 6 weeks. Seven days after the final immunization, exsanguination was carried out and the antiserum was separated.

Western Blot Analysis—Of tissue samples (skin, gill, stomach, intestine, liver, heart, kidney, spleen, ovary and uterine tube), 100 mg was homogenized with 1 ml of PBS and supernatants were obtained. Total protein concentrations in the tissue samples and blood plasma were measured using A_{280} and adjusted to 2 μg/μl with PBS. The samples (20 μg/lane) were subjected to SDS–PAGE and electroblotted onto a PVDF membrane. After blocking with 1% skim milk in PBS containing 1% Tween-20 (PBS-T) for 30 min at room temperature, the membrane was incubated with the antiserum described above or the absorbed antiserum with an excess amount of the antigen at 1:1000 dilution overnight at 4°C. The immune complexes on the membrane were then reacted with peroxidase-conjugated anti-rabbit IgG goat antibody (Sigma–Aldrich) at 1:1000 dilution for 2 h at room temperature. The bound secondary antibody was detected in a substrate solution containing 0.02% 3,3′-diaminobenzidine (DAB) and 0.03% H_2O_2 in 50 mM Tris–HCl (pH 7.5).

Immunohistochemistry—The skin fixed with Bouin's solution was embedded in paraffin and sectioned with a microtome into 8 μm thick slices. After deparaffination, endogenous peroxidase was inactivated by immersion in methanol containing 0.3% H_2O_2 for 30 min. The slides were washed with PBS containing 1% DMSO and 0.5% Triton-X (PBS/DMSO/TX), and then incubated for 18 h at 4°C with the anti-peptide antiserum, the absorbed antiserum with an excess amount of the antigen or the pre-immune serum at 1:1000 dilution. After washing with PBS/DMSO/TX, a peroxidase-conjugated anti-rabbit IgG goat antibody (Sigma–Aldrich) diluted at 1:1000 was applied to the section, which was incubated at room temperature for 2 h. The enzymatic reaction was developed with a substrate solution, as described above.

In Vivo Bacterial Challenge—Formalin-killed *Escherichia coli* K12 (XL1-Blue, Toyobo) was prepared as described elsewhere (*16*). In brief, *E. coli* was cultured in Luria Broth at 37°C for 16 h while being shaken at 200 rpm. The cells were collected by centrifugation at $3000 \times g$ for 1 min and re-suspended in PBS containing 0.5% formalin. After incubation at 4°C for 16 h,

the formalin-killed cells were washed with PBS and then re-suspended in the same buffer.

Fourteen common skates weighing 669 ± 291 g (mean ± SD) were used in the experimental challenge test. Two groups (six fish each) were injected intraperitoneally with either 1 ml of PBS or the formalin-killed *E. coli* suspension (10 mg/ml in PBS) and held in separate FRP tanks. Skins and livers from two fish from each group were sampled after 24, 48 and 72 h. These tissues were also obtained from two non-treated fish, which served as an initial control group.

Northern Blot Analysis—Total RNA from the skin and liver of the challenged skates was obtained using the same method described above. Ten microgram of each RNA sample was run on 1% denaturing agarose gel containing formamide in a 3-morpholinopropanesulfonic acid (MOPS) running buffer, and transferred to a Hybond-N^+ membrane (GE Healthcare) with 20× SSC as the transfer buffer. The blots were hybridized with the probes, which were the same as those used in the Southern blot analysis, at 55°C for 16 h. After stringency washing, the signals were detected using the same method described in the Southern blot analysis.

RESULTS

Agglutination Activity of the Skate Skin Mucus towards Yeast Cells—Yeast cell agglutination was detected in the crude skin-mucus extract from the common skate (result not shown). Lactose clearly inhibited the agglutination, while mannose did not affect the agglutination. These results indicate that the agglutination was induced by a lectin whose ligand was lactose and prompted us to purify the lectin from the common skate skin secretion.

Purification of the Lectin—We used lactose-affinity chromatography to purify the skin mucus lectin. On reduced SDS–PAGE, the affinity-purified fraction yielded a single band at ~25 kDa (Fig. 1A). In contrast, a protein band was detected at ~22 kDa on unreduced SDS–PAGE (Fig. 1A). The difference is suggestive of the existence of internal disulfide bonds in each subunit. It is also suggested that the lectin consists of non-covalently associated subunits. The lectin was positive in PAS staining (Fig. 1B).

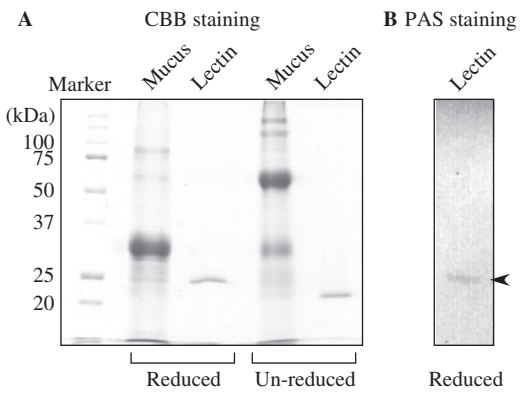

Fig. 1. **SDS–PAGE analysis for skin mucus extract and purified lectin.** Skin mucus extract and the affinity-purified lectin were subject to either un-reduced or reduced SDS–PAGE, and stained with CBB (A) and PAS (B). Numbers on the left represent molecular markers (kDa).

Primary Structure of the Lectin—N-terminal protein sequencing showed 12-amino-acid residues as Ala-Gly-Leu-Met-Gln-Lys-Ser-Val-Ile-Phe-Pro-Thr. Based on the N-terminal amino-acid sequence, we designed four antisense primers and performed 3′ RACE. Among the primers, primer-FC amplified a product with a size of 1.2 kb. Subsequently, 5′ RACE was performed with a specific primer, which was designed based on the nucleotide sequence obtained by 3′ RACE. Figure 2 shows the complete cDNA with 1540 bp and 226 residues of the deduced amino-acid sequence for the lectin. The lectin cDNA included 5′- and 3′-untranslated regions, and the deduced amino-acid sequence was composed of 20 residues of signal peptide and 206 residues of mature protein. The calculated molecular mass of the mature protein was 22780.65 Da, which was slightly smaller than that determined by reduced SDS–PAGE (~25 kDa, Fig. 1A). Because a possible *N*-glycosylation site was present in the protein sequence and the lectin was stained with the PAS reagent (Fig. 1B), the discrepancy could be attributed to the attachment of the sugar chain.

The full-length protein sequence of the lectin showed ~38.4% identity to PTXs in other animals (Fig. 3); the lectin sequence showed the highest amino-acid identity (38.4%) with guinea pig (*Cavia porcellus*) CRP (*19*), with 37.9% identity to human (*Homo sapiens*) SAP (*20*), which was the sequence exhibiting the second highest identity. By using the MOTIF software, one pentaxin family signature, H-x-C-x-[S/T]-W-x-[S/T], was identified at amino-acid positions from His^{112} to Ser^{119} (Figs 2 and 3). These findings indicated that the skin mucus lectin of the common skate belongs to the PTX family; hence, we termed the lectin 'csPTX'.

Tissue Distribution of csPTX Transcripts—The tissue-specific expression of the csPTX gene was analysed by RT–PCR with primer pairs of F1 and R1 (Fig. 4). A strong expression was detected not only in the skin but also in the gill, oesophagus, intestine and liver; on the other hand, a weak expression was observed in the stomach and kidney. In contrast, the heart, spleen, ovary and uterine tube appeared to be negative tissues. Notably, the band size of the PCR product from the liver (~550 bp; Fig. 4, lane 6) was apparently smaller than that from other positive tissues.

cDNA Cloning of the Lectin-Isoform from the Liver—The different band size observed in the liver sample in RT–PCR analysis (Fig. 4) could be explained by the existence of a liver-specific isoform of csPTX, so we tried to sequence this product. Based on the sequence obtained, we determined the complete cDNA of the liver-type isoform using the RACE method. The liver-type isoform is 1416 bp long, including the entire coding region of 226 amino acids, and shares 69.0% identity with the skin-type csPTX (Fig. 5). An alignment analysis of nucleotide sequences of these isoforms showed that there is an ~120 bp deletion in the 3′-untranslated region of the liver-type (Fig. 5), which shows good agreement with the result of RT–PCR analysis (Fig. 4).

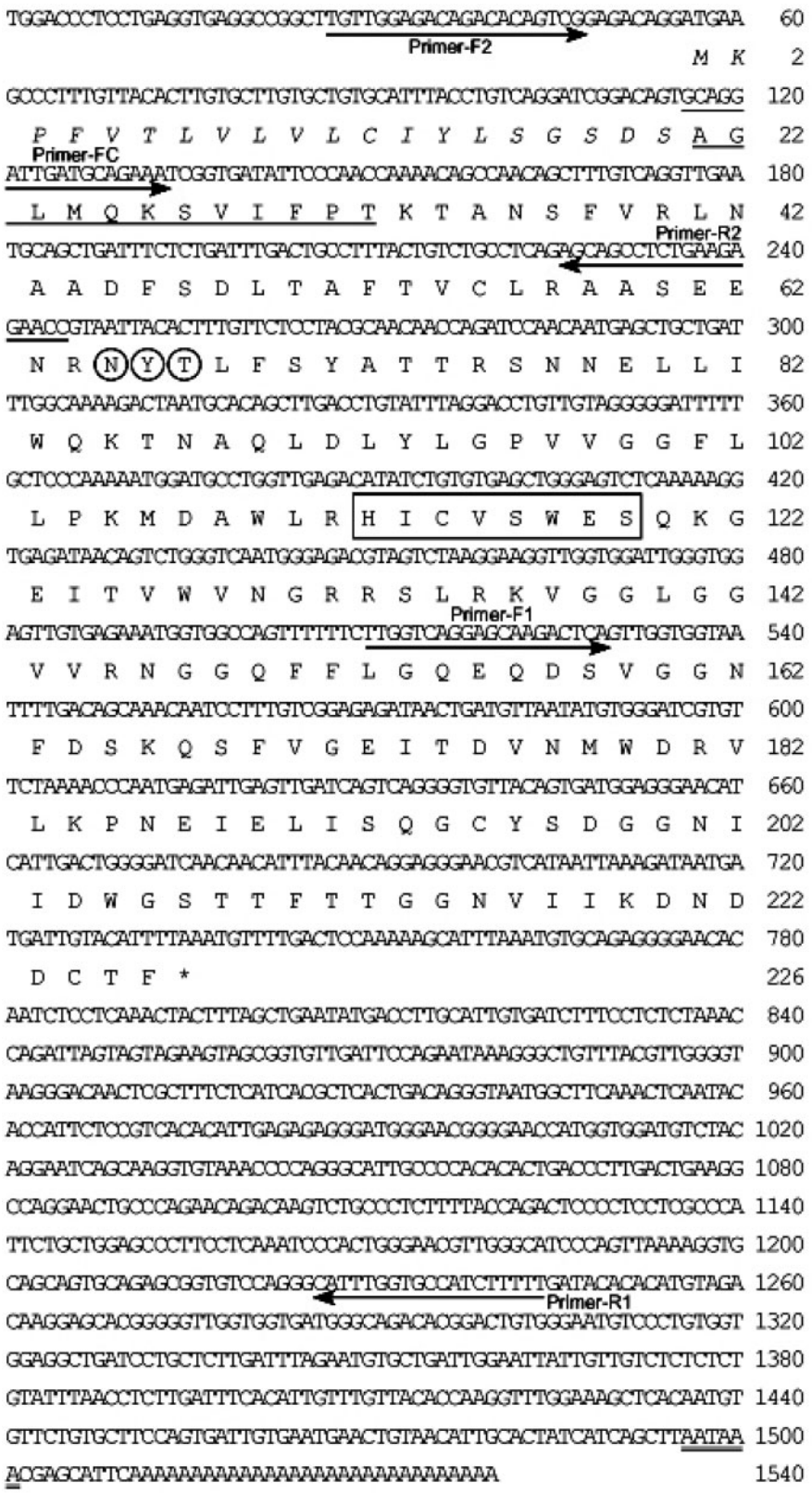

Fig. 2. **Skin mucus lectin cDNA sequence and predicted amino-acid sequence.** Numbers on the right indicate the position of the nucleotide and amino acids. The underlining indicates the N-terminal amino-acid sequence obtained by protein sequencing, and a putative signal sequence is shown in italics. The polyadenylation signal (AATAAA) is double-underlined, and the pentaxin family signature is boxed. A possible *N*-glycosylation site is circled. Arrows indicate the location and orientation of primers.

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G-pig CRP  MAKLLLYFLLLTSLSDVFGGTDMSKKTFVFPKETDNSYVSLKAQLKKPLSAFTVCLHIYT 60
csPTX      MKPFVTLVLVLCIYLSGSDSAGLMQKSVIFPTKTANSFVRLNAADFSDLTAFTVCLRAAS 60
Human SAP  MNKPLLWISVLTSLLEAFAHTDLSGKVFVFPRESVTDHVNLITPLEKPLQNFTLCFRAYS 60
           *   :  . :*    .    :.:  * .:** :: ...* * :   . *  **:*::  :

G-pig CRP  ELFMTRGYSIFSYATEKEANEILIFWSKDRGYILGVGGIEMPFKAPEIPSAPVHICTSWE 120
csPTX      EE--NRNYTLFSYATTRSNNELLIWQKTNAQLDLYLGPVVGGFLLPKMDAWLRHICVSWE 118
Human SAP  DL--SRAYSLFSYNTQGRDNELLVYKERVGEYSLYIGRHKVTSKVIEKFPAPVHICVSWE 118
           :   .* *::*** *    **:*:: .      * :*         :  .   ***.***

G-pig CRP  SVSGIIELWVDGKAQVRKSLQKGYFGVTEAMIILGQDQDSFGGSFDANQSFVGDIGDVNM 180
csPTX      SQKGEITVWVNGRRSLRKVGGLGGVVRNGGQFFLGQEQDSVGGNFDSKQSFVGEITDVNM 178
Human SAP  SSSGIAEFWINGTPLVKKGLRQGYFVEAQPKIVLGQEQDSYGGKFDRSQSFVGEIGDLYM 178
           * .*   .*::*   ::*    * .      :.***:*** **.** .*****:* *: *

G-pig CRP  WDFVLSPKEIDMVYSGGTFS-PNVLSWRSLTYETHGEVFIKPQLWP-- 225
csPTX      WDRVLKPNEIELISQGCYSDGGNIIDWGSTTFTTGGNVIIKDNDDCTF 226
Human SAP  WDSVLPPENILSAYQGTPLP-ANILDWQALNYEIRGYVIIKPLVWV-- 223
           ** ** *::*    .*      *::.* : .:   * *:**
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Fig. 3. **Comparison of the amino-acid sequence of the skin mucus lectin with guinea pig CRP and human SAP.** Numbers on the right indicate the amino-acid position. Identical (*) and similar (':' or '.') residues identified by ClustalW are indicated. Boxed residues represent the pentaxin family signature.

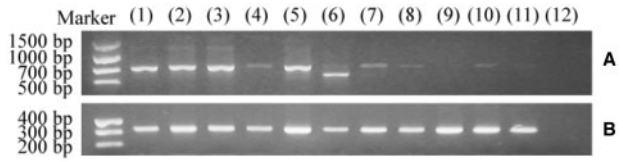

Fig. 4. **Gene expression analysis with RT–PCR.** Identical amounts of total RNA from the skin (*1*), gill (*2*), oesophagus (*3*), stomach (*4*), intestine (*5*), liver (*6*), kidney (*7*), heart (*8*), spleen (*9*), ovary (*10*) and uterine tube (*11*) were isolated and reverse transcribed to produce cDNA. PCR amplifications were then performed with specific primer-F1 and -R1 (A). Corresponding β-actin bands for these samples are shown in (B). (*12*): negative control.

Phylogenetic Tree of PTXs—In phylogenetic analysis, both skin-type and liver-type csPTXs were grouped with the PTXs of teleosts (Fig. 6). Evolutional analyses had proposed that fish have classical PTX, which gave rise to CRP and SAP by gene duplication in the mammalian lineage; thus, fish PTXs could not be classified as strict counterparts of mammalian CRP or SAP (*19*, *21*). Figure 6 supports this hypothesis, because the fish PTXs have been separately grouped from mammalian SAPs and CRPs.

Purification of Serum Lactose-Specific Lectin—Because the liver produces a large amount of plasma proteins, the expression of liver-type csPTX prompted us to examine whether or not the molecule is present in the blood plasma. Figure 7 shows SDS–PAGE patterns of the affinity-purified serum fraction with lactose-binding activity. Among several bands, there was an apparent band at ~25 kDa.

Southern Blot Analysis of csPTX—Genomic DNA blots from a common skate were hybridized with probes specific to the skin-type and liver-type csPTXs. As shown in Fig. 8, both probes hybridized with multiple bands in each digest with four enzymes. This result indicates that the common skate has multiple csPTX genes in its genome. In addition, band patterns produced for the skin-type and liver-type csPTXs were similar. This suggests that the skin-type and liver-type genes are closely located in the genome; these genes might be generated by tandem gene duplication in the process of evolution.

Production of Antiserum and Tissue Distribution of the Lectin Protein—A rabbit polyclonal antiserum was generated for the synthesized peptide containing amino-acid residues corresponding to ^{45}Asp–^{57}Arg in the mature form of skin-type csPTX. The specificity of the antiserum was characterized by the western blot analysis; a single band of 25 kDa was observed when the crude extract of skin mucus was subjected to the analysis (Fig. 9A, lane 12). The absorbed antiserum did not show apparent immune reaction against the skin mucus extract and purified lectin (Fig. 9B, lanes 12 and 13), indicating that the 25 kDa band detected by the antiserum in crude extracts was for sure csPTX.

Tissue distribution of the csPTX was examined by the western blot analysis. A strong signal at 25 kDa was detected in the skin (Fig. 9A, lane 1), and a weak one was observed in the blood plasma (Fig. 9A, lane 11). A few weak bands were detected in the stomach, kidney, heart and uterine tube (Fig. 9A, lanes 3, 6, 7 and 10). But these bands were not considered to be csPTX, because molecular masses of them were much higher than 25 kDa. Other tissues showed negative results in the analysis. These data suggest that csPTX is not a major protein in the common skate, except in the skin and plasma.

csPTX Is Located in the Epidermal Cells in the Skin—The localization of csPTX in the skin was examined by immunohistochemistry using the anti-csPTX

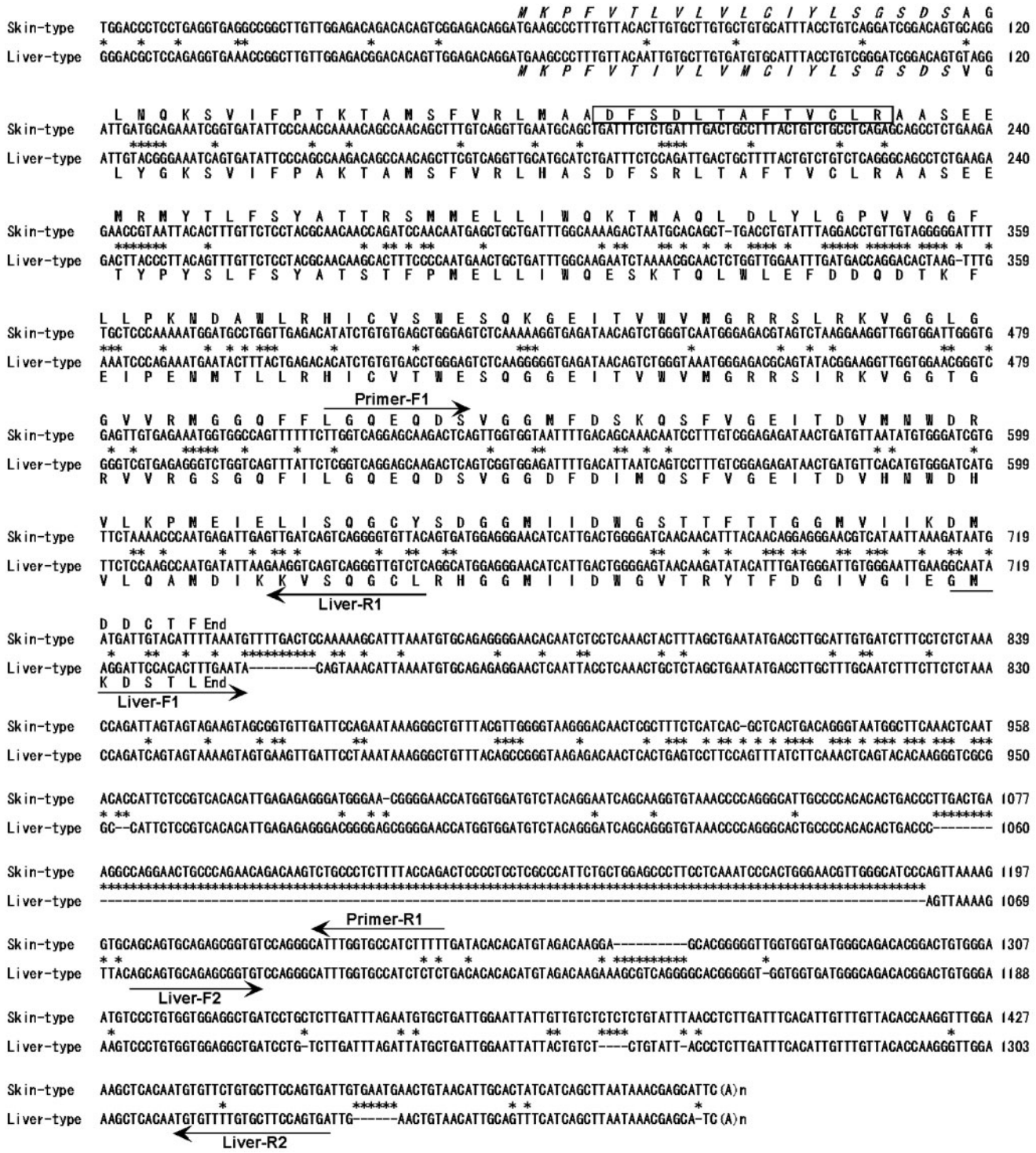

Fig. 5. **Comparison of DNA and protein sequences between skin-type and liver-type csPTX.** Asterisk highlights different bases. Gaps have been introduced to optimize identity. Location of primers used in RT–PCR (primer-F1 and -R1) and cloning of the liver-type (liver-F1, -F2, -R1 and -R2) are shown by arrows. The sequence of antigens for the antibody used in this study is boxed.

peptide antiserum. The antiserum specifically stained epidermal cells (Fig. 10A). No specific staining was observed with the absorbed antiserum or the preimmune serum (Figs 10B and 11C).

Bacterial Injection Does Not Affect the Gene Expression of the Lectins—PTX is known to be an acute phase protein (*5*, *6*); this prompted us to carry out a time course experiment to assess mRNA dynamics in skin-type and

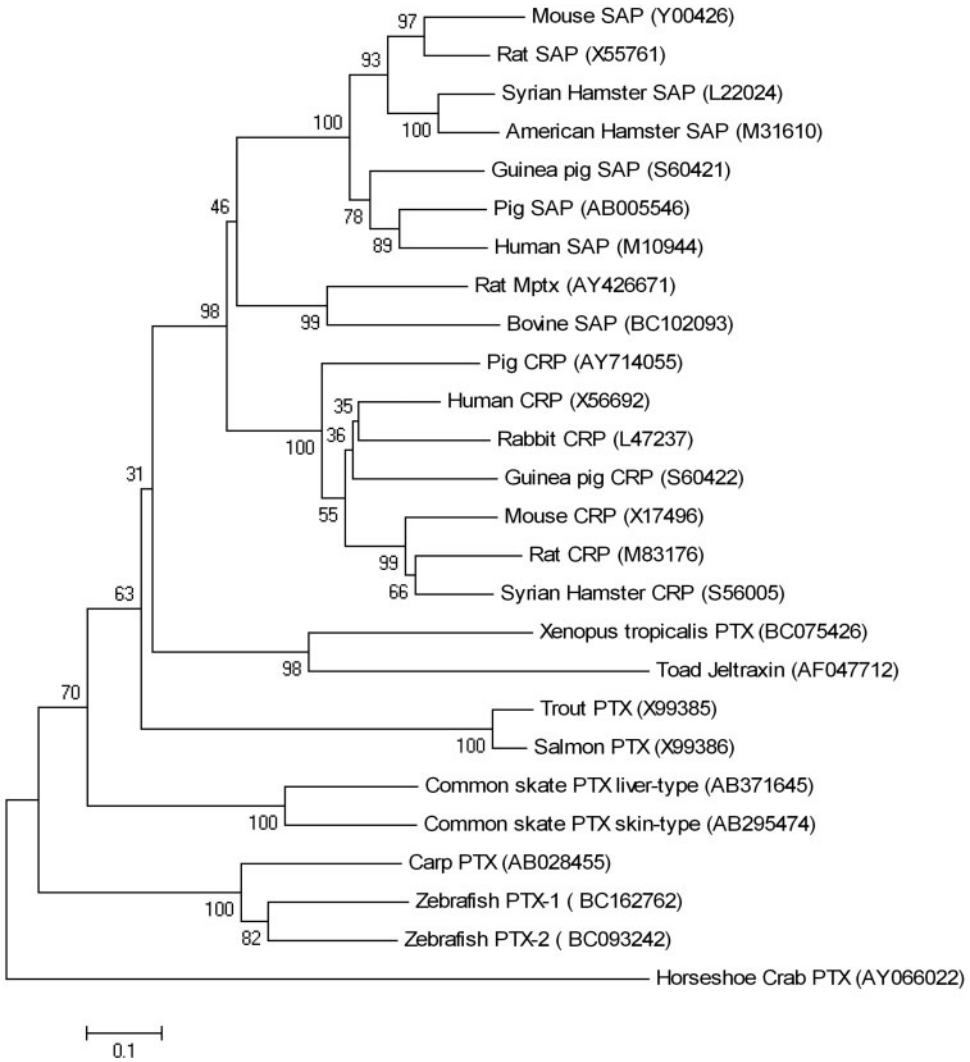

Fig. 6. **Neighbour-joining phylogenetic tree of PTXs.** Node values represent bootstrap analysis of 1000 replicants. The bar (0.1) indicates genetic distance. Molecules from non-mammalian species are described as PTX, for reasons proposed by Rubio *et al*. (*19*) and Seery *et al*. (*21*): lower vertebrates have only one classical PTX and their PTXs could not be classified as strict counterparts of mammalian CRP and SAP. Invertebrate PTX (Horseshoe crab PTX, accession no. AY066022) was used as the outer group.

liver-type csPTXs after bacterial injection. However, no apparent upregulation of the gene expression was observed by the northern blot analysis until 72 h after injection (Fig. 11).

DISCUSSION

PTXs have been found in the serum of large numbers of higher vertebrates. PTX-like proteins have also been isolated from various teleosts, such as plaice *Pleuronectes platessa* (*22*), rainbow trout *Oncorhynchus mykiss* (*23–25*), channel catfish *Ictalurus punctatus* (*26*), snapper *Pagrus auratus* (*27*) and common carp *Cyprinus carpio* (*28*), as well as from an elasmobranch fish species (dogfish *Mustelus canis*) (*29*). All of these proteins are derived from the serum. The current study revealed the presence of PTX for the first time in the skin mucus of fish. Recently, a mucosal pentraxin gene, *Mptx*, was identified in the gut of rats using a DNA microarray (*30*). The expression of the *Mptx* gene was restricted to the intestine (*31*). The human lung epithelial cell line is also shown to be an important source for a PTX3 (*32*). These findings suggest that mucosal PTXs are widely distributed among vertebrates from cartilaginous fish to mammals. Although not a mucosal PTX, jeltraxin, which is a PTX with a calcium-dependent lectin activity against galactose, was identified in the egg jelly of the South American burrowing frog, *Lepidobatrachus laevis* (*33*). Though interactions of serum PTXs with leucocytes, such as the opsonic effect, have been clarified in mammals (*8*, *9*) and fish (*34*), the function of PTXs acting outside the body, such as in mucosal PTXs and jeltraxin, has not yet been clarified. Future studies will involve the functional analysis of these PTXs.

PTXs were originally found as serum proteins in mammals. Their most striking feature is their sudden

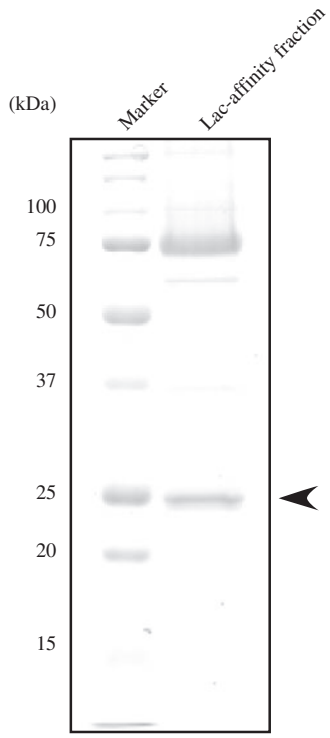

Fig. 7. **Detection of serum lactose-specific lectins on SDS/polyacrylamide gel.** Lactose-binding proteins were subjected to SDS–PAGE and visualized with CBB. Note that the 25 kDa protein was included in lactose-binding lectins.

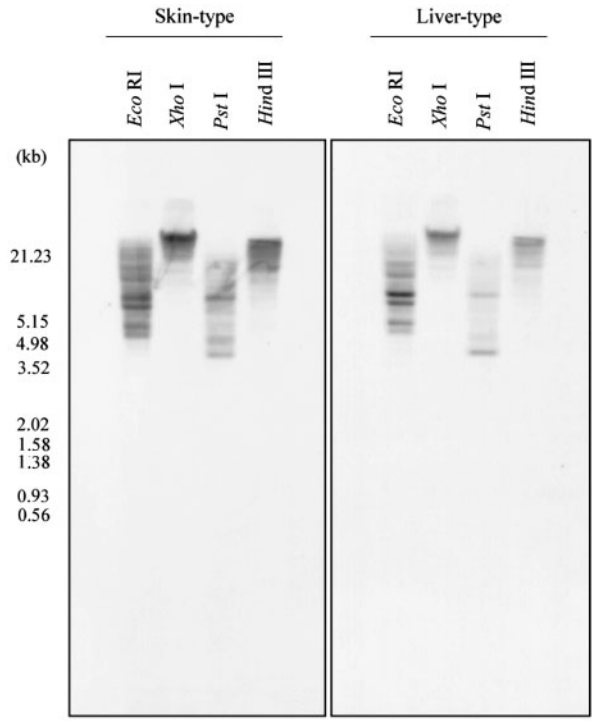

Fig. 8. **Southern blot analysis of the common skate genomic DNA using probes specific for the skin-type and liver-type csPTX.** The restriction enzymes used were *Eco*RI, *Hind*III, *Pst*I and *Xho*I. Numbers on the left indicate the molecular size (kb).

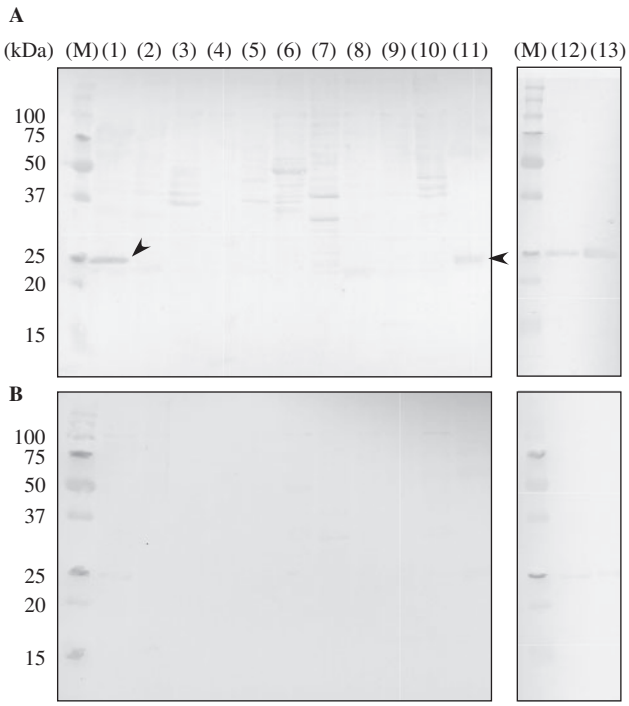

Fig. 9. **Tissue distribution analysis with western blotting.** Identical amounts of soluble protein from the skin (*1*), gill (*2*), stomach (*3*), intestine (*4*), liver (*5*), kidney (*6*), heart (*7*), spleen (*8*), ovary (*9*), uterine tube (*10*), blood plasma (*11*), skin mucus (*12*) and purified skin-type csPTX (*13*) were subjected to western blotting with the anti-lectin antiserum (A) or absorbed antiserum with the excess amount of antigen (B). Prestained molecular weight markers were used in this analysis.

rise in the serum level during the acute phase response to injury, infection and trauma (*5*, *6*). Inductions in the serum have also been reported for some fish PTXs, such as those of arctic char *Salvelinus alpinus* (*35*), Atlantic salmon *Salmo salar* (*36*) and snapper (*27*), although PTX production in fish does not appear to be very acute; the peaks of serum levels of PTXs in LPS-injected snapper and metriphonate-exposed rainbow trout occurred on the second and third days after the experiments (*27*, *37*). However, all PTXs are not acute phase proteins; in mammals, only either CRP or SAP acts as a true acute phase reactant. For example, within a few hours of infection, the plasma level of human CRP dramatically increases to 1000-fold, while human SAP levels stay relatively stable (*6*, *38*). In contrast, the opposite is true in mice: SAP is a major acute phase protein in the serum, whereas CRP is not (*5*, *39*). This may also be the case with fish: plaice has two PTXs, one of which is upregulated while the other decreases during inflammation (*40*). In the present study, the mRNA levels of csPTXs were not altered in the skin and liver after *E. coli* injection (Fig. 11). This finding might indicate that the common skate also possesses the true acute phase PTX other than the present csPTXs.

RT–PCR demonstrated that the csPTX gene was expressed in various tissues, such as the skin, gill, oesophagus, intestine and liver (Fig. 4). Among these tissues, a different type of csPTX gene is transcribed in the

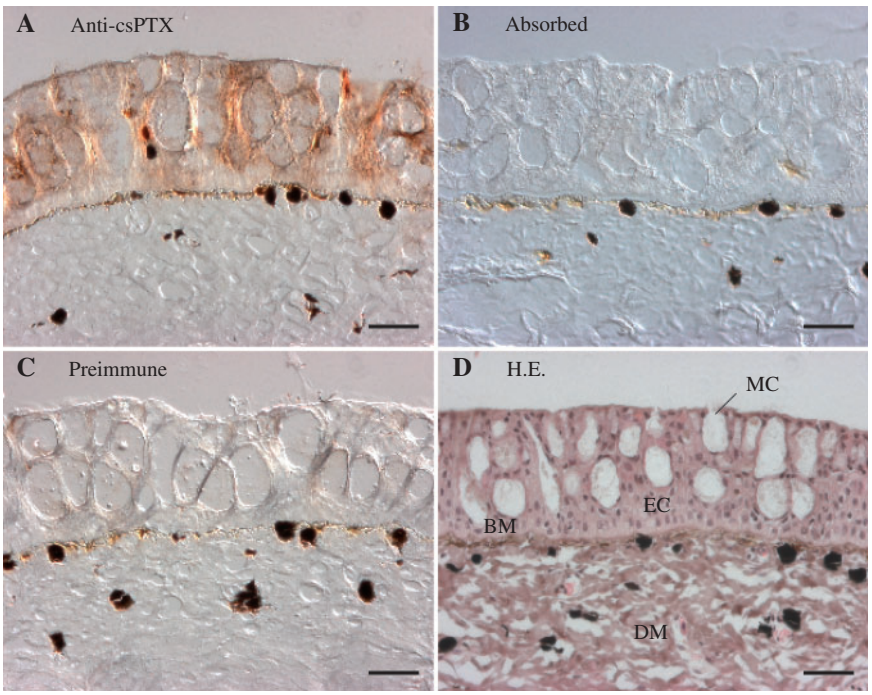

Fig. 10. **Immunohistochemical localization of csPTX in the skin.** Serial sections of the skin were stained with antiserum against csPTX peptide (A), absorbed antiserum (B), preimmune serum (C) and haematoxylin–eosin reagents (D). Clear staining was observed with the antiserum, but not with the absorbed or preimmune serum. MC, mucous cell; EC, epidermal cell; BM, basement membrane; DM, dermis. Scale bars represent 50 μm.

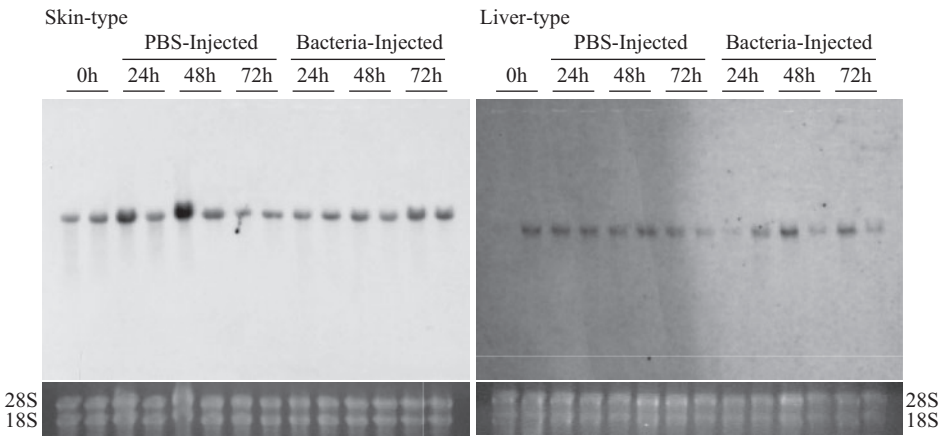

Fig. 11. **Kinetic analysis of skin-type and liver-type csPTX gene expression in bacteria-injected or PBS-injected individuals.** Skates were intramuscularly injected with either PBS or formalin-killed *E. coli* suspension, and the total RNA was isolated from the skin and liver of two individuals separately at the times indicated; the RNA was subjected to northern blot analysis. 18S and 28S rRNA were shown to estimate the relative amounts of RNA loaded in each lane.

liver (Fig. 5). We demonstrated by western blot analysis that csPTX is present in the blood plasma (Fig. 9), and that the common skate possesses ~25 kDa lectin with a lactose-binding activity in the blood plasma (Fig. 7). The liver generally produces a large portion of the plasma proteins, which includes the most frequently reported serum PTXs in animals. Additionally, the sequence of antigens for the antibody used in this study is conserved to a high extent in the liver-type csPTX (Fig. 5). Thus, it is likely that the protein detected in the blood plasma in western blotting and affinity chromatography is the Liver-type csPTX. On the other hand, another protein having a molecular mass of about 75 kDa was also purified from the blood plasma with lactose-affinity chromatography (Fig. 7). It is likely that other lectins are present in the blood plasma in the skate.

Moreover, the present study has reported the fifth type of fish skin mucus lectin, i.e. csPTX. Although many skin mucus lectins have been purified from various fish species, structural information on the lectins has not yet

accumulated (*11*). Fish species whose skin mucus lectins were structurally examined were limited to teleosts; furthermore, only a few orders have been hitherto analysed, which includes anguilliformes (*12–16*), perciformes (*18*) and tetraodontiformes (*17*). In spite of the limited information, these molecules are classified into distinct types: congerin I and II in the conger eel *Conger myriaster* and AJL-1 in the Japanese eel *Anguilla japonica* are galectins (*12–14*), while conCL-s in the conger eel and AJL-2 in the Japanese eel are C-type lectins (*15*, *16*). The ponyfish *Leiognathus nuchalis* possesses another type of lectin PFL (L-rhamnose-binding lectin) (*18*), while the fugu *Takifugu rubripes* possesses pufflectin-s, a novel animal lectin similar to monocotyledonous plant lectins (*17*). In the present study, we discovered a new type of fish skin mucus lectin from the common skate, which is a cartilaginous fish. This finding reinforces the notion that skin mucus lectins have evolved with a marked molecular diversity in fish.

CONFLICT OF INTEREST

None declared.

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