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Molecular Cloning, Expression Analysis and Enzymatic Characterization of Elastase-like Serine Protease from the Olive Flounder (*Paralichthys olivaceus*)

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넙치로부터의 Serine Protease의 분자생물학적 클로닝, 발현, 특성분석

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Abstract

넙치 (*Paralichthys olivaceus)*로부터 elastase-like serine protease (*PoElSp*)를 암호화하는 cDNA를 클로닝하여 그 서열을 분석한 결과, *PoElSp* 유전자는 269 아미노산을 암호화하는 978염기쌍으로 구성되었다. *PoElSp* 유전자의 조직 특이적 발현 양상을 RT-PCR법으로 조사한 결과, 간, 비장 및 소장에서 그 발현이 크게 나타났다. lipopolysaccharide (LPS)로 인위적 세균감염을 유도한 후, 1시간째에 콩팥에서, 3시간째에는 근육에서, *PoElSp* 유전자의 발현이 크게 증가하였다. 또한, 이 유전자의 발현은 비장에서 LPS 주입 후 1-24시간동안 점차로 증가하였다. pro-mature PoElSp (proPoElSp)에 해당하는 cDNA를 pET32a 벡터 시스템을 이용하여 대장균에서 발현시켰다. 이 재조합 proPoElSp 단백질의 활성은 gelatin zymography 방법과 합성형광 Z-Phe-Arg-AMC의 분해법을 이용하여 측정하였다. 단백질 분해효소 활성을 위한 최적 pH는 7.5였다. 실험결과들을 종합하면, PoElSp 단백질은 면역 반응에서 중추적 역할을 하리라 판단된다.

Key words : Elastase-like serine protease, Olive flounder, *Paralichthys olivaceus*, RT-PCR, Zymography

Ⅰ. Introduction

Proteases are currently classified into four major classes, serine, cysteine, aspartic, and metallo proteases (Barrett et al., 1998). Proteases are involved in numerous important physiological processes including protein turnover, digestion, blood coagulation and wound healing, fertilization, cell differentiation, cell signaling, immune response and apoptosis (Barrett et al., 1998; Powers et al., 2002).

Serine proteases are one of the most thoroughly understood enzyme families, and all serine proteases contain an active site termed the catalytic triad, which consists of His, Asp and Ser amino acid residues. They are found ubiquitously in both eukaryotes and prokaryotes. The serine residue at the active site participates in the formation of a

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transient acyl-enzyme intermediate between the substrate and the protease (Kraut, 1977; Parry et al., 1998; Perona et al., 1995; Piao et al., 2007). Serine protease cascades amplify signals from physiological or pathological responses in the extracellular milieu of vertebrate and invertebrates (Piao et al., 2005). In mammals, a complex cascade of coagulation factors, most of which are serine proteases, is triggered upon tissue injury to prevent bleeding. The complement system also employs similar protease cascades in response to microbial infection (O'Brien et al., 1993). Serine proteases fall into two broad categories based on their structures, chymotrypsin-like or subtilisin-like serine proteases. Chymotrypsin-like serine proteases are characterized by a distinctive structure, consisting of two beta-barrel domains that converge at the catalytic active site. These enzymes can be further categorized based on their substrate specificity as either trypsin-like, chymotrypsin-like or elastase-like (Ovaere et al., 2009).

In contrast, elastase-like serine protease has not been completely characterized with regard to its enzymatic properties and physiological functions except its molecular and phylogenetic characterization from teleosts including Atlantic cod (*Gadus morhua*) (Gudmundsdóttir et al., 1996), Japanese flounder (*Paralichthys olivaceus*) (Suzuki et al., 2002) and zebrafish (*Danio rerio*) (Wan et al., 2006). In this study, we described the cDNA cloning, tissue-typical mRNA expression, and enzymatic characterization of the recombinant elastase-like serine protease in the olive flounder (*P. olivaceus*).

Ⅱ. Materials and methods

1. cDNA Synthesis and Rapid Amplification of cDNA Ends (RACE)

The total RNA was isolated from olive flounder using the TRIzol® (Invitrogen) according to the manufacturer's instructions, and cDNA was synthesized from this isolated mRNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). In an effort to identify the elastase-like serine protease of the olive flounder, regions around the highly conserved region of elastase-like serine protease (sense primer, pro-ElSp-F; antisense primer; pro-ElSp-R, [Table 1]) were utilized in the amplification of cDNAs from an olive flounder cDNA mixture. Rapid amplification of cDNA ends (RACE) was used to clone the cDNA containing 5'- and 3'-end of *PoElSp* using a SMART™ RACE cDNA amplification kit (Clontech). The 5'-end of the *PoElSp* gene was obtained by 5' RACE-PCR using the specific primer sets PoElSp-GSP-R1 with Universal Primer Mix (UPM) for first round-PCR and PoElSp-GSP-R2 primer with Nested Universal Primer (NUP) for second nested PCR, respectively. The two primer sets used for 3' RACE-PCR were PoElSp-3'-F1 primer with UPM and PoElSp-3'-F2 primer with NUP [Table 1] for obtaining 3'-end region. After amplification, the RACE products were subcloned into a pGEM® T-Easy vector (Promega), and then transformed into *E. coli* DH5 α competent cells according to the manufacturer's instructions. The *E. coli* clones containing the recombinants were overlaid with 100 μg/ml of ampicillin, 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG) and 40 μg/ml 5-bromo-4-chloro-3-indoly-β-D-galactoranoside (X-Gal) in the Luria-Bertani (LB) agar plate. White colonies were randomly chosen, cultivated and used for extraction of plasmid DNA. Plasmid DNA was prepared from *E.*

coli using a LaboPass™ Plasmid Mini Purification Kit (COSMO GENETECH). DNA sequencing was conducted using T7 promoter/ SP6 primers in the SolGent Co., Ltd.

<Table 1> Oligonucleotide primers used for *PoElSp* amplification and expression studies

Primer name		5`-3` sequence	Information
Universal Primer A Mix (UPM)	UP-Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATC	Universal primers for 5' and 3' RACE
	UP-Short	AACGCAGAGTCTAATACGACTCACTATAGGGC	
Nested Universal Primer (NUP)		AAGCAGTGGTATCAACGCAGAGT	
pro-ElSp-F		CAAGCTCTCCTACAGGGTGT	Gene specific primers for 5' and 3' RACE
pro-ElSp-R		CATGATCAGCCACAGGCATC	
PoElSp-GSP-R1		CCATCCGGTGATGTAGCAGG	
PoElSp-GSP-R2		GAGCAATGTCGTTTCCGAGG	
PoElSp-3'-F1		CAGCTGCACACTGCATCAAC	
PoElSp-3'-F2		GGTTACTCAAAGTCACAGGC	
Po-18s rRNA-real-F		GTTGGTGGAGCGATTTGTCTGG	Primers for expression studies
Po-18s rRNA-real-R		CATCTAAGGGCATCACAGACCTG	
Po-β-actin-real-F		FGACATGGAGAAGATCTGGCA	
Po-β-actin-real-R		ATGTCCTGCTCGAAGTCCAG	
PoElSp-RT-For		CATGAATTCCCGTGTGGTCA	
PoElSp -RT-Rev		CACGAAGATGGAGTTCCATTTCTC	
PoIL1β-F		GGTGCTACCAGACCTTCAACATCCAG	
PoIL1β-R		CAAAGTCTTTCCAGCAGACAGTGGTG	
PoIL6-F		CAGCACTTTCCACAGGAAGATGACG	
PoIL6-R		AGAGGGATGGATGGGTGGAATAATTC	
PoIL8-F		GGGTCAGAAGCCGTTTAAAGACAACTC	
PoIL8-R		GTTAGTTCCCTTCAAACAAGCACAGGC	
BamHI-proPoElSp-F		GCGAGGATCCTGCGGCACCCCATCCATTGA	Primers for Recombinant protein
HindIII-pro-PoElSp-R		GCGAAAGCTTGTTGTTCATCATAACCATGT	

2. Sequence and Phylogenetic Analysis

Nucleotide and predicted amino acid sequences of *PoElSp* were analyzed using DNAsis for Windows version 2.5 (Hitachi software engineering), BioEdit Sequence Alignment Editor (Hall, 1999) and BLAST programs in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The signal sequence and putative cleavage site of *PoElSp* was identified using the SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP).

Predictions of the pro-region cleavage sites and active sites were based on alignment of the elastase-like serine protease sequences with the vertebrate orthologues. Multiple sequence alignments of *PoElSp* with other serine proteases were constructed using BioEdit Sequence Alignment Editor with CLUSTAL W version 1.9 (Thompson et al., 1994). The phylogenetic tree was constructed using the Neighbor-Joining method and plotted with MEGA version 4.1 (Kumar et al., 2008).

3. Expression Studies by RT-PCR and Quantitative Real-Time PCR

In order to visualize the expression patterns of the *PoElSp* mRNA, RT-PCR and quantitative real-time PCR were conducted using total RNAs from brain, eye, gill, heart, gullet, liver, spleen, pyloric ceca, stomach, intestine, kidney and muscle tissues obtained from healthy specimens of *P. olivaceus*. The total RNA was isolated using

TRIzol® (Invitrogen) in accordance with the manufacturer's instructions, and the purified RNA was quantified via optical density at 260 nm using a UV spectrophotometer (Ultrospec 6300 pro, Amersham Biosciences). Two micrograms of total RNA from the *P. olivaceus* tissues was reverse-transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche). The specific primers for olive flounder elastase-like serine protease (*PoElSp*), PoElSp-RT-For and PoElSp-RT-Rev primers were used for RT-PCR and real-time PCR <Table 1>. *P. olivaceus* β-actin was utilized as the internal control (Poβ-actin-real-F and Poβ-actin-real-R primer set for the flounder β-actin, (<Table 1>).

All of the PCR was run as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 20 s and 72 °C 30 s, and a final 7 min of elongation at 72 °C. The resultant PCR products were separated on 1.2% agarose/TAE gels containing ethidium bromide and visualized with a Gel Doc image analysis system (Bio-Rad). The PCR products were purified via agarose gel extraction (QIAquick® Gel Extraction kit) and sequenced (SolGent Co., Ltd., DNA Sequencing Service).

To examine the immune responses and expression regulation of *PoElSp* gene, lipopolysaccharide (LPS) from *E. coli* 0127: B8 (Sigma-Aldrich, USA) was used to induce inflammatory responses in the various flounder tissues (gill, kidney, spleen and muscle). Gene expression in LPS-stimulated *PoElSp* gene was compared with the expression of cytokines, IL-β (PoIL1β-F and PoIL1β-R for the flounder IL-1β), IL-6 (PoIL6-F and PoIL6-R for the flounder IL-6) and IL-8 (PoIL8-F and PoIL8-R for the flounder IL-8) genes, which were utilized as internal controls for inducible gene expression <Table 1>. LPS was intraperitoneally injected at a concentration of 0.02 mg/g body mass. Three fish (average mass 100 g) were sacrificed after 1, 3, 6 and 24 h of injection. Total RNA isolation, reverse transcription, PCR reaction and direct DNA sequencing were performed as described above. Quantitative PCR was conducted using SYBR 480 Real-Time PCR (Roche) with SYBR Green (Roche applied science). Each reaction contained the following: 10 μl of the Power SYBR® Green PCR Master Mix (Roche applied science), 0.2 μM primer sets, 0.5 cDNA (300 ng), and water to a final volume of 20 μl. The PCR parameters were as follows: initial denaturation at 95 °C for 5 min to activate DNA polymerase. The melting curve cycles were conducted under the following conditions: followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C. All of the data were gathered from triplicate experiments and were expressed as fluorescence relative to β-actin. The relative fold change of *PoElSp* expression was determined by the $2^{-\triangle\triangle Ct}$ method as described previously (Giulietti et al., 2001; Livak et al., 2001).

4. Expression and Purification of Recombinant proPoElSp Protein

To prepare an expression vector suitable for the production of recombinant olive flounder elastase-like serine protease in *E. coli*, a 759bp DNA fragment containing the coding sequence for the pro-mature form of *P. olivaceus* elastase-like serine protease (proPoElSp) was generated by PCR amplification. The primers (BamHI-proPoElSp-F, 5'-GCGAGGATCCTGCGGCACCCCATCCATTGA-3'; HindIII-pro-PoElSp-R, 5'-GCGAAAGCTTGTTGT-TCAT CATAACCATGT-3') harbor *BamH*I/*Hind*III restriction sites (underlined), allowing for the cloning of the amplified DNA in a predicted orientation into pET32a vector system (Novagen). Recombinant plasmid (*proPoElSp/pET32a*) was transformed into *E. coli* strain

BL21(DE3). Transformed cells were grown in LB broth containing 100 mg/ml ampicillin at 37°C for approximately 16 hr, diluted 1/100 with the same medium, and grown to an A_{600} of 0.6. Next, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the incubation was continued for 3 hr at 18 °C. Cells were collected by centrifugation, washed, and resuspended in 0.2 volumes of phosphate buffered saline, and then sonicated. After, cell lysates are centrifuged at 20,000 × g for 20 min at 4°C, the supernatant fraction was applied to the His-bind column. After twice washing of column with 1x wash buffer (20 mM Tris/pH 7.9, 60 mM imidazole, 0.5 M NaCl), recombinant proPoElSp protein was eluted in ten fractions with 1x elution buffer (20 mM Tris/pH 7.9, 1 M imidazole, 0.5 M NaCl). The pooled fractions were dialyzed and analyzed by SDS-PAGE, western blotting and enzyme activity assay. Protein concentration was determined with a Bio-Rad protein assay kit.

5. SDS-PAGE, Western Blotting and Zymography

Purified proPoElSp enzyme was analyzed by 12% SDS-PAGE. All samples were denatured in a buffer containing 60 mM Tris/pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue, boiled for 5 min, and separated by 12% SDS-PAGE (Bio-Rad). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. Western blotting was performed using mouse monoclonal anti-His antibody (1: 2000, Santa Cruz Biotechnology). The substrate zymography was performed by a modified procedure using gels with gelatin (Sigma) as described earlier (Heussen el al., 1980) with slight modifications. To prepare the zymography, 30 ul of a protease sample was mixed with 10 ul of 4x SDS-sample buffer (0.5 M Tris/pH 6.8, 10% SDS, 20% glycerol and 0.02% bromophenol blue). The sample was then applied to the gel and electrophoresed using a Bio-Rad Mini-PROTEAN® system (Bio-Rad) with a constant current of 12 mA per gel at 4°C. After electrophoresis, the gels were immersed in 100 ml of 2.5% (v/v) Triton X-100 for 1h to remove SDS and were washed once with incubation buffer (0.1 M Tris/ pH 8.0, containing 1 mM DTT). Next, the gels were immersed in the incubation buffer for 18hr at 37°C. Subsequently, the gels were washed with water and stained in 5% methanol/10% acetic acid/ water containing 0.1% Coommassie Brilliant Blue R-250. Protease bands appeared as clear zones on a blue background.

6. Enzyme Activity Assays

The Serine proteases activity was assayed according to the modified method of Barret et al, (1981). The optimum pH for enzymatic activity of proPoElSp protein was determined using a sodium acetate buffer in pH ranges of 3-10 with Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (Z-FR-AMC; Sigma) as substrates. Briefly, 10 $\mu\ell$ of recombinant proPoElSp protein in 85 $\mu\ell$ of 0.1 M Tris/pH 7.5, containing 1 mM DTT were preincubated at 37°C for 2 hr, and the enzyme reaction was initiated by adding 5 $\mu\ell$ of 1 mM Z-FR-AMC at 37°C for 10 min. The 7-amido-4-methylcoumarin (AMC) was measured using a Microplate Fluorometer (Packard Co. USA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Substrate specificities were investigated using Z-Arg-Arg-AMC (Sigma), Z-Gly-Pro-Arg-AMC (Sigma), Ala-Ala-Phe-AMC (Sigma), Z-Arg-Gly-Phe-Pro-Pro (Sigma), and Suc-Leu-Leu- Val-Tyr-AMC with 0.1 M Tris/ pH 8.0, and containing 1 mM DTT, respectively. Substrates were added to a final concentration of 100 μM.

<Table 2> Sequences used in this study

Gene name	Accession number	Species (common name)
PoElSp	KJ769206	*Paralichthys olivaceus* (olive flounder)
PoELA3	BAA82369	
TrChymotrypsin_C	XP_003963183	*Takifugu rubripes* (pufferfish)
DrELA1	NP_001020645	*Danio rerio* (zebrafish)
DrELA3L	NP_001019579	
DrChymotrypsin	NP_001004582	
DrPlasminogen	NP_958880	
DrHepsin	NP_001091657	
XtELA1	NP_001025669	*Xenopus tropicalis* (western clawed frog)
XtClELA2A	XP_002944523	
XtELA3B	NP_001011421	
XtClELA3A	NP_001011426	
XtClELA3B	XP_004920158	
XtChymotrypsin_C	NP_001008077	
GgClELA1	NP_001096672	*Gallus gallus* (chicken)
GgChymotrypsin_C	NP_001264846	
GgChymotrypsinogen	NP_001264565	
GgPlasminogen	XP_419618	
GgOvochymase-2	XP_001232535	
MmELA1	AAH11218	*Mus musculus* (mouse)
MmELA2A	AAH26552	
MmELA3	AAH61066	
MmChymotrypsin_C	AAI15517	
MmChymotrypsinogen_B1	AAH61083	
MmPlasminogen	AAH14773	
MmHepsin	AAI38810	
HsELA1	AAD28441.1	*Homo sapiens* (human)
HsELA2A	AAH07031	
HsELA2B	AAH69412	
HsELA3A	AAH07028	
HsELA3B	AAH05216	
HsChymotrypsin_C	AAH15118	
HsChymotrypsinogen_B2	EAW95661	
HsPlasminogen	AAN85555	
HsOvochymase-1	NP_899234	
HsHepsin	AAH25716	

7. Effect of Enzyme Inhibitors, Metal Ions and Detergents

The effects of enzyme inhibitors on protease activity were studied using Z-Phe-Arg-AMC as the fluorogenic substrate. The following known proteinase inhibitors were tested: trans-Epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64; Sigma), and Leupeptin (USB Co., USA) for cysteine protease inhibitor; Antipain (Sigma), *N*-ethylmaleimide (NEM; Sigma), Phenylmethylsulphonyl fluoride (PMSF; Sigma), Chymostatin (Sigma), and Aprotinin (Sigma) for serine protease inhibitor; Ethylene diamine tetraacetic acid (EDTA; Sigma), Ethylene glycol-bis (beta-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA;Sigma) and 1,10-Phenanthroline (Sigma) for metalloproteinase inhibitor; Pepstatin A (Sigma) for aspartic protease inhibitor. Also, the effect of various metal ions (1 and 5 mM) on enzyme

activity was investigated using $CoCl_2$, $CaCl_2$, $MgSO_4$, KCl, $ZnSO_4$, $CuSO_4$ and $HgCl_2$. The effects of some surfactants (Brij35, TritonX-100, Tween 20 and SDS) on enzyme stability were also examined. The effects of enzyme inhibitors, metal ions and detergents (0.01 and 0.05%) on the activity of proPoElSp protein were studied at pH 7.5 and 37°C. The values shown in tables and graphical data represent the mean of three assays (±standard deviation). All the analyses were performed using Microsoft Excel and SigmaPlot software package.

Ⅲ. Results and discussion

1. Cloning and Sequence Analysis of Olive Flounder Elastase-like Serine Protease

In order to determine the evolutionary relationship of PoElSp, phylogenetic tree analysis Full-length of *P. olivaceus* elastase-like serine protease (*PoElSp*) cDNA was obtained by combining DNA sequences of cDNA library clones and 5' SMART™ RACE cDNA Amplification PCR product. The full-length

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5'   AA GCA TCA ATC AAC ATG ATC CCC ATT GTG CTG GCC TCA GTG CTC ATC GCT AGC     54
                        M   I   P   I   V   L   A   S   V   L   I   A   S
     GCC CTT GGG TGC GGC ACC CCA TCC ATT GAG CCC ATG AAT TCC CGT GTG GTC AAT   108
       A   L   G   C   G   T   P   S   I   E   P   M   N   S   R   V   V   N
     GGA GTC GAT GCC AAG CCC CAC AGC TGG CCC TGG CAG ATC TCC CTG CAG TAT GAG   162
       G   V   D   A   K   P   H   S   W   P   W   Q   I   S   L   Q   Y   E
     AGG GAC GGT CAA TGG AGG CAC ACG TGT GGG GGA TCT CTG ATT GCT GCC AAC TGG   216
       R   D   G   Q   W   R   H   T   C   G   G   S   L   I   A   A   N   W
     GTC ATG ACA GCT GCA CAC TGC ATC AAC ACC AAG CTC TCC TAC AGG GTG TTT GTG   270
       V   M   T   A   A   H   C   I   N   T   K   L   S   Y   R   V   F   V
     GGC AAA CAC AAC CTG TTG GAG GAG GAG CCT GCC TCT CAG GCC ATC CTG CCT GAG   324
       G   K   H   N   L   L   E   E   E   P   A   S   Q   A   I   L   P   E
     AAG ATG ATT GTC CAT GAG AAA TGG AAC TCC ATC TTC GTG GCC CTC GGA AAC GAC   378
       K   M   I   V   H   E   K   W   N   S   I   F   V   A   L   G   N   D
     ATT GCT CTG ATC AAG CTG TCA GAG CCT GTG ACT TTG AGT AAC CAG GTG CAG CTG   432
       I   A   L   I   K   L   S   E   P   V   T   L   S   N   Q   V   Q   L
     GCA TGT ATC CCT GCT GCC GGC ACT CTT CTC CCC AAC CTA TAC CCC TGC TAC ATC   486
       A   C   I   P   A   A   G   T   L   L   P   N   L   Y   P   C   Y   I
     ACC GGA TGG GGC AGG CTG TAC ACT GGA GGC CCC ATC GCT GAT AAG CTG CAG CAA   540
       T   G   W   G   R   L   Y   T   G   G   P   I   A   D   K   L   Q   Q
     GCT CTG ATG CCT GTG GCT GAT CAT GCC ACC TGC TCC CAG CCT GAC TGG TGG GGT   594
       A   L   M   P   V   A   D   H   A   T   C   S   Q   P   D   W   W   G
     TTT GCT GTC AGG GAC AGC ATG GTG TGT GCC GGC GGG GAT GGA ATC GTG GGT GGA   648
       F   A   V   R   D   S   M   V   C   A   G   G   D   G   I   V   G   G
     TGC AAC GGA GAC TCT GGC GGC CCC CTT AAC TGC AAG AAC AGC CAG GGA GCC TGG   702
       C   N   G   D   S   G   G   P   L   N   C   K   N   S   Q   G   A   W
     GAA GTC CAC GGC ATT GCC AGT TTC GTC TCC GGC CTT GGC TGC AAC TAC GTG AAG   756
       E   V   H   G   I   A   S   F   V   S   G   L   G   C   N   Y   V   K
     AAA CCC ACT GTC TTC ACC CGT GTC TCT GCT TTC AAC GAC TGG ATC GAC ATG GTT   810
       K   P   T   V   F   T   R   V   S   A   F   N   D   W   I   D   M   V
     ATG ATG AAC AAC TAA AGA AGA TGA AGG CAC AAG AAA AAG AGA GAC ACA AAA AAA   864
       M   M   N   N   *
     AAA AAA AAA AAA AAA AAA A   3'                                          882
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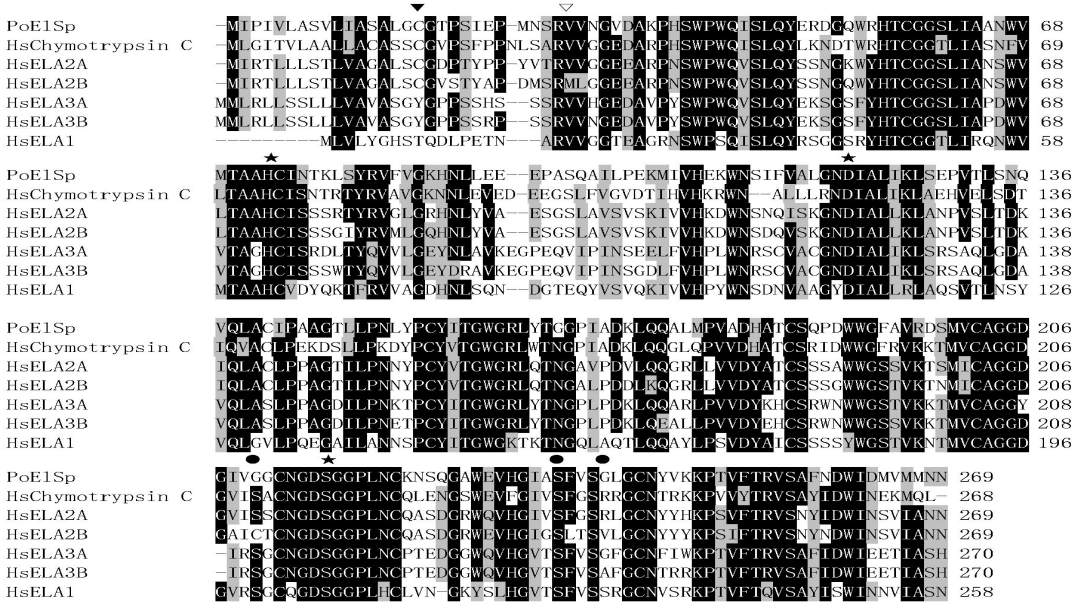
[Fig. 1] Nucleotide and deduced amino acid sequences of Paralichthys olivaceus elastase-like serine proteases (PoElSp). The shaded box and thick underline in the amino acid sequence indicates the putative signal peptide (pre) and the propeptides of PoElSp. The three active site triad residues His45, Asp93 and Ser188 (based on mature PoElSp numbering) are marked with a shaded box and underlined. The amino acid sequences in the open boxes are indicated the substrate binding sites on conserved domain (Gly182, Ser208 and Gly212). The asterisk at the end of the amino acid sequences shows the stop codon.

cDNA yielded a 978 bp sequence comprised a 110 bp 5'-untranslated region (5'UTR) and a 810 bp coding region including a stop codon (TAA), followed by a 58 bp 3'-untranslated region (3'UTR), including the run of poly (A) sequences presumably derived from the poly (A)-rich tail of the mRNA (Proudfoot and Brownlee, 1976) [Fig. 1].

The nucleotide sequence of *PoElSp* was predicted to encode for a pre-pro-mature protein of 269 amino acids, which contained a 16 residue putative signal peptide analyzed with SignalP 4.0 (Petersen et al., 2011), a 12 residue propeptide and the 241 residue mature enzyme with a calculated molecular mass of 29,040 Da. The sequence of *PoElSp* was deposited in GenBank under the accession number KJ769206 [Fig. 1].

[Fig. 2] shows the alignment of PoElSp with different serine proteases of human. A comparison of the amino acid sequence of PoElSp with human chymotrypsin C, chymotrypsin-like elastase family, member 1 (ELA1), ELA2A, ELA2B, ELA3A and ELA3B showed 46.5 - 57.3% identities. As shown in [Fig. 2], each of the catalytic triad residues PoElSp is surrounded by a highly conserved motif. The motif "GDSGGP" surrounds serine, "TAAHC" histidine and DIM/ALL aspartate (Rawlings et al., 1993).

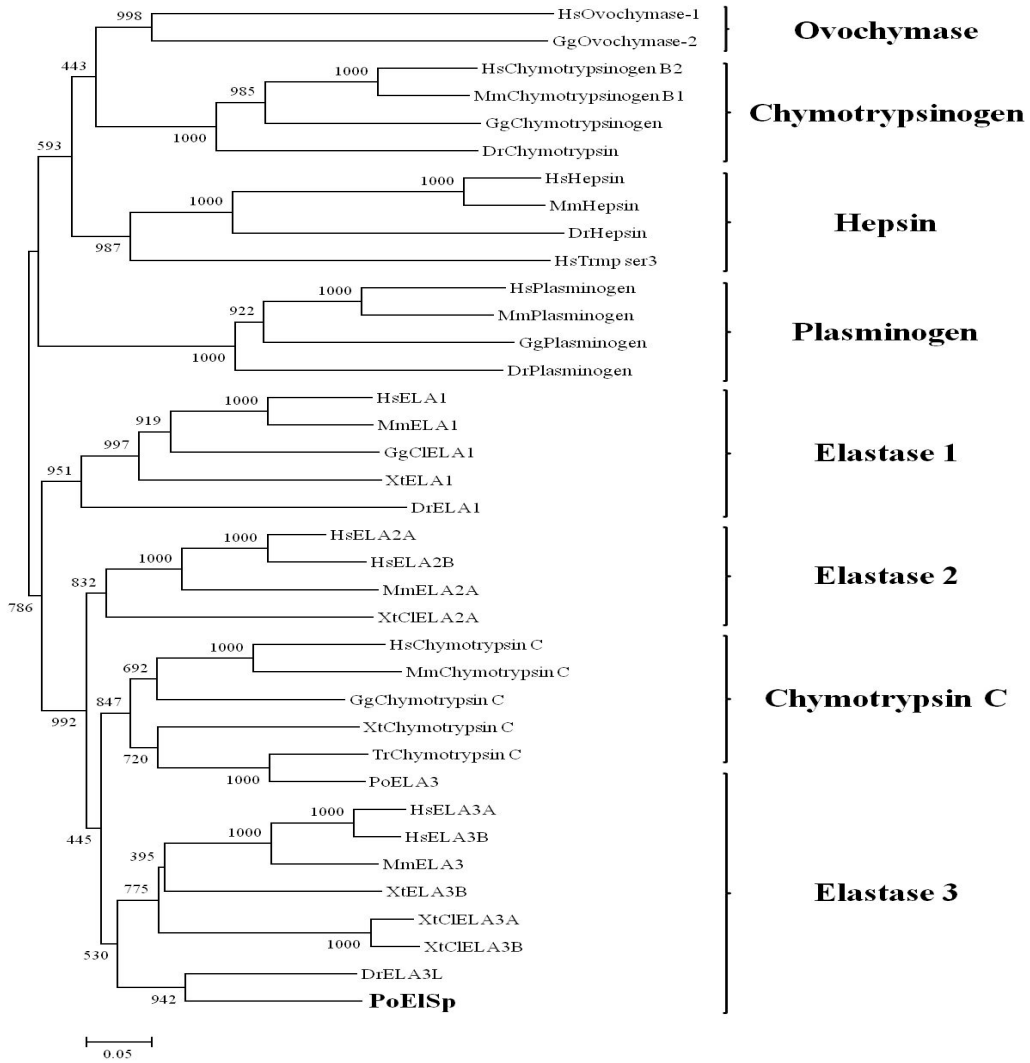
was conducted with the amino acid sequences of vertebrate serine protease families acquired from



[Fig. 2] Multiple amino acid sequence alignment of *P. olivaceus* elastase-like serine protease with various human serine proteases. Identical amino acid residues are darkly shaded, similar amino acids are lightly shaded, unrelated residues have a white background, and amino acid numbers are shown on the right. The filled and open triangle represents the predicted cleavage sites of the pre- and pro-sequences in *Paralichthys olivaceus* elastase-like serine protease, respectively. The serine protease catalytic triad residues (H, D, and S) are shown with an asterisk. The circles are indicated the substrate binding site of PoElSp (G, S and G). GenBank accession numbers of all sequences was presented in [Table 2].

GenBank via neighbor-joining methods (Saitou et al., 1987) [Fig. 3].

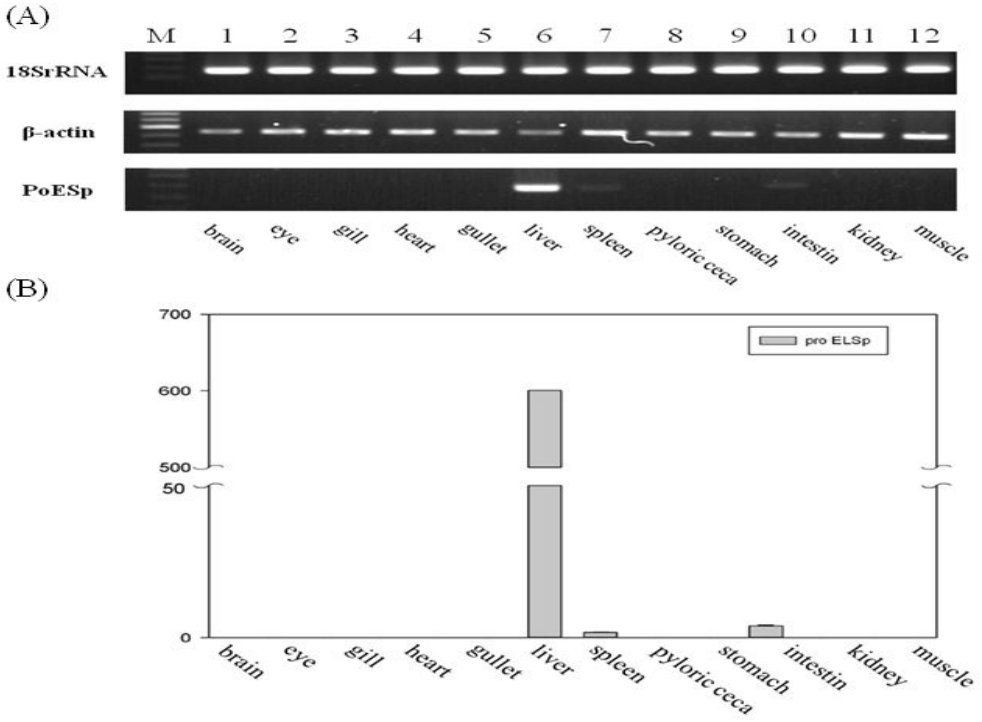
On the basis of a comprehensive phylogenetic analysis, the clades of elastase-like serine protease did not create a monophyletic group. As shown in [Fig. 3], PoElSp was placed closely related vertebrate elastase clades and clustered with a large group of vertebrate serine proteases, including chymotrypsin C, elastase 1, elastase 2, plasminogen, hepsin, chymotrypsinogen and ovochymase. The results of our phylogenetic analysis showed that the PoElSp clustered with elastase 3 from vertebrates.



[Fig. 3] Phylogenetic relationships of PoElSp among representative mammalian and fish groups based on the elastase-like serine protease genes. In this neighbor-joining phylogram, all individuals are represented and the branches are based on the number of inferred substitutions as indicated by the bar. GenBank accession numbers of all sequences was presented in [Table 2].

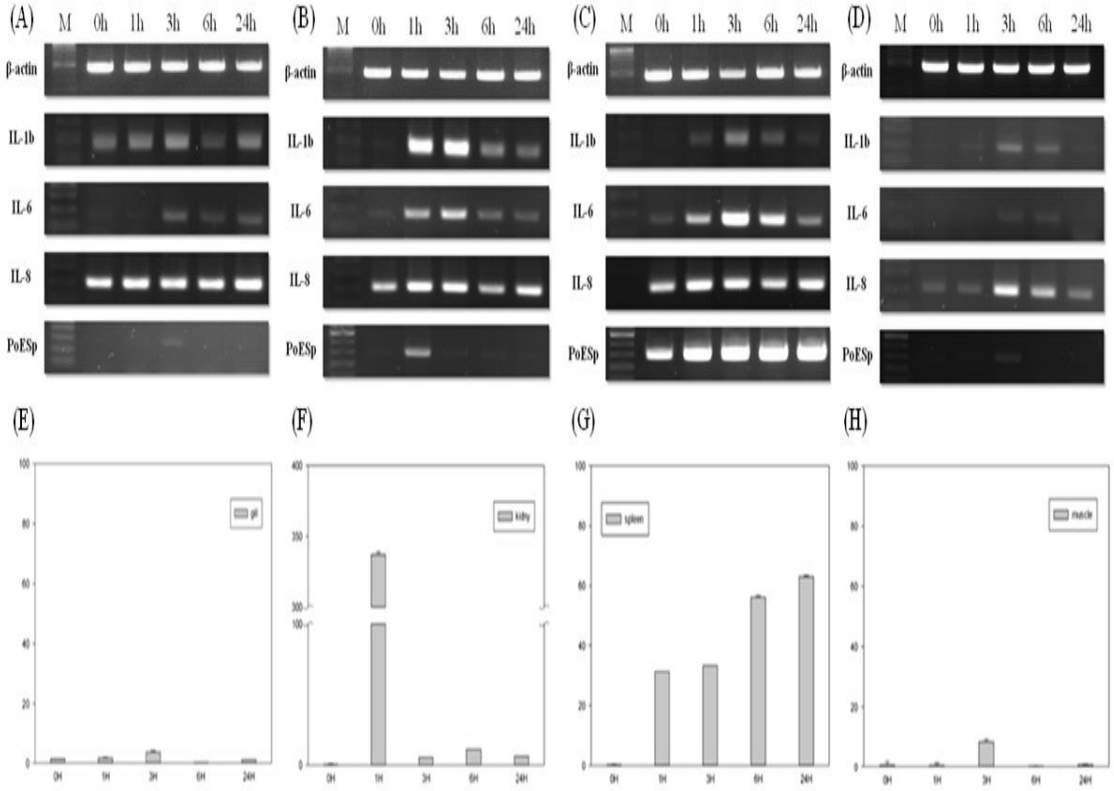
2. Tissue Distribution and expression studies of PoElSp mRNA

To examine the tissue type-specific expression of *PoElSp* mRNA transcripts in olive flounder tissues. RT-PCR was conducted with *PoElSp*-specific primers <Table 1>. Relative *PoElSp* gene expression level was normalized using olive flounder β-actin and 18S rRNA genes. As is shown in [Fig. 4], *PoElSp* mRNA was observed in liver tissue with a high expression level. Also, *PoElSp* mRNA exists in spleen and intestine tissues. Humans have six elastase genes which encode the structurally similar proteins elastase 1, 2, 2A, 2B, 3A, and 3B. Unlike other elastases, elastase 3 has little elastolytic activity, and it has a digestive function in the intestine. Also, elastase may also function in the intestinal transport and metabolism of cholesterol (Tani et al., 1988; Shimada et al., 2002). This result suggests that *PoElSp* may be somewhat linked to digestive function in the intestine. To study the functional characteristics of PoElSp, we compared the changes in the mRNA expression levels in the kidney, spleen, muscle, and gill tissues after in vivo stimulation (up to 0,1, 3, 6 and 24 h) with lipopolysaccharide (LPS) using RT-PCR and real time PCR. The interleukin-1 beta (IL-1β), IL-6 and IL-8 were chosen as the indicator genes, since



[Fig. 4] Tissue distribution of the Pro-form elastase-like serine protease mRNA. (A) Total RNA was isolated from various *Paralichthys olivaceus,* and 0.5μg was subjected to RT-PCR analysis using the amplimers for the *P. olivaceus* Pro-form elastase-like serine protease, β-actin, and 18s rRNA. (B) Quantitative real-time PCR of proPoElSp.

these genes can be induced by LPS, and are important cytokines and good markers of inflammatory responses (Corripio-Miyar et al., 2007; Zhang et al., 2008). After stimulation with LPS, the levels of IL-1β, IL-6, and IL-8 transcripts varied according to the time post-injection and increased significantly in these tissues 1–6 h post-injection (HPI), whereas the β-actin transcripts were expressed at a steady-state level at all times post-injection. The level of PoElSp mRNA was strongly increased expression after 1 HPI in the kidney, 1-24 HPI in the spleen, and 3 HPI in the muscle tissues [Fig. 5]. Therefore, our results showed that PoElSp may be involved in immune defense and responses of olive flounder against bacterial infection (Sonawane et al., 2006).

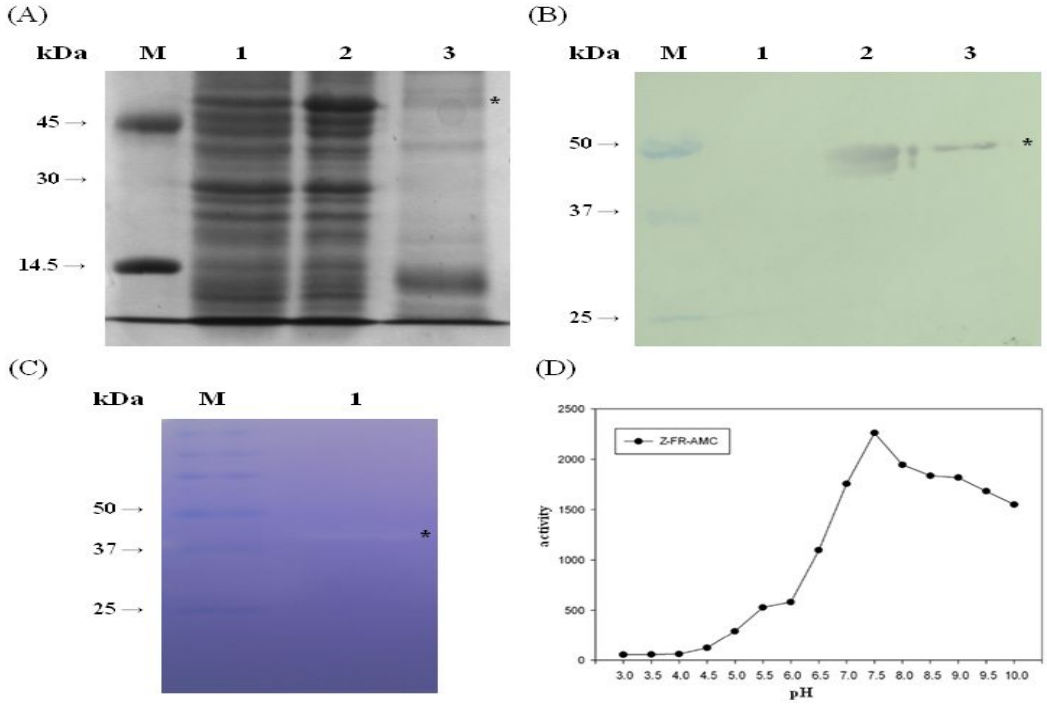


[Fig. 5] Tissue distribution of proPoElSp gene after LPS injection. RT-PCR and real-time PCR analysis after LPS injection between 0, 1, 3, 6, and 24 h from gill (A and E), kidney (B and F), spleen (C and G), and muscle tissues (D and H). Mean mRNA levels in olive flounder tissues were analyzed by realtime PCR, and 2-△△Ct levels were calculated relative to the tissue with 0h set to 1, and normalized against β-actin expression. Each experiment was done in triplicate.

3. Enzymatic Characterization of Recombinant proPoElSp

In order to assess the functional and enzymatic characteristics of PoElSp, the cDNA encoding for pro form of PoElSp was expressed in *E. coli* as a fusion protein with His-tag. The recombinant proPoElSp /pET32a was overexpressed in *E. coli* BL21(DE3) as a 47 kDa fusion protein. The

overproduced soluble His-tag fusion protein (proPoElSp) was then applied to His bind column chromatography. The proPoElSp protein band had a high purity and the correct size by SDS-PAGE and Western blot analysis [Fig. 6]. The purified proPoElSp activity was quantified by measuring the cleavage of a synthetic fluorogenic peptide substrate, Z-FR-AMC, and proteolytic activity of recombinant proPoElSp was determined by gelatin zymography. As can be seen in (Fig. 6), the purified proPoElSp was capable of hydrolyzing 0.1% gelatin, and the recombinant proPoElSp displayed activity over a wide range of pH (pH 7.0 to 10) with optimal activity occurring at pH 7.5.



[Fig. 6] Analysis of enzymatic characterization of recombinant proPoPlSp. (A) Coomassie blue staining after SDS-PAGE. The lanes were labeled as follows: lane M, standard size marker; lane 1, non-induced proPoElSp; lane 2, IPTG-induced proPoElSp (37℃); 3. His tag affinity column purified proPoElSp. (B) Western blot analysis. Lane M, prestained protein size marker; lane 1, non-induced PoElSp (negative control); lane 2, IPTG-induced PoElSp (37℃); lane 3, His tag affinity column purified PoElSp. (C) Gelatin zymography of purified proPoElSp. Lane M, prestained protein size marker; lane 1, purified PoElSp protein. (D) pH dependence of proPoElSp. Proteolytic activity was assayed against Z-Phe-Arg-AMC. The points show the average values of 3 independent experiments.

We also compared proPoElSp activity on various substrates conjugated with AMC as the fluorescent chromophore <Table 3>. The highest levels of AMC release activity were seen from Z-FR-AMC, Z-RR-AMC, and Z-GPR-AMC.

To identify the function of proPoElSp as a serine protease, the effects of enzyme inhibitors on protease activity were studied using Z-Phe-Arg-AMC

<Table 3> Substrate specificity of proPoElSp protein

Substrates	Concentration (μM)	Activity (%)
Z-Phe-Arg-AMC (FR)	50	100.00 ± 0.52
Z-Arg-Arg-AMC (RR)	50	90.55 ± 0.45
Z-Gly-Pro-Arg-AMC (GPR)	50	54.26 ± 0.36
Ala-Ala-Phe-AMC (AAF)	50	9.67 ± 0.18
Z-Arg-Gly-Phe-Pro-Pro (RGFPP)	50	1.95 ± 0.34
Suc-Leu-Leu-Val-Tyr-AMC (LLVY)	50	1.59 ± 0.67

as fluorogenic substrate. The following known proteinase inhibitors were tested: trans-Epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), antipain, and leupeptin as cysteine protease inhibitors, N-ethylmaleimide (NEM, phenylmethylsulphonyl fluoride (PMSF), chymostatin, and aprotinin as serine protease inhibitors, ethylene diamine tetraacetic acid (EDTA), ethylene glycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 1,10-phenanthroline as metalloproteinase inhibitors, and pepstatin A as aspartic protease inhibitor. The enzymatic activity of proPoElSp was significantly reduced or blocked by antipain, NEM and PMSF while the other cysteine, aspartic, and metallo-protease inhibitors had low influence on proPoElSp activity [Table 4]. Additionally, many metal ions (at 1 mM and 5 mM) and detergents (at 0.01% and 0.05%) have been found to have no influence on the activity of proPoElSp, but CoCl2 led to significant induction in protease activity [Table 5].

<Table 4> Effects of various protease inhibitors on the enzymatic activity of the proPoElSp protein. The purified proPoElSp protein was pre-incubated with the indicated inhibitors and assayed for residual activity using Z-Phe-Arg-AMC as the fluorogenic substrate

Inhibitors	Specificity	Concentration (mM)	Inhibition (%)
Control	-	-	0
E-64	All cysteine proteinases	0.1	33.8 ± 3.38
Antipain	Serine/cysteine proteinases	0.1	66.8 ± 9.54
Chymostatin	Chemotrypsin	0.1	13.5 ± 2.33
Leupeptin	Cysteine/trypsin-like Serine proteinases	0.1	32.8 ± 0.67
NEM	Serine proteinases	0.1	41.3 ± 4.17
PMSF	Serine proteinases	0.1	40.1 ± 0.53
Aprotinin	Serine proteinases	0.1	24.6 ± 1.44
EDTA	Metallo proteinases	0.1	23 ± 2.01
EGTA	Metallo proteinases	0.1	25.8 ± 0.89
1,10-phenanthroline	Metallo proteinases	0.1	37.8 ± 0.212
Pepstatin A	Aspartic proteinases	0.1	0 ± 2.89

Here, we report the molecular cloning, sequence analysis, tissue-specific distribution, and enzymatic characterization of a recombinant protein from olive flounder (*P. olivaceus*) elastase-like serine protease

(*PoElSp*). To the best of our knowledge, this is the first report on the molecular characteristics and heterologous expression of elastase 3-like serine protease from fish. The results for the tissue-specific expression and enzymatic activity of PoElSp indicate that may explain their importance in digestive function in intestine, and immune defense and responses in the kidney and spleen against bacterial infection.

<Table 5> Effect of metal ions and various detergents on activity of proPoElSp protein

Agent	Relative activity (%)	
	1mM	5mM
Control	100.00	100.00
CoCl2	254.97 ± 14.70	218.60 ± 12.40
CaCl2	114.36 ± 0.55	78.99 ± 2.37
MgSO4	104.13 ± 2.37	91.23 ± 0.57
KCl	94.19 ± 9.48	84.19 ± 4.35
ZnSO4	91.84 ± 2.84	82.23 ± 5.53
CuSO4	80.17 ± 2.29	53.74 ± 2.53
HgCl2	66.48 ± 8.85	65.08 ± 0.08
	0.01%	0.05%
SDS	134.53 ± 14.22	94.25 ± 0.97
Brij-35	96.31 ± 2.37	70.95 ± 1.83
TritonX-100	92.63 ± 5.69	69.11 ± 2.66
Tween20	86.54 ± 13.83	74.92 ± 2.00

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