Thesis for the Degree of Master of Engineering

Molecular cloning, expression and characterization of serine protease from the olive flounder (*Paralichthys olivaceus*)

by

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The Graduate School

Pukyong National University

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(Paralichthys olivaceus)

넙치로부터의 **serine protease** 의

분자생물학적 클로닝, 발현, 특성분석

Advisor : Prof. Hyung Ho Lee

by

Jin woo Han

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한 진 욱

부 경 대 학 교 대 학 원 생 물 공 학 과

요 약

일반적으로 serine proteases 의 촉매활성은 활성부위에 존재하는 3 개의 아미노산(Ser, His and Asp)나타나게 되며 진핵생물과 원핵생물에서 발견되어진다. Serine proteases 는 구조를 기준으로 chymotrypsin-like 와 subtilisin-like 로 구별할수 있다. Chymotrypsin-like serine proteases 의 경우 두개의 beta-barrel domains 을 가지고 있으며, 기질에 따라서 trypsin-like, chymotrypsin-like, elastase-like 로 나눠진다. Serine protease 는 척추와 무척추 동물에서 세포의 생리적,병리적 신호를 증폭시키는 역활을 한다고 알려져 있다. 포유동물에서는 혈전용해와 미생물감염과 관계가 있는 것으로 보고되고있다.

본 실험에서는 넙치에서 elastase-like Serine protease 를 클로닝하였다.넙치로부터 동정된 serine proteases 는 269 개의 아미노산을 암호화하는 807bp 의 open reading frame 을 가진다.

RT-PCR 을 수행한 결과, 넙치의 간에서 발현되는 것을 확인할 수 있었다.

promature 형태의 유전자를 대장균에서 과발현시켰다. 재조합 단백질은 pH7.5 및 40℃의 배양환경에서 최고 활성을 나타냈으며, 기질은 Z-Phe-Arg-AMC 을 이용하여 측정하였다.

1. Introduction

Proteases are the largest and one of more important group. Proteases are currently classified into four major classes : serine proteases, cysteine proteases, aspartate proteases, and metalloproteases. Proteases are involved in numerous important physiological processes including protein turnover, digestion, blood coagulation and wound healing, fertilization, cell differentiation and growth, cell signaling, the immune response, and apoptosis.(Barrett et al.,1998, James 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 transient acyl-enzyme intermediate between the substrate and the protease.(Perona et al.,1995, Piao et al.,2007, Kraut et al.,1977)

Serine proteases fall into two broad categories based on their structure: chymotrypsin-like or subtilisin-like. Chymotrypsin-like serine proteases are characterised by a distinctive structure, consisting of two beta-barrel domains that converge at the catalytic active site. These enzymes can be

further categorised based on their substrate specificity as either trypsin-like, chymotrypsin-like or elastase-like.(Ovaere et al.,2009)

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, and the complement system also employs similar protease cascades in response to microbial infection .(O'Brien et al., 1993)

Elastase-like proteases have a much smaller S1 cleft than either trypsin- or chymotrypsin-like proteases. Consequently, residues such as alanine, glycine and valine tend to be preferred.

However olive flounder (*Paralichthys olivaceus*) elastase-like serine protease has not been completely characterized with regard to its enzymatic properties and physiological functions. In this study, we described the cDNA cloning, tissue-typic expression, and enzymatic characterization of the recombinant elastase-like Serine protease protein in the olive flounder.

2. Materials and methods

2.1. cDNA synthesis from olive flounder 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) and then used as the template for amplification. 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-ESp F, Table 1; antisense primer, pro-ESp R, Table 1) were utilized in the amplification of cDNAs from a olive flounder cDNA mixture. Rapid amplification of cDNA ends (RACE) was used to clon cDNA containing 5'- and 3'-end of elastase-like serine protease from a cDNA mixture which was prepared from total RNA of olive flounder using a SMART™ RACE cDNA amplification kit (Clontech). The 5'-end of the elastase-like Serine protease was obtained by 5' RACE-PCR using the specific primer PoESp-GSP-R1 with Universal Primer Mix (UPM) for first round-PCR and the second specific primer PoESp -GSP-R2 with Nested Universal Primer (NUP) for second nested PCR, respectively. The two

primer sets used for 3' RACE-PCR were PoESp -3'F1 with UPM and PoESp -3'F2 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* DH5α 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 a T7 promoter/ SP6 primers in the SolGent co, Ltd.

2.2. Sequence and phylogenetic analysis

Nucleotide and predicted amino acid sequences 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 *PoESp* 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 protein sequences with the vertebrate orthologues. Multiple sequence alignments were constructed using CLUSTAL W version 1.9 (Thompson et al., 1994) and adjusted with the BioEdit Sequence Alignment Editor. The phylogenetic tree was constructed using the Neighbor-Joining Method and plotted with MEGA version 4.1 (Kumar et al., 2008).

2.3. LPS-injected espression study by a quantitative method

In order to visualize the patterns of the PoESp, interleukin-1β In an effort to assess the tissue expression of PoESp mRNA, quantitative real-time PCR was conducted using brain, eye, gill, heart, gullet, liver, spleen, pyloric ceca, intestine, kidney and muscle 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 were PoESp-RT-For and

PoESp-RT-Rev (Table 1). P. olivaceus β actin was utilized as the internal control (Po-βactin-real-F and Po-βactin-real-R for the flounder βactin,Table 1). (IL-1β, AB070835), IL-6 (DQ267937) and IL-8 (AF216646) expressions in the Brain, Gill, Spleen, Muscle and Kidney tissue tissues, expression of these genes were assessed following stimulation with lipopolysaccharide (sigma). It was intraperitoneally injection fot each animal with 500 ul LPS (500ug/ml). Three fish (average mass: 50 g) were sacrificed after 1, 3, 6 and 24 h post-injection. Total RNA isolation, reverse transcription, PCR reaction, and direct DNA sequencing were performed as described above. B-actin was utilized as the internal control for the housekeeping gene, and the 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 were utilized as internal controls for inducible gene expression (Table 1).

2.4 Expression studies by RT-PCR & Quantitative real-time PCR.

The specific primers for olive flounder elastase-like serine protease. olive flounder β-actin were utilized as the internal controls (Pobactin-F and Po-bactin-R, for the olive flounder). All of the PCR was run as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 58 °C for 20 s and 72 °C 30 s, and a

final 7 min of elongation at 72 ℃. 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)

Quantitative PCR was conducted using SYBR 480 Real-Time PCR (Roche) with SYBR Green (Roche applied science). βactin was utilized as the internal control for the housekeeping gene in the analysis of the tissue samples (Table 1). Each reaction contained the following: 10 μL of the Power SYBR® Green PCR Master Mix (Roche applied science), 0.2 μMprimer sets, 0.5 μL cDNA (300 ng), and water to a final volume of 20 μL. The PCR parameters were as follows: initial denaturation at 95 ℃ 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 ℃, 10 s at 60 ℃, and 10 s at 72 ℃ . All of the data were gathered from triplicate experiments and were expressed as fluorescence relative to βactin. The relative fold change in gene expression as compared to the controls was determined by the 2−ΔΔCt method as described previously (Giulietti et al., 2001).

2.5. Expression and purification of recombinant pro-PoESp in *E. coli*

To prepare an expression vector suitable for production of recombinant olive flounder elastase-like serine protease in *E. coli*, a 759bp DNA fragment containing the coding sequence for the *Paralichthys olivaceus* pro elastase-like Serine protease (*pro-PoESp*) was generated by PCR amplification.The primers (BamHI-pro-PoESp-F, 5'-GCGAGGATCCCTGCGGCACCCCATCCATTGA-3'; HindIII-pro-PoESp -R,5'-GCGAAAGCTTGTTGTTCATCATAACCATGT-3') harbor BamH/ HidIII restriction sites (underlined), allowing for the cloning of the amplified DNA in a predicted orientation into pET32a (Novagen). Recombinant plasmid (pro-*PoESp/*pET32a) was transformed into *E. coli* strain BL21(DE3). Transformed cells were grown in LB broth (100 ml) 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. Cells were collected by centrifugation, washed, and resuspended in 0.2 volumes of phosphate buffered saline, lysed by using a Sonication cell and centrifuged at 20,000 × *g* for 20 min at 4°C. Expression of pro-*PoELp/*pET32a gene in *E.coli* strain *BL21(DE3)* resulted in a recombinant protein of approximately 47kDa.

Purified pro-PoELp protein was used for SDS-PAGE, western blotting and enzyme activity assay.

2.6. SDS-PAGE, western blotting and zymography

Purified pro-PoELp 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 10% SDS-PAGE (Bio-Rad). Stained molecular weight markers (GE) were run as standards on each gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. Western blotting was performed using mouse monoclonal anti-His-tagged antibody (1: 2000, Santa Cruz Biotechnology). The substrate zymography was performed by a modified procedure using gels with gelatin (Sigma) as described earlier (Heussen and Dowdle, 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) without reducing the agent and without boiling. 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 1 h 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 18 hr 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.

2.7. Enzyme activity assays

The Serine proteases activity was assayed according to the modified method of Barret and Kirschke (1981). The optimum pH for enzymatic activity 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 of recombinant PoESp enzyme in 85 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 ㎕ 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-Gly-Pro-Arg-AMC (Sigma), Ala-

Ala-Phe-AMC (Sigma), Z-Phe-Arg-Glu-AMC (Sigma), Z-Arg-Arg-AMC, Suc-Leu-Leu-Val-Tyr-AMC and Z-Arg-Ala-Phe-AMC with 0.1 M Tris/pH 8.0, containing 1 mM DTT, respectively. Substrates were added to a final concentration of 100 μM.

2.8. 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-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma) and 1,10-Phenanthroline (Sigma) for metalloproteinase inhibitor; Pepstatin A (Sigma) for aspartic protease inhibitor. The effect of various metal ions (1 and 5 mM) on enzyme activity was investigated using $ZnSO_4$, $CuSO_4$, $CoCl_2$, KCl, $MgSO_4$, $CaCl_2$ and $HgCl_2$. The effects of some surfactants (Brij 35, Triton X-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 pro-*PoESp* protease were studied at pH 7.5 and 37°C. All the experiments were replicated three times. 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.

3. Results & Discussion

3.1 Cloning and sequence analysis of A. pectinifera cathepsin L cDNA

Full-length of *Paralichthys olivaceus* elastase-like serine protease (*PoESp*) cDNA was obtained by combining DNA sequences of cDNA library clones and 5' SMART™ RACE cDNA Amplification PCR product. The full-length 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 *PoESp* was predicted to encode for a preproprotein of 269 amino acids, which contained a 16 residue putative signal peptide analyzed with signalIP (Martoglio and Dobberstein, 1998) (Fig. 1).

3.2. Tissue distribution of PoESp

Distribution of *PoESp* mRNA transcripts in different organs was examined by RT-PCR using *PoESp* -specific primers (Table 1). As shown in Fig. 4 expression of *PoESp* was observed in all of the tissues. The expression pattern of *PoESp* was revealed in high levels in the liver.

3.3. Enzymatic characterization of recombinant Pro-PoESp

In order to assess the functional and enzymatic characteristics of PoESp the cDNA encoding for Pro-*PoESp* was expressed in *E. coli* as a fusion protein with His-tag. The recombinant Pro-PoESp /pET32*a* was overexpressed in *E. coli* BL21(DE3) as a 47 kDa fusion protein. The overproduced soluble HIS-tag fusion protein (Pro-PoESp) was then applied to His bind column chromatography, and the Pro-PoESp fusion protein band had a high purity and the correct size by SDS-PAGE and Western blot analysis (Fig. 6).

The purified Pro-PoESp activity was quantified by measuring the cleavage of a synthetic fluorogenic peptide substrate, Z-FR-AMC

As can be seen in Fig. 7 the Pro-PoESp protein was demonstrated to evidence a high level of activity at 40℃. Also proteolytic activity of recombinant Pro-PoESp was determined by gelatin zymography. The purified Pro-PoESp was capable of hydrolyzing 0.1% gelatin at pH 7.5 (Fig. 9). Interestingly, using the synthetic substrate, Z-FR-AMC the recombinant Pro-*PoESp* displayed activity over a wide range of pH (pH 7.0to 10) with optimal activity occurring at pH 7.5 (Fig. 8).

We also compared Pro-PoESp activity on various substrates conjugated

with aminomethylcoumarin as the fluorescent chromophore (Table 2). The highest levels of AMC release activity were seen from Z-FR-AMC, Z-RR-AMC, and Z-GPR-AMC.

Table 1

Oligonucleotide primers used for *PoESp* amplification and expression studies.

Primer name		5`-3` sequence	Information
Universal Primer A Mix (UPM)	UP-Long	CTAATACGACTCACTATAGGGCAAGCAGTGG TATC	Universal primers for 5' and 3' RACE
	UP-Short	AACGCAGAGTCTAATACGACTCACTATAGGGC	
Nested Universal Primer A (NUP)		AAGCAGTGGTATCAACGCAGAGT	
pro-ESp F		CAAGCTCTCCTACAGGGTGT	Universal primers for 5' and 3' RACE
pro-ESp R		CATGATCAGCCACAGGCATC	
PoESp -GSP-R1		CCATCCGGTGATGTAGCAGG	
PoESp -GSP-R2		GAGCAATGTCGTTTCCGAGG	
PoESp -3'F1		CAGCTGCACACTGCATCAAC	
PoESp -3'F2		GGTTACTCAAAGTCACAGGC	
Po-18s rRNA-real-F		GTTGGTGGAGCGATTTGTCTGG	Primers for expressioin studies
Po-18s rRNA-real-R		CATCTAAGGGCATCACAGACCTG	
Po-bactin-real-F		FGACATGGAGAAGATCTGGCA	
Po-bactin-real-R		ATGTCCTGCTCGAAGTCCAG	
PoESp-RT-For		CATGAATTCCCGTGTGGTCA	
PoESp -RT-Rev		CACGAAGATGGAGTTCCATTTCTC	

PoIL1β-F	GGTGCTACCAGACCTTCAACATCCAG	
PoIL1β-R	CAAAGTCTTTCCAGCAGACAGTGGTG	
PoIL6-F	CAGCACTTTCCACAGGAAGATGACG	
PoIL6-R	AGAGGGATGGATGGGTGGAATAATTC	
PoIL8-F	GGGTCAGAAGCCGTTTAAAGACAACTC	
PoIL8-R	GTTAGTTCCCTTCAAACAAGCACAGGC	
Ser-start-Bam	GCGAGGATCCCATGATCCCCATTGTGCTGGC	Primers for Recombinant protein
Ser-pro-Bam	GCGAGGATCCCTGCGGCACCCCATCCATTGA	
Ser-start-Sac	GCGAGAGCTCATGATCCCCATTGTGCTGGC	
Ser-start-Sac	GCGAGAGCTCTGCGGCACCCCATCCATTGA	

Table 2

Substrate specificity of pro-PoESp

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
Z-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

Table 3

Effect of various protease inhibitors on the enzymatic activity of the pro-PoESp. The purified pro-PoESp was pre-incubated with the indicated inhibitors and assayed for residual activity using Z-Phe-Arg-AMC as the fluorogenic substrate.

Inhibitiors	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

Table 4

Effect of metal ions and various detergents on pro-PoESp activity.

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

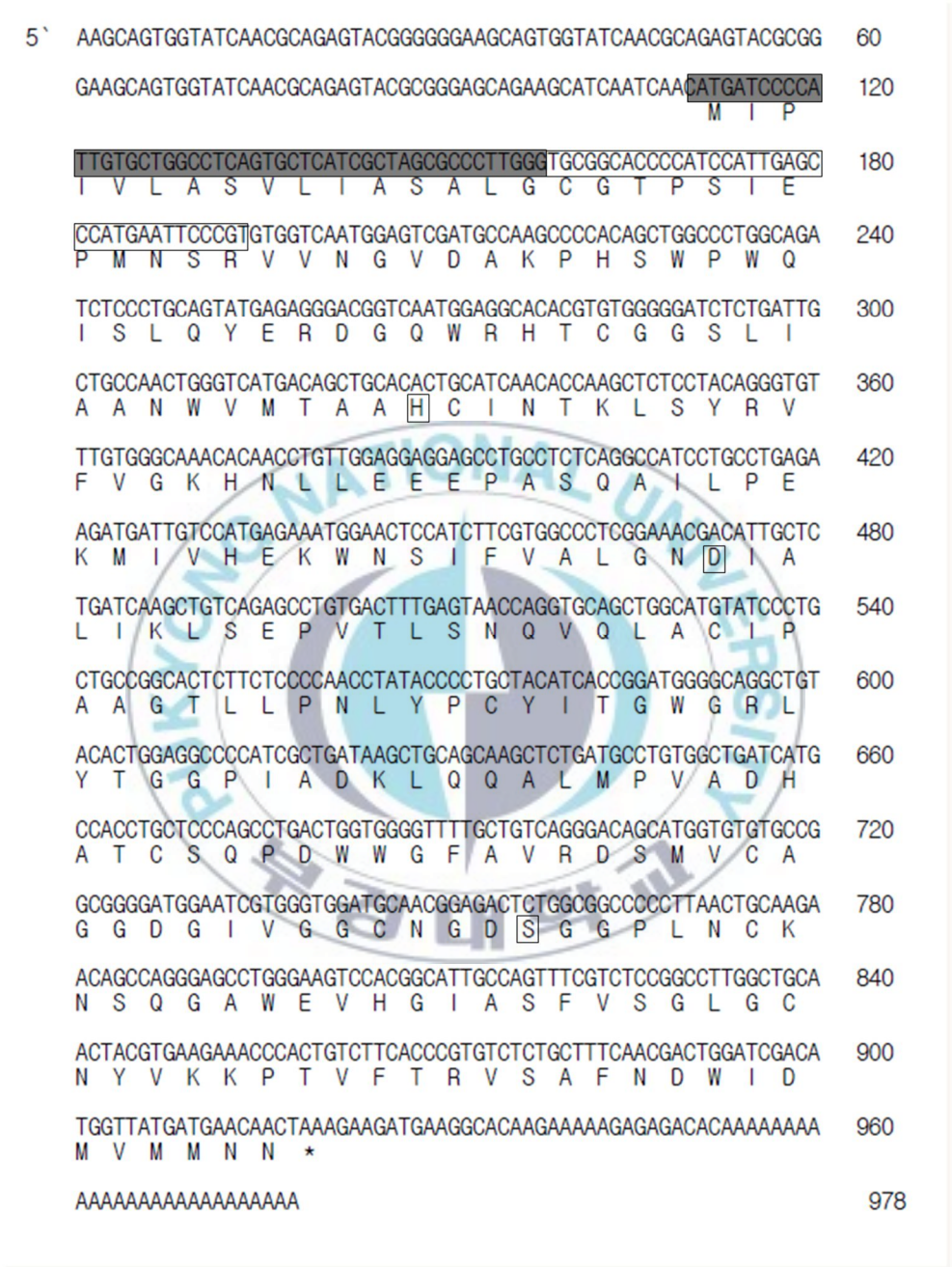


Fig. 1. Nucleotide and deduced amino acid sequence of *Paralichthys olivaceus* elastase-like serine proteases(*PoESp*).

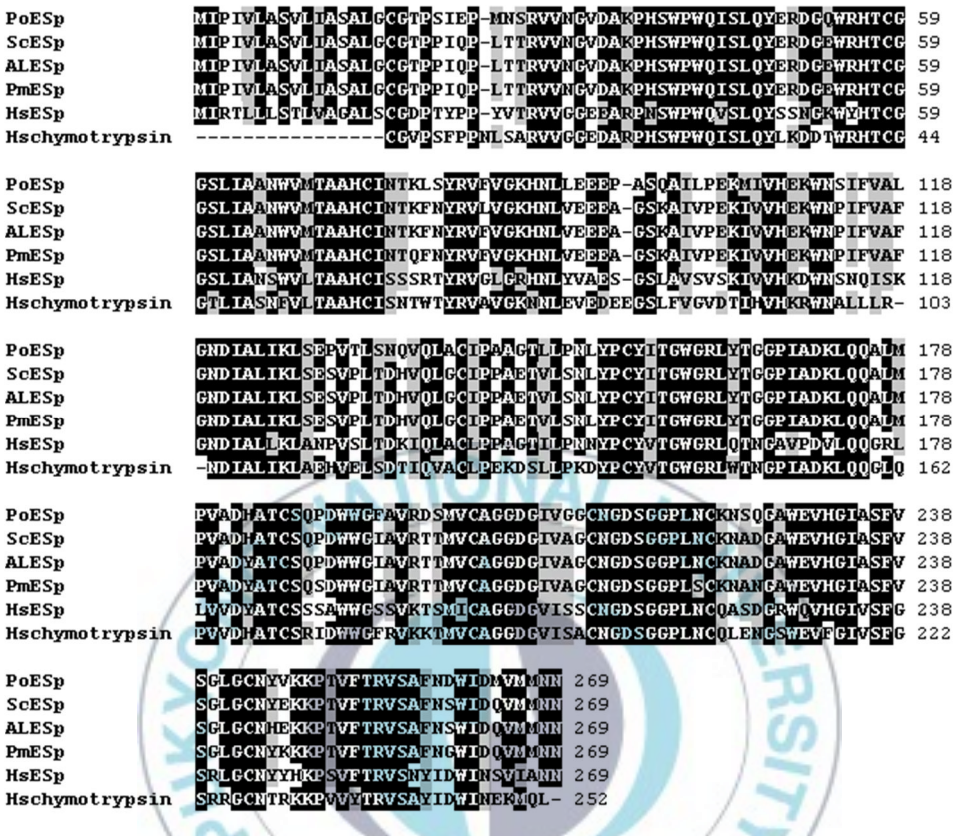


Fig. 2. Multiple amino acid sequence alignment of *Paralichthys olivaceus* elastase-like serine protease(*PoESp*) between different species of serine protease and human serine protease.

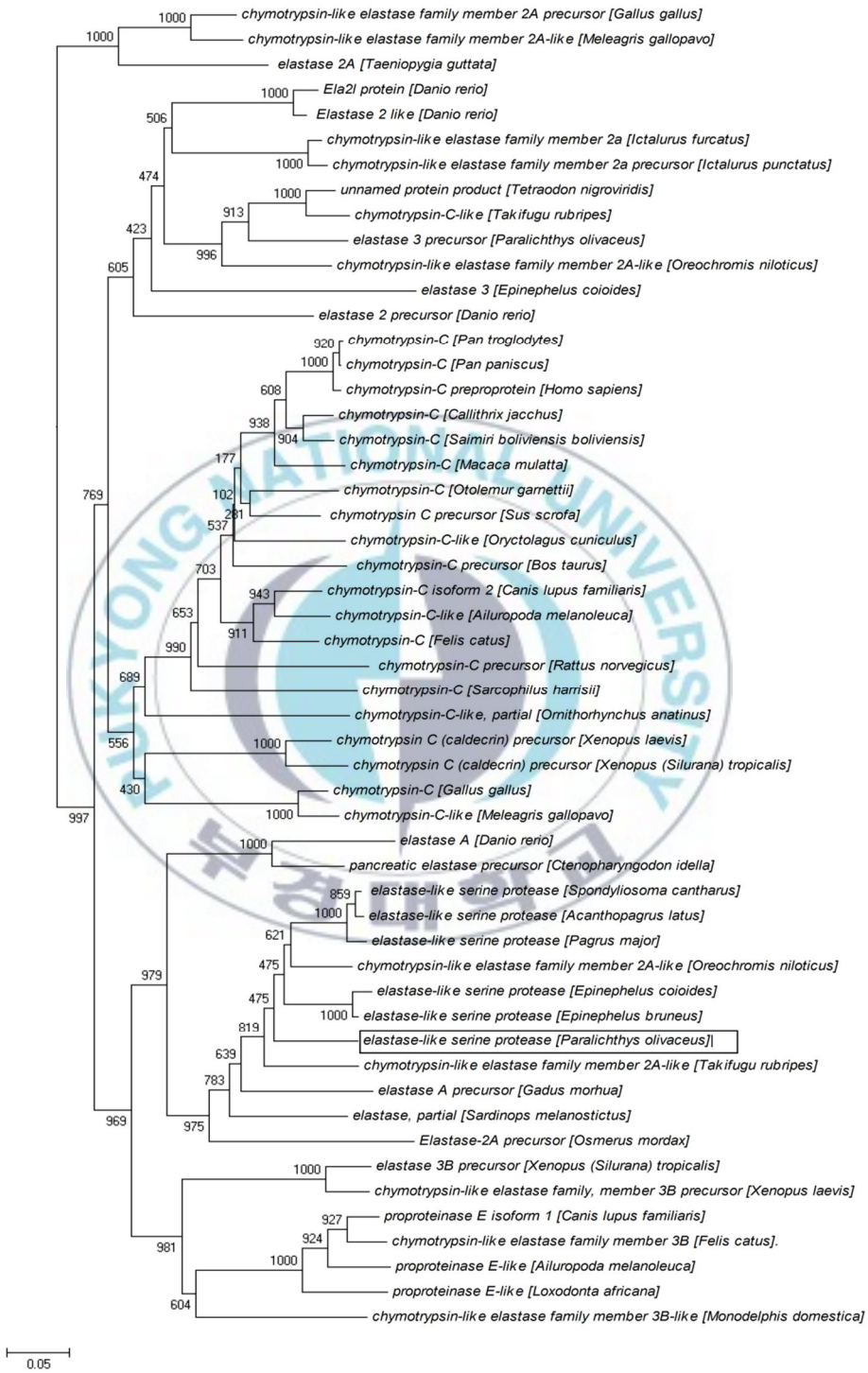

chymotrypsin-like elastase family member 2A precursor [Gallus gallus]
chymotrypsin-like elastase family member 2A-like [Meleagris gallopavo]
elastase 2A [Taeniopygia guttata]
Ela2l protein [Danio rerio]
Elastase 2 like [Danio rerio]
chymotrypsin-like elastase family member 2a [Ictalurus furcatus]
chymotrypsin-like elastase family member 2a precursor [Ictalurus punctatus]
unnamed protein product [Tetraodon nigroviridis]
chymotrypsin-C-like [Takifugu rubripes]
elastase 3 precursor [Paralichthys olivaceus]
chymotrypsin-like elastase family member 2A-like [Oreochromis niloticus]
elastase 3 [Epinephelus coioides]
elastase 2 precursor [Danio rerio]
chymotrypsin-C [Pan troglodytes]
chymotrypsin-C [Pan paniscus]
chymotrypsin-C preproprotein [Homo sapiens]
chymotrypsin-C [Callithrix jacchus]
chymotrypsin-C [Saimiri boliviensis boliviensis]
chymotrypsin-C [Macaca mulatta]
chymotrypsin-C [Otolemur garnettii]
chymotrypsin C precursor [Sus scrofa]
chymotrypsin-C-like [Oryctolagus cuniculus]
chymotrypsin-C precursor [Bos taurus]
chymotrypsin-C isoform 2 [Canis lupus familiaris]
chymotrypsin-C-like [Ailuropoda melanoleuca]
chymotrypsin-C [Felis catus]
chymotrypsin-C precursor [Rattus norvegicus]
chymotrypsin-C [Sarcophilus harrisii]
chymotrypsin-C-like, partial [Ornithorhynchus anatinus]
chymotrypsin C (caldecrin) precursor [Xenopus laevis]
chymotrypsin C (caldecrin) precursor [Xenopus (Silurana) tropicalis]
chymotrypsin-C [Gallus gallus]
chymotrypsin-C-like [Meleagris gallopavo]
elastase A [Danio rerio]
pancreatic elastase precursor [Ctenopharyngodon idella]
elastase-like serine protease [Spondyliosoma cantharus]
elastase-like serine protease [Acanthopagrus latus]
elastase-like serine protease [Pagrus major]
chymotrypsin-like elastase family member 2A-like [Oreochromis niloticus]
elastase-like serine protease [Epinephelus coioides]
elastase-like serine protease [Epinephelus bruneus]
elastase-like serine protease [Paralichthys olivaceus]
chymotrypsin-like elastase family member 2A-like [Takifugu rubripes]
elastase A precursor [Gadus morhua]
elastase, partial [Sardinops melanostictus]
Elastase-2A precursor [Osmerus mordax]
elastase 3B precursor [Xenopus (Silurana) tropicalis]
chymotrypsin-like elastase family, member 3B precursor [Xenopus laevis]
proproteinase E isoform 1 [Canis lupus familiaris]
chymotrypsin-like elastase family member 3B [Felis catus].
proproteinase E-like [Ailuropoda melanoleuca]
proproteinase E-like [Loxodonta africana]
chymotrypsin-like elastase family member 3B-like [Monodelphis domestica]
0.05

Fig. 3. Phylogenetic relationships of *PoESp* 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.

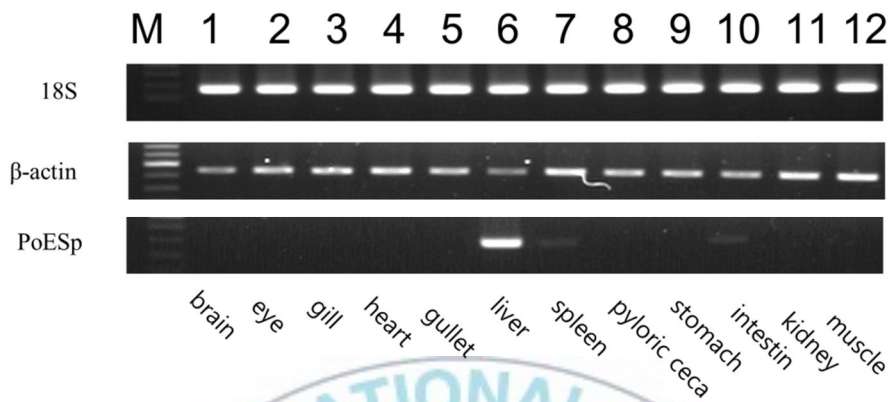
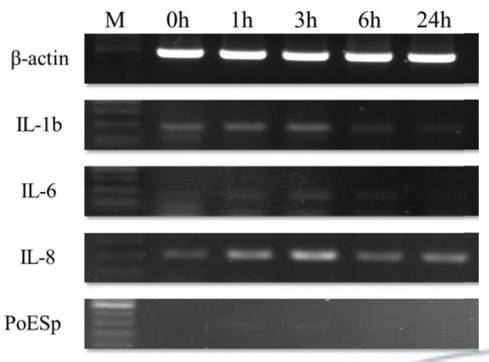
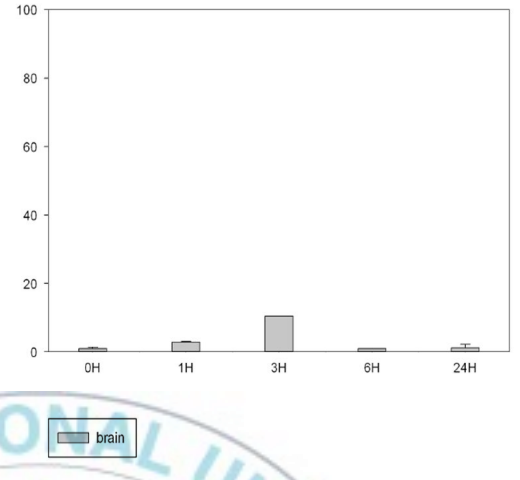


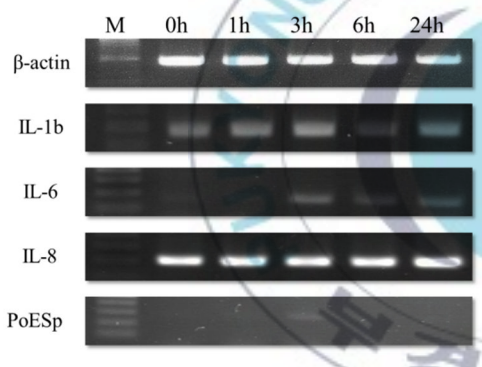
Fig. 4. Tissue distribution of the Pro-form elastase-like serine protease mRNA. Total RNA was isolated from various *Paralichthys olivaceus,* and 0.5μg was subjected to RT-PCR analysis using the amplimers for the p.olivaceus Pro-form elastase-like serine protease, β-actin, and 18sRNA.

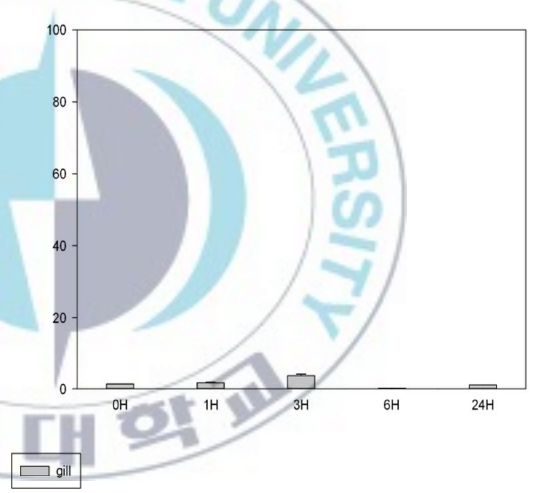
Expression pattern in the Brain



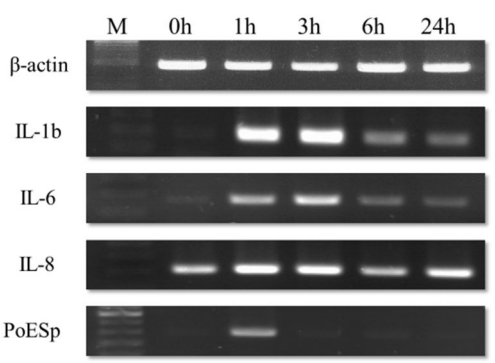


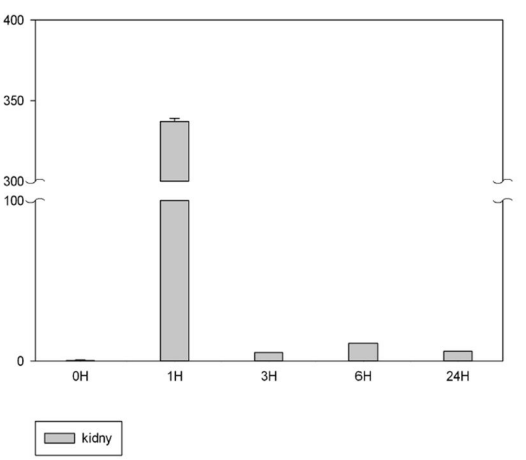
Expression pattern in the Gill





Expression pattern in the Kidney





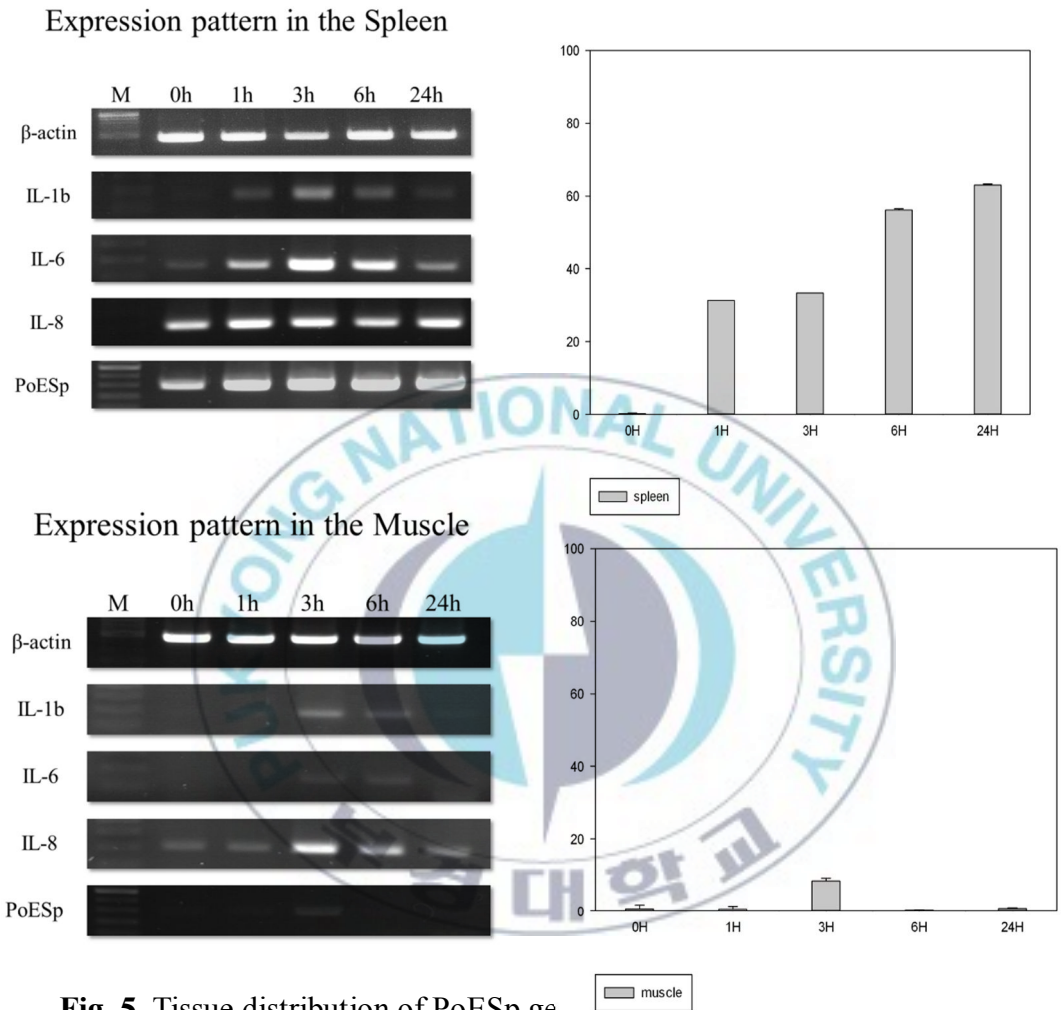
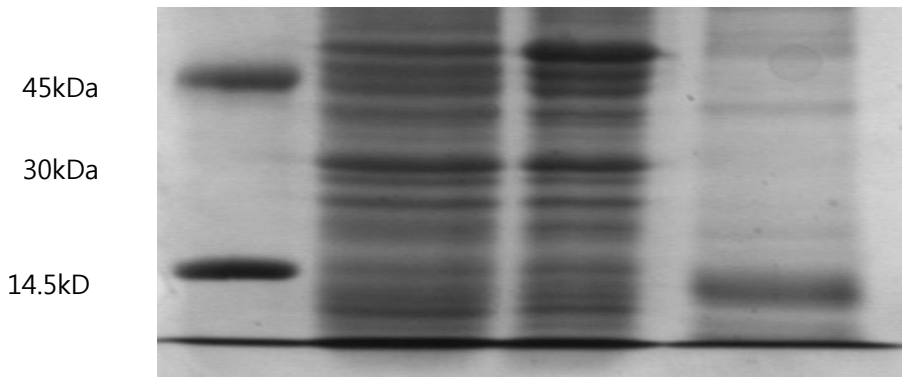


Fig. 5. Tissue distribution of PoESp ge

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, B-actin, and 18sRNA. Real-time PCR alyses after LPS injection between 0, 1, 3, 6, and 24 h from brain, kidney, spleen, muscle and gill tissues. The relative expression of pro ELSp was obtained relative to B-actin gene expression.

A



B

Fig.6. Expression of reconbinant Pro-form elastase-like serine protease. Purification of recombinant PoESp form overexpression in E.coli.

A. Coomassie blue staining after SDS-PAGE. The lanes were labeled as follows: M, standard size marker; 1, non-induced PoESp ; 2. overexpressed PoESp (37℃) ; 3. His tag affinity column purified PoESp

B. Western blot analysis. M: prestained protein size marker, lane 1: non-induced PoESp (negative control), lane 2: overexpressed PoESp (37℃) lane 3: His tag affinity column puried PoESp

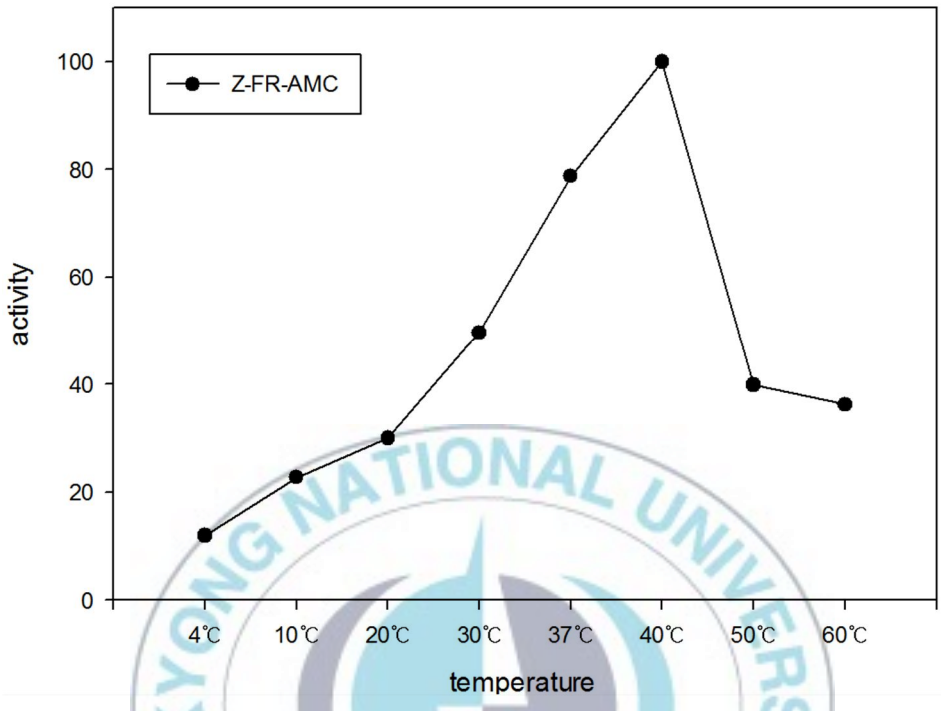


Fig. 7. temperature dependency of pro-PoESp. Proteolytic activity was assayed against Z-Phe-Arg-AMC and was expessed in terms of arbitraty fluorescence units per tine (dF/dT). The points and bars show the average values and S.D. of 3independent experiments, respectively.

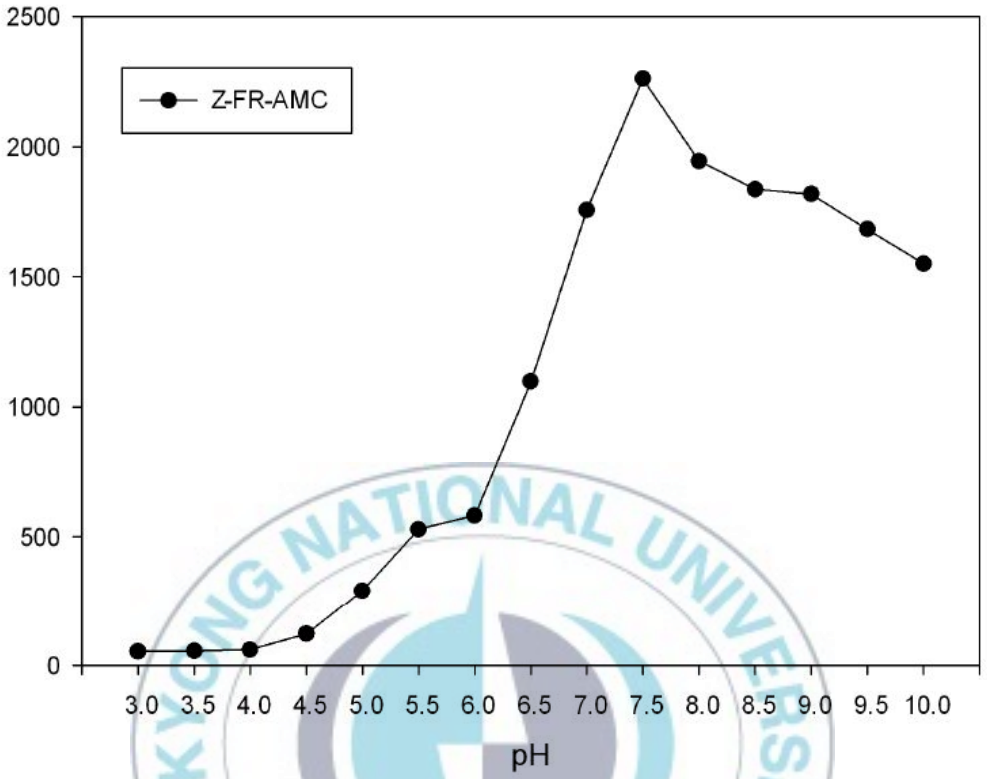


Fig. 8. pH dependency of pro-PoESp. Proteolytic activity was assayed against Z-Phe-Arg-AMC and was expessed in terms of arbitraty fluorescence units per tine (dF/dT). The points and bars show the average values and S.D. of 3independent experiments, respectively.

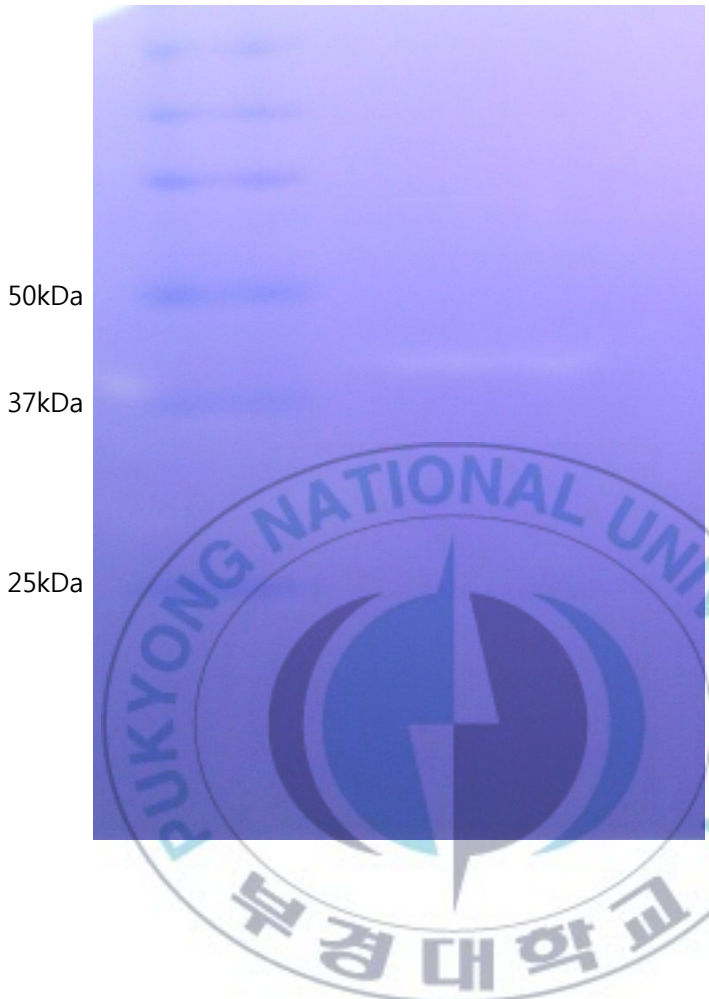


Fig. 9. Gelatin zymography of purified pro- PoESp. The purified enzyme was subjected to electro phoresis on a 10% polyacrylamide gel containing 0.1%gelatin. The gel was incubated overnight in 0.1 M tris/pH 8.0, containing 1mM DTT at 37℃. After staining with 0.1% commassie brilliant blue R-250. Areas ofproteolysis appear as clear regions within the gel. Lane 1, purified PoESp protein.

References

Alexander, C., Rietschel, E. T. (2001) Bacterial lipopolysaccharides and innate immunity. J. Endotoxin Res. 7, 167–202.

Barrett, A.J., Rawlings, N.D., Woessner, J.F. Handbook of Proteolytic Enzymes. Academic press, San Diego, CA. 1998

Dellinger, R. P., Carlet, J. M., Masur, H., Gerlach, H., Calandra, T., Cohen, J., Gea-Banacloche, J., Keh, D., Marshall, J. C., Parker, M. M., Ramsay, G., Zimmerman, J. L., Vincent, J. L., Levy, M. M. (2004) Surviving sepsis campaign guidelines for management of severe sepsis and septic shock. Intensive Care Med. 30, 536–555.

Finberg, R. W., Re, F., Popova, L., Golenbock, D. T., Kurt-Jones, E. A. (2004) Cell activation by Toll-like receptors: role of LBP and CD14. J. Endotoxin Res. 10, 413–418.

Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 25:386–401

Hall T.A., BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41(1995), 95-98.

Hurley, J. C. (2003) Endotoxemia and Gram-negative bacteremia as predictors of outcome in sepsis: a meta-analysis using ROC curves. J. Endotoxin Res. 9, 271–279.

James C. Powers, Juliana L. Asgian, ozlem Dogan Ekici, Karen Ellis James. Irreversible Inhibitors of Serine, Cysteine, and Threonine Proteases.

Chemical Reviews, 2002;102:4639-750

Kraut J. Serine protease: structure and mechanism of catalysis. Annu Rev Biochem 1977;46:331–58.

Kumar S., Tamura K., Nei M., MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5 (2004), 150-163.

O'Brien D, McVey J. Blood coagulation, inflammation, and defense. In: Sim E, editor. The natural immune systems, humoral factors. New York: IRL Press 1993;p. 257–80.

Perona JJ, Craik CS. Structural basis of substrate specificity in the serine proteases. Protein Sci 1995;4:337–60.

Piao S, Kim S, Kim JH, Park JW, Lee BL, Ha NC. Crystal structure of the serine protease domain of prophenoloxidase activating factor-I. J Biol Chem 2007;282:10783–91.

Piao S, Song YL, Kim JH, Park SY, Park JW, Lee BL, et al. Crystal structure of a clip-domain serine protease and functional roles of the clip domains. EMBO J 2005;24:4404–14.

O'Brien D, McVey J. Blood coagulation, inflammation, and defense. In: Sim E, editor. The natural immune systems, humoral factors. New York: IRL Press; 1993. p. 257–80.

Ovaere P, Lippens S, Vandenabeele P, Declercq W. The emerging roles of serine protease cascades in the epidermis. Trends Biochem Sci 2009;34(9):

453–63.

Thompson J.D., Higgins D.G., Gibson T.J., Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22 (1994), 4673-4680

Vogel, S., Hirschfeld, M. J., and Perera, P. Y. Signal integration in lipopolysaccharide (LPS)-stimulated murine macrophages. J. Endotoxin Res. 7: 237, 2001.

Zhang, F. X., Kirschning, C. J., Mancinelli, R., Xu, X. P., Jin, Y., Faure, E., Mantovani, A., Rothe, M., Muzio, M., and Arditi, M. Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. J. Biol. Chem. 274: 7611, 1999.

ABSTRACT

Molecular cloning, expression and characterization of serine protease from the olive flounder

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Abstract

We have cloned a cDNA encoding a Elastase-like serine protease (ELSp) from the olive flounder, Paralichthys olivaceus. The ELSp gene was determined to consist of the 807 bp nucleotide sequence which encodes for a 269-amino acid polypeptide. The tissue-specific pattern of ELSp was examined by RT-PCR. The results of RT-PCR analysis revealed liver, kidney and spleen the entirety of the flounder tissues, and the expression of the ELSrP gene was also examined in brain, gill, kidney, spleen, and muscle at 0,1,3,6, and 24H after induction of an artificial bacterial infection (lipopolysaccharide, LPS). The cDNA encoding enzyme of ELSp was expressed in Escherichia coli using the pET32a vector systems. The activity of ELSp was detected by gelatin zymography and cleaving synthetic fluorogenic Z-Phe-Arg-AMC, a substrate commonly used for functional characterization of serine

protease. The optimal pH for the protease activity was 7.5

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학부 때부터 졸업할때까지 속 많이 썩힌 김 무상 박사님, 논문부터 실험실 생활 까지 많은걸 가르켜 주신 안 상중 박사님과 실험실 행활하면서 큰 힘이 된김 나영 박사님에게도 감사하다는 말을 꼭 전하고 싶습니다. 또한 박 남규 교수님방의 서 정길 박사님과 고 혜진 박사님에게도 말 잘 안들어도 많은걸 가르켜 주셔서 감사하다는 말을 올립니다.

실험실 같이 들어와서 고생한 혜진아 석사생활 한다고 고생했고, 고마워. 덕분히 힘이 났다. 그리고 앞으로 고생할 진영이, 주현이 힘내고, 파이팅 있게 생활 잘 할꺼라고 믿는다. 함께 석사 생물공학과 석사 동기들과 2층 약리방의 미정이, 희영이, 혜인이 그리고 희성이형 함께 생활해서 정말 즐거웠습니다. 앞이 많이 남은 희은이, 성환이와 세환이에게도 힘들어도 재미있길 바란다라는 말을 전하고 싶습니다. 항상 마음속으로 '이런 사람들 또 어디가서 보겠나' 라는 생각을 가지고 있겠습니다.

그리고 나이 먹어도 변하지 않을 초등학교 친구들인 휘문학원애들, 니들이 있어서 힘들때 마다 견디고 항상 웃고 살수 있었다. 고마워.

마지막으로 고집 쎈 자식 때문에 마음 고생하신 아버님과 어머님에게 감사하다는 말을 전하며, 이제 졸업해서 사회생활 잘해나가겠다는 말을 올립니다.