JFMSE, 26(3), pp. 543~552, 2014.
수산해양교육연구, 제26권 제3호, 통권69호, 2014.
www.ksfme.or.kr
http://dx.doi.org/10.13000/JFMSE.2014.26.3.543

Molecular Analysis and Enzymatic Characterization of Cathepsin B from Olive Flounder (*Paralichthys olivaceus*)

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넙치 카텝신 B의 분자생물학적 분석 및 효소학적 특성 연구

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Abstract

Papain family중 하나인 cysteine protease는 근골격계 질환 치료를 위한target molecule로 인식 되어 왔으며 Cathepsin B 는 단백질 분해의 초기과정에 관여하는 cysteine proteases 중 하나이다. 본 연구는 넙치의 cathepsin B 유전자의 발현 양상과 넙치 cathepsin B(PoCtB)의 클로닝, 발현 및 효소특성을 분석하였다. cDNA Library Screening을 이용하여 넙치의 cDNA를 클로닝하였다. 넙치의 동정된 cathepsin B 유전자는 993bp의 open reading frame과 330개의 아미노산으로 이루어져있다. Cathepsin B의 propeptide region 내에 GNFD motif와 occluding loop 가 존재함으로써 이것이 명백하게 cathepsin B group이라는 것을 보여주며, 계통 유전학적 분석 결과 다른 종의 cathepsin B에 비해 초창기에 분화되어 나온 것으로 사료된다. mature enzyme인 *maPoCtB*은 fusion protein인 glutathione S-transferase를 포함하는 pGEX-4T-1 vector에 삽입하여 *E.coli* 균주인 DH5α 내에 발현시켰다. 재조합 단백질인 *PoCtB*을 과발현 시킨 결과 53kDa의 분자량을 가진다. 넙치 cathepsin B 활성은 Z-Arg-Arg-AMC와 같은 fluorogenic 펩타이드 기질을 이용하여 측정되었고 적정 pH는 pH.7.5 이다.

Key words : Molecular analysis, Cathepsin B, Olive flounder, *Paralichtys olivaceus*

Ⅰ. Introduction

Proteases are expressed in various cells and in tissues of numerous species of living organisms, and are classified into large groups including the cysteine, serine, aspartic, and metalloproteases. Cysteine proteases consist of two diverse groups of enzymes which localize mainly to digestive vacuoles or lysosomes (cathepsins) and the cytosol (calpain) (Portnoy et al., 1986; Yoshimura et al., 1984). These enzymes have a variety of biological functions such as intracellular protein degradation or the precise processing of precursor proteins. In some cases, cysteine proteases are secreted into the extracellular environment. Cathepsin B is a papain-like cysteine protease, which is one of the major components of the lysosomal proteolytic system responsible for protein degradation and turnover (Mort and Buttle, 1997 ; Fasciola et al., 1998). This protease also participates in specificcellular processes outside the lysosomes, including prohormone activation and antigen

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* This work was supported by a Research Grant of Pukyong National University (2013 Year)

processing (Mort and Buttle, 1997 ; Katunuma et al., 1998; Jutras and Reudelhuber, 1999; Berdowasa and Siewinski, 2000). Proteolytic enzymes have been suspected to play a role in the immune system of fish skin (Braun et al., 1990; Hjelmeland et al., 1983), but their characteristics and roles remain unclear (Alexander and Ingram, 1992). The existence of skin cysteine proteinases had been previously suggested in some fresh and sea water fish, and two distinct cysteine proteases identified as cathepsin B and L have been reported in the dorsal skin of *A. japonica* (Aranishi and Nakane, 1997). Subsequent reports have research about their defensive role against bacterial infection (Aranishi et al., 1998a), localization in skin epidermal cells (Aranishi et al., 1998b) and bacteriolytic ability toward fish pathogens (Aranishi, 1999). It has been speculated that cathepsins secreted by certain tumor cells play an active extracellular role in cancer metastasis and tissue remodeling (Erickson, 1989). In the previous report, we performed cloning and characterization of cathepsin B from Scuticociliate, *Uronema marinum*, which is invading olive flounder, *paralichthys olivaceus* and leading to high mortalities in cultured olive flounder (Lim et al., 2005). In the parasitic organisms, cysteine protease of cathepsin B-like, as well as cathepsin L, has a variety of biological functions, such as intracellular protein degradation, host-parasite attachment, immunoevasion, excystment/encystment or the precise processing of precursor proteins (Lecaille et al., 2002; McKerrow, 1989; Sajid and McKerrow, 2002). Cathepsin B has been reported from fish muscle (Bonete et al., 1984; Mastsumiya et al., 1989; Kolodziejska and Sikorski, 1995). Cathepsin B and L have been purified from olive flounder skin and reported that they serve as antibacterial proteinases responsible for lysis of Gram-negative pathogenic bacteria in the skin (Aranishi and Mano, 2000). However, these studies did not investigate either gene level or well characterized. Therefore, in order to design novel antiparasitic agents, purification and characterization of cathepsin B from *P. olivaceus* have been performed and the phylogenetic relationship of *P. olivaceus* cathepsin B with other known cathepsins reported.

Ⅱ. Material and Methods

1. mRNA isolation and olive flounder muscle cDNA library construction

Poly(A)$^+$ RNA was isolated from *P. olivaceus* using the PolyA Ttrack® System 1,000 (Promega, USA), according to the manufacturer's instructions. cDNA was synthesized from Poly(A)$^+$ RNA using the λ ZAP cDNA synthesis kit (Stratagene), and size fractionated into a > 0.4-kb pool. The cDNA pool was cloned into the Uni-ZAP vector containing the whole phagemid pBluescript SK sequence (Stratagene) and packed into a λ phage extract (Gigapack gold extract, Stratagene). The cDNA library was constructed with 2.75×10^6 plaque-formingunits/$\mu\ell$ (pfu).

2. cDNA cloning for the complete coding sequence of PoCtB

Degenerate primer sites <Table 1> corresponding to the conversed region of the reported cathepsin B were designed to amplify a cathepsin B in olive flounder muscle cDNA library. The first PCR was performed for 4 min at 94℃, followed by 35 cycles of denaturation for 30 s at 94℃ 30 s of annealing at 45℃ and 1min of extension at 72℃, using the Fca-For1 and Fca-Rev1. The final extension

step was performed at 72℃ for 7 min. Nested PCR were carried out as above, at an annealing temperature of 50℃, using Fca-For2 and Fca-Rev2 primer. The generated PCR products were sequenced and compared with all rep

orted sequences in GeneBank using the BLAST program. Previously constructed cDNA libraries from olive flounder muscle were screened with the positive PCR product. From the available single-pass sequences, internal antisense primer were designed (<Table 1>) These were used in combination with vector-derived primers <Table 1> to amplify the 5′ and 3′ end from the cDNA library using a PCR. To obtain 5′ truncated region, the first PCR was performed for 4 min at 94℃, 30 s of annealing at 48℃ and 1 min of extension at 72℃, using the Po5′ -1 and Reverse primer. The final extension step was performed at 72℃ for 7 min. Nested PCR was performed at 72℃ for 7 min. Nested PCR were carried out as above, at an annealing temperature of 46℃, using Po3′ -2 and T3 primer. The composition of the first and nested PCR reaction mixture was a Bioneer AccuPower PCR PreMix. The amplified products were subcloned into the pEZ-T vector (RNA), using *E. coli* strain DH5α. Finally, by combining the DNA sequences of the partial *PoCtB* and 5′ and 3′ cDNA PCR products, a full-length of the *PoCtB* cDNA sequence was yielded [Fig. 1].

3. Sequence and phylogenetic analysis

DNA and predicted protein sequences were analyzed using DNAsis for Windows, version 2.5 (Hitachi software engineering). Sequencing data were compared with the database using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Deduced protein sequence was analyzed for signal peptide with the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). Predictions of the pro-region cleavage sites, as well as the active sites, were based on alignment of the cathepsin protein sequences with the vertebrate orthologs. Multiple sequence alignments were constructed using CLUSTAL W version 1.8 (Thompson et al., 1994) and adjusted using BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999). Phylogenetic tree was constructed using the Neighbour-Joining Method and plotted with MEGA version 3.0 (Kumar et al., 2000).

<Table 1> Primers used in amplification and sequencing of the cathepsin B gene

Primer name	5'-3' sequence
CatB-F1	TGYGGHTCHTGYTGGGC
CatB-R1	AARWADCCWTTVTCDCCCCA
CatB-F2	TGYGAVCAYCAYGTBRAYGG
CatB-R2	CWRBGYYCCAGGARTTDGC
T7	TAATACGACTCACTATAGGG
T3	ATTAACCCTCACTAAAGGGA
M13(-20)	GTAAAACGACGGCCAGT
M13 R	GGAAACAGCTATGACCATG
Po5′ -1	TGCTTGTCCTGTTTGTAGC
Po5′ -2	AGAGTATCCTGCTTCACAGC
Po3′ -1	CCTTCACCGTCTATGAAGAC
Po3′ -2	GTGTCTGGATCTGTTCTGG
fish-b-actin-F	GACTTCGAGCAGGAGATGGGCAC
fish-b-actin-R	GTGATCTCCTTCTGCATCCTGTC
PoCtB-RT-F1	CCTGCTGGGCATTTGG
PoCtB-RT-R1	TAGGGCCGGCAACCA

4. Expression studies by RT-PCR

In order to analyze the tissue expression of the *PoCtB* mRNA, RT-PCR was conducted using brain, eye, gullet, heart, liver, muscle, stomach, head kidney, body kidney, spleen, pyloric ceca, intestine, and gill tissues from healthy *P. olivaceus* specimens. Total RNA was isolated using TRIzol ® (Invitrogen, USA) in accordance with the manu-

facturer's instructions, and purified RNA was quantified by optical density at 260 nm using a UV spectrophotometer (Ultrospec 6300 pro, Amersham Biosciences, UK). Two micrograms of total RNA from the *P. olivaceus* tissues were reverse-transcribed with an oligo dT_{20} primer and Superscript™ III reverse transcriptase (Invitrogen, USA), in-accordance with the manufacturer's instructions.

The specific primers for olive flounder cathepsin B were *PoCtB*-RT2-F and *PoCtB*-RT2-R (<Table 1>). *P. olivaceus* β-actin was utilized as the internal control. All of the PCR was run as follows: 94 °C for 4 min, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C 30 sec, and a final 7 min of elongation at 72°C. The resultant PCR products were separated on 1% agarose/TAE gels containing ethidium bromide and visualized with a Gel Doc image analysis system (Bio-Rad, USA). The PCR products were purified via agarose gel extraction (QIAquick®Gel Extraction kit, EU) and sequenced (COSMO co, Ltd., DNA Sequencing Service, Seoul, Korea).

5. Expression and purification of recombinant PoCtB in E. coli

In order to prepare an expression vector suitable for the production of recombinant fish cathepsin B in *E. coli*, we first generated a DNA fragment harboring the coding sequence for the *P. olivaceus* cathepsin B (*PoCtB*) by PCR amplification. The primers (*PoCtB*-EcoF, 5'- TCGGAATTCCTCCCCAAATTCGTCGACTACCGC-3'; *PoCtB*-6xHis-XhoR, 5'- GCCAGCTACCCCATCATGCATCATCATCATCATCATTGACTCGAGCGGTA-3') harbor EcoI /XhoI restriction sites (underlined) and 6-histidine-tag (double underlined), allowing for the cloning of the amplified DNA in a predicted orientation into pGEX-4T-1 (Amersham Pharmacia Biotech, USA). Recombinant plasmids (*PoCtB*/pGEX) were transformed into *E. coli* strain DH5αMCR. Transformed cells were grown in LB broth (100 ml) containing 100 μg/ml ampicillin at 37°C for about 16 hr, diluted 1/100 with the same medium, and grown to an A600 of 0.6. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4mM, and the incubation was continued for 3hr. Cells were collected by centrifugation, washed, and resuspended in 0.2 volumes of phosphate buffered saline (PBS), lysed by using a sonicator (Vibracell, Sonics & Materials Inc, USA) ata setting of 40%, and centrifuged at 20,000×g for 20 min at 4°C. The soluble supernatant was subjected to glutathione-Sepharose 4B column (Amersham Pharmacia Biotech, USA), equilibrated with PBS. After washing the column with equilibration buffer, protein was eluted in elution buffer with 50 mM Tris/pH 8.0, 10 mM reduced glutathione (Sigma, USA). The fractions containing sufficient amount to active enzyme were pooled, and then dialyzed and concentrated using centricon 10 concentrators (Amicon, USA). Purified PoCtB protein was used for SDS-PAGE, western blotting and enzyme activity assay.

6. SDS-PAGE, western blotting

Purified *PoCtB* enzyme was analyzed by 12% SDS-PAGE. SDS-PAGE was carried out by the method of Laemmli (1970). All samples were denatured in 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, USA). Stained molecular weight markers (Amersham Pharmacia Biotech, USA) were run as standards on each gel. After electrophoresis, the gel was stained with

Coomassie brilliant blue R-250. Western blotting was performed as previously described (Ahn et al., 2007) using mouse monoclonal anti-GST antibody (1: 2000, Santa Cruz Biotechnology, USA).

7. Enzyme activity assays

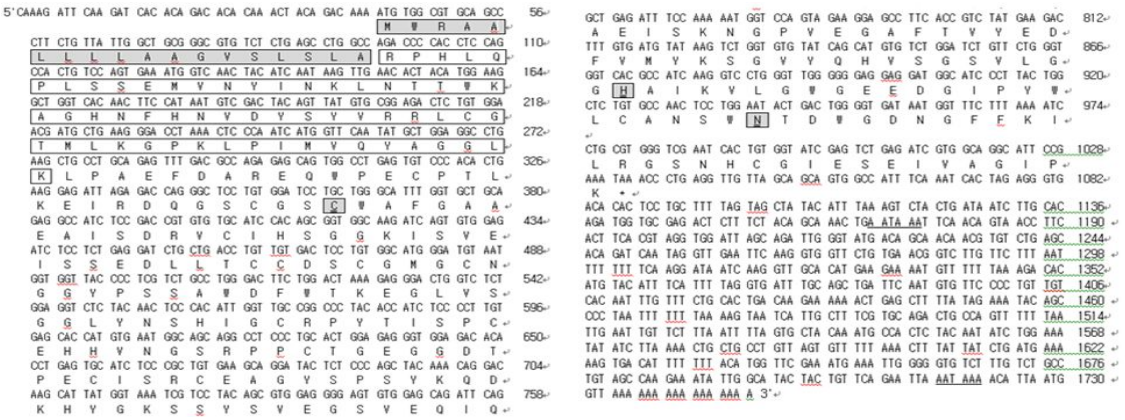
The cathepsin B activity was assayed according to the modified method of Barret and Kirschke (1981). Briefly, 10 ㎕ of recombinant PoCtB enzyme in 85 ㎕ of 0.1 M sodium acetate/ pH 8.0, containing 1 mM DTT were preincubated at 37°C for 2 hr, and the enzyme reaction was initiated by adding 5 ㎕ of Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-Arg-Arg-AMC; Sigma, USA) 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 emission wavelength of 460 nm. The optimum pH for enzymatic activity was determined using sodium acetate buffer in pH ranges of 3-10 with Z-Arg-Arg-AMC as substrate.

Substrate specificity was investigated using Z-Gly-Gly-Arg-AMC, Z-Phe-Arg-AMC, Z-Leu-Leu-Glu-AMC, Z-Val-Val-Arg-AMC, Z-Gly-Pro-Arg-AMC, N-Succinyl (Suc) -Ile-Ala-AMC, Suc-Leu-Tyr-AMC, and Suc-Ala-Ala-Pro-Phe-AMC (Sigma, USA) with 100 mM sodium acetate (pH 7.5) with 2 mM DTT, respectively. Substrates were added to a final concentration of 100 ㎛M.

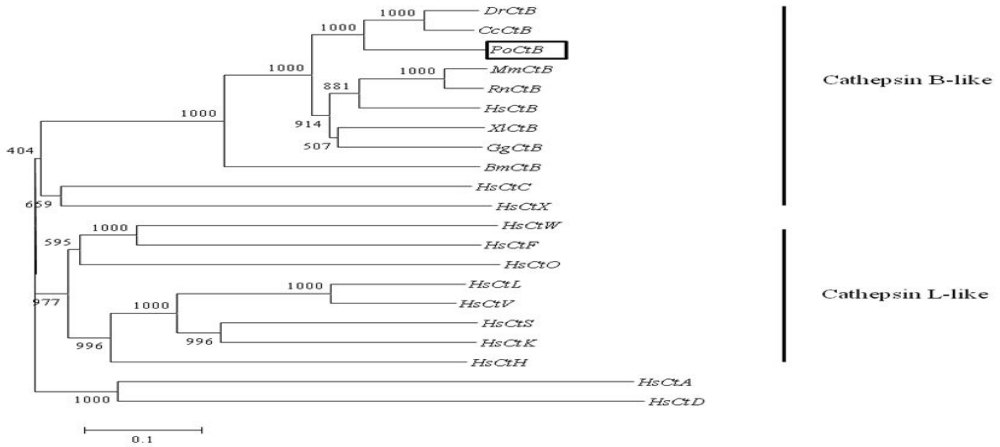
Ⅲ. Results & Discussion

1. Cloning and sequence analysis of P. olivaceus cathepsin B cDNA

In order to isolate full-length *PoCtB*, we conducted 3' cDNA library screening using homologous cathepsin B protein sequences (CGGGYMTNA/GYILMARNRN) from the olive flounder muscle cDNA library. The *PoCtB* cDNA (GenBank accession no. AY686604) consists of 1749 nucleotides with a topcodon flanked by a 41bp 5'-untranslatedregion (UTR) and a 667bp 3'-untranslated region (UTR), including the presumed polyadenylation signal, AATAAA, and the run of poly (A) sequences presumably derived from the poly (A)-rich tail of mRNA (Proudfoot and Brownlee, 1976). The nucleotide sequence of *PoCtB* was predicted to encode for a preproprotein of 330 amino acids, which contained a 18-residue putative signal peptide analyzed with signalIP (Martoglio and Dobberstein, 1998), a 60-residue propeptide and the 252-residue mature enzyme [Fig. 1]. All cysteine proteases harbor in common a conserved active site consisting of a cysteine, a histidine,and an asparagine residue. The cysteine residue(Cys25 based on mature *PoCtB* numbering) is embedded within a highly conserved peptide sequence, CGSCWAFS. The histidine residue (His159;*PoCtB* numbering) is adjacent to small amino acid residues, such as glycine or alanine. Asparagine (Asn175; *PoCtB* numbering) is embedded within a highly conserved peptide sequence, CGSCWAFS. The histidine residue (His159;*PoCtB* numbering) is adjacent to small amino acid residues, such as glycine or alanine. Asparagine (Asn175; *PoCtB* numbering) is a component of the Asn-Ser-Trp (NSW) motif . The interspersed ERFNIN motif, a characteristic feature of cathepsin L-like enzymes (Karrer et al., 1993), was not found in the proregion of *PoCtB*. However, Figure 2 shows an alignment of *PoCtB* with the sequences of other cathepsins. *PoCtB* contains the GNFD motif (GNLD; *PoCtB*) in its proregion, which is generally conserved in most of the cysteine proteases of the papain superfamily (Vernet et al., 1995; Turk et al., 2000). Comparing the *PoCtB* with sequences in the GenBank databases, we determined that *PoCtB* evidences



[Fig. 1] Nucleotide and deduced amino acid sequence of olive flounder (*Paralichthys olivaceus*) cathepsin B cDNA (PoCtB). The shaded box and the open box in the amino acid sequence indicate the putative signal peptide (pre) and the pro peptides of PoCtB, respectively. The active site triad residues Cys25, His159 and Asn175 (papain numbering) are indicated in double underlined and shaded box. Consensus polyadenylation signals (AATAAA) are underline.

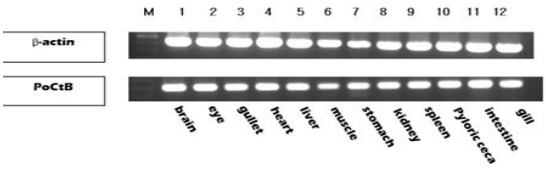


[Fig. 2] Phylogenetic relationships of PoCtB among representative mammalian and piscine groups based on the cathepsin genes. In this neighbor-joining phylogram, all individuals are represented and the branches are based on the number of inferred substitutions as indicated by the bar. GenBank accession numbers not listed previously are as follows: CcCtB, Cyprinus carpio cathepsin B (BAE44111); RnCtB, Rattus norvegicus cathepsin B (NP_072119); BmCtB, Bombyx mori cathepsin B (NP_001036850); HsCtC, H. sapiens cathepsin C (AAQ08887); HsCtX, H. sapiens cathepsin X (AAC39839) HsCtW, H. sapiens cathepsin W (AAH48255); HsCtF, Homo sapiens cathepsin F (NP_003784); HsCtO, H. sapiens cathepsin O (NP_001325); HsCtL, H. sapiens cathepsin L (NP_666023); HsCtV, H. sapiens cathepsin V (B001928); HsCtS, H. sapiens cathepsin S (AAC37592); HsCtK, H. sapiens cathepsin K (P09668); HsCtH, H. sapiens cathepsin H(P09668); HsCtA, H. sapiens cathepsin A (P10619); HsCtD, H. sapiens cathepsin D (AAP35556).

high degrees of identity with other piscine and ma mmalian cathepsin Bs (56-69%). Uniquely, *PoCtB* d idn't exhibit high degree of identity with human ca thepsins (8~9%). However, the cathepsin X have hi gher degrees of identity (24%) with *PoCtB* in hum an cathepsins except *HsCtB*. In order to determine the evolutionary relationship of *PoCtB* with other c athepsin families, a phylogenetic tree was constructe d. Phylogenetic analyses were conducted with the a mino acid sequences of other cathepsins obtained fr om GenBank using neighbor-joining methods [Fig. 2]. On the basis of a comprehensive phylogenetic a nalysis, the enzymes of the Family C1 peptidases (i.e. the papain superfamily of cysteine proteases) c ould be divided into two primary evolutionary bran ches, branches A and B. Branch A includes catheps ins B, C and Z. Branch B includes the cathepsin L -like enzymes, a group including papain, cathepsin L,cathepsin S, cathepsin K, and cathepsin H (Tinga ud-Sequeira and Cerdà, 2007). *PoCtB* is more close ly related to the cathepsin B subfamily (B, C, and X) than to the cathepsin L-like enzymes (L, V, S, K, H, W).

2. Tissue-typic expression of PoCtB

Distribution of *PoCtB* transcripts in different in different organs were examined by RT-PCR. As shown in [Fig. 3], expression of *PoCtB* was observed in all of the tissues. The expression pattern of *PoCtB* was found in high levels in the brain, eyes, gullet, heart, liver, kidney, muscle, stomach, spleen, pyloric ceca, intestine, gill, it was also detectable levels in other tissues. Together with the previously described ubiquitous expression in human and mouse, these results characterize *PoCtB* as a constitutively expressed 'housekeeping gene' like other ubiquitously expressed representatives of the C1 family of cysteine proteases, such as cathepsin F, H and



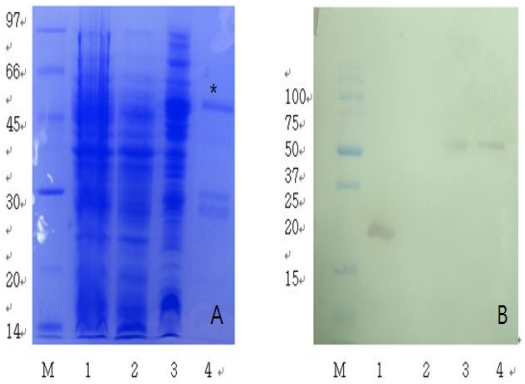
[Fig. 3] Tissue-typic expression of the PoCtB mRNA.

L. But this ubiquitous expression of *PoCtB* is in contrast to the tissue-specific expression of cathepsi ns S, K, and W.

3. Enzymatic characterization of recombinant PoCtB

In order to assess the functional and enzymatic characteristics of *PoCtB*, the cDNA encoding for mature *PoCt*B was expressed in *E. coli* as a fusion protein with glutathione S-transferase (GST). The recombinant *PoCtB*/pGEX was overexpressed in *E. coli* DH5αMCR as a 53kDa fusion protein. The overproduced soluble GST and His-6-tag-fusion protein (PoCtB) was then applied to glutathione-Sepharose 4B column chromatography, and the sample harboring the fusion protein evidenced a high level of purity when analyzed via SDS-PAGE and Western blotting. [Fig. 4].

We also compared the utilization of various substrates conjugated with aminomethylcoumarin as the fluorescent chromophore <Table 2>. The highest levels of AMC release activity were seen from Z-RR-AMC, and the Z-RR-AMC substrate was hydrolyzed 2-fold more efficiently than the Z-FR-AMC.



[Fig. 4] Expression of recombinant *PoCtB*

(A) Coomassie blue staining after SDS-PAGE. The asterisk (*) indicates GST-fused *PoCtB*. The lanes were labeled as follows: M, standard size marker; 1, overexpressed GST protein; 2, non-induced GST-fused *PoCtB*; 3, overexpressed GST-fused proApCtL (37℃); 4, glutathione-Sepharose 4B affinity column purified *PoCtB*.

(B) Western blotting analysis. M: prestained protein size marker, lane 1: expressed GST protein (37℃) reacted with monoclonal anti-GST antibody (positive control), lane 2: non-induced *PoCtB* protein (negative control), lane 3, overexpressed GST-fused PoCtB (37℃); lane 4, glutathione-Sepharose 4B affinity column purified PoCtB

<Table 2> Substrate specificity of PoCtB

Substrates	Concentration (uM)	Activity (%)
Z-Arg-Arg-AMC (RR)	50	100.00
Z-Phe-Arg-AMC (FR)	50	34.12 ± 1.99
Z-Gly-Gly-Arg-AMC (GGR)	50	14.90 ± 1.24
Z-Val-Val-Arg-AMC (VVR)	50	4.51 ± 1.55
Z-Gly-Pro-Arg-AMC (GPR)	50	7.18 ± 0.90
Z-Leu-Leu-Glu-AMC (LLE)	50	7.38 ± 0.64
Suc-Ile-Ala-AMC (IA)	50	3.64 ± 1.80
Suc-Leu-Tyr-AMC (LY)	50	0.00
Suc-Ala-Ala-Pro-Phe-AMC (AAPF)	50	3.92 ± 0.35

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● 논문접수일 : 2014년 04월 18일
● 심사완료일 : 1차 - 2014년 05월 07일
● 게재확정일 : 2014년 05월 30일