Matrix metalloproteinase 2 is involved in the regulation of the antimicrobial peptide parasin I production in catfish skin mucosa

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Abstract A 19-residue antimicrobial peptide parasin I is generated from histone H2A in the skin mucus of catfish by the action of cathepsin D activated by a procathepsin D-processing enzyme induced upon epidermal injury. Here we report the isolation and characterization of the procathepsin D-processing enzyme in the mucus of wounded catfish. Sequence analysis of the cDNA identified the purified procathepsin D-processing enzyme as matrix metalloproteinase 2 (MMP 2). By acting as a procathepsin D convertase upon epidermal injury, MMP 2 is involved in the regulation of parasin I production in catfish skin mucosa.

Key words: Antimicrobial peptide; Cathepsin D; Histone H2A; Matrix metalloproteinase 2; Parasin I

1. Introduction

Fish are in intimate contact with a rich microbial flora and are presumed to use their innate immune system as the first line of defense against microbial invasion [1]. Many antimicrobial peptides have been isolated from a multitude of animals, microorganisms and plant species [2], including fish [3–6], and are recognized as important components of the innate immunity [7]. These peptides exhibit potent antimicrobial activities against a broad range of microorganisms, including bacteria, protozoa, fungi and viruses [8]. They can function intracellularly, as in circulating leukocytes, or in the external environment after release by secretory cells and other granulated epithelia [9]. Most of the antimicrobial peptides are made as gene-encoded preproproteins and are processed by defined pathways to their active peptide forms [10]. However, an increasing number of antimicrobial peptides are found to be derived by proteolysis from larger proteins with other known functions such as lactoferrin [11], ribosomal protein L1 [12] and histone H2A [13,14].

Recently, a potent 19-residue linear antimicrobial peptide named parasin I was isolated from the skin mucus of wounded catfish [4]. The amino acid sequence of parasin I (KGRGKQGGKVRAKAKTRSS) is identical in 17 of the 19 residues to the N-terminal region of histone H2A from calf thymus [15]. In a previous study, we demonstrated that parasin I is generated from unacetylated histone H2A by the action of cathepsin D; cathepsin D is found to exist in the mucus as an inactive procathepsin D and to be activated to mature cathepsin D by an extracellular enzymatic process induced upon epidermal injury [14]. However, little is known about the activation of secreted procathepsin D by extracellular proteases except that the procathepsin D-processing enzyme induced in the mucus of wounded catfish is a metalloprotease [14]. The characterization of the procathepsin D-processing enzyme would greatly aid in the understanding of the regulation of parasin I production. In this study, we identified and characterized the procathepsin D-processing enzyme in the mucus of wounded catfish.

2. Materials and methods

2.1. Materials

pET-16b and *Escherichia coli* BL21(DE3) were obtained from Novagen (Madison, WI, USA). Restriction enzymes and modification enzymes for DNA manipulation were from New England Biolabs (Beverly, MA, USA). Calf thymus histone H2A, leupeptin, pepstatin A, E-64, ethylenediamine tetraacetic acid (EDTA) and *o*-phenanthroline were purchased from Roche (Mannheim, Germany). Phenylmethylsulfonyl fluoride (PMSF), pepstatin A-agarose and isopropyl β-D-thiogalactopyranoside (IPTG) were from Sigma (St. Louis, MO, USA). All other reagents used in this study were of analytical grade.

2.2. Expression and purification of recombinant procathepsin D

Catfish procathepsin D gene was polymerase chain reaction (PCR)-amplified from the cDNA clone [14] with the upstream primer 5′-CCGCTCGAGCTGGTTCGGATTCCTC-3′ and the downstream primer 5′-CGGGATCCCTAAATTGCTTTAGCC-3′. The 1021-bp PCR fragment was digested with *Xho*I and *Bam*HI (restriction sites underlined in the primers) and then subcloned into pET-16b digested with the same restriction enzymes. The resultant vector was named pET-proCAD and used to transform *E. coli* BL21(DE3). The resulting transformants were cultured in 1.5 l of Luria–Bertani (LB) medium containing ampicillin (50 μg/ml) at 37°C. When the absorbance at 600 nm reached 0.6, IPTG was added to a final concentration of 1 mM. 3 h after induction, cells were harvested by centrifugation at 6000×*g* for 10 min at 4°C and resuspended in buffer A (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl and 5 mM imidazole). After lysis of cells by sonication, inclusion bodies were recovered by centrifugation at 13 000×*g* for 30 min at 4°C. The inclusion bodies were denatured and solubilized in buffer A containing 6 M urea (buffer B). The sample was then centrifuged and the supernatant was loaded onto a Ni^{2+} affinity column (His·Bind resin, Novagen) equilibrated with the same buffer. After washing out the unbound proteins with buffer B containing 20 mM imidazole, the procathepsin D was eluted from the column with buffer B containing 1 M imidazole and dialyzed extensively against buffer A. The histidine-tag of the procathepsin D was cleaved by incubating with factor Xa (New England Biolabs) at room temperature for 8 h and then the cleavage products were reapplied to

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the Ni^{2+} affinity column equilibrated with buffer A. The recombinant procathepsin D in the flow-through fraction was collected and the purity of recombinant procathepsin D was assessed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.3. Assay of procathepsin D-processing enzyme activity

Mucus extracts of wounded catfish were prepared as described by Cho et al. [14]. The mucus extracts were acidified to pH 3.5 and applied to a pepstatin A affinity column, which was equilibrated with 50 mM sodium acetate (pH 3.5) at 4°C, to remove endogenous active cathepsin D by binding to the column. The pepstatin A flow-through fraction, in which endogenous active cathepsin D was eliminated, was then concentrated and desalted by using a Centricon microconcentrator (Amicon, Beverly, MA, USA) with a 10-kDa molecular mass cut-off membrane. Proteolytic processing of procathepsin D was examined by incubating recombinant procathepsin D (2 μg) with the pepstatin A flow-through fraction (5 μg of protein) in 50 μl of 20 mM sodium phosphate (pH 6.0) containing 50 mM NaCl. After incubation for 1 h at room temperature, the reaction was stopped by boiling for 5 min and analyzed by 10% SDS–PAGE.

2.4. Purification of a procathepsin D-processing enzyme

The pepstatin A flow-through fraction was loaded into a Superdex 200 (HR 10/30) column (1×30 cm, Pharmacia LKB, Uppsala, Sweden) on a fast protein liquid chromatography (FPLC) system, which was equilibrated with 0.05 M sodium phosphate buffer (pH 7.0), and eluted with the same buffer at a flow rate of 0.2 ml/min. All purification steps were carried out at 4°C. Fractions (2 ml) were collected and analyzed for procathepsin D-processing activity by 10% SDS–PAGE. Active fractions were concentrated and desalted as above. The pooled active fractions were injected into a Waters high pressure liquid chromatography (HPLC) system equipped with a gel permeation column (8×300 mm, Shodex Protein KW-803, Showa Denko, Tokyo, Japan), which was equilibrated with 0.05 M sodium phosphate buffer (pH 7.0), and were eluted with the same buffer at a flow rate of 1 ml/min. The purified enzyme with procathepsin D-processing activity was subjected to the N-terminal sequence analysis by automatic Edman degradation on an Applied Biosystems gas-phase sequencer, Model 477A (Foster City, CA, USA).

2.5. Characterization of the procathepsin D-processing enzyme

The pH optimum of the procathepsin D-processing enzyme was determined using the same assay described above with the following buffer systems: sodium citrate (20 mM, pH 4.0–5.0), sodium phosphate (20 mM, pH 6.0–8.0) and glycine–NaOH (20 mM, pH 9.0–10.0). The effects of protease inhibitors were examined by pre-incubating the procathepsin D-processing enzyme for 1 h at room temperature with each of the following protease inhibitors: PMSF (10 mM), leupeptin (50 μM), E-64 (50 μM), pepstatin A (50 μM), EDTA (5 mM) and *o*-phenanthroline (5 mM). Recombinant procathepsin D (2 μg) was then added in 50 μl of the reaction buffer, and the protease reactions were allowed to proceed for additional 1 h.

2.6. cDNA cloning of the gene encoding the procathepsin D-processing enzyme

A cDNA encoding the procathepsin D-processing enzyme was isolated from the catfish skin cDNA library [14]. The library was screened with a ^{32}P-labeled 1721-nucleotide probe specific to the purified protease, which was generated by 3′-RACE (3′-RACE PCR kit, Life Technologies, Rockville, MD, USA). For the 3′-RACE, a PCR primer (5′-GGNCCNAARCARGARGT-3′, where R = A, G; N = A, C, G, T) was designed based on the amino acid sequence (residues 3–8) of the purified enzyme and used as a gene-specific sense primer. The nucleotide sequence of the selected clones was determined on both strands using universal and sequence-specific primers on an automated DNA sequencer (ABI prism model 377, Perkin Elmer, Foster City, CA, USA).

2.7. Characterization of the processed cathepsin D

Recombinant procathepsin D (10 μg) was incubated for 1 h at room temperature with the purified procathepsin D-processing enzyme (3 μg) in 100 μl of 20 mM sodium phosphate (pH 7.0) containing 50 mM NaCl. The reaction mixture was then separated by 10% SDS–PAGE and blotted onto a polyvinylene difluoride membrane (0.2 μm pore size, Bio-Rad, Hercules, CA, USA) by semidry electrophoretic transfer. The band corresponding to the processed cathepsin D was excised from the membrane for the N-terminal sequence analysis, which was performed on an Applied Biosystems gas-phase sequencer, Model 477A.

3. Results and discussion

3.1. Expression and purification of recombinant procathepsin D

The cDNA encoding a catfish procathepsin D was PCR-amplified and subcloned into pET-16b, which contained an N-terminal tag composed of 10 histidine residues (His-tag) for rapid purification and a factor Xa cleavage site for removing the N-terminal addition from the expressed protein. The fusion protein was expressed as inclusion bodies in *E. coli* BL21(DE3). After solubilization of the inclusion bodies, the expressed protein was purified using Ni^{2+} affinity column chromatography. The fusion protein was then digested with factor Xa to remove the N-terminal His-tag fusion peptide, and reapplied to the Ni^{2+} affinity column. The cleaved procathepsin D was recovered to 98% homogeneity (data not shown).

3.2. Purification and characterization of a procathepsin D-processing enzyme

The mucus extracts were first applied to a pepstatin A-agarose column to remove endogenous active cathepsin D by binding to the column, and the pepstatin A flow-through fraction was purified by FPLC on a Superdex 200 column (Fig. 1A and B). The active fractions with procathepsin D-processing activity (81–90 min) were found to contain three proteins, as confirmed by SDS–PAGE. Therefore, the active fractions were further separated into a single protein band of ~60 kDa by HPLC on a Shodex gel permeation column (Fig. 1C and D). This purified enzyme was subjected to automated Edman degradation for amino acid sequence analysis, which allowed identification of the N-terminal 19 amino acid residues as VLGPKQEVPRAALEQTEAG.

The 60-kDa enzyme showed procathepsin D-processing activity in the pH range of 5.0–8.0 and displayed optimal activity at pH 7.0 (Fig. 2). Table 1 shows the effects of various inhibitors on the activity of the 60-kDa enzyme. Metal-chelating agents such as EDTA and *o*-phenanthroline strongly inhibited the procathepsin D-processing activity whereas serine protease inhibitors such as PMSF and leupeptin, a cysteine protease inhibitor E-64, and an aspartic protease inhibitor pepstatin A did not inhibit. These results clearly indicate that the procathepsin D-processing enzyme induced in the mucus of wounded catfish was a metalloprotease. The size of the processed cathepsin D is ca. 37 kDa which is the same as that of native cathepsin D [14]. To determine the cleavage specificity of the 60-kDa enzyme, we sequenced the N-terminal amino acids of the processed cathepsin D. The first five amino acid residues were identified as GPTPE, which exactly matched with those of mature cathepsin D. On the basis of the above results, we concluded that the 60-kDa enzyme activated procathepsin D to mature cathepsin D by cleaving the Phe^{61}–Gly^{62} bond of procathepsin D.

3.3. cDNA cloning of the purified procathepsin D-processing enzyme

A cDNA encoding the purified procathepsin D-processing enzyme was obtained by screening the catfish skin cDNA library with a specific probe generated by 3′-RACE. DNA

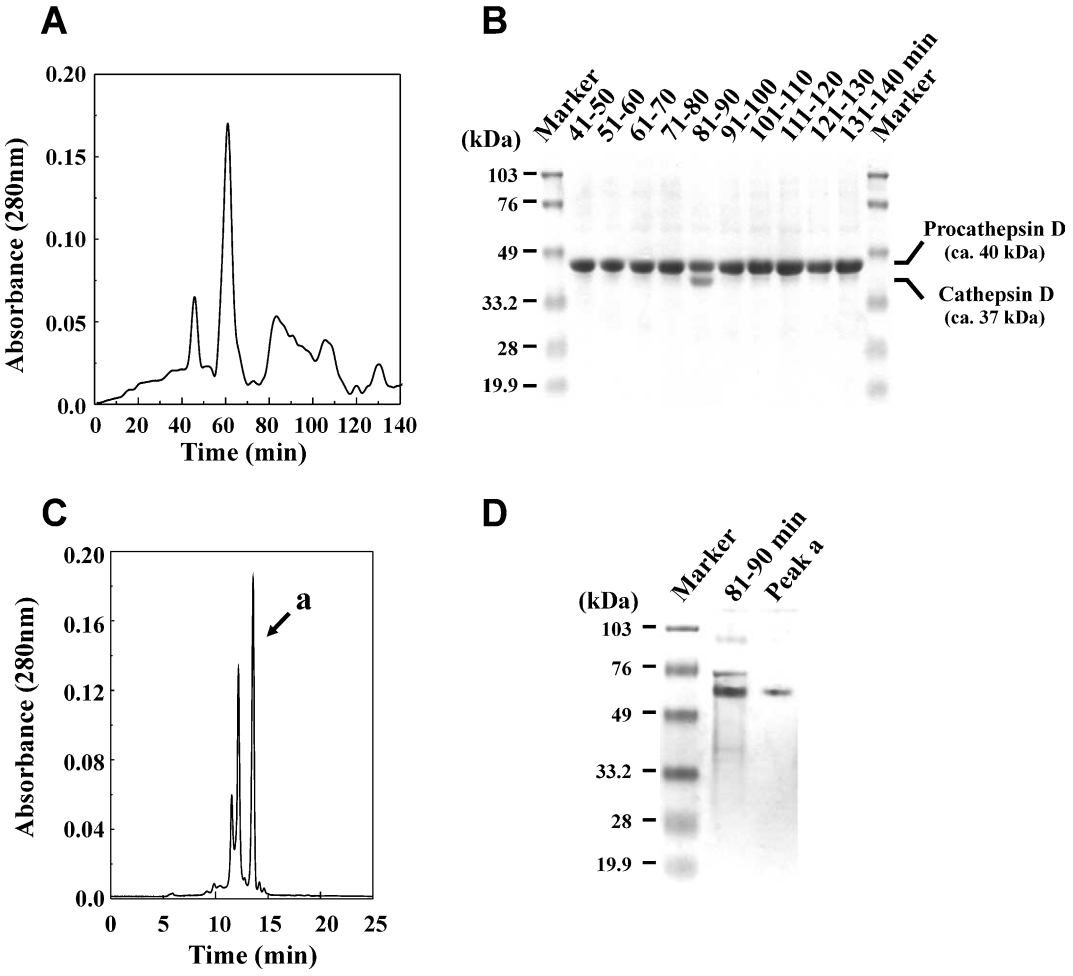

Fig. 1. Purification of a procathepsin D-processing enzyme from the mucus of wounded catfish. A: Chromatography on an FPLC Superdex 200 column of the pepstatin A flow-through fraction in which endogenous active cathepsin D was eliminated. B: Proteolytic cleavage of procathepsin D by the fractions obtained from the Superdex 200 FPLC step. The procathepsin D-processing activity of each fraction (2 ml) was assessed by incubating with recombinant procathepsin D (2 μg, total reaction volume, 50 μl). C: Gel permeation HPLC of the active fractions (81–90 min) obtained from the Superdex 200 FPLC step on a Shodex Protein KW-803 column. D: SDS–PAGE analysis of the purified enzyme. An FPLC fraction containing the procathepsin D-processing activity (indicated by an arrow, a) was subjected to 10% SDS–PAGE.

sequence analysis of a 2052-bp cDNA showed an open reading frame encoding a 638-residue polypeptide chain, which consisted of a putative signal peptide of 18 amino acids, a propeptide of 78 amino acids and a mature enzyme of 542 amino acids (GenBank accession number AF544370). A search of the NCBI data bank through the BLAST network service showed that the procathepsin D-processing enzyme had a significant homology with matrix metalloproteinases (MMPs), especially that of rainbow trout MMP 2 (Fig. 3). The amino acid sequence of the purified enzyme showed 52% identity with the rainbow trout MMP 2 [16], and contained all the characteristics of MMPs such as a highly conserved metal binding motif (HEXXHXXGXXH) in the catalytic domain and a cysteine switch (PRCXXPD) in the propeptide [17]. The above results strongly suggest that the 60-kDa enzyme purified is a catfish homolog of MMP 2.

3.4. Conclusion

In this study, we found that MMP 2 is involved in the enzymatic cascade reaction for the production of the antimicrobial peptide parasin I from histone H2A in catfish skin mucosa by acting as a procathepsin D-processing enzyme. Using recombinant procathepsin D as a substrate, we assayed the mucus extracts of wounded catfish for procathepsin D-processing activity and successfully purified a 60-kDa enzyme that is responsible for the conversion of 40-kDa proca-

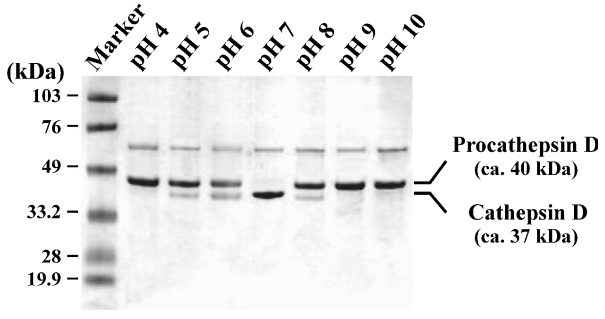

Fig. 2. Effect of pH on the activity of the procathepsin D-processing enzyme. Recombinant procathepsin D (2 μg) was incubated with the purified enzyme (1 μg) in reaction buffers in the pH range of 4.0–10.0 (total reaction volume, 50 μl). After incubation for 1 h at room temperature, the reactions were stopped by boiling for 5 min and analyzed by 10% SDS–PAGE.

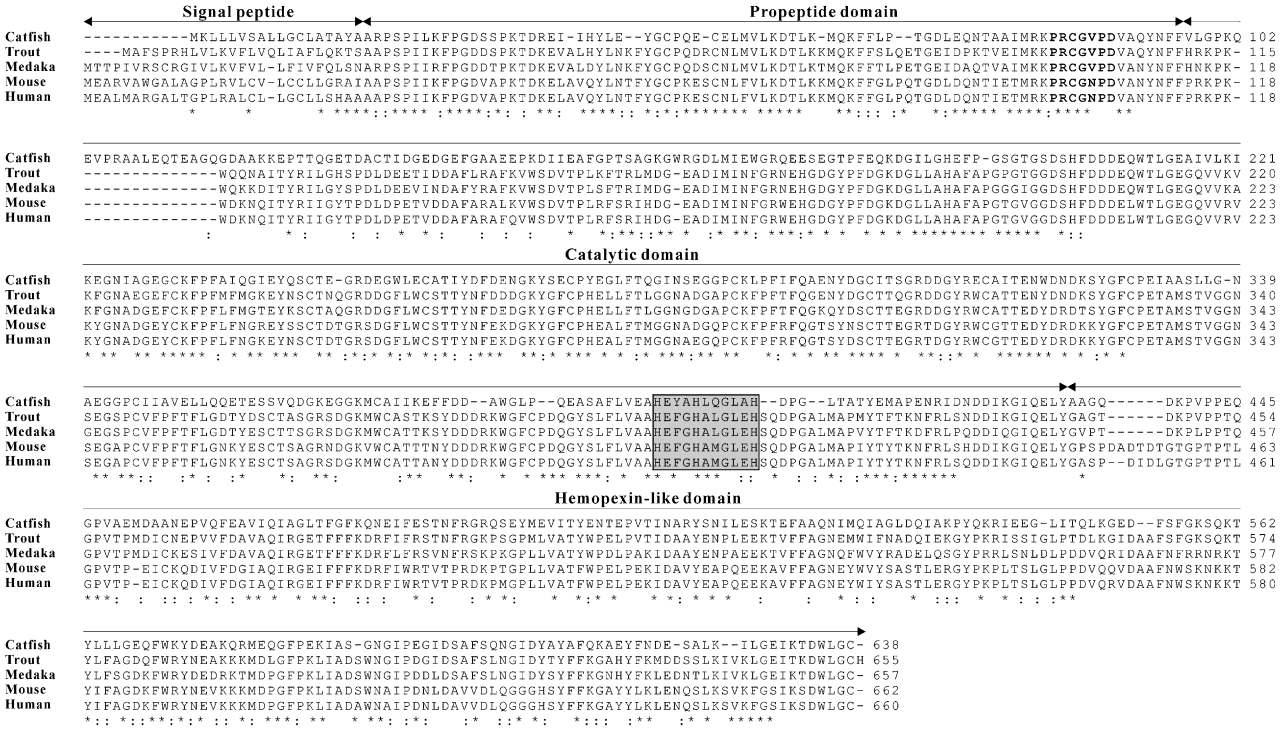

Fig. 3. Alignment of the amino acid sequence of vertebrate MMP 2. The multiple sequence alignment was obtained using the Clustal W program with default parameters. The amino acid sequence of catfish MMP 2 is aligned with those of MMP 2 from rainbow trout, medaka fish, mouse and human. Asterisks and colons indicate perfect matches of amino acids and conservative substitutions, respectively. Each domain is assigned a signal peptide, a propeptide domain, a catalytic and a hemopexin-like domain. The cysteine switch motif (PRCXXPD) in the propeptide domain is shown in bold. The metal binding motif (HEXXHXXGXXH) in the catalytic domain is boxed.

thepsin D to 37-kDa cathepsin D (Fig. 1). Amino acid sequence analysis and cDNA cloning identified the purified procathepsin D-processing enzyme as MMP 2 (Fig. 3). MMP 2 converts procathepsin D to cathepsin D by cleaving the Phe^{61}–Gly^{62} bond of procathepsin D, as confirmed by structural analysis of the processed protein.

In vertebrates, MMPs, including MMP 2, which are expressed or released in response to injury, disease or inflammation, are typically associated with the immune response and tissue repair [18]. As their name suggests, MMPs are thought to be responsible for the turnover and degradation of connective tissue proteins. However, matrix degradation is not the sole function of these enzymes. Several reports have demonstrated that various MMPs are involved in the activation of a variety of non-matrix proteins, including cytokines, chemokines, receptors and antimicrobial peptides [19]. Among them, matrilysin (MMP 7), the expression of which is induced by exposure to bacteria, has been reported to function in the intestinal mucosal defense by regulating the activity of defensins [20]. Our results suggest that the role of MMP 2 may be extended to the innate host defense system beyond connective tissue remodeling, specifically being involved in the regulation of parasin I production by acting as a procathepsin D convertase.

Extracellular proteolytic processing of host defense peptides from larger precursor polypeptides has been emerging as a common theme in innate immunity. In bovine and porcine neutrophils, antimicrobial peptides are liberated by elastase-mediated cleavage of cathelicidins [21,22]. Recently, it has been reported that the antimicrobial peptide buforin I is released from histone H2A by the action of pepsin in the gastric mucosa of toad [13] and human α-defensin 5 (HD5) from proHD5 by the action of trypsin in human Paneth cells [23]. The role played by pepsin in the processing of buforin I is similar to that of cathepsin D in the production of parasin I. But unlike pepsin which is autoactivated by hydrochloric acid, catfish cathepsin D is secreted to the mucosal surface as an inactive proenzyme and activated to the mature enzyme by MMP 2 induced upon epidermal injury. The enzymatic cascade reaction involved in parasin I production is strikingly similar to that of the prophenoloxidase (proPO)-activating system in melanin formation of arthropods. In arthropods, PO is synthesized as an inactive proenzyme, proPO, which is activated by a proPO-activating enzyme (proPO-AE) upon wounding or exposure to certain microbial polysaccha-

Table 1
Effects of protease inhibitors on the activity of the procathepsin D-processing enzyme

Inhibitor	Concentration	Relative activity (%)[a]
None		100
Metalloprotease inhibitor		
EDTA	5 mM	24
o-Phenanthroline	5 mM	11
Serine protease inhibitor		
PMSF	10 mM	100
Leupeptin	50 μM	95
Cysteine protease inhibitor		
E-64	50 μM	93
Aspartic protease inhibitor		
Pepstatin A	50 μM	87

[a]Relative enzyme activities were calculated as the ratio of the amount of processed procathepsin D in the presence of inhibitors to that in the absence of inhibitor.

rides. PO then catalyzes the oxidation of phenols to quinones, which then polymerize non-enzymatically to melanin [24]. Because active PO can produce highly toxic intermediates, its activation is tightly regulated by proPO-AE inhibitors such as serpins [25]. The activation of cathepsin D might be regulated similar to that of PO, although we did not confirm the existence of cosecreted MMP 2 inhibitors in the catfish mucus. Further work will focus on the identification of MMP 2 inhibitors and their expression in vivo.

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