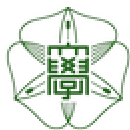
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1 Running title: Structural Characterization of ANISEP

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5 Structural Characterization Reveals the Keratinolytic Activity of an Arthrobacter nicotinovorans Protease

6 Protease ANISEP

7

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20 Keywords: *Arthrobacter nicotinovorans*, cadmium, crystallization, metallothionein, scallops, serine

21 protease

1 **Abstract**

2 Cadmium (Cd) contamination in fishery byproducts is an environmental hazard; enzymatic removal and
3 adsorption of the contaminant may be useful for recycling byproducts as animal feed. We cloned the gene
4 for *Arthrobacter nicotinovorans* serine protease (ANISEP), which was isolated from the hepatopancreas
5 of the Japanese scallop (*Patiopecten yessoensis*) and has been found to be an effective enzyme for Cd
6 removal. The gene is 993 bp in length and encodes 330 amino acids, including Pre (1–30) and Pro (31–
7 111) residues. The catalytic triad consists of His, Asp, and Ser; sequence similarity confirmed ANISEP as
8 a member of the extracellular serine proteases. X-ray crystallography revealed structural similarities
9 between ANISEP and the trypsin-like serine protease NAALP from *Nesterenkonia* sp. Site-directed
10 mutagenesis identified Ser171 as the catalytic residue. The keratinolytic activity of ANISEP was 10-fold
11 greater than that of trypsin. ANISEP digested Cd-bound recombinant metallothionein MT-10a from
12 *Laternula elliptica,* but did not release Cd. These results suggest ANISEP is a trypsin-like serine protease
13 that can cause Cd release from the Japanese scallop hepatopancreas because of its strong keratinolytic
14 activity.

Introduction

Japanese scallop, *Patiopecten yessoensis*, is an important bivalve in Japanese fisheries. The total annual scallop production, including sea catch and aquaculture, is greater than 400,000 tons. Processing includes removal of the hepatopancreas, which is rich in protein and is thus used for animal feed and fertilizers; however, this organ often contains toxic heavy metals such as cadmium (Cd). Therefore, safe and effective utilization of the hepatopancreas requires the elimination of toxic heavy metals.

Heavy metals are usually bound with proteins in the hepatopancreas and are not removed by washing or other simple mechanical methods. Several Cd-removal processes have been proposed, such as treatment with H_2SO_4, but acid treatment reduces protein quality and quantity, and accelerates corrosion of processing equipment [1]. The competitive adsorption method involving insoluble humic acid enables the removal of Cd from the scallop hepatopancreas under mild acidic conditions (pH 5.0) [2]. A subcritical water treatment was recently proposed, but it requires high temperatures and yields insoluble Cd-contaminated waste [3]. An alternative microbial process uses the Gram-negative bacterium *Xanthomonas* sp. as a specific adsorptive agent for Cd, but protease digestion is required to release Cd from the hepatopancreas [4]. Subsequently, Ren et al. isolated a bacterium capable of releasing Cd from the hepatopancreas; the responsible protease was purified, characterized [5]. This enzyme, designated ANISEP (*Arthrobacter nicotinovorans* serine protease), releases Cd more effectively than commercial enzymes at neutral pH and should therefore be suitable for enzymatic Cd release before bacterial adsorption. ANISEP was inhibited strongly by PMSF (phenylmethylsulfonyl fluoride), indicating that it is a serine protease [5].

One of the most well-studied microbial serine proteases is the subtilisin-like protease. These proteases are generally secreted to scavenge extracellular nutrients. Various *Bacillus* species produce enzymes of this class [6]. Most of them exhibit maximal activity at alkaline pH and are thus considered the most important group of commercial proteases for use as laundry detergents [6]. Subtilisin-like proteases are known for broad substrate specificity, although there are a few exceptions [7]. Recently, a

novel alkaline protease from *Nesterenkonia* sp, named NAALP, was found to show strong keratinolytic activity. This enzyme is capable of degrading feathers and has potential for application in feather meal production [8,9]. Keratinolytic protease might be effective for Cd release from the scallop hepatopancreas.

The most well-known heavy metal-binding proteins in marine animals are the metallothioneins (MTs). MTs are low-molecular-weight, cysteine-rich, metal-binding proteins [10]. Reports on the heterologous expression, purification, and Cd-binding nature of bivalve MTs suggest they are the most likely candidates for Cd accumulation in *P. yessoensis* [11,12]. Additional reports of bivalve MT genes suggest their expression is induced upon exposure to Cd and/or environmental contaminates such as heavy metals [13-17]. However, the MTs in *P. yessoensis* have not been characterized and the molecular mechanism of Cd release by ANISEP remains unclear.

The long-term objective of this study was to characterize how ANISEP mediates Cd release from the scallop hepatopancreas. We must first understand the enzyme's primary and higher structure to determine the molecular basis of its activity and substrate specificity. We cloned the protease gene, analyzed the structure of the purified protease, and performed digestion assays with recombinant clam metallothionein MT-10a [11].

Materials and Methods

Bacterial strains and vectors

Arthrobacter nicotinovorans 23-0-11 (AHU1956, [5]) was inoculated from a stock culture. *Escherichia coli* strains TOP10, BL21-AI, and BL21-Star (DE3) and the vectors pENTR-SD-D-TOPO and pDEST14 were purchased from Invitrogen (Carlsbad, CA). The expression vector pET41-MT contains MT-10a fused to GST and was a gift from Dr. Hyun Park (Korea Polar Research Institute, South Korea).

1 *Purification of ANISEP*

2 ANISEP was obtained from the culture supernatant of *A. nicotinovorans* 23-0-11 and purified by
3 two-step ion-exchange chromatography as described by Ren et al. with modifications [5]. Briefly, an
4 isolated colony of *A. nicotinovorans* on nutrient agar was used to inoculate 100 mL Uro medium (scallop
5 hepatopancreas powder, 7 g/L; K_2HPO_4, 1 g/L; $MgSO_4 \cdot 7H_2O$, 1 g/L; pH 7.0). After incubation with
6 shaking at 27°C for 48 h, 10 mL of the culture broth was inoculated into 500 mL Uro medium and
7 incubated for another 48 h at the same temperature with shaking. The culture supernatant was lyophilized,
8 dissolved in 15 mL of 50 mM potassium phosphate buffer (pH 6.0), and desalted with a PD-10 desalting
9 column (GE Healthcare, Buckinghamshire, UK), followed by column chromatography using HiTrapQ FF
10 (GE Healthcare, Uppsala, Sweden) and HiTrap SP FF (GE Healthcare). Column chromatography was
11 performed on an ÄKTA explorer 100 system (GE Healthcare). First, a flow-through fraction was
12 recovered during binding of the crude enzyme to HiTrapQ. The recovered fraction was then applied to a
13 HiTrap SP column and eluted with 50 mM potassium phosphate buffer, pH 6.0, with a linear gradient of
14 NaCl to 1.0 M. Fractions with protease activity were recovered.

15

16 *N-terminal and internal amino acid sequence*

17 Purified ANISEP (240 µl, 0.5 µg/µl in 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 10% glycerol,
18 0.008% bromophenol blue) was heat-denatured and mixed with 10 µl lysylendopeptidase (Wako, Osaka,
19 Japan) or undenatured ANISEP (0.1 µg/µl in 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 5% glycerol, 0.008%
20 bromophenol blue). After incubation at room temperature, samples were separated by SDS-PAGE and
21 transferred by electroblotting onto a Mini Problot PVDF membrane (Applied Biosystems, Foster City,
22 CA). Bands corresponding to digested ANISEP were recovered. The N-terminal amino acid sequences of
23 the undigested enzyme and digested fragments were determined on a Procise 49-HT Protein Sequencer
24 (Applied Biosystems).

25

Degenerate PCR

Degenerate PCR was performed with seven primers designed according to internal N-terminal sequences (Table 1). Reaction mixtures contained 500 pmol primers, 100 ng *A. nicotinovorans* 23-0-11 DNA, 2.5 U Amplitaq DNA polymerase (Applied Biosystems), 5 µl of 10× PCR buffer, 5 µl of 2 mM dNTP mix, and 3.5 µl of 25 mM $MgCl_2$ in a total volume of 50 µl. Cycling conditions were as follows: 95°C for 2 min; 35 cycles of 95°C for 1 min, 45°C for 1 min, and 72°C for 1 min; 72°C for 5 min.

Cloning of the protease gene

A. nicotinovorans DNA was digested with *Sal*I (Takara Bio, Ohtsu, Japan) and self-ligated with T4 DNA ligase (New England Biolabs, Beverly, MA). A pair of inverse PCR primers (InverseF-2-1: 5´-CCTTCGCCGTCATGAACAC-3´ and InverseR-2-1: 5´-CCACTTCCCGTAGGGTGCT-3´) was designed inside the degenerate PCR fragment. PCR was performed with the Expand long template PCR system (Roche, Mannheim, Germany) according to manufacturer instructions. The 3.4-kb amplicon was cloned into pGEM-T easy (Promega, Madison, WI). PCR amplification of the protease gene from *A. nicotinovorans* 23-0-11 DNA was performed with another pair of primers (Forward: 5´-CACCTGTCCGAGGAGGGGTTGTC-3´, Reverse; 5´-CGGCATCACGATGTACTTCAC-3´), designed to amplify a 2.0-kb fragment containing the full-length deduced ORF for the protease, its promoter and terminator, and KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The amplified DNA was cloned into pENTR-D-TOPO (Invitrogen) according to manufacturer instructions.

DNA sequencing and sequence analysis

DNA sequencing reactions were performed with the BigDye terminator 1.1 cycle sequencing kit (Applied Biosystems). Sequencing products were purified with Performa DTR Gel Filtration cartridges (EdgeBio, Gaithersburg, MD) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Genetyx software (Genetyx, Tokyo Japan) was used to analyze the DNA and protein

sequences. DNA sequences were deposited into the NCBI database under accession number AB721406.

Expression of ANISEP in E. coli

Primers cacc990ATG-F (5´-CACCATGACAAAAACCAAGACCCTG-3´) and ORF-r (5´-ACATCAGTTACGAAGCCGCA-3´) were to construct the expression vector. After PCR amplification to generate a modified start codon with the above primers, the protease gene was cloned into pENTR/SD/D-TOPO (Invitrogen). The resulting plasmid, pENTR/SD/990ATG, was then used for the insert exchange reaction with pDEST14 (Invitrogen) using LR clonase II (Invitrogen) to construct the expression vector pDEST14/990ATG. As a negative control, pDEST14/gus, a similar vector that contains the *E. coli* gus (β-glucuronidase) gene was also constructed.

Expression vectors were electroporated into *E. coli* BL21-AI, which can induce T7 promoter activity with arabinose. Single transformant colonies were transferred into 10 mL LB-carbenicillin (NaCl, 10 g/L; yeast extract, 5 g/L; and polypeptone, 10 g/L; carbenicillin [Wako, Osaka, Japan], 50 μg/mL) and incubated with shaking at 37°C to OD_{600} = 0.6. This pre-culture was then used to inoculate 100 mL LB-carbenicillin at OD_{600} = 0.03. Shaking at 25°C or 37°C, L-arabinose solution (20% w/v) was added to a final concentration of 0.2% (w/v), at OD_{660} = 0.3.

E. coli cells were collected by centrifugation, resuspended in 50 mM phosphate buffer (pH 7.0), and disrupted by glass beads (0.1 mm diameter) using a Multi-beads shocker (Yasui kikai, Osaka, Japan) at 4°C, 2500 rpm, 30 sec × 6 times, followed by separation into soluble (supernatant) and insoluble fractions (pellet) by centrifugation. Extracted proteins were visualized by 14% SDS-PAGE.

Further purification of the recombinant protease was attempted. Cell-free extract of *E. coli* BL21-AI/pDEST14/990ATG in 50 mM sodium phosphate buffer (pH 7.0) was prepared from 100 mL culture broth. This extract was then applied to a HiPrep DEAE FF 16/10 column (GE Healthcare) attached to an ÄKTA explorer 100 system (GE Healthcare) and flow-through fractions were recovered.

1 *Protease activity and protein concentration*

2 In a 1.5-mL plastic tube, enzyme samples (0.1 mL) were mixed with 0.9 mL skim milk (Morinaga
3 milk industries, Tokyo, Japan) solution (2% w/v in 50 mM phosphate buffer, pH 7.0) and incubated at
4 30°C. The reaction was stopped by cooling on ice, followed by centrifugation at 15,000 rpm for 2 min.
5 Free amino groups in the supernatant were quantified by ninhydrin colorimetry. Briefly, 250 µl of the
6 supernatant was mixed with 50 µl $SnCl_2$ solution (1.35% in 3 M sodium acetate buffer, pH 5.0) and 1 mL
7 ninhydrin solution (2% in 2-methoxyethanol:3 M sodium acetate buffer, pH 5.0 = 1:1) and boiled for 20
8 min. To this mixture, 2.5 mL 50% (v/v) 2-propanol was added, mixed, and cooled at room temperature.
9 A_{570} of the reaction mixture was measured and the amino group was quantified by using a leucine
10 standard. One unit (U) of protease activity was is defined as the amount of enzyme required to liberate 1
11 µmol of leucine per min. Protein concentration was determined by the method of Lowry et al. [18], with
12 the Protein Assay Lowry kit (Nacalai Tesque, Kyoto, Japan).

13

14 *Cd release from the scallop hepatopancreas*

15 The Cd release assay was performed as described previously [5]. Dried scallop hepatopancreas
16 powder was suspended into 50 mM Tris-HCl (pH 7.0) at 2% (w/v). A 1.8-mL aliquot of scallop
17 hepatopancreas suspension was mixed with 0.2 mL enzyme solution and incubated with gentle shaking at
18 50°C for 10 h. After centrifugation at 4,000 × *g* for 10 min, the supernatant was recovered and filtered
19 through a 0.45-µm filter. The Cd concentration in the supernatant was measured with a polarized Zeeman
20 atomic adsorption spectrophotometer Z-5310 (Hitachi Koki, Tokyo Japan).

21

22 *Crystallization and structural analyses*

23 Prior to the crystallization trials, the purified protein in buffer containing 10 mM Tris-HCl (pH 7.0)
24 and 20 mM NaCl was concentrated to 15 mg mL^{-1} on a Millipore centrifugal filter device (Amicon
25 Ultra-4, 10 kDa cutoff; Millipore, Bedford, MA). Screening for crystallization was performed using

1 Wizard Screen I and II (Emerald Biosciences, Bedford, MA), JCSG Core I–IV, MPD suite, and Classics
2 suite (Qiagen, Valencia, CA) by the sitting-drop vapor diffusion method in 96-well plates (SWISSCI
3 MRC 2 Well, Jena Bioscience, Jena, Germany). A 0.5-μl drop of sample was mixed with an equal volume
4 of reservoir solution and equilibrated against 0.1 mL reservoir solution at 20°C. Crystals were grown
5 from JCSG Core IV #47 (0.1 M HEPES, pH 7.5, 4.3 M sodium chloride). Several conditions were
6 screened further by the hanging-drop method using 24-well VDX plates (Hampton Research, Aliso Viejo,
7 CA) by mixing 1.5 μl protein solution and 1.5 μl reservoir to be equilibrated against 0.5 mL reservoir
8 solution at 20°C.
9 X-ray diffraction data were collected at beamline NE3A of the Photon Factory Advanced Ring
10 (PF-AR, Tsukuba, Japan) using an ADSC CCD detector Q210. Prior to data collection, crystals were
11 cryoprotected by transfer into a solution containing 32% (v/v) sucrose for a few seconds and flash-cooled.
12 The data set was integrated, merged, and scaled using HKL-2000 [19]. The structure was solved by the
13 molecular replacement method using the program Molrep [20]. The structure of NAALP from
14 *Nesterenkonia abyssinica* AL20 (PDBID: 3CP7) was used for the search model. Structure refinement was
15 carried out using Refmac5 [21]. The stereochemical properties of the structure were assessed by
16 Molprobity [22] and COOT [23] and showed no residues in the disallowed or generously allowed regions
17 of the Ramachandran plot. The final model comprises 219 residues and 294 water atoms.
18
19 *Construction of catalytic residue mutants*
20 pDEST14/990ATG was used as a template DNA for the formation of a catalytic residue mutant by
21 *Dpn*I mediated site-directed mutagenesis [24]. Three pairs of primers were designed to introduce the
22 mutation(s) (Table 2). Amplification was performed with pDEST14/990ATG, a pair of primers, and
23 KOD-plus DNA polymerase (Toyobo, Osaka, Japan) as instructed by the manufacturer. The amplified
24 DNA was purified with MicroSpin S-300 HR columns (GE Healthcare) and blunted and kinated with the
25 BKL kit (Takara, Otsu, Japan). After ethanol precipitation, DNA was self-ligated with T4 DNA ligase

1 (New England Biolabs). This DNA was digested with *Dpn*I (New England Biolabs) and used in
2 transformation of *E. coli* TOP10. After confirming the mutation, mutated vectors (pDEST14/S170A,
3 pDEST14/S171A, and pDEST14/S170,171A) were used in expression analyses.
4
5 *Digestion of various proteins*
6 Digestion of keratin was tested using keratin azure (Sigma, St. Louis, MO) as a substrate. In a
7 1.5-mL tube, 10 mg keratin azure, 1.0 mL of 50 mM phosphate buffer (pH 7.0), and 0.5 mL ANISEP
8 solution (1 mg/mL in the same buffer) were added and incubated at 50°C for 12 h. After centrifugation at
9 20,000 × *g* for 1 min, A_{595} of the supernatant was measured. One unit of keratinolytic activity was defined
10 as the amount of enzyme required to increase A_{595} by 0.01 per 30 min.
11 Digestion of other proteins was tested as follows. In a 1.5-mL tube, 0.9 mL of 0.2% protein solution
12 (hemoglobin from bovine blood [Sigma], casein [Sigma] or BSA [Nacalai Tesque] in 50 mM phosphate
13 buffer [pH7.0]) and 0.1 mL ANISEP solution (10 µg/mL in the same buffer) were added and incubated at
14 50°C for 15 min. A 0.6-mL aliquot of the reaction mixture was transferred to a new tube and mixed with
15 0.3 mL of 10% trichloroacetic acid and cooled on ice. After centrifugation at 20,000 × *g* for 3 min, 0.5 mL
16 supernatant was mixed with 2.5 mL of 0.5 M Na_2CO_3, followed by addition of 0.5 mL of 1 M
17 Folin-Ciocalteu reagent. The mixture was incubated at room temperature for 30 min; A_{660} was measured
18 and the amino group was quantified with a tyrosine standard. One unit of protease activity was defined as
19 the amount of enzyme that released 1 µg tyrosine per min.
20 In the assays using trypsin from the porcine pancreas (Wako), the buffer pH was 8.0, and reactions
21 were performed at 37°C. Heat-inactivated enzymes were used in the negative control (blank) reactions.
22
23 *Preparation of recombinant* Laternula elliptica *metallothionein-10a*
24 pET41-MT was introduced into *E. coli* BL21 star (DE3). A 10-mL aliquot of an overnight culture
25 of the transformant in LB containing kanamycin (50 µg/mL) was inoculated into 500 mL fresh medium

1 and shaken at 30°C to OD_{600} = 0.6, when 0.1 mM IPTG was added. Incubation was continued for 5 h.
2 Cells were harvested by centrifugation and resuspended in 15 mL binding buffer (140 mM NaCl, 2.7 mM
3 KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 5 mM dithiothreitol) and disrupted by glass beads (0.1-mm
4 diameter) using a Multi-beads shocker (Yasui kikai) at 4°C, 2500 rpm, 30 sec × 6 times. The soluble
5 fraction was recovered after centrifugation.
6 Affinity chromatography was performed with GSTrap HP columns (GE Healthcare) attached to
7 syringes according to manufacturer instructions. Fractions eluted with glutathione were recovered and
8 desalted on PD-10 columns (GE Healthcare).
9
10 *Reconstruction of Cd-MT-GST*
11 The MT-GST sample was acidified with 0.1 M HCl, mixed with 89 mM $CdCl_2$ in 0.1 M HCl to
12 achieve 6 molar equivalents of MT-GST, and incubated at room temperature for 16 h. An aliquot (0.1 mL)
13 was retained as the pre-neutralization sample. The rest of the Cd/MT-GST mixture was neutralized to pH
14 > 6.0 by dropwise addition of 0.2 M Tris base (neutralized sample). Ice-cold acetone (1 mL) was added to
15 0.1 mL aliquots of both samples and stored at -80°C for 1 h. After centrifugation at 20,000 × *g* for 15 min,
16 1 mL supernatant was recovered, vacuum-dried, and dissolved in 0.1 N HCl. Cd concentration was
17 measured on a polarized Zeeman atomic adsorption spectrophotometer Z-5310 (Hitachi Koki, Tokyo,
18 Japan). The remainder of the neutralized sample was acetone-precipitated, resuspended in 20 mM
19 Tris-HCl (pH 6.0) and stored as Cd-MT-GST.
20
21 *Cd release from Cd-MT-GST*
22 Cd-MT-GST (1 mL; 1 mg/mL) was mixed with protease solution (0.1 mL) and incubated for 12 h
23 at 50°C. Proteases (recombinant enterokinase [Novagen, Darmstadt, Germany], V8 protease [Wako], and
24 ANISEP) were dissolved in 50 mM phosphate buffer (pH 7.0) at 200 µg/mL. HCl (1 N) and MilliQ water
25 were used as positive and negative controls, respectively. Acetone precipitation and measurement of Cd in

the supernatant were performed as described for the Cd-binding assay. The pellets of the acetone precipitation were separated by SDS-PAGE.

Results

Cloning of the protease gene

ANISEP was prepared as described by Ren et al. [5], but the molecular mass was estimated to be 23 kDa, slightly smaller than the original report (27 kDa). We decided to use this newly prepared batch of purified protein for further analysis because it showed Cd-release activity similar to that in the original report (Fig. 2B), and the N-terminal sequence VNQSETPV was the same as the N-terminal sequence of the purified ANISEP in the original report [5]. The internal amino acid sequence of the purified ANISEP was determined after digestion with lysylendopeptidase or the protease itself. The identified sequences were ALYAPTQ, STVSTAGHN, HIGKIFFTLGG, TQGIPEN, and the N-terminal VNQSETPV. These sequences showed high similarity (70–100%) to the hypothetical protein Arth_1091 from *Arthrobacter* sp. FB24 (YP 830585). PCR amplification of the protease gene from *A. nicotinovorans* 23-0-11 using primers designed from the DNA sequence of Arth_1091 was not successful.

Amino acid sequence locations were assigned based on the similarity to Arth_1091, and the gene fragment corresponding to the ANISEP was PCR-amplified with the degenerate primers listed in Table 1. Among every combination of the primers expected for the amplification of protease gene fragment, two combinations, F2-R4 and F2-R5, resulted in successful amplification of DNA bands of the expected size. The F2-R5 fragment was cloned into pGEM-T easy and sequenced, because it includes F2-R4 fragment. The cloned insert consists of 467 bp encoding 154 amino acids and contains two internal sequences (STVSTAGH and ALYAPTQ) derived from the purified protease (Fig. 1A). No similarity was found between the nucleotide sequences of the F2-R5 fragment and the gene for hypothetical protein Arth_1091, but the amino acid sequence encoded by the fragment showed 80% similarity to Arth_1091. These results suggest the amplified DNA is a fragment of the gene encoding ANISEP.

1 In order to clone the full-length protease gene, an inverse PCR was performed with two inverse
2 primers designed in the F2-R5 fragment and *Sal*I-digested, self-ligated genomic DNA of *A.*
3 *nicotinovorans* 23-0-11. The inverse PCR amplified a 3.5-kb *Sal*I fragment containing the protease gene.
4 Based on the sequence of the inverse PCR fragment, two primers were designed, one 1000 bp upstream
5 of the deduced start codon and one downstream of a putative terminator with a stem-loop structure.
6 Amplification using this primer pair from the genomic DNA of *A. nicotinovorans* 23-0-11 yielded a
7 2.1-kb DNA band that was subsequently cloned into pENTR/D-TOPO and sequenced (Fig. 1A). BLAST
8 analysis of the nucleotide sequence of the 2.1-kb region revealed a partial sequence for the isocitrate
9 dehydratase-like sequence (*idh*) in the complementary strand of its 5´ region and an ORF encoding a
10 protease-like protein, containing all the internal amino acid sequences determined in ANISEP. The ORF
11 shared similarity with hypothetical proteins in the database, including Arth_1091 of *Arthrobacter* sp.
12 FB24 and AAur_1199 from *Arthrobacter aurescens* TC1. Alignment of these amino acid sequences
13 indicated the putative start codon is GTG located 333 bp upstream of the N-terminus of the purified
14 protease. The putative ANISEP gene is 993 bp long, encoding 330 amino acids. Following the stop codon,
15 a putative terminator with a stem-loop structure (Fig. 1A) was located. SignalP predicted cleavage of the
16 signal sequence between two alanine residues at positions 30 and 31, and the N-terminus of the mature
17 protease was found at position 112. These data indicate residues 1–30 and 31–111 correspond to Pre and
18 Pro sequences required for secretion and folding of the protease, respectively [25]. The molecular mass of
19 the mature enzyme was estimated to be 22.8 kDa, corresponding to the size of the purified protease (23
20 kDa). Alignment with two well-characterized serine proteases mpr1 [26] and NAALP [27] revealed that
21 the catalytic triad was conserved and consisted of His 57, Asp 102, and Ser 195 (Fig. 1B), indicating that
22 ANISEP belongs to Clan PA serine protease family [28]. Ser 195 was located in a GGSSG motif (residues
23 192–196), corresponding to the conserved motif of serine proteases, GXSYG (X and Y for any amino
24 acids). These results confirmed prior data indicating ANISEP is an extracellular serine protease.
25

Heterologous expression of the ANISEP in E. coli

In order to characterize the ANISEP further, heterologous expression of the cloned protease gene was attempted. The gene was expressed in *E. coli* by cloning the full-length ORF (993 bp) into pDEST14. The GTG start codon was changed to ATG for stable expression in *E. coli*. The expression vector pDEST14/990ATG was introduced into *E. coli* BL21-AI (Invitrogen). Expression at 25°C enabled the expression of the recombinant protease in the soluble fraction, with the same molecular mass as the mature protein (Fig. 2). The fraction exhibited proteolytic activity and Cd-releasing activity from the scallop hepatopancreas. The fraction was purified by chromatography. Collection of the flow-through fraction of HiPrep 16/10 DEAE FF (GE Healthcare) increased the specific activity to 2.0 U/mg, but further purification by gel filtration was not successful, probably due to autolysis of the protein. Expression of the protein with other modified methods such as expression in mature form or addition of tags for affinity purification resulted in low expression or abnormal folding. However, the features of the recombinant protein described above have proven that the cloned gene corresponded to ANISEP.

Crystallization and structural analysis

To analyze the structural basis for the digestion of scallop hepatopancreas proteins by ANISEP, crystallization of the purified protein from *A. nicotinovorans* was attempted. The best crystallization condition for ANISEP was 0.1 M HEPES pH 7.0, 4.1 M sodium chloride, and 4% sucrose (Fig. 3). The crystal diffracted up to 1.6 Å. The ANISEP crystal belonged to space group *I*23, with unit-cell parameters $a = b = c = 103.02$ Å. Based on the value of the Matthews coefficient (V_M) [29], it was estimated that there was one molecule in the asymmetric unit with $V_M = 1.98$ A^3/Da (Vsolv = 38.0%). Details of the data collection and processing statistics are given in Table 3.

The structure of ANISEP was similar to that of NAALP from *Nesterenkonia abyssinica* AL20 (PDBID: 3CP7, superimposed with the root mean square deviation (rmsd) value of 0.89Å using 206 Cα atoms), which shares 50.1% sequence identity with ANISEP and strong activity toward hemoglobin and

keratin, as well as casein (Fig. 3c) [9,27]. ANISEP was screened for its strong ability to release Cd from the scallop hepatopancreas. These proteases might have similar enzymatic features. Structural similarity indicated that the catalytic residues of ANISEP are Ser170 or Ser171. The catalytic residue was further investigated by replacement with alanine. Mutations were introduced by *Dpn*I cloning into pDEST14/990ATG. The resulting three vectors (pDEST14/S170A, pDEST14/S171A and pDEST14/S170,171A), positive control vector pDEST14/990ATG, and negative control pDEST14/gus were introduced into *E. coli* BL210AI. Protein was extracted from each cell free-extract and semi-purified by collecting the flow-through fraction by anion exchange column chromatography (HiTrapQ FF). Protease activity in the eluted fractions is shown in Fig. 4. S170A protease activity was comparable to that of the original recombinant protein, but S171A and S170,171A proteases had no activity. Therefore, the catalytic residue of ANISEP was identified as Serine 171.

Digestion of various proteins by ANISEP

As shown above, ANISEP has a structure similar to that of NAALP from *N. abyssinica* AL20, which exhibits strong activity toward hemoglobin and keratin, as well as casein. In order to determine whether ANISEP has similar proteolytic activity, purified ANISEP was used to digest hemoglobin and keratin-azure, in addition to casein and BSA. ANISEP exhibited similar hydrolytic activity against casein and BSA in comparison to porcine trypsin, a representative protease of the Clan PA family [28]. However, ANISEP showed significantly higher activity on hemoglobin and keratin-azure than trypsin (Fig. 5). Keratinolytic activity of ANISEP was about 10-fold higher than that of trypsin.

No Cd release activity against recombinant metallothionein-10a from L. elliptica

Heavy metals in bivalves are known bound to metallothionein, a family of cysteine-rich, low-molecular-weight proteins. The specific Cd-release activity of ANISEP may be due to the substrate specificity for metallothioneins. To test this, a Cd-release assay using pure metallothioneins would be

ideal; however, there are no data on the molecular weight or metal chelating ability of scallop metallothioneins. A metallothionein gene was cloned from bivalve *L. elliptica* and expressed in *E. coli* [11]. The same recombinant protein was expressed and used as a model metallothionein substrate in this study.

The vector pET41-MT, which contains the gene for metallothionein MT-10a from *L. elliptica* fused to the C terminal of glutathione *S*-transferase (GST), was introduced into *E. coli* BL21 Star (DE3). The GST-fused metallothionein MT-10 (GST-MT) protein was recovered from the cell-free extract after IPTG induction followed by affinity chromatography using GSTrap HP (Fig. 6). Further recovery of MT-10 by recombinant enterokinase (rEK) was unsuccessful, probably due to the undesirable digestion of MT-10 by rEK.

Cd binding was attempted using GST-MT, by releasing metals bound during recombinant expression in 0.1 M HCl followed by neutralization with excess Cd. The amount of absorbed Cd was estimated to be 38.4 μg/mg protein by comparing the free Cd concentration before and after the Cd-binding assay. Cd binding by GST was negligible, because no absorption was detected when pure GST was used in the same assay (data not shown).

Cd-bound GST-MT was then used as a substrate for the Cd-release assay with ANISEP. After incubation at 50°C for 12 h, HCl treatment released detectable amounts of Cd. Almost no Cd was released by digestion with ANISEP, although SDS-PAGE indicated effective digestion of the same amount of GST-MT by ANISEP as a faint protein band (Fig. 6). These results suggest Cd release from the scallop hepatopancreas by ANISEP may not be due to substrate specificity for the metallothionein.

Discussion

ANISEP from *A. nicotinovorans* has a structure similar to that of NAALP from *Nesterenkonia* sp. AL20. From the structural feature revealed by X-ray crystallography, these proteases belong to clan PA, trypsin-like serine proteases [28]. The sequence similarity of these proteases is 50.1%.

Nesterenkonia sp. AL 20 was identified as a feather-degrading microorganism from a natural alkaline environment [8]. *A. nicotinovorans* 23-0-11 was isolated from soil for its Cd-releasing activity in the scallop hepatopancreas [5]. The producing organisms are both Micrococcaceae in the Actinobacteria.

In addition to their similar structures, the proteases are also Ca^{2+} independent in their activity and stability. Both showed strong keratinolytic activity. They also exhibited some differences. Sensitivity to PMSF is not conserved in NAALP [8]. Activity and stability under varying pH and temperatures also differ; NAALP is more stable and active at higher pH and temperature [8].

The most important feature revealed in this study of ANISEP is its high keratinolytic activity. Most keratinolytic proteases (keratinases) are serine proteases, and several keratinases have been identified from *Streptomyces* [30]. Although the molecular characteristics of these keratinases have not been defined, with the exception of one from *Streptomyces fradiae* [31], there may be a specific group of trypsin-like, keratinolytic serine proteases in Actinobacteria.

ANISEP degraded recombinant metallothionein MT-10 from *L. elliptica*, but did not release Cd bound in the metallothionein (Fig. 5), whereas the enzyme effectively released Cd from the scallop hepatopancreas. One possible explanation for this is that the binding status of Cd to metallothionein differs between *L. elliptica* and *P. yessoensis*. ANISEP cannot digest the critical residue for Cd binding in the *L. elliptica* metallothinein, but can digest it in *P. yessoensis* metallothionein. We could not test this hypothesis because molecular information about metallothionein in *P. yessoensis* is not available. In addition, the fact that primary structure and the Cd-binding properties of metallothioneins are conserved between species should be considered [10]. Another possibility is that Cd binds metallothioneins and other keratin-like, digestion-resistant proteins in *P. yessoensis*. If the binding affinity for Cd is higher in the latter, unknown proteins than in the former, the explanation might be convincing. Unfortunately, such proteins have not been identified, but their existence is suggested by studies on the molecular mass of Cd-binding proteins obtained from scallop hepatopancreas specimens with naturally occurring Cd contamination [32]. Further studies on Cd-binding proteins in Japanese scallop are necessary to

characterize the mechanism of Cd removal by ANISEP.

Conclusion

This study revealed the structure of ANISEP and its high keratinolytic activity, which may related to the Cd released from the scallop hepatopancreas. Further structural studies on substrate specificity and the thermodynamic profile will help elucidate the Cd-removal mechanism and inform enzymatic modifications for applications in the microbial removal of Cd from fishery byproducts obtained from scallops and other mollusks.

Abbreviations

Cd = cadmium

Cd-MT-GST = Cd-bound MT-GST

MTs = metallothioneins

MT-GST = metallothionein MT-10 fused to glutathione-S-transferase tag

PCR = polymerase chain reaction

PMSF = phenylmethylsulfonyl fluoride

PVDF = Polyvinylidene difluoride

rmsd = root mean square deviation

SDS-PAGE = sodium dodecyl sulfate – polyacrylamide gel electrophoresis

1

Conflict of interest

3 The authors confirm they have no conflicts of interest to declare.

4

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7 vector pET41-MT.

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9

10

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14
15 Received: June 26, 2014 Revised: August 26, 2014 Accepted: September 11, 2014

Figure Legends

Fig. 1. Structure of the ANISEP gene. A, Structure of the 2.1-kb DNA containing the ANISEP gene. Numbers above and below the horizontal line indicate position in bp and amino acid residues in the protease, respectively. The solid box indicates the partial gene encoding *idh.* A terminator-like downstream structure is indicated as a stem-loop. The open box indicates the ANISEP gene. Amino acid regions corresponding to Pre, Pro, and the mature ANISEP are indicated. The striped bar indicates the degenerate-PCR amplified fragment F2-R5, and arrows indicate the positions of inverse primers. Assignment of amino acid sequences revealed by sequencing is shown by speech balloons. B, Alignment of ANISEP, NAALP, and MPR1 flanking the catalytic triad. Numbers following m indicate the position of the N-terminal amino acid residue in each region, in the mature form. Catalytic residues are indicated by **bold** letters.

Fig. 2. Heterologous expression of ANISEP in *E. coli*.

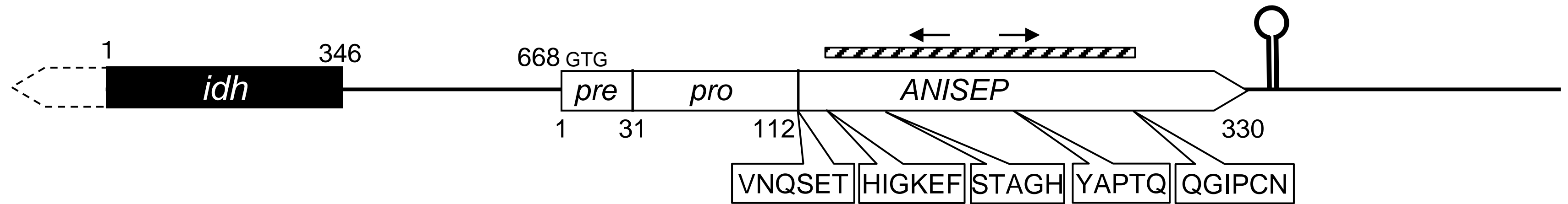
A, SDS-PAGE of crude protein. Soluble and insoluble fractions of *E. coli* harboring pDEST14/990ATG and the control vector pDEST14/GUS were analyzed. Sizes of the GUS protein and mature ANISEP are indicated on the right. B, Protease activity (black bars, left axis) and Cd-release activity (white bars, right axis) of purified native, crude recombinant, and semi-purified recombinant ANISEP. All data are presented as the average of duplicate experiments.

Fig. 3. Crystallization and structural analysis of ANISEP

A, Crystals of ANISEP. B, Structure of ANISEP, solved by X-ray crystallography, presented in rainbow color with the N- and C-termini as red and purple, respectively. C, Superimposed structures of ANISEP (yellow) and *N. abyssinica* NAALP (green).

1 **Fig. 4.** Identification of the catalytic residue by directed point mutation. SDS-PAGE (A) and relative
2 protease activity (B) of uninduced, negative control (pDEST14/gus), native (pDEST14/990ATG), and
3 mutants S170A, S171A, and S170171A are shown. All data are presented as the average of duplicate
4 experiments.
5
6 **Fig. 5.** Proteolytic activity of ANISEP with various substrates. Protease-specific activity (left axis, to
7 casein, hemoglobin, and BSA) and keratinolytic activity (right axis, to keratin azure) of ANISEP and
8 trypsin are shown. All data are presented as the average of duplicate experiments with standard deviation.
9
10 **Fig. 6.** Cd binding and release of recombinant metallothionein MT-10 of *L. elliptica.* SDS-PAGE of
11 cell-free extract of recombinant *E. coli* expressing MT-10 fused to GST (cell-free extract), eluted fraction
12 of GST-affinity column chromatography (eluted fraction) are shown on the left side. The right side of the
13 panel represents SDS-PAGE of protein samples after the Cd-binding/release assay of MT-GST (MT-10
14 fused to GST). Cd released after treatment is shown below each lane. 1 N HCl, acid denaturation by 1N
15 HCl; rEK, recombinant enterokinase digestion; V8 protease, V8 protease digestion; ANISEP, ANISEP
16 digestion.

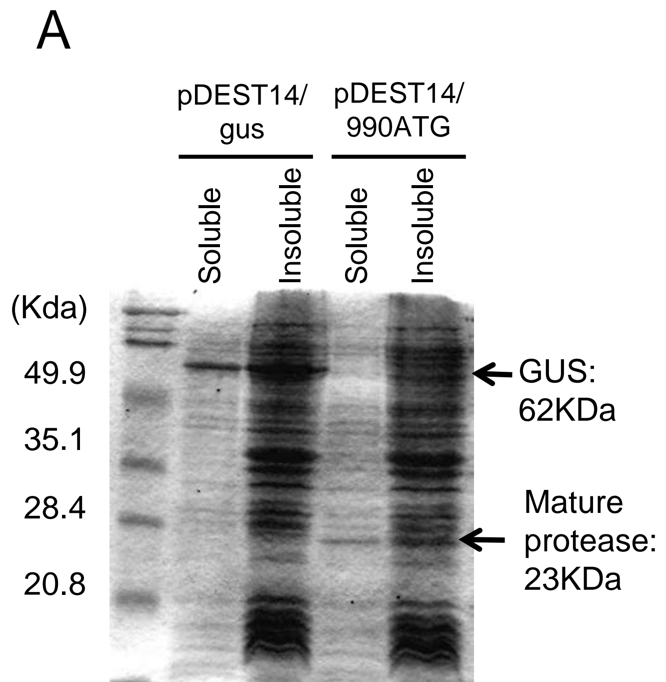
A

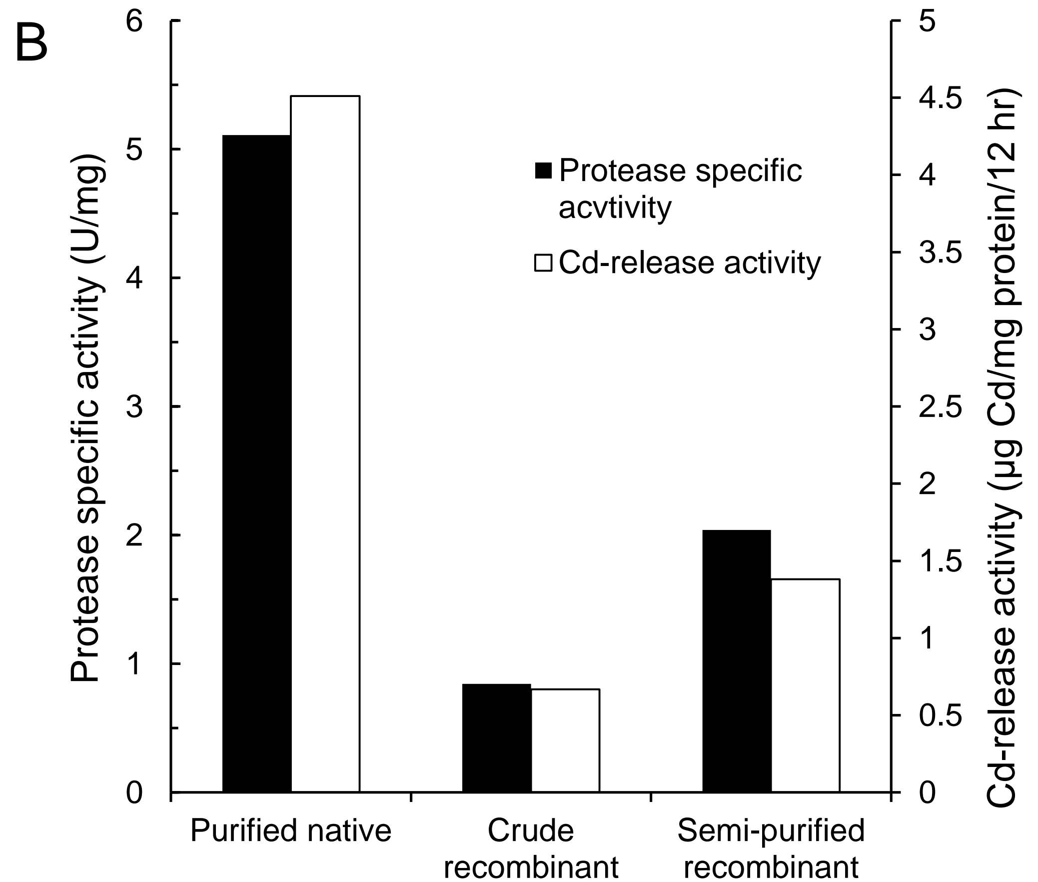


B

	His[57] region		Asp[102] region		Ser[195] region	
ANISEP	m37	TVSTAG**H**CLN	m88	NMQY**D**TAFA	m166	NMTGGSS**S**GGP
NAALP	m35	TVATAG**H**CLH	m87	DFEH**D**YAFA	m163	NMTGGSS**S**GGP
MPR1	m47	TVVTAG**H**CVY	m97	DTNY**D**YGAI	m173	-----Q**S**GSP

Sone – Fig. 1

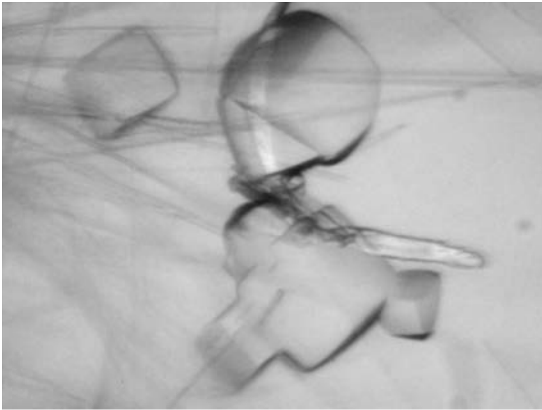




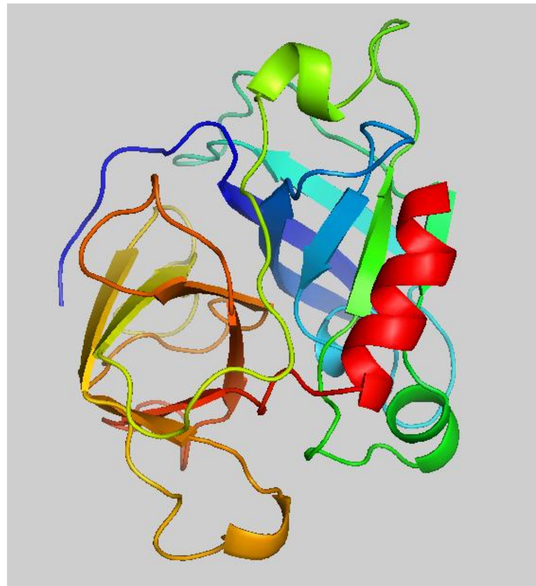
Sone – Fig. 2

COLOR Printing

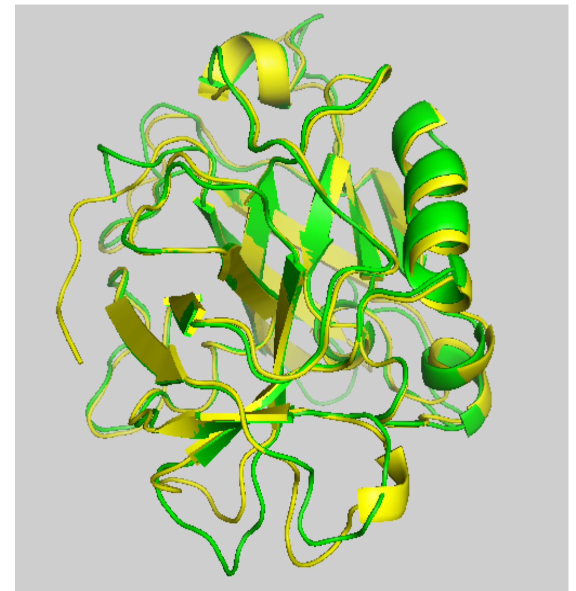
A



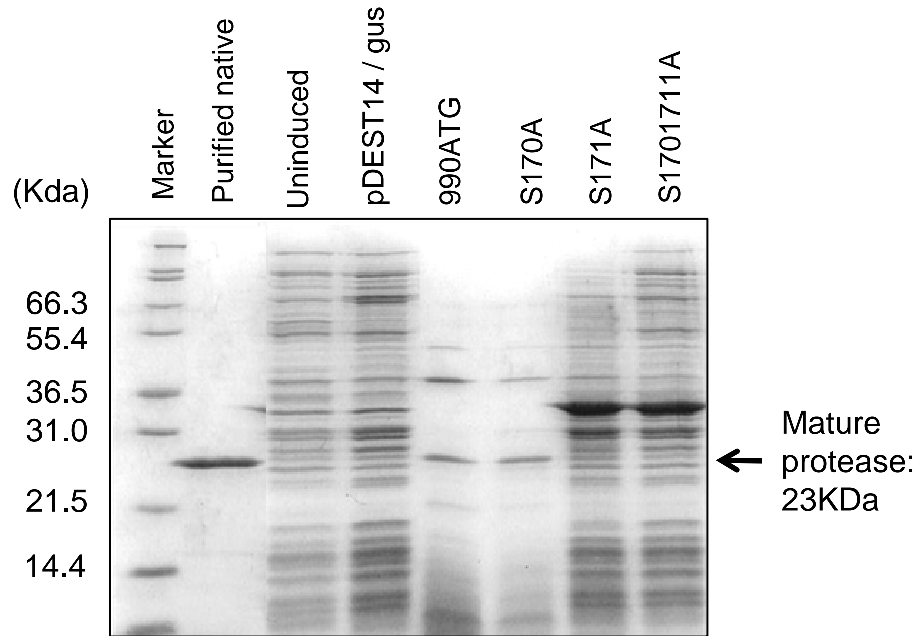
B

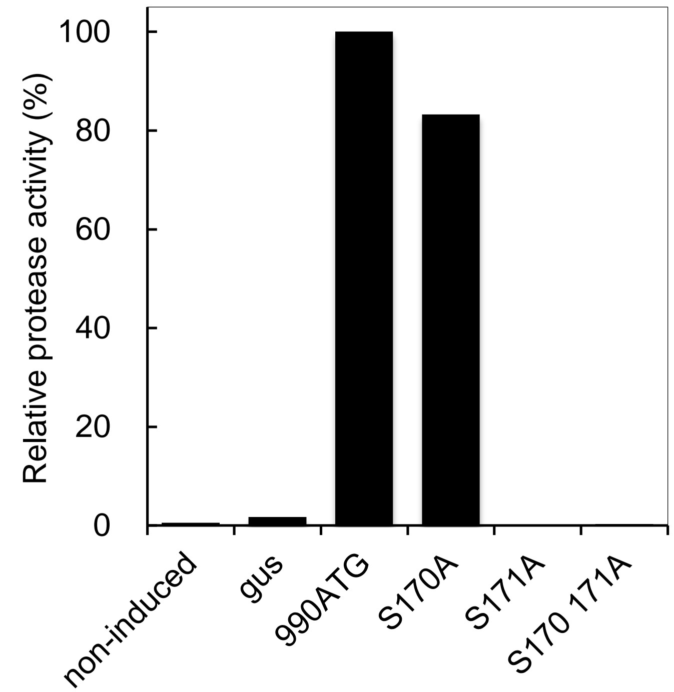


C

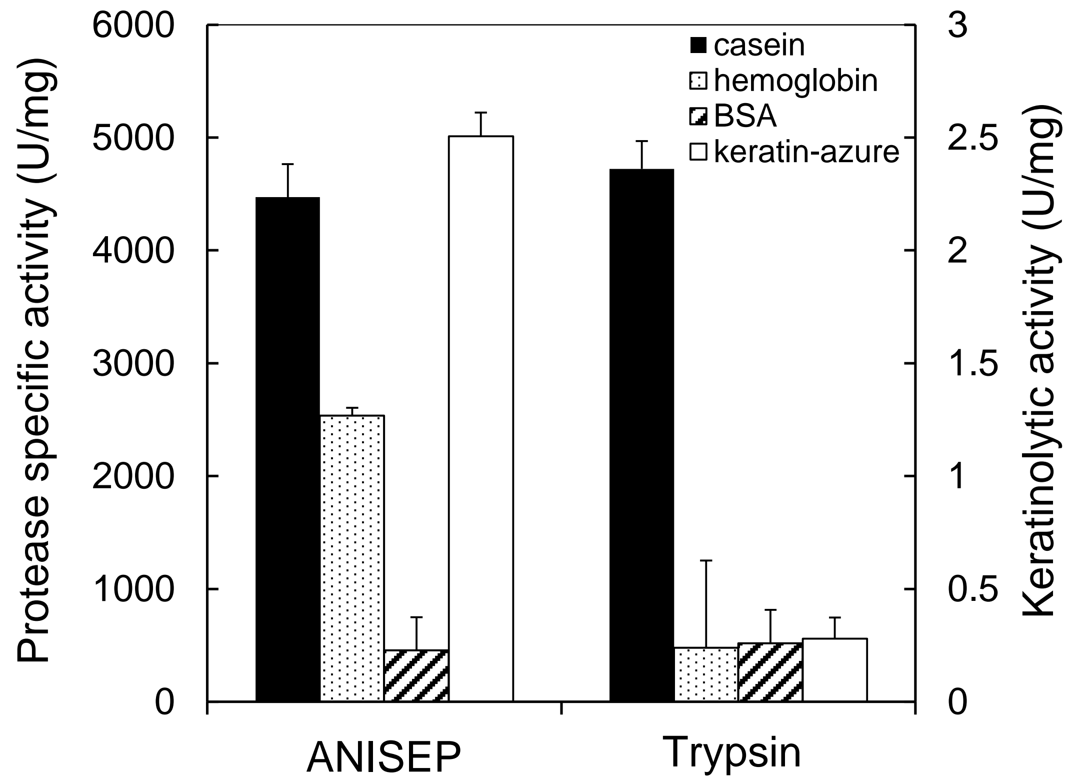


Sone – Fig. 3

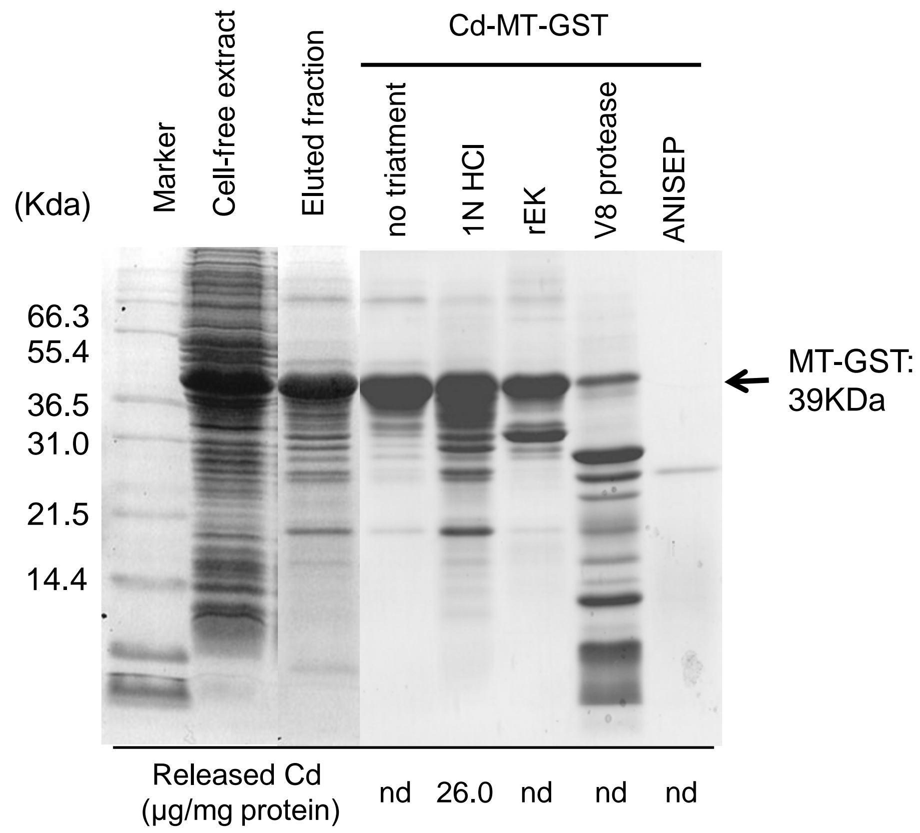




Sone – Fig. 4



Sone – Fig. 5



Sone – Fig. 6

Table 1. Partial amino acid sequences and degenerate primers

Primer	Amino acid sequence	Nucleotide sequence (5´ - 3´)	Degeneracy
F1	VNQSET	GTN AAY CAR WSY GAR AC	256
F2	HIGKFF	CAY ATH GGN AAR ATH TTY TT	288
F3	STAGHN	ACN GCN GGN CAY AAY GA	256
R3	STAGHN	TCR TTR TGN CCN GCN GT	256
F4	YAPTQ	TAY GCN CCN ACN CA	128
R4	YAPTQ	TGN GTN GGN GCR TA	128
R5	QGIPEN	TTY TCN GGD ATN CCY TG	192

Table 2. Data statistics for X-ray structure analysis.

PDB ID	3WY8
Data colleciton	PF-AR NE3A
Wavelength (Å)	1.000
Resolution range (Å)	50-1.70 (1.73-1.70)
Space group	*I*23
Unit-cell parameters (Å)	*a* = *b*= c= 103.02
No. of observations	447140
No. of unique reflections	20158 (1001)
Completeness (%)	100.0 (100.0)
Multiplicity	22.2 (22.0)
Averaged I/σ (I)	44.9(7.2)
R_{merge} [a]	0.076 (0.500)
Wilson B factor ($Å^2$)	20.68
Refinement	
Protein atoms	1606
water atoms	294
Resolution range (Å)	50-1.70 (1.74-1.70)
R_{work} [b]	0.142 (0.188)
R_{free} [c]	0.182 (0.230)
R. m. s. deviation	
Bond lengths (Å)	0.006
Bond angles (˚)	1.04

Values in the parentheses are for the highest resolution shell.

[a] $R_{merge}=\Sigma_h\Sigma_j|<I>_h-I_{h,j}|/\Sigma_h\Sigma_j I_{h,j}$, where $<I>_h$ is the mean intensities of symmetry-equivalent reflections.

[b] $R_{work}=\Sigma_h|F_o-F_c|/\Sigma_h F_o$, where F_o and F_c are the observed and calculated structure factor amplitudes respectively.

[c] R_{free} value was calculated for R factor, using only a test set of reflections (5% of the total) not used in the refinement.

Table 3. Primers for PCR mutagenesis

Mutation	Primer name	Nucleotide sequence (5´ - 3´)
S170A	990ATG_S170A-F	GCC TCG GGC GGT CCG TGG
	990ATG_S170A-R	ACC ACC GGT CAT GTT GCA GGG
S171A	990ATG_S171A-F	GCC GGC GGT CCG TGG TTC A
	990ATG_S171A-R	GGA ACC ACC GGT CAT GTT GCA GG
S170, 171A	990ATG_S170, 171A-F	GCC GCC GGC GGT CCG TGG TTC
	990ATG_S170A-R	ACC ACC GGT CAT GTT GCA GGG