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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
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21 protease

1 Abstract

 $\mathbf{2}$ 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 $\mathbf{5}$ 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 (31-7111) 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 12Laternula elliptica, but did not release Cd. These results suggest ANISEP is a trypsin-like serine protease 13that can cause Cd release from the Japanese scallop hepatopancreas because of its strong keratinolytic 14activity.

1 Introduction

2	Japanese scallop, Patiopecten yessoensis, is an important bivalve in Japanese fisheries. The total
3	annual scallop production, including sea catch and aquaculture, is greater than 400,000 tons. Processing
4	includes removal of the hepatopancreas, which is rich in protein and is thus used for animal feed and
5	fertilizers; however, this organ often contains toxic heavy metals such as cadmium (Cd). Therefore, safe
6	and effective utilization of the hepatopancreas requires the elimination of toxic heavy metals.
7	Heavy metals are usually bound with proteins in the hepatopancreas and are not removed by
8	washing or other simple mechanical methods. Several Cd-removal processes have been proposed, such as
9	treatment with H ₂ SO ₄ , but acid treatment reduces protein quality and quantity, and accelerates corrosion
10	of processing equipment [1]. The competitive adsorption method involving insoluble humic acid enables
11	the removal of Cd from the scallop hepatopancreas under mild acidic conditions (pH 5.0) [2]. A
12	subcritical water treatment was recently proposed, but it requires high temperatures and yields insoluble
13	Cd-contaminated waste [3]. An alternative microbial process uses the Gram-negative bacterium
14	Xanthomonas sp. as a specific adsorptive agent for Cd, but protease digestion is required to release Cd
15	from the hepatopancreas [4]. Subsequently, Ren et al. isolated a bacterium capable of releasing Cd from
16	the hepatopancreas; the responsible protease was purified, characterized [5]. This enzyme, designated
17	ANISEP (<u>Arthrobacter nicotinovorans serine protease</u>), releases Cd more effectively than commercial
18	enzymes at neutral pH and should therefore be suitable for enzymatic Cd release before bacterial
19	adsorption. ANISEP was inhibited strongly by PMSF (phenylmethylsulfonyl fluoride), indicating that it is
20	a serine protease [5].
21	One of the most well-studied microbial serine proteases is the subtilisin-like protease. These
22	proteases are generally secreted to scavenge extracellular nutrients. Various Bacillus species produce
23	enzymes of this class [6]. Most of them exhibit maximal activity at alkaline pH and are thus considered
24	the most important group of commercial proteases for use as laundry detergents [6]. Subtilisin-like
25	proteases are known for broad substrate specificity, although there are a few exceptions [7]. Recently, a

1 novel alkaline protease from Nesterenkonia sp, named NAALP, was found to show strong keratinolytic $\mathbf{2}$ activity. This enzyme is capable of degrading feathers and has potential for application in feather meal 3 production [8,9]. Keratinolytic protease might be effective for Cd release from the scallop 4 hepatopancreas. $\mathbf{5}$ The most well-known heavy metal-binding proteins in marine animals are the metallothioneins 6 (MTs). MTs are low-molecular-weight, cysteine-rich, metal-binding proteins [10]. Reports on the 7heterologous expression, purification, and Cd-binding nature of bivalve MTs suggest they are the most 8 likely candidates for Cd accumulation in P. yessoensis [11,12]. Additional reports of bivalve MT genes 9 suggest their expression is induced upon exposure to Cd and/or environmental contaminates such as 10 heavy metals [13-17]. However, the MTs in P. yessoensis have not been characterized and the molecular 11 mechanism of Cd release by ANISEP remains unclear. 12The long-term objective of this study was to characterize how ANISEP mediates Cd release from 13the scallop hepatopancreas. We must first understand the enzyme's primary and higher structure to 14determine the molecular basis of its activity and substrate specificity. We cloned the protease gene, 15analyzed the structure of the purified protease, and performed digestion assays with recombinant clam 16 metallothionein MT-10a [11]. 1718 **Materials and Methods** 19Bacterial strains and vectors 20Arthrobacter nicotinovorans 23-0-11 (AHU1956, [5]) was inoculated from a stock culture. 21Escherichia coli strains TOP10, BL21-AI, and BL21-Star (DE3) and the vectors pENTR-SD-D-TOPO 22and pDEST14 were purchased from Invitrogen (Carlsbad, CA). The expression vector pET41-MT 23contains MT-10a fused to GST and was a gift from Dr. Hyun Park (Korea Polar Research Institute, South

24 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 ₂ HPO ₄ , 1 g/L; MgSO ₄ ·7H ₂ O, 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).

1 Degenerate PCR

2	Degenerate PCR was performed with seven primers designed according to internal N-terminal
3	sequences (Table 1). Reaction mixtures contained 500 pmol primers, 100 ng A. nicotinovorans 23-0-11
4	DNA, 2.5 U Amplitaq DNA polymerase (Applied Biosystems), 5 μ l of 10× PCR buffer, 5 μ l of 2 mM
5	dNTP mix, and 3.5 μ l of 25 mM MgCl ₂ in a total volume of 50 μ l. Cycling conditions were as follows:
6	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.
7	
8	Cloning of the protease gene
9	A. nicotinovorans DNA was digested with SalI (Takara Bio, Ohtsu, Japan) and self-ligated with T4
10	DNA ligase (New England Biolabs, Beverly, MA). A pair of inverse PCR primers (InverseF-2-1:
11	5'-CCTTCGCCGTCATGAACAC-3' and InverseR-2-1: 5'-CCACTTCCCGTAGGGTGCT-3') was
12	designed inside the degenerate PCR fragment. PCR was performed with the Expand long template PCR
13	system (Roche, Mannheim, Germany) according to manufacturer instructions. The 3.4-kb amplicon was
14	cloned into pGEM-T easy (Promega, Madison, WI). PCR amplification of the protease gene from A.
15	nicotinovorans 23-0-11 DNA was performed with another pair of primers (Forward:
16	5'-CACCTGTCCGAGGAGGGGTTGTC-3', Reverse; 5'-CGGCATCACGATGTACTTCAC-3'),
17	designed to amplify a 2.0-kb fragment containing the full-length deduced ORF for the protease, its
18	promoter and terminator, and KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The amplified DNA
19	was cloned into pENTR-D-TOPO (Invitrogen) according to manufacturer instructions.
20	
21	DNA sequencing and sequence analysis
22	DNA sequencing reactions were performed with the BigDye terminator 1.1 cycle sequencing kit
23	(Applied Biosystems). Sequencing products were purified with Performa DTR Gel Filtration cartridges
24	(EdgeBio, Gaithersburg, MD) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied
25	Biosystems). Genetyx software (Genetyx, Tokyo Japan) was used to analyze the DNA and protein

1

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

 $\mathbf{2}$

3 Expression of ANISEP in E. coli 4 Primers cacc990ATG-F (5'-CACCATGACAAAAACCAAGACCCTG-3') and ORF-r $\mathbf{5}$ (5'-ACATCAGTTACGAAGCCGCA-3') were to construct the expression vector. After PCR 6 amplification to generate a modified start codon with the above primers, the protease gene was cloned 7 into pENTR/SD/D-TOPO (Invitrogen). The resulting plasmid, pENTR/SD/990ATG, was then used for 8 the insert exchange reaction with pDEST14 (Invitrogen) using LR clonase II (Invitrogen) to construct the 9 expression vector pDEST14/990ATG. As a negative control, pDEST14/gus, a similar vector that contains 10 the *E. coli* gus (β-glucuronidase) gene was also constructed. 11 Expression vectors were electroporated into E. coli BL21-AI, which can induce T7 promoter 12activity with arabinose. Single transformant colonies were transferred into 10 mL LB-carbenicillin (NaCl, 1310 g/L; yeast extract, 5 g/L; and polypeptone, 10 g/L; carbenicillin [Wako, Osaka, Japan], 50 µg/mL) and 14incubated with shaking at 37° C to OD₆₀₀ = 0.6. This pre-culture was then used to inoculate 100 mL 15LB-carbenicillin at OD₆₀₀ = 0.03. Shaking at 25°C or 37°C, L-arabinose solution (20% w/v) was added to 16a final concentration of 0.2% (w/v), at $OD_{660} = 0.3$. 17E. coli cells were collected by centrifugation, resuspended in 50 mM phosphate buffer (pH 7.0), 18 and disrupted by glass beads (0.1 mm diameter) using a Multi-beads shocker (Yasui kikai, Osaka, Japan) 19at 4° C, 2500 rpm, 30 sec × 6 times, followed by separation into soluble (supernatant) and insoluble 20fractions (pellet) by centrifugation. Extracted proteins were visualized by 14% SDS-PAGE. 21Further purification of the recombinant protease was attempted. Cell-free extract of E. coli 22BL21-AI/pDEST14/990ATG in 50 mM sodium phosphate buffer (pH 7.0) was prepared from 100 mL 23culture broth. This extract was then applied to a HiPrep DEAE FF 16/10 column (GE Healthcare) 24attached 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 μ l 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	A570 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	μ mol 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	DpnI 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

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 DpnI (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	$20,000 \times g$ 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 μ g/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 $20,000 \times g$ 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 $1 \ \mu g$ 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 μ g/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 ₂ PO ₄ , 1.8 mM KH ₂ PO 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 $20,000 \times g$ 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 μ g/mL. HCl (1 N) and MilliQ water

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.

3

4 **Results**

5 Cloning of the protease gene

6 ANISEP was prepared as described by Ren et al. [5], but the molecular mass was estimated to be 7 23 kDa, slightly smaller than the original report (27 kDa). We decided to use this newly prepared batch of 8 purified protein for further analysis because it showed Cd-release activity similar to that in the original 9 report (Fig. 2B), and the N-terminal sequence VNQSETPV was the same as the N-terminal sequence of 10 the purified ANISEP in the original report [5]. The internal amino acid sequence of the purified ANISEP 11 was determined after digestion with lysylendopeptidase or the protease itself. The identified sequences 12were ALYAPTQ, STVSTAGHN, HIGKIFFTLGG, TQGIPEN, and the N-terminal VNQSETPV. These 13sequences showed high similarity (70–100%) to the hypothetical protein Arth_1091 from Arthrobacter sp. 14FB24 (YP 830585). PCR amplification of the protease gene from A. nicotinovorans 23-0-11 using 15primers designed from the DNA sequence of Arth_1091 was not successful. 16 Amino acid sequence locations were assigned based on the similarity to Arth_1091, and the gene 17fragment corresponding to the ANISEP was PCR-amplified with the degenerate primers listed in Table 1. 18 Among every combination of the primers expected for the amplification of protease gene fragment, two 19combinations, F2-R4 and F2-R5, resulted in successful amplification of DNA bands of the expected size. 20The F2-R5 fragment was cloned into pGEM-T easy and sequenced, because it includes F2-R4 fragment. 21The cloned insert consists of 467 bp encoding 154 amino acids and contains two internal sequences 22(STVSTAGH and ALYAPTO) derived from the purified protease (Fig. 1A). No similarity was found 23between the nucleotide sequences of the F2-R5 fragment and the gene for hypothetical protein Arth_1091, 24but the amino acid sequence encoded by the fragment showed 80% similarity to Arth_1091. These results 25suggest 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 SalI-digested, self-ligated genomic DNA of A.
3	nicotinovorans 23-0-11. The inverse PCR amplified a 3.5-kb SalI 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.

Heterologous expression of the ANISEP in E. coli

2	In order to characterize the ANISEP further, heterologous expression of the cloned protease gene
3	was attempted. The gene was expressed in <i>E. coli</i> by cloning the full-length ORF (993 bp) into pDEST14.
4	The GTG start codon was changed to ATG for stable expression in E. coli. The expression vector
5	pDEST14/990ATG was introduced into E. coli BL21-AI (Invitrogen). Expression at 25°C enabled the
6	expression of the recombinant protease in the soluble fraction, with the same molecular mass as the
7	mature protein (Fig. 2). The fraction exhibited proteolytic activity and Cd-releasing activity from the
8	scallop hepatopancreas. The fraction was purified by chromatography. Collection of the flow-through
9	fraction of HiPrep 16/10 DEAE FF (GE Healthcare) increased the specific activity to 2.0 U/mg, but
10	further purification by gel filtration was not successful, probably due to autolysis of the protein.
11	Expression of the protein with other modified methods such as expression in mature form or addition of
12	tags for affinity purification resulted in low expression or abnormal folding. However, the features of the
13	recombinant protein described above have proven that the cloned gene corresponded to ANISEP.
14	
15	Crystallization and structural analysis
16	To analyze the structural basis for the digestion of scallop hepatopancreas proteins by ANISEP,
17	crystallization of the purified protein from A. nicotinovorans was attempted. The best crystallization
18	condition for ANISEP was 0.1 M HEPES pH 7.0, 4.1 M sodium chloride, and 4% sucrose (Fig. 3). The
19	crystal diffracted up to 1.6 Å. The ANISEP crystal belonged to space group I23, with unit-cell parameters
20	a = b = c = 103.02 Å. Based on the value of the Matthews coefficient (V _M) [29], it was estimated that
21	there was one molecule in the asymmetric unit with $V_{\rm M} = 1.98 \text{ A}^3/\text{Da}$ (Vsolv = 38.0%). Details of the data
22	collection and processing statistics are given in Table 3.
23	The structure of ANISEP was similar to that of NAALP from Nesterenkonia abyssinica AL20
24	(PDBID: 3CP7, superimposed with the root mean square deviation (rmsd) value of 0.89Å using 206 C α

1	keratin, as well as casein (Fig. 3c) [9,27]. ANISEP was screened for its strong ability to release Cd from
2	the scallop hepatopancreas. These proteases might have similar enzymatic features. Structural similarity
3	indicated that the catalytic residues of ANISEP are Ser170 or Ser171. The catalytic residue was further
4	investigated by replacement with alanine. Mutations were introduced by DpnI cloning into
5	pDEST14/990ATG. The resulting three vectors (pDEST14/S170A, pDEST14/S171A and
6	pDEST14/S170,171A), positive control vector pDEST14/990ATG, and negative control pDEST14/gus
7	were introduced into E. coli BL210AI. Protein was extracted from each cell free-extract and semi-purified
8	by collecting the flow-through fraction by anion exchange column chromatography (HiTrapQ FF).
9	Protease activity in the eluted fractions is shown in Fig. 4. S170A protease activity was comparable to
10	that of the original recombinant protein, but S171A and S170,171A proteases had no activity. Therefore,
11	the catalytic residue of ANISEP was identified as Serine 171.
12	
13	Digestion of various proteins by ANISEP
14	As shown above, ANISEP has a structure similar to that of NAALP from <i>N. abyssinica</i> AL20,
14 15	As shown above, ANISEP has a structure similar to that of NAALP from <i>N. abyssinica</i> AL20, which exhibits strong activity toward hemoglobin and keratin, as well as casein. In order to determine
14 15 16	As shown above, ANISEP has a structure similar to that of NAALP from <i>N. abyssinica</i> 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
14 15 16 17	As shown above, ANISEP has a structure similar to that of NAALP from <i>N. abyssinica</i> 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
14 15 16 17 18	As shown above, ANISEP has a structure similar to that of NAALP from <i>N. abyssinica</i> 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,
14 15 16 17 18 19	As shown above, ANISEP has a structure similar to that of NAALP from <i>N. abyssinica</i> 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).
 14 15 16 17 18 19 20 	As shown above, ANISEP has a structure similar to that of NAALP from <i>N. abyssinica</i> 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.
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1 ideal; however, there are no data on the molecular weight or metal chelating ability of scallop $\mathbf{2}$ metallothioneins. A metallothionein gene was cloned from bivalve L. elliptica and expressed in E. coli 3 [11]. The same recombinant protein was expressed and used as a model metallothionein substrate in this 4 study. $\mathbf{5}$ The vector pET41-MT, which contains the gene for metallothionein MT-10a from L. elliptica fused 6 to the C terminal of glutathione S-transferase (GST), was introduced into E. coli BL21 Star (DE3). The 7 GST-fused metallothionein MT-10 (GST-MT) protein was recovered from the cell-free extract after IPTG 8 induction followed by affinity chromatography using GSTrap HP (Fig. 6). Further recovery of MT-10 by 9 recombinant enterokinase (rEK) was unsuccessful, probably due to the undesirable digestion of MT-10 by 10 rEK. 11 Cd binding was attempted using GST-MT, by releasing metals bound during recombinant 12expression in 0.1 M HCl followed by neutralization with excess Cd. The amount of absorbed Cd was 13estimated to be 38.4 µg/mg protein by comparing the free Cd concentration before and after the 14Cd-binding assay. Cd binding by GST was negligible, because no absorption was detected when pure 15GST was used in the same assay (data not shown). 16 Cd-bound GST-MT was then used as a substrate for the Cd-release assay with ANISEP. After 17incubation at 50°C for 12 h, HCl treatment released detectable amounts of Cd. Almost no Cd was released 18 by digestion with ANISEP, although SDS-PAGE indicated effective digestion of the same amount of 19GST-MT by ANISEP as a faint protein band (Fig. 6). These results suggest Cd release from the scallop 20hepatopancreas by ANISEP may not be due to substrate specificity for the metallothionein. 2122Discussion 23ANISEP from A. nicotinovorans has a structure similar to that of NAALP from Nesterenkonia sp. 24AL20. From the structural feature revealed by X-ray crystallography, these proteases belong to clan PA, 25trypsin-like serine proteases [28]. The sequence similarity of these proteases is 50.1%.

1	Nesterenkonia sp. AL 20 was identified as a feather-degrading microorganism from a natural
2	alkaline environment [8]. A. nicotinovorans 23-0-11 was isolated from soil for its Cd-releasing activity in
3	the scallop hepatopancreas [5]. The producing organisms are both Micrococcaceae in the Actinobacteria.
4	In addition to their similar structures, the proteases are also Ca ²⁺ independent in their activity and
5	stability. Both showed strong keratinolytic activity. They also exhibited some differences. Sensitivity to
6	PMSF is not conserved in NAALP [8]. Activity and stability under varying pH and temperatures also
7	differ; NAALP is more stable and active at higher pH and temperature [8].
8	The most important feature revealed in this study of ANISEP is its high keratinolytic activity. Most
9	keratinolytic proteases (keratinases) are serine proteases, and several keratinases have been identified
10	from Streptomyces [30]. Although the molecular characteristics of these keratinases have not been defined,
11	with the exception of one from Streptomyces fradiae [31], there may be a specific group of trypsin-like,
12	keratinolytic serine proteases in Actinobacteria.
13	ANISEP degraded recombinant metallothionein MT-10 from L. elliptica, but did not release Cd
14	bound in the metallothionein (Fig. 5), whereas the enzyme effectively released Cd from the scallop
15	hepatopancreas. One possible explanation for this is that the binding status of Cd to metallothionein
16	differs between L. elliptica and P. yessoensis. ANISEP cannot digest the critical residue for Cd binding in
17	the L. elliptica metallothinein, but can digest it in P. yessoensis metallothionein. We could not test this
18	hypothesis because molecular information about metallothionein in P. yessoensis is not available. In
19	addition, the fact that primary structure and the Cd-binding properties of metallothioneins are conserved
20	between species should be considered [10]. Another possibility is that Cd binds metallothioneins and
21	other keratin-like, digestion-resistant proteins in <i>P. yessoensis</i> . If the binding affinity for Cd is higher in
22	the latter, unknown proteins than in the former, the explanation might be convincing. Unfortunately, such
23	proteins have not been identified, but their existence is suggested by studies on the molecular mass of
24	Cd-binding proteins obtained from scallop hepatopancreas specimens with naturally occurring Cd
25	contamination [32]. Further studies on Cd-binding proteins in Japanese scallop are necessary to

1	characterize the	mechanism	of Cd remova	al by ANISEP.

 $\mathbf{2}$

3	Conclusion
4	This study revealed the structure of ANISEP and its high keratinolytic activity, which may related
5	to the Cd released from the scallop hepatopancreas. Further structural studies on substrate specificity and
6	the thermodynamic profile will help elucidate the Cd-removal mechanism and inform enzymatic
7	modifications for applications in the microbial removal of Cd from fishery byproducts obtained from
8	scallops and other mollusks.
9	
10	
11	Abbreviations
12	
13	Cd = cadmium
14	Cd-MT-GST = Cd-bound MT-GST
15	MTs = metallothioneins
16	MT-GST = metallothionein MT-10 fused to glutathione-S-transferase tag
17	PCR = polymerase chain reaction
18	PMSF = phenylmethylsulfonyl fluoride
19	PVDF = Polyvinylidene difluoride
20	rmsd = root mean square deviation
21	
22	SDS-PAGE = sodium dodecyl sulfate – polyacrylamide gel electrophoresis
23	
24	
25	

1	
2	Conflict of interest
3	The authors confirm they have no conflicts of interest to declare.
4	
5	Acknowledgments
6	We thank Dr. Hyun Park (Korea Polar Research Institute, South Korea) for providing the expression
7	vector pET41-MT.
8	This work was supported by a MEXT Grant-in-Aid for Scientific Research (C) 16580049.
9	
10	
11	References
12	[1] Sakuta, Y.; Tomita, K.; Fujishima, K. Processing and Utilizing Technology of Scallop Wastes (Part
13	III). Reports of the Hokkaido Industrial Research Institute 1993, 292, 9–12.
14	[2] Seki, H.; Suzuki, A. A New Method for the Removal of Toxic Metal Ions from Acid-Sensitive
15	Biomaterial. J Colloid Interface Sci 1997, 190, 206–211.
16	[3] Umeki, S.; Yoshii, S.; Saito, M.; Konma, N.; Tokuda, M. Removal of cadmium from scallop
17	[Mizuhopecten yessoensis] wastes including mid-gut glands by subcritical water treatment. Nippon Suisan
18	Gakkaishi 2010 , 76, 204–209.
19	[4] Obara, T.; Sawaya, T.; Hokari, K.; Umehara, Y.; Mizukami, M.; Tomita, F. Removal of cadmium
20	from scallop hepatopancreas by microbial processes. Biosci. Biotechnol. Biochem. 1999, 63, 500-505.
21	[5] Ren, S.; Tomita, F.; Yokota, A.; Asano, K. Isolation of a cadmium-releasing bacterium and
22	characterization of its novel protease. Biosci. Biotechnol. Biochem. 2004, 68, 1627-1633.
23	[6] Gupta, R.; Beg, Q. K.; Lorenz, P. Bacterial alkaline proteases: molecular approaches and industrial
24	applications. Appl. Microbiol. Biotechnol. 2002, 59, 15–32.

- [7] Perona, J. J.; Craik, C. S. Structural basis of substrate specificity in the serine proteases. *Protein Sci.* 1995, 4, 337–360.
- 3 [8] Gessesse, A.; Hatti-Kaul, R.; Gashe, B. A.; Mattiasson, B. Novel alkaline proteases from alkaliphilic
- 4 bacteria grown on chicken feather. *Enzyme Microb. Technol.* **2003**, *32*, 519–524.
- 5 [9] Bakhtiar, S.; Estiveira, R. Substrate specificity of alkaline protease from alkaliphilic feather-degrading
- 6 Nesterenkonia sp. AL20. Enzyme Microb. Technol. 2005, 37, 534–540.
- 7 [10] Klaassen, C. D.; Liu, J.; Choudhuri, S. Metallothionein: an intracellular protein to protect against
- 8 cadmium toxicity. Annu. Rev. Pharmacol. Toxicol. 1999, 39, 267–294.
- 9 [11] Park, H.; Ahn, I.-Y.; Choi, H. J.; Pyo, S. H.; Lee, H. E. Cloning, expression and characterization of
- 10 metallothionein from the Antarctic clam *Laternula elliptica*. *Protein Expr. Purif.* **2007**, *52*, 82–88.
- 11 [12] Grattarola, M.; Carloni, M.; Dondero, F.; Viarengo, A.; Vergani, L. Expression, purification and
- 12 preliminary characterization of mussel (*Mytilus galloprovincialis*) metallothionein MT20. *Mol. Biol. Rep.*
- **13 2006**, *33*, 265–272.
- 14 [13] Wang, L.; Song, L.; Ni, D.; Zhang, H.; Liu, W. Alteration of metallothionein mRNA in bay scallop
- 15 Argopecten irradians under cadmium exposure and bacteria challenge. Comp. Biochem. Physiol. C
- 16 *Toxicol. Pharmacol.* **2009**, *149*, 50–57.
- 17 [14] Andreani, G.; Carpenè, E.; Capranico, G.; Isani, G. Metallothionein cDNA cloning, metallothionein
- 18 expression and heavy metals in *Scapharca inaequivalvis* along the Northern Adriatic coast of Italy.
- 19 Ecotoxicol. Environ. Saf. 2011, 74, 366–372.
- 20 [15] Liu, F.; Wang, W.-X. Differential roles of metallothionein-like proteins in cadmium uptake and
- elimination by the scallop *Chlamys nobilis*. Environ. Toxicol. Chem. 2011, 30, 738–746.
- 22 [16] Aceto, S.; Formisano, G.; Carella, F.; De Vico, G.; Gaudio, L. The metallothionein genes of Mytilus
- 23 galloprovincialis: genomic organization, tissue expression and evolution. Mar Genomics 2011, 4, 61–68.

- 1 [17] Paul-Pont, I.; Gonzalez, P.; Montero, N.; de Montaudouin, X.; Baudrimont, M. Cloning,
- 2 characterization and gene expression of a metallothionein isoform in the edible cockle Cerastoderma
- 3 *edule* after cadmium or mercury exposure. *Ecotoxicol. Environ. Saf.* **2012**, 75, 119–126.
- 4 [18] Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin
- 5 phenol reagent. *J Biol Chem* **1951**, *193*, 265–275.
- 6 [19] Otwinowski, Z.; Minor, W. Processing of X-ray diffraction data collected in oscillation mode.
- 7 *Methods in Enzymology* **1997**, 276, 307–326.
- 8 [20] Vagin, A.; Teplyakov, A. Molecular replacement with MOLREP. Acta Crystallogr. D Biol.
- 9 Crystallogr. 2010, 66, 22–25.
- 10 [21] Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. Refinement of macromolecular structures by the
- 11 maximum-likelihood method. Acta Crystallogr. D Biol. Crystallogr. 1997, 53, 240–255.
- 12 [22] Davis, I. W.; Leaver-Fay, A.; Chen, V. B.; Block, J. N.; Kapral, G. J.; Wang, X.; Murray, L. W.;
- 13 Arendall, W. B. I.; Snoeyink, J.; Richardson, J. S.; Richardson, D. C. MolProbity: all-atom contacts and
- structure validation for proteins and nucleic acids. *Nucleic acids research* **2007**, *35*, W375–W383.
- 15 [23] Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. Features and development of Coot. Acta
- 16 Crystallogr. D Biol. Crystallogr. 2010, 66, 486–501.
- 17 [24] Sambrook, J.; Russell, D. W. Molecular Cloning, 3rd ed.; CSHL Press: New York, 2001.
- 18 [25] Inouye, M. Intramolecular chaperone: the role of the pro-peptide in protein folding. *Enzyme* **1991**, 45,
- 19 314–321.
- 20 [26] Okamoto, H.; Fujiwara, T.; Nakamura, E.; Katoh, T.; Iwamoto, H.; Tsuzuki, H. Purification and
- 21 characterization of a glutamic-acid-specific endopeptidase from *Bacillus subtilis* ATCC 6051; application
- 22 to the recovery of bioactive peptides from fusion proteins by sequence-specific digestion. Appl. Microbiol.
- 23 Biotechnol. 1997, 48, 27–33.

- 1 [27] Yang, N.; Nan, J.; Brostromer, E.; Hatti-Kaul, R.; Su, X.-D. Crystal structure of an alkaline serine
- 2 protease from *Nesterenkonia* sp. defines a novel family of secreted bacterial proteases. *Proteins* 2008, 73,
- 3 1072–1075.
- 4 [28] Page, M. J.; Di Cera, E. Serine peptidases: classification, structure and function. *Cell. Mol. Life Sci.*

5 2008, *65*, 1220–1236.

- 6 [29] Matthews, B. W. Solvent content of protein crystals. *J Mol Biol* **1968**, *33*, 491–497.
- 7 [30] Brandelli, A. Bacterial keratinases: useful enzymes for bioprocessing agroindustrial wastes and
- 8 beyond. Food Bioprocess Technol 2007, 1, 105–116.
- 9 [31] Meng, K.; Li, J.; Cao, Y.; Shi, P.; Wu, B.; Han, X.; Bai, Y.; Wu, N.; Yao, B. Gene cloning and
- 10 heterologous expression of a serine protease from *Streptomyces fradiae* var.k11. *Can. J. Microbiol.* 2007,
- 11 53, 186–195.
- 12 [32] Nakayama, K.; Kazuo, J.; Tsuzuki, T. Studies on cadmium accumulated in hepatopancreas of scallop
- 13 Mizuhopecten yessoensis. Res. Rep. Hokkaido Inst. Public Health 1995, 45, 13–20.
- 14
- 15 Received: June 26, 2014 Revised: August 26, 2014 Accepted: September 11, 2014

1

2 Figure Legends

3	Fig. 1. Structure of the ANISEP gene. A, Structure of the 2.1-kb DNA containing the ANISEP gene.
4	Numbers above and below the horizontal line indicate position in bp and amino acid residues in the
5	protease, respectively. The solid box indicates the partial gene encoding <i>idh</i> . A terminator-like
6	downstream structure is indicated as a stem-loop. The open box indicates the ANISEP gene. Amino acid
7	regions corresponding to Pre, Pro, and the mature ANISEP are indicated. The striped bar indicates the
8	degenerate-PCR amplified fragment F2-R5, and arrows indicate the positions of inverse primers.
9	Assignment of amino acid sequences revealed by sequencing is shown by speech balloons. B, Alignment
10	of ANISEP, NAALP, and MPR1 flanking the catalytic triad. Numbers following m indicate the position
11	of the N-terminal amino acid residue in each region, in the mature form. Catalytic residues are indicated
12	by bold letters.
13	
14	Fig. 2. Heterologous expression of ANISEP in <i>E. coli</i> .
15	A, SDS-PAGE of crude protein. Soluble and insoluble fractions of <i>E. coli</i> harboring pDEST14/990ATG
16	and the control vector pDEST14/GUS were analyzed. Sizes of the GUS protein and mature ANISEP are
17	indicated on the right. B, Protease activity (black bars, left axis) and Cd-release activity (white bars, right
18	axis) of purified native, crude recombinant, and semi-purified recombinant ANISEP. All data are
19	presented as the average of duplicate experiments.
20	
21	Fig. 3. Crystallization and structural analysis of ANISEP
22	A, Crystals of ANISEP. B, Structure of ANISEP, solved by X-ray crystallography, presented in rainbow
23	color with the N- and C-termini as red and purple, respectively. C, Superimposed structures of ANISEP

24 (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 <i>L. elliptica</i> . 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.





COLOR Printing





Sone – Fig. 4





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 1. Partial amino acid sequences and degenerate primers

PDB ID	3WY8	
Data colleciton	PF-AR NE3A	
Wavelength (Å)	1.000	
Resolution range (Å)	50-1.70 (1.73-1.70)	
Space group	123	
Unit-cell	a = b = c = 103.02	
parameters (Å)		
No. of observations	447140	
No. of unique reflections	20158 (1001)	
Completeness (%)	100.0 (100.0)	
Multiplicity	22.2 (22.0)	
Averaged $I/\sigma(I)$	44.9(7.2)	
$R_{ m merge}{}^{ m a}$	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)	
$R_{ m work}{}^{ m b}$	0.142 (0.188)	
$R_{\rm free}^{\ \ c}$	0.182 (0.230)	
R. m. s. deviation		
Bond lengths (Å)	0.006	
Bond angles (°)	1.04	

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

Values in the parentheses are for the highest resolution shell.

 ${}^{a}R_{merge} = \Sigma_{h}\Sigma_{j} | \langle I \rangle_{h} - I_{h,j} | / \Sigma_{h}\Sigma_{j}I_{h,j}$, where $\langle I \rangle_{h}$ 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