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1 **Characterization and role of a metalloprotease induced by chitin in**
2 ***Serratia* sp. KCK**
3
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Abstract

A metalloprotease induced by chitin in a new chitinolytic bacterium *Serratia* sp. Strain KCK was purified and characterized. Compared with other Serratia enzymes, it exhibited a rather broad pH activity range (pH 5.0-8.0), and thermostability. The cognate ORF, *mpr*, was cloned and expressed. Its deduced amino acid sequence showed high similarity to those of bacterial zinc-binding metalloproteases and a well conserved serralysin family motif. Pretreatment of chitin with the Mpr protein promoted chitin degradation by chitinase A, which suggests that Mpr participates in, and facilitates, chitin degradation by this microorganism.

Keywords: metalloprotease · chitin · *Serratia* · chitinase A

1 Introduction

2

3 The polysaccharide chitin, a polymer of β-1,4-linked *N*-acetylglucosamine
4 (GlcNAc) residues that are highly cross-linked by hydrogen bonds, is the second most
5 abundant organic compound in nature. It is a major constituent of cell walls of fungi,
6 exoskeletons of insect and zooplankton, and shells of crustaceans. It is degraded and
7 re-modelled by chitinases (EC 3.2.1.14) which hydrolyse the glycosidic bonds between
8 GlcNAc residues [9,14]. Chitin in crustacean cuticles is associated with protein,
9 inorganic salts and lipids, and it has been reported that a combination of chitinase and
10 protease results in more efficient degradation of crustacean cuticles than chitinase
11 alone [20,21].

12 Microbial proteases are classified into four groups, according to the catalytic
13 residue functioning in their active sites: serine proteases, cysteine proteases, aspartate
14 proteases and metalloproteases [10]. Metalloproteases have the HEXXH zinc-binding
15 motif and can be further classified into thermolysin, serralysin, and neurotoxin families,
16 according to the location of the third zinc ligand.

17 Bacteria belonging to the genus *Serratia* are frequently found to produce chitinases,
18 and we have recently isolated from the traditional Korean fermented cabbage product
19 kimchi a novel *Serratia* strain producing a chitinase with unusually broad substrate, pH
20 activity and thermostability spectra [15]. Although there are a number of reports of
21 metalloproteases produced by various strains of *Serratia marcescens* [3,6,18,19,24,26],
22 so far there is no report of chitinase:metalloprotease interactions in the degradation of
23 chitin by *Serratia*.

24 In this study, we purified and characterized metalloprotease excreted in the

1 presence of chitin. Furthermore, we cloned the gene coding for metalloprotease and

2 determined its nucleotide sequence. After that, we have investigated its role in

3 chitinolytic system.

1 **Materials and methods**

2

3 **Strains, plasmids and culture conditions**

4 *Serratia* sp. KCK isolated kimchi juice [15] was cultivated and used as the source
5 of metalloprotease. For the production of metalloprotease, a mineral medium (2 g
6 NH_4Cl, 3 g KH_2PO_4, 2 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.01 g
7 $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4 \cdot 7H_2O$, 0.001 g $MnCl_2 \cdot 4H_2O$ per liter of water at pH 7.2)
8 containing 5 g/l colloidal chitin as sole carbon source was inoculated with strain KCK
9 and incubated at 30°C. Colloidal chitin was prepared by the method of Imanaka et al
10 [12]. *Escherichia coli* DH5α (Stratagene, La Jolla, CA, USA) was used as hosts for
11 cloning of the metalloprotease gene and was cultured in Luria-Bertani (LB) medium at
12 37 °C. Plasmid pGEM-Teasy (Promega, Madison, WI, USA) was used as the cloning
13 vector. Ampicillin (100 μg/ml) was added to media used for strains harboring plasmids.

14

15 **Enzyme assay and protein determination**

16 Metalloprotease was assayed by a slight modification of the method of Salamone
17 and Wodzinski [26]: 0.2 ml of enzyme solution was added to 0.8 ml of reaction mixture
18 containing 1.0% (w/v) casein in Tris-HCl buffer (100 mM, pH 8.0) and 1 mM $MgCl_2$.
19 After 1 h at 30 °C, the reaction was terminated by addition of 0.5 ml 10% (w/v)
20 trichloroacetic acid (TCA) and incubation at room temperature for 30 min. The reaction
21 mixture was then centrifuged at 10,000 *g* for 15 min and the A_{280} of the supernatant
22 measured. One unit of proteolytic activity was defined as the amount of enzyme that
23 produced an increase of absorbance at 280 nm of 0.1 under the assay conditions. The
24 protein concentration was measured by the Bradford method with bovine serum

albumin as a standard.

Purification of metalloprotease

All purification procedures were carried out 4 °C in Tris-HCl buffer containing 1 mM $MgCl_2$ to prevent complexing of the zinc metal [26]. After cultivation of *Serratia* sp. KCK in minimal medium containing colloidal chitin for 7 days, the cells were removed by centrifugation at 10,000 *g* for 20 min. The culture medium (200 ml) was fractionated by successive precipitation with ammonium sulfate, up to 80% saturation. The concentration at which most of the activity was precipitated was the 50-65% saturation cut, and this precipitate was collected by centrifugation (16,000 *g*, 30 min), and dissolved in 50 mM Tris-HCl buffer (pH 8.0). This material was fractionated further by successive acetone precipitation between concentrations 20-80%. Acetone was slowly added and the mixture stirred for 1 h. The precipitates resulting from each acetone addition were centrifuged (12,000 *g* 15 min) and the pellets dissolved in 50 mM Tris-HCl buffer (pH 8.0). The majority of activity was precipitated in the acetone cut of 55-60% concentration, which was dialyzed overnight at 4 °C against the same buffer and concentrated to 1ml by ultrafiltration on a Centricon YM-10 membrane (Amicon, Milipore, Billerica, MA, USA).

SDS-PAGE and activity staining

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the method of Laemmli [17], and activity staining was done by a slight modification of the method of Ried and Collmer [25]. After electrophoresis, the gel was incubated for 4 h at 37 °C in 50 mM Tris-HCl buffer (pH 8.0) containing 1% (v/v)

Triton X-100 to remove SDS [16], and washed with distilled water. It was then overlaid with molten agar-substrate consisting of 2% (w/v) Noble agar in 100 mM Tris-HCl (pH 8.0) buffer containing 5% (w/v) skimmed milk. The overlay covered, the gel incubated at 37 °C 5 h, and was then submerged in a 1% (w/v) trichloroacetic acid (TCA) solution. The region containing the proteolytic activity appeared as a clear area in the opaque background.

Enzyme kinetics

The kinetics experiments were performed with different concentrations of casein (2.5-50 mg/ml) dissolved in 50 mM Tris-HCl buffer (pH 7.0) at 30 °C with the standard method. The K_m value was determined from the Michaelis-Menten curve and V_{max} was calculated from the Lineweaver-Burk double reciprocal plot. Turnover number (k_{cat}) and catalytic efficiency value (k_{cat}/K_m) were calculated.

pH and temperature optima

The buffers used to determine the pH optimum of the enzyme were 50 mM sodium acetate buffer (pH 3.0-6.0), 50 mM Tris-HCl buffer (pH 7.0-9.0) and 50 mM glycine-NaOH buffer (pH 10.0-12.0). The optimum temperature for enzyme activity was measured with the standard assay in the range of 20-80 °C. Temperature stability was determined by incubation of the enzyme solution in 50 mM Tris-HCl buffer (pH 7.0) at temperatures of 40-80 °C for different periods of time. The residual activity was then assayed with the standard assay.

Protein sequencing by Q-TOF

After SDS-PAGE, the enzyme spot of about 50 kDa was excised the from gel, and the protein digested *in situ* with trypsin (sequencing grade modified trypsin, Promega, Madison, WI, USA), and peptide extraction performed as described previously [8]. 2005). Peptide samples were analyzed by protein sequence using quadrupole time-of-flight (Q-TOF) mass spectrometry (MS/MS peptide sequencing) at the Helmholtz Center for Infection Research (HZI, Braunschweig, Germany) Structural Research Facility. Q-TOF analysis was performed to conveniently obtain the exact sequences of internal fragments of peptides.

Cloning of the gene coding for the metalloprotease

The gene coding for metalloprotease was amplified using the two oligonucleotide primers, 5′-AACACACTGCCGGTAACG-3′ and 5′-CTGCCATCCGCCCGCAGC-3′, designed from the polypeptide sequence determined by Q-TOF, and conserved amino acids in enzymes from *Serratia marcescens* (X55521) and *Serratia proteamaculans* (AY818193). Genomic DNA of *Serratia* sp. KCK was isolated by using a genomic DNA purification kit (Qbiogen, Heidelberg, Germany). The PCR amplification was carried out using genomic DNA obtained from *Serratia* sp. KCK as the template under following conditions: 94 °C for 2 min of initial denaturation; 35 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min. The PCR product was cloned into pGEM-Teasy vector (Promega, USA), and this construct was transformed into *E. coli* DH5α by electroporation. For the selection of PCR insert-containing recombinants, transformants were spread on Luria-Bertani (LB) plate containing ampicillin (100μg/ml), isopropyl-ß-D-thiogalactopyranoside (IPTG; 1 mM), and 5-bromo-4-

chloro-3-indolyl-*β*-D-galactopyranoside (X-Gal; 50 μg/ml). After incubation for 24 h at 37 °C, a white colony was purified on an LB plate containing ampicillin. The plasmid was extracted and sequenced with the two M13 Forward/Reverse universal primers, forward (5′-TGTAAAACGACGGCCAGT-3′) and reverse (5′-CAGGAAACAGCTATGACC-3′). The nucleotide and amino acid sequences of the putative protease ORF (open reading frame) were analyzed using BLAST server of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The multiple sequence alignments of related or retrieval enzymes were performed with the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/).

Effect of metalloprotease on chitinolytic activity

The effect of the metalloprotease on chitinolytic activity was assayed with a slight modification of the method of Miyamoto et al. [20]. Crude powdered chitin (10 mg) was suspended with 500 μl of 50 mM Tris-HCl buffer (pH 7.0) containing 2 μg of purified metalloprotease and incubated at room temperature for 1 h. The reaction mixture was centrifuged, and the protein removed from the chitin was assayed by the method of Bradford. The residual chitin was washed with 1 ml of 50 mM Tris-HCl buffer (pH 7.0) and resuspended in 500 μl of the buffer containing 1.5 μg of purified chitinase A (ChiA) from *Serratia* sp. KCK. The reaction mixture was incubated at 30 °C for 6 h, samples were taken at 1 h intervals, and chitinolytic activity was measured as described by Imoto and Yagashita [13].

Nucleotide sequence accession numbers

The nucleotide and amino acid sequence of metalloprotease was deposited in

1 GenBank, with accession numbers EF191201.

Results

Purification and characterization of the metalloprotease

An extracellular metalloprotease produced by *Serratia* sp. KCK was purified by ammonium sulfate saturation, acetone precipitation and dialyzed concentration. The purity and the apparent molecular mass of the enzyme were analyzed by SDS-PAGE. As shown in Fig. 1, the analysis of SDS-PAGE and active staining showed a single polypeptide active form which was estimated to be about 50 kDa. This result indicated that this protein is active in the monomeric form. The steps of the purification are summarized in Table 1. The enzyme was purified 16 fold with a recovery of 20 %. The K_m and V_{max} values for casein were determined to be 11.16 mg/ml and 1.80 ΔA_{280}/h/ml, respectively. On the basis of Lineweaver-Burk calculation, the k_{cat} was found to be 0.196/h, and the catalytic efficiency ratio k_{cat}/K_m to be 0.018/h/mg/ml.

The enzyme was active over a broad pH range between pH 5.0 and 8.0, with an optimum at pH 7.0. Activity dropped steeply below pH 5.0 and above pH 8.0 (Fig. 2). The optimal temperature of enzyme activity was found to be 30 °C at pH 7.0 (Figure 3 A); activity rapidly declined above 40°C and no activity was detected at 80°C. When the enzyme was incubated at 50°C and 60°C for 1 h, prior to assaying, the enzyme lost about 50% and 80% of its activity, respectively; incubation at 70°C resulted in 90% loss in activity after 15 min and 100% loss in 30 min (Fig. 3B).

Cloning of the metalloprotease gene

To clone the chitinase gene coding from *Serratia* sp. KCK, the purified enzyme was first sequenced by Q-TOF. It displayed high sequence similarity to that of a

metalloprotease of *Serratia proteamaculans* (Table 2). Primers based on conserved sequences of metalloproteases of *Serratia* species reported in GenBank were designed and used to amplify an approximately 1.9 kb fragment from genomic DNA of strain KCK, which was then cloned and sequenced. It consisted of 1936 nucleotides and translates into a protein of 487 amino acids with a predicted molecular weight of 52 kDa and a theoretical isoelectric point (PI) value of 4.44.

Metalloproteases of *Serratia* have N-terminal propeptides [22,24] and, as shown in Figure 4A, the region encoding the mature protease was similarly preceded by a region coding for a 16 amino acid propeptide. Thus, the size of 50 kDa estimated for the purified protein corresponds well with the 471 amino acid polypeptide lacking the N-terminal propeptide deduced from the gene sequence.

The deduced amino acid was compared to the NCBI database using the BLAST program to search for homologous protein and found to display sequence homology to the sequences of metalloprotease from *Serratia proteamaculans* AY818193 (100% identity), SMP from *Serratia marcescens* X55521 (92% identity), EprB from *Erwinia chrysanthemi* AY919873 (56% identity), VVP from *Vibrio vulnificus* U50548 (10% identity), Mpr from *Aeromonas sobria* DQ784565 (10% identity), thermolysin from *Bacillus thermoproteolyticus* X76986 (9% identity) and MprI from *Alteromonas* sp. AB063611 (8% identity). The enzyme contains a core region HEXXH Zinc-protease motif conserved in many microbial metalloproteases (Fig. 4B). The internal amino acid sequence determined by Q-TOF analysis coincided precisely with the sequence starting from the Phe^{97} residue of the deduced amino acid sequence. Therefore, we concluded that the deduced protein was a metalloprotease and was designated Mpr.

Effect of metalloprotease on chitinolytic activity

We investigated the role of Mpr on the chitinolytic activity of *Serratia* sp. KCK. As shown in Figure 5A, the metalloprotease released substantial amounts of protein from powdered chitin. Pretreatment of chitin with Mpr increased the chitinolytic activity of ChiA from *Serratia* sp. KCK throughout the reaction period (Fig. 5B), as has been reported in *Alteromonas* sp. [20]. Mpr had no effect on chitinolytic activity when deproteinized chitin was used as substrate (data not shown).

1 **Discussion**

2

3 Metalloproteases are ubiquitous enzymes with diverse functions, including
4 pathogenicity in some microorganisms such as *Vibrio* sp. [5,11], *Aeromonas*
5 *hydrophila* [4] and *Pseudomonas aeruginosa* [30]. Metalloproteases of *Serratia* have
6 been studied extensively as an anti-inflammatory agent, a diagnostic tool for
7 taxonomic classification, and a model in studies of secretion of extracellular proteins
8 [3,10,18,19].

9 Here we report the characterization of the metalloprotease Mpr of *Serratia* sp
10 KCK, a chitinolytic strain isolated from kimchi. Like metalloproteases of the serralysin
11 family [22,24], it has propeptide consisting of 16 amino acids, not a signal peptide on
12 the basis of homology analysis (Fig. 4A). Such enzymes possess the HEXXH zinc-
13 binding motif - the zincins superfamily [22] and the zinc ion is coordinated by the first
14 and second histidine resides in HEXXH motif. Also, the third histidine of the motif is
15 conserved in this sequence and this residue acts as a third zinc ligand [2,27]. The
16 serralysin family is grouped in the metzincin metalloprotease superfamily [1] that
17 includes astacins, matrix metalloproteases (collagenases) and snake venom proteases
18 [2]. The metzincins are characterized by the zinc-binding consensus sequence
19 HEXXHXXGXXHP, with a proline residue instead of glutamic acid/glutamine [26],
20 which is conserved in Mpr of *Serratia* sp KCK. In addition, the serralysin family is
21 characterized by the conserved SXMSY motif [22], which is also found in Mpr (Fig.
22 4B), the tyrosine residue of which, located 41 residues from the HEXXH motif
23 towards the C terminal, seems to be a potential ligand. These results suggest that Mpr
24 is a member of the serralysin family. The deduced amino acid sequence of Mpr is

identical to that of the metalloprotease of *Serratia proteamaculans* (AY818193), although the gene sequences are different (data not shown). We have already reported that strain KCK was considered to represent a new species of *Serratia* [15]. Thus, based on homology relationships, we suppose that the metalloprotease of *Serratia proteamaculans* may have similar functions as a member of the serralysin family.

The treatment of powdered chitin with Mpr resulted in increased chitinolytic activity by ChiA. In the case of the marine bacterium *Alteromonas* sp. Strain O-7, it was reported that the third metalloprotease, MprIII, weakly promoted chitin degradation, presumably through hydrolysis of constituent proteins on the surface of chitin cuticles [20]. Mpr presumably plays the same role in the chitinolytic system of *Serratia* sp. KCK and facilitates access between chitinase A and chitin molecules by removal of sterically-hindering chitin-associated proteins [21,29]. In *Alteromonas* sp, it also was reported that chitin hydrolysis was significantly promoted by the fourth chitin binding protease, AprIV, which improves hydrolysis of chitin more effectively than MprIII [21], and whose activity is additive with that of MprIII [20]. It has been reported that chitin binding proteases of *Vibrio*, *Bacillus* and *Pseudomonas* strains promote attachment of bacterial cells to substrates containing chitin [7,23,31]. In addition, chitin binding protein, CBP21, produced by *Serratia marcescens* has been reported to be an essential factor in chitin degradation [28,29], and is, with chitinase A, one of the major proteins excreted into the culture supernatant when this bacteria is cultivated in the presence of chitin [28]. It remains to be seen if other excreted proteins induced in *Serratia* sp. KCK by growth in the presence of chitin include a chitin-binding protein and another metalloprotease that is able to promote chitin degradation.

The *Serratia* metalloproteases have been mainly used for medical purposes. The

1 Mpr metalloprotease induced by chitin in *Serratia* sp. KCK promotes hydrolysis of
2 chitin. It may therefore be useful for biological applications in relation to chitin
3 degradation.

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1
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Table 1 Purification of metallopeptidase from *Serratia sp*. KCK

Purification step	Total protein (mg)	Total activity (U)	Specific Activity (U/mg)	Yield (%)	Purification factor
Culture supernatant	1,567.3	226.7	0.15	100.0	1.0
Ammonium sulfate Precipitation	294.5	92.3	0.31	40.7	2.1
Acetone precipitation	42.7	67.4	1.58	29.7	10.5
Dialyzed concentrate	18.9	46.2	2.44	20.4	16.3

Table 2 Peptides of 50 kDa band from SDS-PAGE gel (Fig. 1) by Q-TOF protein sequencing and BlastP search

Protein	Peptide fragments sequence	*E*-value
Metalloprotease (ZP_01535592) (*Serratia proteamaculans* 568)	FSAEQQQQAK	0.56

Figure legends

Fig. 1 SDS-PAGE analysis and activity staining of purified metalloprotease from *Serratia* sp. KCK. Lane M, Standard proteins, from the top: myosin (185 kDa), phosphorylase B (98 kDa), glutamic dehydrogenase (52kDa), carbonic anhydrolase (31 kDa), myoglobin blue (19 kDa), myoglobin red (17 kDa), lysozyme (11 kDa), aprotinin (6 kDa), insulin (3 kDa); lane 1, Coomassie Brilliant Blue R250 staining of the purified metalloprotease; lane 2, activity staining of the purified metalloprotease

Fig. 2 Effect of pH on the activity of the purified metalloprotease from *Serratia* sp. KCK

Fig. 3 (A) Effect of temperature on the activity of the purified metalloprotease from *Serratia* sp. KCK. (B) The curves show stability at selected temperatures. The residual activity was determined using the standard assay after incubating the enzyme at 40 °C (empty triangles), 50 °C (filled squares), 60 °C (filled triangles), 70 °C (filled circles) and 80 °C (filled diamonds) for different time intervals

Fig. 4 (A) Diagram of domain structure of Mpr in *Serratia* sp. KCK. (B) Comparison of amino acid sequences of the core region of the catalytic domain in *Serratia* sp. KCK metalloprotease with those in other microbial metalloproteases. Highly conserved amino acids are highlighted in black. The residue numbers of the first and last amino acid in each line was shown on the left and right. The HEXXH motif is lined on the top, and the SXMSY motif of serralysin is indicated by the box. The third histidine of the

motif is marked with an asterisk. GenBank accession numbers are provided in parenthesis. SpMpr, *Serratia* sp. KCK Mpr (EF191201); SmSmp, *Serratia marcescens* Smp (X55521); EcEpr, *Erwinia chrysanthemi* EprB (AY919873); VvVvp, *Vibrio vulnificus* Vvp (U50548); AsMpr, *Aeromonas sobria* Mpr (DQ784565); AsMprI, *Alteromonas* sp. MprI (AB063611); BtNpr, *Bacillus thermoproteolyticus* thermolysin (X76986)

Fig. 5 Effect of Mpr on the chitinolytic system. (A) The protease activity was examined using powdered chitin. The control (a) and (b) are without enzyme and powered chitin, respectively. (B) The effect of Mpr on chitinolytic activity was measured with powdered chitin at ChiA (filled circles); ChiA plus Mpr (filled triangles). The samples from reaction mixture were taken at the indicated times, and chitinolytic activity was measured. The relative activity was compared to the value after incubation for 6 h using ChiA plus Mpr. Each analysis was performed with three independent experiments.