Ann Microbiol (2011) 61:757–764
DOI 10.1007/s13213-010-0192-3

ORIGINAL ARTICLE

Cloning, characterization and molecular analysis of a metalloprotease from *Proteus mirabilis*

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Received: 29 September 2010 / Accepted: 22 December 2010 / Published online: 9 January 2011

Abstract *Proteus mirabilis* is an important pathogen that is usually found in complicated urinary tracts infection. It possesses a metalloprotease, ZapA, that acts as a virulence factor. The gene encoding ZapA was cloned from *P. mirabilis* Pm7—a strain isolated from marine environments—and conditionally expressed in *Escherichia coli*. Zn^{2+} and Co^{2+} exhibited an apparently positive effect on the enzyme activity of the 54-kDa protease. Ag^{+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, EDTA and sulfhydryl reagents including β-mercaptoethanol and dithiothreitol exhibited an apparently negative effect on enzyme activity. Enzyme activity analysis revealed that the optimum temperature and pH for purified recombinant ZapA were approximately 40°C and 8.0, respectively. Enzyme activity and western immunoblotting analysis were used for the determination of the extracellular location of ZapA. The simultaneously depressed expression of *zapA* and swarming motility of Pm7 in the presence of glucose were determined by real-time PCR and swarming motility measurements, respectively. Furthermore, the outer membrane proteins of two bacteria (*Enterobacter* sp. T41 and *Edwardsiella tarda* strain TX1—a fish pathogen) were found to be substrates of ZapA proteolysis.

Keywords *Proteus mirabilis* · ZapA · Enzyme activity · Swarming motility · Proteolysis

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Introduction

Proteus mirabilis is an opportunistic pathogen that can cause urinary tract infection (UTI) (Mobley and Belas 1995). However, investigation of pyelonephritis and renal calculus diseases proved that *P. mirabilis* is not a common cause of UTI in normal hosts and instead infects a much higher proportion of patients with complicated UTI (Mobley 1996). *Proteus mirabilis* possesses various potential virulence factors including urease, deaminase, hemolysin, protease, and lipopolysaccharide, in addition to its swarming motility (Coker et al. 2000; Fraser et al. 2002). *Proteus mirabilis* undergoes swarming—a multicellular behavior during which long, differentiated, aseptate, hyperflagellated swarm cells migrate over surfaces—in response to changes in cell density, surface contact, and certain physiological signals (Fraser and Hughes 1999; Fraser et al. 2000; Stankowska et al. 2008). Although the fundamental role of swarming motility in *P. mirabilis* remains unknown, it has been observed that active swarming bacteria facilitate indwelling in the kidney or bladder and elevate resistance to certain antibiotics in comparison to vegetative *Salmonella typhimurium* cells (Allison et al. 1994; Kim et al. 2003; Phan et al. 2008). However, the specific signals and mechanisms that drive swarm cell differentiation remain to be elucidated at both the physiological and molecular levels.

ZapA, an IgA metalloprotease, is considered to be a virulence factor of the opportunistic bacterium *P. mirabilis* (Senior et al. 1987; Phan et al. 2008). During swarming behavior, expression of *zapA* is coordinately upregulated though quorum sensing systems, lon protease and *p*-nitrophenylglycerol (PNPG) (Walker et al. 1999; Liaw et al. 2000; Clemmer and Rather 2008). ZapA has been purified as a single 54-kDa protein, and the gene encoding this enzyme has been cloned (Wassif et al. 1995). The

activity of the 54-kDa enzyme was stimulated by divalent cations and inhibited by EDTA (Loomes et al. 1992; Wassif et al. 1995). Based on the deduced amino acid sequence, ZapA belongs to the serralysin metalloprotease family of zinc metalloproteases, which are affiliated with a larger group of virulence proteins from pathogenic strains of *Actinobacillus*, *Bordetella*, enterohemorrhagic *Escherichia coli*, *Neisseria*, *Pasteurella* and *Pseudomonas* sp. (Bode et al. 1993; Ghigo and Wandersman 1994). To our knowledge, previously reported biochemical characterization of ZapA from *P. mirabilis* did not examine the effects of environmental factors, such as temperature, pH, heavy metals, and sulfhydryl reagents, on the enzyme activity of recombinant ZapA (Loomes et al. 1992; Wassif et al. 1995). The influence of these environmental factors may be very important to the enzyme activity of ZapA and thus affect the infection processes caused by *P. mirabilis*.

Metalloprotease ZapA from *P. mirabilis* degrades a broad spectrum of substrates, including IgA and antimicrobial peptides (Loomes et al. 1990; Almogren et al. 2003; Belas et al. 2004). The substrate specificity of ZapA had been characterized using fluorescent peptides derived from bioactive peptides and the oxidized β-chain of insulin. As one of the most important virulence factors of *P. mirabilis*, the role of ZapA in pathogenesis was probably due to the destruction of IgA, antimicrobial peptides, bioactive molecules, and structural components of the host cells (Fernandes et al. 2000; Anéas et al. 2001; Belas et al. 2004).

In this report, we describe the isolation and identification of *P. mirabilis* strain Pm7 from marine environments, and the cloning and expression of the *zapA* metalloprotease gene in *E. coli* BL21(DE3). Our aim was to investigate the effects of temperature, pH and heavy metals on the enzyme activity of recombinant ZapA. Furthermore, we intended to examine whether some new substrates of ZapA could be determined from bacterial matrix proteins.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli strains Top10 (TaKaRa, Dalian, China), BL21(DE3) (Tiangen, China), and *P. mirabilis* strain Pm7 (isolated from marine environments) were cultured in Luria-Bertani broth (LB) medium (Sambrook et al. 1989) at 37°C (for *E. coli*) and at 28°C (for *P. mirabilis*). If necessary, kanamycin (kan) was supplemented at a concentration of 50 mg/l. To detect the effects of glucose on the growth and swarming motility of Pm7 and the enzyme activity of the supernatant, *P. mirabilis* strain Pm7 was cultured in LB medium amended with 20 mM glucose.

DNA and molecular manipulation

The plasmid preparation kit was purchased from Omega Bio-Tek (http://www.omegabiotek.com/) and plasmid preparations were performed according the manufacturer's instructions. DNA extraction from agarose gels was done according to the instructions of the Sangon kit (Shanghai, China). All the restriction endonucleases and modifying enzymes were purchased from Fermentas (Shenzhen City, China). The TA cloning plasmid pMD18-T was purchased from TaKaRa (Dalian, China). The 16S rRNA gene of Pm7 was PCR amplified with primers 8F (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGT TACGACTT-3′) according to the method described by Lane et al. (1985).

Measurement of swarming motility

Assessment of swarming motility of Pm7 was carried out according to previously described methods with minor modifications (Stankowska et al. 2008). Briefly, Pm7 (2 μl inoculum containing $1–1.5\times10^8$ CFU) was inoculated onto solid LB medium with or without 20 mM glucose and measurements were taken every hour. The speed of migration was measured (in millimeters) from the center of the inoculated bacteria to the last zone of swarming growth.

Cloning of *zapA* gene and generation of *zapA* variant

Chromosomal DNA of Pm7 was extracted according to the method described previously (Syn and Swarup 2000). *zapA* was cloned from Pm7 with primers F1 (5′-GACGCA TATGGGATCTTTTTTATTAAAAA-3′) and R1 (5′-CACTCGAGAACAATAAAATCAGTTTCG-3′). *Zap27*, a *zapA* variant bearing a deletion of the C-terminal region, was generated by PCR with primers F1 and R7 (5′-ATACTCGAGACCAGGATGCATAAGACC-3′).

Plasmid and strain construction

To construct plasmids pETZap and pETZap27, *zapA* and its variant were digested with *Nde*I–*Xho*I and ligated into pET28 between the *Nde*I/*Xho*I sites. Both pETZap and pETZap27 were transformed into the BL21(DE3) strain.

Purification and reconstitution of the recombinant proteins

BL21(DE3) transformants harboring pETZap and pETZap27 plasmids individually were grown in LB medium to an OD_{600} of 0.5, and expression of *zapA* and its variant were induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the cultures. After an additional growth of 6 h,

the cells were harvested by centrifugation. The recombinant ZapA and Zap27 proteins were purified with nickel-nitrilotriacetic acid agarose (Ni-NTA agarose) under denaturing conditions according to the recommendations of the manufacturer (Qiagen, Valencia, CA). The denatured proteins were reconstituted to retrieve the enzyme activity by the methods described by Hu et al. (2009). Briefly, the cell pellet was suspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris-Cl, and 8 M urea; pH 8.0) for 1 h at room temperature, and the lysate was centrifuged at 10,000 *g* for 30 min at room temperature to pellet the cellular debris. The lysate obtained was added to the 50% Ni-NTA agarose (4:1; v/v) and mixed gently by shaking for 1 h at room temperature. The supernatant was removed after centrifugation at 10,000 *g* for 30 min and the Ni-NTA agarose was washed twice with an equal volume of buffer B. The ZapA- and Zap27-bound Ni-NTA agarose were dialyzed against reconstitution buffer containing 50 mM Tris-Cl (pH 8.0), 200 mM KCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM β-mercaptoethanol (β-ME), 1 mM EDTA , 20% glycerol, and decreasing concentrations of urea (4, 2, 1, 0.5, 0.25, and 0 M). After dialysis for 48 h, proteins were eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole; pH 8.0). The proteins were concentrated by centrifugation using Amicon Ultra centrifugal filter devices (Millipore, Bedford, MA). The reconstituted ZapA and Zap27 proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assay

Enzyme activities of the supernatants of Pm7 (prepared as described in Zhang et al. 2008b) and of the reconstituted ZapA and Zap27 proteins were measured using azocasein as a substrate under standard conditions. Extracellular proteins ZapA and Zap27 were independently incubated with azocasein (2.5 mg/ml) in standard phosphate buffer (pH 7.4) at 37°C for 1 h. The reaction was stopped by adding an equal volume of 10% trichloroacetic acid followed by incubation of the mixture at 4°C for 15 min. The samples were then centrifuged at 13,000 *g* for 5 min to remove unhydrolyzed azocasein and supernatants were collected for measurement of absorbance at 350 nm. One unit of enzyme activity was defined as the amount of enzyme that caused an increase of 0.001 at 350 nm in 1 min (Zhang et al. 2009).

Effects of temperature, pH and metal ions on the activity of recombinant ZapA

The effects of temperature and metal ions on the activity of the purified protease ZapA were determined by incubating the enzyme with azocasein (2.5 mg/ml) under standard assay conditions. The effect of pH on the activity of ZapA was determined by the method described by Zhang et al. (2008b), in which a combination of three different buffering systems were used: 50 mM citric acid-sodium phosphate (pH 4.0–6.0), 50 mM phosphate buffer (pH 6.0–9.0) and 50 mM glycine-NaOH (pH 9.0–11.0). To determine the thermostability, ZapA aliquots were incubated at temperatures ranging from 20 to 80°C for 1 h, after which azocasein was added to trigger the reaction in standard assay buffer at the optimum temperature. pH stability was determined as described above for thermostability except that ZapA aliquots were incubated at different pH points varying from pH 4.0 to pH 11.0.

Antisera

Antisera to recombinant ZapA were prepared as described previously (Zhang et al. 2008b): 50 μg purified recombinant protein was mixed in complete Freund's adjuvant and injected subcutaneously in a rat (5 months; purchased from Luye Pharma, Yantai, China). After 3 weeks, the rat was boosted with half the amount of protein in incomplete Freund's adjuvant. The rat was bled 7 days after the boosting and blood was collected. The antisera were obtained by centrifugation for further use after being kept at 4°C overnight.

Western immunoblotting analysis

Bacteria were cultured to an OD_{600} of 4.0 in LB medium in the presence and absence of 20 mM glucose, and supernatants were collected by centrifugation. The extracellular proteins were prepared by concentrating the supernatant approximately 100 times using Amicon Ultra-4 centrifugal filter devices (Millipore). The collected proteins were then electrophoresed in 0.1% SDS/12% polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes. Western immunoblotting was performed as described previously (Martin et al. 2004) using rat anti-ZapA polyclonal antibodies contained in the antisera.

Real-time PCR

Cells were grown to an OD_{600} of 2.0 in LB medium with and without 20 mM glucose, and total RNA was extracted. Real-time PCR was carried out as described previously with minor modifications (Zhang et al. 2008a). The primers used for real-time PCR were RTF1 (5′-GAAATTGGTCATA CACTGGGTCTT-3′) and RTR1 (5′-GCGCCTGTTTCA TATTCATCCC-3′). 16S rRNA amplified with 933F (5′-GCACAAGCGGTGGAGCATGTGG-3′) and 16SRTR1 (5′-CGTGTGTAGCCCTGGTCGTA-3′), was used as internal control.

Protein substrate analysis

The outer membrane proteins (OMPs) of two bacteria, *Enterobacter* sp. T41 and *Edwardsiella tarda* strain TX1, were extracted as described by Hantke (1981) and used as substrates to analyze the proteolysis of ZapA. Recombinant protease ZapA was incubated with these OMPs at 37°C in 50 mM sodium phosphate buffer in a reaction volume of 100 μl. After 1 h of reaction, the samples were boiled for 5 min and electrophoresed in a 0.1% SDS/12% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue. The loss of the substrate band or the presence of a new digestion product band after incubation with ZapA indicated that the protease had degraded the protein.

Database search and nucleotide sequence accession numbers

Searches for nucleotide and amino sequence similarities were conducted with the BLAST programs from the National Center for Biotechnology Information (NCBI). The nucleotide sequences of the 16S rRNA gene of Pm7, and *zapA* have been deposited in the GenBank database under accession numbers HM217132 and HM217133.

Results

Sequence characterization of ZapA homologue of *P. mirabilis* Pm7

The gene encoding ZapA was cloned from *P. mirabilis* Pm7—a strain isolated from marine environments. The gene was obtained by PCR amplification with primers F1 and R1. It encoded a protein of 491 amino acids with an estimated molecular mass of 54-kDa. ZapA from Pm7 shares 99% identity in overall sequence with ZapA proteins from other *P. mirabilis* strains, with one or two amino acid residue changes. Tyrosine^{167} in ZapA from Pm7 was different from histidine^{167} in sequences of ZapA under accession numbers AF064762, U25950 and AM942759. Another two amino acid residues, tyrosine^{308} and threonine^{406} in ZapA from Pm7, replaced histidine^{308} and serine^{406} in sequences of ZapA under accession numbers AF064762 and AM942759, respectively.

Subcellular location of ZapA from Pm7

To detect whether ZapA from Pm7 could be secreted into extracellular spaces, both enzyme activity and western immunoblotting assays using supernatants from culture of Pm7 were performed. When Pm7 was cultured in standard LB medium, its extracellular enzyme activity increased with growth, reaching a maximum at late exponential/early stationary phase (Fig. 1a). Extracellular proteins were collected and western immunoblotting analysis was performed using rat anti-ZapA polyclonal antibodies. ZapA in the extracellular proteins reacted with anti-ZapA antibodies and one band at approximately 54-kDa was detected in the extracellular samples (Fig. 2, lane 2); immunoblotting with preimmune serum showed no band of the corresponding size (Fig. 2, lane 5). These results suggested that *zapA* encoded an extracellular protein that exhibited high proteolytic activity.

Purification and reconstitution of recombinant ZapA and its variant

ZapA and *zap27* were introduced into the Novagen pET expression system and conditionally expressed in *E. coli* strain BL21(DE3) as C-terminally His-tagged recombinant proteins. After induction, the recombinant proteins were expressed mostly as a non-active polymer and thus were purified under denaturing conditions. The purified proteins were reconstituted to recover bioactivity. Enzymatic analysis showed that the purified recombinant ZapA protein exhibited apparent enzyme activity (18 U μl^{-1}), while

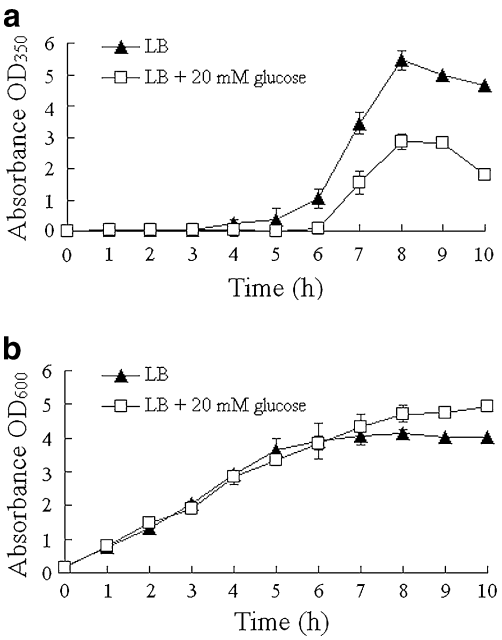

Fig. 1a,b Extracellular enzyme activity of *Proteus mirabilis* Pm7 grown in the presence and absence of glucose, and effect of glucose on the growth of Pm7. **a** Supernatants were collected from Pm7 cultured in LB medium in the presence and absence of glucose at different densities and assayed for enzyme activity using azocasein as the substrate. **b** Pm7 was cultured in LB medium in the presence and absence of glucose; aliquots were taken at different time points for the measurement of absorbance at OD_{600}. Data are from at least three independent experiments and are presented as mean ± SE

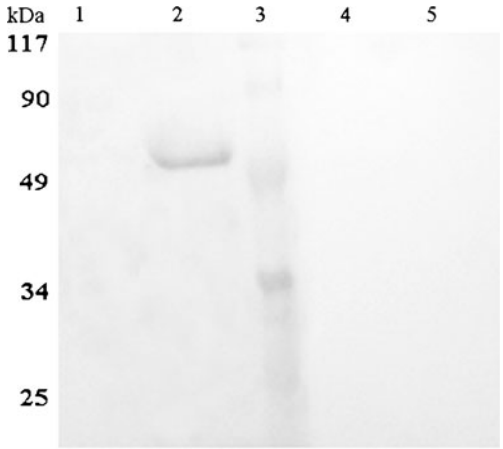

Fig. 2 Subcellular location of ZapA from Pm7. Cells were cultured in LB medium to an OD_{600} of 4.0 and used for the preparation of extracellular proteins. Equal amounts of proteins were electrophoresed in a 0.1% SDS/12% polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane and reacted with anti-ZapA antibodies or preimmune serum. Lanes: *1* extracellular proteins of Pm7 grown in the presence of glucose blotted with anti-ZapA antibodies; *2* extracellular proteins of Pm7 grown in the absence of glucose blotted with anti-ZapA antibodies; *3* prestained protein molecular weight marker; *4* extracellular proteins of Pm7 grown in the presence of glucose blotted with preimmune serum; *5* extracellular proteins of Pm7 grown in the absence of glucose blotted with preimmune serum.

Zap27 showed no obvious enzyme activity. These results suggested the possibility that the C-terminal motif was essential to the enzyme activity of ZapA.

Effect of temperature on activity of recombinant ZapA

Temperature was found to have a strong impact on the activity of recombinant ZapA. As shown in Fig. 3, within the range of temperatures tested, the activity of ZapA increased with temperature until the latter reached 40°C, at which the enzyme exhibited maximum activity. Once the temperatures exceeded 50°C, the activity of the protease decreased precipitously to barely detectable levels. The protease exhibited more than 50% of the maximum activity in the temperature range of 20 to 50°C. Thermostability analysis showed that recombinant ZapA was stable over the temperature range of 10 to 35°C and retained nearly 100% activity after incubation at 40°C for 1 h (Fig. 3).

Effect of pH on activity of recombinant ZapA

To examine the effect of pH on the activity of recombinant ZapA, a combination of three different buffering systems were employed. As shown in Fig. 4, the enzyme activity of ZapA exhibited more than 50% of the maximum activity over a pH range of 7.0–9.0. Deviations from this window led to drastic reductions in enzyme activity. The optimum

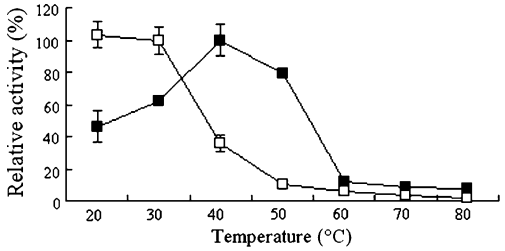

Fig. 3 Effect of temperature on the activity of purified recombinant ZapA and thermostability of activity of ZapA. The effect of temperature on activity (■) was determined under standard assay conditions at temperatures between 20 and 80°C with azocasein (2.5 mg/ml) as substrate. Thermostability (□) was determined by preincubating ZapA in the standard assay buffer at the indicated temperature for 1 h before initiating the enzymatic reaction by the addition of azocasein. Data are from at least three independent experiments and are presented as mean ± SE

pH for the recombinant protease was approximately 8.0. pH stability analysis showed that purified ZapA was stable over the pH range of 7.0–9.0 and retained more than 50% activity after incubation at pH 7.0 for 1 h (Fig. 4).

Effects of cations and sulfhydryl reagents on activity of recombinant ZapA

To examine the effects of various cations, EDTA and sulfhydryl reagents such as β-ME and dithiothreitol (DTT) on the enzyme activity of recombinant ZapA, standard enzyme assays were performed at 40°C in 50 mM sodium phosphate buffer (pH 7.0) supplemented with the tested cation at two different concentrations (1 and 10 mM; for Hg^{2+} the concentrations were 0.05 mM and 0.5 mM). The results (Table 1) showed that, in contrast to several metal ions tested (Na^{+}, K^{+} and Ca^{2+}), which exhibited no apparent effects on the enzyme activity of ZapA, Zn^{2+} and Co^{2+} at concentrations of 1 mM had a clear positive effect on the enzyme activity of ZapA. Heavy metal ions,

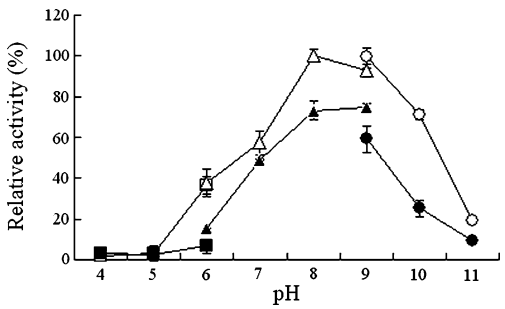

Fig. 4 Effect of pH on the activity of purified recombinant ZapA and pH stability of activity of ZapA. The effect of pH on the activity of ZapA (□, △, ○) and pH stability (■, ▲, ●) were determined in three different buffers using azocasein as substrate: 50 mM citric acid-sodium phosphate (pH 4.0–6.0; *squares*), 50 mM sodium phosphate (pH 6.0–9.0; *triangles*), and 50 mM glycine-NaOH (pH 9.0–11.0; *circles*)

Table 1 Effects of different cations, EDTA and sulfhydryl reagents on the enzyme activity of the purified recombinant ZapA. Activities of the purified proteins were determined by using azocasein as a substrate and are expressed as percentages of the enzyme activity measured in 50 mM citric acid-sodium phosphate buffer (pH 6.0) alone. Data are from three independent experiments and are presented as mean ± SE. *β-ME* β-mercaptoethanol, *DTT* dithiothreitol

Cation, EDTA or reducing reagent	Relative activity (%) of ZapA at cation concentration of:	
	1 mM	10 mM
Na^{+}	104±2.8	107±3.9
K^{+}	105±1.9	108±4.1
Ca^{2+}	97±10.0	101±9.3
Co^{2+}	136±9.6	88±6.9
Mg^{2+}	101±3.3	87±9.3
Mn^{2+}	114±3.8	91±5.4
Zn^{2+}	138±4.8	107±7.7
Cu^{2+}	109±3.2	60±2.0
Cd^{2+}	86±4.6	23±2.9
Pb^{2+}	74±3.8	63±5.6
Ag^{+}	94±4.3	9±2.5
Hg^{2+} [a]	14±4.5	2±2.1
EDTA	74±4.5	20±9.0
β-ME	98±2.7	73±3.6
DTT	88±4.3	33±3.1

[a] Concentrations of Hg^{2+} used in the test were 0.05 mM and 0.5 mM, respectively

including Cd^{2+}, Cu^{2+}, Ag^{+}, Pb^{2+}, and, to a certain extent, Mg^{2+} and Mn^{2+}, showed negative effects on enzyme activity at concentrations of 1 mM or 10 mM. The effect of Hg^{2+} on the enzyme activity of ZapA was detectable even at concentrations of 0.05 mM. Sulfhydryl reagents β-ME and DTT, which can reduce S–S bonds to –SH intramolecularly, and the divalent chelating agent EDTA, appeared to have a range of negative effects on the enzyme activity of ZapA.

Effects of glucose on swarming motility of Pm7 and expression of *zapA*

Swarming motility of Pm7 was measured in millimeters from the center of the inoculated bacteria to the last zone of swarming growth. After incubation on the plate for 5 h, Pm7 began to grow outward in a bull′s-eye pattern formed by consecutive waves of rapid swarming. However, on a plate supplied with 20 mM glucose, motility of Pm7 was inhibited completely. Extracellular enzyme activity of Pm7 cultured in LB supplied with glucose exhibited similar trends to that of Pm7 cultured in LB without glucose, but was lower in the measured value at each of the selected time points after an OD_{600} of 3.5 (Fig. 1a). Western immunoblotting with extracellular proteins using anti-ZapA polyclonal antibodies indicated that glucose depressed the production capacity of ZapA (Fig. 2, lanes 1 and 2). Total RNA was extracted from cells grown for 5 h in LB medium supplied with different concentrations of glucose and used for real-time PCR to analyze the expression of *zapA*. The result showed that addition of glucose at a final concentration of 20 mM caused a 5-fold depression in the mRNA levels of *zapA*. To ensure that these reductions were not due to the retardation of growth, Pm7 was cultured in LB medium in the presence or absence of glucose. Figure 1b shows that addition of glucose had no effect on the growth of Pm7. All these results suggested that both expression of *zapA* and swarming motility of Pm7 were regulated by glucose.

ZapA degrades bacterial OMPs

To test the hypothesis that ZapA hydrolysis is not limited solely to IgA, IgG and antimicrobial peptides, an SDS-PAGE method was carried out to qualitatively assess the substrates hydrolyzed by this metalloprotease. OMPs were extracted from bacterial strains T41 and TX1 and used as

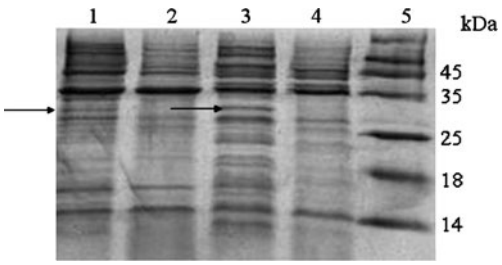

Fig. 5 Qualitative measurements of the degradation of proteins by ZapA. A qualitative assessment of protein degradation by ZapA was made by an SDS-PAGE assay in which protein substrates alone and protein substrates degraded by ZapA were compared. The assay was performed in standard assay buffer at 37°C for 1 h. After electrophoresis, the gel was stained with Coomassie blue. Lanes: *1* Outer membrane proteins (OMPs) of T41; *2* OMPs of T41 digested by ZapA; *3* OMPs of TX1; *4* OMPs of TX1 digested by ZapA; *5* protein molecular weight marker

substrates to analyze the broad protease substrate specificity of ZapA. After electrophoresis, the proteins were viewed after staining with Coomassie blue. Figure 5 showed that, although OMPs treated with ZapA exhibited similar protein profiles when compared with OMPs alone, there were proteins intermediate in size between 25-kDa and 35-kDa from OMPs of both bacteria obviously digested by ZapA. However, the results of SDS-PAGE analysis showed that ZapA did not prefer a distinct peptide bond.

Discussion

Many strains of *P. mirabilis* from a variety of sources have been reported to secrete EDTA-sensitive metalloproteases (Loomes et al. 1992; Wassif et al. 1995; Anéas et al. 2001). In this study, ZapA from Pm7—a strain isolated from marine environments—was reconstituted and purified from BL21(DE3) for the first time. The biochemical characteristics of the recombinant ZapA were also determined. ZapA exhibited optimum enzyme activity at pH 8.0, which indicated that the weakly alkaline conditions of infected urine tract would permit the protease ZapA to act efficiently. This finding was consistent with the fact that Pm7, the source strain of ZapA, was a marine inhabitant. Similar to the protease purified from *P. mirabilis* strain BB2000, the 54-kDa ZapA from Pm7 was inhibited by EDTA, whereas Ca^{2+} at concentrations of 1 mM or 10 mM had no obvious effects on the enzyme activity of ZapA. This was probably due to the substitution of histidine by tyrosine at position 167 in the amino acid sequence of ZapA from Pm7. Since proteases have been implicated in several physiological and pathogenic processes, including acquiring nutrition and hydrolyzing the defense factors of their hosts (Häse and Finkelstein 1993; Rao et al. 1998), the negative effects of heavy metals including Ag^{+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} and sulfhydryl reagents suggested that Pm7 may not survive under severe environments (Mobley and Belas 1995).

Expression of *zapA* was coordinately regulated during swarming cell differentiation (Allison et al. 1992a, b), but the varied, strain-dependent levels of ZapA and swarming motility of *P. mirabilis* strains are worth emphasizing (Stankowska et al. 2008). In this study, adding glucose at a final concentration of 20 mM showed inhibitory effects on both swarming mobility of Pm7 and expression of *zapA* at the transcriptional level. These observations were in accordance with studies of *P. mirabilis* L-68 and PRM1 variants, respectively (Bonato et al. 1982; Rauprich et al. 1996). This result suggested that glucose could prevent infection caused by *P. mirabilis*. Experiments aimed at elucidating the role of glucose in the expression of *zapA* and swarming motility of Pm7 are in progress.

Metalloprotease ZapA is known to have low substrate selectivity. In addition to the exhaustively studied immunoglobulin proteins, ZapA is also believed to destroy the structural components of cells matrix (collagen, fibronectin, and laminin) and cytoskeletal proteins (actin and tubulin) (Loomes et al. 1990; Belas et al. 2004). Our studies verified that ZapA could degrade OMPs from bacteria that could cause serious diseases in aquaculture. The broad substrate spectrum of ZapA provided *P. mirabilis* with a certain selective advantage to enable its survive in the environment.

Acknowledgments We thank Dr. L. Sun of Institute of Oceanology, Chinese Academy of Sciences for her gift of the bacterial strain TX1. This work was supported financially by the National Natural Science Foundation of China (NSFC) grant 20975089, the Department of Science and Technology of Shandong Province of China grant 2008GG20005005, the Department of Science and Technology of Yantai City of China grant 2010235, the Innovation Projects of the Chinese Academy of Sciences grant KZCX2-EW-206 and KZCX2-YW-Q07-04, the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences grant Kf201012, and the 100 Talents Program of the Chinese Academy of Sciences.

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