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ORIGINAL PAPER

Isolation and characterization of a serine protease-producing marine bacterium *Marinomonas arctica* PT-1

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Abstract A serine protease-producing marine bacterial strain named as PT-1 was isolated and identified as a family of *Marinomonas arctica,* based on molecular characterization of 16S rRNA gene sequence, phylogenetic tree, and fatty acid composition analyses. Optimized culture conditions for growth of the bacterium PT-1 and production of protease (ProA) were determined to be pH 8.0 in the presence of 5 % NaCl, at 37 °C during 24 h of incubation in the presence of 1.0 % skim milk. The molecular weight of the purified ProA was estimated to be 63-kDa as a major band by SDS-PAGE. We were intrigued to find that the activity of ProA was not inhibited by pepstatin A, chymostatin, and leupeptin known as inhibitors for cysteine protease. However, phenylmethylsulfonyl fluoride (PMSF) completely inhibited protease activity, suggesting that the ProA is like a serine protease. To the best of our knowledge, this is the first report on serine protease of *Marinomonas* species.

Keywords Serine protease · Substrate specificity · *Marinomonas* species · Marine bacterium

Introduction

Among the different industrial enzymes, proteases, which can be obtained from plants, animals, and microbial sources, the latter such as bacteria [1, 2] account for up to 60 % of protease sources in various industries including food, detergents, production of nutritionally important amino acids, and pharmaceutical manufacture applications. Extracellular proteases secreted into culture medium by many bacteria differ from one another in their biochemical properties [3, 4]. Therefore, the practical use of microbial proteases has led to the isolation of hyperactive strains, the development and characterization of novel biochemical properties including substrate specificity, thermo-stability, and detergent resistance.

Until recently, microbial sources, in particular the genus of *Bacillus* known as one of the most important producers of extracellular proteases from *B. subtilis* [5], *B. halodurans* [6], and *B. laterosporus* [7], have been extensively studied by many researchers. Beside bacterial proteases, fungal keratinase [8] has also attracted the attention of environmental biotechnologists since fungi secrete large amount of enzymes into the culture medium, which facilitates downstream processing for industrial production and applications [6]. Among the genus of many fungi known to exhibit high keratinolytic activities, *Aspergillus* keratinase activity from *A. niger* [9] has been extensively studied. Due to the high industrial demand for proteases, researchers continue to isolate and indentify potent protease producers. Earlier studies have demonstrated that many bacterial and fungal species secrete a substantial amount of proteolytic enzymes including cold-adaptive proteases [10, 11]. However, there are no reports on the production and characterization of protease from *M. arctica* species. In the present study, we communicate the isolation and characterization of marine bacterium *M. arctica* (KTCC 12465BP) producing a halophilic extracellular protease, isolated from the sea-mud called Getpearl in South Korea.

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Materials and methods

Chemicals

Azocasein, casein, skim milk, gelatin, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pepstatin A, phenylmethylsulfonyl fluoride (PMSF), chymostatin, leupeptin used as inhibitors for cysteine protease, and Coomassie Brilliant Blue R250 were also purchased from Sigma Chemical Co. (St. Louis, MO, USA). Columns of HiPrep 16/10 Q XL and Superdex^{TM} 75 HR 10/30 were purchased from GE Healthcare (GE Healthcare Science AB, Sweden). Other protein sources such as bean powder, fishery powder, and keratins were kindly provided by Prof. Yu Jin Hwang (Gachon University, Korea). All the other chemicals used were of analytical grade manufactured in Korea.

Gene sequencing and phylogenetic tree analyses

A marine bacterial strain named as PT-1 that produces halophilic protease exclusively was selected and subjected to the 16S rDNA sequences and phylogenetic analyses to identify the microbial species. The 16S rDNA gene sequences determined was aligned based on the secondary structure of the ribosomal RNA, and estimated % similarity through the DNA data bank. A phylogenetic tree analysis based on 16S rDNA gene sequences data to classify the isolate was constructed using the CLUSTAL W program and the neighbor-joining method, following the previous study [12].

Analysis of fatty acid methyl esters composition

Bacterial cells were centrifuged and lyophilized using a freeze-dryer (FDUT-6002, Operon, Gimpo, Korea) for fatty acid methyl esters (FAMEs) analysis. Briefly, dried samples were dissolved in 2 mL of solution freshly prepared by mixing acetyl chloride and methanol (5:100, v/v) with nonadecanoic acid (19:0) as an internal standard, making a concentration of 1 mg/L. Trans-esterification reaction was performed at 80 °C for 1 h under pure nitrogen gas stream and darkness. FAMEs obtained by trans-esterification reaction were extracted by addition of 1 mL hexane. FAMEs were then analyzed by using a gas chromatograph equipped with flame ionization detector (Acme 6000 GC, Younglin, Seoul, Korea) with HP-INNOW WAX column (length = 100 m, diameter = 0.25 mm, and film thickness = 0.2 μm). The FAMEs were identified by comparing their retention time with those of FAMEs standards mixture (F.A.M.E. Mix C4-C24, SUPELCO, Bellefonte, USA).

Bacterial strain and culture conditions for protease production

A potent protease producer *M. arctica* PT-1 (KTCC 12465BP) which revealed hydrolyzing activity on solid-agar plate containing 1.0 % skim milk was isolated from the sea-mud (called Getpearl) in South Korea and used for this study. This marine bacterial strain PT-1 was further cultured for protease production in culture medium (250 mL) consisting of $MgSO_4$ (0.05 %), yeast extract (0.05 %), peptone (0.05 %), and dibasic sodium phosphate (0.05 %) in sea-water for 24 h at room temperature, with constant shaking at 150 rpm (JEIO TECH Co., Korea). The crude culture filtrate obtained by filtration through 0.2 μm filter membrane (Toyo Roshi Kaisha, Ltd, Japan) was used as enzyme source for purification.

Purification of protease

The enzyme in the cell-free culture filtrate (250 mL) was precipitated with ammonium sulfate (20 % saturation) and then centrifuged at 13,000 rpm for 30 min to remove the precipitates and dialyzed against the same buffer for 24 h at 4 °C. Continuously, the dialyzed sample was further brought to 50 % (w/v) saturation with ammonium sulfate and precipitated overnight at 4 °C. The precipitate was then collected by centrifugation at 13, 000 rpm for 30 min, dissolved, and replaced with the same buffer using Amicon ultra filters ranging from 30 to 100 K (Millipore Ireland Ltd, Ireland). The dialyzed solution (22 mL) loaded onto a column (2.8 × 30 cm) of DEAE-Sepharose (GE Healthcare, Uppsala, Sweden) was equilibrated with 25 mM Tris–HCl buffer (pH 8.0) containing 10 mM sodium chloride. The purity and apparent molecular weight of the purified enzyme was estimated by Superdex 75 10/300 GL column chromatography and SDS-PAGE analysis, using a 4–25 % gradient polyacrylamide gel and stained using Coomassie Brilliant Blue or a silver staining kit (ELPIS Biotech Inc., Taejon, Korea). The molecular mass of the enzyme was determined using a known standard protein molecular weight marker ranging from 10 to 120-kDa protein ladder (Bio-Rad Laboratories, Hercules, CA, USA). The concentration of the purified enzyme was determined according to the method of Bradford using BSA as standard [13].

Protease assays

ProA activity was measured by using skim milk, casein, and gelatin as the substrate. The reaction mixture contained 1.0 mL of 25 mM Tris–HCl buffer (pH 8.5), 0.01 mL of enzyme solution, and 10 mg of substrate. Incubation was

carried out at 37 °C for 30 min with constant agitation at 150 rpm. The enzyme reaction was quenched by the addition of 0.5 mL of 10 % (w/v) TCA and then kept on ice for 30 min. After centrifugation at 13,000 rpm for 10 min, the absorbance of the supernatant was measured at 280 nm. One unit (U) of ProA activity was estimated as an increase in absorbency of 0.1 at 280 nm under the standard conditions for the assay.

Effects of pH and temperature on ProA activity

For measurement of the optimum pH of ProA activity, the enzyme activity was estimated using casein as substrate at 37 °C at various pHs ranging from pH 3.0 to 6.0, using 50 mM acetate buffer, pH 6.0–8.0 using 50 mM potassium phosphate buffer, pH 8.0–10.0 using 50 mM sodium borate, and pH 10.0–11.0 using 50 mM sodium carbonate, respectively. The optimum temperature ProA activity was measured by performing the enzyme reaction at temperatures between 20 and 60 °C in Tris–HCl buffer (pH 8.5). For thermo-stability of ProA, the enzyme was incubated at 60 °C for 1 h and the residual activity retained was measured at the optimum temperature.

Kinetic characteristics of ProA

The kinetic constants, K_m and V_{max}, were calculated using Woolf–Augustinsson–Hofstee plot, as described in the previous study [14]. Effects of detergents, such as DMSO, EDTA, Triton X-100, and SDS and divalent metal ions, on ProA activity were tested; detergents were added to a final concentration of 0.1–5.0 % and at a concentration of 1.0 mM.

Statistical analysis

Results obtained from the experiments were expressed as the mean ± SD, unless representative data were indicated as the means of averages from the experiments. *P* values obtained from the experiments less than 0.05 were considered significant.

Results and discussion

Identification of proteolytic strain

To expand the effective utilization and enzymatic processing of protease (ProA), we have isolated and identified a gram-negative marine microbial strain *Marinomonas arctica* from sea–soil called Getpearl collected in InCheon-city, South Korea. Among nine different types of strains which appeared on the solid-agar plate containing skim milk, one named as PT-1 was further selected as for the strongest ProA-producing strain. Consequently, the action of ProA may be directly related to the degradation of skim milk used as the sole carbon and nitrogen source, as shown in Fig. 1a. Interestingly, the bacterium PT-1 was able to degrade colloidal chitin used as a substrate to elucidate other biological activity of PT-1 (see Fig. 1b). The bacterial strain PT-1 that showed the highest protease activity was further isolated and characterized to be a Gram-negative, short rod, with a single-long polar flagellum as shown by a TEM (see Fig. 1c). As earlier studies demonstrated, the high number and phylogenetic diversity of bacterial strains collected in different seasons were observed in the phylogenetic composition differences of sea bacterial communities [15, 16]. The various species

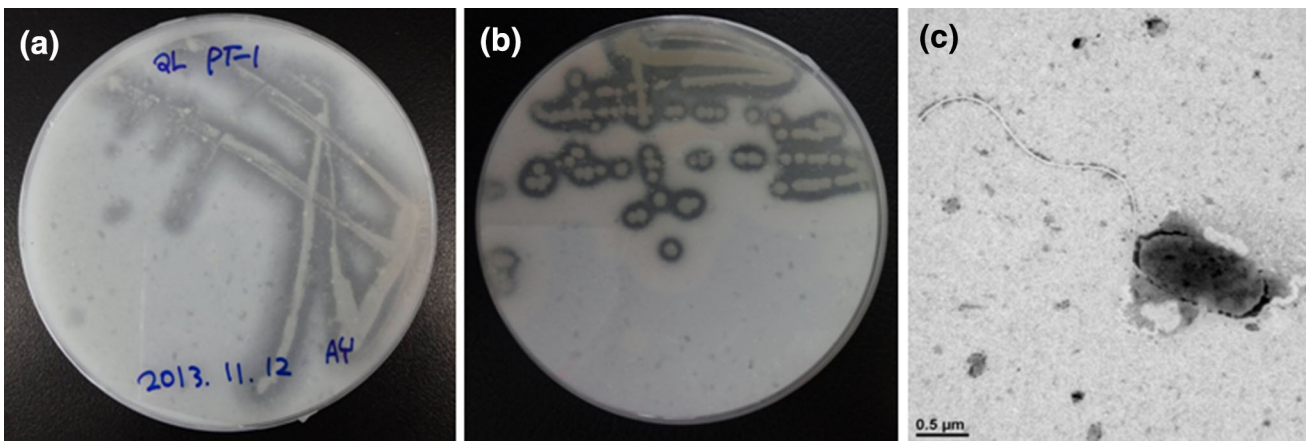

Fig. 1 Visible enzyme activities of *Marinomonas arctica* PT-1. The bacterium was grown on solid-agar medium containing colloidal chitin (**a**) and skim milk (**b**) for chitinolytic and proteolytic enzymes, respectively, at 37 °C, for 3 days. The proteolytic bacterium *M. arctica* PT-1 was negatively stained and its morphology was observed at 80 kV using a transmission electron microscope (TEM) (**c**)

isolated from the sea-ice or arctic sea-ice known to date were routinely grown at between 20 and 27 °C. Upon the culture of the strain PT-1 in the presence of NaCl or KCl ranged from 0 to 20 % in the culture media to assess the salt resistance, maximal growth 5.0 % NaCl, with a wide range from 2.5 to 10 % was observed (data not shown). These results indicate that the strain PT-1 is one of the halophilic marine bacteria. Taken together, PT-1 strain, deposited in the Korean Type Culture Collection under the accession number KCTC 12465BP, was first identified and characterized as a strain for a serine protease and chitinsolytic enzyme producer associated with the viability of bacterial counts under halotolerant conditions.

16S rDNA sequence and phylogenetic tree analysis

A potent ProA producer PT-1 was identified and characterized based on the gene 16S rDNA sequences with phylogenetic tree analysis (see Fig. 2). The blast search of 939 bp of 16S rDNA gene from the strain PT-1 indicated 98.1 % pair-wise similarity toward the species of *Marinomonas*. Based on the blast search, we found that PT-1 exhibited over 96.7–97.9 % sequence similarity with the partial sequences of various microbial strains such as *M. pollencensis* [17], *M. arctica* [18], and *M. pontica* [19]. These results indicate that the strain PT-1 has considerable sequence homology with a wide variety of *Marinomonas* species, no particular genus of *Marinomonas* species has been exploited for the production of halophilic protease with wide variety of substrate specificities. In addition, it is worthy to note that the strain PT-1 can actively hydrolyze skim milk, casein, and gelatin used as protein sources at 4 °C, suggesting that PT-1 is thus a potentially important source for cold-active or cold-adaptive proteases.

Fatty acid composition of *M. arctica* PT-1

Bacterial lipid membranes are widely used as important indicators for the classification of the bacterial cell type. Our results show that the fatty acid composition of *M. arctica* PT-1 consists of C16:0 and C-18:1 accounting for approximately 96 % in total fatty acids as major fatty acids, and of these about 36 % were isomers (see Table 1). These results quite differ from other data base obtained from *Pseudoalteromonas* [20, 21], *Bacillus* [22], and *Marinomonas* species [17, 18] known as proteolytic enzyme producers. Taken together, based on the results of 16S rRNA sequence, phylogenetic tree, and major fatty acid composition analyses, we concluded that *M. arctica*

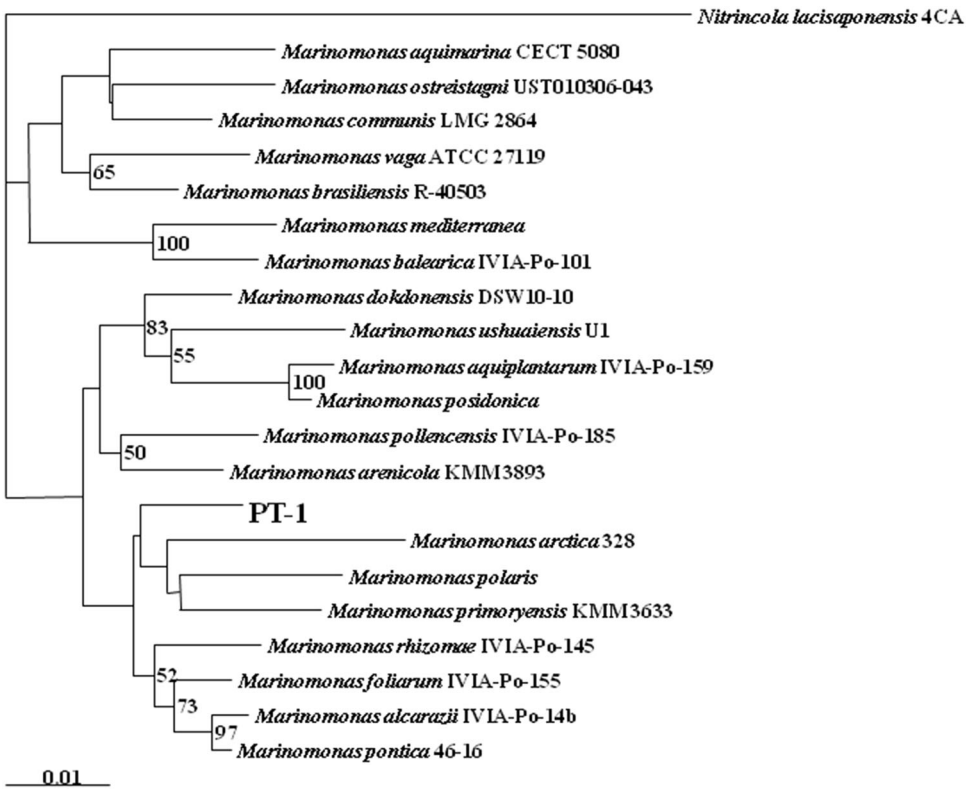

Fig. 2 Phylogenetic position of the strain *M. arctica* PT-1. The partial 16S rDNA gene sequence (939 bp) of the strain *M. arctica* PT-1 was determined and its homology was compared with the complete 16S rDNA gene sequences from other related genera deposited in data bank with 1000 replicates. *Bars* indicate 1 nucleotide substitution per 100 nucleotides

Table 1 Major compositions of fatty acids of *Marinomonas arctica* PT-1

Fatty acids	Average content (%)
C12:0	0.09 ± 0.003
C14:0	0.05 ± 0.009
C16:0	0.50 ± 0.024
C16:1	0.14 ± 0.007
C18:0	0.15 ± 0.011
C18:1	0.49 ± 0.026
C18:3	0.01 ± 0.004
Unidentified	2.33 ± 0.176

PT-1 can be classified as a novel marine bacterial strain isolated as a specific ProA producer.

Enzyme purification

An extracellular ProA was partially purified from the culture filtrate of *M. arctica* PT-1. (see Table 2). Molecular weight of the purified ProA from the culture filtrate of *M. arctica* PT-1 was determined to be about 63-kDa by silver staining as major bands (Fig. 3). It is worthy to note that our study provides the first report on the production and purification of a native *Marinomonas arctica*'s proteolytic enzyme.

Optimum pH and temperature of ProA

The effect of pH on ProA activity was investigated under varying pH values ranging from 3 to 11. Optimum pH of the purified ProA toward skim milk showed a typical bell-shaped curve with the maximum activity at pH 8.0 in 20 mM Tris–HCl buffer. These results suggest that the purified ProA in this study was confirmed as alkalophilic protease, which has been extensively studied by many researchers due to its importance in industries [8, 23]. In addition, the partially purified ProA showed an optimum

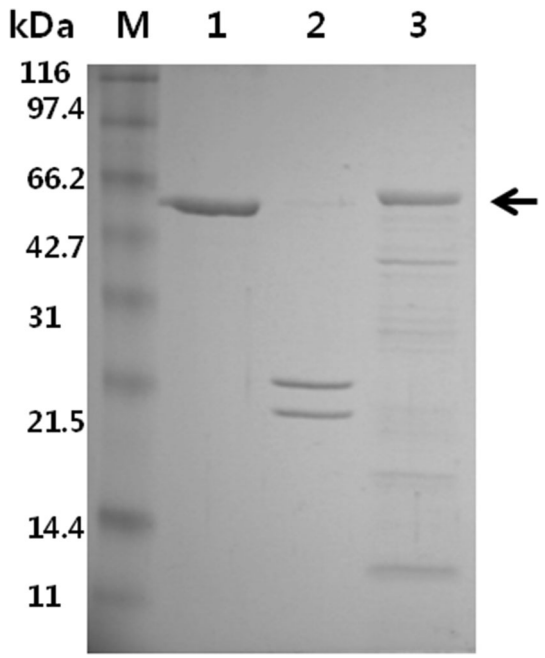

Fig. 3 SDS-PAGE analysis of ProA. *M* molecular weight markers, *lane 1* purified ProA using DEAE-Sepharose, *lane 2* Amicon ultra filters filtrates (<30 kDa), *lane 3* ammonium sulfate precipitates. The gel was stained using Coomassie Brilliant Blue

activity at 37 °C, and the enzyme activity retained similar activity up to 64 °C. Also 50 % of relative activity was retained after incubation at 50 °C for 2 h tested under the standard assay. Of note, considering about the wide variety of substrate specificities such as skim milk, casein, and gelatin used as protein sources at even 4 and 64 °C, our results suggest that *M. arctica* PT-1 is a potentially important source for cold-adaptive proteases with its thermal stability.

Inhibitory effects on ProA activity

The effect of protease inhibitors on ProA activity was determined using cocktail containing different protease

Table 2 Purification steps of protease from *Marinomonas arctica* PT-1

Step	Total vol. (mL)	Protein (mg/mL)	Total protein (mg)	Activity (mg/mL)	Specific activity (U/mg of protein)	Total activity (unit)	Purification (fold)
Cell culture supernatant	250.0	2.7	675.0	0.8	0.3	202.5	1.0
Ammonium sulfate precipitation	22.0	1.2	26.4	2.5	2.1	55.4	7.0
Amicon ultra filters	5.0	0.7	3.5	4.4	6.3	22.1	21.0
DEAE-sepharose chromatofocusing	1.5	0.4	0.6	5.1	12.8	7.7	42.7

Table 3 Inhibitory effect of chemicals on proteolytic activity of ProA

Compounds	Concentration	Relative activity (%)	
		Skim milk	Gelatin
None	0	100.0	100.0[a]
Ions and metal ions (mM)			
NaCl	1	98.8	103.3
KCl	1	95.5	102.4
$CaCl_2$	1	97.5	100.0
$MnCl_2$	1	33.3	98.4
$MgCl_2$	1	97.7	97.3
$ZnSO_4$	1	95.3	98.2
Chemical compounds (w/v, %)			
EDTA	0.5	3.6	97.7
SDS	0.1	70.6	129.6
	0.5	17.7	193.2
Triton-X100	0.1	105.5	66.7
	0.5	104.9	69.5
DMSO	1.0	83.9	67.0
	5.0	72.1	74.2

[a] Values are averages of three independent experiments

inhibitors at varying concentrations of 0.1–4 mM. Purified ProA was completely inhibited by the cocktail containing different protease inhibitors including serine protease inhibitor PMSF, and cysteine protease inhibitors such as chymostatin, leupeptin, and pepstatin A. However, no significant inhibitory activity by chymostatin, leupeptin, and pepstatin A was observed, when these reagents (0.1 mM) were used individually. Only PMSF, which completely inhibited enzyme activity, clearly indicates that the purified ProA from *M. arctica* PT-1 is a serine protease. Similar response to PMSF was reported for the keratinases [22, 24] and some of the bacterial proteases from *Bacillus* species [25]. Subsequently, since no inhibition of the ProA activity with chymostatin even at 0.1 mM was observed, these results suggest that cysteine residues may not be possibly involved in the catalytic process of the degradation of outsourcing protein (see Table 3).

In addition, about 30 % of enzyme activity decreased by addition of Triton X-100 and DMSO at 5 %, whereas, SDS showed of 82.3 % inhibitory activity at 0.5 % concentration when skim milk was used as substrate, not for gelatin. These results demonstrate that enzyme activity of ProA in the presence of EDTA or SDS is obviously depending on the primary structure of substrate which may affect the mode of enzyme actions. Additionally, effect of various metal ions at 1 mM on the ProA activity was investigated. Among the metal ions tested, Mn^{2+} significantly inhibited about 67 % of enzyme activity, whereas the ProA was found to be stable toward most of the metal ions tested compared to the control. The decreased ProA activity by the divalent metal ion Mn^{2+} may be due to its role in destabilizing complex of enzyme (see Table 4).

Table 4 Determination of substrate specificity of ProA

Substrate	Specific activities (U/μg)[a]
Azocasein	$0.56 \times 10^{-5} \pm 0.8 \times 10^{-7}$
Casein	$0.12 \times 10^{-5} \pm 0.8 \times 10^{-7}$
Gelatin	$0.44 \times 10^{-4} \pm 0.6 \times 10^{-7}$
Skim milk	$0.77 \times 10^{-3} \pm 0.9 \times 10^{-7}$

[a] Values are averages of three independent experiments

Kinetic parameters

The K_m and V_{max} values of enzyme activity toward skim milk and gelatin were determined to be 1.16×10^{-3} mg/mL and 7×10^{-5} U, and 7.60×10^{-4} mg/mL and 0.2×10^{-3} U, respectively (see Fig. 4a, b). The observed K_m value of this enzyme for gelatin was found to be higher than that of the K_m values of skim milk, casein, and azocasein used as substrates, respectively. These values indicated that ProA has a relatively high affinity toward gelatin for the substrate, since it needed a much lower concentration of substrate. Since proteolytic products are commercially important bioactive materials especially in functional foods [26, 27], nutraceuticals [27], and pharmaceuticals [28], further studies will be set to elucidate the ecological and physiological significances of the isolate *M. arctica* PT-1 to apply in biotechnology. Overall, our results provide evidence to support the further investigation of proteases for producing bioactive peptides for developing an alternative biological tool useful in controlling and maintaining food quality.

Conclusions

In this paper, isolation and production of a serine protease from a marine bacterial strain was proposed. The strain named as PT-1 was isolated and deposited as a novel species of the genus *Marinomonas* based on molecular characterization of 16S rRNA gene sequence, phylogenetic tree, and fatty acid composition analyses. A serine protease from the genus *Marinomonas* was purified and kinetically

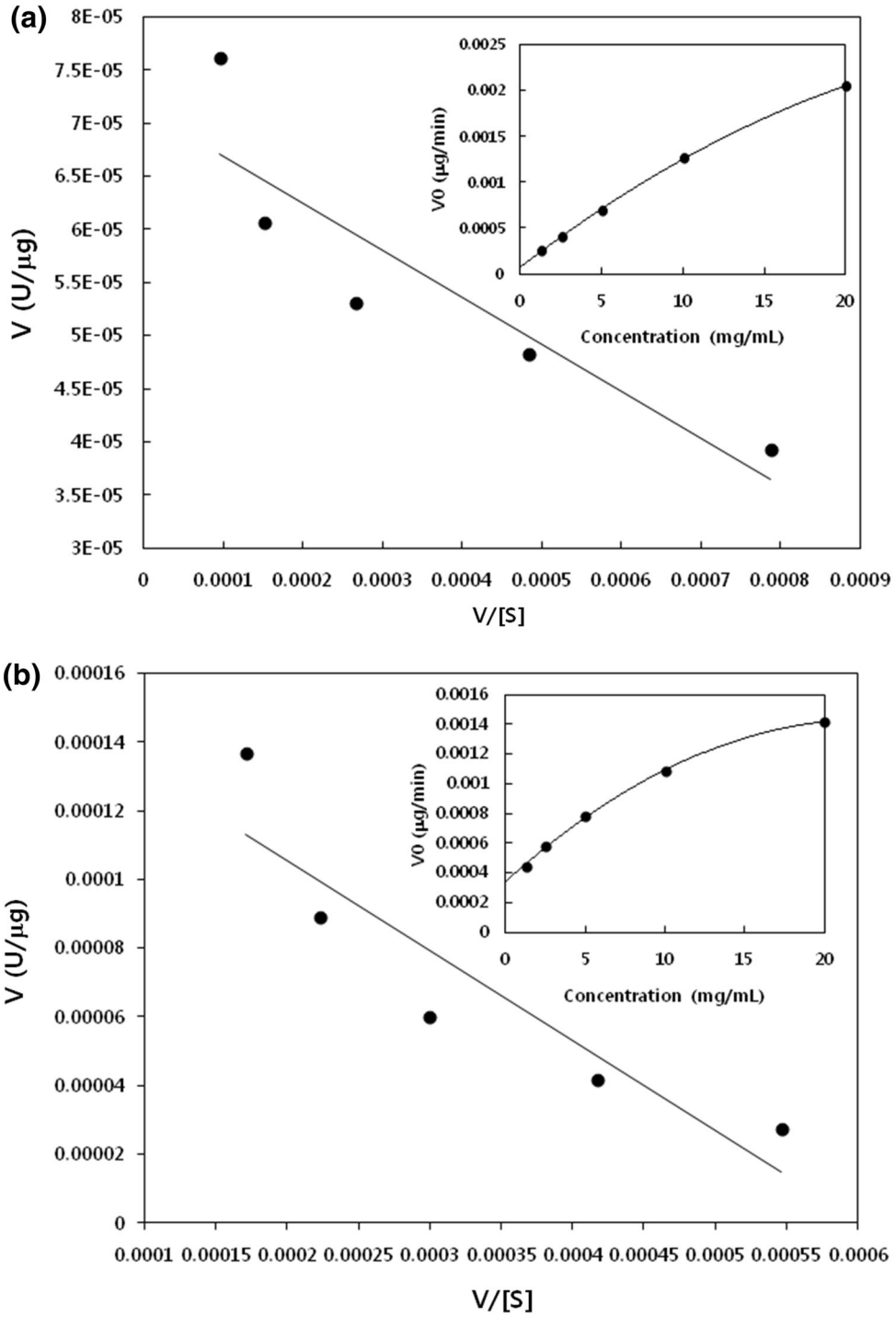

Fig. 4 Effect of substrate concentrations on the ProA toward skim milk (**a**) and gelatin (**b**). The initial rate of activity is plotted versus substrate concentration using 20 mg/mL, respectively, in Woolf–Augustinsson plot

characterized to elucidate the properties of the enzyme. The result proved the technical feasibility of the proposed process that could efficiently produce and utilize the enzyme for the production of bioactive peptides in industry of biological fermentation. This is the first report on the production and characterization of a serine protease from *Marinomonas* species.

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Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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