African Journal of Microbiology Research Vol. 6(9), pp. 2211-2221, 9 March, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.921
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Isolation and characterization of a keratinolytic protease from a feather-degrading bacterium *Pseudomonas aeruginosa* C11

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Accepted 27 December, 2011

A feather-degrading bacterium *Pseudomonas aeruginosa* C11 was isolated from feather dumping soil with quick plate screening technology and identified based on morphological and biochemical characterization and analysis of 16S rDNA sequences. The keratinolytic protease from *P. aeruginosa* C11 was purified 17.4-fold through ammonium sulphate precipitation, a sephadex G-75 gel filtration column and a DEAE sepharose fast-flow column. The purified keratinolytic protease could deeply degrade raw feathers within 24 h at 37°C. The relative molecular mass of the protease was estimated to be 33 kDa by SDS–PAGE. The protease was stable in pH range 5 to 10 and at temperature below 50°C with optimum pH of 7.5 and optimum temperature of 60°C. Mn^{2+} stimulated the keratinolytic activity by 21% at 2 mM, while Co^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+} and Fe^{2+} inhibited the keratinolytic activity. The keratinolytic activity was strongly inhibited by EDTA, but not by PMSF, which indicated the protease was a metalloprotease. The isolated protease was different from any reported keratinolytic metalloprotease and was worthy of further investigation.

Key words: feather-degrading bacterium, keratinolytic protease, enzymatic characterization, *Pseudomonas aeruginosa*.

INTRODUCTION

Keratin is known for its strong stability against chemical reagents such as acids, alkalis and common proteolytic enzymes like trypsin, papain and pepsin (Mabrouk 2008), and it represents the main constituents of the skin of vertebrates and appendages such as nails, hair, feathers, and wool (Brandelli et al., 2010; Chojnacka et al., 2011).

The polypeptide of keratin is densely packed which is strongly stabilized by several hydrogen bonds and hydrophobic interactions. Furthermore, cross-linking of protein chains resulted by disulfide bonds are also responsible for its high degree of recalcitrance in degradation processes (Brandelli et al., 2010; Jeong et al., 2010a).

Worldwide, feathers are produced from poultry-processing plants in excess of 5 million tons per year as a waste product (Poole et al., 2009); Considering that these keratin wastes have high protein content, they could be used as a source of protein and amino acids for animal feed and any other applications. Microorganisms which produce keratinolytic protease may have important use in hydrolysis of keratin-containing wastes from leather and poultry industries. That represents an attractive alternative method for efficient bioconversion and improving the nutritional value of keratin wastes by developing economically and environmentally friendly

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technology (Gupta et al., 2006; Brandelli et al., 2010; Prakash et al., 2010). The potential applications of such microbial keratinases have been recently reported (Gupta et al., 2006; Brandelli, 2008; Brandelli et al., 2010; Chojnacka et al., 2011).

The aim of this study was to obtain a keratinolytic protease with high ability to degrade the keratinous wastes, especially native feathers, and to discover the enzymatic characterization of keratinolytic protease for the future application in waste resources.

MATERIALS AND METHODS

Reagents

Folin-Ciocalteu reagent was from Shanghai Generay Biotech Co., Ltd. (China). Pyrobest Taq polymerase was from Sangon Biotech (Shanghai) Co., Ltd. Phenylmethylsulphonyl fluoride (PMSF), dithiothreitol (DTT) and casein was from Sigma Co. (USA). Other reagents were of either analytical or biological grade.

Feather powder

The fresh feathers from local market were washed with tap water, cut into sections of approximately 5 mm and sterilized by autoclaving at 121°C for 15 min, then further dried at 60°C for 24 h. The dried feathers were milled into powder for isolation of the keratinolytic strains on selective feather meal agar plates and determination of keratinolytic activity.

Screening and isolation of the bacterial keratinolytic strains

Samples were isolated from feather dumping soil in Huaian, China, and 5 g of soil sample was added to 100 mL 0.85% sterile saline solution. The mixture was used to spread on selective feather meal agar plates containing (g/L, pH 7.5): feather powder, 20.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; agar, 18 which were incubated at 30°C for 2 days. Colonies that formed hydrolysis zone were inoculated into liquid broth at 30°C for 4 days on a rotary shaker at 180 rpm. The liquid culture medium contained (g/L, pH 7.5): feather, 20.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2. The keratinolytic strains were confirmed by three different ways, that is, determination of keratinolytic activity using feather powder as substrate, measurement of the size of hydrolysis zone around the single colony on solid agar plate containing feather powder, and observation of the degradation of feathers in liquid culture.

Morphological, biochemical and molecular identification of the keratinolytic bacterial strain

The morphological and biochemical characteristics of the isolated bacterium were studied according to Bergey's Manual of Systematic Bacteriology (Brenner et al., 2004). Further identification of the microorganism was performed through 16S rDNA sequencing. Chromosomal DNA was extracted from 18 h culture cells using a CTAB method (Xu et al., 2007). The 16S rDNA was amplified by PCR using a DNA thermal cycler (Prism, MWG-Biotech). The bacterial 16S rDNA primers used for gene amplification and sequencing were 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). After initial denaturation at 98°C for 5 min, the PCR reaction was carried out using Pyrobest Taq polymerase. The conditions for each cycle were as follows: denaturation at 95°C for 35 s, annealing at 55°C for 35 s, and extension at 72°C for 90 s. A final extension step for 8 min at 72°C was performed at the end of the 35 cycles. The amplified PCR products were sequenced by Sangon Biotech (Shanghai) Co., Ltd. Nucleotide sequence of the amplified PCR products was compared with other deposited sequences in the Genbank via the online programme BLAST (basic local alignment search tool). The 16S rDNA gene sequences were aligned by using multiple-sequence alignment software Clustal X and trimmed, and phylogenetic trees were constructed with the neighbour-joining method according to the Kimura two-parameter model implemented in MEGA4.

Protein estimation and keratinolytic activity determination

The *P. aeruginosa* C11 was cultured at 30°C for 4 days on a rotary shaker at 180 rpm for the production of keratinolytic protease. The culture medium (g/L, pH 7.5) was supplemented with feather powder, 30; trypton, 5; $MgSO_4$, 0.1; yeast extract, 5; K_2HPO_4, 1 and distilled water. Protein concentration was determined at 750 nm according to Lowry method (Scopes 1982) using bovine serum albumin (BSA) as the standard. The keratinolytic activity was determined spectrophotometrically using a modified Folin-Ciocalteu method (Margesin et al., 1991).The enzyme solution (0.5 ml) incubated with 1% (w/v) feather powder (1.5 ml), in 50 mmol/L barbital/HCl buffer (pH 7.5) at 50°C for 2 h. The reaction was terminated with 3 ml of 10% (w/v) trichloroacetic acid (TCA) and then the reactants were allowed to stand for 30 min. After centrifugation at 10,000×g for 10 min, 1 ml of the supernatan was added with 5 ml of 0.55 mol/L Na_2CO_3 and 1 ml of Folin-Ciocalteu reagent, and then the mixture was incubated at 40°C for 15 min. The keratinolytic activity was measured at 750 nm with a 721 visible spectrometer (China). One keratinolytic activity unit (U) was defined as an increase of 0.01 OD at 750 nm in 1 h.

Purification of the keratinolytic protease

The culture supernatant was obtained by centrifugation at 10,000×g for 5 min, then solid ammonium sulfate was gradually added to 30% saturation. The precipitate formed was removed by centrifugation at 10,000×g for 5 min. After further addition of solid ammonium sulfate to supernatant until 50% saturation, the resulting precipitate was collected by centrifugation at 10,000×g for 5 min. The collected precipitate was dissolved in Tris–HCl buffer (25 mM, pH 7.8), dialyzed against 10 mM of the same buffer for 24 h, and concentrated. Next purification step of the concentrated sample was carried out through a sephadex G-75 gel filtration column equilibrated with 10 mM Tris–HCl buffer (pH 7.8). The keratinolytic active fractions were eluted with the same buffer, pooled and concentrated. Insoluble substances were removed by centrifugation at 10,000×g for 5 min, and the sample was applied to a DEAE sepharose fast-flow column equilibrated with 10 mM Tris–HCl buffer (pH 7.8). Elution was performed with a linear gradient of Tris–HCl buffer (10 mM, pH 7.8) containing NaCl from 0 to 0.5 M. The fractions possessing keratinolytic activity were pooled and concentrated. The active fractions eluted from DEAE sepharose fast-flow column were used for keratinolytic activity analysis. All purification steps were performed at 4°C. To examine the purity and determine the molecular weight of the enzyme, discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed with 12% polyacrylamide gel as described (Laemmli et al., 1970). The protein bands were stained with a solution of Coomassie blue R-250.

Figure 1. Zone of hydrolysis around the bacterial growth on selective feather meal agar plates at 30°C for 2 days.

Effect of pH and temperature on keratinolytic activity and stability

The optimal pH for keratinolytic activity was determinated over a pH range of 6.0–9.5. To determine the pH stability, the purified enzyme were preincubated in buffers over a pH range of 3.0–11.0 at 50°C for 60 min. Citric acid/sodium phosphate buffer was used for pHs between 3.0 and 7.5; barbital/HCl for pHs between 7.0 and 9.0 and sodium carbonate buffer for pHs between 9.0 and 11. The optimal temperature for the purified keratinolytic protease was determined over the range of 40–70°C in barbital buffer (pH 7.5). To determine the thermostability, the enzymes were preincubated in barbital buffer (pH 7.5) at temperatures over the range of 50-80°C.

Effect of metal ions, additives and protease inhibitors on keratinolytic activity

The purified protease were preincubated in barbital buffer (pH 7.5) at 30°C for 10 min with Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, Fe^{2+}, Li^{+}, ethylenediaminetetracetic acid (EDTA), 2-mercaptoethanol, dithiothreitol (DTT) and phenylmethylsulfonyl fuoride (PMSF) at 2.0 and 10 mM, and with gycerol and dimethyl sulfoxide (DMSO) at 1% and 5% (v/v). The activity of the control without addition of any additive was taken as 100%.

RESULTS

Screening for feather-degrading microorganisms

More than 200 microorganisms were obtained, among which, strain C11 was found to have good keratinolytic activity when inoculated on solid agar plate at 30°C for 2 days by measuring the diameter of hydrolysis zone (Figure 1). Feathers could be completely degraded by strain C11 in liquid broth at 30°C for 4 days (Figure 2) and raw feathers be deeply degraded by the purifed keratinolytic protease at 37°C for 24 h (Figure 3).

Characterization of the feather-degrading strain C11

Morphological and biochemical characteristics of strain C11 were summarized in Table 1, which indicated that it belonged to *P. aeruginosa* according to Bergey's Manual of Systematic Bacteriology (Brenner et al., 2004). 16S rRNA gene sequence of strain C11 was submitted to GenBank and given accession numbers JQ083279. Phylogenetic tree of 16S rRNA gene from the strain C11 is showed in Figure 4, and the 16S rRNA sequence of strain C11 showed highest similarity to that of *P. aeruginosa* ATCC 10145^{T}.

Purification of keratinolytic protease

With the purification procedure (Table 2), the keratinolytic protease from *P. aeruginosa* C11 culture was purified 17.4-fold and the purified enzyme showed a specific activity of 170.4 U/mg. Only one peak of keratinolytic activity was eluted from sephadex G-75 gel filtration

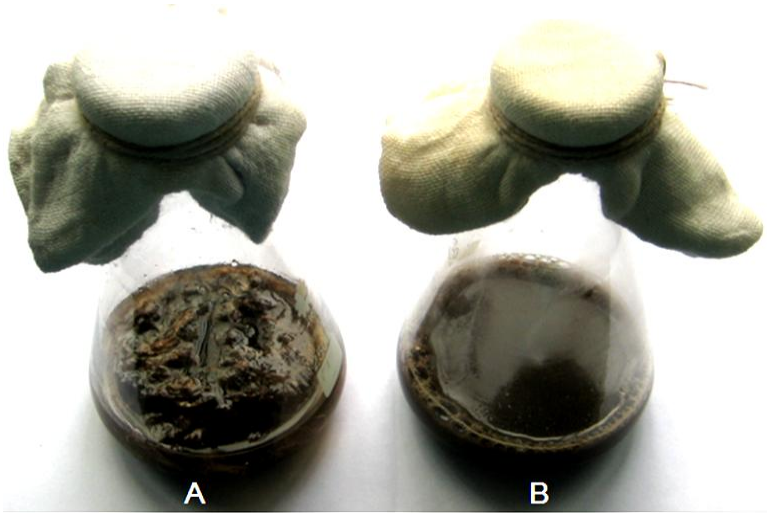

Figure 2. Complete degradation of feathers by *P. aeruginosa* C11 when cultured at 30°C for 4 days. (A): uninoculated feathers medium (control) remained unchanged. (B): complete hydrolysis of feathers

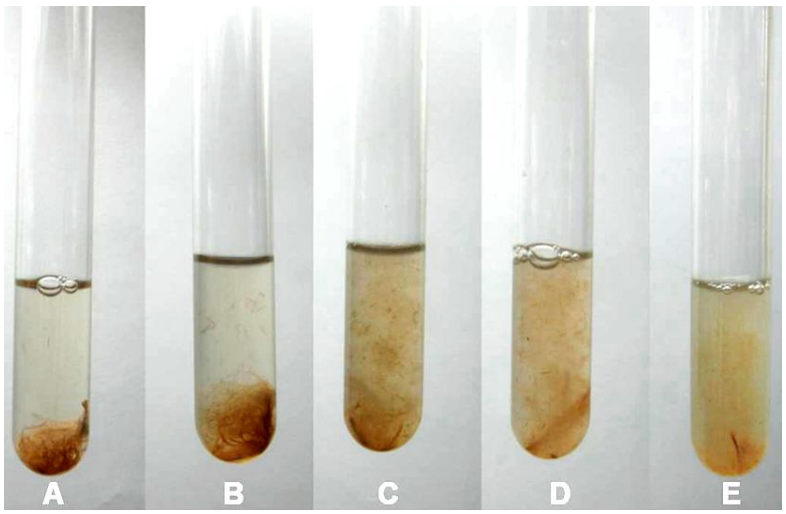

Figure 3. Feather degradation by purifed keratinolytic protease (151 U/mL) from *P. aeruginosa* C11 in Tris–HCl buffer (10 mM, pH 7.8) after (A) 0, (B) 8, (C) 12, (D) 16 and (E) 24 h at 37°C.

chromatography (Figure 5a). The pooled fractions with activity were then charged on a DEAE sepharose fast-flow column and eluted. The active peak was subsequently analyzed by SDS-PAGE and a unique band was observed. The relative molecular mass of the protease was approximately 33 kDa, compared to standard molecular markers (Figure 6, lane 2).

Effect of temperature and pH on the activity and stability of the keratinolytic protease

The influence of pH and temperature on the enzyme activity and stability was presented in Figure 7. The keratinolytic protease was active at pH between 7.0 and 8.5 with the optimum activity at pH 7.5 (Figure 7a). The keratinolytic protease was also active in the temperature range of 50 to 70°C with the optimum activity at 60°C (Figure 7c). The enzyme was quite stable at pH between 5 and 10, retaining more than 80% of its initial activity for 1 h at 50°C (Figure 7b). There was an abrupt fall in stability when the pH was lowered to 5 or increased to 10. As for thermostability, the enzyme was stable below 50°C. With the increase in temperature, the enzyme was inactivated considerably. The enzyme maintained 87% of

Table 1. Morphological and physiological characteristics of strain C11.

Morphological characteristics	
Form	Rods
Gram stain	Negative
Spore	Non-sporulating
Cultural characteristics	
colonies	Yellow color, circular, smooth, moist
Physiological characteristics	
Motility	+
Growth at pH4.0	-
Growth at 4 °C	-
Growth at 42 °C	+
Growth with 8.5% NaCl	-
Catalase	+
Oxidase	+
Voges-Proskauer test	-
Methyl Red test	-
Indole test	-
Oxidation-fermentation test	Oxidative
Nitrate reduction	+
Gelatin liquefaction	+
Starch hydrolysis	-
Tween esterase	+
Phenylalanine deaminase	-
Tryptophan deaminase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Pyocyanin	+
Pyoverdin (King B medium)	+
Denitrification	+
Lecithinase	-
H_2S production	+
Assimilation of	
d-Fructose	+
d-Mannitol	+
Gycerol	+
Sorbitol	-
m-Inositol	-
Sucrose	-
d-Tartrate	-
α-Aminobutyrate	-
Creatine	-
Acetamide	+

Symbols: + = positive; -= negative.

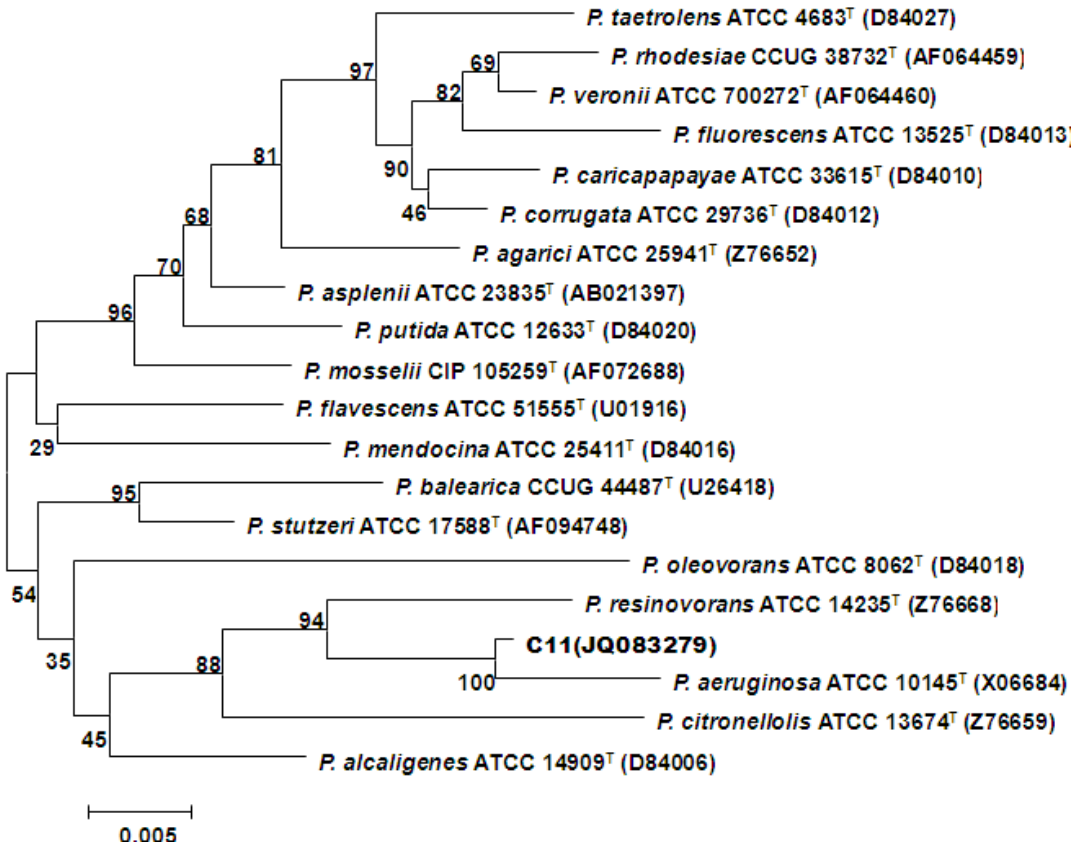

Figure 4. Phylogenetic tree showing relationship between strain C11 and other members of genus *Psuedomonas*. The accession number of each reference type strain (GenBank) is shown in parenthesis. There were 1361 nucleotides in the comparison dataset. Bootstrap values were calculated based on 1000 resamplings. The scale bar represents 0.005 substitutions per nucleotide position.

Table 2. Purification of the keratinolytic protease from *P. aeruginosa* C11.

Purification step	Protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg)	Purification (folds)
Culture filtrate	5.3	52.0	9.8	1.0
$(NH_4)_2SO_4$ precipitation	11.6	359	30.9	3.2
G-75 sephadex	1.2	189	157.5	16.1
DEAE sepharose fast-flow	0.71	121	170.4	17.4

its initial activity after 1 h at 60°C, and was mostly inactivated within the first 1 h of incubation at 80°C (Figure 7d).

Effect of metal ions, additives and protease inhibitors on keratinolytic protease

As shown in Table 3, metal ions exhibited three different effects on the enzyme activity. Mn^{2+} (2 mM) enhanced the enzyme activity by 21.14%. Ca^{2+}, Mg^{2+} and Li^{+} almost exerted no obvious effects on it, while Co^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, and Fe^{2+} inhibited the enzyme activity. The maximum inhibition was observed by Hg^{2+} and Cu^{2+}. 96.9% and 94.9% of the initial activities were inhibited by Hg^{2+} (10 mM) and Cu^{2+} (10 mM), respectively. EDTA caused strong inhibition to the enzyme activity, while PMSF did not, indicating that the keratinolytic protease may be a metalloproteinase. The keratinolytic protease activity was stable in the presence of reducing agents, such as 2-mercaptoethanol and DTT, but not enhanced noticeably by these reducing agents. DMSO stimulated the keratinolytic protease by 11.34% at 5% (v/v), while gycerol exerted no significant influence upon the enzyme

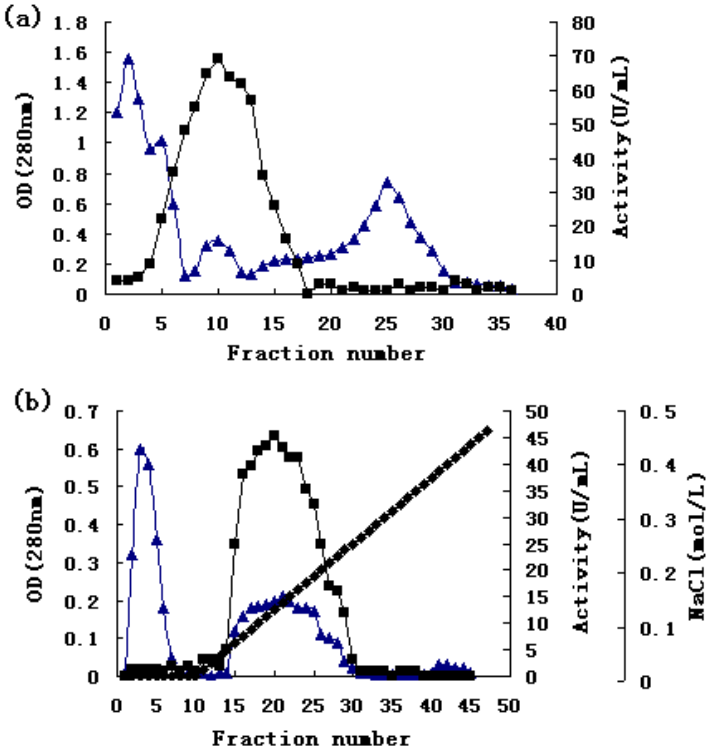

Figure 5. Purification of keratinolytic protease from *P. aeruginosa* C11. (a) Gel filtration chromatography on a Sephadex G-75 of precipitated crude enzyme. (b) DEAE sepharose fast-flow column-chromatography of pooled fractions with keratinolytic activity from gel filtration chromatography. ▲ absorbance at 280nm, ■ the keratinolytic activity of the protease, and ◆NaCl concentration.

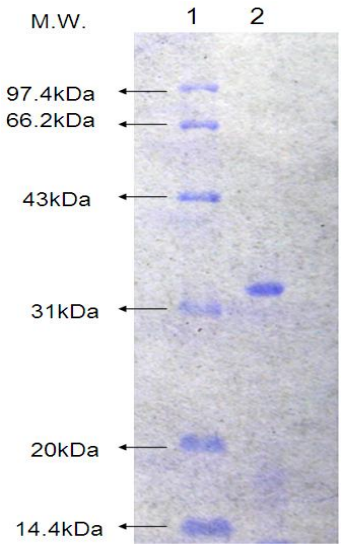

Figure 6. SDS-PAGE of the purifed keratinolytic protease. Lane 1: molecular mass standards (Shanghai Generay Biotech Co., Ltd.); Lane 2: the purifed keratinolytic protease.

activity.

Hydrolysis of soluble and insoluble protein

The activities of the keratinolytic protease against different protein substrates were tested and the results were presented in Table 4. Casein hydrolysis was considered as control for the estimation of relative activities against other protein substrates. The purified keratinolytic protease could hydrolyze a broad range of protein substrates like feather, collagen, gelatine and casein, but showed no activity against human hair powder.

DISCUSSION

As well known, keratin degradation is mostly performed by gram-positive bacteria (Gupta et al., 2006), although there are a few reports on feather-degrading gram-negative bacteria (Brandelli et al., 2010; Jeong et al., 2010b; Tork et al., 2010; Bach et al., 2011). In this paper,

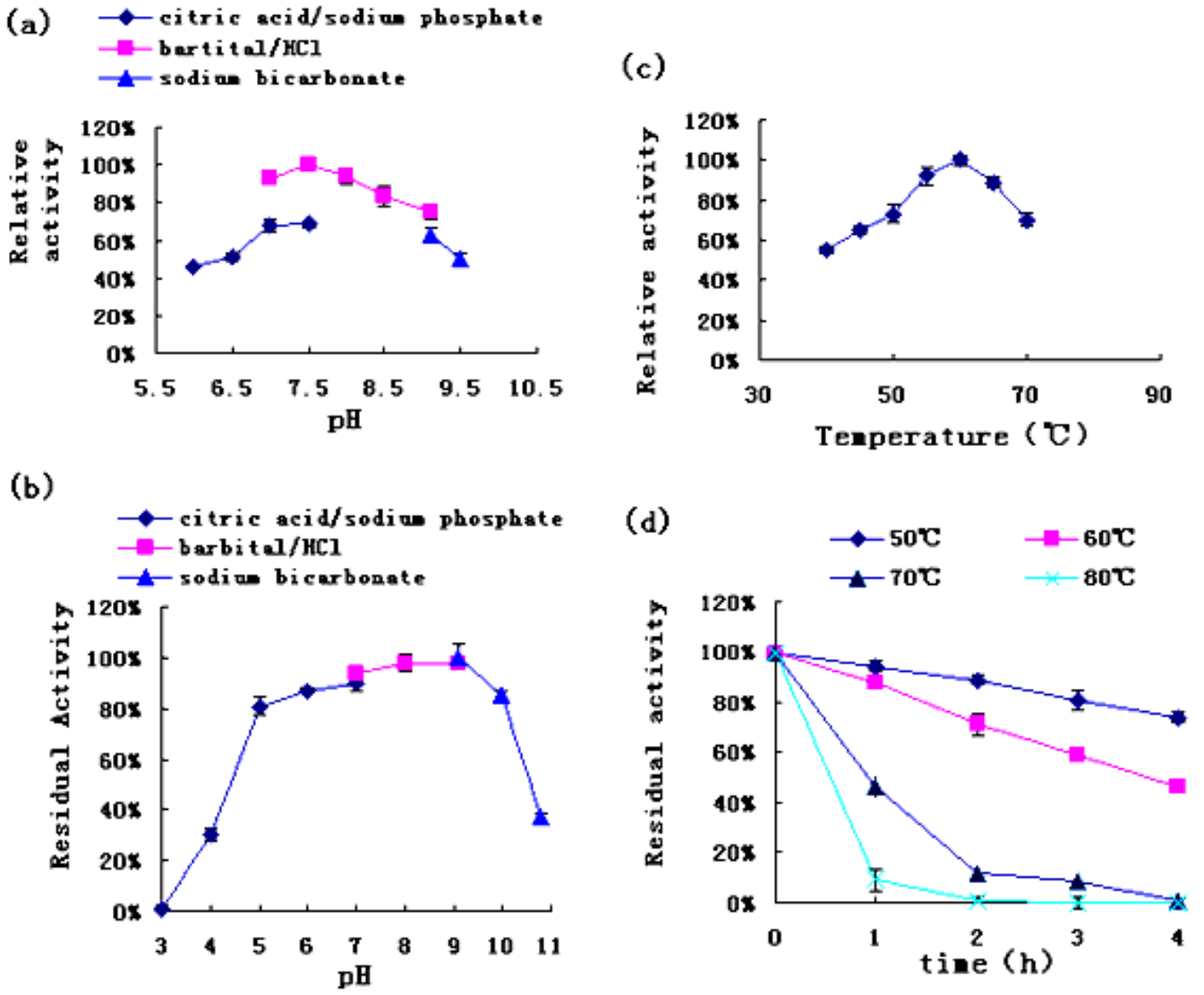

Figure 7. Effect of pH and temperature. (a) Determination of optimal pH of the enzyme activity. The keratinolytic activity tested at pH 7.5 in barbital/HCl buffer was regarded as 100%. (b) Determination of pH stability of the enzyme at 50 °C for 1 h. The residual activity tested at pH 9.0 in barbital/HCl buffer was regarded as 100%. (c) Determination of optimal temperature of the enzyme activity. Optimal temperature was determined by performing the enzyme assay from 40 to 70°C in presence of pH buffers 7.5. The keratinolytic activity tested at 60°C was regarded as 100%. (d) Thermostability of the enzyme was evaluated by measuring the residual activities after incubating in various temperatures at pH 7.5. Each point represents the mean ± standard deviation (n = 3).

a feather-degrading gram-negative bacterium (strain C11) was isolated and identified as *P. aeruginosa*. The isolated keratinolytic protease was found to be a metalloprotease, while a majority of the keratinases reported to date have been found to be serine proteases (Brandelli et al., 2010). The reported keratinolytic metalloproteases are generally produced by gram-negative bacteria (Riffel et al., 2002, 2007; Thys et al., 2006; Wang et al., 2008), so was the isolated keratinolytic protease from *P. aeruginosa* C11. We have only found one 33 kDa keratinolytic metalloprotease reported to date which is also from *P. aeruginosa*, and DTT, β-mercaptoethanol, Zn^{2+}, Ca^{2+} and Mg^{2+} stimulate its activity (Lin et al., 2009a, b, c). However, DTT, β-mercaptoethanol, Ca^{2+} and Mg^{2+} exterted no positive effect on the isolated protease, and more importantly, Zn^{2+} greatly inhibited its activity, which differred it from the reported one. So the isolated protease was different from any reported keratinolytic proteases and was worthy of further investigation.

The isolated protease was stable over a wide pH range with optimum pH at 7.5, which is consistent with the reports where keratinases show broad pH stability and are alkaline in nature (Gupta et al., 2006; El-Gendy,

Table 3. Effect of metal ions, additives and protease inhibitors on the purified protease activity.

Effectors	Relative activity (100%)[b]	
	2 mM	10 mM
control	100	
$MnSO_4$	121.14±5.74	[a]
$CaCl_2$	101.66±8.62	96.49±0.19
$MgSO_4$	97.75±3.28	96.09±6.19
$CuSO_4$	13.32±0.47	5.10±3.66
$CoCl_2$	79.32±1.41	72.37±1.50
$HgCl_2$	69.32±0.19	3.11±0.28
$FeCl_2$	90.06±5.89	47.78±0.47
$ZnSO_4$	22.53±1.50	11.27±1.31
$LiCl_2$	103.18±2.34	102.19±4.87
PbAC	77.00±8.43	[a]
EDTA	23.12±2.31	20.22±3.19
PMSF	97.95±3.78	99.09±4.19
2-mercaptoethanol	94.67±0.48	103.57±6.59
DTT	89.89±8.32	91.74±3.60
	1%(v/v)	5%(v/v)
Gycerol	102.52±2.32	100.94±8.32
DMSO	105.98±3.42	111.34±8.32

[a] The concentration of metal ions added in the reaction mixture caused interference on Folin-Ciocalteu reagent.
[b] Each value represents the mean ± standard deviation (n = 3).

Table 4. The activities of the keratinolytic protease from *P. aeruginosa* C11 against different substrates.

Substrates[a]	Relative activity (100%)[b]
Casein	100
Gelatin	89.78±3.21
Collagen type I	54.21±2.87
BSA	96.54±3.71
Feather powder	9.54±2.01
Human hair powder	0.34±0.21

[a] The concentration of the substrates was 1% (w/v). [b] Each value represents the mean ± standard deviation (n = 3).

2010; Mazotto et al., 2010, 2011; Sharma et al., 2010; Tork et al., 2010). Most of keratinolytic proteases are stimulated by Mn^{2+} (Gupta et al., 2006; El-Gendy, 2010; Brandelli et al., 2010; Mazotto et al., 2011), as was the isolated keratinolytic protease, although a few keratinolytic proteases are inhibited by Mn^{2+} (Rai et al., 2009; Bach et al., 2011). Ca^{2+} and Mg^{2+} generally enchance the activity of keratinases (Gupta et al., 2006; Riffel et al., 2007; Cao et al., 2009; El-Gendy, 2010; Fakhfakh-Zouari et al., 2010; Mazotto et al., 2011), on the contrary, these two divalent metal ions had no influence on the isolated protease. Cu^{2+}, Hg^{2+}, and Pb^{2+} had negative effect on the isolated protease, which is consistent with performances of most keratinases (Brandelli, 2008; Brandelli et al., 2010; Riffel et al., 2007; Cao et al., 2009; Prakash et al., 2010; Fakhfakh-Zouari et al., 2010). The isolated protease was inhibited by Zn^{2+}, which is in accordance with the reports where inhibitions of keratinases from gram-negative bacteria by Zn^{2+} are observed (Sangali et al., 2000; Riffel et al., 2003, 2007; Cao et al., 2009; Bach et al., 2011).

The purified isolated protease exhibited a broad range of substrate specificity, hydrolyzing soluble proteins more effectively than insoluble proteins. That is quite similar to early reports (Brandelli et al., 2010). On the basis of secondary structural confirmation, keratins have been

classified into α-keratins and β-keratins and hair is formed primarily with α-keratins, while feather with β-keratins (Akhtar et al., 1997). The result that the purified isolated protease was much more active against feather powder than hair powder is in accordance with the reported observation that β-keratins from feather is much more easily to been hydrolysed than α-keratins from hair and wool by keratinolytic proteases (Brandelli, 2005; Pillai et al., 2008; Cao et al., 2009; Moreira-Gasparin et al., 2009).

P. aeruginosa is an opportunistic pathogen and can produce many toxic substances during fermentation, so the protease gene should been heterologously expressed in a more safe and efficient host. Further work on cloning and expression of recombinant protease from *P. aeruginosa* C11 in *Picha pastoris* will been carried out in our laboratory.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Grant No. 21176105) and the Fundamental Research Funds for the Central Universities (Grant No. JUSRP111A24).

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