J Ind Microbiol Biotechnol (2009) 36:181–188
DOI 10.1007/s10295-008-0469-8

ORIGINAL PAPER

Characterization of a novel *Stenotrophomonas* isolate with high keratinase activity and purification of the enzyme

Zhang-Jun Cao · Qi Zhang · Dong-Kai Wei · Li Chen · Jing Wang · Xing-Qun Zhang · Mei-Hua Zhou

Received: 3 November 2007 / Accepted: 3 September 2008 / Published online: 10 January 2009

Abstract A feather-degrading bacterium was isolated from poultry decomposition feathers in China. The strain, named L1, showed significant feather-degrading activity because it grew and reproduced quickly on basal medium containing 10 g/L of native feather as the source of energy, carbon, and nitrogen. According to the phenotypic characteristics and 16S rRNA profile, the isolate belongs to *Stenotrophomonas maltophilia*. Keratinase activity of the isolate was determined during cultivation on raw feathers at different temperatures and initial pH. Maximum growth and feather-degrading activity of the bacterium were observed at 40°C and initial pH ranging from 7.5 to 8.0. The crude enzyme was purified by ammonium sulphate precipitation, Sephadex G-100 chromatographic and ceramic hydroxyapatite (CHT) chromatographic. Its molecular mass estimated as 35.2 kDa in SDS-PAGE. The enzyme had an optimum activity at the pH was 7.8 and the temperature was 40°C. The keratinase was wholly inhibited by a serine protease inhibitor, PMSF. Its activity was activated or inhibited by different metal ions. The keratinase activity of enzyme from strain L1 functioned on different keratins, such as feather, hair, wool, horn, and so on.

Z.-J. Cao · D.-K. Wei · X.-Q. Zhang · M.-H. Zhou (✉)
Key Laboratory of Science and Technology of Eco-Textile,
Donghua University, Ministry of Education,
201620 Shanghai, China
e-mail: mhzhou@dhu.edu.cn

Z.-J. Cao · D.-K. Wei · X.-Q. Zhang
College of Chemistry,
Chemical Engineering and Biotechnology,
Donghua University, 201620 Shanghai, China

Q. Zhang · L. Chen · J. Wang · M.-H. Zhou
College of Environmental Science and Engineering,
Donghua University, 201620 Shanghai, China

Keywords Keratin-degrading bacterium · Feather · 16S rRNA · Cultivated conditions · Keratinase characteristics

Introduction

Feathers, almost pure keratin protein (90% or more), are produced as wastes or byproducts at poultry processing plants, reaching millions of tons per year worldwide [1]. The keratin chain is tight packed into a super coiled polypeptide chain as α-helix or β-sheet and cross-linking of protein chains by cysteine bridges, resulting in mechanical stability and resistance to proteolytic enzymes such as pepsin, trypsin, and papain. Therefore, feathers are currently limited to use as dietary protein supplement for animal feed because feather meal production is an expensive process, requiring significant amounts of energy. Furthermore this process destroys certain kinds of amino acids, yielding a product with poor digestibility and variable nutrient quality. Nevertheless, feathers do not accumulate in nature, since structural keratin can be degraded in environment by some microorganisms [2, 3]. The current investigation has been focused on proteolytic microorganism; however, reduction of cysteine bridges may significantly influence keratin degradation.

Keratinolytic enzymes have important utilities in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes [3]. After hydrolysis, the feathers can be converted to feedstuffs, fertilizers, glues, films and as the source of the rare amino acids, such as serine, cysteine and proline [2]. Currently, most keratinases are produced with some mesophilic fungi and some thermophilic species of *Bacillus* producing feather-degrading enzymes [2]. The use of keratinase to increase the nutritional

value of feather meal has been described [4]. The utilization of a *Bacillus licheniformis* feather-lysate with amino acid supplementation in test diets fed to growing broilers produced a weight gain identical to that achieved with soybean meal [1]. The use of crude extract of keratinase significantly increases the amino acid digestibility of raw feathers and commercial feather meal [5].

Besides the application of keratinase in traditional industry such as detergent, medicine, cosmetics, leather, it was also used in many newer fields like prion degradation for treatment of the dreaded mad cow disease [6–9], biodegradable plastic manufacture and feather meal production and thus can be aptly called "modern proteases".

The aim of this study was to identify a new kind of keratinolytic bacterium isolate showing high feather degradation at lower temperature, with potential application in biotechnological processes. Such microorganisms will be less energy consuming than the thermophilic strains usually used in feather processing. This report characterized a novel high keratinase activity of feather-degrading bacterial isolate L1 and purification of the keratinase.

Materials and methods

Isolation of feather keratin-degrading bacterium

Feathers were collected from several sites at a local poultry plant. Feathers were flooded in peptone broth (5 g/L) and incubated at 37°C for 24 h. The suspension was used to streak feather-meal agar plates (feather-meal 20 g/L, NaCl 5 g/L, K_2HPO_4 3 g/L, KH_2PO_4 4 g/L, and agar 20 g/L, pH 7.4–7.6) which were incubated at 37°C for 3 days. Single colony was isolated and screened for their ability to hydrolyze keratin in feather powder agar plates [10]. Colonies producing clear zones in this medium were selected for further analysis. The morphology of the bacterium was determined by a transmission electron microscope (TEM, H-800; Hitachi, Tokyo).

Molecular phylogenetic studies

Genomic DNA was extracted as follows. Briefly, the strain, which had the highest enzyme activity, was inoculated into beef extract/peptone/NaCl medium and incubated with shaking at 40°C for 16 h. Cultures (1 mL) were centrifuged at 7,000 rpm for 10 min, supernatant was removed and the pellet was resuspended in 10 µL lysozyme (50 mg/mL) and 0.5 mL sterilized deionized water, followed by mixing with 500 µL DNA extraction buffer (4% SDS 100 mM Tris–HCl, 10 mM EDTA, pH 8.0) at 65°C for 1 h. The suspension was extracted by mixture of phenol and chloroforms (1:1 volume) and then was extracted by pure chloroform again. DNA was precipitated by the adding of two volumes of pre-cooling 100% ethanol. The pellet was then dissolved into 50 µL distilled water and quantified by spectrophotometer.

The 16S rRNA gene was amplified by the polymerase chain reaction (PCR) with the primers AGA GTT TGA TCC TGG CTC AG (Position 8-27), AAG GAG GTG ATC CAG CCG (Position 1541–1522) (http://www.psb.ugent.be/rRNA/). The PCR reaction mixtures (50 µL) contained: each dNTPs 200 µmol/L, 10 × PCR buffer 10 µL, 1.5 mM $MgCl_2$, 50 pmol each primers, Taq DNA polymerase 1.5 U and genomic DNA 0.1–2 µg. The PCR was carried out with initial denaturation of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 30 s, extension at 72°C for 90 s and a final extension at 72°C for 10 min. Amplified DNA fragments were detected by horizontal electrophoresis in 1.0% agarose gel containing ethidium bromide (0.5 µg/mL) at 100 V for 0.5 h in 1 × TAE buffer with 3 µL aliquots of PCR products. PCR product with the expected insert size was cloned into pGEM-T Easy Vector system I (Promega) as the manufacturer recommended.

The sequences were analyzed by DNASTAR. The BLAST algorithm was used to search for homologous sequences in GenBank. Molecular phylogenetic studies of the 16S rRNA were conducted using MEGA [11].

Bacteria growth and feather degradation conditions

In the experiments of bacteria growth and feather degradation, initial pH was changed from 5.5 to 8.5 with space 0.5 while the temperature was kept at 37°C; temperature varied in the range of 25, 35, 40, and 50°C while initial pH was set at 7.5. The isolate from a 10^6 colony forming units (CFU) mL^{-1} culture was cultivated in whole-feather medium solution (feather powder 20 g/L, NaCl 5 g/L, K_2HPO_4 3 g/L, KH_2PO_4 4 g/L, and agar 20 g/L). A measure of 100 mL the medium containing 10 mL isolate was added into 500 mL flasks, which was put in an orbital shaker at 150 rpm for 72 h. The bacterial culture was diluted to 10 or 20× by deionized water and bacterial growth was monitored with measuring the absorbance in 600 nm. Protein concentration was measured by the method of Bradford [12], which involves the binding of Coomassie Brilliant Blue G-250 to protein, using bovine serum albumin (Sigma, USA) as the standard.

Purification of keratinase

The bacterium was cultivated in 200 mL of medium in a 500 mL triangular flask, and grown at 40°C at 150 rpm for 72 h. The crude extraction was prepared by centrifugation of the cultures at 4°C, 10,000×*g* for 10 min. Protein in the

liquid was precipitated by adding solid ammonium sulphate with gentle stirring until the concentration of ammonium sulphate reached 10%, and then stand for 2 h and centrifuged at 4°C, 10,000×*g* for 10 min. The precipitate was dissolved in deionized water. The crude enzyme from the solution was applied to a Sephadex G-100 gel filtration (Pharmacia, USA), equilibrated and eluted with 0.2 M PBS (pH 7.5) at a flow rate of 0.4 mL/min. The keratinase active fractions were pooled, concentrated and desalted by dialysis. Subsequently the enzyme solution was applied on ceramic hydroxyapatite (CHT) chromatography (BioLogic LP, Bio-Rad, USA). The column was equilibrated with 5 mM phosphate buffer (pH 7.5) containing 30 mM NaCl (CHT-A) at a flow rate of 0.5 mL/min. After loading the crude extraction, the column was eluted with buffer CHT-A for 2 min at a flow rate of 1 mL/min; and 0.4 M phosphate buffer (pH 7.5) (CHT-B) with a linear gradient from 0 to 100% for 4 min; then buffer CHT-B for 20 min. Fractions of 1 mL from each step were collected and analyzed for proteolytic activity as described above. The purified enzyme was stored in aliquots at −20°C.

Keratinase characteristic

Molecular weight determination

SDS-PAGE was carried out according to Laemmli [13] on a 0.75 mm thick polyacrylamide slab gel consisting of 12% separating and 4% stacking gels by using the minigel system (Mini-Protean) of Bio-Rad. The electrophoresed protein gels were stained with Coomassie brilliant blue.

Effect of pH and temperature on enzyme activity and stability

The effect of pH on activity of the enzyme was investigated by measurements at 40°C in buffers of various pH at 100 mM (HAc–NaAc, pH 3.6–4.0; Na_2HPO_4–KH_2PO_4, pH 5.0–8.0; glycine–sodium hydroxide buffer, pH 8.0–10.0). For pH stability, residual activity was measured at pH 7.8 after 2 h of incubation at 40°C in different pH buffers. The effects of temperature on its activity were investigated by having a constant pH (7.8) with varying temperatures from 20 to 70°C. The temperature stability and the residual activity was measured at 40°C after an incubation period of every 0.5 h in Tris–HCl buffer pH 7.8 at different temperature levels.

Effect of enzyme inhibitors and metal ions

The enzyme activity was tested by incubating the enzyme solution with 1, 10, 100 mM specific protease inhibitors phenylmethane-sulphonyl fluoride (PMSF), and 1, 10, 100 mM metal ions for 1 h.

Substrate hydrolysis

Various types of native keratins: feather (fowl), feather (pigeon), wool, hair (human), horn (cattle), horn (caprine), Japanese dock root, finger nail (human) were utilized to keratinase hydrolysis. These materials were defatted, washed and dried. After grinding, the powder was sifted and weighed 10 mg to use as an enzyme substrate. The enzyme activity was assayed by the following procedure [10]. The reaction mixture containing 1 mL of enzyme, which was obtained from above culture, cultivated 72 h, and 2 mL of 10 mg keratin powder in 0.05 mol/L Tris–HCl (pH 7.8) buffer. The mixture was incubated at 40°C for 1 h, and the reaction was stopped by adding of trichloracetic acid to a final concentration of 200 g/L. After centrifugation at 10,000 rpm at 4°C for 10 min, the absorbance of supernatant was determined at 280 nm. One unit of enzyme activity corresponds to the amount of enzyme that cause a change of absorbance of 0.1 at 280 nm at 40°C for 1 h.

Results

Isolation of feather keratin-degrading bacterium

Distinct colony morphologies were observed on feather agar plates. These isolates were characterized for the purpose of their keratin-degrading properties. They were cultured on feather meal agar plates and transferred to feather meal broth plates for monitoring the activity of keratin degradation. The isolate, L1, presented the highest feather-degrading activity among these isolates. Feather barbules were completely degraded and rachis was also attacked by L1 after 72 h at 40°C (Fig. 1).

Characterization of keratinase-producing L1

Strain L1 was selected for identification by molecular phylogenetic studies and morphology observation. The results of taxonomic properties studies on strain L1 are summarized in Table 1 and Fig. 2. The 16S rRNA sequence of strain L1 (DQ327729) showed high similarities to a group consisting of several *Stenotrophomonas maltophilia* strains, for example, *Stenotrophomonas maltophilia* strain c6 with a similarity of 99.2%. Bootstrap analysis resulted in relatively high values for the branching of L1 within the *Stenotrophomonas maltophilia* cluster. Other related members of the *Pseudomonas*, *Xanthomonas* shared 95–98% sequence similarity with strain L1 (Fig. 3). It showed that the isolate belongs to *Stenotrophomonas maltophilia.* Cell and colony morphology, growth characteristics of the isolate were also coincident with the properties of *Stenotrophomonas*

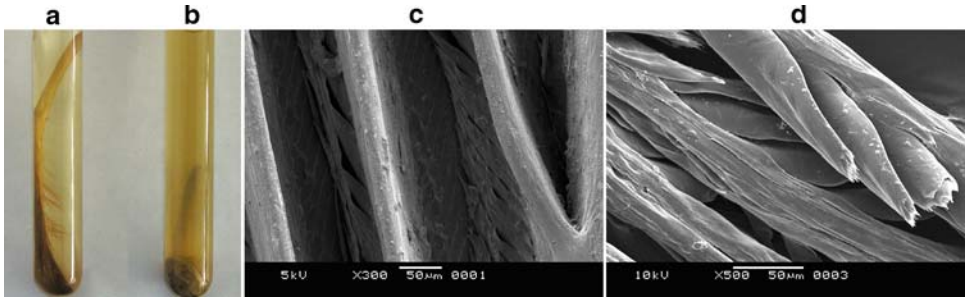

Fig. 1 Photographs and images of scanning electron microscopy (SEM) of feather degradation by L1. **a**, **b** were photographs of native feathers autoclaved and incubated at 40°C for 72 h by strain L1 absence or presence, respectively; **c**, **d** were SEM images of feather degradation absence or presence L1 at 40°C for 24 h

Table 1 Morphological characteristics of keratinase-producing bacterium L1

Morphological characteristics				Cultural characteristics
Form	Size (μm)	Gram stain	Spore	
Rods	0.3–0.6 × 0.8–1.5	Negative	Non-sporulating	Feather meal agar colonies yellow color, circular, smooth, convex, moist

Fig. 2 Transmission electron micrograph of strain L1 (×5,000)

maltophilia (describe a little detail, Fig 2 and Table 1). We named it *Stenotrophomonas maltophilia* DHHJ.

Optimal bacteria growth and feather degradation conditions

The effect of initial pH and temperature on bacterial growth and keratinase production was shown in Figs. 4a and 5a. As shown in Fig. 4a, the bacterial quantity and keratinase activity of the newly isolated strain increased until the value respectively reached 0.44OD and 12.8U/mL at pH 7.5 and then decreased with increasing the initial pH. From Fig. 5a, it was observed that the bacterial quantity and keratinase activity, respectively increased to 0.72OD and 32.0U/mL at 40°C and then declined with getting up temperature. From these figures, it was shown that changes of keratinase activities were acuter than those of bacteria growth. Maybe the reason was that not only the amount of enzyme producing lowered, but also enzyme lost its part activities in these conditions.

The effect of initial pH and temperature on protein and amino acid concentration were investigated and shown in Figs. 4b and 5b. It displayed in Fig. 4b that soluble protein concentrations had the tiptop point 16 mg/ml at initial pH 7.5 and amino-acid concentrations enhanced with increase initial pH value. This phenomenon could be explained that the soluble protein was decomposed to amino acid when the pH increasing. Fig. 5b revealed that maximum soluble protein and amino-acid concentrations were, respectively 5.8 and 0.22 mg/mL at temperature 40°C.

Kerainase characteristic

The crude enzyme was stable below 50°C, so all operations were done at room temperature. Protein in culture medium of strain L1 was precipitated after adding 10% concentration of ammonium sulphate and then dialyzed to concentrate 3-fold with 42% yield. Further keratinase was purified by Sephadex G-100 gel filtration and CHT chromatography. These steps gave a purified keratinase with 27% yield and an overall purification of 17-fold. The final purified keratinase was shown single band by SDS-PAGE (Fig. 6), indicating that the enzyme was purified to homogeneity. The keratinase seemed to be a monomer, and molecular mass was estimated as 35.2 kDa.

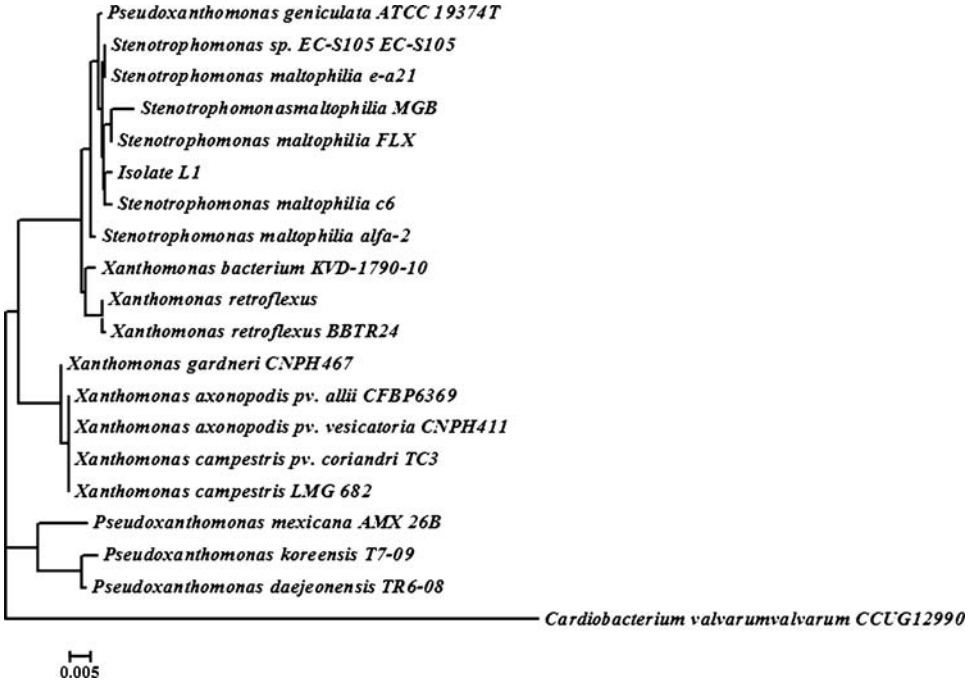

Fig. 3 Phylogenetic tree of 16S rRNA of strain L1 within the genus *Stenotrophomonas maltophilia* and allied bacteria. The branching pattern was generated by the neighbor-joining method. Bar Jukes-Cantor distance of 0.005. *Cardiobacterium valvarum* was used as an out group

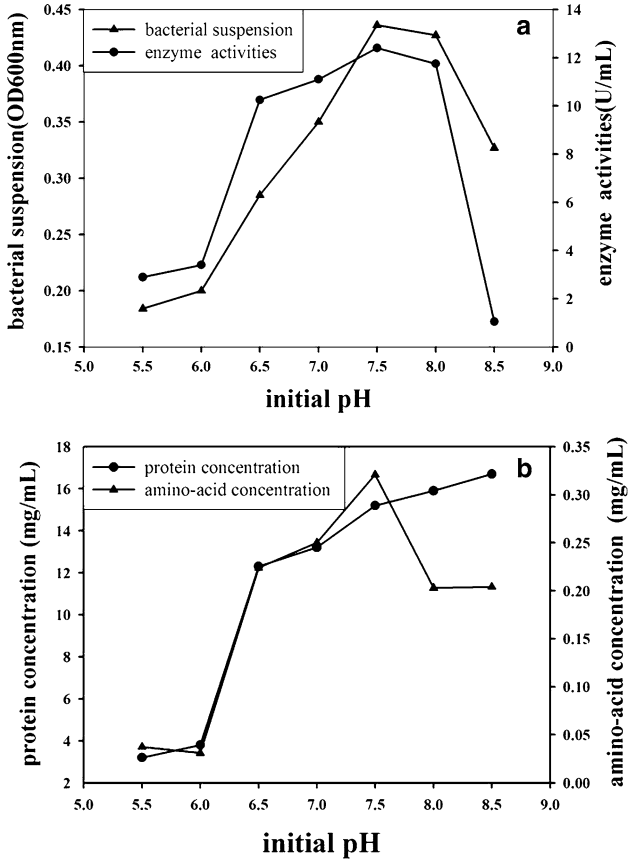

Fig. 4 Effects of initial pH on bacteria growth, keratinase production and feather degradation. **a** *Filled inverted triangle* for bacterial concentration and *open square* for keratinase activity; **b** *filled inverted triangle* for protein concentration and *open square* for amino acid concentration. (Each point represents the mean of three independent replicates)

The enzyme was active with wide range (from 5.0 to 9.0), and it had an optimum activity at the pH of 7.8. The activity of keratinase was more stable in the pH range of 5.0–9.0 for 2 h at 40°C (Fig. 7a, b). The enzyme had an optimum activity at the temperature of 40°C and rapidly inactivated at higher than 50°C (Fig. 7c, d). Above 80°C for 2 h, the keratinase was no longer active. The enzyme was unstable at high temperatures but was stable at moderate temperature ranges.

The keratinase was wholly inhibited by a serine protease inhibitor, PMSF. It indicates that the enzyme is in the serine protease family. Its activity was activated or inhibited by different metal ions as shown in Table 2. Ca^{2+}, Ba^{2+}, Cu^{2+}, Na^{+}, K^{+} and Mg^{2+} may increased enzyme activity, but higher ion concentration may inhibited the enzyme activity to some extend. Other ions, Zn^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, may inhibit the enzyme activity not only lower (1 mM) but also higher (10 and 100 mM) metal ion concentration.

Different native keratins from human and animal keratinous materials were used as hydrolysis substrates of the keratinase from strain L1 and the results were shown in Fig. 8. It was exhibited that the keratinase from strain L1 could degraded all keratinous materials we prepared, α-keratins as well as β ones. However, the degrading capacities of the keratinases were different to various keratins.

Discussion

A novel isolate of feather-degrading bacterium (strain L1) was isolated from a poultry-processing industry in this

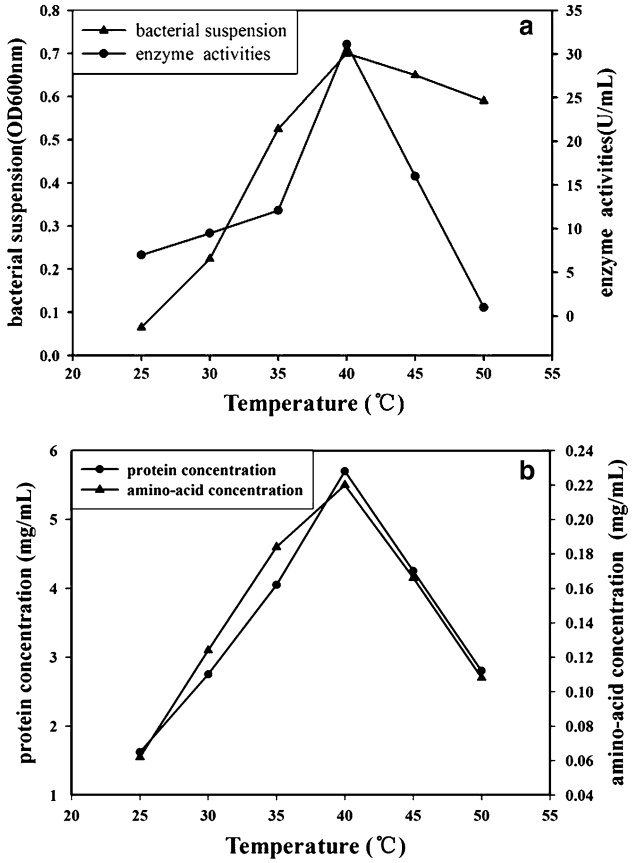

Fig. 5 Effects of culture temperature on bacteria growth, keratinase production and feather degradation. **a** *Filled triangle* for bacterial concentration and *filled square* for keratinase activity; **b** *filled inverted triangle* for protein concentration and *filled diamond* for amino acid concentration. (Each point represents the mean of three independent replicates)

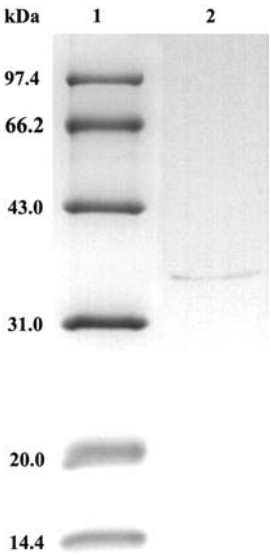

Fig. 6 SDS-PAGE of the purified keratinase. *Lane 1* molecular mass marker proteins. Rabit Phosphorglase B (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20 kDa), hen egg white lysozyme (14.4 kDa); *Lane 2* purified keratinase

work. Based on phenotypic characteristics and phylogenetic relationships, strain L1 belongs to the *Stenotrophomonas* and was identified as *Stenotrophomonas maltophilia* DHHJ.

Initially, feather degradation by bacteria had been described only in gram-positive bacteria such as *Bacillus* and *Streptomyces* species [3, 14–16]. Later, fewer gram-negative bacteria had been reported degrading feather [17–19]. In some earlier studies, two *Stenotrophomonas* isolates were discovered that they could solubilize autoclaved deer fur. The organisms resembled *Stenotrophomonas,* however, had not been clearly classified [20, 21] up to now. Moreover, the optimal temperature of production enzyme they reported was 20°C. In our work, the maximal production of keratinolytic enzymes by L1 was at 40°C, it is different from those studies before. On the other hand, the molecular mass of keratinase from strain L1 (35.2 kDa) was similar to those of *Bacilllus licheniformis* PWD-1 and *B.licheniformis* (35 kDa) [22], and was different from those of *Streptomyces albidoflavus* (18 kDa) [23], *Fervidobacterum pennavorans* (130 kDa) [24]. *Stenotrophomonas* was gram-negative bacteria and *Bacilllus* was gram-positive bacteria. Therefore, the keratinase from *S. maltophilia* DHHJ was guessed to be a new kind of keratinase. Its sequences are been striving to determine. As we known, this is the first purified keratinase from gram-negative bacteria. The keratinase from strain L1 was induced by feather meal, so it had an extracellular and inducible nature. Compared with the few researches reported in Gradisar et al. [25], the keratinase from strain L1 possessed wider substrate degradation. Therefore, it may be used to not only degrade feather industries, but also to other areas, for example, modification of protein fibers in finishing treatment of wool fiber.

The keratinase from L1 was wholly inactivated by either lower or higher concentration PMSF. Moreover, some metal ions activated the keratinase activity of L1. This phenomenon, especially Ca^{2+}, is typical for a serine protease. This keratinase is likely a serine protease. Metal ions probably act as a salt or ion bridge to maintain the structure conformation of the enzyme or to stabilize the binding of the substrate and enzyme complex. Metal ions play important role for protease thermal stability as well. Zn^{2+}, activated the most keratinases activity, inhibited this keratinase activity. Just as most of keratinases [2], the enzyme activity had been inhibited by heavy metal ions (Pb^{2+}, Cd^{2+}, Hg^{2+}). This enzyme activity was decreased with metal ions concentration increase, and higher metal ions concentration maybe affects the enzyme active center.

Microbe nature of degrading keratin is still an enigma in the world of protease. Most of microbial keratinases are extracellular, which grow on keratinous substrates. However, a few cell-bound and intracellular keratinases have also been reported. The intracellular fraction mainly contributes to disulfide reductases, sulfite or thiosulfate, which synergistically assists the extra cellular keratinases to degrade keratin by opening the disulfide bonds of keratin.

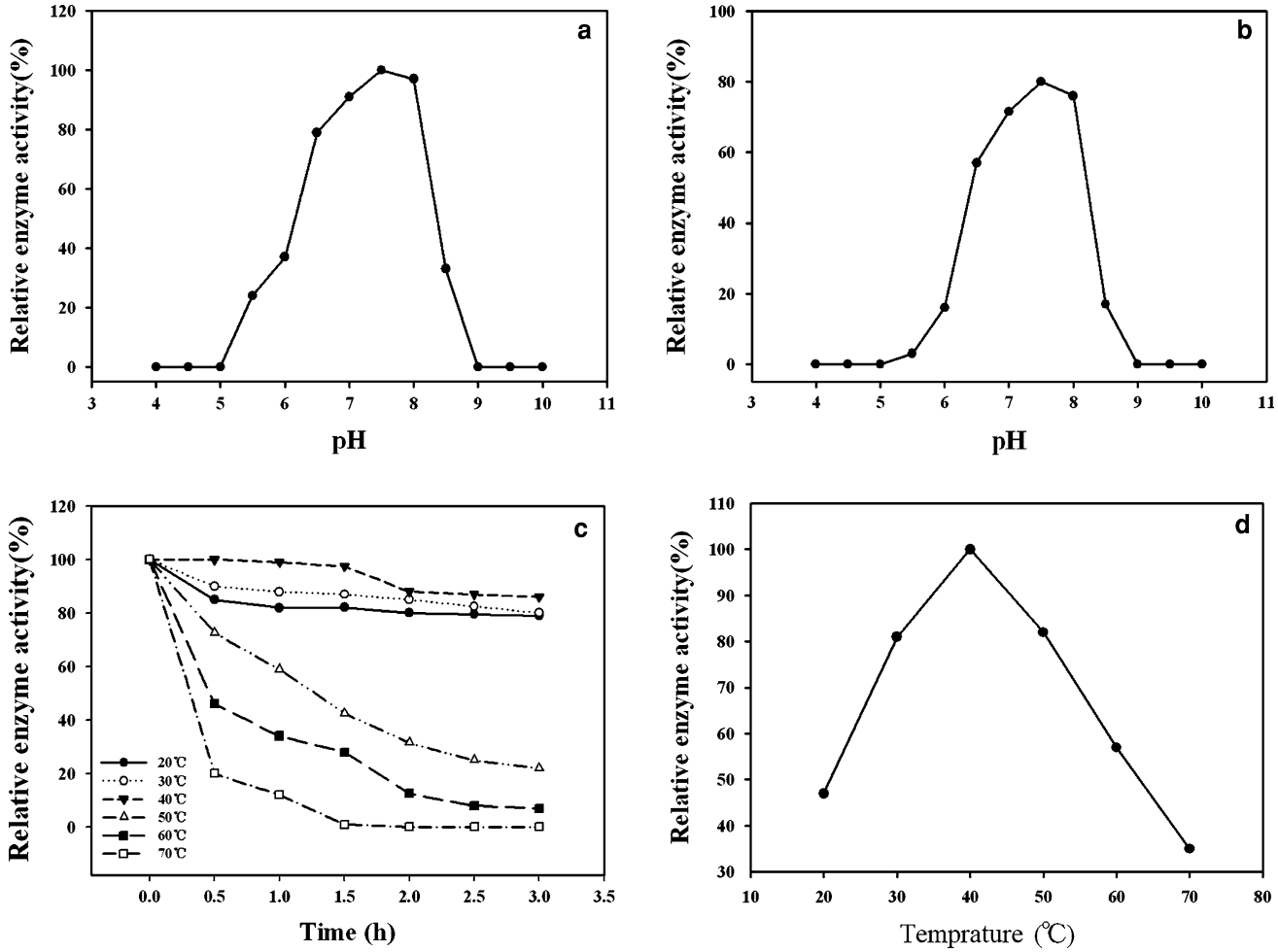

Fig. 7 Effect of pH and temperature on enzyme activity and stability. **a** pH stability; **b** effect of pH on enzyme activity; **c** temperature stability; **d** effect of temperature on enzyme activity

Table 2 Effects of inhibitors and metal ions on keratinase activity

Compound	Concentration (mM)		
	1	10	100
CK	100.00	100.00	100.00
$CaCl_2$	170.11	120.33	116.98
$BaCl_2$	136.16	121.77	92.62
$CuSO_4$	125.83	104.40	90.78
NaCl	122.14	110.70	100.00
KCl	117.34	97.05	87.09
$MgSO_4$	114.02	96.68	81.55
$MnCl_2$	100.00	91.88	70.85
$ZnCl_2$	93.36	85.61	63.10
$PbCl_2$	32.84	18.45	8.85
$CdCO_3$	61.99	34.69	19.89
$HgCl_2$	71.20	44.28	29.10
PMSF	0.00	0.00	0.00

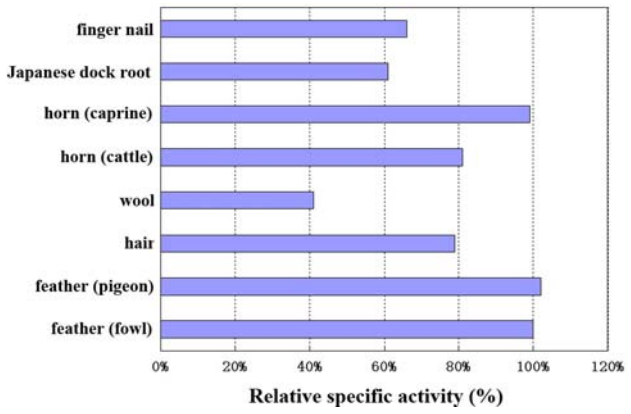

Fig. 8 Hydrolysis of different keratinous substrates with keratinase of L1. (Each point represents the mean of three independent replicates)

From these points of view, it could be put forth that there are two steps in keratinolysis:sulfitolysis or opening in disulfide bonds and proteolysis. But intracellar keratinase

was not purified and cloned, so the order of these events and their exact nature are debatable.

Strain L1 was able to complete feather degradation, indicating its strong keratinolytic character. In addition, the keratinase produced by strain L1 was active over a wide range of pH values and temperatures, and was relatively heat stable. These are interesting properties for industrial applications of the enzyme.

Acknowledgments This work was partially supported by Program for Changjiang Scholars and Innovative Research Team in University (No. IRT0526) and partially financed by Shanghai Leading Academic Discipline Project (No. B604).

References

1. Williams CM, Lee CG, Garlich JD, Shih JCH (1991) Evaluation of a bacterial feather fermentation product, feather-lysate, as a feed protein. Poultry Sci 70:85–94
2. Gupta R, Ramnani P (2006) Microbial keratinases and their prospective applications: an overview. Appl Microbiol Biotechnol 70:21–33
3. Onifade AA, Al-Sane NA, Al-Musallam AA, Al-Zarban S (1998) A review: potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresour Technol 66:1–11
4. Zaghloul TI, Haroun MA, El-Gayar K, Abdelal A (2004) Recycling of keratin-containing materials (chicken feather) through genetically engineered bacteria. Polym Plast Technol Eng 43:1589–1599
5. Lee CG, Ferket PR, Shih JCH (1991) Improvement of feather digestibility by bacterial keratinase as a feed additive. FASEB J 5:596–596
6. Langeveld JPM, Wang JJ, Van de Wiel DFM, Shih GC, Garssen GJ, Bossers A, Shih JCH (2003) Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. J Infect Dis 188:1782–1789
7. Mitsuiki S, Hui Z, Matsumoto D, Sakai M, Moriyama Y, Furukawa K, Kanouchi H, Oka T (2006) Degradation of PrPSc by keratinolytic protease from *Nocardiopsis* sp TOA-1. Biosci Biotechnol Biochem 70:1246–1248
8. Wang JH, Borwornpinyo R, Odetallah N, Shih JCH (2005) Enzymatic degradation of a prion-like protein, Sup35NM-His6. Enzyme Microb Technol 36:758–765
9. Yoshioka M, Miwa T, Horii H, Takata M, Yokoyama T, Nishizawa K, Watanabe M, Shinagawa M, Murayama Y (2007) Characterization of a proteolytic enzyme derived from a *Bacillus* strain that effectively degrades prion protein. J Appl Microbiol 102:509–515
10. Gradisar H, Kern S, Friedrich J (2000) Keratinase of *Doratomyces microsporus*. Appl Microbiol Biotechnol 53:196–200
11. Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5:150–163
12. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
13. Laemmli UK (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227:680–685
14. Bockle B, Galunsky B, Muller R (1995) Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. Appl Environ Microbiol 61:3705–3710
15. Kim JM, Lim WJ, Suh HJ (2001) Feather-degrading *Bacillus* species from poultry waste. Process Biochem 37:287–291
16. Zaghloul Ti (1998) Cloned *Bacillus subtilis* alkaline protease (aprA) gene showing high level of keratinolytic activity. Appl Biochem Biotechnol 70–2:199–205
17. Riffel A, Brandelli A (2002) Isolation and characterization of a feather-degrading bacterium from the poultry processing industry. J Ind Microbiol Biotechnol 29:255–258
18. Lucas FS, Broennimann O, Febbraro I, Heeb P (2003) High diversity among feather-degrading bacteria from a dry meadow soil. Microb Ecol 45:282–290
19. Sangali S, Brandelli A (2000) Isolation and characterization of a novel feather-degrading bacterial strain. Appl Biochem Biotechnol 87:17–24
20. Yamamura S, Morita Y, Hasan Q, Rao SR, Murakami Y, Yokoyama K, Tamiya E (2002) Characterization of a new keratin-degrading bacterium isolated from deer fur. J Biosci Bioeng 93:595–600
21. Yamamura S, Morita Y, Hasan Q, Yokoyama K, Tamiya E (2002) Keratin degradation: a cooperative action of two enzymes from *Stenotrophomonas* sp. Biochem Biophys Res Commun 294:1138–1143
22. Suntornsuk W, Tongjun J, Onnim P, Oyama H, Ratanakanokchai K, Kusamran T, Oda K (2005) Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium. World J Microbiol Biotechnol 21:1111–1117
23. Bressollier P, Letourneau F, Urdaci M, Verneuil B (1999) Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. Appl Environ Microbiol 65:2570–2576
24. Friedrich AB, Antranikian G (1996) Keratin Degradation by Fervidobacterium pennavorans, a novel thermophilic anaerobic species of the order thermotogales. Appl Environ Microbiol 62:2875–2882
25. Gradisar H, Friedrich J, Krizaj I, Jerala R (2005) Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. Appl Environ Microbiol 71:3420–3426