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ORIGINAL PAPER

Substrate specificity characterization of a thermostable keratinase from *Pseudomonas aeruginosa* KS-1

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Abstract A feather-degrading strain of *Pseudomonas aeruginosa* KS-1 was used in the present study. Its crude cell-free fermentation broth completely degraded chicken feather within 12 h, in the absence of disulphide reductase activity. Keratinase from its extracellular broth was purified and characterized, assuming that it would be a potential β-keratin-degrading enzyme with prospective applications in degradation of β-plaques of prions. The keratinase was purified by using Q-Sepharose anion exchange chromatography and its molecular weight, as determined by SDS–PAGE analysis, was 45 kDa. It was an alkaline, serine protease with pH and temperature optima of 9 and 60°C, respectively. The enzyme was highly thermostable with a $t_{1/2} > 2$ h at 80°C and had a very high K to C (keratinolytic to caseinolytic) ratio of 2.5. Besides feather keratin, it also hydrolyzed a variety of other complex substrates including fibrin, gelatin and meat protein. Its activity on synthetic substrates revealed that it efficiently cleaves them in the order phenylalanine > lysine > alanine > leucine *p*-nitroanilides. It also cleaved insulin B chain between Val^{12}-Glu^{13}, Ala^{14}-Leu^{15}, Gly^{20}-Glu^{21} and Arg^{22}-Gly^{23} residues.

Keywords Keratinase · Thermostable · Substrate specificity · Characterization · *Pseudomonas aeruginosa*

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Introduction

Keratinases are a special group of proteases capable of degrading recalcitrant proteins such as feather, hair, nail and hoof that comprise of α- or β-keratins and are characterized by the presence of a high degree of cross-linking disulfide bonds along with hydrophobic interactions. By virtue of their ability to attack hard-to-degrade proteins, keratinases occupy a special niche among proteases [6]. They find immense biotechnological applications in the leather and cosmetic industries, where they have the advantage of being used in non-polluting processes [2]. Lately, it has been reported that keratinases that degrade feather could be potential catalysts for dissolution of prion plaques since both these proteins have similarly aggregated β-sheet structures [7]. Compendia of microorganisms are known to produce keratinases, the majority of which are able to degrade feather only in the presence of a suitable redox agent, which can be either in the form of a chemical or live-cell reductant [13]. Till now, cell-free feather degradation has not been reported. The aim of the present investigation was to purify keratinase from the fermentation broth of a bacterial strain of *Pseudomonas aeruginosa* KS-1 that was capable of completely degrading feather in cell-free conditions.

Materials and methods

Bacterial strains

A feather-degrading strain of *Pseudomonas aeruginosa* KS-1 was isolated from garden soil of South Campus, New Delhi, India. One gram soil sample was inoculated in 50 ml feather peptone medium containing 1% glucose,

1% peptone, 1% phosphate, 0.5% feather at pH 7 ± 0.2 [12]. The flasks were incubated at 37°C and 200 rpm in a New Brunswick Scientific shaker (Edison, NJ, USA) till complete degradation of chicken feather was observed. The enrichment was repeated three times. Appropriate dilutions of the enriched cultures were spread-plated on milk agar plates and incubated at 37°C for 24 h. Colonies were selected on the basis of a clear zone of hydrolysis and the isolates were further tested for feather degradation. A strain which completely degraded feather within 24 h was selected and identified by using complete 16S rDNA sequencing. The complete 16S rDNA sequence (1,491 bp) has been submitted to the GenBank database (Accession no. GQ203616). Stock cultures of the strain were maintained in 50% (v/v) glycerol and stored at −80°C.

Source of keratin substrate

Chicken feather for keratinase production was obtained from local poultry plants. Feather was washed thoroughly with detergent and distilled water and finally autoclaved at 121°C for 20 min. Autoclaved feather was dried in an oven at 80°C and stored in sterile bags till further use.

Keratinase production and cell-free degradation of chicken feather

For keratinase production, feather peptone medium was used [12]. This medium (50 ml, pH 7) was dispensed in 250-ml Erlenmeyer flasks and sterilized by autoclaving at 121°C for 20 min. Two per cent of a 24-h-old culture grown in nutrient broth was used as inoculum. The flasks were incubated at 37°C under shaking (250 rpm) in a New Brunswick Scientific shaker (Edison, New Jersey). After 24 h of incubation, the cells were harvested by centrifugation at 8,000*g* (Sigma Centrifuge, Germany) for 10 min. The supernatant was concentrated by passing through a 10-kDa centricon. The concentrated enzyme was then filtered through a 0.2-μm membrane filter (MDI, India). A total of 5,000 keratinolytic units of this cell-free crude enzyme was then added to 0.5% (w/v) chicken feather in 50 ml phosphate buffer (pH 7) and incubated at 37°C and 200 rpm for 24 h, or till complete degradation. Feather degradation was also analysed by scanning electron microscopy (LEO 435VP SEM, Carl-Zeiss NTS, GmbH, Germany).

Analytical methods

Keratinase assay

Keratinase activity was measured as described by Dozie et al. [5] with some modifications. The assay mixture containing 1 ml of appropriately diluted enzyme, 4 ml glycine–NaOH buffer (50 mM, pH 10) and 20 mg of chicken feathers was incubated at 60°C for 60 min. The reaction was terminated with 4 ml of 5% (w/v) trichloroacetic acid, and the tubes were incubated at room temperature (25°C) for 1 h. Feather and insoluble residues were removed by filtration through glass wool, and the filtrate was centrifuged at 5,000*g* for 5 min. An enzyme control was prepared in a similar manner, except that 1 ml of 5% trichloroacetic acid and 3 ml of the buffer were added, instead of 4 ml of the buffer used in the test. Substrate control was prepared by adding only 20 mg feather in 5 ml buffer. Proteolytic products in the supernatant were determined by observing absorbance at 280 nm. An increase in absorbance of 0.01 was considered as 1 U enzyme activity.

Protein assay

Protein concentration was determined by the method of Bradford [3] taking bovine serum albumin (BSA) as standard. The protein concentration was determined spectrophotometrically at 595 nm.

Purification of keratinase

The cell-free culture broth was first lyophilized. One gram of the lyophilized enzyme was dissolved in 5 ml of 10 mM Tris–HCl buffer (pH 8) and loaded on a Q-Sepharose column (Sigma–Aldrich, USA) equilibrated with 10 mM Tris–HCl buffer (pH 8). The column was washed with the same buffer and a 15-ml fraction was collected at a flow rate of 2 ml/min. Bound protein was eluted in a linear salt gradient (0.1–1 M NaCl). Twenty 2-ml fractions were collected for each salt concentration. Protein elution was monitored by measuring absorbance at 280 nm and keratinolytic activity was determined as described earlier. Purity of the protein was determined by SDS–PAGE analysis and by HPLC (C18 column, mobile phase acetonitrile/water 90:10, flow rate 1 ml/min, UV detector).

Zymogram analysis

The zymogram of the purified keratinase was carried out with a slight modification to the protocol followed by Najafi et al. [10]. The keratinase samples were mixed with the electrophoresis sample buffer without heat denaturation prior to electrophoresis. Native PAGE was carried out by using 10% polyacrylamide gel. After electrophoresis, the gel was washed with glycine–NaOH buffer (50 mM, pH 10.0) for 10 min on a rocker. The gel slab was then overlaid onto a 1% w/v casein agar plate prepared in glycine–NaOH buffer (50 mM, pH 10.0) and incubated at

60°C for 18 h. After incubation, the plate was flooded with 5% trichloroacetic acid.

Biochemical characterization of keratinase

Effect of pH and temperature on the keratinolytic activity

The effect of pH on the keratinolytic activity was studied by carrying out the keratinase assay in the pH range of 2.0–11.0 using buffers (50 mM) of varying pH viz. citrate phosphate buffer (pH 2.0–6.0), sodium phosphate buffer (pH 7.0), Tris–HCl buffer (pH 8.0–9.0), glycine–NaOH buffer (pH 10.0), phosphate hydroxide buffer (pH 11.0). Similarly, the effect of temperature on keratinase activity was determined by incubating the reaction mixture at different temperatures ranging from 40 to 70°C at pH 9. Activity was expressed as percentage relative activity with respect to maximum activity, which was taken as 100%.

Effect of pH and temperature on the stability of keratinase

pH stability was determined by incubating the enzyme in buffers of varying pH (2.0–11.0) for 1 h at room temperature (25 ± 1°C) and thereafter determining the residual activity at the optimum pH and temperature. The temperature stability was determined by incubating the enzyme samples at various temperatures ranging from 50 to 80°C at different time intervals up to 2 h (30, 60, 90 and 120 min). The residual activity was determined at pH 9 and 60°C.

Effect of inhibitors and metal ions on the keratinolytic activity

The purified enzyme was pre-incubated at room temperature (25 ± 1°C) for 15 min with various inhibitors viz. phenylmethylsulfonylfluoride (PMSF), ethylenediamine tetraacetate (EDTA), bromoacetic acid, iodoacetic acid and β-mercaptoethanol, dithiothreitol (DTT) (Sigma–Aldrich, USA; ICN chemicals, USA) at a final concentration of 10 mM. Similarly, the effect of metal ions was studied by pre-incubating the purified enzyme with metal ions viz. Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} at a final concentration of 10 mM for 1 h. The residual activity was measured at pH 9 and 60°C and was expressed in terms of percentage activity relative to control without the inhibitor which was taken as 100%.

Substrate specificity of keratinase

Proteolytic activity on complex substrates

The proteolytic activity of the keratinase was studied by using soluble and insoluble substrates. Complex substrates included casein, gelatin, elastin, feather keratin, fibrin and nail keratin (substrates were either purchased locally from CDH (Central Drug House) and SRL (Sisco Research Laboratories Pvt Ltd) or from Sigma–Aldrich, USA). The reaction mixture was set up by using 20 mg of each of the substrate in 1 ml of Tris–HCl buffer (50 mM, pH 9) and 1 ml of appropriately diluted enzyme prepared in the same buffer. The reaction was incubated at 60°C for 1 h. The reaction was stopped by the addition of 4 ml of 5% (w/v) trichloroacetic acid. The contents were centrifuged after 1 h at 1,006 g for 10 min. Folin–Ciocalteau's reagent (0.5 ml) was added to 1 ml of the supernatant and the optical density of the samples was recorded at 660 nm against appropriate substrate and enzyme blanks. One unit of protease was equivalent to the amount of enzyme required to release 1 μg of tyrosine ml^{-1} h^{-1} under standard assay conditions.

Activity on synthetic substrates

Activity of keratinase was examined by using 10 mM stock solutions of *p*-nitroanilide substrates viz. *N*-Suc-Ala-Ala-Pro-Phe-*p*NA (AAPF), *N*-Suc-Ala-Ala-Ala-*p*NA (AAA), *N*-Bz-Tyr-*p*NA, *N*-Bz-Phe-*p*NA, *N*-Bz-Arg-*p*NA, *N*-Suc-Ala-Ala-Pro-Leu-*p*NA and *N*-Suc-Gly-Gly-Phe-*p*NA (Sigma–Aldrich, USA; ICN chemicals, USA). Activity was also checked on the synthetic substrate of plasmin i.e. D-Val-Leu-Lys-*p*NA. Reaction mixture contained 100 μl of synthetic substrate and 900 μl of appropriately diluted enzyme prepared in Tris–HCl (50 mM, pH 9.0) buffer. The reaction mix was incubated at optimum temperature for 10 min. The hydrolyzed product was measured at 405 nm using a UV–Vis spectrophotometer (UV 1700 Shimadzu, Japan). The molar extinction coefficient for *p*NA was taken to be 9,900 M^{-1} cm^{-1}. In addition, kinetic constants for keratinase activity were determined on *N*-Suc-Ala-Ala-Pro-Phe-*p*NA (0.1–1 mM) and D-Val-Leu-Lys-*p*NA.

Hydrolysis of insulin B chain and mass spectrometry

The substrate specificity of the keratinase was also determined on the basis of hydrolysis of insulin B chain (Sigma, cysteine residues oxidized). Hundred microlitres of purified enzyme was incubated with 100 μl of insulin B chain (1 mg/ml in 10 mM Tris–HCl buffer, pH 9). The mixture was incubated at 60°C for 16 h, after which 40 μl of 0.1% (v/v) trifluoroacetic acid was added to inactivate the enzyme. Identification of the cleavage products was performed by liquid chromatography followed by electrospray mass spectrometry (LC-ESI/MS) using an LCT mass spectrometer (Waters/Micromass, USA). The ion trap was operated in the positive ionization mode with a flow rate of 50 μl/min and capillary and cone voltage of 3,500 and

45 V, respectively. Solvent used was acetonitrile/water (1:1) with 0.1% formic acid. The data were treated with the Mass Lynx (version 4.0) software. The *m/z* values ranged between 650 and 2,100. Hydrolysis sites were determined by using FindPept, a part of the Expasy software package.

Enzymatic degradation of feather using purified keratinase

A total of 5,000 keratinolytic units of purified keratinase was added to 0.5% (w/v) chicken feather present in 50 ml phosphate buffer (pH 7) and incubated at 37°C and 200 rpm for 24 h, and percentage feather degradation was measured by using the weight loss method. The dry mass of feather was obtained by washing the residue twice with distilled water and the residue was dried on a pre-weighed aluminium cup in an air-circulating oven at 60°C until constant weight. Feather degradation was determined by subtracting the weight of feather before the inoculation from the weight of the residue after degradation.

Statistical analysis of data

All the above experiments were repeated three times in triplicates and the final values have been presented as mean ± standard deviation.

Results

Keratinase production and cell-free degradation

A feather-degrading strain of *Pseudomonas aeruginosa* KS-1 produced 1,200 U/ml of keratinase when grown in feather peptone medium. This strain degraded chicken feather within 24 h of incubation at 37°C and 200 rpm. Its microfiltered, cell-free crude fermentation broth completely degraded chicken feather within 12 h. No disulfide reductase activity was detected in this broth. SEM analysis of the degraded feather also depicted complete degradation with loosening of the keratin fibrils within 6 h of incubation (Fig. 1).

Purification of keratinases

Keratinase was purified from the culture broth of *Pseudomonas aeruginosa* KS-1 with a yield of 64.35% (Table 1). One major keratinase peak termed KP1 was observed from the Q-Sepharose column in the 0.6 M NaCl fraction and a minor keratinase peak termed KP2 was eluted in the 0.1 M fraction (Fig. 2a). The homogeneity of the purified keratinase KP1 was revealed by SDS–PAGE, showing a single protein band at 45 kDa (Fig. 3). Purity was also confirmed by HPLC where a single major peak having a retention

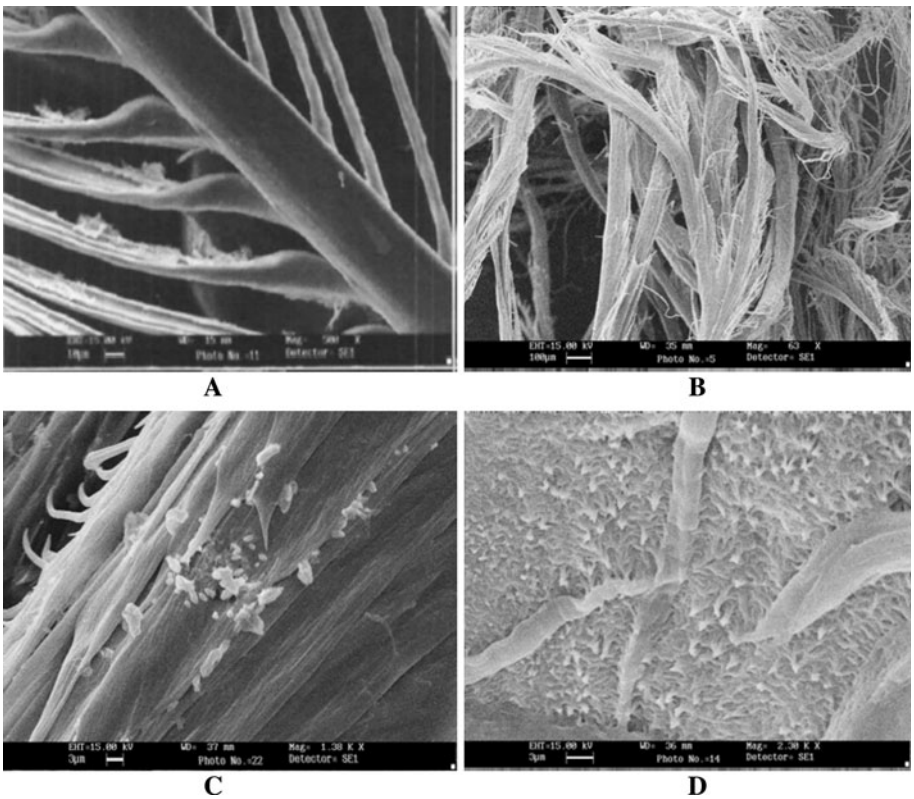

Fig. 1 SEM analysis of degradation of feather using cell-free extract at pH 7 and 37°C. **a** Uninoculated control feather, **b** after 6 h, **c** after 9 h, **d** after 12 h (*bar* represents 10 μm in **a**, 100 μm in **b**, 3 μm in **c** and **d**; magnification of **a** ×500, **b** ×63, **c** $\times 1.38 \times 10^3$ and **d** $\times 2.38 \times 10^3$)

Table 1 Purification scheme of keratinase KP1 on Q-Sepharose anion exchange column

Purification step	Total protein (mg)	Total keratinolytic activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (*n*-fold)
Crude	14	968	69.14	100	1
Q-Sepharose	3.8	623	163.94	64.35	2.37

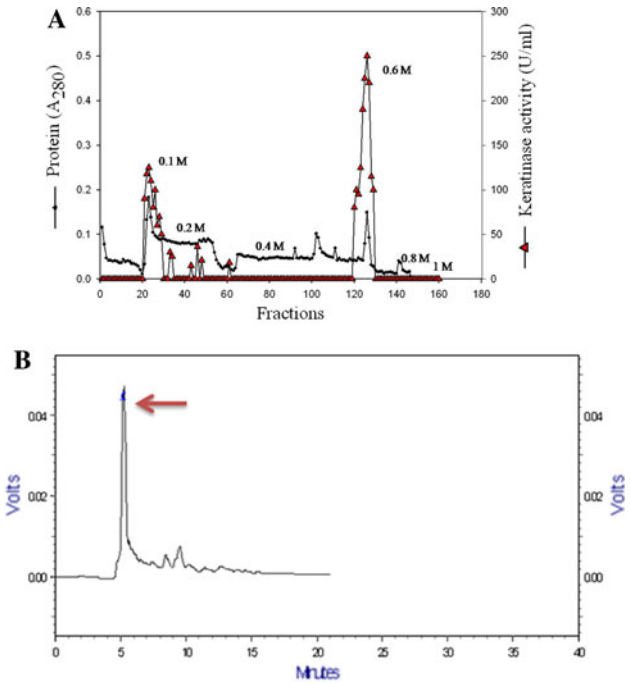

Fig. 2 **a** Purification profile of keratinase KP1 on Q-Sepharose anion exchange column. Profile was repeated three times. **b** HPLC chromatogram of purified keratinase KP1 on a C18 reversed-phase column using a UV detector (mobile phase acetonitrile/water (90:10), UV detector)

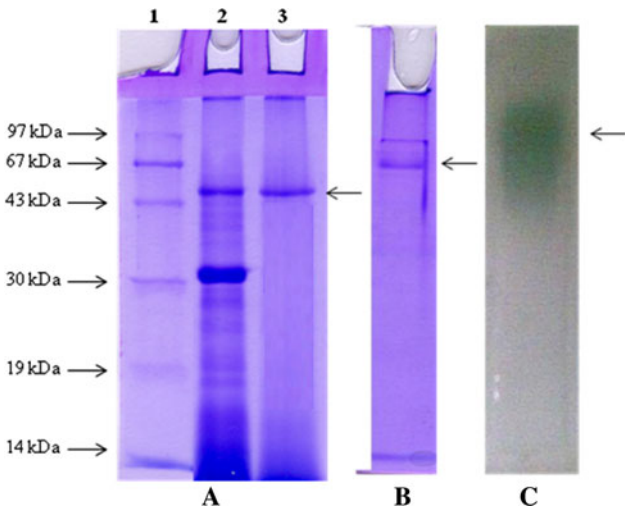

Fig. 3 SDS–PAGE analysis and zymogram of purified keratinase KP1. *A* SDS–PAGE; *1* molecular weight ladder, *2* crude broth, *3* purified keratinase KP1. *B* native PAGE. *C* zymogram on casein agar plate

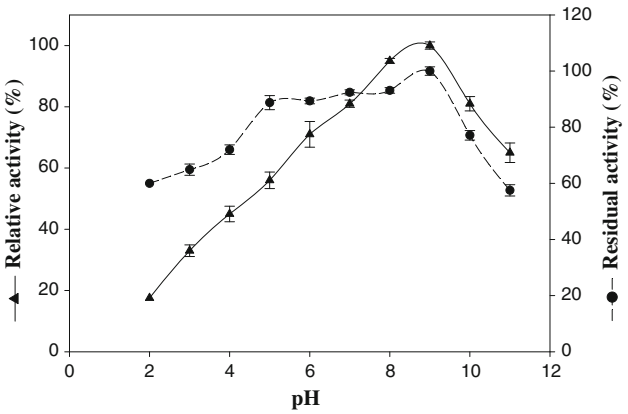

Fig. 4 Effect of pH on the keratinolytic activity and stability of KP1 (100% activity corresponded to 84 U/mg protein on feather keratin as substrate). Data points and error bars indicate averages and standard deviation of triplicate measurements in three replicates

time of 5.2 min was observed when analysed on a C18 column using a UV detector (Fig. 2b). On zymogram analysis, the band for keratinase appeared as a translucent zone against a white background (Fig. 3).

Biochemical characterization of keratinase KP1

Effect of pH and temperature on the keratinolytic activity of KP1

The purified keratinase KP1 was found to be active over a pH range of 8–10 with optimal activity at pH 9 (Fig. 4). It was active over a broad range of temperature ranging from 40 to 80°C with maximal activity at 60°C (Fig. 5a).

Effect of pH and temperature on the stability of KP1

KP1 was highly stable over a pH range of 5–9 with 88% residual activity at pH 5 (Fig. 4). It was highly thermostable with $t_{1/2}$ of more than 2 h at 80°C (Fig. 5b).

Effect of inhibitors and metal ions on the keratinolytic activity of KP1

The effect of various protease inhibitors and chelators on keratinase activity is shown in Table 2. KP1 was completely inhibited by 10 mM PMSF followed by 40% residual activity in presence of 10 mM EDTA. Among the

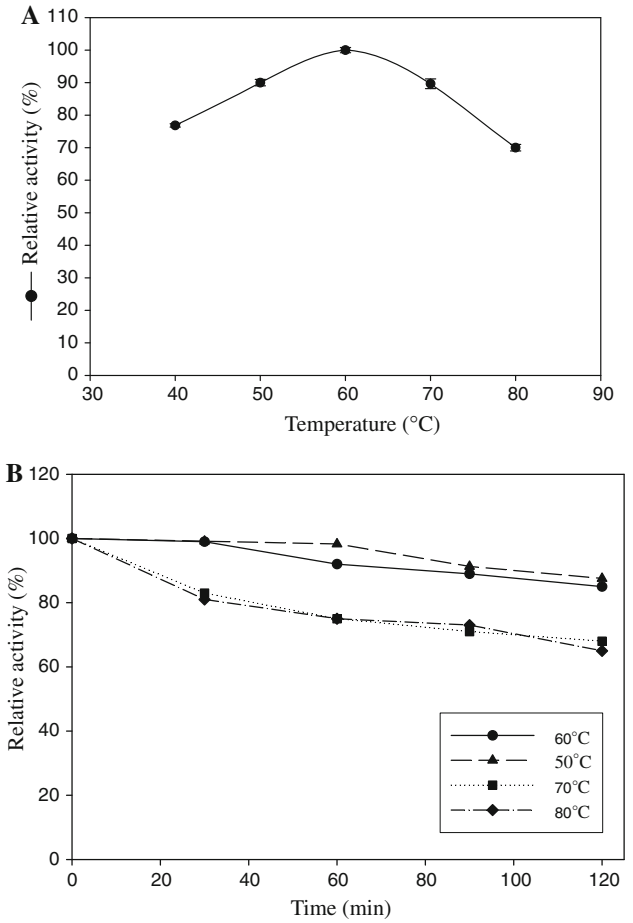

Fig. 5 Effect of temperature on activity (**a**) and stability (**b**) of keratinase KP1 at pH 9 (100% activity corresponded to 80 U/mg protein on feather keratin as substrate)

metal ions, Mn^{2+} showed a stimulatory effect on KP1 whereas the rest of the metal ions, namely Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+} and Zn^{2+}, did not affect the enzyme activity (data not shown).

Substrate specificity of keratinase KP1

Keratinase KP1 exhibited broad substrate specificity as it could hydrolyze a variety of substrates in the order: meat protein > gelatin > nail keratin > fibrin > chicken feather > elastin > casein. It was noteworthy that keratinase KP1 had a very high keratinolytic to caseinolytic (K to C) ratio of 2.5 (Fig. 6a).

Among various *p*-nitroanilide substrates, KP1 showed maximum activity for cleaving the hydrophobic residue of *N*-Suc-Ala-Ala-Pro-Phe-*p*NA followed by 96% relative activity on plasmin substrate i.e. D-Val-Leu-Lys-*p*NA. KP1 could also hydrolyze *N*-Suc-Leu-*p*NA efficiently. KP1 was much less active on *N*-benzoyl-DL-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA and *N*-Suc-L-Phe-*p*NA (Fig. 6b). The K_m for

Table 2 Effect of inhibitors on the activity of keratinase KP1

Inhibitors (10 mM)	Residual activity (%)
PMSF	10
β-Mercaptoethanol	95
EDTA	40
Bestatin	97
Bromoacetic acid	93
Iodoacetic acid	98
DTT	110

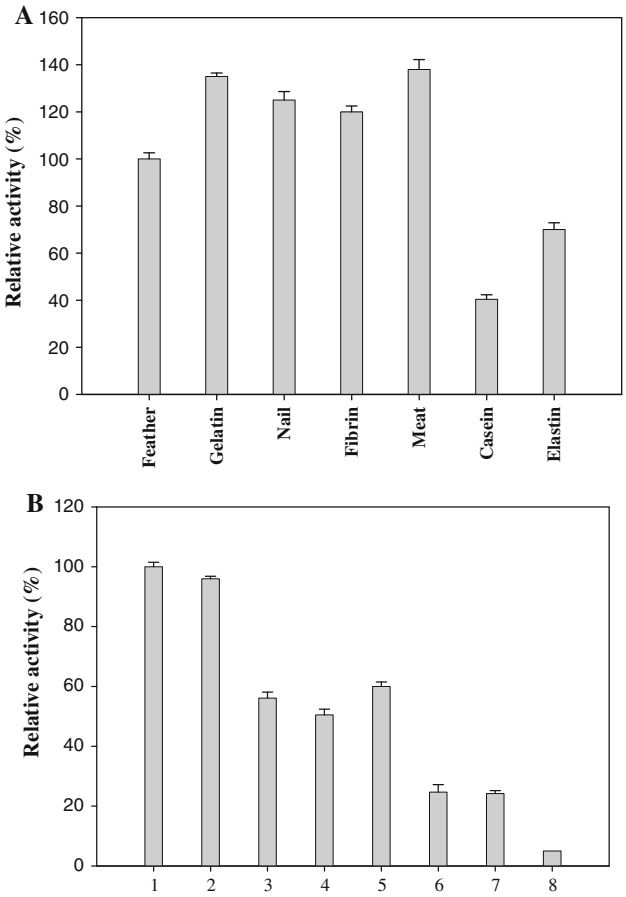

Fig. 6 **a** Substrate specificity of keratinase KP1 on insoluble, complex substrates. **b** Activity of keratinase KP1 on various synthetic substrates. (100% activity corresponds to 20 U/mg protein) *1* *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, *2* D-Val-Leu-Lys-*p*NA, *3* *N*-Suc-Ala-Ala-Ala-*p*NA, *4* *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, *5* *N*-Suc-Gly-Gly-Phe-*p*NA, *6* *N*-benzoyl-DL-Arg-*p*NA, *7* *N*-benzoyl-L-tyrosine-*p*NA, *8* *N*-Suc-L-Phe-*p*NA

N-Suc-Ala-Ala-Pro-Phe-*p*NA and D-Val-Leu-Lys-*p*NA was 1.66 and 2.85 mM, respectively (Table 3). LC-ESI/MS analysis of the cleaved insulin B chain revealed the cleavage sites to be between Val^{12}-Glu^{13}, Ala^{14}-Leu^{15}, Gly^{20}-Glu^{21} and Arg^{22}-Gly^{23} (Fig. 7).

Table 3 Kinetic parameters for keratinase KP1 activity on synthetic substrates

Substrate	K_m (mM)	V_{max} (μmol ml^{-1} min^{-1})	K_{cat} (s^{-1})
N-Suc-Ala-Ala-Pro-Phe-*p*NA	1.66	3.1	1.12×10^2
D-Val-Leu-Lys-*p*NA	2.85	2.5	5.28×10^1

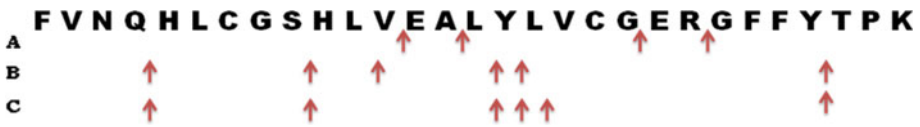

Fig. 7 Hydrolysis of insulin B chains (with oxidized cysteine residues) by keratinase KP1. *Arrows* point at cleavage sites. *A* keratinase KP1; *B* subtilisin Carlsberg; *C* subtilisin Novo

Enzymatic degradation of feather using keratinase KP1

Only 30% degradation of feather was obtained when 5,000 keratinolytic units of keratinase KP1 were used at 37°C and 200 rpm for 24 h.

Discussion

A feather-degrading strain of *Pseudomonas aeruginosa* KS-1 was isolated from garden soil of South Campus, New Delhi, which completely degraded chicken feather within 24 h at 37°C and 200 rpm. A wide spectrum of bacteria are known to degrade feather, most of which are gram-positive including *Bacillus* species and *Streptomyces* species [4, 6]. However, only a few gram-negative keratinase producers are known, namely *Vibrio* sp., *Chryseobacterium* sp., *Burkholderia* sp. and *Pseudomonas aeruginosa* [8, 14, 15]. The present strain of *Pseudomonas aeruginosa* KS-1 was not only a potential feather degrader but its fermentation broth could also degrade more than 90% of feather within 12 h in cell-free conditions. This is in contrast to earlier reports where cell-free degradation has never been reported to exceed 10–20% [13]. In addition it has also been reported that complete feather degradation requires external redox either in the form of chemicals or live cells [13]. Hence, the fermentation broth in *Pseudomonas aeruginosa* was worthy of further investigation for its keratinolytic enzyme.

Keratinase KP1 was purified by anion exchange chromatography in 0.6 M NaCl concentration. SDS–PAGE analysis revealed it to be a 45-kDa monomer. This is in contrast to the earlier report of keratinase from *Pseudomonas aeruginosa* of 33 kDa [8]. Usually the molecular weight of keratinases from common keratinolytic bacteria including *Bacillus* and *Streptomyces* sp. is around 20–50 kDa [16].

Keratinase KP1 was an alkaline, serine protease with maximal activity at pH 9 and broad pH stability, with more than 50% relative activity over a range of pH 3–11. The majority of keratinases reported are alkaline in nature [2]. It is assumed that alkaline conditions aid in the breaking of disulfide bonds and assist rapid feather degradation. The fact that keratinase KP1 was highly thermostable with a $t_{1/2}$ of more than 2 h at 80°C was remarkable. It was active over a wide temperature range with more than 70% relative activity over a range of 30–70°C. This is in contrast to the earlier reported keratinase from *Pseudomonas aeruginosa* KS-1 with pH and temperature stability within the range of pH 6–9 and 10–50°C [8]. This broad range of stability of the present keratinase makes it suitable for wide applications in the industrial sector.

The present keratinase was inhibited by PMSF and thus it is a serine protease, in accordance with most of the reported keratinases [4]. It efficiently cleaved a number of complex proteins viz. fibrin, gelatin, nail keratin and meat protein besides feather keratin. It was noteworthy that among all the protein substrates, casein was the least hydrolyzed, bringing the K to C ratio to 2.5. This is in contrast to most of the keratinases and also to the known keratinase from *Pseudomonas aeruginosa* where either the assay is casein-based, or keratinase has high caseinolytic activity [18, 19]. Generally, K to C ratio is used to assess the potential of a keratinase. A protease with a K to C ratio of more than 0.5 is considered as a potential keratinase [11]. By and large, K to C ratio for keratinases is generally reported to be between 0.2 and 0.5.

Keratinase KP1 exhibited specificity for hydrophobic amino acid residues such as phenylalanine, alanine and leucine as demonstrated by using synthetic substrates. This is in accordance with earlier reports on keratinase from *Nesterenkonia* sp. AL20 and *Nocardiopsis* sp. [1, 9]. The amino acid amides viz. *N*-benzoyl-DL-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA and *N*-Suc-L-Phe-*p*NA were hydrolyzed at

very low rates. Among *N*-Suc-Ala-Ala-Pro-Phe-*p*Na and *N*-Suc-Gly-Phe-*p*NA, the latter was cleaved more efficiently indicating the P1 preference of the keratinase KP1 for proline instead of glycine. Keratinase KP1 also efficiently cleaved the synthetic substrate of plasmin i.e. D-Val-Leu-Lys-*p*NA. This was substantiated by high activity on fibrin. This suggests that the present keratinase also has the potential to become an efficient fibrinolytic enzyme. There are only few reports on keratinases with high fibrinolytic activity [17]. Further cleavage of the insulin B chain revealed the cleavage sites to be at valine, alanine, glycine and arginine. Keratinase KP1 therefore cleaved at different sites compared with subtilisin Carlsberg and subtilisin Novo. This may be a reason for the low caseinolytic activity of and thus the high K to C ratio for keratinase KP1.

In an attempt to study feather degradation by keratinase KP1, it was observed that 30% degradation of chicken feather was achieved which was in contrast to the complete degradation obtained in the case of the crude fermentation broth. This suggests that feather degradation is a result of the action of a consortium of enzymes rather than a single enzyme, which is in keeping with the fact that during purification one more keratinase was isolated as a smaller peak. To validate this hypothesis, detailed study on the minor keratinase KP2 is being undertaken.

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