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A Novel Serine Metallokeratinase from a Newly Isolated *Bacillus pumilus* A1 Grown on Chicken Feather Meal: Biochemical and Molecular Characterization

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Abstract A keratinolytic enzyme (KerA1) secreted by a newly isolated *Bacillus pumilus* strain A1 cultivated in medium containing chicken feather meal was purified and characterized, and the gene was isolated and sequenced. The molecular mass of the purified enzyme was estimated to be 34,000 Da by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and gel filtration. The optimum pH and temperature for the purified keratinase were 9.0 and 60 °C, respectively, using keratin as a substrate. KerA1 showed a high stability towards nonionic surfactants. It was found to be relatively stable toward the strong anionic surfactant (SDS). The deduced amino acid sequence of the keratinase KerA1 differs from both the organic solvent tolerant protease of *B. pumilus* 115b and the dehairing protease of *B. pumilus* UN-31-C-42 by one and nine amino acids, respectively. These results suggest that this keratinase may be a useful alternative and ecofriendly route for handling the abundant amount of waste feathers and for applications in detergent formulations.

Keywords *Bacillus pumilus* · Keratinase · Purification · Molecular characterization

Introduction

Keratins are stable and insoluble structural proteins found in the epidermis of vertebrates and their appendages, such as feathers and hair. The α-helix (α-keratin) or β-sheet (β-keratin) keratin chains are tightly packed into a supercoiled polypeptide chain extensively cross-linked with disulfide bridges, hydrogen bonds, and hydrophobic interactions, resulting in the mechanical stability of keratin and its recalcitrance to common proteolytic enzymes such as pepsin, trypsin, and papain [1].

Keratin-rich wastes, mainly in the form of feathers and hair, are generated in high amounts as by-products of agro-industrial processing. The accumulation of such residues

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could lead to environmental problems [2]. However, despite its recalcitrance, keratin can be efficiently hydrolyzed by keratinolytic enzymes produced by a multitude of bacteria and fungi and particularly the bacterial genus *Bacillus* which seems to be a prominent keratinolytic microorganism [3, 4].

The microbial degradation of the insoluble keratin depends on the secretion of enzymes with the ability to act on the surface of these substrates. In this sense, keratinases are reported to be mainly extracellular, and the production of such enzymes is generally induced by keratinous substrates such as feathers [2]. These enzymes have many applications in the feed, fertilizer, detergent, leather, and pharmaceutical industries [2]. For example, the feather hydrolysate of *Bacillus licheniformis* [5] can be used as supplementation on animal diets. Indeed, addition of the crude keratinase from *B. licheniformis* PWD-1 improved the poultry growth [6]. Also, the keratinase can degrade the infectious form of prion (PrP^{sc}) in the presence of detergents at high temperature [7], which could be important for the utilization as animal feed. Finally, the keratinase from *Bacillus pumilus* exhibits a remarkable dehairing function, which shows a great utilization potential in leather industry [8].

Many of these enzymes have been purified from different microorganisms, such as fungi [9], bacteria [10, 11], and *Streptomyces* species [12–14]. The purified enzymes act as proteinases and have a high level of activity on insoluble keratin. Nevertheless, there is little information on the characterization and purification of keratinase from *B. pumilus*.

The aim of this study was to purify and characterize the keratinase KerA1 produced by *B. pumilus* A1 strain. Besides, it attempted to perform the nucleotide sequence of the *kerA1* gene. This enzyme seems to have an excellent effect on the degradation of feathers.

Materials and Methods

Isolation and Identification of the Bacterial Strain

An alkaline keratinase producing bacterium A1 was isolated from the wastewater effluent released from a slaughter house in Sfax, Tunisia. Keratinolytic strains were screened for their ability to hydrolyze keratin in chicken feather meal agar plates containing (grams per liter): chicken feathers meal prepared in our laboratory, 5; NaCl, 0.5; $MgSO_4$ (7 H_2O), 0.1; KH_2PO_4, 0.7; K_2HPO_4, 1.4; and agar, 30; pH 11.0. The plates were incubated for 72 h at 37 °C. Several keratinolytic strains were isolated, and A1, which exhibited a large clear zone of hydrolysis in the medium, was selected.

The isolate A1 was identified on the basis of the 16S ribosomal DNA (rDNA) sequence analysis. Sequence comparison with the databases was performed using the program Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information (NCBI), National Institutes of Health, USA).

Culture and Growth Conditions

Production of keratinolytic enzymes by the newly isolated strain *B. pumilus* A1 was carried out in an optimized medium containing (grams per liter): chicken feather meal, 30; soy peptone, 3.151; KH_2PO_4, 0.5; K_2HPO_4, 0.5; NaCl, 2; KCl, 0.1; and $MgSO_4$ (7 H_2O), 0.1; pH 6.0. Inocula were routinely grown in Luria–Bertani broth medium (grams per liter): peptone, 10.0; yeast extract, 5.0; and NaCl, 5.0; pH 7.0. Media were autoclaved for 20 min at 120 °C. The keratinolytic enzyme was produced in 250 ml Erlenmeyer flasks containing

25 ml culture medium maintained for 48 h at 30 °C and 250 rev/min. The culture medium was centrifuged for 30 min at 13,000 rev/min and at 4 °C. The supernatant containing the keratinolytic activity was collected.

Enzyme Assay

Keratinolytic and caseinolytic activities were determined by the modified methods of Takiuchi et al. [15] and Kembhavi et al. [16], respectively. One unit of keratinolytic activity was defined as an increase of corrected absorbance at 280 nm (A_{280}) of 0.1 under the experimental conditions. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions. The data presented are the mean value of two parallel determinations.

Determination of Protein Concentration

Protein concentration was determined by the method of Bradford [17] using the Bio-Rad assay reagent (Bio-Rad, Munich, Germany) and bovine serum albumin (BSA) as the standard. During the course of purification, the protein concentration was determined by measuring the absorbance at 280 nm.

Enzyme Purification

The optimized culture medium used for producing keratinolytic protease was centrifuged for 30 min at 13,000 rev/min and 4 °C. The supernatant (200 ml) was subjected to CM-Sephadex cation exchange chromatography column (3×45 cm) equilibrated with buffer A (25 mM Tris-HCl, pH 8.0). Elution of keratinase was performed with the same buffer A at 80 ml/h. The bound proteins were eluted with a linear gradient of sodium chloride in the range of 0–0.5 M in the equilibrating buffer. The fractions containing keratinase activity were combined then applied to a stirred ultrafiltration cell (Millipore 8400) using a 10,000-Da MM cutoff membrane (PBGC membrane, Millipore). The concentrate was loaded on a gel-filtration Sephadex G-100 column (2.6×150 cm) equilibrated with buffer B (25 mM Tris-HCl, pH 8.0, 0.5‰ Triton X-100). Fractions of 5.5 ml were collected at a flow rate of 27 ml/h with the same buffer and the keratinase activity was determined. The fractions showing keratinase activity were pooled and then loaded to a CM-Sephadex column (2×25 cm) which was equilibrated with buffer A. After being washed with the same buffer A, the bound proteins were eluted with a linear gradient of sodium chloride in the range of 0–0.5 M in the buffer A. Fractions of 5.5 ml were collected at a flow rate of 95 ml/h and analyzed for keratinase activity. The fractions showing keratinase activity were pooled concentrated to a small volume by ultrafiltration as described previously and stored at −20 °C for further analyses. All purification steps were performed at 4 °C.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the control of the purity and determination of molecular mass of the enzyme as described by Laemmli [18], using a 5% (*w*/*v*) stacking and a 12% separating gels. Samples were heated for 5 min at 100 °C before electrophoresis. The molecular mass of the purified enzyme was estimated using a low molecular mass calibration kit (Amersham Biosciences). The molecular mass markers used were phosphorylase b (97,000 Da), albumin (66,000 Da),

ovalbumin (45,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da).

Detection of Keratinolytic Activity on Polyacrylamide Gels (*Keratinase Zymogram*)

Zymography was performed in conjunction with SDS-PAGE according to the method of Garcia-Carreno et al. [19] with a slight modification. Samples were not heated before electrophoresis. After electrophoresis, the gel was submerged in 100 mM glycine–NaOH buffer (pH 9.0) containing 2.5% Triton X-100 for 60 min, with constant agitation to remove SDS. Triton X-100 was then removed by washing the gel three times with 100 mM glycine–NaOH buffer (pH 9.0). The gel was then incubated with 2% (*w*/*v*) keratin in 100 mM glycine–NaOH buffer (pH 9.0) for 60 min at 50 °C. Finally, the gel was stained with Coomassie Brilliant Blue R-250 for zymography analysis. The development of a clear zone on the blue background of the gel indicated the presence of keratinase activity.

N-terminal Amino Acid Sequence of the Purified Enzyme

The purified enzyme was transferred from SDS-PAGE to a polyvinylidene difluoride (PVDF) membrane. The PVDF band corresponding to the keratinase was excised from the membrane, and the N-terminal amino acid sequence was determined by the Edman degradation method on an ABI Procise 494 protein sequencer (Applied Biosystems).

Biochemical Properties of the Purified Enzyme

Effect of pH on the Activity and Stability of the Purified Enzyme

The optimum pH of the purified enzyme was studied over a pH range of 6.0–13.0 at 60 °C using casein 1% (*w*/*v*) and keratin 0.8% (*w*/*v*) as substrates. The effect of pH on enzymes stability was examined by incubating the enzymes in buffers of different pH values in the range of pH 6.0–13.0 for 1 h at 40 °C. The residual keratinolytic activity was determined under standard assay conditions. The following buffer systems were used: 100 mM sodium acetate buffer for pH 6.0, 100 mM potassium phosphate buffer for pH 7.0, 100 mM Tris-HCl buffer for pH 8.0–8.5, 100 mM glycine–NaOH buffer for pH 9.0–10.5, and 100 mM KCl-NaOH for pH 11.0–13.0.

Effect of Temperature on the Activity and Stability of the Purified Enzyme

To investigate the effect of temperature, the caseinolytic and keratinolytic activities were tested at different temperatures between 40 °C and 70 °C at pH 10.0 and pH 9.0 using casein and keratin, respectively, as substrates. Thermal inactivation was examined by incubating the purified enzyme at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, and 80 °C for 60 min in the absence then in the presence of 5 mM $CaCl_2$. The remaining keratinolytic activity was determined at pH 9.0 and 60 °C. The non-heated enzyme was considered as control (100%).

Effects of Metal Ions and Inhibitors on the Activity of the Purified Enzyme

The effects of various metal ions (5 mM) on enzyme activity was investigated by adding the monovalent (Na^+ and K^+) or divalent metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Ba^{2+}, and

Hg^{2+}) to the reaction mixture. Relative activity was determined using keratin as a substrate. The activity of the enzyme without any metallic ions was considered as 100%.

The effects of enzyme inhibitors on protease activity were studied using phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol, and dithio-bis-nitrobenzoic acid (DTNB). The purified enzyme was preincubated with inhibitors for 30 min at 25 °C, and then the remaining enzyme activity was determined at pH 10.0 and 65 °C or at pH 9.0 and 60 °C using casein 1% (*w*/*v*) or keratin 0.8% (*w*/*v*), respectively, as substrates. The activity of the enzyme assayed in the absence of inhibitors was taken as 100%.

Effects of Surfactants and Oxidizing Agent on the Stability of the Purified Enzyme

The effects of some surfactants (SDS, Triton X-100, Tween 20, Tween 80) and oxidizing agent (sodium perborate) on enzyme stability were studied by pre-incubating the enzyme for 1 h at 30 °C. The residual activity was measured at 60 °C and pH 9.0 using keratin as a substrate. The activity of the enzyme without any additive was taken as 100%.

Hydrolysis of Soluble and Insoluble Proteins

All the substrates used in this study were purchased from Sigma-Aldrich Chimie except for fibrin bovine which was purchased from MP Biomedical Co (St. Louis, MO, USA). The substrate specificity of KerA1 enzyme was determined using natural (casein, BSA, egg albumin, gelatin, fibrin, and keratin) and modified protein substrates (azocasein and azokeratin). Protease activity was assayed by mixing 0.5 ml of the diluted enzyme with 0.5 ml of buffer (glycine–NaOH 100 mM, pH 9.5) containing 1% (*w*/*v*) of each substrate. After 30 min incubation at 60 °C, the reactions were stopped by adding 0.5 ml of trichloroacetic acid (TCA) 20% (*w*/*v*) and allowed to stand at room temperature. After 15 min, they were centrifuged for 15 min at 13,000 rev/min. The increase in absorbance due to the hydrolysis and release of peptide was measured under the corresponding wavelength of the initial substrate. The relative protease activity toward casein was taken as a control.

Kinetic Studies

The activity of the purified keratinase KerA1 was evaluated with different final concentrations of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilic (SAAPNA; specific substrate for chymotrypsin-like protease), ranging from 25 to 100 μM. Amidase activity of KerA1 was evaluated according to Tsai et al. [20], with slight modifications: A 10 μl of enzyme solution was mixed with 990 μl of SAAPNA in 100 mM glycine–NaOH buffer (pH 9.5) and incubated at 25 °C. Production of *p*-nitroaniline was measured by monitoring the increment in absorbance at 410 nm every 30 s for 10 min. SAAPNA hydrolysis units (*U*) were calculated with the following equation: $U=[A_{(410)}/\text{min}\times 1{,}000\times 1]$ divided by 8,800, where 8,800=*p*-nitroaniline molar extinction coefficient in per molar per centimeter and 1,000 is the factor to convert molars to micromoles in 1 ml which is the volume of the reaction mixture.

The determinations were repeated twice, and the respective kinetic parameters including K_m and V_{max} were calculated from Lineweaver–Burk plots. The value of the turnover number (k_{cat}) was calculated from the following equation: $k_{cat}=V_{max}/[E]$, where $[E]$ is the active enzyme concentration and V_{max} is the maximal velocity.

Amplification and Determination of the Nucleotide Sequence of the *kerA1* Gene

Genomic DNA was extracted from *B. pumilus* A1 cells using the Wizard® genomic DNA purification kit (Promega). Four oligonucleotides, designed on the basis of the published sequence of the protease gene of *B. pumilus* [21], were synthesized and used for isolation and determination of the keratinase gene sequence. The complete *kerA1* gene and its flanking regions were amplified using the upstream primer BPE1 (5′-TCAGGTCTACTCT TATTTGC-3′) and the downstream primer BPE2 (5′-ATGATTCTCTCCATCATCG-3′) to generate a fragment of 1,199 bp. The DNA region encoding the internal sequence (655 bp) was amplified using upstream primer BPI1 (5′-GAACACGACCCTAGCATTGC-3′) and downstream primer BPI2 (5′-AGTACAGTACCAAGCAGTGG-3′). Amplification of DNA was carried out using the ThechGene® TC-312 ThermalCycler (TECHNE, Cambridge, England), under the following condition: two cycles of denaturation at 94 °C for 2 min, annealing to 58 °C for 45 s, and extension at 72 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 2 min. Amplified polymerase chain reaction (PCR) products were analyzed on an agarose gel and purified by the QIAquick Gel Extraction Kit (QIAGen) and were directly sequenced as described above. Three independent PCR products were sequenced in both directions to obtain a reliable sequence. The BLAST program (NCBI) was used to search for similar sequences in the databases. Alignment of protein sequences were carried out using CLUSTALW program at the European Bioinformatics Institute server (http://www.ebi.ac.uk/clustalw).

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the 16S rDNA and the *kerA1* gene reported in this study were assigned to the GenBank accession numbers EU719191 and FJ619651, respectively.

Results and Discussion

Isolation and Identification of the Microorganism

The strain used in this study was isolated from the wastewater effluent released from a slaughter house. Samples collected were plated onto chicken feather meal agar plates. Plates were incubated for 72 h at 37 °C, and clear halos around colonies gave an indication of keratinase producing strains. Several keratinolytic strains were isolated, and A1, which exhibited a large clear zone of keratin hydrolysis, was selected and identified as *B. pumilus* on the basis of the 16S ribosomal DNA (rDNA) sequence analysis. *B. pumilus* A1 strain is available in the strain collection of “Center of Biotechnology, Sfax-Tunisia” and assigned an accession number CTM 50290.

Purification of *B. pumilus* A1 Keratinase (KerA1)

Keratinase synthesis was strongly induced when *B. pumilus* A1 was grown in media containing chicken feather meal as carbon source. Complete feathers degradation was achieved after 48 h of culture at 30 °C. Indeed, the loss of dry weight was determined after filtration through Whatman no. 3 filter paper, washing and drying at 105 °C to constant weight. Interestingly, a loss of 94% of feathers weight was obtained.

Keratin zymography of the crude extract showed five clear zones, indicating that at least five major keratinases are produced by the strain *B. pumilus* A1 (Fig. 1, lane 4). This is in line with previous finding [22] dealing with the crude supernatant of *Bacillus mojavensis* A21 which showed at least six clear zones on casein zymography. However, a single protease band was detected upon zymogram analysis with the crude enzyme produced by the strain *Bacillus subtilis* P13 [23].

KerA1 was purified by a three-step procedure. In the first step, the culture supernatant was subjected to CM-Sephadex cation exchange column pre-equilibrated and washed with buffer A. Binding proteins were eluted with a linear gradient of NaCl in the range of 0–0.5 M (Fig. 2). It is noteworthy to note that two keratinases detected only by zymography analysis were eluted during the wash step of the CM-Sephadex column (Fig. 1, lane 5). Active fractions from 102 to 111 were pooled, concentrated by ultrafiltration, and then subjected successively to gel filtration on a Sephadex G-100 column and to a second CM-Sephadex. The purified keratinase was homogenous on SDS-PAGE, and its molecular weight was estimated to be 34,000 Da (Fig. 1, lane 2), corresponding with that determined by gel filtration. Purity of the enzyme was also evaluated using zymogram activity staining. As shown in Fig. 1 lane 3, a unique clear band of keratin hydrolysis was observed in the gel, indicating the homogeneity of the purified keratinase and its keratinolytic activity. At this stage, the other keratinases produced by *B. pumilus* A1 were probably unstable and they were lost during purification steps. The results of the purification procedure are summarized in Table 1. After the final purification step, KerA1 was purified 25-fold with a recovery of 35% and a specific activity of 6,320 U/mg protein using keratin as a substrate. The molecular mass of KerA1 was similar to those of the alkaline proteases of *B. pumilus* strains [4, 8, 24, 25]. The NH_2-terminal sequencing of the purified keratinase allowed the identification of 11 residues and was found to be: AQTVPYGIPQI which is 100% identical to that of the dehairing protease (DP) from *B. pumilus* strain UN-31-C-42 [21]. The NH_2-terminal sequence of KerA1 differs from that of subtilisin KerRP from *B. licheniformis* RPk [3] by only one amino acid. Residue Gln_{10} in KerA1 was replaced by Leu_{10} in KerRP enzyme.

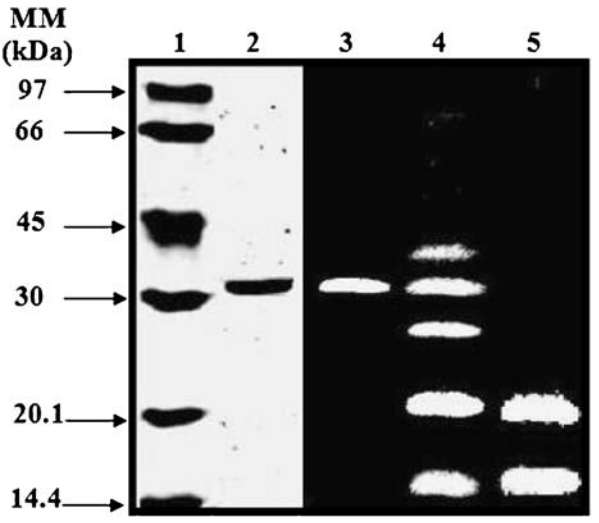

Fig. 1 SDS-PAGE and activity staining of the purified keratinase from *B. pumilus* A1. *Lane 1* molecular mass markers, *lane 2* purified keratinase, *lane 3* activity staining of the purified keratinase, *lane 4* activity staining of the crude supernatant of *B. pumilus* A1, *lane 5* activity staining of two keratinases eluted during the wash step of the CM-Sephadex column

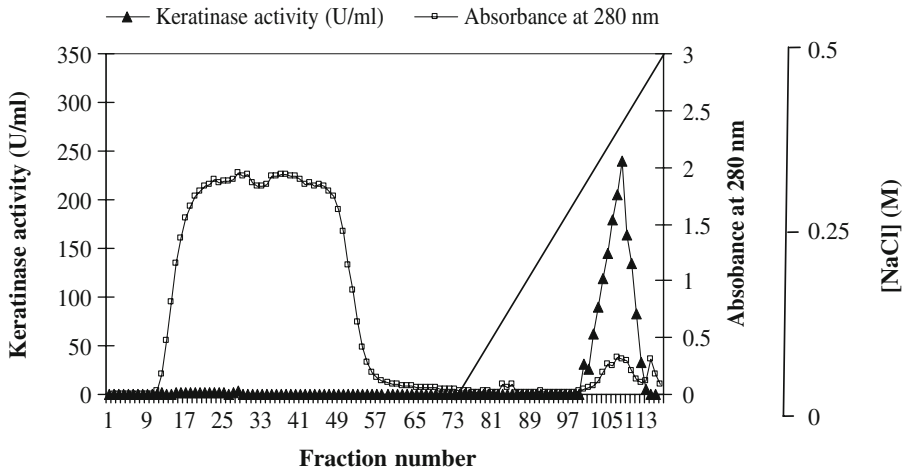

Fig. 2 Elution profile of *B. pumilus* A1 keratinase from a CM-Sephadex column. The crude extract was applied to a 3×45-cm column, equilibrated with buffer A (25 mM Tris–HCl, pH 8.0). The enzyme was eluted with a linear gradient of NaCl (0–0.5 M) in buffer A at a flow rate of 80 ml/h

Effect of pH on Activity and Stability of the Purified Enzyme

The optimal pH of protease activity was determined by varying the reaction pH at 60 °C. The purified enzyme has a maximum activity at pH 9.0 and pH 10.0 using keratin and casein, respectively, as substrates (Fig. 3a). These finding were also observed with keratinase of *B. licheniformis* PWD-1 that shows a maximum activity at pH 8.0 with casein and 7.5 with keratin as substrates [26]. Optimum pH of KerA1 is in accordance with several earlier reports showing pH optima between 10.0 and 11.5, using casein for proteases of *B. pumilus* strains [4, 8, 24, 25].

The pH stability of the enzyme was studied by assaying the residual activities of the enzyme after incubation for 1 h at 40 °C in buffers of various pH values. The caseinolytic and keratinolytic activities have the same pH stability profile. Interestingly, KerA1 was highly stable over a wide pH range, maintaining more than 85% of its initial activity between pH 6.0 and 12.0 (Fig. 3b). KerA1 was found to be more stable at high pH values than protease Q from *B. pumilus* which retained only 40% of its initial activity after 120 min incubation at 25 °C and pH 12.0 [24].

Table 1 Summary of the purification of KerA1 keratinase from *B. pumilus* A1.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	16,240	64.3	252.5	100	1
CM-Sephadex	13,521	6.48	2,086.5	83.2	8.26
Sephadex G-100	7,116.8	2.46	2,893	43.8	11.45
CM-Sephadex	5,688	0.9	6,320	35	25

All operations were carried out at 4 °C

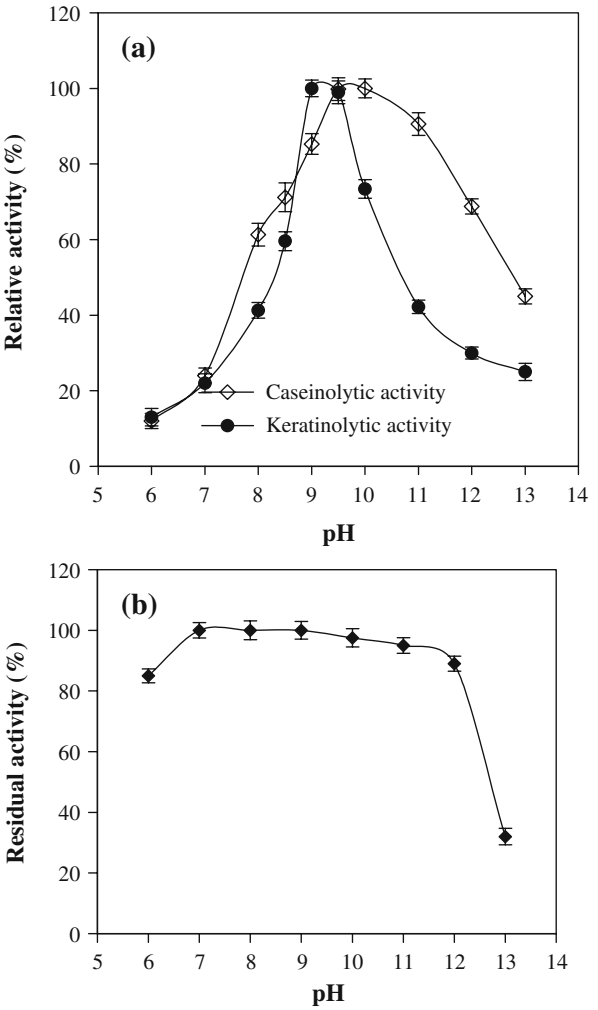

Fig. 3 Effect of pH on activity (**a**) and stability (**b**) of the purified keratinase KerA1. The protease activity was assayed in the pH range of 6.0–13.0 using buffers of different pH values at 60 °C. The maximum activity obtained at pH 9.0 and pH 10.0 using keratin and casein, respectively, as substrates was considered as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme in different buffers (pH 6.0–13.0) for 1 h at 40 °C, and the residual activity was measured at pH 9.0 and 60 °C using keratin as a substrate. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in "Materials and methods" section

Effect of Temperature on Activity and Stability of the Purified Enzyme

The influence of temperature on the protease activity was examined at pH 9.0 and pH 10.0, using keratin and casein, respectively, as substrates. The results showed that the enzyme was active between 50 °C and 70 °C with an optimum temperature around 60 °C with keratin and 65 °C with casein (Fig. 4a). In fact, this difference is most likely due to differences in substrate solubility and steric hindrance. The relative keratinolytic activities at 40 °C and 70 °C were about 45.7% and 55.7%, respectively, of that at 60 °C. Temperature optima at 60 °C were reported for proteases from *B. pumilus* strains [4, 24]. However, temperature optima of 50–55 °C were reported for subtilisin-like alkaline protease BPP-A [25] and serine protease DP [8] from *B. pumilus* strains.

The thermostability of the purified protease was examined by incubating the enzyme at different temperatures in the absence and then in the presence of 5 mM $CaCl_2$ (Fig. 4b). This was explained by previous studies which established the fact that divalent metal ions

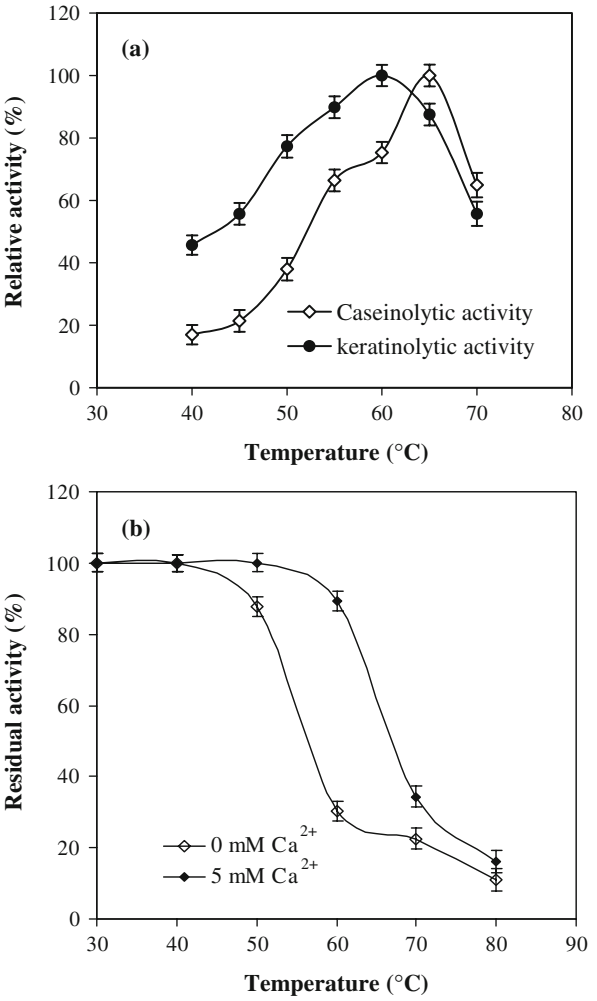

Fig. 4 Effect of temperature on activity (**a**) and stability (**b**) of the purified keratinase KerA1 protease. The temperature profile was determined by assaying protease activity at temperatures between 40 °C and 70 °C, using casein or keratin as substrates. The activity of the enzyme at 60 °C and 65 °C using keratin and casein, respectively, as substrates was taken as 100%. The temperature stability was determined by incubating the purified enzyme in the absence and then in the presence of $CaCl_2$ (5 mM) for 60 min at temperatures from 30 °C to 80 °C. The residual enzyme activity was measured under the standard conditions assay. The original activity before preincubation was taken as 100%

stabilize enzymes at high temperatures [27]. Indeed, the metal ions were found to act as salt or ion bridges to maintain the conformation of the enzyme. The caseinolytic and keratinolytic activities have the same thermostability profile. The enzyme was highly stable at temperatures below 40 °C. The enzyme retained 100% of its initial activity after 1 h incubation at 30 °C and 40 °C. At 50 °C and 60 °C, KerA1 was less stable and retained about 87.7% and 30.3% of its initial activity, respectively, in the absence of Ca^{2+}. As shown in Fig. 4b, the stability of the enzyme was enhanced in the presence of 5 mM $CaCl_2$, and the enzyme retained 100% and 89.3% of its initial activity after 1 h incubation at 50 °C and 60 °C, respectively. These results are in line with several works which reported that calcium ions play an important role in the stabilization of enzymes at higher temperatures [3, 28]. The improvement in protease thermostability in the presence of calcium may be explained by the strengthening of interactions inside protein molecules and by the binding of calcium to autolysis sites. Furthermore, many studies showed that subtilases (the subtilisin superfamily of protease) contain Ca^{2+} binding sites which play an important role in stabilizing the enzyme

Table 2 Effect of various metal ions on protease activity.

Metal ions	Relative activity (%)
Control	100
Ca^{2+}	123
Mg^{2+}	100
Mn^{2+}	51
Cu^{2+}	18
Ba^{2+}	100
Hg^{2+}	28
Na^{+}	135
K^{+}	100

The activity of the kerA1 was determined by incubating the enzyme in the presence of various metal ions (5 mM) for 60 min at 60 °C and pH 9.0, using keratin and casein as substrates

against thermal denaturation and autodegradation, and the removal of Ca^{2+} from these binding sites was associated with a significant reduction in thermal stability [29, 30].

Effects of Metal Ions on the Activity of the Purified Enzyme

The effects of various metal ions (5 mM), on the activity of the purified keratinase, was studied at pH 9.0 and 60 °C by the addition of the respective cations to the reaction mixture (Table 2). All the tested ions had the same effect on both caseinolytic and keratinolytic activities. The addition of Ca^{2+} and Na^{+} enhanced KerA1 activity by 123% and 135% of the control, respectively. However, Mn^{2+}, Hg^{2+}, and Cu^{2+} inhibited the KerA1 activity by 51%, 28%, and 18%, respectively. Mg^{2+}, Ba^{2+}, and K^{+} showed no influence on the enzyme activity.

Effects of Enzyme Inhibitors on the Activity of the Purified Enzyme

In order to determine the nature of the purified KerA1 enzyme, the effect of some enzyme inhibitors and reducing agents on enzyme activity was investigated (Table 3). Our results showed that thiol reagent (DTNB) had no effect on the enzyme activity. Nevertheless, the enzyme was completely inhibited by the serine-protease inhibitor (PMSF, 5 mM).

Table 3 Effects of inhibitors on protease activity.

Inhibitors	Concentration (mM)	Remaining caseinolytic activity (%)	Remaining keratinolytic activity (%)
Control	0	100	100
PMSF	2	35	27
	5	0	0
EDTA	2	62	57
	5	8	6
DTNB	5	100	100
β-Mercaptoethanol	2	100	129
	5	100	192

Enzyme activity measured in the absence of any inhibitor was taken as 100%. The remaining protease activity was measured after preincubation of enzyme with each inhibitor at 25 °C for 30 min

PMSF phenylmethylsulfonyl fluoride, *EDTA* ethylenediaminetetraacetic acid, *DTNB* dithio-bis-nitrobenzoic acid

Furthermore, the purified KerA1 was also highly inhibited by the metalloprotease inhibitor (EDTA, 5 mM) retaining only 6% of its initial activity. From these results, KerA1 can be considered as a serine metalloprotease since it was inhibited by both PMSF and EDTA. This is in line with previous finding dealing with a serine metallokeratinase from *Streptomyces* sp. [13]. However, most of the serine proteases from *B. pumilus* strains are slightly affected by metalloprotease inhibitors [4, 8, 24, 25].

β-Mercaptoethanol did not affect the caseinolytic activity since cysteine residues are absent in the purified keratinase. This was confirmed by KerA1 amino acid sequence (accession number ACM47735). The increased activity of purified keratinase in the presence of β-mercaptoethanol confirms the requirement of reducing condition for optimal keratinase activity. The maximum increase in keratinase activity (192%) was achieved in the presence of 5 mM β-mercaptoethanol. Several reports showed that purified keratinases are generally unable to degrade keratin in the absence of reducing agents which help in sulfitolysis by breaking disulfide bonds [10, 12]. Interestingly, KerA1 enzyme was able to degrade keratin in the absence of reducing agent. This is in line with previous reports dealing with keratinases from *B. licheniformis* PWD-1 [31] and *B. licheniformis* RPk [3].

Effects of Surfactants and Oxidizing Agent on Stability of the Purified Enzyme

In addition to activity and stability at high pH range and various temperatures, enzymes incorporated into detergents formulations must be compatible and stable with all commonly used detergent compounds like surfactants, bleaches, and other additives. As shown in Table 4, the strong anionic surfactant (SDS) at 0.2% and 1% caused a moderate inhibition of KerA1 activity 24.5% and 39.4%, respectively. The stability of KerA1 enzyme against SDS was higher than BM2 protease and alkaline serine protease from *B. mojavensis* A21, which retained about 49.32% and 50% of their initial activity after a 1-h incubation at 30 °C in the presence of 1% SDS [22, 32]. Furthermore, the purified keratinase was more stable than protease from *B. pumilus* MK6-5 strain which retained about 78% of its initial activity after 30 min incubation with 0.1% SDS at 30 °C [4]. In addition, the KerA1 was highly stable in the presence of nonionic surfactants such as Tween 20, Tween 80, and Triton X-100. However, the KerA1 enzyme was influenced by oxidizing agent, retaining 50.7% and 23.2% of its initial activity after a 1-h incubation at 30 °C in the presence of 0.2 and 1% sodium perborate, respectively. The stability of the enzyme in the presence of surfactants is a very important characteristic for its potential use in detergent formulations.

Table 4 Effect of surfactants and oxidizing agents on keratinase stability.

Reagents	Concentration (%)	Remaining keratinolytic activity (%)
Control	0	100
SDS	0.2 (*w*/*v*)	75.5
	1	60.6
Sodium perborate	0.2 (*w*/*v*)	50.7
	1	23.2
Tween 20	5 (*v*/*v*)	106
Tween 80	5 (*v*/*v*)	113
Triton X-100	5 (*v*/*v*)	84.5

Hydrolysis of Soluble and Insoluble Proteins

The protease activity using different protein substrates was investigated and the results are shown in Table 5. Hydrolysis of casein was considered as control for the estimation of relative activities on protein substrates. Using keratin as a substrate, KerA1 showed 45% relative activity. Comparatively, the enzyme exhibited 41.6% and 44% relative activities using keratin azure and BSA, respectively. The relative activity of the keratinase was 80% on azocasein, while a low hydrolysis level was observed with gelatin and egg albumin. Similarly, keratinase from *B. licheniformis* MKU3B was reported to exhibit highest activity toward casein [27].

Kinetic Studies

SAAPNA is the preferred substrate for several keratinolytic serine proteases [24, 33, 34]. The kinetic parameters, K_m and k_{cat}, of the purified *B. pumilus* keratinase were determined using Lineweaver–Burk plots. The K_m and k_{cat} of the purified enzyme, using SAAPNA, were 0.26 mM and $0.32\times10^5\,min^{-1}$, respectively. In comparison with keratinolytic subtilisin-like serine protease from *Microsporum canis* (K_m of 0.37 mM) [34], KerA1 has 1.4-fold affinity to the used substrate.

Nucleotide Sequence of the *kerA1* Gene of *B. pumilus* A1 and Deduced Amino Acid Sequence

Analysis of the nucleotide sequence of the *kerA1* gene and its flanking DNA regions (accession number FJ619651) revealed the presence of an open reading frame (ORF) of 1,149 bp, starting with an ATG codon at nucleotide position 1 and terminating with a TAA stop codon at the position 1,149 bp. Analysis of the ORF revealed a codon usage typical of *B. pumilus* with a G + C content in the *kerA1* gene of 43.87%, close to the value of 41.59% reported for *B. pumilus* genome [35]. The nucleotide sequence of the *kerA1* gene showed 99% and 90% homology with those encoding the organic solvent tolerant protease of *B. pumilus* 115b [36] and the dehairing protease of *B. pumilus* [21], respectively.

Table 5 Hydrolysis of soluble and insoluble proteins.

Substrate	Monitoring wavelength (nm)	Relative activity (%)
Casein	280	100
BSA	280	44
Fibrin	280	24.6
Albumin (egg)	280	2
Gelatin	280	10
Keratin	280	45
Azokeratin	595	41.6
Azocasein	410	80

Protease activity was assayed by mixing 0.5 ml of the diluted purified enzyme and 0.5 ml of buffer (glycine–NaOH 100 mM, pH 9.5) containing 1% (*w*/*v*) of each substrate. After 30 min of incubation at 60 °C, the reaction was stopped by adding 0.5 ml of TCA 20% (*w*/*v*) and allowed to stand at room temperature for 15 min. The increase in absorbance due to the hydrolysis and release of peptides was measured under the corresponding wavelength of the initial substrate. The relative protease activity towards casein was taken as a control (100%)

The amino acid sequence deduced from the nucleotide sequence contained 383 amino acids (accession number ACM47735) including a putative signal peptide of 29 amino acids that is comparable to other *Bacillus* signal sequences, prosequence of 79 amino acids, and the mature protease of 275 amino acids. The cleavage sites of the pre- and the propeptide were conserved among other *B. pumilus* proteases. The 11 residues of the N-terminal amino acid sequence of the purified enzyme perfectly matched the deduced amino acid sequence of mature protease, confirming that the isolated gene encoded the purified enzyme.

The mature enzyme has a predicted molecular mass of 27,800 Da as determined using ExPASy server. The predicted molecular mass is different from the value determined by SDS-PAGE, estimated to be 34,000 Da. This difference between predicted molecular mass and that estimated by SDS-PAGE was reported in previous works [8, 25].

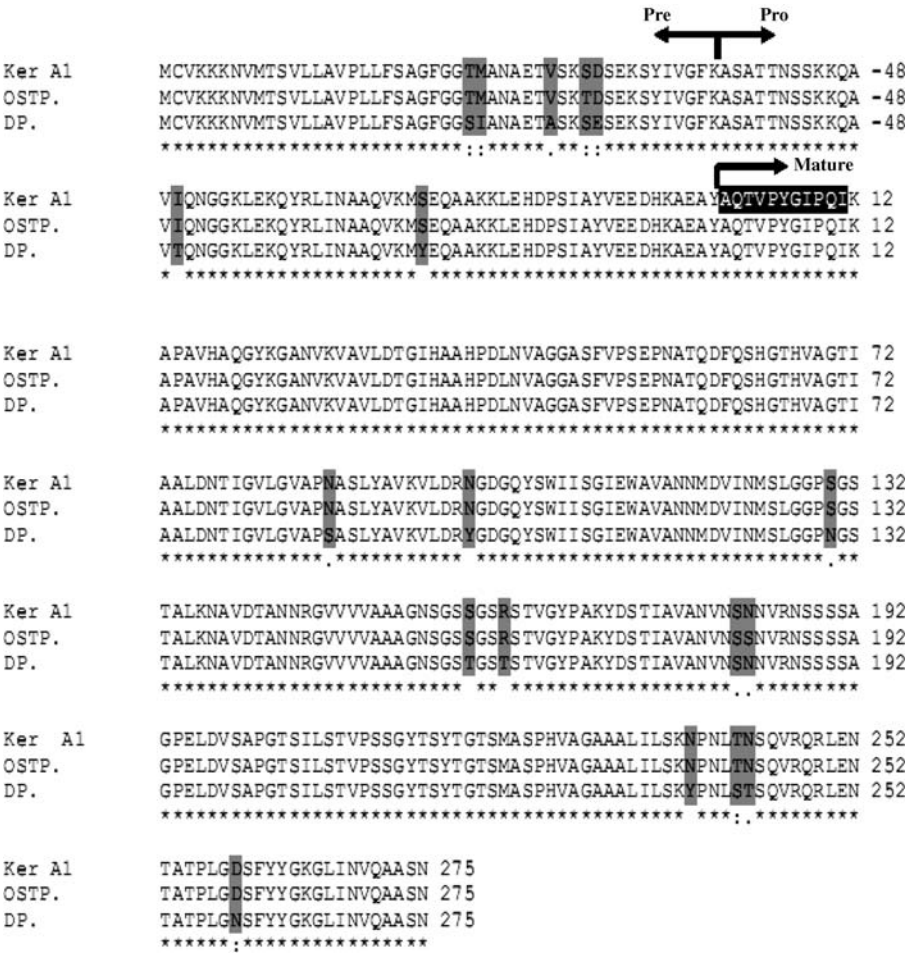

Fig. 5 Amino acid sequence alignment of KerA1 with organic solvent tolerant protease (*OSTP*) of *B. pumilus* 115b [36] and the dehairing protease (*DP*) of *B. pumilus* [21]. Identical amino acids are marked by an *asterisk*. *Gray-shaded letter* shows amino acid changes between the KerA1, OSTP, and DP. The first amino acid of the mature enzyme, Ala, is counted as +1. The *black box* indicates the NH_2 terminal amino acid sequence of the purified enzyme KerA1

Although KerA1 enzyme shows a high sequence homology with a number of other proteases, detailed sequence analysis revealed point mutations throughout the sequence (Fig. 5). KerA1 shares 99% homology with the sequence of organic solvent tolerant protease (OSTP) of *B. pumilus* 115b [36] and 96% homology with the DP of *B. pumilus* [21] (Fig. 5). KerA1 and OSTP differ only in one amino acid. Asn_{183} in KerA1 sequence was replaced by Ser_{183}. Nine amino acids (Asn_{87}, Asn_{99}, Ser_{130}, Ser_{159}, Arg_{162}, Asn_{238}, Thr_{242}, Asn_{243}, Asp_{259}) in the mature enzyme of KerA1 were replaced by Ser_{87}, Tyr_{99}, Asn_{130}, Thr_{159}, Thr_{162}, Tyr_{238}, Ser_{242}, Thr_{243}, and Asn_{259} in DP sequence. These amino acids modifications could explain the differences in their biochemical properties. In fact, OSTP enzyme retaining only 70% of its activity after incubation 30 min at 50 °C, while DP enzyme had a shorter half-life time of 30 min at 50 °C compared to KerA1 which maintained 87.7% of its activity after 1 h of incubation at 50 °C. In addition, KerA1 is distinguished by its optimum temperature around 65 °C using casein as a substrate. However, temperature optimum of serine protease DP from *B. pumilus* strain was only 55 °C [8].

Conclusion

In this study, we report the purification and characterization of a new keratinolytic enzyme named KerA1 from *B. pumilus* A1 strain. The enzyme was purified 25-fold with a specific activity of 6,320 U/mg and 35% recovery. The molecular mass of KerA1 was estimated to be 34,000 Da by SDS-PAGE. The purified enzyme was highly stable and active at high pH. The optimum pH and temperature for the keratinolytic activity were 9.0 and 60 °C, respectively. The activity of the enzyme was completely inhibited by PMSF and highly inhibited by EDTA, which suggests that the purified enzyme is a serine metalloprotease. The thermostability of the enzyme was considerably enhanced in the presence of Ca^{2+} ions at temperatures ≥50 °C. KerA1 was stable toward anionic and nonionic surfactants. The keratinase KerA1 differs from the organic solvent tolerant protease of *B. pumilus* 115b and the dehairing protease of *B. pumilus* UN-31-C-42 by one and nine amino acids, respectively. Considering the high keratinolytic activity and stability in high alkaline pH and in the presence of various detergent compounds, the *B. pumilus* A1 keratinase shows a potential use in biotechnological processes involving keratin hydrolysis, leather industry, and in laundry detergent.

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