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Purification and biochemical characterization of two novel extracellular keratinases with feather-degradation and hide-dehairing potential

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ABSTRACT

Two novel extracellular keratinases were produced by *Actinomadura keratinilytica* strain Cpt20. Both enzymes were purified to homogeneity using heat-treatment (60 °C for 30 min) and ammonium sulfate salt fractionation (40 %–70 %), followed by anion-exchange chromatography with fast protein liquid chromatography (FPLC) system. The purified keratinases, designated as KERA-71 and KERB-19, are monomeric and named according to their molecular masses of 71 kDa and 19 kDa, respectively, as estimated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), zymography, and high-performance liquid chromatography (HPLC). N-terminal residues of both enzymes exhibited high identity with other *Actinomadura* keratinases. Their hydrolytic activities were significantly inhibited by phenylmethylsulfonyl fluoride (PMSF) and di-iodopropyl fluorophosphates (DFP), classifying them in the serine proteases family. While KERA-71 was ideally active at 50 °C and pH 8, KERB-19 illustrated optimum activity at 40 °C and pH 7. The thermo-activity and thermo-stability of both enzymes were improved with 10 mM Ca^{2+}. Interestingly, the KERA-71 displayed broader substrate specificity, higher catalytic efficiency (k_{cat}/K_m), and a high degree of hydrolysis (DH) than actinobacterial keratinases, including KERB-19, KERDZ, KERAK-29, Actinase E, and KERAB. Interestingly, both enzymes exhibited effective keratinase activities with high potential, illustrating their possibility to be used in the process of keratin-containing wastes valorization and leather industry.

1. Introduction

The agro-industrial fields engender huge quantities of by-products of diverse nature during processing, which may also be channeled into numerous bioeconomy sectors with the crucial objectives of environmental safety, waste reduction, and sustainable production [1,2]. Strikingly, a few agro-wastes are recalcitrant to degradation due to their structural convolution, particularly those emanating from poultry conversion farms and the leather processing industry [3]. Compared to other keratinous bio-wastes, avian feathers are exclusively omnipresent, with millions of tons produced annually due to the increased demand for poultry meat [4]. Keratinous materials, e.g., feathers, wool, and hair, are insoluble fibrous sulfur-rich proteins and are the major element of skin, hair, teeth, hooves, nails, scales, horns, and claws. They are produced by keratinocytes and resistant to proteolytic degradation due to their extensive cross-linking of S—S and H-bonds, hydrophobic interactions, and rigid wadding of the protein chains either into α-helix (called α-keratins) or β-sheet (called β-keratins) structures [5]. Predominantly, feathers represent over 90 % protein, the major component being α-keratin, a fibrous and insoluble structural protein extensively cross-linked by disulfide, hydrogen, and hydrophobic bonds. For that reason, the valorization of non-food renewable biomasses into high-value products is crucial from the environmental and economic perceptions. Given the waste valorization concept, the microbial-directed keratinolytic process has been envisaged as an effective,

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sustainable, and profitable advance for feather waste valorization [6]. Owing to their insoluble nature, feathers are resistant to degradation by common microbial proteases. In addition, the dehairing process, which includes a complex set of operations, such as soaking, unhairing, bating, and tanning, has undergone a dramatic increase. It is one of the extremely polluting procedures. The usual lime-sulfide method used for dehairing produces a huge amount of sulfide, which poses serious toxicity and ecological problems [3].

Keratinolytic enzymes, so-called keratinases (EC 3.4.21/24/99), are highly active on keratin, making them particularly suitable for industrial and biotechnological applications [7–11]. This is primarily due to their maximum stability in harsh conditions besides their remarkable ability to degrade various keratinous substrates. In recent years, keratinases have gained attention in bioremediation due to their vital role in keratin resource management and keratin waste degradation [12]. Currently, keratinases belong to at least 14 different protease families in the MEROPS database (http://www.ebi.ac.uk/merops/) and are the unique group of proteases with a wide temperature and pH range that allow the whole degradation of recalcitrant and complex proteins [11,13]. The exclusive characteristic that distinguishes keratinases from other proteases is the ability to bind to the complex and insoluble substrates (feathers, wool, silk, collagen, elastin, horns, stratum corneum, hair, keratin azure, and nails) [11,13]. Various studies have been performed recently to biochemically classify keratinases produced by diverse microorganisms such as fungi, i.e., *Alternaria* [15] and *Microsporum* [16], and bacteria, such as *Proteus vulgaris* strain EMB-14 [14], *Bacillus* [17,18], *Pseudomonas* [19], and *Streptomyces* [20,21]. In comparison, rare works conducted on keratinases from the *Actinomadura* genus are far from being fully explored [22–24]. Higher performance keratinases are preferred for biotechnological applications, namely feather and leather processing industry, detergent, wastewater treatment, and others [10,11,25]. The constant changes in process parameters for keratin-containing waste degradation make it difficult to apply enzymes from actinobacteria in scale-up processes because of their low activity and stability under these changing conditions. The conventional methods of leather treatment encompass different steps (soaking, dehairing, bating, and tanning), which implicate the consumption of various harmful substances, particularly sodium sulfide (Na_2S), chromium (Cr), and sulfate (SO_4^{2-}), which generate harmful waste [22,26]. Hence, a safer and more environmentally-friendly approach to the classical chemicals used is imperative to minimize the detrimental effects.

In a previous study, the authors described the purification and physico-chemical characterization of highly thermostable xylanase [27], thermoalkaliphilic pectate lyase [28], and the statistical optimization of keratinase production [29] from *A. keratinilytica* strain Cpt20 isolated from Algerian poultry compost. The results revealed that inexpensive medium compositions considered as waste could be used to increase the enzyme production using chicken-feathers waste, a very copious waste as the source of carbon, nitrogen, and energy for the production of keratinases [29]. Furthermore, the study also showed the high biotechnological ability and potential of *A. keratinilytica* strain Cpt20 for the biodegradation and solubilization of the keratin waste from chicken-feathers [29]. The current work was conducted to explore the purification and biochemical characterization of the native molecular masses of 71 kDa and 19 kDa keratinases designated as KERA-71 and KERB-19, respectively produced by *A. keratinilytica* strain Cpt20. The KERA-71 and KERB-19 keratinases were comparatively studied against other actinobacterial keratinolytic proteases (KERDZ, KERA-29, and KERAB) and the commercial actinobacterial keratinase (Actinase E). This study provides information regarding the potential role of KERA-71 and KERB-19 as candidates for application in the feather and leather industry.

2. Materials and methods

2.1. Materials

Except otherwise indicated, all compounds were of analytical grade purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Feathers from chicken and duck were gathered from slaughterhouses, repeatedly washed with water and sterilized by alkaline (0.1 N NaOH)-autoclave pre-treatment at 121 °C for 20 min [30–32]. The bright-haired goat, sheep, and bovine skins were acquired from Annaba municipal slaughterhouse (Algeria). The actinobacterial enzymes used for comparison were KERDZ keratinase produced by *Actinomadura viridilutea* strain DZ50 [24]; KERAK-29, a detergent stable keratinase from *A. keratinilytica* strain Cpt29 [22]; Actinase E, a commercial protease of *Streptomyces griseus* (Sigma, MO, USA); and KERAB, keratinase of *Streptomyces* sp. strain AB1 [21]. The Hammarsten casein, UNO Q-12 column (15 mm × 68 mm), and ProBlott polyvinylidene fluoride (PVDF) membrane were brought from Merck (Darmstadt, Germany), Bio-Rad Laboratories Inc. (Hercules, CA, USA), and Applied Biosystems (Foster City, CA, USA), respectively.

2.2. Keratinase-producing strain and culture conditions

The present study is carried out using a previously isolated and identified thermophilic actinobacterial strain Cpt20 [27] (GenBank accession no.: GQ205433). It was tested for keratinolytic and proteolytic activity under optimal medium conditions [29]. The feather-based-medium (FBM) was composed of (in g/L): chicken-feather-meal (CFM, 5), NaCl (0.5), $MgSO_4{\cdot}7H_2O$ (0.5), $CaCO_3$ (3), K_2HPO_4 (1), and agar (20) at pH 7.

2.3. Keratinase production

The microorganism was cultivated in 0.5 L baffled flasks containing 100 mL of whole-feather medium (g/L): whole chicken-feathers (20), NaCl (0.5), $MgSO_4{\cdot}7H_2O$ (0.5), $CaCO_3$ (3), K_2HPO_4 (1), $FeSO_4$ (0.1), and trace salt solution (0.1 %, *v/v*), which was composed of (in g/L): $ZnCl_2$ (0.4), $FeSO_4{\cdot}7H_2O$ (2), H_3BO_3 (0.065), and $MoNa_2O_4{\cdot}2H_2O$ (0.135) at pH 7 [29,33]. The media was then autoclaved for 20 min. After fermentation, the biomasse was harvested by centrifugation for 35 min at 10,000 × *g*, and the supernatant was analyzed for keratinase and protease activities.

2.4. Keratinase activity

The keratinase activity was ascertained with keratin azure [8], whereby 1 mL of keratin azure (10 g/L) was suspended in 50 mM Tris−HCl buffer at pH 8 (buffer A, for KERA-71) and pH 7 (buffer B, for KERB-19). The substrate was used for keratinase activity by the following method: a mixture of 1 mL of keratin azure solution (10 g/L) combined with 1 mL of keratinase solution was heat-treated at 50 °C (for KERA-71) and 40 °C (for KERB-19) for 30 min with 200 rpm shaking and then kept for 10 min at 0 °C. One keratinase activity unit corresponds to the quantity of enzyme that causes a 0.01 increase in absorbance ($A_{440\ nm}$) under the standard assay conditions in 1 min. A similar activity assay was applied to measure enzymatic activities with azo-casein and azo-albumin.

2.5. Protease activity

The protease activity was calculated at $A_{660\ nm}$ with casein as a substrate using the Folin-Ciocalteu method [34]. One protease activity unit corresponds to the quantity of the enzyme that produced 1 μg of tyrosine in 1 min at 50 °C and pH 8 (KERA-71) and 40 °C and pH 7 (KERB-19). A same test, at the same concentrations (20 g/L) and under the same conditions (at 40 °C and pH 7), was applied to ascertain

protease activity using substrates such as gelatin, elastin, fibrin, albumin, and hemoglobin.

2.6. Disulfide bond-reducing activity

The disulfide bond-reducing activity was measured at $A_{412\ nm}$ by assessing the yellow-colored sulfide solution resulting from the decrease of 5,5'-dithio-bis-2-nitro benzoic acid (DTNB), as reported by Jaouadi et al. [35]. One disulfide bond-reducing activity unit is defined as the quantity of enzyme that produces 1 mM of sulfide per minute.

2.7. Protein quantification

Protein concentration was ascertained by the method of Bradford [36] using the Dc protein assay kit from Bio-Rad Laboratories (Inc., Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

2.8. Purification of keratinases KERA-71 and KERB-19

The cell-free culture supernatant was subjected to heat treatment at 60 °C for 30 min, followed by precipitation between 40 % and 70 % of ammonium sulfate saturation in an ice-cooled bath. It was then centrifuged for 35 min at 10,000 × *g*. The obtained precipitate was suspended in the smallest volume of piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) buffer at 50 mM and pH 6.1 (buffer C) and dialyzed 12 h at 4 °C alongside repeated buffer C changes. The resulting insoluble materials were then separated from the enzyme solution by centrifugation (10,000 × *g* for 15 min). The obtained supernatant was loaded into a UNO Q-12 column pre-equilibrated with buffer C using the FPLC system. Besides the un-adsorbed protein, the adsorbed material was eluted with a linear gradient of NaCl (0–500 mM) at a rate of 1 mL/min in buffer C. After washing with buffer C, the fractions of every peak (from adsorbed [KERA-71] and un-adsorbed [KERB-19] proteins) were collected for protein measurement at $A_{280\ nm}$ and keratinase activity at $A_{440\ nm}$. The protein purity was determined by SDS-PAGE and gel-filtration chromatography OH-40 using HPLC system pre-equilibrated with buffer C and protein markers of 670–13.5 kDa. Isocratic elution at a flow rate of 0.5 mL/min with buffer C was applied to separate the proteins, detected at $A_{280\ nm}$. The purified KERA-71 and KERB-19 keratinolytic activities were eluted at retention times (Rt) of 7.712 min and 4.300 min. The pooled fractions containing keratinase activity were concentrated in centrifugal micro-concentrators (Amicon Inc., Beverly, MA, USA) with 10 kDa cut-off membranes and were stored at -20 °C in a 20 % glycerol (*v*/*v*) solution and then used for the determination of the biochemical properties.

2.9. Electrophoresis and zymography

Analytical 10 % SDS-PAGE was performed following the method of Laemmli [37]. Protein bands were visualized with Bio-Safe™ Coomassie Brilliant Blue G-250 staining (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The discontinuous substrate native-PAGE (zymogram analysis) for the crude extract and purified keratinases were performed with a 4 % stacking gel, except that 200 µL of 10 g/L keratin azure as a substrate was incorporated into the 10 % separating gel and resolved using the Mini-PROTEAN® system from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Electrophoresis was performed at a constant current of 25 mA under non-reducing conditions. The gels were then gently washed and incubated at 40 °C for 2 h in 50 mM Tris−HCl buffer at pH 7.5 supplemented with 10 mM $CaCl_2$, producing a keratin azure cleared zone at the location of the keratinase band of each enzyme. A clear zone was visualized by fixing the gel with ice-cold trichloroacetic acid 20 % (*w*/*v*) for 1 h, staining it with 0.1 % Bio-Safe™ Coomassie Brilliant Blue G-250 in water/methanol/acetic acid 60:30:10, and destaining it in the same solution without dye [24].

2.10. N-terminal sequencing of KERA-71 and KERB-19

The bands representing the purified KERA-71 and KERB-19 enzymes were separated from the SDS-PAGE gels, and electro-transferred to the ProBlott membrane and their N-terminal sequence analyses were performed using the automated Edman's degradation through the Applied Biosystem sequencer (Model 473A).

2.11. Effects of pH and temperature on KERA-71 and KERB-19 activities and stabilities

The effect of pH on the activities of KERA-71 and KERB-19 was tested in the pH range of 2–13. The pH stability of both enzymes was determined by pre-incubation of each enzyme for 36 h at 37 °C in pH ranging from 4 to 9 (for KERB-19) and 6–11 (for KERA-71) using the suitable buffers at 100 mM concentration (pH 2–4 glycine−HCl, pH 4–6 sodium acetate, pH 7–8 Tris−HCl, and pH 9–11 glycine-NaOH). The effect of temperature on KERA-71 and KERB-19 was tested between 20 °C and 80 °C. Thermal stabilities were tested by incubating both keratinases separately for 36 h at 30 °C, 40 °C, and 50 °C (for KERB-19) and 40 °C, 50 °C, and 60 °C (for KERA-71) with and without 10 mM $CaCl_2$. Samples were collected, and the residual activity was tested at different time intervals to determine the residual activity under the standard assay conditions.

2.12. Effect of selected chemical compounds on keratinase stability

The influence of different specific keratinase inhibitors, at various concentrations, such as: soybean trypsin inhibitor (SBTI, 5 mg/mL), *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK, 1 mM), *N*α-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK, 1 mM), 2-mercaptoethanol (2-ME, 5 mM), DL-dithiothreitol (DL-DTT, 2 mM), *N*-ethylmalemide (NEM, 2 mM), iodoacetamide (5 mM), DTNB (10 mM), leupeptin (50 µg/mL), pepstatin A (10 µg/mL), EDTA (10 mM), EGTA (2 mM), PMSF (5 mM), and DFP (5 mM), and various metal ions at concentration of 10 mM on KERA-71 and KERB-19 activities were examined after incubation of each enzyme for 1 h in the presence of each chemical compound at room temperature (23° ± 2 °C). The keratinase activity was measured under the standard conditions.

2.13. Substrate specificity

The KERA-71 and KERB-19 substrate specificities were determined using the complex natural (keratin, gelatin, casein, elastin, albumin, fibrin, and hemoglobin) and synthetic (keratin azure, azo-casein, and azo-albumin) proteins. Their activity on these protein substrates was determined by the Folin–Ciocalteu method [34] and also using synthetic peptides (*N*-succinyl-L-Tyr-L-Leu-L-Val-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Val-L-Ala-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Val-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Phe-*p*-nitroanilide, and *N*-benzoyl-L-tyrosine *p*-nitroanilide [BAPNA]). For the determination of the activity on synthetic peptides, the quantity of released *p*-nitroanilide (*p*-NA) was measured at $A_{410\ nm}$ [38]. One activity unit on synthetic peptides corresponds to the quantity of enzyme liberating 1 µmole of *p*-NA under experimental conditions. The mixture-reaction was executed for 15 min in assay buffer.

2.14. Kinetic measurements

Kinetic parameters of KERA-71, KERB-19, KERDZ, KERAK-29, Actinase E, and KERAB were calculated using the Lineweaver-Burk plots from the initial rate activities with keratin and *N*-Suc-Tyr-Leu-Val-pNA with increasing concentrations (0.1–10 mM). The initial velocities were measured on the linear section of the kinetics plots and the apparent K_m and V_{max} were calculated from an hyperbolic regression analysis using

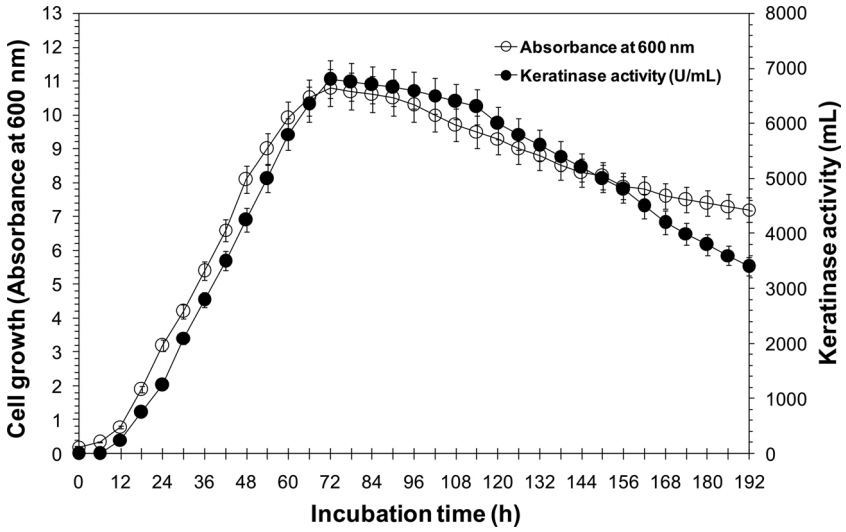

Fig. 1. Kinetic activity of keratinase from strain Cpt20. The time course of *A. keratinilytica* strain Cpt20 cell development (○) and keratinases activity (●). Cell growth was examined by calculating the absorbance at $A_{600\ nm}$.

the software Hyper32 version 1.0.0 provided by the University of Liverpool (http://hompage.ntlword.com/john. easterby/hyper32.html). The value of the turnover number (k_{cat}) was calculated by the following equation:

$$k_{cat} = \frac{V_{max}}{[E]} \quad (1)$$

Where [E] refers to the active enzyme concentration and V_{max} to the maximal velocity.

2.15. DH determination

The DH of keratin was assessed under the following conditions: pH 8 at 50 °C (for KERA-71), pH 7 at 40 °C (for KERB-19), pH11 at 80 °C (for KERDZ), pH 10 at 70 °C (for KERAK-29), pH 11 at 60 °C (for Actinase E), and pH 11.5 at 75 °C (for KERAB). Approximately 10 g of CFM and duck-feather-meal (DFM) were suspended in 100 mL of each assay buffer and incubated with 1,000 U/mL of each purified keratinase. The quantity of NaOH required to keep the pH at a constant value was relative to the DH. The reaction mixtures were stopped when the DH became constant. The DH was measured for every assay from the quantity of the NaOH provided to maintain the pH to a constant value throughout hydrolysis [39, 40] as follows:

$$DH(\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{a} \times \frac{1}{h_{tot}} \times 100 \quad (2)$$

Where *B, Nb*, *MP*, and α refer respectively to the quantity of the base required (mL) to maintain the pH constant through the enzymatic reaction, normality of the base, mass (g) of protein (N × 6.25), and average dissociation degree of the α-NH_2 groups released in hydrolysis defined as:

$$a = \frac{10^{pH-pK}}{1 + 10^{pH-pK}} \quad (3)$$

Where, *pH* and *pK* refer to the values at which the enzyme was achieved. Total peptide bond number (h_{tot}) in the CFM or DFM proteins was determined as 8.1 meq/gas [39,40].

2.16. Keratin-biodegradation by KERA-71 and KERB-19

The keratin-degrading capability of KERA-71 and KERB-19 was determined in the presence of various keratin sources, CFM, chicken-feathers, DFM, and duck-feathers. Approximately 10 g/L of substrates were added to the medium for KERA-71 at pH 8 at 50 °C and pH 7 at 40 °C (for KERB-19) and incubated on the shaker for 12 h. The keratin hydrolysis was evaluated after the filtration (0.45 μm) and the remaining substrate's heat-dry process. Ellman's reactive was applied for the calculation of the sulfhydryl groups released at $A_{412\ nm}$. Free amino-acids with cysteine, methionine, and cystine were identified using the UHPLC C18 column, with isocratic mode (0.5 mL/min) and 70/30 (*v/v*) acetonitrile/sodium acetate (10 mM at pH 4.9).

2.17. Dehairing activity of KERA-71 and KERB-19

The dehairing efficacy of KERA-71 and KERB-19 was conducted using bright-haired goat, sheep, and bovine skins, which have been cut and carefully washed to eliminate waste and blood. The portion of cut skin (10 cm × 10 cm size) was added to 100 mL of KERA-71 or KERB-19 (1,000 U/mL each) assay buffers [8]. After incubation (30 °C for 12 h), the skins were extracted, and the hair was hand-dragged to determine whether it can be gently pulled. The dehairing efficiency was evaluated in the proportion of the skin's depilated area at the end of the procedure. The quality and efficacy of the dehaired skin were assessed by the naked eye after the 12h treatment. The dehaired skin is assessed by the elevated amount and efficacy of clear grain structure and spotless hair pore without collagen damage.

2.18. Statistical analyses

All assays were replicated at least three times, and the control test devoid of the enzyme was performed according to the similar assays. The data obtained correspond to the mean of the replicate assessment and standard deviation (mean ± SD). The statistical meaning was reviewed with t-tests for 2-sample evaluation. The statistically significant results were presented by P values < 0.05.

3. Results and discussion

3.1. Keratinase production by strain Cpt20

The previously isolated Cpt20 strain [27] tested for keratinolytic activity under optimal conditions showed a large clear zone on FBM agar meduim after one week of incubation at 30 °C. For the sterilization, the

Table 1
Production and purification procedures of the two keratinases KERA-71 and KERB-19 from *A. keratinilytica* strain Cpt20.

Purification step[a]		Total protein (mg) [a,b]	Total activity (units)[a] × 10^3	Specific activity (U/mg of protein)[a]	Purification factor (*n*-fold)	Activity yield (%)
Crude extract		1645 ± 39	3,295 ± 66	2,067 ± 88	1.0	100
Heat-treatment (60 °C for 30 min)		298 ± 11	3,024 ± 43	10,120 ± 123	4.9	91.77
$(NH_4)_2SO_4$ fractionation (40 %−70 %)-dialysis		50 ± 5	1,507 ± 23	30,147 ± 265	14.5	45.74
Anion-exchange chromatography (UNO Q-12 FPLC)	KERA-71	9.1 ± 0.7	880 ± 21	96,700 ± 398	46.8	26.70
	KERB-19	4.9 ± 0.1	209 ± 5	42,653 ± 175	20.6	6.34

[a] The assays were determined in triplicate and the ± SE is indicated.
[b] The protein quantity was estimated using Bio-Rad assay reagent kit.

feather culture medium was prepared by alkaline-autoclave pre-treatment to improve the solubility and digestibility of raw feathers to enhance the enzymatic hydrolysis of feathers to produce a high protein content hydrolysate [30–32]. The keratinase production by *A. keratinilytica* strain Cpt20 was performed in an optimum FBM medium, and it was growth-dependent, attaining the optimal keratinase activity of 6,800 U/mL after 72 h of incubation at 30 °C and pH 7 (Fig. 1). The FBM medium was optimized using statistical experimental design [29]. The optimization of culture conditions and the composition of the culture medium led to the enhancement of keratinase activity and decreased enzyme production cost since chicken-feather is considered a inexpensive and readily available raw substrate [29].

The crude keratinolytic extract was subjected to a series of purification steps to assess the particular characteristics of the studied enzymes, every purification step allowed a significant improvement of purity level, and two extracellular keratinases were purified to homogeneity and biochemically characterized.

3.2. Purification procedures of KERA-71 and KERB-19

The purification procedures of KERA-71 and KERB-19 are listed in Table 1. A concentrated crude enzyme was obtained after treatment for 30 min at 60 °C and selective precipitation of proteins with ammonium sulfate at 40 %–70 %. The resulting protein pellet was dialyzed overnight and then used as initial material for the next purification step. After filtration using a 0.45-μm membrane filter, the dialyzed enzyme solution was recovered by centrifugation, and the retained clear-supernatant was loaded on a UNO Q-12 column using the FPLC system previously equilibrated with buffer C (Fig. 2). In this condition, KERA-71, the major keratinase activity, was adsorbed onto the cationic support and eluted at 170–250 mM NaCl. Meanwhile, KERB-19, the minor keratinase activity, was not adsorbed and eluted during the washing phase with the same buffer C (Fig. 2A). These two different peaks (KERA-71 and KERB-19) demonstrated that the *A. keratinilytica* strain Cpt20 could produce two keratinolytic enzymes (Fig. 2B) with different isoelectric points (pI) of 5.20 for KERA-71 and 9.2 for KERB-19 (data not shown). The overall purification factors achieved are 46.8-fold (for KERA-71) and 20.6-fold (for KERB-19), and the yield is 26.70 % (for KERA-71) and 6.34 % (for KERA-71). When using keratin as substrate, the purified enzyme, KERA-71, showed one of the highest reported specific activities with 96,700 U/mg, while KERB-19 only exhibited a specific activity of 42,653 U/mg.

3.3. Molecular masses of KERA-71 and KERB-19

The purity of KERA-71 and KERB-19 was confirmed by SDS-PAGE and zymogram activity assay (native-PAGE), which represent a sensitive and rapid assay method for the analysis of keratinase activity. A unique band was obtained for each of the active pooled fractions corresponding to the purified keratinases. The purified KERA-71 and KERB-19 have an estimated molecular masses of ~71 kDa (Fig. 2D) and ~19 kDa (Fig. 2G), respectively, which can be compared to the earlier described keratinases from actinobacteria [21,22,24,34,41]. The revelation of the zymography activity disclosed a single band of keratinolytic activity for the KERA-71 (Fig. 2E) and the KERB-19 (Fig. H). The purity of both keratinases KERA-71 and KERB-19 was also confirmed by gel filtration chromatography OH-40 using HPLC, where single peaks having Rt values of 7.71 min for KERA-71 (Fig. 2C) and 4.31 min for KERB-19 (Fig. 2F) were observed.

These observations suggested that both KERA-71 and KERB-19 from *A. keratinilytica* strain Cpt20 are monomeric, similar to those previously reported for actinobacterial keratinases [21,22,24,34,41]. Although most reported keratinases have a monomeric structure, multimer organization of keratinases has also been cited [42,43]. Keratinolytic enzymes produced by actinobacteria have low to medium molecular masses, with the molecular weight of ~13 kDa and ~15 kDa [45] for keratinases secreted by *Streptomyces* sp. and *Streptomyces albidoflavus* strains [44]. High molecular mass keratinolytic enzymes are frequently linked to keratinases with metallic ions or those of a thermophilic nature [46,47].

3.4. N-terminal amino-acid sequences of KERA-71 and KERB-19

The first 29 and 22 N-terminal amino-acids of KERA-71 and KERB-19 determined (Table 2) demonstrated consistency, signifying their pure forms. The comparisons with accessible amino-acid sequences in the GenBank-NR protein database resulted in homology with other keratinases isolated from actinobacterial strains, with up to 64.00 % and 90.91 % identities to the S8 family serine peptidases from *Actinomadura rubrobrunea* strain NRBC 15,275 and *A. viridilutea* strain DZ50 (Table 2). These results strongly suggest that the KERA-71 and KERB-19 are new keratinases.

3.5. Effect of selected chemical compounds on KERA-71 and KERB-19 enzymes

The addition of 10 mM $CaCl_2$ enhanced KERA-71 activity to 162 %, 206 %, and 175 % at 45 °C, 50 °C, and 55 °C, respectively compared to the activities without $CaCl_2$. Similar behavior is observed for keratinase KERB-19 (Table 3). These observations suggest that the metallic ion is implicated in stabilizing each keratinase (A or B) alongside thermal denaturation and the conservation of their conformation [48]. Another obvious enhancement of the keratinase activity of KERA-71 up to 126 % and 108 % is shown with Mg^{2+} and Mn^{2+} at 50 °C, respectively. While, KERB-19 is enhanced up to 120 % and 117 % with the addition of Zn^{2+} and Fe^{2+} at 40 °C, respectively (Table 3). The addition of Mn^{2+}, Ca^{2+}, and Mg^{2+} earlier reported to increase the activities of *Streptomyces* sp. [20,49] and *Actinomadura* [22] keratinases. The Ni^{2+}, Hg^{2+}, and Cd^{2+} are demonstrated to have strong inhibition on each keratinase activity, whereas Cu^{2+} and NH^{4+} slightly reduce the enzyme activity (Table 3).

Enzyme activities were observed to be completely abolished by

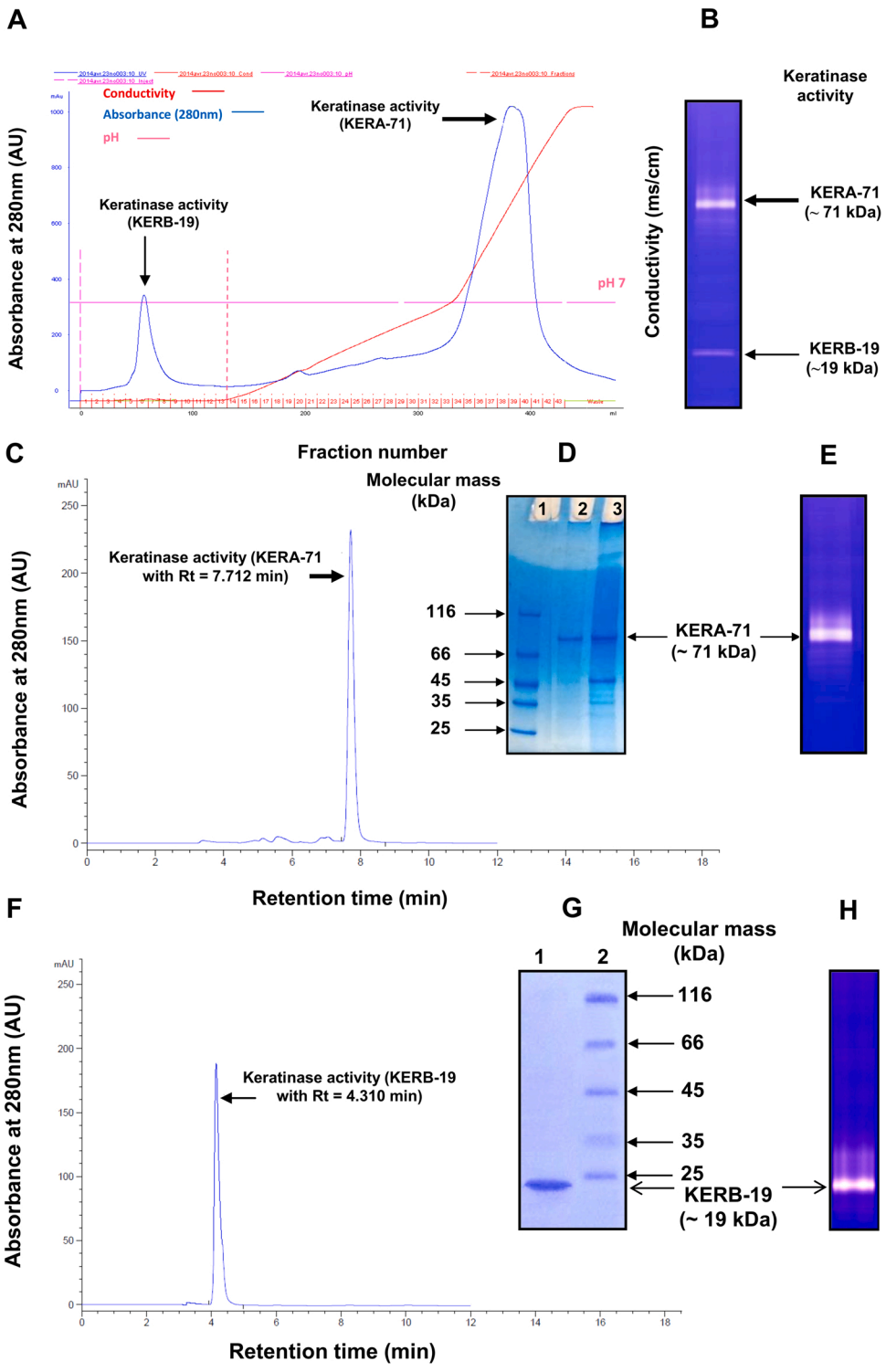

Fig. 2. Purification and identification of KERA-71 from strain Cpt20. (A) Anion-exchange chromatography profile of the purified adsorbed keratinase KERA-71 on UNO-Q12 Sepharose column using FPLC system. **(B)** Zymography with keratin azure staining of two keratinase activities (adsorbed keratinase: KERA-71 and unadsorbed keratinase: KERB-19) on UNO-Q12 Sepharose column using FPLC system (30 µg). **(C)** Size exclusion chromatography profile of the purified KERA-71 (with Rt =7.712 min) with OH-40 column using HPLC system. **(D)** 10 % SDS-PAGE of KERA-71. Lane 1, Amersham low molecular weight protein marker. Lane 2, purified KERA-71 (30 µg) obtained after anion-exchange chromatography FPLC UNO-Q12 Sepharose (fractions 170-250 mM NaCl). Lane 3, sample after ammonium sulfate (40%-70 %)-dialysis. **(E)** Zymography with keratin azure staining of high molecular mass keratinase activity: KERA-71. Lane 1, purified KERA-71 (50 µg). **(F)** Size exclusion chromatography profile of the purified KERB-19 (with Rt =4.310 min) with OH-40 column using HPLC system. **(G)** 10 % SDS-PAGE of KERB-19. Lane 1, purified KERB-19 (30 µg) unadsorbed keratinase on FPLC UNO-Q12 Sepharose column. Lane 2, LMW. **(H)** Zymography with keratin azure staining of low molecular mass keratinase activity: KERB-19. Lane 1, purified KERB-19 (50 µg). Three independent experiments were performed, each with two technical replicates.

PMSF and DFP, signifying that these enzymes belong to the serine keratinase family. Other inhibitors did not show any inhibitory effects (Table 3). The thiol (2-ME, DL-DTT, NEM, DTNB, leupeptin, and iodoacetamide) and acid reagents (pepstatin A) did not exhibit any effect on keratinase activities. Each keratinase retained 95 % (for KERA-71) and 101 % (for KERB-19) of their activities with the addition of EDTA (10 mM) indicating that no metallic cofactors are necessary.

3.6. *Influence of different pH and temperature on the keratinase activity*

The keratinase activities of the KERA-71 and KERB-19 enzymes were determined from the data of a wide range of pH values, with an optimum pH at 8 and 7, respectively (Fig. 3A). *A. keratinilytica* strain Cpt20 produced two extracellular keratinases that can be effective in alkalophilic and neutral environments; a good advantage as the two keratinases can be used in different industrial processes. In the presence of 10 mM $CaCl_2$, KERA-71 and KERB-19 displayed activities over a large temperature

Table 2
N-terminal sequence alignment of the KERA-71 and KERB-19 keratinases with other N-terminal sequences of actinobacterial keratinases.

Enzyme	Origin	N-terminal amino-acid sequence [b]	Identity (%)
KERA-71 (this work)	*Actinomadura keratinilytica* Cpt20	MSQAGRRLPARLGATLSALTLTAVFVFPV[a]	–
S8 family serine peptidase (WP_067916364)	*Actinomadura rubrobrunea* NBRC 15,275	MSHADRRLPVRFGAALSAVAFAAVF	64.00
S8 family serine peptidase (WP_165975702)	*Actinomadura* sp. H3C3	MSQAGRRRTRRSRPAARLGARSTATLAVLTLTA	60.61
S8 family serine peptidase (WP_132160220)	*Actinomadura* sp. 7K507	MSEAGRRRPARARPATRLGARTTAALTAL	59.26
KERB-19 (this work)	*Actinomadura keratinilytica* Cpt20	ADIDAGLAYTMGGRCSVGFAAT[a]	–
KERDZ	*Actinomadura viridilutea* DZ50	ADIRAGLAYTMGGRCSVGNAATNAS	90.91
Serine protease NAPase (Q6K1C5)	*Nocardiopsis alba* TOA-1	ADIIGGLAYTMGGRCSVGFAAT	90.91
S1 family peptidase (WP_017586902)	*Nocardiopsis ganjiahuensis* DSM 45,031	ADIIGGLAYTMGGRCSVGFAAT	90.91
Peptidase S1 (WP_017583158)	*Nocardiopsis valliformis* DSM 45,023	ADIIGGLAYYMGGRCSVGFAAT	86.36

[a] These sequences were submitted to BLASTP in order to identify the top hits to the query sequences.
[b] The residues not matching with KERA-71 and KERB-19 keratinases are in bold.

Table 3
Influence of different chemical compounds on the keratinases activities.

Compounds	Residual keratinase activity (%) [a,b]	
	KERA-71	KERB-19
Metal ions (10 mM)		
None	100 ± 2.5	100 ± 2.5
Ca^{2+} ($CaCl_2$)	206 ± 4.6	150 ± 3.1
Mg^{2+} ($MgSO_4$)	126 ± 2.9	107 ± 2.6
Mn^{2+} ($MnSO_4$)	108 ± 2.6	97 ± 2.4
Zn^{2+} ($ZnSO_4$)	102 ± 2.5	120 ± 2.8
Fe^{2+} ($FeSO_4$)	95 ± 2.3	117 ± 2.7
NH_4^+ ($NH_4{\cdot}SO_4$)	78 ± 1.8	93 ± 2.2
Cu^{2+} ($CuSO_4$)	66 ± 1.4	88 ± 2.1
Co^{2+} ($CoCl_2$)	60 ± 1.3	65 ± 1.4
Ba^{2+} ($BaCl_2$)	73 ± 1.5	77 ± 1.8
Ni^{2+} ($NiCl_2$)	0 ± 0.0	0 ± 0.0
Hg^{2+} ($HgCl_2$)	0 ± 0.0	0 ± 0.0
Cd^{2+} ($CdCl_2$)	0 ± 0.0	0 ± 0.0
K^+ (KCl)	101 ± 2.5	110 ± 2.6
Li^+ ($LiSO_4$)	99 ± 2.5	105 ± 2.6
Inhibitors /reducing agents		
None	100 ± 2.5	100 ± 2.5
SBTI (5 mg/mL)	102 ± 2.5	101 ± 2.5
TPCK (1 mM)	92 ± 2.2	95 ± 2.3
TLCK (1 mM)	98 ± 2.5	99 ± 2.5
2-ME (5 mM)	65 ± 1.4	70 ± 1.5
DL-DTT (2 mM)	59 ± 1.2	64 ± 1.3
NEM (2 mM)	97 ± 2.4	96 ± 2.4
Iodoacetamide (5 mM)	61 ± 1.3	66 ± 1.4
DTNB (10 mM)	43 ± 0.7	51 ± 1.0
Leupeptin (50 μg/mL)	101 ± 2.5	102 ± 2.5
Pepstatine A (10 μg/mL)	100 ± 2.5	98 ± 2.5
EDTA (10 mM)	95 ± 2.3	101 ± 2.5
EGTA (2 mM)	71 ± 1.5	85 ± 2.0
PMSF (5 mM)	0 ± 0.0	0 ± 0.0
DFP (5 mM)	0 ± 0.0	0 ± 0.0

[a] The values corresponds to mean of 4 replicates and ± SE are indicated.
[b] The residual enzyme activity was considered for 1 h after incubating (23 ± 2 °C) each purified enzyme with inhibitor or metallic ions. Residual activity was calculated at the described test conditions. Keratinase activity measured without any additive where the non-treated keratinase to which 5 mM EDTA were supplemented was taken as 100 %.

range (20 °C – 80 °C), with the highest activity at 50 °C (for KERA-71; Fig. 3B) and 40 °C (for KERB-19; Fig. 3B), while the maximum temperature for KERA-71 is 45 °C and KERB-19 is 35 °C, in the absence of calcium (Fig. 3B).

The KERA-71 and KERB-19 enzymes are stable at pH ranging from 6–11 (Fig. 3C) and from 4 to 9, respectively (Fig. 3D). As shown in Fig. 3C, KERA-71 half-life at pH 6, 7, 8, 9, 10, and 11 are 30 h, 26 h, 22 h, 16 h, 10 h, and 2 h, while the half-life of KERB-19 at pH 4, 5, 6, 7, and 8 are 32 h, 28 h, 24 h, 20 h, 16 h, and 10 h, respectively (Fig. 3D).

These findings are similar to other proteases of microbial origins that have an optimum pH in the neutral to the alkaline range [24,50]. The pH stability profile of KERA-71 and KERB-19 would render their applications in industrial sectors that require alkaline and neutral conditions.

The thermo-stability of each enzyme was evaluated at pH 8 (for KERA-71) and pH 7 (for KERB-19) and 36 h of incubation using keratin azure as the substrate at three different temperatures. Both enzymes retained 100 % of their activity for more than 30 h at 30 °C. The KERA-71 half-life times without Ca^{2+} are 24 h and 9 h at 50 °C and 60 °C, respectively (Fig. 3E), while their half-life times are increased to 32 h and 14 h, with 10 mM $CaCl_2$. Similarly, at 40 °C and 50 °C, the KERB-19 half-life times without Ca^{2+} are 28 h and 9 h (Fig. 3F), while their half-life times are increased to 36 h and 16 h, with 10 mM $CaCl_2$, respectively.

Ca^{2+} was formerly described to enhance the activity by maintaining the molecular conformation needed for enzyme activity [51,52]. The results indicate that the thermo-activities and thermo-stabilities of KERA-71 and KERB-19 are in the usual range for keratinases produced by actinobacteria [24,53].

3.7. Kinetic study

The KERA-71 displayed broad substrate specificity, as it hydrolyzed various proteins in the following order: keratin > gelatin > hemoglobin > albumin > casein > elastin, and > fibrin (Table 4). Meanwhile, KERB-19 displayed broad substrate specificity in the following order: keratin > casein > gelatin > hemoglobin > albumin > elastin, and > fibrin. The KERA-71 and KERB-19 showed an elevated keratinolytic to the caseinolytic ratio of 1.17 and 1.09, respectively. In addition, the KERB-19 demonstrated a higher relative activity against azo-casein and azo-albumin than KERA-71 (Table 4). Among various synthetic peptides, KERA-71 displayed a higher specificity than KERB-19 for hydrophobic residues such as phenylalanine, alanine, and leucine. Maximum activity was noted in the cleaving of *N*-Suc-Tyr-Leu-Val-*p*NA and *N*-Suc-Ala-Ala-Ala-*p*NA, followed by *N*-Suc-Ala-Ala-Phe-*p*NA. The results reported here are similar to what was observed for the keratinases produced by *Nesterenkonia* sp. AL20 [54] and *Nocardiopsis* sp. 28ROR strains [55]. KERA-71 and KERB-19 are less active on *N*-Suc-Ala-Ala-Pro-Phe-*p*NA and *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, suggesting the preference of KERA-71 and KERB-19 for alanine and leucine instead of proline (Table 4).

KERA-71 and KERB-19 displayed typical Michaelis-Menten kinetics and Lineweaver–Burk plots for keratin (Supplementary data, Fig. S1A and S1B) and *N*-Suc-Tyr-Leu-Val-*p*NA (Supplementary data, Figs S1C and S1D) substrates. When keratin was used as a protein substrate, KERA-71 displayed a Michaelis constant (K_m) of 0.68 times than KERAB (Fig. 5A) and catalytic efficiencies (k_{cat}/K_m) of 1.74, 2.14, 2.35, 3.75, and 9.87-times superior to those of KERDZ, KERB-19, KERAK-29, Actinase E, and KERAB, respectively (Fig. 5B). When *N*-Suc-Tyr-Leu-Val-*p*NA was used as a synthetic substrate, KERA-71 exhibited a K_m of 0.49 times than KERAB (Fig. 5C) and a k_{cat}/K_m of 1. 47, 1.68, 2.22, 2.72, and 3.63-times superior to those of KERDZ, KERB-19, KERAK-29, Actinase E, and KERAB, respectively (Fig. 5D).

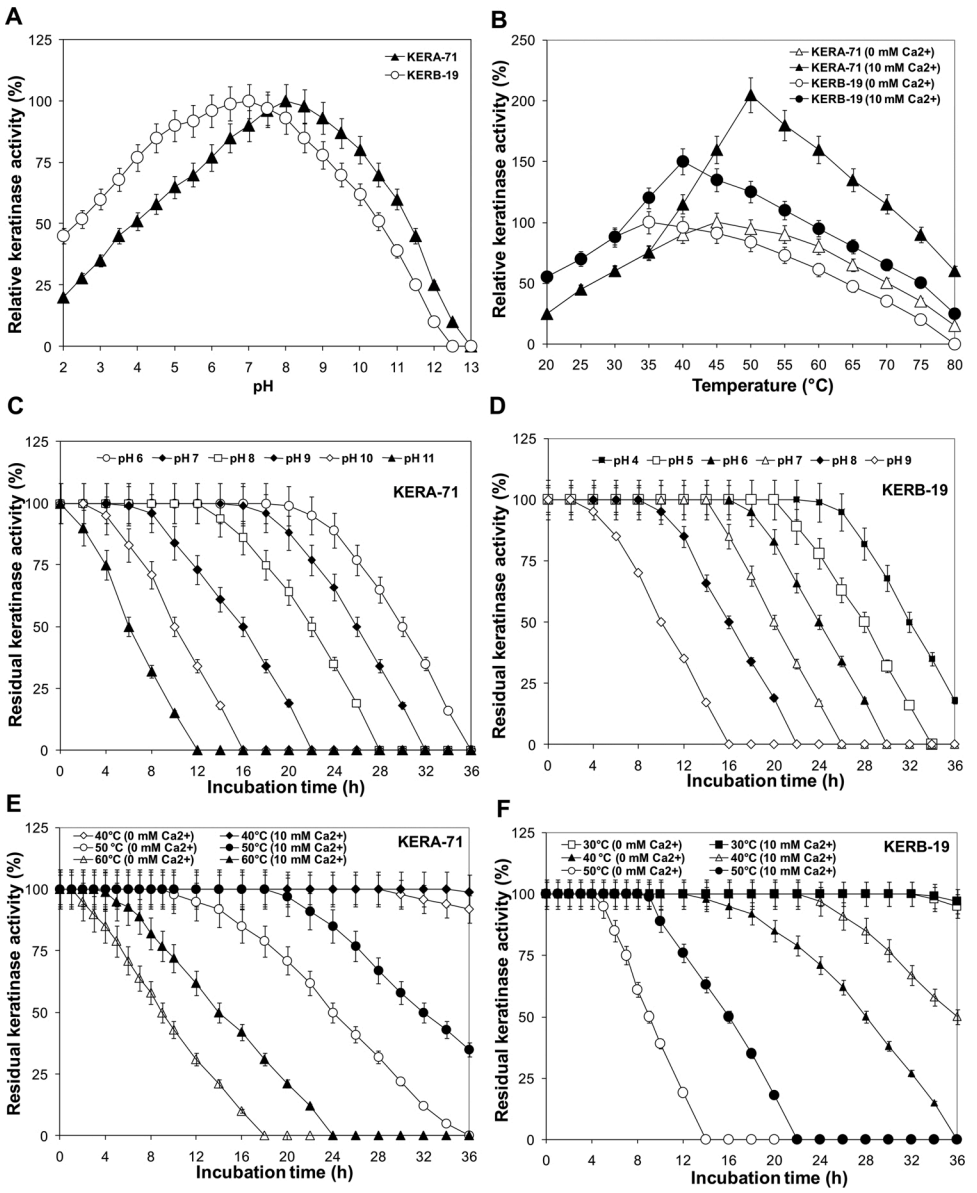

Fig. 3. Biochemical characterization of KERA-71 and KERB-19. Evaluation of the effect of different pH on the activity **(A)** and thermo-activity **(B)** of KERA-71 and KERB-19. Evaluation of the effect of different pH on the stability of KERA-71 **(C)** and KERB-19 **(D)** and thermostability of KERA-71 **(E)** and KERB-19 **(F)**. The keratinase KERA-71 was incubated with and without $CaCl_2$ at 20 °C to 80 °C. Remaining keratinase activity was ascertained (0 h–36 h) at 2 h periods in the absence or presence of 10 mM $CaCl_2$. The activity of the non-heated keratinase was considered as 100 %. Vertical bars designate ± SE of the mean (n = 3). All results are expressed as mean of at least three independent assays.

3.8. DH for both keratinases

The DH curves of CFM and DFM proteins behind the 3 h-incubation period are shown in Fig. 4. Both KERA-71 and KERB-19 were applied at a similar activity level (1,000 U/mL) in their respective optimal conditions for protein hydrolysates production from CFM and DFM and to compare their DH efficiencies. The CFM and DFM were demonstrated to reach high DH rates throughout the initial 60 min, followed by a steady-state phase characterized by no obvious hydrolysis. In addition, KERA-71 is the most efficient enzyme, with 47 % (DH) for the CFM hydrolysis, followed by KERDZ (44 %), KERB-19 (37 %), KERAK-29 (32 %), and Actinase E (26 %), with KERAB being the least efficient (15 %; Fig. 4A). However, KERB-19 exhibited the best efficiency, with 43 % when DFM is used, then KERDZ (35 %), KERA-71 (30 %), KERAK-29 (27 %), and Actinase E (20 %), with KERAB being the enzyme with the lowest efficiency (11 %; Fig. 4B). These findings indicate that KERA-71 and KERB-19 keratinases could be used to improve the dietary value of chicken and duck feather-meals.

3.9. Keratin biodegradation

KERA-71 and KERB-19 keratinases could degrade and solubilize 10 g/L of CFM, DFM, chicken-feather, and duck-feather at 1,000 U/mL (Table 5). Among the mentioned substrates, CFM is the best substrate (100 %), followed by DFM (96 %), chicken-feather (92 %), and duck-feather (81 %) for KERA-71; whereas for KERB-19, DFM is the best substrate (100 %), followed by CFM (95 %), duck-feather (87 %), and chicken-feather (82 %). These behaviors consolidate the data obtained in Fig. 4 regarding the DH of CFM and DFM. Therefore, the most excellent feather-degrading activity was achieved after 12 h of incubation at 50 °C and pH 8 (for KERA-71) and at 40 °C and pH 7 (for KERB-19). Both soluble proteins and free thiol (S—S) groups were increased in association with the augmentation of keratin degradation (Table 5).

These data affirmed that KERA-71 and KERB-19 have the potential to reduce disulfide bonds. A variety of free amino-acids are detected in the cell-free supernatants of hydrolyzed keratin substrates from the KERA-71 and KERB-19 (Table 5). The analysis of free residues in the supernatant exposed an apparent augment of free amino-acids after 12 h of incubation. The obtained profile comprising aliphatic amino-acids

Table 4
Substrate specificity on the keratinases activities.

Substrate	Concentration	Relative keratinase activity (%)	
		KERA-71	KERB-19
Natural protein			
Keratin	20 g/L	100 ± 2.5	100 ± 2.5
Gelatin	20 g/L	92 ± 2.2	88 ± 2.1
Hemoglobin	20 g/L	83 ± 2.0	80 ± 1.9
Albumin	20 g/L	71 ± 1.5	75 ± 1.7
Casein	20 g/L	85 ± 2.0	92 ± 2.3
Elastin	20 g/L	34 ± 0.6	51 ± 1.1
Fibrin	20 g/L	23 ± 0.5	33 ± 0.7
Synthetic protein			
Keratin azures	30 g/L	100 ± 2.5	100 ± 2.5
Azo-casein	30 g/L	86 ± 2.0	95 ± 2.4
Azo-albumin	30 g/L	77 ± 1.7	81 ± 1.9
Synthetic peptide			
N-Suc-Tyr-Leu-Val-*p*NA	5 mM	100 ± 2.5	100 ± 2.5
N-Suc-$(Ala)_3$-*p*NA	5 mM	98 ± 2.5	97 ± 2.5
N-Suc-$(Ala)_2$-Phe-*p*NA	5 mM	85 ± 2.0	82 ± 2.0
BAPNA	5 mM	70 ± 1.5	65 ± 1.4
N-Suc-$(Ala)_2$-Val-*p*NA	5 mM	62 ± 1.3	59 ± 1.3
N-Suc-$(Ala)_2$-Val-Ala-*p*NA	5 mM	55 ± 1.2	51 ± 1.1
N-Suc-$(Ala)_2$-Pro-Phe-*p*NA	5 mM	25 ± 0.5	22 ± 0.5
N-Suc-$(Ala)_2$-Pro-Leu-*p*NA	5 mM	22 ± 0.5	20 ± 0.4

Values correspond to the means ± SE after 3 measurements.

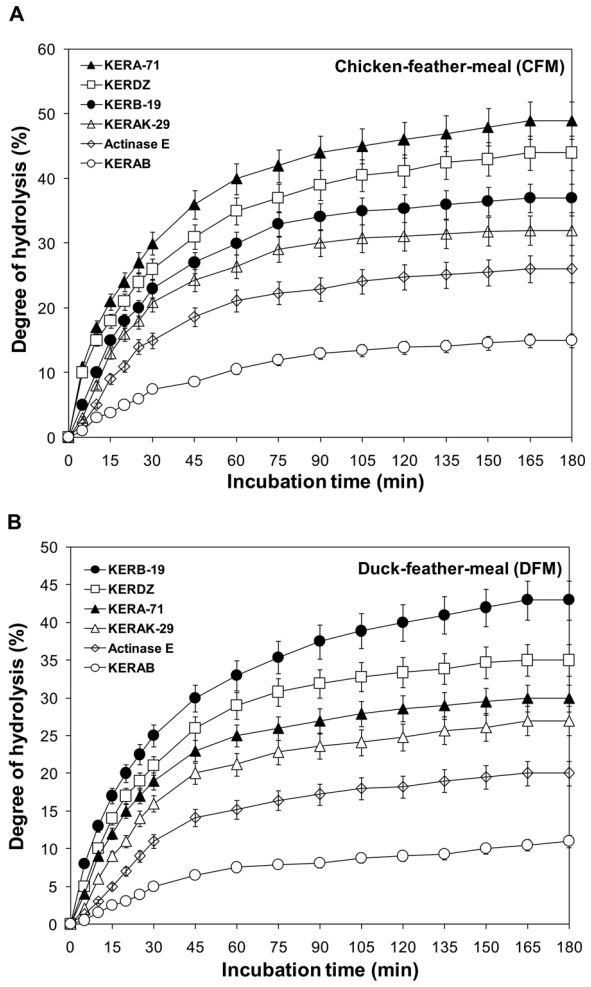

Fig. 4. DH curves of keratins treated 3 h with some actinobacterial keratinases. KERA-71, KERDZ, KERAK-29, Actinase E, and KERAB using CFM **(A)** and DFM **(B)** feather-meals. Every point indicates the mean of 3 independent tests. Vertical bars designate ± SE of the mean (n = 3).

(isoleucine, leucine, valine, and alanine) and aromatic residues in tryptophan and phenylalanine are the mainly detected free amino-acids. Moreover, the profiles of amino-acid release by KERA-71 and KERB-19 are very similar to those cited before for keratinase SAPB from *Bacillus pumilus* strain CBS [22], keratinase KERAB from *Streptomyces* sp. strain AB1 [21], KERUS from *Brevibacillus brevis* strain US575 [3], and KERZT-A and KERZT-B from *Bacillus amyloliquefaciens* strain S13 [43].

3.10. Dehairing activity of KERA-71 and KERB-19

KERA-71 and KERB-19 were used on bright-haired goat, sheep, and bovine skins for evaluating their dehairing efficacy. Results illustrated that after a 12 h-incubation with KERB-19 than KERA-71 at 30 °C, the hairs of each of the animal skin are effortlessly removed compared to their corresponding controls. The results also showed that collagen did not sustain any visible damage (Fig. 6).

These findings provide evidence that the individual KERA-71 and KERB-19 keratinases could achieve the entire bioprocess of dehairing. The dehairing activity of leather is usually achieved in a relatively elevated pH value (6–9) [56], and KERA-71 and KERB-19 demonstrated the required characteristics to function under these conditions. Relatively similar data was attained by KERUS from *B. brevis* strain US575 on sheep, goat, and bovine skins at pH 8 and 40 °C [3]. Keratinase from *B. pumilus* strain KER1 [57] and keratinases KERZT-A and KERZT-B from *B. amyloliquefaciens* strain S13 [43] were described to exhibit high levels of keratinolytic activities, allowing them to accomplish the entire bioprocess of animal (bovine, cowhides, and goat) skin dehairing.

4. Conclusion

This study described two extracellular serine keratinases of high and low molecular masses that have been purified, characterized, and their potential for possible industrial application evaluated. The KERA-71 and KERB-19 keratinases with high and low molecular masses of 71 kDa and 19 kDa, respectively which has not been reported before, especially for a thermophilic actinobacterial strain. The data revealed that both enzymes are highly active and stable at relatively elevated temperatures and neutral to alkaline pH. In addition, KERA-71 showed high catalytic efficiency and an elevated degree of hydrolysis compared to other actinobacterial keratinases (KERDZ, KERB-19, KERAK-29, Actinase E, and KERAB). Additionally, KERB-19 demonstrated higher dehairing ability than KERA-71 on the goat, sheep, and bovine skins after 12 h-incubation (30 °C). Overall, the findings indicate that KERA-71 and KERB-19 have some attractive properties rendering them potential candidates to numerous prospects to improve resourceful, cost-effective, efficient, and environmentally-friendly alternatives to the harmful products being used in numerous industrial applications. To further study the interesting properties of KERA-71 and KERB-19, additional work is now underway in our laboratory to essentially accomplish the 3D molecular structure of both enzymes after expressing the two genes encoding for KERA-71 and KERB-19 to have a better understanding and discover their structure-functions relationships and eventual site-directed mutagenesis.

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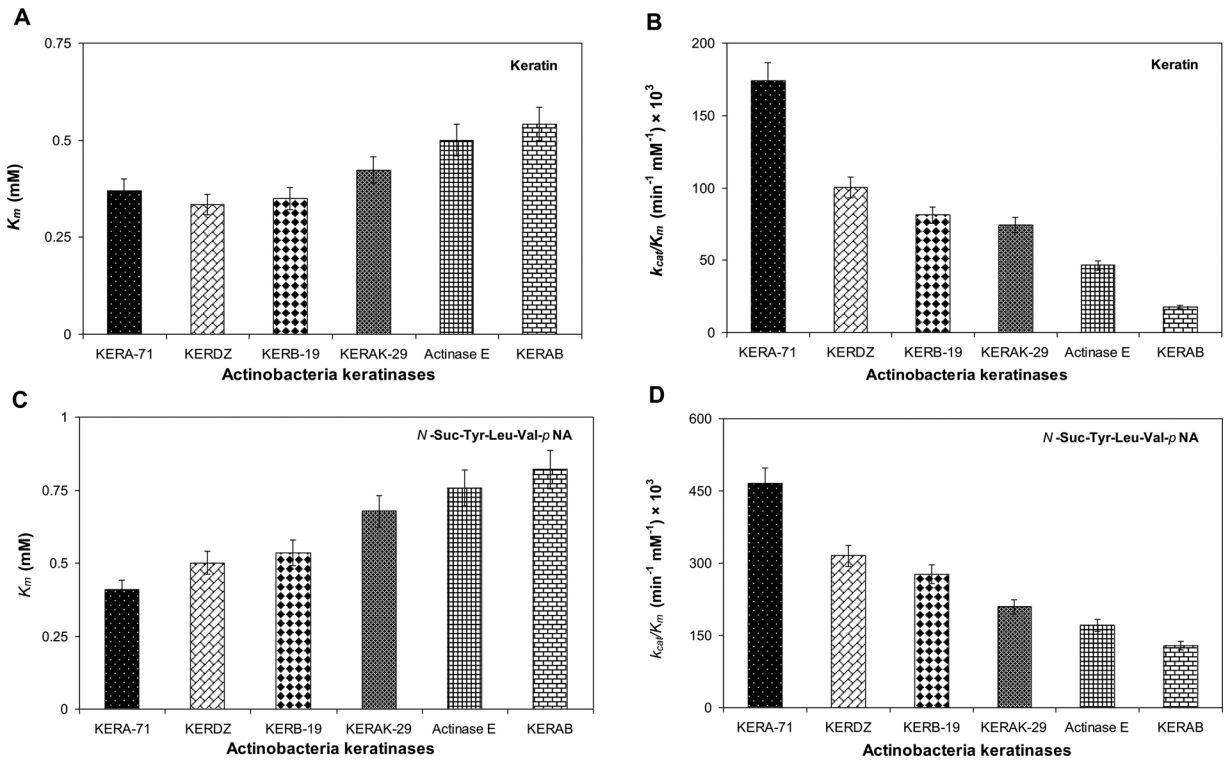

Fig. 5. Kinetic parameters of the purified actinobacterial keratinases for the hydrolysis of natural protein and synthetic peptide substrates. KERA-71, KERDZ, KERAK-29, Actinase E, and KERAB using keratin and *N*-Suc-Tyr-Leu-Val-*p*NA. **(A)** and **(B)** the K_m and k_{cat}/K_m values using keratin as substrate, respectively. **(C)** and **(D)** the K_m and k_{cat}/K_m values using *N*-Suc-Tyr-Leu-Val-*p*NA as substrate, respectively. Every point indicates the mean of 3 independent tests. Vertical bars designate ± SE of the mean (n = 3).

Table 5
Influence of substrates on the KERA-71 and KERB-19 after 12 h-incubation.

Keratin substrate	Enzyme	Soluble protein (mg/mL)	Sulfhydryl groups (μM)	Keratin degradation (%)	Free amino-acids
Chicken-feather-meal (CFM)	KERA-71	94 ± 0.7	7.4 ± 0.3	100 ± 2.5	Ala, Val, Leu, Ile, Met, Trp, Phe, Cys
	KERB-19	92 ± 0.6	7.0 ± 0.3	95 ± 2.4	Ala, Val, Leu, Ile, Met, Phe, Cys
Duck-feather-meal (DFM)	KERA-71	88 ± 0.5	6.8 ± 0.3	96 ± 2.3	Ala, Val, Leu, Ile, Met, Trp, Phe, Cys
	KERB-19	90 ± 0.6	6.9 ± 0.3	100 ± 2.5	Ala, Val, Leu, Ile, Met, Trp, Cys
Chicken-feather	KERA-71	5.57 ± 0.3	6.1 ± 0.2	92 ± 2.1	Ala, Val, Leu, Ile, Trp, Phe, Cys
	KERB-19	5.11 ± 0.3	5.5 ± 0.2	82 ± 1.9	Ala, Val, Leu, Ile, Trp, Cys
Duck-feather	KERA-71	4.51 ± 0.2	5.0 ± 0.2	81 ± 1.9	Ala, Val, Leu, Ile, Trp, Phe
	KERB-19	4.75 ± 0.2	5.6 ± 0.2	87 ± 2.1	Ala, Val, Leu, Ile, Trp

Values correspond to the means ± SE after 3 determinations.

Campus France: 43791TM & code PHC: 01MAG20); and the Algerian-Tunisian R&I Cooperation for the Mixed Laboratories of Scientific Excellence 2021-2024 (Hydro-BIOTECH, code LABEX/TN/DZ/21/01).

Declaration of Competing Interest

The authors report no declarations of interest.

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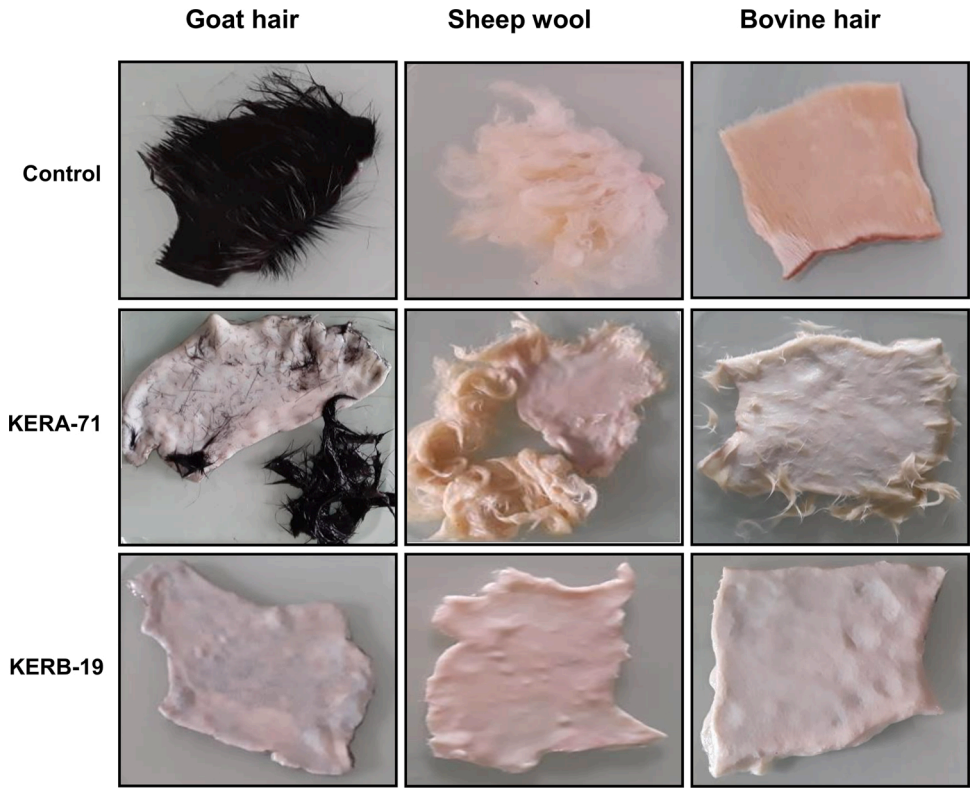

Fig. 6. Dehairing activity of the purified keratinases from Cpt20 on animal hides. The KERA-71 and KERB-19 were incubated with different samples (goat, sheep, and cow hides) at 30 °C for 12 h and were compared to the control (t = 0). For all examination a control was carried out without providing keratinases.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2021.04.009.

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