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# Novel serine keratinase from *Caldicoprobacter algeriensis* exhibiting outstanding hide dehairing abilities

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ABSTRACT

The current paper reports on the purification of an extracellular thermostable keratinase (KERCA) produced from *Caldicoprobacter algeriensis* strain TH7C1$^T$, a thermophilic, anaerobic bacterium isolated from a hydrothermal hot spring in Algeria. The maximum keratinase activity recorded after 24-h of incubation at 50 °C was 21000 U/ml. The enzyme was purified by ammonium sulfate precipitation-dialysis and heat treatment (2 h at 50 °C) followed by UNO Q-6 FPLC anion exchange chromatography, and submitted to biochemical characterization assays. Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI–TOF/MS) analysis revealed that the purified enzyme was a monomer with a molecular mass of 33246.10 Da. The sequence of the 23 N-terminal residues of KERCA showed high homology with those of bacterial keratinases. Optimal activity was achieved at pH 7 and 50 °C. The enzyme was completely inhibited by phenylmethanesulfonyl fluoride (PMSF) and diiodopropyl fluorophosphates (DFP), which suggests that it belongs to the serine keratinase family. KERCA displayed higher levels of hydrolysis and catalytic efficiency than keratinase KERQ7 from *Bacillus tequilensis* strain Q7. These properties make KERCA a potential promising and eco-friendly alternative to the conventional chemicals used for the dehairing of goat, sheep, and bovine hides in the leather processing industry.



## 1. Introduction

Leather processing industries are gradually increasing every year. Annually approximately 5.5 million tons of wet salted rawhides were processed and produced 460,000 t of heavy leathers and 940 million square meters of light and split leathers through worldwide. Europe alone produced about 74,000 t of heavy leathers and 240 million square meters of light and split leathers [1]. Conventional leather processing involves serial operations that are classified into three groups. First pre-tanning or beamhouse-operations steps involves the cleaning of hides or skins, second step is tanning which enduringly stabilizes the skin matrix and the last one is post-tanning or finishing operations, where an esthetic value is added [2]. The pre-tanning steps are the most polluting steps in leather processing which generate about 70% of the effluents produced [3]. Maximum major corrosive sulfide were emitted from dehairing operations [4]. Additionally, chrome-tanning step generates large emissions of chromium and sulfate [5]. In order to overcome the hazards caused by the tannery effluents and to improve the quality of the end product, the use of keratinolytic enzymes as viable alternative has been resorted to in pre-tanning operations such as dehairing. Thus, the search for more efficient, safe and eco-friendly strategies for raw hide preservation and leather processing has always been a major challenge in the leather industry and an area of continued research in the scientific community [6].

Microbial keratinolytic proteases have particularly been reported to constitute a resourceful class of enzymes with promis-

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ing industrial applications [7]. Of special interest, keratinases are a group of serine or metallo peptidases that can degrade the insoluble structure forming keratin substrates. Keratin is an insoluble structural protein of feather and wool and is known for its high stability [8]. Increased attention has been devoted to these enzymes because of their several potential uses associated with the hydrolysis of keratinous substrates and other applications [9].

By now, a fairly large number of mesophilic keratinolytic microorganisms have been studied from bacteria, yeasts, and fungi [7,10]. Some of them produce keratinases also active at higher temperatures ranging from 70 to 85 °C [10,11]. Thermophilic bacteria have gained a significant attention to industrial scale as they possess enzymes that are active and stable at high temperatures. Hot springs are natural habitats of peculiar interest for searching such thermopiles [12–15]. The discovery of thermo-, neutral-, and alkalistable enzymes is still of importance in order to increase catalytic efficiency throughout industrial leather processes due to variable conditions and treatment temperatures. A large number of proteases has been discovered in thermophilic and hyperthermophilic bacteria [13]. Nevertheless, few data are currently available on the purification and characterization of keratinases from thermophilic anaerobic bacteria [13]. Keratinases have found application in various steps of leather processing, *e.g.*, neutral keratinase in soaking [16], alkaline keratinase in dehairing [17,18], and alkaline keratinase in bating [19,20]. Dehairing enzymes from bacteria have also been reported in the literature [18,21–24]. Interestingly, a recent patent proposed a concrete mixture of dehairing enzymes from *Bacillus subtilis* and *Bacillus cereus* with sodium carbonate, caustic soda, and thioglycolic acid [25].

Considering the attractive properties of keratinases and the promising opportunities that they might open for the development of efficient and eco-friendly leather manufacturing processes, the present work aimed to study the purification and biochemical characterization of a novel keratinase (KERCA) from the thermophilic anaerobic bacterium *Caldicoprobacter algeriensis* strain TH7C1$^T$, isolated from a hydrothermal hot spring in Guelma (Algeria). The hide dehairing capacities of the enzyme were also demonstrated.

## 2. Materials and methods

### 2.1. Substrates, chemicals and native leather dehairing enzyme

Unless specified otherwise, all substrates, chemicals, and reagents were of the analytical grade or highest available purity, and purchased from Sigma Chemical Co. (St. Louis, MO, USA). KERQ7, a proposed serine neutral keratinase, from *Bacillus tequilensis* strain Q7 for dehairing of hides and skins, was used as comparative keratinase [20].

### 2.2. Bacterial identification and growth conditions

*C. algeriensis* strain TH7C1$^T$ = DSM 22661$^T$ = JCM 16184$^T$ (T = type strain) used in this study was isolated from water samples collected at a terrestrial hot spring in Guelma, Algeria (70°25′E, 36°27′N) and cultivated under anaerobic conditions as previously described elsewhere [26]. It was identified on the basis of 16S rRNA gene sequencing and biochemical properties as well as phenotypic, phylogenetic, and genetic characteristics [27,28].

The present study used a modified non-glucose containing medium (TH) [27,28] consisting of (g/l): chicken feather-meal, 10; yeast-extract, 2; $NH_4Cl$, 0.5; $K_2HPO_4$, 1; $KH_2PO_4$, 1; KCl, 0.3; $MgCl_2{\cdot}6H_2O$, 0.5; $CaCl_2{\cdot}2H_2O$, 0.2; NaCl, 1.5; biotrypcase, 2; cysteine–HCl, 0.5; together with sodium acetate (2 mM) and a Balch trace element solution (10 ml) at pH 7.4 [29]. The Media were autoclaved for 20 min at 121 °C. The production of keratinase in the culture medium was recorded after 24 h of incubation at 50 °C and pH 7.4. Before assay, the cells were separated by centrifugation at 10,000 × *g* for 30 min. The clear supernatant was used as crude enzyme preparation in subsequent studies.

### 2.3. Determination of enzyme activity

Keratinolytic activity was determined using keratin azure as a substrate [30]. Unless otherwise stated, 1 ml of 10 g/l keratin azure, suspended in 100 mM HEPES buffer and supplemented with 2 mM $CaCl_2$ at pH 7 (Buffer A), was mixed with 1 ml of a suitably diluted enzyme solution. The sample was incubated with shaking for 30 min at 50 °C and 200 rpm. One keratin azure unit was defined as the amount of enzyme causing an increase of 0.01 in absorbance at 595 nm in one min under the experimental conditions described.

Keratinolytic activity was also measured using the Folin–Ciocalteu method and as previously described elsewhere [31] with keratin as a substrate. One keratin unit was defined as the amount of enzyme that hydrolyzed the substrate and that produced 1 μg of amino acid equivalent to tyrosine per min at 50 °C and pH 7 in buffer A.

### 2.4. Purification of the KERCA from C. algeriensis strain TH7C1$^T$

An amount of five hundred ml of a 24-h old culture of *C. algeriensis* strain TH7C1$^T$ was centrifuged for 30 min at 10,000 × *g* and 4 °C to remove microbial cells. The supernatant containing extracellular keratinase was used as the crude enzyme preparation and was submitted to the following purification steps. The supernatant was precipitated between 35 and 55% ammonium sulfate saturation. The precipitate was then recovered by centrifugation at 9000 × *g* for 25 min, resuspended in a minimal volume of 50 mM HEPES buffer containing 2 mM $CaCl_2$ at pH 6.2 (Buffer B), and dialyzed by replacement with another new solution buffer B, against at least three times for 14 h. Insoluble material was removed by centrifugation at 9000 × *g* for 25 min. The supernatant was heat-treated for 2 h at 50 °C. The clear supernatant was loaded and applied to a UNO Q-6 FPLC (Bio-Rad Laboratories, Inc., Hercules, CA, USA) equilibrated with buffer B. The column was rinsed with 500 ml of the same buffer. Adsorbed material was eluted with a linear NaCl gradient (0–500 mM) in buffer B at a rate of 60 ml/h. The column (12 × 53 mm) was extensively washed with buffer B until the optical density of the eluent at 280 nm became zero. Fractions of 5 ml each were collected at a flow rate of 60 ml/h and analyzed for keratinase activity and concentration of protein content. Pooled fractions containing keratinase activity were concentrated in centrifugal micro-concentrators (Amicon Inc., Beverly, MA, USA) with 10-kDa cut-off membranes and stored at −20 °C for further analysis.

### 2.5. Protein concentration and analytical methods

Protein concentration was determined by the method of Bradford [32] using a Dc protein assay kit purchased from Bio-Rad Laboratories (Hercules, CA, USA), with bovine serum albumin as a reference. The analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed following the method of Laemmli [33]. The protein bands were visualized with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) staining. Keratin azure zymography staining was performed as previously described by Jaouadi et al. [30]. The molecular mass of purified KERCA was analyzed in linear mode by MALDI–TOF/MS using a Voyager DE-RP instrument (Applied Biosystems/PerSeptive Biosystems, Inc., Framingham, MA, USA). Data were collected with a Tektronix TDS 520 numeric oscillograph and analyzed using the GRAMS/386 software (Galactic Industries Corporation, Salem, NH, USA). Bands of