Isolation and Partial Characterization of Extracellular Keratinase from a Novel Mesophilic Streptomyces albus AZA

Mona A. Esawy

Department of Chemistry of Natural and Microbial Products, National Research Centre, Cairo, Egypt.

Abstract: The novel mesophilic marine Streptomyces albus AZA strain was investigated for its ability to produce constitutive and inducible extracellular keratinase. In this study different substrates were used as the sole carbon and nitrogen source for keratinase production under solid state fermentation conditions (SSF). Maximum keratinase production was achieved (1103 U/g solid substrate) with wheat flour 10 g/flask at 150% moisture content after 5 days at 30 ºC. The partially purified enzyme showed its maximum activity at 65 ºC, pH 10 and complete stability after one hour incubation at 70 ºC. Zn²⁺ and Ca²⁺ played a significant role in enzyme stability. This keratinolytic enzyme offers an interesting potential for the hydrolysis of different keratin wastes to be used as feed supplement or bioconversion to added-value products. The enzyme is unique in that unlike most other microbial serine proteases known so far, it did not show hydrolytic activity towards casein and BSA. Microscopic analysis showed complete lyses of the native cow horn particles.

Keywords: Isolation, characterization, extracellular keratinase and Streptomyces albus

INTRODUCTION

Keratins (KRTs) are the most abundant proteins in mammalian epithelial cells and the major components of skin, nail, hair, horn, feather, and wool. Two types of KRTs, α-KRTs and β-KRTs, consist of tightly packed protein chains in α-helices and β-sheets, respectively[32]. Furthermore, Keratin filament structures are stabilized by their high degree of cross-linking of disulfide bonds, hydrophobic interactions, and hydrogen bonds[32]. Due to their extremely rigid structures, KRTs are insoluble and hard to degrade. However, certain microorganisms synthesize keratinases that are able to degrade keratins. Keratinolytic activity has been reported for different bacterial genera especially Streptomyces. sp[9,34] These enzymes enable keratinolytic bacteria and fungi to degrade waste keratin in nature[21]. Keratinases have several potential applications: in detergent formulations for eliminating horny epithelial cells adhered to textile fibers[4] ecologically friendly leather processing[9,36] waste chicken feather degradation[5,26], nutritional improvement of waste feather for livestock feed[29] and production of protein hydrolysis's from keratinous waste materials[21]. Further, their prospective application in the challenging field of prion degradation would revolutionize the protease world in the near future[15].

The properties of microbial keratin-degrading enzymes appear to differ according to the producing species of microorganism. Some reports have described thermoactive keratinolytic proteases produced by mesophilic microorganisms[10,27]. These enzymes show keratinolytic activity at temperatures above 70ºC, whereas most of the other keratinases from mesophilic bacteria and fungi are active at a rather alkaline pH but show optimal activity at lower temperatures[10,37]. The optimum activity at moderate temperature will be less energy consuming than the currently used thermophilic bacteria and therefore meets the increasing consciousness for energy conservation. Most thermoactive keratinases from mesophilic bacteria and dermatophytes producing keratinases are mostly pathogenic, they are undesirable for application[13]. So it is necessary to peruse a greater variety of save mesophilic microorganisms producing characteristic proteases with broad substrate specificity and thermostable activity in order to establish the utility of mesophilic microorganisms for the degradation of hard proteins.

Although the production of proteases in complex growth media often promotes exuberant growth and high enzyme yield[26,19], their expensive cost makes them unsuitable for a large-scale production. Some less expensive substrates, such as soybean meal, have been successfully used[41,19]. It seems more adequate to use raw materials like some wastes from the food industry as a basis of the culture media.
In this study, a marine isolated mesophilic *Streptomyces albus* AZA produced inducible and constitutive α, β keratinase from different substrates under solid state fermentation (SSF) conditions. A keratinolytic activity was partially purified and characterized in terms of its biochemical properties and was found to have broad substrate specificity and it can hydrolyze not only keratin but also a large variety of insoluble proteins. The enzyme was characterized by a good thermal stability, which is a prerequisite for biotechnological applications.

**MATERIALS AND METHODS**

**Microorganism and Growth Condition:** An aerobic mesophilic saprophytic bacterium was isolated from a Mediterranean Sea (20-35°C) nearby sea shore, Alexandria. Isolation and enrichment of the keratin-degrading microorganism was performed at 30 °C and pH 8.5 in a medium containing (g/l): 0.2 g KCl, 0.2 g MgSO₄·7H₂O, 1 g (NH₄)Cl and 1 g native chicken feather in 250 Erlenmeyer flasks containing 50 ml sea water. For the enzyme production, the bacterium was grown for 5 days in the same medium without NH₄Cl. The culture was centrifuged at 4 °C and 10,000 rpm for 15 min in order to harvest clear supernatant contain keratinase. The basal medium (BM) contained (1°distilled water): 5 g peptone, 1 g yeast extract, 2 g gelatin, 10 g starch. The pH was adjusted to 7.5 with 0.1 M NaOH.

**Identification of the Keratinolytic Microorganism:**
The strain was identified in Taxonomy departement, Ain Shams University, Cairo, Egypt.

**Sanning Electron Microscopy:** Two ml of culture were centrifuged at 14,000 rpm for 10 min. Carefully washed cell pellets were fixed by adding 2.5% glutaraldehyde (v/v) for 2 h and 1 % osmium tetraoxide (w/v) for 1 h before being washed with 50 mM phosphate buffer pH 6.8. Dehydration was performed in 50% acetone for 10 min and 70% concentration for 18 h. Further dehydration was obtained with 85%, 95% and 100% absolute alcohol. Solid state fermentation:

These studies on *Streptomyces albus* ASA were performed using commercially available minced cow horn, human hair, chicken feather, banana skin, ground barely, salmolina flour, popcorn, wheat bran, or rice stalk as the solid substrate. Ten grams of each substrate in 250 Erlenmeyer flasks were mixed with 8 ml of the production medium (BM) contained (1°sea water): 0.2 g KCl, 0.2 g MgSO₄·7H₂O, thoroughly and autoclaved at 121 °C for 30 min. They were cooled to room temperature and each flask was inoculated with 2 ml inoculum (BM). The influence of temperature on growth and production of protease was studied at 25°C, 30°C, 35°C and 40°C. Keratinase production was also investigated in wheat flour medium with initial pH adjusted to 7.0, 7.5, 8.0, 9.0 and 9.5. The cultivation was carried out at 30°C.

**Extraction and Enzyme Recovery:** Keratinase was extracted from the substrate by the simple method of extraction using distilled water as extracting agent[1]. Ten volumes of distilled water per gram substrate (based on initial dry weight of the substrate) was added to the fermented media and the extraction was performed by agitation at room temperature in a rotary shaker for 60 min at 150 rpm. The slurry was then squeezed through wound sheet and clarified by centrifugation at 5,000 rpm at 4 °C for 15 min. The clear supernatant was used for assay of enzyme activity and protein content.

**Assay of Enzyme Activity:** Keratinase activity was determined spectrophotometrically according to the method of[4] with a modification. The reaction mixture consisted of 1 ml diluted enzyme solution and 0.1 g keratin in 5 ml borate buffer (0.05 M, pH 9) was incubated for 4 h at 40 °C. The reaction was stopped with 1 ml of 10% trichloroacetic acid (TCA) for 30 min at room temperature. This mixture was centrifuged and the released amino acids measured as tyrosine by Lowry method.

One unit of keratinase activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine under the specified conditions.

Enzyme activity was expressed as units per gram initial dry substrate (U/g WB) in the solid state experiments and (U/ml) in the others.

Caseinase, gelatinase and BSA activity: were estimated according to the method of[3] by determining the rate of hydrolysis of 1 ml of 1.5% (w/v) casein, gelatin or BSA solution in glycine NaOH buffer 0.05M at pH 8.6 and 0.05M citrate phosphate buffer pH 6 with 1.0 ml diluted enzyme after incubation for 30 min at 40 °C.

One unit (U) of enzyme activity was taken as the amount of enzyme that liberated 1 µg of tyrosine per ml per minute.

Enzyme activity was expressed as units per gram initial dry substrate (U/g WB) in the solid state experiments.

Keratin azure activity: Keratinase activity was determined by incubating the enzyme with 4 mg of keratin azure in 1 ml of 50 mM Tris–HCl buffer (pH 9.0) at 65 °C for 1 h with constant agitation using a rotary shaker at 240 rpm. The samples were centrifuged at 12,000 rpm for 10 min and the absorbance of the supernatant was measured at 595 nm.
One unit of keratinase activity was defined as the amount of the enzyme that gives rise to an increase in absorbance at 595 nm of 0.01 after the reaction with keratin azure for 1 h at pH, 8.0 and 65 °C.

Preparation of Reused and Treated Hair: Reused hair was prepared using commercially available native human hair where ten grams of it in 250 Erlenmeyer flasks were mixed with 8 ml of the production medium (BM) contained (l’sea water): 0.2 g KCl, 0.2 g Mg SO₄.7H₂O, thoroughly and autoclaved at 121 º C for 30 min Thereafter each flask was inoculated with 2 ml inoculum of keratinacious bacterial sp isolated from Sharm el Shaih 30 meter depth, After 48 h the cultivated hair was separated from the culture, washed carefully, and dried in the oven.

Treated hair was prepared by the following procedure: native human hair was immersed in 0.1 N Na OH, then autoclaved at 121 º C for 10 min, the hair was washed vigorously until the neutral pH was reached.

Optimization of Process Parameters for Protease Production: The parameters studied included initial moisture content (50-150%), incubation time (24-168h), different concentrations of wheat flour (5-25 g) and incubation temperature (25-40 °C), pH (5-9).

Partial Purification of the Enzyme: Ammonium sulfate was added to 100 ml of the culture filtrate at different concentrations to obtain various fractions at 25, 30, 40, 50, 60, 70 and 80% saturation. Each precipitate was dissolved in a certain amount of distilled water and dialyzed against distilled water using a cellophane bag until the water outside the bag gave no precipitation with BaCl₂ solution. After complete dialysis 2 g of bentonite was mixed with 100 ml enzyme in 0.05 M borate buffer. The mixture was stirred for 15 min at 4 °C, centrifuged and the activity was detected in the filtrate.

Hydrolysis of Protein Substrates: Enzyme activity with various protein substrates including: insoluble proteins: minced cow horn, sheep wool, chicken feather, keratin azure, treated hair and reused hair were assayed by incubating 50 (U/g) of keratinase in 5 ml borate buffer containing (20 mg substrates /ml). After incubation at 65 °C for 24 h, the enzyme activity was estimated as proteolytic activity and compared with that of the control containing keratin.

Investigation under the Inverted Microscope: Native minced bovine horn 0.1 g and of treated hair 0.1 g were incubated with 50 U/g enzyme in 5 ml buffer for 24 h at 65 °C. The samples were incubated with distilled water. The samples were washed carefully with distilled water, dried at 50 °C and examined under the inverted microscope.

Thermal Stability: Profile was studied by incubating the enzyme preparation at various temperatures, (50-80 °C) in glycine NaOH buffer 0.05M, pH 10 for different incubation period (15-60min) and the residual activity was measured at 70 ºC.

RESULTS AND DISCUSSIONS

This studies reported in the isolation of a novel mesophilic bacterial sp from a Mediterranean Sea Alexandria (30-40 °C) nearby sea shore, Egypt. Following taxonomic, morphological, enzymatic, examination under the inverted microscopy and SEM analyses (Fig.1) of AZA strain, the reference isolate was identified as Streptomyces albus AZA. The morphological analysis showed that the Streptomyces albus was a filamentous rod-shaped gram positive forming chain of spores which showed an extracellular keratinolytic activity expressed in the culture broth containing one gram chicken feather after 5 days at 30 °C. Degradation of substrate was visually inspected where the strain disintegrated feather barbules but not all raehis and reported keratinase activity in minced horn keratin (10 U/ml) and keratin azore (2 U/ml).. Yoshioka et al.[40], showed that the keratinases produced by B. subtilis, B. amyloliquefaciens and B. velesensis were active as azokeratin and degradation of feather barbules was observed.

The novel mesophilic Streptomyces albus AZA strain was investigated for its ability to produce constitutive and inducible extracellular keratinase. In the following study the keratinaceous and the non keratinaceous materials were tested as the sole carbon and nitrogen sources under solid state fermentation (SSF) conditions for keratinase production. This medium has no extraneous sources of organic carbon and nitrogen that might interfere with the keratinase production and the keratin utilization assay[18]. The recent study on keratinase synthesis by Streptomyces albus AZA indicated that the major regulatory mechanism is constitutive. The keratinase achieved its maximum keratinase production activity with wheat flour (800 U/g) (Fig. 2). Gushterovaet et al.[18] showed that a 9-fold increase in keratinase activity of the Streptomyces flavis in the presence of 5 g starch/l. The recent study on keratinase synthesis by Streptomyces albus AZA indicated that the major regulatory mechanism is constitutive Son et al.[15] reported that keratinolytic protease from Bacillus
Fig. 1: *Streptomyces albus* AZA. under the inverted microscopy (A) and scanning electron microscopy (B)

![Streptomyces albus AZA](image1.png)

Fig. 2: Effect of different substrates on keratinase production from *Streptomyces albus* AZA grown at 30 °C under SSF condition

![Effect of different substrates on keratinase production from Strepotmyces albus AZA grown at 30 °C under SSF condition](image2.png)

*pumilis* was produced in the absence of feather (9.4 U/mg). Previous studies have shown that the synthesis of extracellular keratinase is constitutive or partially inducible\(^\text{(7,27)}\). On the contrary\(^\text{(18)}\) showed that keratinase synthesis by *Thermoactinomyces candidus* could not be detected in media lacking keratin.

Maximum keratinase production was (902 U/g wheat flour) at 30 °C as expected for a micro-organism of environmental origin, with initial pH adjusted to pH 9 after 4-5 days. Although keratinolytic bacteria often display optimal growth and activity at higher temperatures\(^\text{(24,22)}\). This is consistent with optimum values described for *Streptomyces* sp krt6 that showed high keratin degradation at room temperature\(^\text{(19)}\). The stimulus of keratinase activity by alkaline pHs suggests a positive biotechnological potential. This observation is based on the fact that keratin degradation involves deamination reactions, which result in an increase in pH. Alkaline proteases have been extensively used to formulate detergents, in leather industry and in bioremediation processes\(^\text{(14)}\).

The optimum keratinase production (992 U/g wheat flour) was observed at 10 g/ flask wheat flour at 150% moisture level (Figs. 3 and 4). High substrate concentrations may cause substrate inhibition or repression of keratinase production. This was observed
when soy meal was used as substrate\textsuperscript{[11]} similar to that described by Joo et al.,\textsuperscript{[19]} during protease production by Bacillus horikoshii.

The concentrated partially purified enzyme obtained after a selective precipitation of soluble proteins with 50–70 % was applied to bentonite. These two steps removed approximately 70 % of total proteins maintaining 50% of the enzyme activity and recorded 22 purification fold.

The Partially purified enzyme proved to be active over a broad range of temperature (40–80 °C) and pH values (pH 6.5–11.0); the optimum temperature for enzyme activity was 65°C (Fig. 5) and the optimal pH around 10.0 (Fig. 6). The optimum pH and temperature for Bacillus pseudofirmus keratinase activity were pH 8.8-10.3 and 60°C, respectively\textsuperscript{[12]}. Some thermostable keratinases have been found with the activity up to 90 °C\textsuperscript{[28]}.

The results of the present investigation showed that the enzyme was fairly stable to heat treatment in absence of the substrate. At 70 °C the enzyme retained its complete stability for one hour. The high
The thermostability of the enzyme allows performance of the experiments at 65-70 °C which minimizes the risk of microbial contamination.

The enzyme activity decreased gradually at 80°C. Zn$^{2+}$ and Ca$^{2+}$ play a significant role in improving the thermal stability of the enzyme (Fig. 7). A stabilizing effect of divalent metal ions has already been reported for many proteinases.$^{[3]}$

Effect of some metals ions on the enzyme activity (Fig. 8) showed that Zn$^{2+}$ increased the activity more than 3 fold. The increased of keratinase activity in the presence of Zn$^{2+}$ implies that the cation plays an important role in the regulation of enzyme active conformation and in this way increases keratinolytic activity. The lack of an inhibitory effect of metalloproteinase inhibitor (EDTA) on keratinlytic activity might have been caused by a competition between the excess of metals present at non-active sites. All the used metals increased the hydrolytic activity except FeCl$_3$, LiSO$_4$. Keratinolytic metalloproteases may have great biotechnological promise, acting as secondary keratinases they may overcome the limited proteolysis on the surface of insoluble keratin particles because of restricted enzyme–substrate interaction.$^{[1]}$
Fig. 7: Effect of Zn$^{2+}$ and Ca$^{2+}$ on the stabilization of partially purified Streptomyces albus AZA keratinase at 80 °C. (●) enzyme+2mM Zn$^{2+}$ (■) enzyme +2mM Ca$^{2+}$ (●) enzyme only

Fig. 8: Influence of metal ions on the partially purified keratinase from Streptomyces albus AZA

*Streptomyces albus* AZA has broad substrate specificity where it can hydrolyze a large variety of insoluble protein substrates. (Fig. 9). Disulfide bridges are an important structural feature of native keratin. Cleavage of these disulfide bonds seems to be indispensable to make the proteins available for the hydrolytic enzymes. A disulfide reductase was purified from the culture of a keratinolytic strain of *Stenotrophomonas* sp.[39] and increased thiol formation was detected during cultivation of *Chryseobacterium* sp kr$^6$ on feather keratin[39]. On contrary the enzyme was not able to hydrolyze soluble protein substrates (casein and BSA) whereas proved gelatin was less susceptible. Although data from soluble proteins cannot be compared with those from insoluble protein, it seems that the activity against the latter may be a function of surface area. The results indicate that the proteolytic activity is mainly directed towards the insoluble protein.

The inspection of the hydrolyzed hair and horn under inverted microscope (Fig. 10 a and b) showed that most of the hair was disintegrated and the horn particles approximatly lyses completely. All the previous work referred to the novelty of *Streptomyces albus* AZA keratinase.
Fig. 9: Hydrolysis of different keratinacious substrates by partially purified *Streptomyces albus* AZA keratinase

Fig. 10a: Treated human Hair before (A) and after treatment with keratinase (B) (Inverted microscope picture)

Fig. 10b: Cow horn keratin before (C) and after treatment with keratinase (D) (Inverted microscope picture).
Conclusion: The results showed that the *Streptomyces albus* AZA enzymes belong to the most active proteases and was found to have a broad substrate specificity against the insoluble proteins and a good thermal stability, which is a prerequisite for biotechnological applications. Both the partial purification procedure set up and the characterization study of the keratinase were important to foresee potential production and uses of this enzyme.

ACKNOWLEDGMENTS

Many thanks to Prof. Ahmed F. Abd El-Fattah, Prof. Nafisa M.A El-Shyeb, Departement of chemistry of natural and microbial products for their supporting the work.

REFERENCES


