



PURIFICATION AND CHARACTERIZATION OF KERATINASE FROM HAIR-DEGRADING STREPTOMYCES ALBUS

Sreenivasa Nayaka, Gireesh Babu K and GM Vidyasagar*

Department of Botany, Gulbarga University, Gulbarga - 585 106, Karnataka-India

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Abstract: The keratinase waste produced in large quantities all over the world from animals and birds including human beings. As the physiological and chemical methods of keratin degradation are not easy possible, the biological method has gained importance. The present study investigated purified keratinase from Keratinolytic *Streptomyces albus*. The cell-bound keratinolytic enzyme was purified 32.72-fold by gel filtration chromatography. The enzyme was characterized as a serine protease with a molecular mass of 29-35kD. Optimal activity pH and Temperature was measured at 7.0 and 40°C furthermore, the various inhibitors had different effect on enzyme activity. PMSF and heavy metal ion HgCl₂ were the most potent inhibitors and EDTA induced the activity by more than 135%, 2-mercaptoethanol did not show any impact on the enzyme, where pCMB, KCN, 8-hydroxyquinoline and cystine inhibited activity moderately.

Keywords: Keratin, *Streptomyces albus*, Hair, Keratinase, Purification

INTRODUCTION

To hydrolyze the keratin by synthesizing specific class of extracellular enzymes called keratinases, which degrade keratin into small peptide that can be utilized by the cells. Keratin occurs in nature mainly in the form of hair, horn, nails and confirmed tissue (Kual and Sumbali, 1999). Several feather and hair degrading *Streptomyces* have been isolated from soil, poultry wastes, hair, debris and animal skin. Keratinases a group of serine metallo proteases, release the free amino acids from keratinous proteins. Keratin is an insoluble, high stable protein found mostly in feathers, wool, nails and hairs of vertebrates (Shih, 1993). Keratin is resistant to the common proteolytic enzymes, papain, pepsin and trypsin (Papadopoulos et al., 1986). The high resistance of keratin to proteases may be attributed to the molecular conformation of their structural amino acids, that is tightly packed in the α -helices (hairs) and β -sheets (feather) in the presence of cystine disulfide bonds, hydrogen bonds and hydrophobic interactions (Parry and North, 1998). The enzyme has received particular attention for its relevant applications in various types of agro and biotechnological industries. After treatment with keratinase feather can be used as feeders, fertilizers and insoluble polymers (Yamauchi, 1996). Feathers consist of about 5-7% of total weight of mature chicken from poultry processing plants, approximated by about million tons produced annually, worldwide. Feather from the poultry processing plant is the common source for the accumulation of more than 90% of keratinous proteins in the environment, causing pollution (Onifade, 1998). A number of keratinolytic microorganisms have been reported, including some species of fungi such as *Microsporium* (Essien et al., 2009), *Trichophyton* (Anbu et al., 2008) and from the

bacteria *Bacillus* (Macedo et al., 2005; Pillai and archana, 2008; Cai and Zheng, 2009) and *Streptomyces* (Szabo et al., 2000; Tatini et al., 2008 Syed et al., 2009) and actinomycetes (Young and Smith, 1975; Bockle et al., 1995). Increase in keratinolytic activity is also found to be associated with thermophilic organisms, which require high energy inputs to achieve maximum growth and the decomposition of keratin wastes (Friedrich and Antranikian, 2002). The present paper reports on the optimization of methodology for keratinase production and its characterization using locally isolated *Streptomyces albus* a thermo tolerant actinomycete.

MATERIALS AND METHODS

Microorganism and inoculum preparation:

From a hot soil zone of the Gulbarga and Bellary, novel thermophilic actinomycete was isolated which was identified as *Streptomyces albus*, which was used for keratinase production. The spore suspension was prepared by scrapping the spores of *Streptomyces albus* from 7 days old culture grown on aspergine agar medium. The spore concentration was adjusted to 5X10⁶/ml.

Determination of keratinase activity:

The keratinase activity was assayed by the modified method of Cheng et al. (1995) by using keratin as a substrate with slight modification. The reaction mixture was prepared by mixing 1ml of 1% keratin in phosphate buffer (pH 8.0) and 0.5 ml of enzyme solution and incubated at 30°C for 30 min. After incubation, the reaction was terminated by adding 2 ml of 10% trichloroacetic acid (TCA). After the separation of untreated keratin precipitate by centrifugation, 1ml

*Corresponding Author:

Dr. Sreenivasa Nayaka,
Department of Botany,
Gulbarga University,
Gulbarga - 585 106, Karnataka-India



of clear supernatant was mixed with 5ml of 0.4 M Na_2CO_3 and 0.5 ml of Folin-Ciocalteu's phenol reagent. After 30 min, absorbance was measured at 660 nm against blank. All assays were done in triplicate. One unit of keratinase activity was defined as the amount of enzyme that released one microgram of tyrosine per minute under the standard assay conditions.

Determination of Protein content:

The protein content of the enzyme extract was determined by Folin-Phenol reagent (Lowry et al., 1951), using bovine serum albumin as a standard.

Purification and Characterization of Keratinase:

The *Streptomyces albus* were grown in optimized starch casein agar media and incubated in static condition at room temperature. The culture fluids of hair and feather were harvested on the 20th and 35th day of incubation, the point of view maximal enzyme activity.

Ammonium sulphate precipitation and dialysis:

The crude extract fluids (200 ml) were concentrated by 80% saturation using ammonium sulphate. The protein precipitate obtained was separated by centrifugation at 10,000 rpm for 10 min and the pellet was dissolved with minimum volume of phosphate buffer (56 mM, pH 7.8). The dissolved sample was dialyzed (Cellophane membrane, Sigma) against 5 mM phosphate buffer (pH 7.8) for 8 h.

Gel filtration: After dialysis, the sample (1.0 ml) was subjected to gel filtration fractionation with a sephadex G-100 column that had been equilibrated with 0.056 M phosphate buffer (pH 7.8). Elution was conducted at a flow rate of 15ml/h and 3ml of fractionation collected. The major peaks of keratinase were detected and the fractions containing these peaks were pooled separately. These pools were lyophilized for further purification.

Polyacrylamide gel electrophoresis (PAGE):

Preparative polyacrylamide gel electrophoresis with 7.5% gel was conducted to detect keratinase in pooled purified fractions. Protein bands were visualized with silver staining.

Effect of pH and temperature on keratinase activity: Keratinolytic activity of purified enzyme was measured in the range of pH 4 to 11 using following buffers: sodium acetate (pH 4.0-6.0), sodium phosphate (pH 7.0-8.0), and Tris-NaOH buffer (pH 9.0-11.0). The optimum temperature was determined by incubating reaction mixture at different temperature range from 20°C to 80°C for 20 min.

Effect of inhibitors: Purified *Streptomyces albus* keratinase was pre-incubated with each inhibitor at pH

7.0 for 1 h at 40°C and then assayed for residual activity was measured. The inhibitors used were: pCMB, EDTA, HgCl_2 , KCN, and 2-mercaptoethanol, 8-hydroxyquinoline, cystine and PMSF.

RESULT AND DISCUSSION

Purification of Keratinase:

The keratinase from hair was subjected for ammonium sulphate precipitation, dialysis and gel filtration chromatographic purification. In the hair sample, the crude enzyme exhibited 8.8×10^{-3} U/mg of specific activity (Table-1.1). In the ammonium sulphate saturated enzyme 15.3×10^{-3} U/mg activity was found and the specific activity of enzyme after dialysis was 21.2×10^{-3} U/mg. The gel filtration purification led to 28.8 U/mg keratinase. Over all purification fold achieved was 32.72. Similar purification protocols were used for keratinase from the solid cultures of bacterial species such as *Bacillus licheniformis* (Lin et al., 1992), *Chryso bacterium* sp. Kr6 (Riffel et al., 2003, 2007) and *Streptomyces* sp. (Bockle et al., 1995).

Table-1.1: Purification profile of *Streptomyces albus* keratinase

Sl. No	Fractionation step	Total protein (mg)	Total activity (kU)	Specific activity $\times 1000$ (U/mg)	Recovery (%)	Fold Purity
1	Crude culture fluid	67.0	0.59	8.8	100.0	1
2	Ammonium sulphate precipitation	32.0	0.49	15.3	47.7	1.73
3	Dialysis	16.0	0.34	21.2	23.8	2.40
4	Sephadex G-100	2.5	0.72	28.8	3.7	32.72

The elution pattern of gel filtration and purification of keratinase from hair samples clearly demonstrated that, the enzyme was eluted in early fractions (Fig.2.1). The sharp peak of activity was found along with the highest protein eluted samples. It was interesting to determine the elution of the enzyme in single peak in hair samples. The SDS-PAGE analysis of purified keratin from hair showed protein bands of 29-35 kD respectively (Fig.2.2). Keratinase activity (2.45kU/m) in crude culture extracts was similar to that of *Microsporium canis* (Tikiuchi et al., 1982) and *Trichophyton rubrum* (Sanyal et al., 1985). Concentration of the culture fluid by vacuum evaporation resulted decrease in keratinase activity. A similar result was observed following dialysis of enzyme samples with phosphate buffer (pH 7.8), even though the specific activity was increased. The elution pattern of the keratinase isolated from hair and feather displayed a single peak of activity (Malviya et al., 1992). The PAGE analysis of pooled eluents from gel filtration chromatography showed the presence of single band;

the keratinase purified by Malviya et al., (1992) had two subunits of 50 and 29 kD.

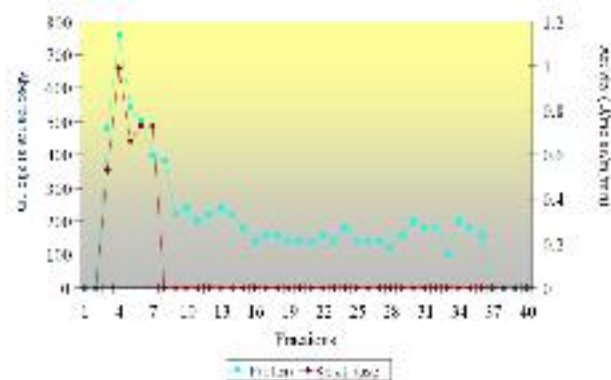


Fig 2.1: Gel filtration purification profile of keratinase from hair

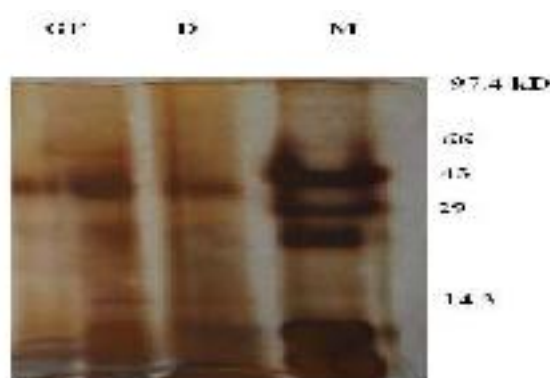


Fig 2.2: SDS- PAGE of purified keratinase from *S. albus*
M: (Protein) Molecular weight marker (medium range, Bangalore Genei, Bengaluru); D- Dialyzed keratinase; GF- gel filtration purified keratinase

The optimum pH for the activity of keratinase isolated was of 7.0 (Fig 2.3). This result is in agreement with those described for most feather-degrading *Bacillus* (Wang and Shih, 1999; Suntornasuk and Suntornasuk, 2003). For production of keratinase by *B. licheniformis* and a recombinant *B. subtilis*, uncontrolled pH operation was more favorable than the controlled pH operations (Wang and Shih, 1999). The same was observed for alkaline protease production by *B. licheniformis* (Çalik et al., 2002). The keratinase from *Chryseobacterium* sp. had optimum pH of 6.0 to 8.0 (Brandelli and Riffel, 2005). The enzyme samples, showed a gradual increase in the activity with increasing pH up to the optimum, followed by a gradual fall in the activity. The keratinase studied by Malviya et al., (1992) was also showed an optimum pH at 7.8.

The enzyme stability studied at various pH ranging from 4.0 to 11.0 showed a significant increase in the stability up to pH 8.0 (Fig 2.3), which declined thereafter. 100 percent stability was recorded at pH 7.0 to 8.0. The keratinase subunits analyzed by Malviya et al., (1992) were stable between pH 5.0-7.8. However, the

keratinase of *S. brevicaulis* were most active at alkaline pH. The phenomenon was similar to dermatophytic keratinase that degrade human hair keratin (Sanyal et al., 1985), wool (Weary et al., 1967), bovine hoof and horn (Meevootisom and Niederpruem, 1979).

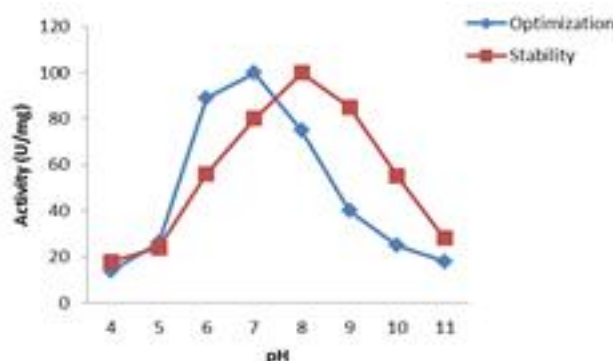


Fig 2.3: Effect of pH optimization and stability on activity of keratinase from *S. albus*

The effect of temperature on keratinase was studied from 20°C to 80°C. The activity increased from 20°C up to 80°C, but optimum temperature for the activity recorded at 40°C respectively (Fig 2.4). Proteases from *Chryseobacterium* sp. are often produced at mesophilic temperatures. A metalloprotease of *C. indologenes* Ix9a (Venter et al., 1999) and an endopeptidase of *Chryseobacterium* sp. (Lijnen et al., 2000) were produced during cultivation in nutrient broth at 25°C and 28°C. Although those conditions were considered satisfactory to produce proteolytic activity and other settings were not investigated. Although keratinolytic bacteria often display optimal growth and activity at higher temperatures (Lin et al., 1999; Kim et al., 2001), this is consistent with optimum values described for keratinolytic gram-negatives such as, *Vibrio* sp. kr2 (Sangali and Brandelli, 2000), *Lysobacter* sp. (Allpress et al., 2002) and *Stenotrophomonas* sp. D-1 (Yamamura et al., 2002), which showed optimum temperature for growth and keratinolytic enzyme production ranging from 20°C to 30°C. The keratinase from *Chryseobacterium* sp. had optimum temperature of 30°C (Brandelli and Riffel, 2005). The optimum temperature for keratinase activity reported by Malviya et al., (1992) was 40°C and 35°C, though K-I was more stable over a broader temperature range than K-II. The temperature stability decreased rapidly as temperatures increased above 40°C, similar to our observations.

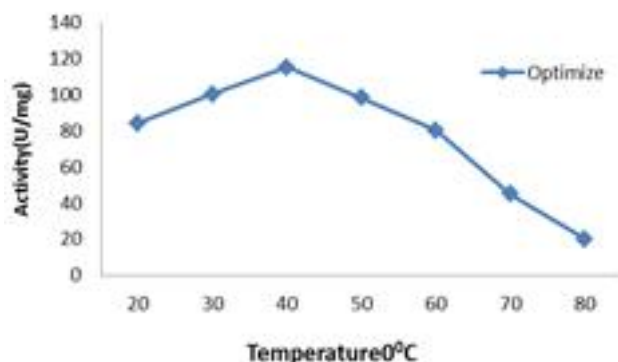


Fig 2.4: Effect of temperature optimization on activity of keratinase from *S. albus*

The various inhibitors had different effect on enzyme activity (Table 3.1). PMSF was the most potent inhibitor, as it completely killed the activity of enzyme, indicating serine residues at the active site of the Keratinase (Rajak et al., 1992). Similar observations were made by Malviya et al., (1992) for one of the subunits of keratinase. The production of serine proteases by keratinophilic fungi has been reported by several workers (Sanyal et al., 1985; Grzywnowicz et al., 1989; Rajak et al., 1992). It signifies that the keratinase is a protease, as PMSF is known to act only on proteases. The heavy metal ion $HgCl_2$ also inhibited the activity by 100%, signifying the enzyme is a serine protease. EDTA induced the activity of the enzyme by more than 135% stating that keratinase is a metallo-enzyme. Keratinase from a *Trichophyton* species has shown metal dependence for optimal keratinase activity. But reports of Malviya et al., (1992), Tikiuchi et al., (1982) and Meevotism and Niederpruem (1979), reported weak inhibition of keratinase by EDTA indicating keratinase is non-metal dependent enzyme. 2-mercaptoethanol did not show any impact on the enzyme, where pCMB, KCN, 8-hydroxyquinoline and cystine inhibited the activity moderately. Close scrutiny of the properties of keratinase revealed that their properties were in many respects identical to the enzymatic activities of dermatophytes (Sanyal et al., 1985; Grzywnowicz et al., 1989). Keratinases have enormous potential applications in processing waste in the poultry and leather industries. The recent finding showed that the keratinase of *B. licheniformis* PWD-1 cause enzymatic breakdown of prion protein Pr PSc (Langeveld et al., 2003) leave open a novel relevant application for broad range keratinases. The present study adds to the knowledge of keratinase to take up further study for efficient applications in industries and environmentally.

Table-3.1: Effects of various inhibitors on the activity from *S. albus*

Inhibitor	Concentration (mM)	Residual activity (%)
		Hair
PMSF	1	0
$HgCl_2$	1	0
EDTA	5	135
2-Mercaptoethanol	2	100
pCMB	1	69
KCN	5	73
8-hydroxyquinoline	1	61
Cystine	5	74

Enzyme activity without the addition of inhibitor was considered 100%. All the values are the mean of three independent assays.

CONCLUSION

The study reveals the potentiality of the local isolate *streptomyces albus* to degrade keratinous substrates producing keratinase was purified and characterization. The microbial source has important to the bio-treatment particularly for dehairing and removing of substance like hair, feather and wool causes environmental pollutants. Further work is focused industrial and also solves the waste disposal problem of poultry waste and with limited resources recycling of Keratinaceous waste used in different fields environmentally.

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