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# A keratinolytic actinomycete

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An actinomycete was isolated in this laboratory from the soil samples collected from various waste protein dumped areas of West Bengal. The actinomycete (TMA-13) degraded human hair, chicken feather, and wheat bran and produced 7.4, 7.6 and 6.0 specific activity of protease respectively after 12 day incubation at 28⁰C. Using chicken feather as substrate, the inducible keratinolytic protease was isolated, purified and characterised. After DEAE-cellulose column chromatography the enzyme activity was increased by 8.7 fold. The molecular weight of the enzyme was 50,000 dalton. In presence of 5 mM $Ca^{2+}$ the activity of the keratinolytic protease was incresed by 43 %. EDTA completely inhibited the enzyme activity. The partly purified enzyme hydrolysed whole chicken feather completely at 37⁰C. The actinomycete was later identified as *Streptomyces* sp. (TMA-13). When solid state fermentation was carried out, more than 4 fold production of the enzyme was noted.

**Key words :** *Streptomyces* sp., keratinolytic protease, purification, solid state fermentation

## INTRODUCTION

Keratin is characterized by its compact structure, usually insoluble and high content of cystine. Keratin is not degraded by all organisms but it does not get accumulated in nature. Many keratinophilic fungi (Bahuguna and Kushwaha, 1989), dermatophytes (Asahi *et al.*, 1985), few bacteria (Lin *et al.*, 1992) and very few actinomycetes (Nickerson *et al.*, 1963) have been reported to degrade keratinous materials despite its sturctural rigidity. Those microbes produce keratinolytic proteases which play key role in keratin hydrolysis. Keratinolytic proteases are reported to play important roles in the biotechnological applications like improvement of feather meal (ingredient of poultry feed), production of keratin hydrolysates (amino acids and peptides) and dehairing of hides and skins (in leather industry). Besides it can also play the role of an active constituent of the depilatory formulations of human use.

One actinomycete has been isolated in this laboratory amongst 122 microbial isolates and has been designated as TMA-13. It has been later identified as *Streptomyces* sp. This streptomycete degrades human hair, chicken feather and wheat bran suspended in basal salts medium and produces 7.4, 7.6 and 6.0 specific activity units of protease respectively after 12 days incubation at 28°C. The keratinolytic proteae has been tested for its ability to degrade waste whole chicken feather, purufied and characterized. For the production of higher amount of keratinolytic protease solid state fermentation has been carried out.

## MATERIALS AND METHODS

### *Organism*

*Streptomyces* sp. TMA-13, a proteolytic soil isolate was maintained on yeast extract malt extract agar medium (Pridham *et al.*, 1956/57).

The protease production medium contained (g/l) glucose, 20 ; $K_2HPO_4$, 1.5 ; $MgSO_4$, $7H_2O$, 0.05 ; $CaCl_2$, 0.25 ; $FeSO_4$, $7H_2O$, 0.015 ; $ZnSO_4$, $7H_2O$, 0.005 ; $KNO_3$, 5.0 ; pH-7.0. Fifty ml of the medium was distributed in 250 ml flasks and sterilized at 121°C for 20 min. Human hair, chicken feather and wheat bran were sterilized successively for 3 days at 108°C for 5 min. Five g/ml of the substrates were added separately to each flask, inoculated with approximately $4\times10^7$ spores from 5 days old

cultures of the actinomycete and incubated on a shaker at 28°C for 12 days. For purification chicken feather was added as substrates since in its presence the actinomycete produced more specific activity units of the protease.

The extra-/intracellular nature of the enzyme was determined by the method of Mukhopadhyay and Chandra (1990). The final pH of the culture filtrates was determined by a pH meter. Keratinase and caseinase activities were assayed following the methods of Yu *et al.,* 1972 and Yoshida and Noda (1965) respectively.

### *Purification of the extracellular enzyme*

The culture filtrate was separated from the cell mass and residual feather by centrifugation at 10,000 rpm for 15 min. The supernatant was saturated with 80 and 100 % $(NH_4)_2SO_4$, allowed to stand for overnight in a refrigerator and centrifuged at 10,000 rpm for 30 min. The pellet was dissolved in 0.1M potassium phosphate buffer (pH-7.8), dialysed against 0.01M of the same buffer for 16 h with several changes of the dialysate and assayed for caseinase and keratinse activities. Degradation of the whole feathers were noted by dipping sterilized chicken feathers aseptically in tubes containing 5 ml sterilized 0.1M K-phosphate buffer and 1 ml enzyme solution containing 93.4 μg protein and incubating at 30°C for 48 h. The fraction showing highest keratinolytic activity was loaded on a DEAE-cellulose column eqilibrated with phosphate buffer, followed by three washes successively with the buffer containing 0.1, 0.25 and 0.5 M NaCl. Total 100 fractions of 3 ml were collected. Keratinase and caseinase activities were assayed and the protein of each fraction was measured by the method of Lowry *et al.* (1951). The fractions possessing maximum keratinase activity were pooled, dialysed and subjected to polyacrylamide gel electrophoresis (PAGE).

PAGE of the pooled fractions showing maximum keratinase activity after DEAE-cellulose column chromatography was carried out according to the method of Davis (1964) and in the same way as reported by Mukhopadhya and Chandra (1990).

### *Determination of molecular weight*

Molecular weight was determined by SDS-gel electrophoresis following the method of Laemli (1970), using carbonic anhydrase (29,000), egg albumin (45,000), bovine serum albumin (66,000) and phosphorylase b (97,400) as standards. Effect of metal ions, modifiers and other parameters like temperature, pH, incubation period and substrate concentration was studied following the methods of Mukhopadhyay and Chandra (1990).

### *Solid state fermentation (SSF)*

Waste chicken feathers were collected from poultry, cleaned and defatted in the mixture of chloroform and methanol (3 : 1) for 30 min. The defatted feathers were then washed several times with distilled water and air-dried. Feathers were then cut into small pieces of 1.5-2.0 cm. 5 g of feather pieces were taken into 250 ml flasks, sterilized at 108°C for 5 min for successive 3 days. In each flask 10 ml production medium was added. Flasks were inoculated with approximately $4\times10^7$ spores/ml from 5 day old cultures, incubated at 28°C for 12 days in stationary condition. After incubation 25 ml 0.1M K-phosphate buffer (pH-7.8) was added and the flasks were shaken vigorously on a shaker for 30 min. The contents of the flasks were then collected and centrifuged at 10,000 rpm for 15 min. The pellet was discarded and supernatant was assayed for protein, keratinase and caseinase.

## RESULTS AND DISCUSSION

Native keratin is not degraded by usually available proteases. Keratinase is defined as the proteinase with keratinolytic activity on a native keratin (Noval and Nickerson, 1959). Keratinolytic activity of the enzyme was measured by spectrophotometric method (Yu *et al.,* 1972).

The actinomycete degraded human hair, chicken feather and wheat bran and produced 7.4, 7.6 and 6.0 specific activity Units of protease respectively. The final pH of the culture filtrate rose to 8.2, 8.4 and 8.0 from 7.0 with the substrates added separately to the cultures in the same sequence. Change of pH of culture filtrate was an indication of degradation of protein (Kunert, 1989). Chicken feather was used as the inducer of the enzyme for further studies.

The specific activities of culture filtrate and cell free extract were 7.6 and 0.06 keratinase Units respectively which indicated that the enzyme was inducible and extracellular. The supernatant when treated with $(NH_4)SO_4$, maximum activity for keratinase was obtained at 0-80 % saturation (Table

1). The enzyme of this fraction showed its ability to hydrolyse the whole feather at 37-50° C (Fig. 1) Column chromatography with DEAE cellulose of this fraction and elution with phosphate buffer (0.1M, pH-7.8) containing 0.1, 0.25 and 0.5 M NaCl resulted the distribution of keratinase activities in the fractions 70-89 (Fig. 2) eluted with 0.25 and 0.5 M NaCl. In other fractions keratinase activity could not be detected. The highest keratinase activity was found in fraction 76. The elution profile showed that caseinase activity was present in fractions 30-63. Polyacrylamide gel electrophoresis of the pooled and dialysed fractions 70-89 produced single band. SDS-polyacrylamide gel electro-phoresis of the pooled and dialysed fractions 70-89 also produced single band (Fig. 3.) which indicated the absence of subunit and the molecular weight was determined to be 50,000. It was slightly higher than the keratinase produced by *Trichophyton mentagrophytes* (48,000) (Yu *et al.,* 1972) and *Streptomyces* sp. $A_{11}$ (49,000)

**Table 1** : Purificatin of keratinolytic protease produced by the *Streptmyces* sp. TMA-13.

| Purification step | Total volume (ml) | Total protein (mg) | Keratinase[a] activity U mg$^{-1}$ | | | Caseinase[b] activity U mg$^{-1}$ | | |
|---|---|---|---|---|---|---|---|---|
| | | | Specific | Total | % Yield | Specific | Total | % Yield |
| Culture filtrate | 250 | 375 | 7.6 | 2850 | 100 | 3.0 | 1125 | 100 |
| $(NH_4)_2$ $SO_4$ ppt. | | | | | | | | |
| 0 — 80%[c] | 30 | 125 | 14.2 | 1575 | 62.3 | 2.8 | 350 | 31.1 |
| 80 — 100% | 10 | 9.0 | 0.5 | 4.5 | 0.15 | 1.1 | 99 | 8.3 |
| 0 — 80% saturation Charged n DEAE-Cellulose column (fractions 74-80) | 21 | 16.1 | 66 | 1062.6 | 37.2 | Nil | Nil | Nil |

[a] Keratinase Units/mg protein (1 keratinase Unit = 0.1 corrected absorbance at 280 nm) ; [b] Absorbance at 273 nm/h/mg protein ; [c] Saturation

**Table 2** : Effect of modifiers at 3 and 5 mM concentrations on the activity of the keratinolytic protease produced by the *Streptomyces* sp. (TMA-13)

| Compound | % Activity | |
|---|---|---|
| | 3 mM | 5 mM |
| None | 100 | 100 |
| EDTA | 00 | 00 |
| Reduced glutathione | 27 | 22 |
| Para chloromercurie benzoate | 56 | 42 |
| Phenylmethylsulfonylfluoride | 31 | 29 |
| 2-mercaptoethanol | 32 | 21 |

**Table 3** : Effect of metal ions at 3 and 5 mM concentrations on the activity of the keratinolytic protease

| Metal ions | % Residual activity | |
|---|---|---|
| | 3 mM | 5 mM |
| None | 100 | 100 |
| $Ca^{2+}$ | 115 | 143 |
| $Mg^{2+}$ | 103 | 98 |
| $Zn^{2+}$ | 95 | 97 |
| $Fe^{2+}$ | 107 | 106 |
| $Co^{2+}$ | 58 | 40 |
| $Mn^{2+}$ | 78 | 67 |
| $Pb^{2+}$ | 28 | 25 |
| $Hg^{2+}$ | 21 | 19 |
| $Ba^{2+}$ | 17 | 11 |
| $Ag^{2+}$ | 09 | 05 |

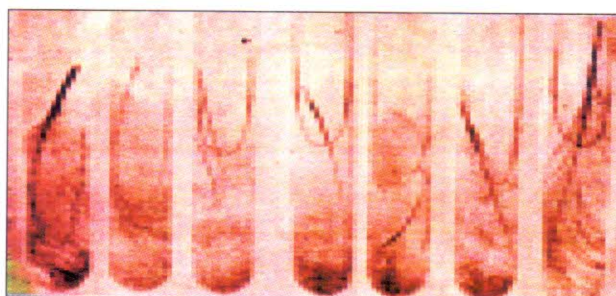

**Fig. 1** : Hydrolysis of whole chicken feather by the partly purified keratinolytic protease produced by the *Streptomyces* sp. (TMA-13). Each tube, except the control, was added with 93.4 µg protein and incubated at various temperatures for 48 h.

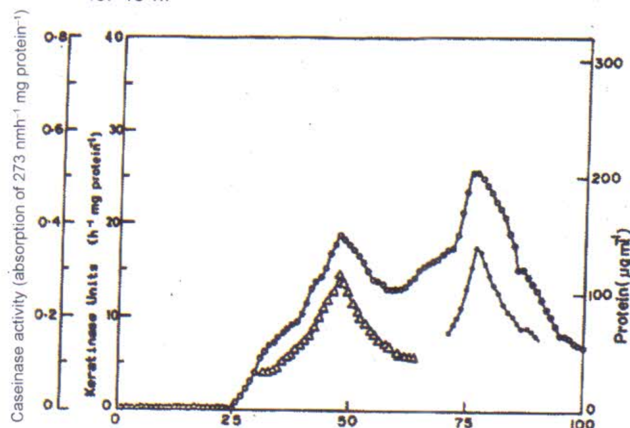


**Fig. 2** : Elution profile of protein, 0-0 ; keratinase, •-• and caseinase, Δ-Δ.

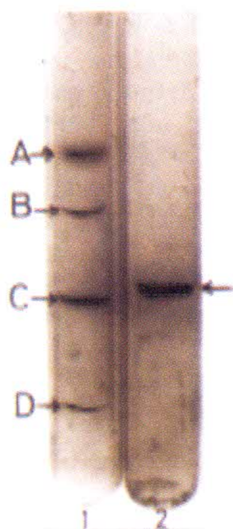


**Fig. 3 :** SDS-Polyacrylamide gel electrophoresis of standard proteins and purified keratinase of *Streptomyces* sp. (TMA-13). **Lane 1 :** A. Phosphorylase b (97, 400) ; B. Bovine serum albumin (66,000) ; C. Egg albumin (45,000) ; D. Carbonic anhydrase (29,000) ; **Lane 2 :** Purified keratinase. ( indicates the position of band).

(Mukhopadhyay and Chandra, 1990). However, the molecular weight of the keratinolytic serine protease of *S. pactum* DSM40530 was 30,000 where there was another peak of keratinolytic protease after elution (Bockle *et al.,* 1995). Among the modifiers tested EDTA completely inhibited the enzyme activity. Reduced glutathione, phenyl methyl sulphonyl fluoroide and 2-mercaptoethanol at 5 mM concentrations reduced the enzyme activity to 22,29 and 21 % respectively (Table 2). $Ca^{+}$ at 5 mM concentration increased the enzyme activity by 43 % but $Pb^{2+}$, $Ba^{2+}$, $Hg^{2+}$ and $Ag^{2+}$ strongly inhibited the enzyme activity (Table 3). The optimal activity of the purified enzyme was found at 30°C, pH 7.8, incubation for 2 h and the substrate concentration of 10 mg/ml.

After the solid state fermentation production of the keratinolytic protease was much more than the liquid culture. When the enzyme was extracted, saturated with 80 % $(NH_2)SO_4$, dialysed and assayed 59.28 specific activity units of the keratinolytic protease was obtained. Escalation of the enzyme production by SSF was reported earlier (Mukhopadhyay and Chandra 1994).

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