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A streptomycete collagenase

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Streptomyces sp. A_{11}, a collagenolytic soil isolate hydrolysed different types of collagen. The enzyme was isolated and purified. The enzyme was found to be extracellular and inducible. The specific activity units of the culture filtrate and the purified enzyme were 5.0 and 37.1 respectively. The molecular weight of the enzyme was 74,000. With bovine Achilles tendon, human placenta, calf skin and rat tail tendon collagen, the Km values obtained at pH 7.8 and 30°C were 2.3, 3.6, 3.7 and 2.7 mg/ml respectively. With synthetic peptide Km value was 31×10^{-5} M at the same pH and temperature. In the presence of 5 mM Ca^{2+}, collagenase activity increased by 10.7%. With silver nitrate as non competitive inhibitor, the Ki was found to be 1.5×10^{-3}M.

Key words: *Streptomyces* sp., collagenase, Km value

INTRODUCTION

Collagenases are responsible for the degradation of native collagen at physiological pH. The enzyme is useful in clinics and laboratories (Mandl, 1961; Mauro *et al.,* 1984). Many microorganisms are reported to produce collagenase. *Clostridum histolyticum* has been reported to produce collagenase (Jennison, 1945). Later, the effect of divalent metal ions on the collagenase produced by *C. histolyticum* has been reported (George *et al,* 1991). The collagenolytic activity of 110 aerobic, halotolerant strains, isolated from cured hides has been reported (Thompson *et al.,* 1972). *Achromobacter iophagus,* one of the halotolerant strains has showed rapid aerobic degradation of collagen. Changes in temperature, pH, NaCl and collagen concentration affected either the maximum rate of collagen hydrolysis by the organism or lag before this rate of hydrolysis has attained on both (Welton and Woods, 1973). The collagenolytic enzyme produced by *Candida albicans* has been isolated and characterized (Kaminishi *et al.,* 1986). *Aspergillus niger* LCF9 synthesises a new semialkaline proteinase with high collagenolytic activity. The enzyme hydrolyses nine commercially available collagens and liberated low molecular weight peptides of potential interest in therapeutics (Chantel *et al.,* 1992). The collagenolytic activity of extracellular vesicles of *Bacteroides gingivalis* W50 and an avirulent variant W 50/BE 1 has also been reported (Smalley *et al.,* 1989). *Streptomyces madurae* is reported to produce an extracellular collagenase, specific for native collagen and its gelatin (Rippon, 1968). A collagenase, active against native, insoluble collagen is isolated from the culture filtrate of *Streptomyces* sp. C-51, grown in media containing gelatin (Endo *et al.,* 1987). Collagenase activity in a *Streptomyces* sp. is reported and the enzyme is observed after 72-96 h of cultivation when gelatin is added as an inducer (Demina and Lysenko, 1992).

We have isolated *Streptomyces* sp. A_{11}, a keratinolytic soil isolate. The keratinase is purified and characterized (Mukhopadhyay and Chandra, 1990). The streptomycete is also found to degrade bovine Achilles tendon (BAT) (type-I), calf skin (CS) (type-III) and human placenta (HP) (type-VI) collagen.(purchased from Sigma Chemical Co., USA). Collagen extracted from carp swim bladder (CSB) and rat tail tendon (RTT) (Gallop and Seifter,

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1963; Bermann *et al,* 1973) in the laboratory are also degraded. Here the extraction, purification and characterization of the collagenase produced by the streptomycete is reported.

MATERIALS AND METHODS

Organism-Streptomyces sp. A_{11}, a soil isolate

Growth and enzyme production–The organism was maintained in yeast extract and malt extract agar (Pridham *et al.*,1956/57). Collagen was sterilized by exposure to 1 ml ethylene oxide for 12 h per liter space of desicator. A medium containing (g/l) distilled water K_2 H PO_4, 1.5; $MgSO_4.7H_2O$, 0.05; $FeSO_4.7H_2O$, 0.015; $CaCl_2$, 0.25; $ZnSO_4.7H_2O$, 0.005; Glucose, 30.0; L-lysine, 2.5; was adjusted to pH 7.5. Two grams of sterilized BAT collagen was added aseptically to 100 ml of the medium, inoculated with 5×10^6 spores obtained from 5 days old cultures of the streptomycete and incubated with shaking at 28° C for 6 days. The culture supernatants and cell free extracts were prepared and used as extra-and intracellular enzymes.

Collagenase assay—Collagenase was assayed by mixing 4 mg BAT collagen, 0.2 ml of culture filtrate/ purified enzyme and 0.8 ml Tris-HCl buffer (0.1M, pH 7.8) and incubating at 30° C for 2 h. After incubation, 0.5 ml of 5% TCA was added. The mixtures were centrifuged and the supernatants were assayed for glycine equivalents with ninhydrin (Spies 1957). Specific activities were calculated as glycine equivalents released/min/mg protein. Hydrolysis of synthetic peptide (Pz-L-Pro-L-Leu-Gly-L-Pro-D-Arg) purchased from Sigma Chemical Co.USA, was carried out according to the method described (Wunsch and Heidrich, 1963).

Caseinase assay—Caseinase activity was assayed by the procedure of Yodhida and Noda (1965) and calculated as micromoles tyrosine liberated/h/mg protein.

Estimation of protein—Protein estimation was done according to the method of Lowry *et. al* (1951) using bovine serum albumine as a standard.

Purification of extracellular collagenase—All experiments were performed at 4°C. After 6 days incubation, the culture broth was centrifuged, the supernatant saturated to 30, 60, and 80% with $(NH_4)_2SO_4$, allowed to settle for overnight and centrifuged at 12000 g for 30 min. The sediment was dissolved in Tris HCl buffer (0.1 M, pH 7.8), dialised against 0.01 M of the buffer for 16 h (with several changes of the dialysate) and assayed for collagenase and caseinase activities. The fraction showing the highest collagenase activity was loaded on a 1.2 × 29 cm DEAE cellulose column equilibrated with Tris HCl buffer (0.1M, pH 7.8). The column was then washed several times with the same buffer and adsorbed proteins were eluted with 180 ml of the buffer containing 0.1, 0.25 and 0.5 M NaCl. The flow rate was adjusted to 15 ml/h. A total of 100 fractions of 4 ml each were collected. Protein content of each fraction was estimated and collagenase and caseinase activities were assayed.

Polyacrylamide gel electrophoresis (PAGE)—The fractions possessing maximum collagenase activity were pooled, dialysed and subjected to PAGE (Davis. 1964). Electrophoresis was carried out in Tris-glysine buffer (pH 8.2-8.4) for 3 h with a current of 2.5 mA per gel tube. Gels were stained with Coomassie blue (0.1% v/v methanol : acetic acid : water, 5 : 5 : 1) for 2 h and destained with a solution containing 7.5% acetic acid and 5% methanol in water. Three gels were kept unstained and sliced. The enzyme from the slices (1 mm thick) was extracted with Tris-HCl buffer (0.1 M, pH 7.8) and assayed for collagenase activity.

Determination of molecular weight

Hedrick-Smith method—The molecular weight of the enzyme was determined by the method of Hedrick, and Smith, (1968). Disc gel electrophoresis was used for this study. The enzyme and bovine serum albumine (BSA), a standard protein were run in various concentrations on native—PAGE. Relative mobilities of the protein bands of BSA and active band of the enzyme were measured, and the molecular weight of the enzyme was determined.

SDS-PAGE–This was done according to the method of Laemli (1970). The marker proteins (Sigma, USA) carbonic anhydrase (29,000), egg albumine (45,000), bovine serum albumine (66,000) and phosphorylase b(97,400) as well as the purified

collagenase were added separately to the sample buffer and treated for 2 min in a boiling water bath. The treated samples were charged on the top of the gel and 2.5 mA current/tube passed for 2 h and 30 min. Gels were removed, fixed in a fixative (containing 10% acetic acid and 50% methanol) overnight, stained with Coomassie blue for 2 h and destained by repeated washing.

Effect of pH on enzyme stability–The purified enzyme was treated for 16 h at pH 4.8 to 8.4 (pH 4.8 to 6.8 in 0.1M Tris-maleate and pH 7.0 to 8.4 in 0.1 M Tris-HCl buffers) and then collagenase and caseinase activities were assayed.

Effect of temperature on enzyme stability–The enzyme was treated for 30 min at temperature ranging from 20 to 50°C and then the assay of the enzymes were carried out.

Effect of substrate concentration on enzyme activity–Different types of collagen were added separately to the reaction mixture at 2, 4, 6, 8, 10, 12 and 14 mg/ml at optimum pH, temperature and incubation period. The Km and Vmax values were calculated from Lineweaver Burk and Michaelis Menten plots respectively.

Determination of inhibitor constant (Ki)–The final concentrations of $AgNO_3$ in the collagenase assay mixtures were 1, 2, 3, 4, 5 mM. BAT collagen was tested at 4 and 6 mg/ml concentrations. Determination was done according to the method of Dixon (1954).

RESULTS AND DISCUSSION

The collagenolytic activity of the culture filtrate was

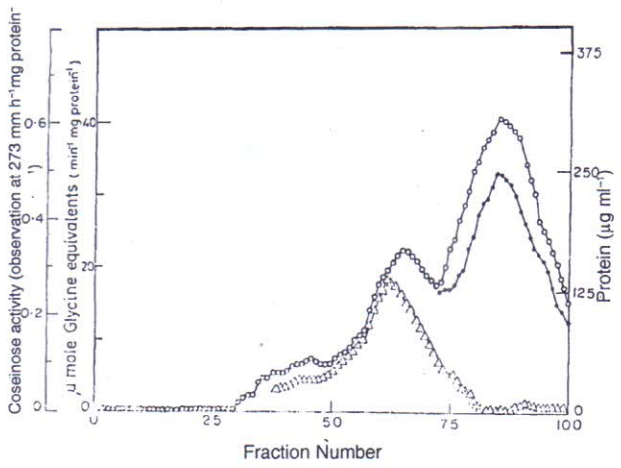

Fig. 1: Elution profile of proteins, 0; collagenase, caseinase. Δ;

maximum at pH 7.8 and at 30°C. A 7.4 fold increase of collagenolytic activity was obtained after DEAE-cellulose column chromatography (Table 1, Fig. 1). The enzyme showed single band after PAGE (Fig 2). The corresponding regions of the band on the other gels, when the enzyme was eluted and assayed after they were sliced, showed collagenolytic activity. It produced single protein band and its molecular weight was calculated to be

Table 1. Purification of collagenase produced by *Streptomyces* sp. A_{11}.

Purification Step	Volume (ml)	Total Protein (mg)	Collagenase activity			Caseinase activity			Hydrolysis of synthetic peptide		
			Specfic[a]	Total	Yield (%)	Specific[b]	Total	Yield (%)	Specifc[c]	Total	Yield (%)
Culture filtrate	250	375	5.0	1875	100	0.06	22.5	100	0.04	15	100
$(NH_4)_2SO_4$ ppt.											
0—30%[d]	12	20.4	5.2	106	5.6	0.09	1.8	8	0.15	3.6	24
30—60%	18	36	16.1	579.6	30.9	0.30	10.8	48	0.2	7.2	48
60—90%	10	10	4.9	49	7.6	0.08	3.9	17.3	0.1	1	66
DEAE-cellulose column chroma-tography (fraction 86-88)	12	15	37.1	536.5	29.6	0.03	0.45	2	0.38	5.7	38

a. micromoles glycine equivalents/min/mg protein
b. micromole tyrosine/h/mg protein
c, Pz-L-Pro-L-Leu-Gly-Pro-D-Arg, pkat/ml/sec/mg protein
d, Saturation.

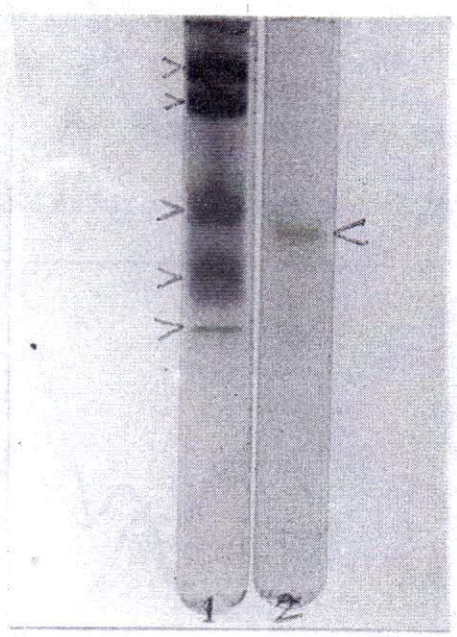

Fig. 2. Polyacrylamide gel electrophoretic patterns of collagenase produced by *Streptomyces* sp. A_{11}.
1) 30-60% $(NH_4)SO_4$ saturated.
2) Purified (> indicates the position of band)

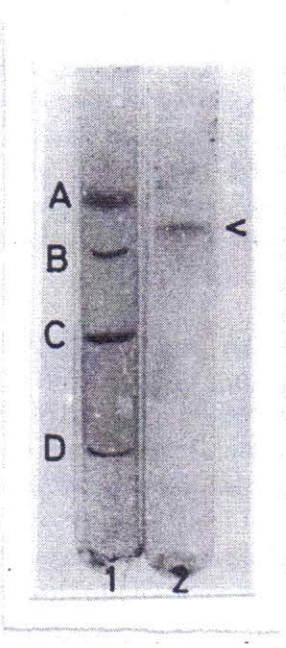

Fig. 3. SDS-Polyacrylamide gel electrophoresis of standard proteins and purified collagenase of *Streptomyces* sp. A_{11}.
Lane 1. A. Phosphorylase b (97,400)
B. Bovine serum albumin (66,00)
C. Egg albumin (45,000)
D. Carbonic anhydrse (29,000)
Lane 2. Purified collagenase.

about 74,000 when studied by PAGE. SDS-PAGE of the collagenase also produced single band (Fig. 3) and the molecular weight was calculated to be about 74,000.

The purified enzyme, when treated at pH 4.8 to 8.4 was found to be stable from pH 7.2 to 8.2 with maximum activity at 7.8. At pH 7.8, 487 µg glycine equivalents/ml were released. The enzyme was active at pH range of 7.5 to 8.2 with maximal activity at pH 7.8. The collagenase showed optimal activity at 30°C and at this temperature 475µg glycine equivalents were released/ml reaction mixture.

Table 2. Effect of inhibitors on the activity of collagenase produced by *Streptomyces* sp. A_{11}

Inhibitor	Residual activity* (%)	
	3 mM	5 mM
Sodium azide	70.1	41.3
Ethylenediamine tetraacetic acid (EDTA)	19.0	00.0
2-mercaptoethanol	61.4	54.0
Reduced glutathione	71.3	65.3
L-cysteine	68.6	51.6
p-chloromercuriebenzoate (pCMB)	20.8	15.8
Phenylmethylsulphonylfluoride (PMSF)	42.3	27.4
8-Hydroxyquinoline	33.8	32.6

* Collagenase activity without addition of inhibitors was taken as 100%

Five mM concentration of EDTA inhibited the enzyme activity completely, Sulphydryl compounds, like reduced glutathione and 2-mercaptoethanol inhibited the enzyme activity by 34.7 and 16% respectively. The chelating agent 8-hydroxyquinoline inhibited collagenase activity by 67.4%. Compounds known to affect –SH enzymes, p-chloromercuriebenzoate and sodium azide were inhibitory at 5 mM and 84.2 and 58.7% of the activity were inhibited respectively. Enzyme activity was inhibited by 72% with 5 mM Phe $MeSO_2F$ (Table 2).

Addition of glycine, L-proline and L-hydroxproline resulted in decrease in enzyme activity to 79.3, 79.8 and 71.4% respectively. Increase in concentrations of these 3 amino acids resulted in further decrease in enzyme activity (Table 3). Separate control sets with the amino acids at the mentioned concentrations in distilled water was reacted with ninhydrin. Amounts obtained were subtracted from

Table 3. Effect of amino acids on the activity of collagenase produced by *Streptomyces* sp. A_{11}.

Amino acids	Residual activity* (%)					
	1 mM	3 mM	5 mM	7 mM	11 mM	13 mM
Glycine	93.5	86.6	79.3	71	59.8	51
L-Proline	89.1	85.7	77.8	70.6	62.3	47.3
L-Hydroxyproline	90	84.1	71.4	64.8	58.7	48
L-Leucine	95	89.7	80.7	—	—	—
L-Isoleucine	100	99.1	99.1	—	—	—
L-Alanine	98	99	99	—	—	—
L-Glutamic acid	100	100	100	—	—	—
L-Lysine	100	100	100	—	—	—

*Collagenase activity without addition of amino acids was taken as 100%; –Not done

ninhydrine positive materials obtained from the assay mixture.

Among the metal ions Ca^{2+} stimulated collagenase activity to 110% and Mg^{2+} decreased it to 9.4% at 5 mM concentrations. All other metal ions tested were inhibitory to collagenase activity (Table 4).

Table 4. Effect of metal salts on the activity of collagenase produced by *Streptomyces* sp. A_{11}.

Metal salts	Residual activity* (%)		
	1 mM	3 mM	5 mM
$CuSO_4$	42.6	32.3	26.1
$AgNO_3$	40.2	40	38.3
$HgCl_2$	27	11.1	9.4
$MgSO_4$	45	33	25
$MnCl_2$	34.4	45.5	62.6
$ZnSO_4$	52.3	30.3	21.1
$CaCl_2$	100	103	110.7
$CoCl_2$	91.1	55	42.6
$CdCl_2$	32.3	50	54.1
$SnCl_2$	78.8	43.2	41.4

*Collagenase activity without metal salts was taken as 100%

Ten mg of BAT. HP and CS collagen/ml reaction mixture were found to be the optimum substrate concentration for collagenolytic activity. The specific activity units obtained were 22, 16.6 and 18.5 respectively, RTT collagen at 8 mg/ml produced specific activity of 9.8 units (Table 5). Eighty milimole of Pz-L-Pro-L-Leu-Gly-L-Pro-D-Arg was the concentration of the synthetic peptide at which optimal activity was noted. The Km values obtained at pH 7.8 and 30°C with BAT, HP, CS and RTT collagen were 2.3, 3.5, 3.7 and 2.6 mg/ml respectively. The Km value obtained with the synthetic peptide was 3.1×10^{-5} M. The Ki was estimated to be 1.5×10^{-3}M with $AgNO_3$.

Table 5. Km values of collagenase produced by *Streptomyces* sp. A_{11} with various substrates

Substrates	Vmax*	Km
Bovine Achilles tendon collagen	22	2.5
Human Placenta collagen	16.6	3.6
Calf skin collagen	18.5	3.7
Rat tail tendon collagen	9.8	2.6
Synthetic peptide (Pz-L-Pro-L-Leu-Gly-L-Pro-D-Arg)	0.230**	3.1×10^{-5}

* micromole glycine equivalent/min/mg protein

**, absorption at 320 mm/h/mg protein

Most of the collagenases are extracellular with few exceptions. The collagenase of *Bacteroides melaninogenicus* and *B. gingivalis* were not extracellular (Gibbons and Mc Donald, 1961). The most important problem during the purification of a collagenase is to avoid the formation of artifact caused by proteolysis because many microorganisms secrete other extracellular proteolytic enzymes into the medium along with collagenase. A notable example of such secretions is the report in *Clostridium histolyticum* which produced collagenase (EC.3.4.24.3) and clostripain (EC 3.4.22.8). So in the purification of our collagenase, extreme care was taken to eliminate other enzymes. After DEAE-cellulose column chromatography, maximum collagenase and no caseinase activity was in the fractions 86-88 (Fig. 1). Collagenase activity of fractions 30-70 was not measured because of the presence of caseinolytic activity.

When fractions 86-88 were pooled, dialysed and subjected to PAGE, one band was obtained indicating homogeneous nature of the enzyme. The corresponding regions of the bands on the other gels, when sliced, eluted and assayed, showed collagenolytic activity. Since native, insoluble

collagen was used for induction, this enzyme could be established as a true collagenase. The extraction of collagenase in homogeneous states has also been reported (Endo *et. al,* 1987). Since hydrolysis was measured with insoluble BAT collagen, the ability of the enzyme to achieve optimum hydrolysis within 2 h was noteworthy. Of special significance for bacterial collagenase was the absence of sulfur containing amino acids particularly, cyst(e)ine (Keil, 1989). This matter is of fundamental importance in the studies on the inactivation by sulfhydryl containing agents. The activity of the collagenase produced by *Streptomyces* sp. A_{11} was also inhibited by sulfhydryl compound, cysteine (Table 2). Cysteine inhibited the collagenase produced by *Clostridium histolyticum.* This inhibition was attributed to metal sequestration (Mandl, 1961)

Most of the metals were inhibitory at all concentrations tested. Of these Ca^{2+} exerted the stimulating effect. The stimulating effect of Ca^{2+} was also found for collagenase activities of other microorganisms (Lin *et al,* 1987).

Silver nitrate was a strong inhibitor of the enzyme activity. From a Dixon plot it was found that $AgNO_3$ was a non competitive inhibitor.

The molecular weight of the streptomycete collagenase was 74,000 (Fig. 3). Proteins of such high molecular weights have the possibility of being composed of smaller subunits. However, molecular weights of collagenase determined by SDS-PAGE and Hedrick-Smith methods were similar to each other and therefore, the enzyme had no subunit.

The parameters of an enzyme to be collagenase as laid down by other authors were checked repeatedly and the enzyme produced by *Streptomyces* sp. A_{11} was found to be a true collagense. By its high molecular weight, sensitivity to chelating agent and Ca^{2+} requirement for activity, the streptomycete collagenase could be included in metalloproteinase group (Keil, 1979).

The collagenase produced by *Streptomyces* sp. A_{11} was compared with collagenase produced by streptomycetes reported earlier. Collagenase of this report and other reported earlier were inhibited by EDTA. The optimum pH for the collagenase of this report were 7.5-8.2 whereas pH optima for two other streptomycete collagenases were reported to be 8-9 and 7.0. The molecular weight of *Streptomycs* sp. A_{11} collagenase was 74,000. But molecular weights of the collagenases produced by *Streptomyces* C-51 and *S. madurae* were 100,000 and 35,000 respectively. Cysteine completely inhibited the collagenase isolated from *S. madurae* but 48.4% of the collagenase of present study was inhibited in presence of 5 mM cysteine.

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