

# Purification and Properties of Individual Collagenases from *Streptomyces* sp. Strain 3B

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Received 26 April 2005

Revised version 20 December 2005

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**ABSTRACT.** *Streptomyces* strain 3B constitutively secreted collagenolytic enzymes during the post-exponential growth phase. Purification is described here leading to two collagenases (I and II) with specific activity of 3350 and 3600 U/mg, respectively, the highest activity obtained as yet for any streptomycete collagenase. Analysis of the purified enzymes by the method of zymography revealed that both I and II were homogeneous, with molar mass 116 and 97 kDa, respectively. Both collagenases were identical in their pH (7.5) and temperature optimum (37 °C). The inhibition profile of the enzymes by EDTA and 1,10-phenanthroline confirmed these enzymes to be metalloproteinases. By testing the activity with insoluble collagen, acid soluble collagen, gelatin, casein, elastin and Pz-PLGPR it was established that I and II are very specific for insoluble collagen and gelatin, showing a high activity toward acid soluble collagen and Pz-PLGPR. However, collagenases I and II failed to hydrolyze casein and elastin; they belong to true collagenases and resemble the clostridial enzymes.

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Collagen is a structure protein of the extracellular matrix of the connection tissue in higher vertebrates. Collagenase has an important role in connective tissue metabolism (Dresden 1971) and is produced by specific cells involved in repair and remodeling processes (Wahl *et al.* 1975; Ravanti and Kähäri 2000). In general the microorganisms producing collagenase are host-invasive, and presumably the enzymes contribute to pathogenicity by allowing the organisms to penetrate a connective tissue barrier. The degradation of this connective tissue during the infection by *Clostridium histolyticum* was first described by Weinberg and Seguin (*cited by* Mandl 1961). Since then, different microbial collagenases have been isolated and a few were characterized in detail, like the collagenases of *C. histolyticum* (Yoshida and Noda 1965; Bond and Van Wart 1984; Bicsak and Harper 1985) and *Achromobacter iophagus* (Welton and Woods 1975; Lecroisey *et al.* 1975).

Although collagenase production by bacteria is well documented, little attention has been paid to surveying the actinomycetes known as antibiotic producers. A variety of actinomycetes are actively involved in the processes of degradation of native collagen under natural conditions (De and Chandra 1979; Chakraborty and Chandra 1982; Kabadjova and Vlahov 1997; Gousterova *et al.* 1998; Goshev *et al.* 2005). Besides, they are harmless for humans.

At present a few reports of collagenases from *Streptomyces* are known (Chakraborty and Chandra 1987; Endo *et al.* 1987; Demina and Lysenko 1996; Kabadjova *et al.* 1997).

Here we describe the purification and some properties of collagenases isolated from culture filtrate of strain *Streptomyces* sp. 3B.

## MATERIALS AND METHODS

**Microorganism.** The original actinomycete strain *Streptomyces* sp. 3B, isolated from Bulgarian soil sample (Kabadjova and Vlahov 1995) was used as a producer of collagenolytic enzymes.

**Media and cultural conditions.** *Streptomyces* sp. 3B was maintained on mineral medium 1 (MM 1; Gause *et al.* 1957), containing (in g/L): agar 30, soluble starch 20, KNO<sub>3</sub> 1, K<sub>2</sub>HPO<sub>4</sub> 0.5, MgSO<sub>4</sub> 0.5, NaCl 0.5, ZnSO<sub>4</sub> 1 µg.

For collagenase production, the strain was cultured aerobically in liquid MM 1 inoculated with 10 % (*V/V*) vegetative inoculum obtained after a 2-d cultivation. Mycelium was grown in a flask on a rotary shaker (3.7 Hz) at 28 °C. Collagenase activity was maximum after 3 d of growth in MM 1.

*Collagenase activity* was measured with insoluble collagen according to Endo (1986). The standard reaction mixture, containing 25 mg collagen (type I, bovine Achilles tendon) in 5 mL 50 mmol/L Tris-HCl buffer was incubated with 1 mL enzyme sample at 37 °C. The amount of free amino groups released was measured by the ninhydrin method of Rosen (1957). One activity unit (U) is defined as the number of  $\mu\text{mol}$  L-leucine released as a result of the action of 1 mL culture filtrate containing collagenase, for 1 h at 37 °C. The specific activity was expressed as  $\mu\text{mol}$  of leucine per min per mg protein. Hydrolysis of synthetic peptide 4-phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-Arg (PZ-PLGPR) was assayed according to Wunsch and Heidrich (1963).

*Other proteolytic activity.* Elastolytic activity and caseinolytic activity were measured according to Nangton and Sanger (1961) and Kunitz (1947), respectively.

*Protein concentration* was determined according to Lowry using bovine serum albumin as substrate.

*Enzyme purification.* The mycelium was removed by centrifugation (10 000 g, 20 min) and the supernatant was used as a source of the enzyme. Culture filtrate was concentrated by ultrafiltration in a *Millipore* system using *Amicon* membranes with a pore size of 10, 30 and 50 kDa. Permeates (P1, P2 and P3) and concentrates (C1, C2 and C3) obtained after each step of ultrafiltration were analyzed for collagenase, elastase and caseinase activity and protein.

Diammonium sulfate was added to 70 % saturation and the precipitate was collected by centrifugation (20 000 g, 1 h, 4 °C). The pellet was dissolved in 20 mL of 50 mmol/L Tris-HCl buffer (pH 7.5), 4 mmol/L  $\text{CaCl}_2$  and the sample was dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-Sephadex G-25 column (22  $\times$  500 mm) equilibrated with 50 mmol/L Tris-HCl (pH 7.5), containing 4 mmol/L  $\text{CaCl}_2$ . The column was washed with the same buffer followed by a linear gradient of 0.8 mol/L NaCl at a flow rate of 0.5 mL/min.

Active fractions from DEAE-Sephadex G-25 column were pooled and concentrated by ultrafiltration. The concentrate was put onto an ion-exchange fast protein liquid chromatography column (Mono Q; *Pharmacia LKB Biotechnology*, Sweden), which was pre-equilibrated with 25 mmol/L Tris-HCl buffer (pH 9.0) and 4 mmol/L  $\text{CaCl}_2$ . Protein fractions were eluted with a linear 1 mol/L NaCl gradient in the same buffer. The pooled collagenase fractions were concentrated and placed on a Polyanion  $\text{Si}^{\text{TM}}$  (*Pharmacia*) column. The column was developed with 25 mmol/L Tris-HCl buffer (pH 9.0) and 4 mmol/L  $\text{CaCl}_2$ . After extensive washing with the equilibration buffer, elution was carried out stepwise using 30, 45, 52 and 70 % saturated NaCl in 25 mmol/L Tris-HCl. The pooled collagenase fractions were applied to a size exclusion column (Superose 12; *Pharmacia*). The column was run in 67 mmol/L phosphate buffer (pH 7.4).

*Determination of the molar mass* of the collagenases by gel filtration chromatography was done using Superose 12 column with detection at 280 nm. The following molar mass standards were used: cytochrome *c* (12 kDa), ovalbumin (43), bovine albumin (67), and bovine  $\gamma$ -globulin (150). The establishment of the molecular homogeneity of the purified enzymes by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). The method of zymography was used to detect enzyme activity and determine the molar mass of protein fractions (Kabadjova *et al.* 1996).

*Effect of pH and temperature on enzyme activity and stability.* Experiments were performed using the standard collagenase assay with insoluble collagen as substrate. The optimum pH was determined at 37 °C with the following buffer systems: 10 mmol/L sodium acetate (pH 4.0–5.5), sodium phosphate (6.0–7.5), Tris-HCl (8.0–9.5) and glycine-NaOH (10.0–11.0), pH stability being also determined in these buffers. The solutions of collagenase I and collagenase II were incubated with the buffers at various pH values for 30 min and the residual collagenase activity was determined subsequently.

For determination of the optimum temperature, the activity was measured at 18–70 °C in 10 mmol/L Tris-HCl buffer using gelatin as the substrate. Native collagen was not used as substrate here, since it was denatured above 50 °C. The temperature stability was determined by measuring the residual activity after a 1-h incubation of the collagenase solutions in 10 mmol/L Tris-HCl buffer at 37, 42 and 47 °C.

*Inhibition of collagenases.* For determination of inhibitor sensitivity, the purified collagenase solutions were incubated at room temperature for 15 and 30 min in 10 mmol/L Tris-HCl buffer (pH 7.5) with the following inhibitors: phenylmethanesulfonyl fluoride (PMSF), diisopropylfluorophosphate (DFP), tosyl-L-phenylalanyl-chloromethylketone (TPCK), sodium tosyl-L-lysine-chloromethylketone (TLCK), iodoacetamide (IA), iodoacetic acid (IAA), pCMB (4-chloromercuribenzoate), ethylenediaminetetraacetate (EDTA), 1,10-phenanthroline, pepstatin. The residual activity was determined as a percentage of the activity in control sample without reagent. The influence of metal ions (10 mmol/L final concentration) activity was determined using native collagen as substrate (as described *above* for proteinase inhibitors).

## RESULTS AND DISCUSSION

*Production of collagenolytic enzymes.* Streptomycetes secrete a large number of hydrolytic enzymes and many of them are inducible by the substrate (Peczynska-Croch and Mordarski 1988). However, the high level of collagenase production by *Streptomyces* sp. 3B was observed in MM 1, whereas in other streptomycetes, collagenase production is generally associated with induction by the substrate (Endo *et al.* 1987; Demina and Lysenko 1996; Kabadjova and Vlahov 1997). The maximum collagenase activity was detected at the end of exponential growth phase, like in other *Streptomyces* producers (Demina and Lysenko 1996; Kabadjova *et al.* 1996). The collagenolytic activity in the culture supernatant was due to the production of several collagenases and a trace of other kinds of proteinases.

*Collagenase purification.* The enzymes were purified to homogeneity in six steps. The first stage was concentration of the culture filtrate by a three-step ultrafiltration with simultaneous separation of impurities and proteins with molar mass <10 kDa. Caseinase and elastase had a maximum concentration in 10–30 and 30–50 kDa fractions, respectively. The concentrate obtained after ultrafiltration by a membrane with a molar size of 50 kDa containing the highest collagenase activity was used for further purification. After the third step of ultrafiltration about 1.65-fold concentration of collagenase was established.

During the second stage of purification by 70 % saturation with diammonium sulfate, the enzyme was concentrated 11.7-fold. The material was applied to a DEAE Sephadex G-25 column. The proteins were eluted in three separate peaks (one non-adsorbed and two adsorbed fractions). The collagenolytic activity was detected in the second peak. The pooled active fractions were chromatographed on a Mono Q column. Four peaks appeared, but only the fractions containing collagenase activity were used for further purification. The activity was separated in the first two peaks with a very close charge of molecules. The collagenases were completely separated in two peaks after chromatography on a Polyanion Si<sup>TM</sup> column. They were designated as collagenase I and collagenase II, respectively.

These results were confirmed by data obtained by chromatography on a Superose 12 gel-filtration column. The eluted profile showed two peaks containing collagenase activity.

This purification protocol produced highly purified products (Table I). Collagenase I was purified 43.1-fold with a 5 % yield. The overall purification factor for collagenase II was about 46.2-fold and the yield was 6 %. The purified enzymes were free of caseinolytic and other proteolytic activities, showing that the collagenases are specific for collagen. The final products (collagenase I and II) had specific activity of 3350 and 3600 U/mg, respectively.

**Table I.** Purification of *Streptomyces* sp. 3B collagenases

Stage	Total protein mg	Collagenase activity		Caseinolytic activity		Yield %	Purification -fold
		total U	specific U/mg protein	total U	specific U/mg protein		
Culture filtrate	2880	224 000	77.8	370	0.128	100	1
Ultrafiltration	1390	178 000	128	206	0.148	79	1.65
Ammonium sulfate (70 %)	162	147 000	909	48	0.30	66	11.7
DEAE Sephadex G-25	58	85 200	1470	20	0.34	38	18.9
Mono Q	16	34 000	2125	6	0.38	15	27.3
Polyanion Si <sup>TM</sup>	7.63	20 900	2740	–	–	9	35.2
Superose 12, collagenase I	3.18	10 650	3350	–	–	5	43.1
Superose 12, collagenase II	3.47	12 500	3600	–	–	6	46.2

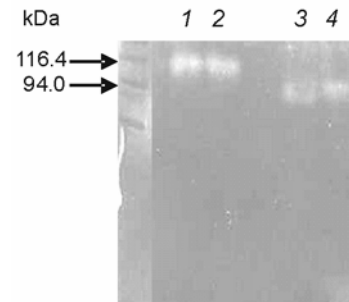
Purified collagenases showed the highest specific activity of all streptomycete collagenases as yet described. They are  $\approx 30$ – $50\times$  more active than the collagenases of *Streptomyces* sp. C 51 (Endo *et al.* 1987) and almost  $3.5\times$  than the pure collagenase of *Streptomyces lavendulae* (Demina and Lysenko 1996). This can partly be ascribed to the initial high activity of the crude enzyme, partly to the efficient purification procedure resulting in the removal of nonspecific proteolytic activities. Apart from other collagenases, *Streptomyces* sp. 3B constitutively secretes strongly collagenolytic enzymes with traces of nonspecific proteinases. *Streptomyces* sp. C-51 produces collagenase in measurable amounts only in the presence of an inducer in the medium and many nonspecific proteinases are produced together with collagenases (Endo *et al.* 1986).

*Molar-mass determination.* Collagenases from *Streptomyces* sp. 3B were purified to homogeneity. The molar mass estimated by the zymogram method was 116 kDa for collagenase I and 97 kDa for collagenase II.

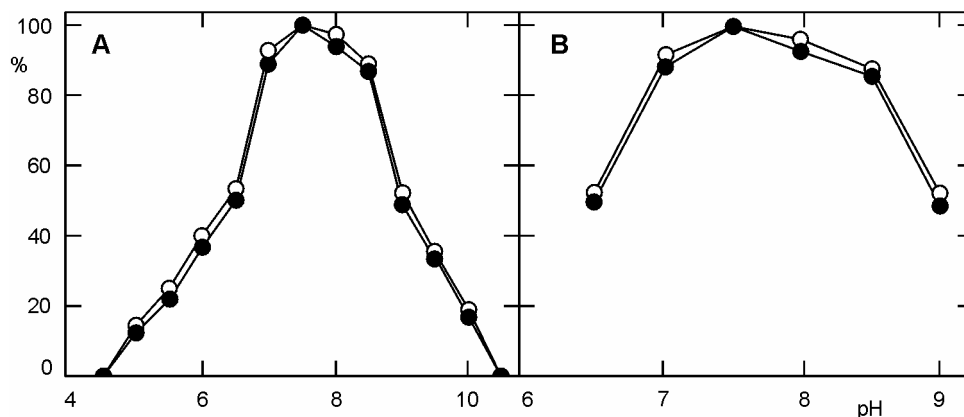
ase II (Fig. 1). Almost the same values were obtained by Superose 12 gel filtration (112 and 95 kDa). It seems that *Streptomyces* sp. 3B synthesized multiple active forms of collagenase. Such multiple collagenases produced by *C. histolyticum* have been also observed by Yoshida and Noda (1965), Kono (1968), Bond and Van Wart (1984), Susagawa and Harper (1984) and Bicsak and Harper (1985). Multiple forms of collagenase were also reported with *Vibrio* B-30 (Merkel and Dreisbach 1978) and many *Streptomyces* strains (Endo *et al.* 1987; Kabadjova *et al.* 1996).

**Optimum pH and pH stability.** Maximum collagenolytic activity was observed at pH 7.5 for both collagenases in 10 mmol/L Tris-HCl buffer (Fig. 2A). Consequently, both collagenases belong to the group of neutral proteinases. More than 50 % of the maximum activity was detected for these enzymes between pH 6.5 and pH 9.0 (Fig. 2B). Complete inactivation of collagenase activity was observed between pH 4.5 and 10.5. Generally, bacterial collagenases are reported to be metal proteinases with an optimum at neutral or slightly alkaline pH that are inhibited by chelators such as EDTA (Yoshida and Noda 1965; Matsushita *et al.* 1994; Demina and Ly-senko 1996; Sasagawa *et al.* 1993; Endo *et al.* 1987).

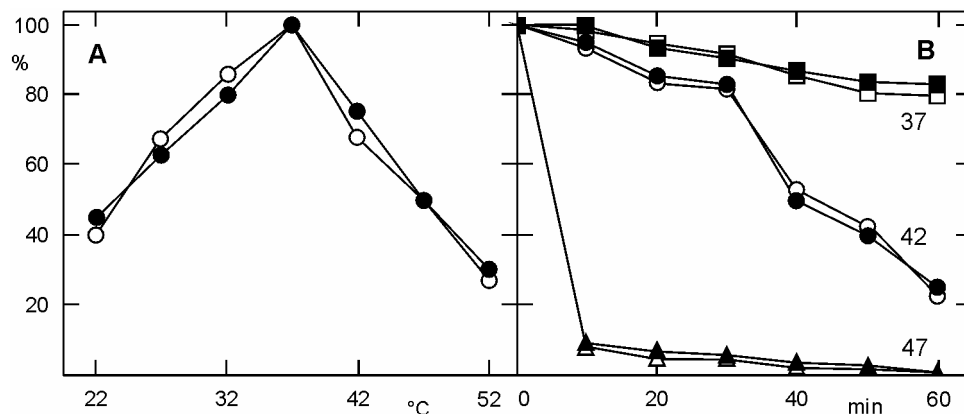
**Maximum temperature and heat stability.** Collagenases I and II were active at 20–50 °C with an optimum at 37 °C (Fig. 3A). Similarly, a maximum temperature of 37 °C was recorded for a col-



**Fig. 1.** SDS-PAGE collagen zymography and molar-mass determination of collagenolytic fractions from *Streptomyces* sp. 3B; 1, 2 – collagenase I; 3, 4 – collagenase II; molar-mass standards (kDa):  $\beta$ -galactosidase 116.4, phosphorylase b 94.0.



**Fig. 2.** pH optimum (relative activity, %; A) and pH stability (residual activity, %; B) of collagenase I (closed symbols) and collagenases II (open symbols); the pH activity profiles were determined at 37 °C; pH stability was determined at pH 7.5 after incubation of enzymes for 30 min at various pH.



**Fig. 3.** A: Effect of temperature on enzyme activity (relative activity, %); maximum activity observed was set as 100 % of relative activity. B: Effect of temperature on enzyme stability (residual activity, %); solutions of collagenases were treated at 37, 42 and 47 °C for 1 h and the residual activity was measured at 37 °C. The highest activity is denoted as 100 %; closed symbols – collagenase I, open symbols – collagenase II.

lagenase from *S. lavendulae* (Demina and Lysenko 1996) and 35–40 °C for *Streptomyces* sp. C51 (Endo *et al.* 1987). *Streptomyces* sp. 3B collagenases showed good stability in response to heat treatment for 1 h at 37 °C and retained 50 % of their activity after treatment at 42 °C for 40 min. Drastic loss of activity was observed at 47 °C after 20 min (Fig. 3B).

*Effect of proteinase inhibitors and metal ions* (Table II). Collagenase I and II were strongly inhibited by metal chelators such as EDTA and 1,10-phenanthroline, but were not inactivated by the inhibitors of serine proteinases (EC 3.4.21), DFP and PMSF, as well as by TPCK and TLCK, specific inhibitors of chymotrypsin and trypsin, respectively. The inhibition by IA, IAA and pCMB was small ( $\leq 20$  %) even when using high concentration (5 mmol/L) and 30 min of incubation, pepstatin having no inhibitory activity. These results suggested that the collagenases I and II belong to the family of metalloproteinases (EC 3.4.24.3).

**Table II.** Effect of inhibitors and divalent metal ions on residual collagenase activity (%) after 15 and 30 min

Inhibitor	Concentration mmol/L	Collagenase I		Collagenase II		Metal ion 10 mmol/L	Collagenase I	Collagenase II
		15 min	30 min	15 min	30 min			
PMSF	1	102	97	100	95	Mg <sup>2+</sup>	101	100
	5	99	95	97	92			
DFP	1	103	101	100	100	Ca <sup>2+</sup>	116	119
	5	100	99	98	97			
TPCK	1	103	105	105	100	Mn <sup>2+</sup>	56	61
	2.5	101	105	100	100			
TLCK	1	101	97	100	100	Fe <sup>2+</sup>	4	5
	2.5	100	96	100	99			
IA	1	100	90	100	93	Co <sup>2+</sup>	99	98
	5	96	87	100	87			
IAA	1	100	93	100	89	Cu <sup>2+</sup>	0	0
	5	92	84	99	80			
pCMB	1	101	98	95	92	Zn <sup>2+</sup>	0	0
	5	98	95	93	81			
EDTA	1	0	0	0	0	Sn <sup>2+</sup>	26	20
	2.5	0	0	0	0			
	10	0	0	0	0			
1,10-Phenanthroline	1	5	0	3	0	Ba <sup>2+</sup>	103	102
	2.5	4	0	3	0			
	10	0	0	0	0			
Pepstatin	1	106	101	110	103	Pb <sup>2+</sup>	18	21
	5	106	100	108	99			

**Table III.** Substrate specificity of collagenase I and II (U/mg protein)

Substrate	Collagenase I	Collagenase II
Collagen, type I	3351	3596
Collagen, acid soluble	2831	2180
Gelatin	3168	2965
Casein	0	0
Elastin	0	0
Pz-PLGPR	1560	2288

Divalent cations (except for Ca<sup>2+</sup>, Mg<sup>2+</sup>, Ba<sup>2+</sup> and Co<sup>2+</sup>) inhibited the enzyme activity (Table II). Fe<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup> and Hg<sup>2+</sup> ions showed maximum inhibition of both enzymes. The inhibitory effect of heavy metal ions is well documented in the literature (Valee and Ulmer 1972). The enzyme activities were activated by  $\approx 16$ –19 % by 10 mmol/L Ca<sup>2+</sup> ions, which stabilize collagenases (Matsushita *et al.* 1994).

*Substrate specificity.* Among protein substrates, native collagen (type I) was found to be the most suitable substrate for the enzymes from *Streptomyces* strain 3B. Collagenases I and II showed high activity toward gelatin and acid soluble collagen but no activity toward casein and another insoluble protein, elastin (Table III). They resemble fraction I and II of *Streptomyces* sp. C51 (Endo *et al.* 1987), because collagenase I was more active against substrates with higher molar mass than collagenase II.

Comparison with both collagenases with analogous enzymes as regards substrate specificity is difficult because of the use of enzyme preparations with different degree of purity and, in particular, different conditions for assays of proteinase activities and in the accessibility of the enzymes to the splitting points.

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