

## Characterization of Alkaline Protease from *Thermoactinomyces* sp.

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### Abstract

*Thermoactinomyces* sp. was isolated from fermented composed sample. The bacterial isolate was identified according to the cultural, microbiological and biochemical characteristics. The bacterial cells secreted the extracellular protease in alkaline agar medium that contained 1% Hammerstein casein and liquid culture. The enzyme was partially purified 40-fold from culture filtrate by sequential steps including salting out with Ammonium sulfate precipitation (80% saturation) subsequently ion exchange chromatography with DEAE-cellulose column and then CM- Cellulose column which increased the specific activity of the enzyme up to 1880 U/mg proteins with 72% recovery.

Characterization study of the partially purified enzyme was revealed that the enzyme had an optimum activity at pH of 10 and the activity was stable in the alkaline pH range from 8 to 12 for 30 min. Enzyme activity toward casein increased with increasing temperature and the maximal activity at 60° C. The enzyme was stable at temperature under 55°C and approximately 75% of the activity abolished by incubation of the enzyme at 75 ° C for 30 min. Alkaline protease activity was completely inhibited by PMSF and DFP; while EDTA showed no inhibition on protease activity that is suggested that the enzyme is a serine protease.

### الخلاصة

عزلت السلالة *Thermoactinomyces* sp. من عينة السماد الحيواني المخمر وتم تشخيص العزلة بالاعتماد على الصفات المزرعية و المجهرية و الكيموحيوية للبكتريا. تفرز خلايا البكتريا البروتينيز الخارجي في الوسط القاعدي الصلب المحتوي على ١% كازين والوسط السائل. تم تنقية الانزيم جزئيا ٤٠ مرة من راسح المزرعة

بعده خطوات متسلسلة مثل الترسيب بملح كبريتات الامونيوم ( ٨٠% تشبع) ثم كروموتوغرافيا التبادل الايوني بطريقة العمود باستخدام المبادل DEAE-cellulose وبعد ذلك بالمبادل cellulose-CM. تم دراسة بعض صفات الانزيم، إذ وجد ان الانزيم المنقى جزئيا يمتلك فعالية مثلى في اس هيدروجيني ١٠ وكان الانزيم ثابتا في مدى من الاس الهيدروجيني القاعدي من ٨ الى ١٠ لمدة ٣٠ دقيقة. تزداد الفعالية الانزيمية اتجاه الكازين بارتفاع درجات الحرارة و الفعالية العظمى له في ٦٠ م. وكان الانزيم ثابتا في درجات حرارية ادى من ٥٥ م و يفقد ٧٥% من الفعالية بعد حضنه بدرجة حرارة ٧٠ م لمدة ٣٠ دقيقة. يثبط البروتين القاعدي بشكل كامل بوساطة PMSF و DFP. في حين لم يظهر EDTA أي تثبيط على الفعالية الانزيمية لذلك يعتقد بان الانزيم من نوع بروتينيز السيرين.

(Keywords: *Actinomyces sp.*, alkaline protease, partial purification, characterization)

## Introduction

Actinomycetes are Gram-positive bacteria but are distinguish from other bacteria by their morphology, by DNA rich in guanine plus cytosine and based on nucleic acid sequencing. The genus *Thermoactinomyces*, although producing a mycelium and considered are actinomycete, is more closely related to the Bacillaceae than other actinomycetes and produces endospore in an analogous way to *Bacillus* and *Clostridium* species<sup>(1)</sup>. Actinomycetes produce antibiotics and enzymes that degrade proteins and complex molecules, especially cellulose, lingo cellulose and lignin, which make them particularly important in composting<sup>(2)</sup>.

Proteases with high activity and stability in the regions of high alkali and temperature are interesting for bioengineering and biotechnological applications as well as protein chemistry. Alkaline protease added to laundry detergents to facilitate the release of pertinacious materials in stains such as those due blood and milk. To withstand the alkaline conditions in detergents, many alkaline serine proteases have been isolated from strains of *Bacillus*<sup>(3, 4, 5)</sup> which they used in hide batting and dehairing or laundry detergents. Alkaline serine proteases from alkalophiles are

generally stable in the highly alkaline region, but they are not very stable to heat. On the other hand, the proteases from thermophiles are generally stable to heat but unstable at high pH. Extremely thermostable alkaline proteases with high pH stability from alkalophilic *Bacillus sp. No.AH-101*<sup>(5)</sup> and extremely thermophilic *Thermus aquaticus YT-1*<sup>(6)</sup> activity have been reported. In addition, the protease from alkalophilic *Thermoactinomyces sp.HS682*<sup>(7, 8)</sup> is more stability in both regions of high alkali and high temperature than thermostable proteases that produced from alkalophilic *Bacillus sp. No.AH-101* and extremely thermophilic *Thermus aquaticus YT-1* strains.

The current study focuses on the partial purification of alkaline protease from *Actinomyces sp.* and study of some properties of this enzyme.

## Material and methods

### *Isolation and identification of the organism:*

The microorganism was isolated from a fermented composed sample according to the methods of Amner *et al.*<sup>(9)</sup> and Athalye *et al.*<sup>(10)</sup>. Microbiological, cultural and biochemical characteristics of the isolates were examined by the methods of lacey and Cross<sup>(11)</sup>.

The isolate was grown in alkaline agar plate to test alkaline protease production, the medium composed of (w/v):

1% glucose, 0.2% yeast extract (Difco); 1% Hammerstein casein (Merck), 0.1%  $\text{KH}_2\text{PO}_4$ , 0.02%  $\text{MgSO}_2 \cdot 7\text{H}_2\text{O}$ , 1%  $\text{Na}_2\text{CO}_3$  (separately autoclaved), and 1.5% agar (pH 10.5)<sup>(8)</sup> that induce protease production.

#### ***Enzyme production:***

The organism was propagated at 55 °C for 2 days, with shaking, in 50ml aliquots of an alkaline medium in 500ml flasks. The production medium contained from 0.5% yeast extract (BDH) , 1% maltose , 0.5% Hammerstein casein, 0.2% NaCl, 0.1%  $\text{KH}_2\text{PO}_4$ , and 1%  $\text{Na}_2\text{CO}_3$  (pH 10)<sup>(8)</sup> . After removal of cells by centrifugation (8000 xg, 20min) at 4°C, the supernatant was used as the crude extract for purification of the enzyme.

#### ***Purification of the enzyme:***

The supernatant (one liter) was concentrated by 80% saturation of ammonium sulfate precipitation at 4°C<sup>(12)</sup>. The precipitate was separated by centrifugation and dissolved in 20 ml of 5mM Tris-buffered pH 8. Then the concentrate was dialyzed against 5mM Tris-HCl (pH 8) that contained 2 mM  $\text{CaCl}_2$  for 48 h with change the buffer each three hours<sup>(8)</sup>.

The retentate (55ml that contained 245 mg protein) was loaded on a column of DEAE-cellulose (2.5x16 cm) that had equilibrated with 10mM Tris -HCl buffer (pH8) plus 2mM  $\text{CaCl}_2$ . The column was washed with the same buffer and then eluted with 10mM glycine-NaOH buffer (pH10) plus 2mM  $\text{CaCl}_2$ . Fractions of 4ml were collected with flow rate 44 ml/h from the beginning of column washing.

The unadsorbed fractions that contained alkaline protease activity were combined

(36 ml, fractions 21-29). Subsequently the enzyme (36 ml contained 75.6 mg protein) was applied to a CM- cellulose column (2.5x 16 cm) that had equilibrated with 10mM Tris-HCl buffer (pH 8) plus 2 mM  $\text{CaCl}_2$ . The column was washed with the equilibrating buffer and proteins were eluted first with a 100 ml of 25 mM KCl second with a 100 ml of 50 mM KCl in the same buffer and then with a 100 ml Of 75mM KCl in the same buffer. Fractions of 4 ml were collected with flow rate 44ml/h from the beginning of column washing. The fractions of the enzyme were combined; dialysis against the equilibrating buffer, and the retentate was concentrated by cooled acetone precipitation. The partial purified enzyme was stored in 20% (v/v) glycerol at -20 °C<sup>(8)</sup>.

#### ***Enzyme assay:***

Protease activity was assayed by the modified method of Kunitz<sup>(13)</sup>. Enzyme solution (0.1 ml) was mixed with 0.9 ml of glycine-NaOH buffer (0.1M, pH 9) that contained 5 mg Hammerstein casein (Merck). After incubation at 55°C for 10 min, 2 ml of 5% trichloroacetic acid (TCA) was added to terminate the reaction. The mixture was passed through No.2 filter paper (Watman) to remove denatured proteins. The absorbency of the supernatant fraction was read at 275 nm. Blanks stopped with the addition of TAC before the addition of the enzyme to be tested. One unit of protease activity was defined as the amount of enzyme required to produce an increase 0.001 in the absorbency at 275 nm per min under assay conditions. Specific activity was expressed as enzyme units per mg protein. The protein content was estimated by the method of Lowry et al.<sup>(14)</sup>, with bovine serum albumin (Sigma) as the protein standard.

### ***Effect of pH on the activity and stability of alkaline protease:***

The reaction between partial purified alkaline protease (2.5 µg) and 0.5% casein substrate were assayed in triplicate for 10 min in solutions ranging from pH 6 to 13<sup>(8)</sup>. The effect of acidic pH (< 5) could not be determined, since the casein precipitates at acid pH conditions. Blanks consisted of the casein substrate at pH values tested without alkaline protease and the enzyme was added after the addition of trichloroacetic acid solution. The absorbance of the supernatant fraction was read at 275 nm.

To determine the effect of pH on the stability of the enzyme, alkaline protease (2.5 µg) had been preincubated in triplicate at 50°C for 30 min in various pH values ranging from 3 to 13. By using the following buffers (20 mM); citrate-phosphate (pH 4.0- 7.0); phosphate (pH 6.0- 8.0); glycine-NaOH (pH 8-10.5) and carbonate (pH 11-12.3) and the carbonate buffer adjusted to pH 13 by 1N NaOH. Then the residual activity was assayed with casein at 55°C for 10 min<sup>(8)</sup>.

### ***Effect of temperature on the activity and stability of alkaline protease:***

Effects of temperature on the enzymatic activity were determined by the reactions between partial purified alkaline protease (2.5 µg) and 0.5% casein substrate were assayed in triplicate for 10 min in 20mM glycine-NaOH buffer (pH10) at various temperature (0- 85°C), each reaction was stopped by addition of trichloroacetic acid solution. Blanks consisted of the casein substrate in the same buffer without alkaline protease where the enzyme was added after the addition of trichloroacetic acid solution<sup>(8)</sup>. The absorbance of the supernatant fraction was read at 275 nm.

To determine the effects of temperature on the stability of the enzyme, alkaline protease (2.5 µg) had been preincubated in

triplicate for 30 min at various temperature (0-85°C) in 20mM glycine-NaOH buffer (pH10). Cooling in ice bath to stopped heating of the enzyme, and then the residual activity was assayed at 55°C for 10 min. Blanks consisted of the casein substrate in the same buffer without alkaline protease where the enzyme was added after the addition of trichloroacetic acid solution<sup>(8)</sup>. The absorbance of the supernatant fraction was read at 275 nm.

### ***Inhibition studies:***

The inhibition studies were performed as described by Yan et al.<sup>(15)</sup> with some modification. The inhibitors were prepared separately as stocks: 10mM of phenylmethylsulfonyl fluoride dissolved in 1-propanol, 50 mM of Diisopropyl fluorophosphate dissolved in 2-propanol, 10mM of EDTA-Na<sub>2</sub> dissolved in distilled water and 500mM of EDTA-Na<sub>2</sub> dissolved in distilled water. Preincubation of protease (50 µg in 10µl of 50 mM glycine-NaOH, pH 9.5) and inhibitor (10µl of the inhibitor from stock, 70µl of 50mM glycine-NaOH at pH9.2 and 10µl of 150mM NaCl) was carried out at room temperature for 30 min before the substrate (Hammerstein casein) was added. The final concentration of the inhibitors used was 1mM of phenylmethylsulfonyl fluoride, 5mM Diisopropyl fluorophosphate, and EDTA (1 and 50 mM) respectively. Assays were performed in triplicate. An appropriate blanks, in which the inhibitor was replaced with solvent, were assayed in parallel. The activity of each inhibitor used was confirmed by demonstrating its activity against susceptible enzymes such as trypsin (Sigma) and alkaline protease of *Bacillus subtilis*<sup>(16)</sup>.

## Results

The isolate was Gram-positive bacilli cells and it produced endospore which was negatively stained with endospore stain. The bacilli cells form true mycelium and the single conidia formed within one week. The isolate grow on mineral agar I and form white aerial mycelium and yellow substrate mycelium, colony produce brown water soluble pigment, colony diameter 2-3mm, entire colony margin, convex colony elevation. The isolate able to assimilate eleven carbon sources (D-glucose, L-arabinose, D-xylose, inositol, D- mannitol, D-fucose, L-rhamnose, sucrose, rhamnose, maltose and lactose). And could not assimilate cellulose, D-sorbitol, dulcitol. The best growth was shown on media with maltose, Fructose, rhamnose and xylose. The isolate grew very well on different culture media. The optimum temperature for cultivation was 55 °C. According to *Bergey`s Manual of systematic Bacteriology* <sup>(11)</sup>. The isolate was *Thermoactinomyces sp.*

*Thermoactinomyces sp.* was found to be a producer alkaline protease on alkaline plate agar. The active extracellular alkaline protease was secreted into the culture medium during late logarithmic phase of bacterial growth. The enzyme was concentrated with 80% saturation of ammonium sulfate precipitation and dialyzed. Protease was partially purified from concentrated supernatant fraction by two steps of ion exchange chromatography. The enzyme was applied on anionic exchanger DEAE-cellulose column to eliminate most impurities by which the specific activity was increased to 3.47 folds by only one-step (Table.1). The enzyme protein was unadsorbed on the anionic exchanger (Fig.1) and the active fractions of the enzyme were

combined. The specific activity of the partial purified enzyme obtained by the next cationic exchange chromatography (CM-cellulose column) was 1880 U/mg protein, with 72% recovery (Table 1). The protein of enzyme adhered to the cation exchange resin (Carboxymethyl Cellulose). A peak of protease activity (fractions 94-103) was eluted by Tris-HCl buffer with 75 mM KCl at pH 8 (Fig.2).

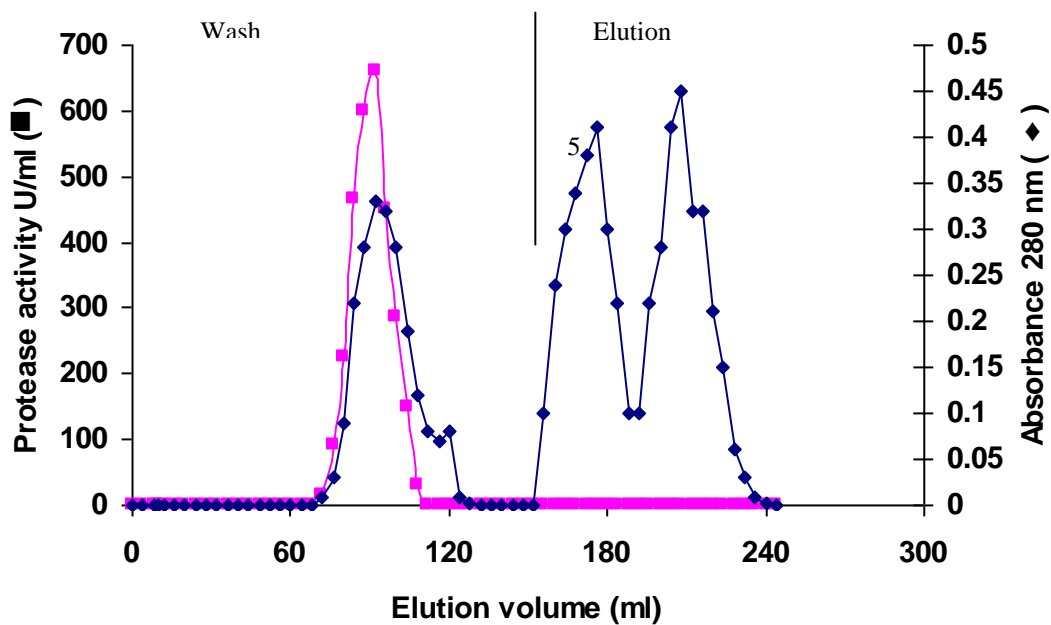
Enzyme assays conducted at eight different pH values indicated the maximal protease activity occurred at pH 10 (Fig.3). Protease activity increased from pH 6 to 10; pH 12 demonstrated approximately 50% of maximal activity, but there was no activity at pH  $\geq$  13. Studies of the pH stability of protease demonstrated that approximately 55% of the activity was abolished by incubation of the enzyme at pH 5 but the enzyme retained its activity at pH 8 to 11 for 30 min (Fig.3).

Enzyme activity of protease increased with temperature from 10 to 60° C with maximal activity occurring at 60°C (Fig.4). Studies of the thermal liability of protease demonstrated that the enzyme was stable at temperature under 55° C and approximately 75% of the activity was abolished by incubation of the enzyme at 75 ° C for 30 min (Fig.4).

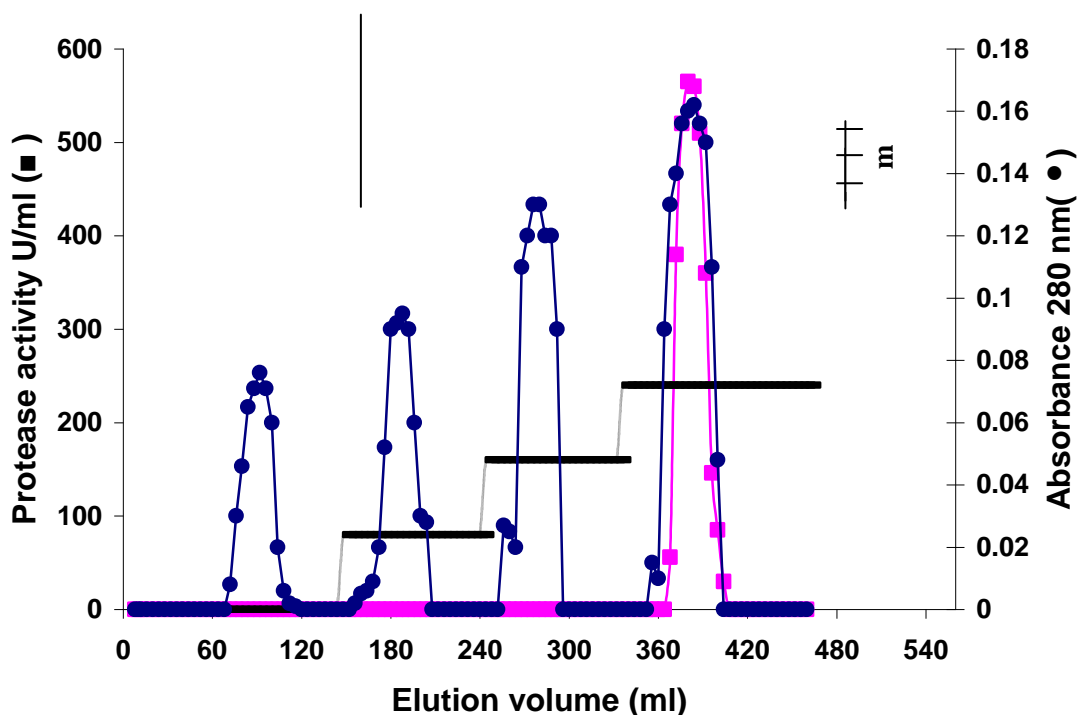
Three enzyme inhibitors were tested for their ability to block the hydrolysis of casein. The Serine protease inhibitors, 1mM of phenylmethylsulfonyl fluoride (PMSF) and 5mM of Diisopropyl fluorophosphate (DFP), demonstrated complete inhibition on protease activity and both concentrations of metalloprotease inhibitor (1mM and 50mM of EDTA) could not inhibit protease activity (Table.2).

**Table 1. Partial purification of alkaline protease from *Thermoactinomyces sp.***

Purification step	Total vol. ml	Protein conce. mg/ml	Total protein conc. mg	Enzyme activity U/ml	Total activity U	Specific activity U/mg protein	Purification Fold	Recovery (%)
Crude filtrate	1000	0.34	340	16	16000	47	1	100
Ammonium sulfate precipitation (80% saturation)	50	5.32	266	301	15050	56.58	1.2	94
Wash: DEAE-cellulose Chromatography (Column 2.5x16 cm)	36	2.10	75.6	342.5	12330	163	3.47	77
Elution: CM-cellulose Chromatography (Column 2.5x16 cm)	36	0.17	6.12	319.6	11505.6	1880	40	72

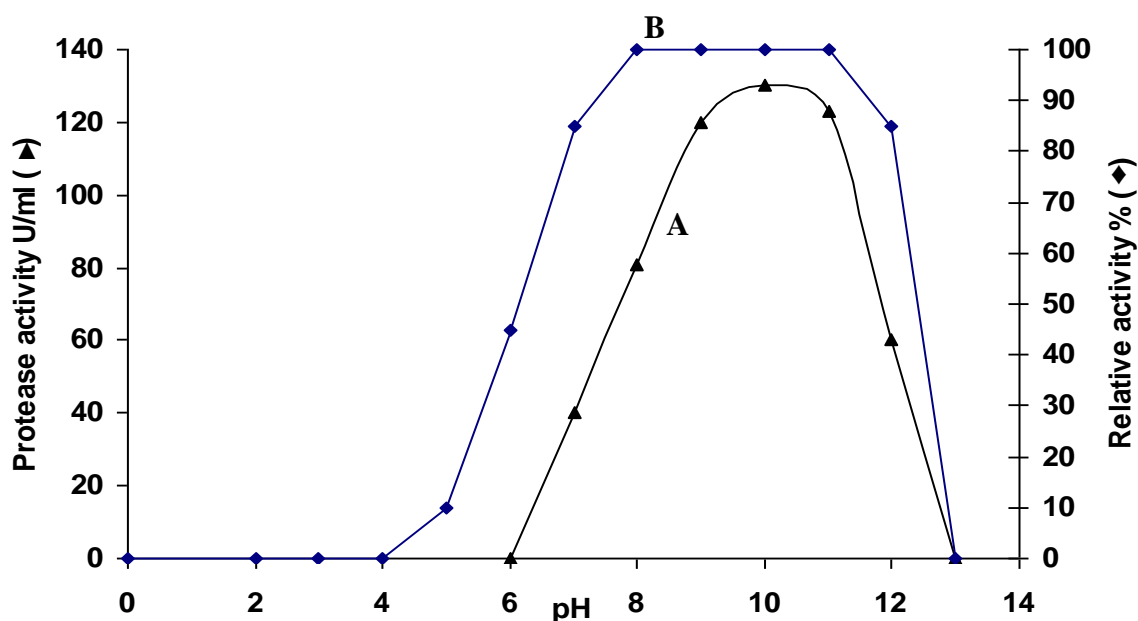
**Fig.1. Chromatography of *Thermoactinomyces sp.* protease on DEAE-cellulose column.**

The preparation obtained after ammonium sulfate precipitation and dialysis (266 mg total protein) was applied on a DEAE-cellulose column and washed with 10mM Tris-HCl buffer (pH8), then eluted with 10mM glycine-NaOH buffer (pH10) plus 2mM CaCl<sub>2</sub>. Fractions of 4ml were collected at 44 ml/h from beginning of column washing.



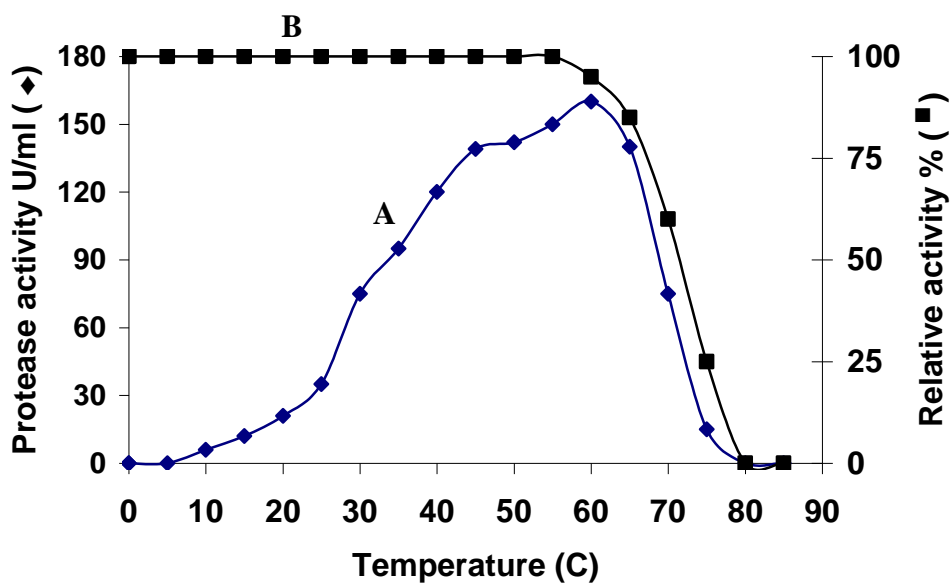
**Fig.2. Chromatography of *Thermoactinomyces* protease on CM-cellulose column.**

The concentrated enzyme preparation (75.6 mg total protein) obtained after DEAE-cellulose chromatography was applied on a column of CM-cellulose (2.5x16cm). The protein was eluted with 10mM Tris-HCl buffer (pH8) plus 2mM CaCl<sub>2</sub> with stepwise gradient of a 100 ml of 25mM KCl, 100ml of 50 mM KCl and then 100ml of 75mM KCl in the same buffer. Fractions of 4ml were collected at 44ml/h from the start.



**Fig.3. The effect of pH on protease activity and stability**

**A.** Effect of pH on enzyme activity. The caseinolytic activity was measured in total volume of 1.0 ml that contained 2.5  $\mu\text{g}$  of protease in various buffers (20mM) [citrate-phosphate (pH 4.0 - 7.0); phosphate (6.0 - 8.0); glycine - NaOH (8-10.5) and carbonate (pH11.0- 13)] at 55°C for 10 min. **B.** Effect of pH on the stability of the enzyme. After protease (2.5  $\mu\text{g}$ ) had been preincubated at 50°C for 30 min in the same buffers (20mM), then the residual activities were measured with casein under assayed conditions.



**Fig.4. Effect of temperature on protease activity and stability**



- A.** Effect of temperature on enzyme activity. Protease (2.5  $\mu\text{g}$ ) has been assayed with casein substrate in 20mM glycine buffer (pH9) at various temperature (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85 $^{\circ}$ C) for 10 min. **B.** Effect of temperature on the stability of enzyme. Protease solutions (2.5  $\mu\text{g}$  in 20mM glycine buffer at pH10)) had been preincubated for 30 min at various temperatures (0-85 $^{\circ}$ C). Cooling in an ice bath to stopped heating of the enzyme, and then the residual activities were measured with casein substrate at 55 $^{\circ}$ C for 10 min.

**Table.2. Inhibitor with Alkaline Protease from *Thermoactinomyces sp.***

<sup>a</sup> Inhibitor class refer to those proteases known to be susceptible to each compound tested.

<sup>b</sup> Enzyme reactivity refer to the specific enzymes (Trypsin and alkaline protease from *Bacillus subtilis*) used to demonstrate the activity of the inhibitor preparation.

<sup>c</sup> These values represent the change in protease activity measured as units of activity relative to inhibitor- free protease assayed using casein as substrate.

## Discussion

Actinomycetes are undoubtedly numerous in composts and have important roles in their decomposition. Many of the species are thermophilic such as *Thermoactinomyces*, with temperature optima at about 55 $^{\circ}$ C, and depend on thermogenesis within the compost piles to provide temperatures suitable for their growth

<sup>(17)</sup>. Re`sz *et al.* isolated *Thermoactinomyces vulgaris*, *Thermomonospora alba*, *Thm. currata* and

against casein, gelatin, collagen and elastin and, to a lesser extent, against keratin<sup>(18)</sup>.

The present study demonstrated that the protease from *Thermoactinomyces sp.* was partially purified from culture supernatant by only two steps of ion exchange chromatography, on DEAE-cellulose and CM-cellulose columns and increased the specific activity 40 times with recovery of 72%. Tsuchiya *et al.* purified and characterized the protease of alkalophilic *Thermoactinomyces sp.* HS682 from culture supernatant by only

Inhibitor	Inhibitor class <sup>a</sup>	Enzyme reactivity <sup>b</sup>	Inhibitor conc.	Percent. activity of control <sup>c</sup> %	Percent. activity of alkaline protease from <i>Thermoactinomyces</i> %
None	-	-	-	100	100
Phenylmethylsulfonyl fluoride	Serine	Trypsin	1.0 mM	0	0
Diisopropyl fluorophosphate	Serine	Trypsin	5.0 mM	0	0
EDTA	Metallo	Alkaline protease	1.0 mM	98	100
EDTA	Metallo	Alkaline protease	50 mM	86	100

*Thm.lineata* from German domestic waste composts. These mostly grow best close to 50 $^{\circ}$ C, with maxima at 60-65 $^{\circ}$ C, and showed proteolytic enzyme activity

two chromatography, on Butyl-Toyopearl 650M and Sp-Toyopearl 650S columns and increased the specific activity 6.8 times with recovery of 67%. They found the enzyme has a molecular weight of

25,000 according to gel filtration on a Sephadex G-75 column and SDS-PAGE and an isoelectric point above 11<sup>(7)</sup>. A serine protease from the keratin-degrading *Streptomyces pactum* DSM 40530 was purified by casein agarose affinity chromatography. The enzyme had a molecular weight of 30,000 and isoelectric point of 8.5<sup>(19)</sup>.

The protease in the present study is heat stable and active over wide range (10-50°C). The protease is stable and active over wide range of neutral and alkaline pH values (7-11) with maximal activity at pH 10, whereas the protease of alkalophilic *Thermoactinomyces sp. HS682* showed optimum pH at pH11.5-13.0 (37°C) and optimum temperature at 70 °C, pH 11.5. The serine protease from *Streptomyces pactum* DSM 40530 was optimally active in pH range from 7 to 10 and at temperatures from 40 to 75°C<sup>(19)</sup>.

However, our overall inhibitor data suggested that the extracellular protease of *Thermoactinomyces sp.* (alkaline protease) is a serine protease due to the proteolytic activity of the enzyme was abolished by active-site inhibitor of serine protease (DFP and PMSF) while the metalloprotease inhibitor (EDTA) had not any effect on it. Phenylmethylsulfonyl fluoride and Diisopropyl fluorophosphate are irreversibly and specifically react with active site serine residues<sup>(20)</sup>.

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