PARTIAL PURIFICATION AND SOME PROPERTIES OF PROTEASE FROM *MICROSPORUM CANIS*

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ABSTRACT

Clinical strain *Microsporum canis* was isolated from specimen of patient with Tinea corporis in arm and it has ability to produce extra cellular keratinolytic protease in a broth containing human hair. The enzyme was partial purified 35.8-fold from culture filtrate by sequential steps through salting out with ammonium sulfate precipitation (80% saturation), ion exchange Chromatography by batch wise ion exchange by CM- Cellulose cation resin and anion resin by DEAE-cellulose column.

The partially purified enzyme had an optimum activity at pH 9 and maximum activity at pH11. The activity was stable in the alkaline pH 9 for 30 min at 25°C. Enzyme activity toward casein increased when temperature raised more than 20°C and maximal activity attained at 55°C. The enzyme was stable at temperature under 25°C and approximately 80% of its activity abolished by incubation of the enzyme at 60 °C for 30 min. Protease had activation energy equal 3.314 Kcal/Mole that to be able to transform casein to product. On the other hand, the activation energy for denaturation was equal to 49.675 Kcal/Mole. The result of this experiment demonstrated that the enzyme is heat labile.

Keywords: *Microsporum canis*, Protease, purification, properties
التقنية الجزئية وبعض صفات البروتين من

*Microsporum canis*

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الخلاصة

عزلت السلالة السريرية عززم من عينة السعفة الحلقة في الذراع للانسان. تفرز خلايا الفطر البروتيزيز الخارجي إلى الوسط السائل المحتوي على شعر الإنسان. تم تنقية البروتين جزئيا 35.8 مرة من راشح المزرعة بعدها عبارة عن التسريب مثبطات كريتيت الأمونيوم (80% اشبع) ثم CMC-cellulose وكوروموتوغرافيا المتبادل الآبوني بطريقة الوجهة بواسطة المتبادل الآبوني الموجود بطريقة العمد. المتبادل الآبوني السائل DEAE- cellulose

تم دراسة بعض صفات البروتين المنقى جزئياً، إذ وجد أنه يمتلك فعالية مثلى في الرقم الهيدروجيني 9.0 وعلى فعالية له عند الرقم الهيدروجيني 11.0، هناك ثانتو في الرقم الهيدروجيني القاعدي 9 لمدة 30 دقيقة بدرجة حرارة 25 م. تزداد الفعالية الزيتية اتجاه الكازين بارتفاع درجات الحرارة أكثر من 20 م، وكانت الفعالية العظمى له عند 55 م. ويتكون البروتين ثانياً في درجات حرارية أدنى من 20 م ويفقد 80% من الفعالية بعد حصين بدرجة حرارة 60 م لمدة 30 دقيقة. يمتلك الانتي طاقة تنشيط تحويل الكازين إلى الناتج بمقدار 14.3 كيلو سعرة / مول وطاقة التنشيط لمسخ البروتين بمقدار 49.67 كيلو سعرة / مول، مما يشير أن البروتين حساس للحرارة.
INTRODUCTION

Dermatophytes produced proteases that play an important role in the pathogenesis of fungal infections in-vivo. Given the ability of dermatophytes to invade and to be essentially confined to keratinize structures, it can assume that keratinolytic proteases (keratinase) might be significant virulence factors. Microsporum spp. produced proteolytic enzymes such as aspartic, cysteine and metalloproteinases and both M. canis and M. cookei expressed metalloelastinolytic proteinases (1). M. canis secreted keratinolytic protease in a broth containing human hair and the enzyme was purified 134-fold from the culture filtrate by ion-exchange chromatography using DEAE-Sephacel, CM-Sephadex C-50, and by Sephadex G-75 gel filtration. The molecular mass of the purified enzyme is 33 kDa, it had an optimum activity at pH 8, and the activity was stable in the alkaline pH range. Enzyme activity increased with temperature up to 35°C and was stable up to 45°C (2). Mignon et al. purified and characterized two M. canis keratinases, a 31.5 kDa subtilisin-like protease and a 43.5 kDa metalloprotease produced in vitro in a minimal feline keratin-enriched medium (3,4). To better understanding of dermatophytic infection pathogenesis and subsequently of the host-fungus relationship, therefore the purification and characterization of keratinolytic protease seems to be a major step. This paper describes the partial purification and characterization of protease from M. canis, which isolated from patient infected with Tinea corporis in arm.

MATERIAL AND METHODS

Bacterial Strain and Culture:

M. canis strain used in this study was isolated from clinical specimen of patient with Tinea corporis in arm and it has ability to produce extracellular keratinolytic protease in a broth containing human hair (5).

Protease Production:

One liter of culture supernatant of M. canis was prepared as described by Lee et al. with some modification [2]. Briefly, the fungus mycelium was grown in Sabouraud’s dextrose agar at 25°C for 8 days to prepare 5mm discs for using as inoculums. The culture medium for protease induction contained glucose, 0.5g; MgSO4.7H2O, 0.6g;
inositol, 0.05g; thiamine.HCl, 0.01g and pyridoxine. HCl, 0.01g in 1L of 28 mM phosphate buffer, pH 9. As nitrogen source, human scalp hairs added to the culture medium in final concentration of 0.52 g/L. The sterilized medium (each 50ml broth in500ml flasks) inoculated with three discs of fungal growth for each flask; the cultures were allowed to stand for 5 days at 35°C and then were shaken (150 rpm) for 5 days. At the end of the growth period, the fungal mycelium and the residual hair were removed from the flask by filtration and the supernatant was sterilized by filtration through a filter with 0.22 µm pores. The culture filtrates were retained for purification of the enzyme. The casinolytic activity and protein concentration of culture supernatant was determined.

**Enzyme Assay:**

Casinolytic activity of protease was measured as follows: 0.1 ml of the enzyme was mixed with 0.9 ml of phosphate buffer (0.2M, pH 8) which contained 5 mg Hammerstein casein (Merck). After incubation at 37°C for 20 min, 2ml of 5% trichloroacetic acid (TCA) was added to terminate the reaction. The mixture was passed through Watman No.2 filter paper to remove denatured proteins. The absorbancy of the supernatant fraction was read at 280 nm. Blanks were stopped with the addition of TCA before the addition of the enzyme. One unit of activity was defined as the amount of enzyme required to produce an increase 0.001 in the absorbancy at 280 nm per min (6). Specific activity expressed as enzyme units per mg protein. The protein content was estimated by the absolute method of Whitaker and Granum (7).

**Partial Purification of the Enzyme:**

The purification of protease was performed as described by Lee et al.(2). Briefly, Proteins from culture supernatant (one liter) were fractionated with ammonium sulfate 40, 45, 50, 60, 65, 70, 75 and 80% saturation at 4º C respectively (2, 8). The precipitation was removed from culture supernatant by centrifugation at 6000 xg for 20 min at 4º C for each step. The precipitates were dissolved in small amounts of 5mM glycine-NaOH-buffered pH 9 separately, then the enzyme activity detected in each fraction and supernatant. The fraction with protease activity (80% saturation) was then dialyzed overnight against 5 mM glycine-NaOH saline (pH 9). The dialyzed supernatant (12ml after adjusted to pH 6) was applied to Carboxymethyl Cellulose – Cation exchange (Bio-Rad). The cation exchange matrix washed with 5mM phosphate buffer pH 6 and eluted by the addition of 5 ml of 5mM phosphate buffer with 0.1 M NaCl at pH 8.
Then the protein of washed and eluted fractions was detected at 280 nm absorbance and the casienolytic activity of washed and eluted fractions was assayed. The protease fractions was combined, concentrated with cooled acetone (one vol enzyme: two vol acetone), dialyzed and determined protease activity and total protein concentration. The concentrated enzyme was applied on DEAE-Cellulose – anion exchange column (0.5x7 cm). The DEAE-cellulose matrix (Bio-Rad) was washed with 50 ml of 5mM Tris-HCl buffer pH 7.4. Then the column was eluted by the addition of 100 ml of 5mM Tris-HCl buffer with stepwise gradient of 0- 0.5 M NaCl at pH 8 at flow rate of 16ml/h, and 1ml fractions were collected. The protein of washed and eluted fractions was detected at 280 nm absorbance as well as protease activity in all of the fractions. The fractions with protease was combined, concentrated with cooled acetone (one vol enzyme: two vol acetone), dialyzed and determined protease activity and total protein concentration.

**Effect of pH on the Activity and Stability of Protease:**

The reaction between partially purified protease and 0.5% casein substrate was assayed in triplicates for 20 min in solutions ranging from pH 6 to 12. 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 protease, enzyme was mixed with substrate after the addition of trichloroacetic acid solution. The absorbance of the supernatant fraction was read at 280 nm.

To determine the effect of pH on stability of protease, protease was preincubated in triplicates at 25°C for 30 min in various pH values ranging from 3 to 12. 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). Then the residual activity was measured with casein at 37°C for 20 min.

**Effect of Temperature on the Activity and Stability of Protease:**

Effect of temperature on enzymatic activity was determined by the reactions between partially purified protease and 0.5% casein substrate assayed (in triplicates) for 20 min in 20mM glycine-NaOH buffer (pH9) at various temperatures ranging from 20 to 80 °C, each reaction was stopped by addition of trichloroacetic acid solution. Blanks consisted of the casein substrate in the same buffer without protease and the enzyme was mixed after the addition of trichloroacetic acid solution. The absorbance of the supernatant fraction was read at 280 nm.

To determine the effect of temperature on the stability of the enzyme, protease had been preincubated (in triplicate) for 30 min at various temperature (0-70°C) in 20mM
glycine-NaOH buffer (pH9). Cooling in ice bath stopped heating of the enzyme, and then the residual activity was measured at 37°C for 20 min. Blanks consisted of the casein substrate in the same buffer without protease and the enzyme was mixed after the addition of trichloroacetic acid solution. The absorbance of the supernatant fraction was read at 280 nm.

RESULTS

The active extracellular protease secreted into the culture medium containing human hair. The enzyme was fractionated by 40-80% saturation of ammonium sulfate to remove impurity protein. The enzyme precipitated at 80% saturation and dialyzed. Protease was partially purified from concentrated supernatant fraction (12ml) by ion exchange chromatography. The batch wise purification of protease by CM-cellulose showed 21.3-fold increase in specific activity that was 1215 U/mg protein. The enzyme returned in wash of the cation exchange resin (CMC) by 5mM phosphate buffer at pH 6. The enzyme was further purified using DEAE-Cellulose column. The protein of enzyme adhered to the anion exchange resin (DEAE cellulose) and it eluted from the resin by 5mM Tris-HC buffer with 0.5 NaCl at pH 8. The stepwise purification of the enzyme in more than a 35.8fold increase in specific activity (2043 U/mg protein) as summarized in Table (1).
Table (1): Partial Purification of Protease from *Microsporum canis*

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Vol. ml</th>
<th>Activity U/ml</th>
<th>Total activity U</th>
<th>Protein concen. mg/ml</th>
<th>Total protein concen. mg</th>
<th>Specific activity U/mg protein</th>
<th>Purification Fold</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude filtrate</td>
<td>100</td>
<td>15</td>
<td>1500</td>
<td>0.262</td>
<td>26.2</td>
<td>57</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation (80% saturation)</td>
<td>12</td>
<td>102</td>
<td>1224</td>
<td>0.122</td>
<td>1.47</td>
<td>836</td>
<td>14.7</td>
<td>81.6</td>
</tr>
<tr>
<td>Batch wise CM-Cellulose Chromatography (Wash)</td>
<td>4</td>
<td>260</td>
<td>1040</td>
<td>0.214</td>
<td>0.856</td>
<td>1215</td>
<td>21.3</td>
<td>69.3</td>
</tr>
<tr>
<td>DEAE-Cellulose Chromatography (Elution)</td>
<td>4</td>
<td>233</td>
<td>932</td>
<td>0.114</td>
<td>0.456</td>
<td>2043</td>
<td>35.8</td>
<td>62.1</td>
</tr>
</tbody>
</table>

Enzyme assays conducted at nine different pH values indicated that the maximal protease activity occurred at pH 11 Fig. (1).
A. Effect of pH on enzyme activity (●). The caseinolytic activity was measured in total volume of 1.0 ml 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- 12.3)] at 37°C for 20 min. B. Effect of pH on the stability of the enzyme (♦). After protease had been preincubated at 25°C for 30 min in the same buffers, the residual activity was measured with casein under assayed conditions.

Protease activity increased from pH 7 to 11; pH 12 demonstrated approximately 22% of maximal activity. Studies of the pH stability of protease demonstrated that approximately 80% of the activity abolished by incubation of the enzyme at pH 5 and 12 but the enzyme retained its activity at pH 9 and retained more than 90% of its activity at pH 8-10 for 30 min at 25°C Fig. (1).

Enzyme activity of protease increased with temperature from 20 to 55°C with maximal activity occurring at 55°C Fig. (2). Studies of the thermal liability of protease demonstrated that approximately 27% and 80% of the activity was abolished by incubation of the enzyme at 50 and 60 °C for 30 min Fig. (2). Thus, the enzyme is heat labile. We can considered the optimum pH of activity and stability at pH 9 and optimum
temperature activity at 25-30 °C because of 100-93% of the enzyme activity is stable at the same temperature.

**Fig.(2): The Effect Temperature on protease activity and stability from *Microsporum canis*.

A. Effect of temperature on enzyme activity (●). Protease has been assayed with casein substrate (0.5%) in 20mM glycine-NaOH buffer (pH9) at various temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60 and 65) for 20 min. B. Effect of temperature on the stability of enzyme (♦). Protease had been preincubated for 30 min at various temperatures (0-65°C) in 20mM glycine buffer (pH9). Cooling in an ice bath stopped heating of the enzyme, and then the residual activity was measured with casein substrate (0.5%) at 37°C for 20 min.

The Arrhenius curve Fig. (3) revealed that the activation energy to transform the substrate to product by partial purified protease from *M.canis* equal approximately 3.314
Kcal/ Mole. This curve also revealed that the value of activation energy for denaturation of enzyme that equal approximately 49.675 Kcal/ Mole. This value denoted that the enzyme is efficient catalyst to transform the substrate (casein) to product as reported by Whitaker (15). The curve also demonstrated the value of activation energy for denaturation of the enzyme is approximately equal to 49.675 Kcal/Mole. This value denoted that the enzyme is heat labile as reported by Whitaker (15).

Fig.(3): Arrhenius curve for determined activation energy to partial purified protease from Microsporum canis

Slope= -Ea/ R
Slope = – 1.66784
Ea = 3.314 KCaMole of Activation

Slope=Ea/R
Slope= 25
Ea = 49.675 KCaMole of Denaturation

$\frac{1}{T} (\text{K}) \times 10^3$

2.303 log k

0 1.5 3 4.5

0 2 3 3.1 3.2 3.3 3.4 3.5
DISCUSSION

Keratinolytic proteases secreted by dermatophytes are likely to be virulence-related factors. *M. canis*, the main agent of dermatophytosis in dogs and cats, causes a zoonosis that is frequently reported. Many researchers in the past have attempted to assess role of proteinases and effects of mechanical forces exerted by the invading microorganism on the host keratinized tissue. Some groups of researchers were able to isolate enzymes from culture medium *in vitro* containing hairs. They observed that this enzyme caused degradation and degeneration of human and guinea pig hair used as substrates *in-vitro* and because of this specific actions by these enzymes they were termed as Proteinases or Keratinases (9,10). Samdani and Al-Bitar suggested that the secretion of proteinases together with the mechanical force of the invading organisms *in-vitro* might be playing part in the inversion of the organisms (10). There is evidence that some dermatophytes enzyme give rise to hydrolytic activity against keratin substance such as *Trichophyton mentagrophytes, T. rubrum, M. gypseum, M. canis* (11,12,13). In present paper, we purified protease partially from the human hair containing culture medium of *M. canis* by ammonium sulfate fractionation followed by batch -wise ion exchange by CM-cellulose and then DEAE-cellulose column and raises the specific activity 35.8 times with recovery of 62 %. Lee *et al.* purified a Keratinolytic protease of *M. canis* from the culture containing human hair to apparent homogeneity. The enzyme was purified 134-fold to a specific activity of 123 unit/mg by ion-exchange chromatography using DEAE-Sephacel, CM-Sephadex C-50 and Sephadex G-75 gel filtration (2). Whereas Mignon *et al.* purified tow *M. canis* keratinases, a 31.5 kDa subtilisin-like protease and a 43.5 kDa metalloprotease, from culture filtrate by affinity chromatography on bacitracin-agarose and by hydrophobic chromatography on octyl-agarose (3, 4).

Characteristic of the extracellular protease from *M. canis* in the present study is active over wide range of neutral and alkaline pH values (7-12) with maximal activity at pH 11 and the optimum pH of activity and stability at pH 9 for 30 min at 25°C. The protease is active over wide range of temperatures (20-55°C) with maximal activity occurring at 55°C and optimum temperature activity at 25- 30 °C because of 100- 93% of the enzyme activity is stabile at the same temperature. Lee *et al.* reported that the keratinolytic activity of protease from *M. canis* has optimum pH at 8 and the optimum temperature activity at 30-35 °C (2,14).
REFERENCES


