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TWO CELL-BOUND KERATINASES OF *TRICHOPHYTON MENTAGROPHYTES**

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ABSTRACT

Two cell-bound keratinases, II and III, of *Trichophyton mentagrophytes* were extracted from mycelium and purified. The purified keratinases, II and III, had a specific keratinolytic activity of 36.4 and 39.4 KU/mg respectively. The molecular weights of keratinases II and III were 440,000 and 20,300 respectively. Immunodiffusion analysis showed that these two cell-bound keratinases, II and III, were not identical to each other nor to extracellular keratinase I of the same species.

The isolation and purification of an extracellular keratinase (keratinase I) of *Trichophyton mentagrophytes* var. *granulosum* (= *T. granulosum* Sabouraud, 1909) has been reported previously (1). This keratinase I showed remarkable keratinolytic activity as demonstrated by rapid hydrolysis of unautoclaved guinea pig hair at pH 7.0 (2). The amino acid composition of keratinase I has been determined and studies of substrate specificity showed that this enzyme also hydrolyzes casein, collagen, elastin, fibrin, fibrinogen, gelatin, hemoglobin, insulin and ovalbumin (3).

The present paper describes the isolation and purification of two cell-bound keratinases, II and III, from the mycelium of the same species that excretes keratinase I when grown under the same conditions.

MATERIALS AND METHODS

Substrate. Guinea pig hair was prepared as described previously (2).

DEAE- and CM-Cellulose columns. DEAE-cellulose (Whatman DE1) and CM-cellulose (Whatman CM1) were pretreated according to Whatman Data Manual and Catalog 2000 (Reeve Angel & Co., Inc., Clifton, New Jersey). The DEAE-cellulose column (4.2 × 30 cm) and CM-cellulose column (4.2 × 38 cm) were prepared with 0.028 M phosphate buffer, pH 7.0 and 6.5 respectively. A chromatographic DEAE-cellulose column (DE52, microgranular 2.5 × 34 cm) was prepared with 10 mM phosphate buffer, pH 7.8.

Gel filtration. Sephadex G-100 and G-200 and Sepharose 2B were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey. The gel filtration columns of Sephadex G-100 and G-200 were prepared in 7 mM phosphate buffer, pH 7.8, with or without 1 NaCl according to an instruction booklet supplied by the company. A Sepharose 2B column (1.5 × 88 cm) was prepared with 7 mM phosphate buffer, pH 7.8.

Purification of keratinases. Trichophyton mentagrophytes var. *granulosum* (= *T. granulosum* Sabouraud, 1909) was grown in six 6-l Erlenmeyer flasks, each containing 4.5 liters of keratin medium composed of (per liter): horse hair, 2.5 g; glucose, 0.9 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; thiamine HCl, 0.01 g; pyridoxine HCl, 0.01 g; and inositol, 0.05 g; in 7 mM phosphate buffer, pH 7.8. Media ingredients were sterilized at 121° and 15 psi for 20 minutes. After inoculation, the cultures were allowed to stand for 5 days at 25° and then were shaken for 7 days. At the end of the growth period, the mycelium and residual hair were separated from the culture fluid by filtration.

The mycelium and residual hair were washed with cold water (200 ml), then soaked in 1 M NaCl in 0.028 M phosphate buffer, pH 7.8, (1 l), at room temperature for 1 hour. The mixture was filtered and the filtrate was dialyzed against 1 mM phosphate buffer, pH 7.8 (12 l) at 4° for 16 hours. The dialyzed mycelial extract (1,102 ml, pH 7.8) of *T. mentagrophytes* was passed through a DEAE-cellulose column. The effluent, adjusted to pH 6.5 with phosphoric acid, was passed through a CM-cellulose column. The crude keratinase II adsorbed on the DEAE-cellulose was eluted with 1 M NaCl in 0.01 M phosphate buffer, pH 7.5 (200 ml), dialyzed against deionized water (12 l), lyophilized and purified by gel filtration on Sephadex G-100 (Fig. 1). The crude keratinase III adsorbed on the CM-cellulose was eluted with 1 M NaCl in 0.01 M phosphate buffer, pH 7.8 (200 ml), dialyzed against deionized water (12 l), lyophilized and purified by gel filtration on Sephadex G-100 (Fig. 4). All purification was carried out at 4°.

Keratinase assay. Unsterilized guinea pig hair

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TABLE

Purification of keratinases II and III

Procedure	Volume (ml)	Total protein (mg)	Total units ($\times 10^3$ KU)	Specific activity (KU/mg)	Activity (%) of total mycelial extract
1. Mycelial extract	1,102	153	3.05	19.9	100
Keratinase II					
2A. DEAE-cellulose	215	19.6	0.46	23.4	15.1
3A. Sephadex G-100	12	8	0.29	36.4	9.5
Keratinase III					
2B. CM-cellulose	329	49	1.43	29.1	46.9
3B. Sephadex G-100	53	23	0.91	39.4	29.8

(3–5 mm long, 50 mg) was suspended in 0.05 M phosphate buffer containing 1 mM $MgSO_4$, pH 7.0 to which 50 μg of enzyme material was added; final volume 5 ml. Controls included enzyme material in buffer and hair in buffer. The reaction mixtures were incubated at 37° for 1 hour, then filtered. Corrected absorbance values of the reaction fluid at 280 nm were converted to keratinase units (1 KU = 0.100 corrected absorbance) and the specific activity was expressed as KU per milligram of protein (2). The assays were performed in duplicate. Protein concentrations of solutions were estimated spectrophotometrically at 280 and 260 nm in a Beckman Spectrophotometer, Model DB, using the formula suggested by Layne (4) and also by the Folin-Ciocalteu method (5).

Disc electrophoresis. Disc electrophoresis was performed at room temperature in a Model 6 Canalco Apparatus (Canal Industrial Corporation, Rockville, Maryland) using 7% polyacrylamide gel at pH 9.5, 6.0 and 4.3 according to Davis (6) and Williams and Reisfeld (7). The treatment of enzyme samples with mercaptoethanol was carried out as follows: the sample was dissolved in 0.01 M phosphate buffer containing 8 M urea, pH 7.7 (25 μl), and 1 M mercaptoethanol (25 μl) was added. The mixture was kept at 4° for 16 hours before electrophoresis.

Molecular weight. The molecular weight of the keratinases was estimated on Sephadex G-100 and G-200 according to the methods of Andrews (8) and Whitaker (9). Molecular weight markers were purchased from Mann Research Laboratories, Inc., New York, New York.

Antisera to keratinase II. Two white adult rabbits, New Zealand strain, were injected subcutaneously and intramuscularly; each injection comprised 2.5 mg purified salt-free keratinase II in 0.5 ml Freund's incomplete adjuvant. Three weeks later, 5 mg of enzyme in 1 ml Freund's incomplete adjuvant was injected intramuscularly. Two additional intramuscular injections were given two weeks and four weeks later. They consisted of 5 mg enzyme in 1 ml saline. The antiserum used for this analysis was obtained by cardiac puncture seven days after the last injection. Sera were screened for precipitating antibody by immunodiffusion analyses according to the method of Ouchterlony (10).

RESULTS

Keratinase II

1. *Isolation and purification.* Crude keratinase II was separated completely from keratinase III by passing the dialyzed mycelial extract through a DEAE-cellulose column (see Table). Whereas

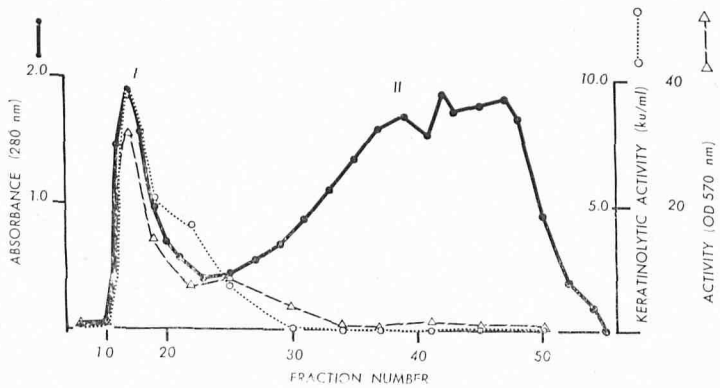

FIG. 1. Gel filtration of crude keratinase II on Sephadex G-100. A sample (184 mg) of lyophilized crude keratinase II was dissolved in 7 mM phosphate buffer, pH 7.8 (20 ml), centrifuged, and the clear supernatant was layered onto a Sephadex G-100 column (2.5 × 90 cm). The column was eluted with the buffer and the eluate collected at 40 ml/hr, 9.7 ml per tube. Symbols: ●——●, absorbance at 280 nm; ○---○, keratinolytic activity (KU/ml); △---△, activity by ninhydrin assay, OD at 570 nm, (2).

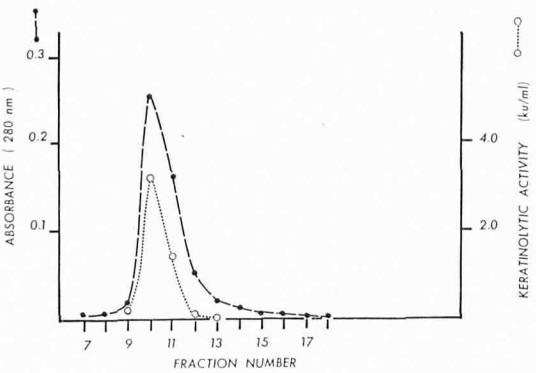

FIG. 2. Gel filtration of purified keratinase II on Sephadex G-200. A sample (5 mg) of lyophilized fraction I (see Fig. 1) was dissolved in 7 mM phosphate buffer, pH 7.8 (0.9 ml) and layered onto a Sephadex G-200 column (1.5 × 86 cm). The column was eluted with buffer, and the eluate collected at 20 ml/hr, 5.3 ml per tube. Symbols: ●---●, absorbance at 280 nm; ○---○, keratinolytic activity (KU/ml).

keratinase III was not retained by DEAE-cellulose, keratinase II was completely adsorbed. The crude keratinase II, adsorbed on the DEAE-cellulose, was eluted with 1 M NaCl in buffer, dialyzed and lyophilized. This DEAE-cellulose eluate showed a specific activity of 23.4 KU/mg (see Table). Further purification of keratinase II was achieved by gel filtration of the eluate on Sephadex G-100. The elution diagram of the gel filtration is shown in Figure 1. Fraction I contained the major part of the keratinolytic activity as shown by keratinase assays. This fraction showed a specific activity of 36.4 KU/mg. A second gel filtration of fraction I on Sephadex G-200 showed a single, symmetrical peak but resulted in a slight loss of activity (Fig. 2). Fraction I from the first gel filtration (Fig. 1) was therefore taken as purified keratinase II.

The purified keratinase II appeared to be homogeneous on DEAE-cellulose (DE52, microgranular) column chromatography when the column was eluted with 1 M NaCl in 0.01 M phosphate buffer, pH 6.5.

2. *Disc electrophoresis.* The purified keratinase II gave a single band when stained with amido black on disc polyacrylamide electrophoresis run at pH 9.5 (Fig. 3A). However, no keratinolytic activity could be detected in the gel fraction which coincided with this protein band or in any other gel fraction. When the electrophoresis was run at pH 6 with or without 10 mM mercaptoethanol, the purified keratinase II showed a major band and traces of four other slower moving bands (Fig. 3B). The three gel fractions coinciding with the major and two slowest-moving protein bands showed keratinolytic activities.

3. *Molecular weight.* The molecular weight of keratinase II estimated on Sephadex G-200 was approximately 440,000. An attempt to estimate molecular weight of keratinase II on Sepharose 2B was not successful.

Gel filtration of keratinase II on Sepharose 2B gave a major and a minor component with no measurable keratinolytic activity.

Keratinase III

1. *Isolation and purification.* The effluent from the DEAE-cellulose column was passed through

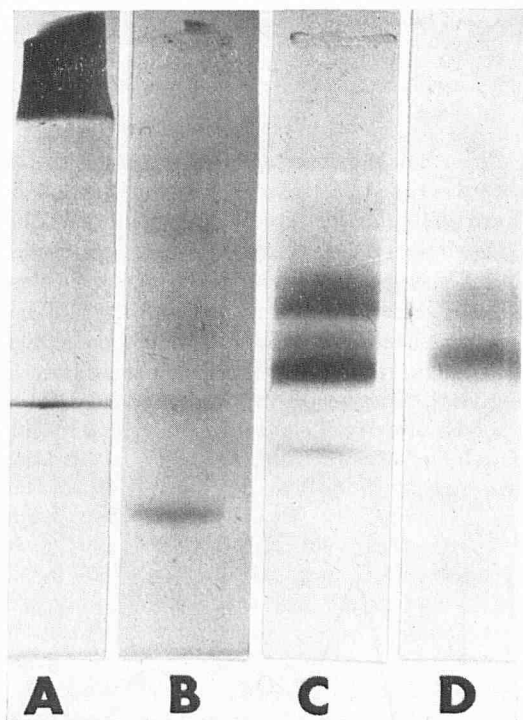

FIG. 3. Disc electrophoreses of purified keratinases in 7% polyacrylamide gel. (A) keratinase II, 456 μg, run at pH 9.5 for 3 hours, (B) keratinase II, 182 μg, run at pH 6 for 2.5 hours, (C) keratinase III, 275 μg, run at pH 6 for 2.5 hours, (D) keratinase III, 413 μg treated with 0.5 M mercaptoethanol, run at pH 6 for 2.5 hours. After electrophoresis, the gels were cut in half. One half was stained with amido black and the other half was cut into 14 fractions by using a Canalco lateral gel slicer. Each fraction was extracted with 1 M NaCl in 0.01 M phosphate buffer, pH 7.8 (1 ml) for 4 hours and the extracts were assayed for keratinolytic activity using guinea pig hair as a substrate (Table I).

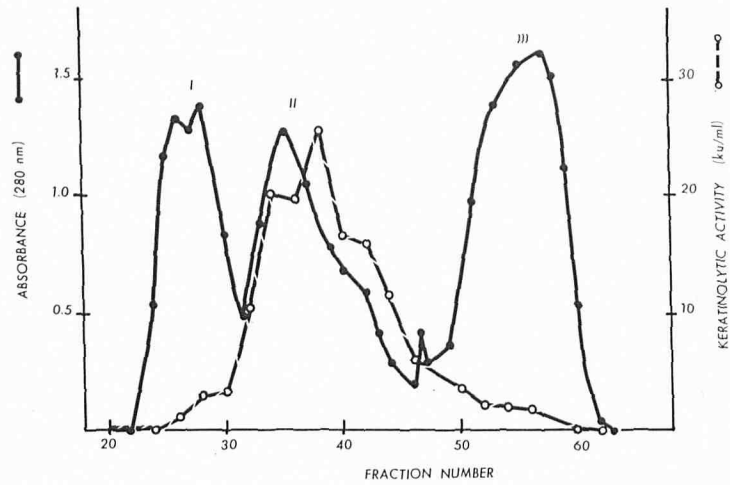

FIG. 4. Gel filtration of crude keratinase III on Sephadex G-100. A sample (173 mg) of lyophilized crude keratinase III was dissolved in 7 mM phosphate buffer, pH 7.8 (15 ml), centrifuged, and the clear supernatant was layered onto a Sephadex G-100 column (2.5 × 90 cm). The column was eluted with buffer and the eluate collected at 58 ml/hr, 9.7 ml per tube. Symbols: ●——●, absorbance at 280 nm; ○- - -○, keratinolytic activity (KU/ml).

a CM-cellulose column. Crude keratinase III adsorbed on the CM-cellulose was eluted with 1 M NaCl in buffer, dialyzed and lyophilized. This CM-cellulose eluate showed a specific activity of 29.1 KU/mg. Further purification of keratinase III was achieved by gel filtration of the CM-cellulose eluate on Sephadex G-100. The elution diagram of the first gel filtration is shown in Figure 4. Fraction II included the major keratinolytic activity. This fraction showed a specific activity of 39.4 KU/mg. A second gel filtration of fraction II gave, in addition to this fraction, a fragment IIA with a specific activity of 8.7 KU/mg (Fig. 5). The weight ratio of fraction II and the fragment IIA was 66:34. A third gel filtration of fraction II gave a similar elution diagram to that of the second gel filtration. The weight ratio of the two fractions in the third gel filtration was 65:35. Gel filtration of fragment IIA did not include any trace of fraction II; the fragment IIA was recovered unchanged. Gel filtration of fraction II on Sephadex G-200 gave a similar elution diagram to that on Sephadex G-100.

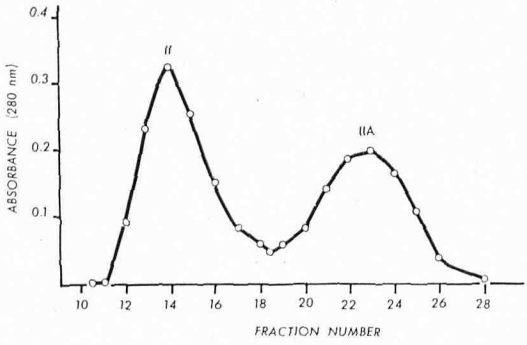

FIG. 5. Gel filtration of purified keratinase III on Sephadex G-100. A sample (6.5 mg) of lyophilized fraction II (see Fig. 4) was dissolved in 7 mM phosphate buffer, pH 7.8 (1 ml) and was layered onto a Sephadex G-100 column (1.5 × 85 cm). The column was eluted with buffer, and the eluate collected at 20 ml/hr, 5.3 ml per tube. Symbols: ○——○, absorbance at 280 nm.

Since fraction II in solution yields its fragment IIA and the presence of the latter in solution appeared to stabilize the former, fraction II from the first gel filtration (Fig. 4) was taken as a purified keratinase III.

2. *Disc electrophoresis.* The purified keratinase III with or without 10 mM mercaptoethanol gave two major bands and a trace of a third band when stained with amido black on disc polyacrylamide electrophoresis run either at pH 6 (Fig. 3C) or at pH 4.3. Three gel fractions coinciding with the protein bands showed keratinolytic activities. The purified keratinase III after treatment with 0.5 M mercaptoethanol gave a major and a minor band when stained with amido black on electrophoresis run at pH 6 (Fig. 3D). However, neither the gel fractions

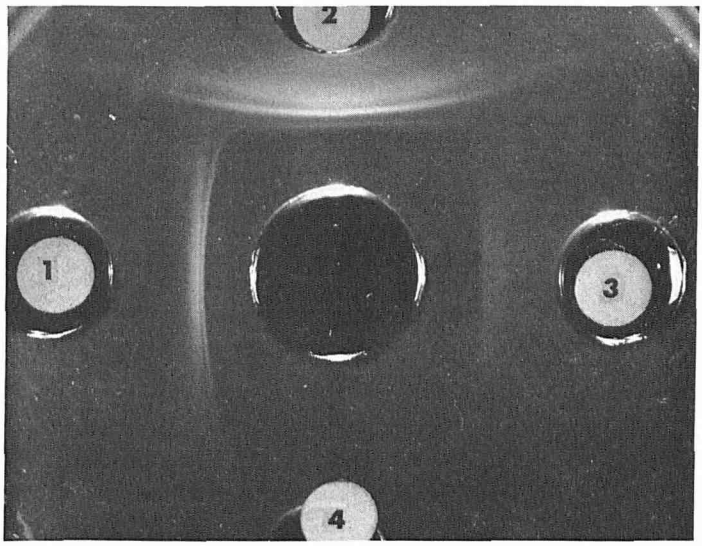

FIG. 6. Immunodiffusion analysis. Peripheral wells: 1. keratinase I. 1.0 mg/ml (1), 2. keratinase II, 1.0 mg/ml, 3. keratinase III, 1.0 mg/ml and 4. saline. Central well: rabbit antiserum to keratinase II.

coinciding with the protein bands nor any other gel fractions showed any keratinolytic activity.

3. *Molecular weight.* The molecular weight of keratinase III (fraction II) estimated on either Sephadex G-100 or G-200 was approximately 20,300.

Immunodiffusion analysis

Figure 6 shows that the antigenic structure of keratinase II (well no. 2) was only partially identical to that of keratinase I (well no. 1) and of keratinase III (well no. 3). Keratinase II gave six precipitin bands with the antiserum. The three strongest bands were not present with keratinase I and III. The precipitin bands, obtained with each of the enzymes which appeared to be identical, did not represent the dominant antigenic determinants of keratinase II.

DISCUSSION

Two cell-bound keratinases were isolated and purified from a mycelial extract of *Trichophyton mentagrophytes*. The purified keratinases II and III had specific activities of 36.4 and 39.4 KU/mg respectively.

Although the purified keratinase II gave a single band on disc polyacrylamide electrophoresis run at pH 9.5, the enzyme became denatured under such conditions. Keratinase II showed multiple forms when the electrophoresis was run at pH 6.

Keratinase III appeared to consist of a major component (fraction II) and a fragment (fraction IIA). The weight ratio of these two fractions was approximately 2:1. It appears that fraction II easily converts to its fragment IIA.

Not only the specific activities, but also the molecular weights of the cell-bound keratinase II and III were different from that of the extracellular keratinase I which was isolated from the culture fluid of the same species (1, 3). Whereas keratinase I had a molecular weight of 48,000, keratinases II and III had MW of approximately 440,000 and 20,300 respectively. It is likely that keratinase II may have an even higher MW than that determined by gel filtration on Sephadex G-200 since the elution volume of this enzyme was close to the exclusion volume.

Immunodiffusion analysis confirmed that the three keratinases are not identical.

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