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Purification and partial characterization of two extracellular keratinases of *Scopulariopsis brevicaulis*

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Abstract

Two extracellular keratinases of *Scopulariopsis brevicaulis* were purified and partially characterized. The enzymes were isolated by the techniques of gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These keratinases (K I & K II) were purified approximately 33 and 29 fold, respectively. SDS-PAGE of the products of gel filtration chromatography (K I & II) produced only one band each, suggesting homogeneity. The optimum pH for both keratinases was 7.8, while the optimum temperatures were 40 °C (K I) and 35 °C (K II). Estimated molecular weights were 40–45 KDa and 24–29 KDa for K I & K II respectively. Both keratinases were inhibited by phenylmethylsulfonyl fluoride which suggests a serine residue at or near an active site.

Introduction

The role of proteolytic keratinases in the virulence and pathogenesis of dermatomycoses has been postulated by several mycologists [1–3]. Moreover, keratinolytic enzymes from several species of dermatophytes have been isolated and characterized [4–6]. Our studies on a number of different fungal isolates showed considerable variation in their ability to hydrolyze keratin [3, 7, 8]. *Scopulariopsis brevicaulis*, a known etiology of human onychomycoses [9], has received little attention with regard to its ability to hydrolyze keratin [3]. The purpose of this study was to purify and partially characterize the extracellular keratinases of this fungus.

Materials and methods

S. brevicaulis (Sacch) Bainier (IMI 313131) [10] was maintained on Sabourauds agar at 28 ± 1 °C. Human hair was sterilized with chloroform : methanol (1 : 1, v/v) for 24 h and washed with distilled water and air-dried. Additionally, the hair (500 mg) was incubated at 80 °C for 30 min on three successive days with intermediate incubation at 30 °C [11]. The basal salts medium contained: KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.025; $CaCl_2$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.015 and $ZnSO_4 \cdot 7H_2O$, 0.005 as g/L of glass distilled water. Erlenmeyer flasks (150 ml) containing 50 ml of the basal salt medium were sterilized at 121 °C for 15 min in an autoclave. After steriliz-

ation, hair (500 mg) was added to the flasks and the flasks were inoculated with 10^8 spores from a suspension obtained by agitating a 10 day old culture with glass beads and sterile saline. The flasks were incubated as stationary cultures at 28 ± 1 °C. Keratinase activity was assayed by the methods of Noval [12] and protein concentrations were determined according to Lowry et al. [13]. One unit of enzyme activity (KU) was defined as the amount of enzyme that could liberate products having an absorbance of 0.1 under the conditions described. The specific activity was the number of units of activity per mg protein.

Keratinase purification

Culture fluids were harvested on the 40th day of incubation – the point of maximal enzyme activity.

Lyophilization and dialysis. Crude culture fluids (200 ml) were concentrated *in vacuo* and dialyzed (Cellophane membrane, Sigma, USA) against 0.056 M phosphate buffer for 48 h with a single change of buffer after 24 h.

Gel filtration. A dialyzed sample (1.0 ml) was subjected to gel filtration fractionation with a Sephadex G-100 (LKB, Sweden) column (74 × 1.5 cm) that had been equilibrated with 0.056 M phosphate buffer (pH 7.8). Elution was conducted at a flow rate of 15 ml/h and 3 ml fractions were collected. Two major peaks, keratinase I & II or K I & II, were detected and the fractions containing these peaks were pooled separately. These pools were lyophilized for further purification.

Polyacrylamide gel electrophoresis. Preparative polyacrylamide gel disc electrophoresis with 7.5% gels was conducted to detect keratinase activity in purified fractions. Reference protein bands were visualized with Coomassie brilliant blue and homologous regions (bands) in unstained gels were excised and homogenized in water (5 ml) at 0–4 °C with glass homogenizer. The suspension was centrifuged at 3000 g for 20 min and keratinase activity of the supernatant fluid was assayed.

Molecular weight determinations. The molecular weights of purified keratinases were determined by gel filtration chromatography with Sephadex G-100 (14) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15) by using standard molecular weight markers (Sigma, USA).

pH optimum and stability. Buffers included: HCl/KCl, pH 2–3; sodium acetate/acetic acid, pH 4–6; sodium phosphate, pH 7–8 and Tris/NaOH, pH 9–12. The optimum pH for the fungal keratinases was measured by adding purified proteins to a solution containing hair and buffer (0.056 M). The stability of the keratinases to pH was determined by first incubating the purified proteins for 1 h at 37 °C in each of the various buffers (0.056 M) and then assaying for residual activity at pH 7.8.

Temperature optimum and stability. The temperature optimum for the keratinases was measured by incubation of hair and keratinase in phosphate buffer (pH 7.8, 0.056 M) at temperature between 10–90 °C for 1 h. The thermal stability of the keratinases was determined by incubating keratinases in the same buffer for 2 h at temperatures between 10–90 °C and then assaying for residual activity at 37 °C.

Effect of inhibitor. Purified fungal keratinases were preincubated with each inhibitor at pH 7.8 for 1 h at 37 °C and then assayed for residual activity. The inhibitors were: *p*-chloromercuribenzoate in NaOH/Tris buffer (pH 10); phenylmethylsulfonyl fluoride (PMSF) in 100% methanol; cysteine in NaOH; ethylenediaminetetraacetic acid (EDTA), $HgCl_2$, KCN, and 2-mercaptoethanol dissolved separately in distilled water.

Table 1. Summary of purification steps of extracellular keratinases of *S. brevicaulis*

S. No.	Fractionation step	Volume (ml)	Total protein (mg)	Total activity (10^3 KU)	Specific activity (KU/mg)	Recovery (%)
1.	Crude culture fluid	200	64.0	0.49	7.6	100.0
2.	Vacuum evaporation	20	29.0	0.42	14.4	85.7
3.	Dialysis	25	14.0	0.30	21.4	71.4
4.	Pooled Sephadex G-100 fraction I	20	2.0	0.60	100.0	66.6
	Homogenous fraction after PAGE I	5	0.4	0.10	250.0	50.0
5.	Pooled Sephadex G-100 fraction II	20	1.8	0.21	116.0	70.0
	Homogenous fraction after PAGE II	5	0.5	0.11	220.0	52.3

Total purification fold K I – 32.67 and K II – 28.75.

Results and discussion

Results of purification of the extracellular keratinase of *S. brevicaulis* are summarized in Table 1. Keratinase activity (2.45 KU/ml) in crude culture extracts on day 40 was similar to that reported for *Microsporum canis* [5] and *Trichophyton rubrum* [6]. Concentration of the culutre fluid by vacuum evaporation resulted in a decrease in keratinase activity. A similar result was observed following dialysis of enzyme samples with phosphate buffer (pH 7.8), even though the specific activity increased. Gel filtration chromatography of concentrated culture filtrates produced two peaks that had keratinase activity (Fig. 1.), and were denoted K I and K II. Further purification of the keratinases by PAGE resulted in the identification of a single band of activity for each enzyme (Fig. 2). Keratinase I and II were purified 32.6 and 28.7 fold, respectively. Enzyme recovery after PAGE was 50% (K I) and 52.3% (K II) of the original activity in the culture filtrates. The molecular weights of K I and K II by gel filtration chromatography were 45 kDa and 24 kDa, respectively, while SDS-PAGE suggested molecular weights of 50 kDa (K I) and 29 kDa (K II).

The effects of pH and temperature on keratinase activity of the two enzymes are shown in Table 2. The optimum pH for both K I and II

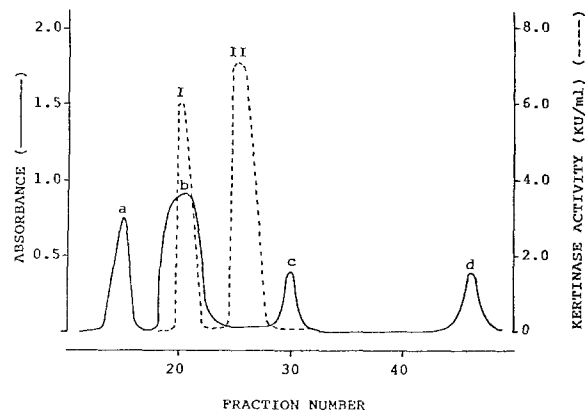

Fig. 1. Gel filtration elution profiles of the crude dialized extracellular keratinase of *S. brevicaulis* and molecular weight markers. (a) Albumin bovine serum (66 kDa), (b) Albumin chicken egg (45 kDa), (c) Carbonic anhydrase (29 kDa) and (d) α-lactalbumin (14.2 kDa). Elution was done with 0.056 M phosphate buffer (pH 7.8).

was 7.8, but the activity declined rapidly as the pH increased above the optimum. Both K I and II were stable between pH 5.0–7.8, however, the keratinases of *S. brevicaulis* were most active at alkaline pH. The phenomenon was similar to that reported for dermatophytic keratinase that degrade human hair keratin [6], wool [16], bovine hoof and horn (17). The optimum temperatures for keratinase activity were 40 °C (K I) and 35 °C (K II), though K I was more stable over a broader temperature range than K II. The temperature

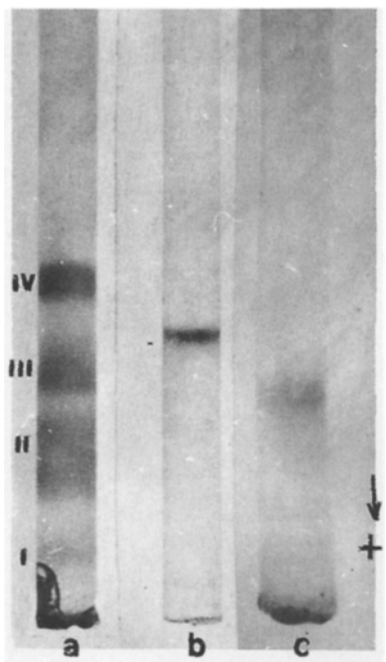

Fig. 2. Separation of proteins by SDS-PAGE. Lane a: I α-lactalbumin (14.2 kDa), II Carbonic anhydrase (29 kDa), III Albumin chicken egg (45 kDa), IV Albumin bovine serum (66 kDa); Lane b: Keratinase I and Lane c: Keratinase II.

stability decreased rapidly as temperatures increased above 40 °C.

K I was completely inhibited by PMSF while partial inhibition (74%) was observed with K II (Table 3). Because of this finding, it is likely that the active sites of K I and K II contain serine residues [7]. However, K II was distinctly different than K I based on our inhibition studies. The production of serine proteases by keratinophilic fungi has been reported in several studies [6, 7, 18]. The activity of K I was stimulated by EDTA, but K II activity was unaffected. Such findings exclude the liklehood of metal dependence of K I and K II for activity. Our results were similar to the observations of others [5, 17], who reported weak inhibition of keratinase by EDTA. However, at least one keratinase from a *Trichophyton* species has shown metal dependence [18] for optimal keratinase activity. Close scrutiny of the properties of keratinase I & II revealed that their properties were in many respects identical to the enzymatic activities of dermatophytes [6, 18]. We have named the keratinolytic enzymes of *S. brevicaulis* 'keratinases' in common with

Table 2. Properties of purified keratinases of *S. brevicaulis*

Fractions	Optimum pH	Optimum temp. (°C)	pH stability	Temp. stability (°C)	Molecular weight (kDa)
Keratinase I	7.8	40	5.0–8.0	15–40	40–45
Keratinase II	7.8	35	4.0–8.0	30–40	24–29

Table 3. Effects of various inhibitors on the activity of keratinases of *S. brevicaulis*

Inhibitor	Concentration (mM)	Keratinase activity (%) K I	K II
pCMB	1	73	88
PMSF	1	0	26
EDTA	5	140	100
8-Hydroxyquinoline	1	68	60
2-Mercaptoethanol	2	100	90
$HgCl_2$	1	0	10
KCN	5	80	100
Cysteine	5	76	60

Enzyme activity without the addition of inhibitor was considered 100%. All the values are the mean of three independent estimations.

previous authors [6, 17]. We found in protease assays that purified keratinases I and II hydrolyzed nonkeratin proteins, including bovine serum albumin (data not shown).

The elaboration of extracellular keratinases could be a significant factor in the pathogenesis of *S. brevicaulis* during onychomycosis [9, 19]. These keratinases could be important for the initial, and later, chronic colonization of host tissues. We are currently examining the role of keratinases in the penetration of skin and subcutaneous tissues during infections of *Scopulariopsis brevicaulis*.

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