Original Article

Characterization of an Extracellular Keratinase from *Microsporum canis*

Taizo Hamaguchi, Nobuaki Morishita, Rieko Usui, Iwao Takiuchi

Department of Dermatology, Showa University, Fujigaoka Hospital

[Received : 14, December 1999. Accepted : 18, April 2000]

Abstract

Extracellular keratinase (Ekase) 48-, 34- and 31.5-kDa polypeptides, which were isolated from *Microsporum canis* and examined by immunoblotting reacted with a monoclonal antibody against Ekase of *M. canis*.

We analyzed the amino acid and determined the first 17 amino acid NH₂-terminal sequences of the 48-, 34- and 31.5-kDa polypeptides. These polypeptides had a high aspartic acid, glycine and alanine content, respectively. The first 17 amino acid residues of the 34-kDa polypeptide were homologous to those of thermomycolin. This indicated that the 34-kDa polypeptide of Ekase is homologous to the thermomycolin produced by *Malbranchea pulchella*. Furthermore, Ekase was very heat-stable in the presence of 50 mM CaCl₂ at 55°C, since 50% of the initial activity remained. In contrast, no activity was detected after heating in the absence of CaCl₂. These results indicate a close relationship between dermatophytes and *M. pulchella*.

Key words : keratinase, *Microsporum canis*, NH₂-terminal sequences, thermomycolin, *Malbranchea pulchella*

Introduction

Dermatophytes are known to possess extracellular and two cell-bound keratinolytic enzymes, the so-called keratinases^{1, 2}. Extracellular keratinase (Ekase), a serine protease³, can dissolve not only the cytoplasm of horny cells but also squamous cells and the dermis⁴. It can also digest α -type fibrous proteins and membranous fractions isolated from human stratum corneum³. Microscopic observations confirmed the disappearance of tonofilaments and cell membranes, including a marginal band of horny cells around fungi^{5, 6}. The presence of Ekase in the horny cells of *tinea pedis* has also been confirmed by immuno-electron-microscopy⁷.

Caseinolytic activity substantially decreases in Ekase incubated with a mouse monoclonal antibody (mAb) against Ekase⁸. After conjugating Ekase to mAb-coupled CNBr-Sepharose 4B gel, some hair-degrading activities were detected by an assay using guinea pig hairs. These results

indicate that the mAb is specific for the Ekase from *Microsporum canis*.

We previously isolated Ekase from *M. canis* using two steps of ion exchange chromatography and gel filtration on Sephadex G-75⁹. Under our conditions, the enzyme was not absorbed on DEAE-cellulofine, but was transferred to CM-cellulofine. The enzyme activity was eluted in the second protein peak from Sephadex G-75⁹. The present paper describes a further purification of Ekase from *M. canis* using high performance liquid chromatography (HPLC), as well as amino acid analyses and NH₂-terminal amino acid sequences of the Ekase polypeptide molecules. The results of a protein homology search for Ekase molecules are also described.

Materials and Methods

Organisms and cultivation methods

Microsporum canis (kDog-0114-98) was isolated from clinical material at our hospital and cultured as described previously^{4, 9}.

Enzyme assay

The keratinolytic activity of the enzyme using guinea pig hairs as the substrate was determined as described previously^{4, 9}. Briefly, fifty mg of guinea pig hairs was added to an enzyme solution

Address correspondence to: Iwao Takiuchi

Department of Dermatology, Showa University,
Fujigaoka Hospital

1-30 Fujigaoka Aobaku Yokohama, Japan, 227-8501

and shaken in a water bath at 37°C for 2 hrs. After incubation, the remaining hairs were removed and absorbance values of the filtrate were measured spectrophotometrically at 280 nm.

Protein measurement

Protein was measured according to the method of Lowry *et al.*¹⁰⁾, using bovine serum albumin as a standard.

Enzyme purification

Ekase of the organisms was purified according to previously described procedures^{4,9)}. Briefly, culture filtrates were passed through DEAE-cellulofine (Seikagaku Kogyo, Tokyo) and the eluate was transferred onto CM-cellulofine (Seikagaku Kogyo). Crude enzyme from CM-cellulofine was concentrated by ultrafiltration on an Amicon YM 3 membrane. These materials were fractionated by chromatography on a column containing Sephadex G-75 (Pharmacia Fine Chemicals) and eluted with 28 mM phosphate buffer (pH 7.8). The fractions containing enzyme activity were combined and concentrated. Samples (20 μ l) were examined by HPLC (Bio-Gel SEC-XL, 7.8 $\times$ 300 mm, Bio-Rad) and eluted with the same buffer at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and assayed for Ekase activity.

Immunization of mice and mAb production

A mouse monoclonal antibody to Ekase from *M. canis* was generated as described⁸⁾. In brief, BALB/c mice were immunized using purified Ekase. Four days after the final injection, spleens were removed aseptically. Spleen cells from the immunized mice were fused with a mouse myeloma cell line by standard methods^{11,12)}. Culture supernatants from hybridomas were screened using an enzyme immunoassay. One hybridoma culture that remained positive was cloned by limiting dilution.

SDS-PAGE and immunoblotting

Polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) was conducted according to the method of Laemmli and Favr¹³⁾ using microslab gel equipment (Marysol, Tokyo). Five microliters of the purified Ekase solution (200 μ g/ml) was incubated at 100°C for 2 min in 2% SDS and 5% 2-mercaptoethanol (2-ME), then applied to slots in the 1.0 cm slab gel. Polypeptides were separated using Tris-glycine buffer containing 0.1% SDS at 10 mA for 90 min at room temperature, and compared with molecular weight standards (LMW calibration kit; Pharmacia Fine Chemicals) preincubated at 100°C for 2 min. Peptide fragments separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes (PVDF; Bio-Rad) by

electrophoresis at 40 V for 2 hrs. The control membrane was stained with 7% acetic acid and 50% methanol containing 0.25% Coomassie brilliant blue R-250 for 30 min and destained in 30% methanol, 10% acetic acid. The membrane was then incubated for 12 hrs at 4°C in 5% bovine serum albumin (Sigma) in phosphate-buffered saline (PBS). Blots were washed in PBS containing 0.05% (v/w) Tween 20 (TW); and mAb diluted 1:1,000 in PBS-TW was layered onto the membrane. After incubation at 37°C for 1 h, blots were washed once again. Horseradish peroxidase-conjugated anti-mouse IgG (goat, Zymed) was added and incubation at 37°C was continued for 1 h. The blots were washed, then the bound mAb was detected using 3, 3'-diaminobenzidine 4 HCl as a substrate.

Heat stability of Ekase

Purified Ekase in 500 μ l of 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM CaCl₂ was incubated in a sealed tube for 10 min at the designated temperature, then rapidly cooled in ice water. The samples were thereafter incubated with 100 μ l of casein (2.5 mg, Hammarsten casein; Merck, Darmstadt) at 37°C for 10 min in a shaking water bath. The reaction was stopped by adding 60 μ l of 50% trichloroacetic acid. After chilling in ice water for 15 min, the reaction mixtures were centrifuged at 1,000 g for 15 min, and soluble aminopeptides obtained from the casein were assayed¹⁰⁾. Control samples without CaCl₂ and blank tubes without Ekase were simultaneously processed.

Amino acid analysis and NH₂-terminal amino acid sequencing

Purified Ekase from *M. canis* was incubated at 100°C for 2 min in SDS and 2-ME, resolved by SDS-PAGE, and blotted onto PVDF membranes. The blots were washed in distilled water and stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 50% methanol for 15 min. The membrane was finally rinsed in deionized water for 10 min, air dried, then the 48-, 34- and 31.5-kDa protein bands corresponding to Ekase were excised. Pieces of the membrane were sequenced on a peptide sequencer (Shimadzu) PSQ-1 Edman degradation at the Takara Shuzo Research Center (Ohtsu, Japan). For the control, the 12-kDa portion of the PVDF with no bands was cut and sequenced. Portions of the polypeptides were also heated at 100°C for 24 hrs in 6 N HCl in a sealed tube. The hydrolyzates were analyzed using the ninhydrin reaction in a model L 8500 Hitachi amino acid analyzer at Takara Shuzo Research Center.

Protein homology search

Proteins homologous to the 48-, 34- and 31.5-

Fig. 1. High performance liquid chromatographic separation of Ekase.

Enzyme (50 μ l) extracted from the culture broth by ion-exchange chromatography and gel-filtration was examined by HPLC and eluted with 28 mM phosphate buffer (pH 7.8). Fractions (500 μ l) were collected and assayed for Ekase activity.

kDa proteins were searched at the Toray Research Center (Tokyo) using NBRF-PIR (National Biomedical Research Foundation-Protein Identification Resource; R. 30.0 September, 1991) and SWISS-PROT (European Molecular Biology Laboratory; R. 20.0 November, 1991).

Results

The HPLC profiles in Fig. 1 shows that Ekase activity eluted from the column as a single peak. When SDS-PAGE was performed using denatured Ekase, polypeptides of 48-, 34- and 31.5-kDa were stained in the gel as shown in Fig. 2-A. Each of these polypeptides reacted with the mAb (Fig. 2-B). Table 1 shows the amino acid composition of the polypeptides. Tryptophan was completely destroyed and cysteine was partially destroyed by hydrolysis. No detectable peak was demonstrated in the 12-kDa portion of the PVDF membrane. The NH₂-terminal amino acid sequences of the three polypeptides determined by degradation are presented in Table 2. The first 17 amino acid residues of the 34-kDa polypeptide resembled thermomycin (Table 2). However, no homology to the 48- and 31.5-kDa

Fig. 2. SDS-PAGE profile and Western blot analysis of Ekase.

(A) Purified Ekase (1 μ g) was boiled at 100°C for 2 min in 2% SDS and 5% 2-ME, separated by SDS polyacrylamide gel (10%) electrophoresis and stained with Coomassie brilliant blue. Molecular weight is indicated in kilodaltons. (B) 48-kDa, 34-kDa and 31.5-kDa polypeptides reacted with the mAb.

polypeptides was found.

Figure 3 shows that the initial enzyme activity without CaCl₂ was completely suppressed by a 10 min incubation at temperatures above 65°C.

Discussion

Keratinase is currently defined as a protease with keratinolytic activity on native keratin¹⁴⁾ Noval and Nickerson¹⁴⁾ reported that natural wool seems to be acceptable as native keratin and they used wool to isolate keratinase from *Streptomyces fradiae*. Yu *et al.*¹⁾ and Day *et al.*¹⁵⁾ initially described keratinase from dermatophytes and they measured its activity using guinea pig hairs as the substrate. In this paper, we refer to this keratinolytic enzyme as "keratinase", in accordance with other authors^{1, 14, 15)}.

When Ekase fractions from Sephadex G-75 were subjected to a native PAGE, a single

Table 1. Amino acid composition of the 48-, 34- and 31.5-kDa polypeptides and thermomycolin

	48-kDa		34-kDa		31.5-kDa		Thermomycolin
	Pmol	%	Pmol	%	Pmol	%	%
Asp	2,806	16.6	683	14.2	352	11.9	12.0
Thr	1,259	7.4	370	7.6	221	7.5	4.9
Ser	792	4.7	424	8.7	256	8.7	10.2
Glu	1,064	6.3	328	6.8	186	6.3	6.4
Gly	2,089	12.3	810	16.7	456	15.5	17.7
Ala	1,992	11.3	766	15.8	516	17.5	13.9
Cys	N.D.	0.0	12	0.2	N.D.	0.0	0.0
Val	545	3.2	467	9.6	280	9.5	7.4
Met	469	2.8	N.D.	0.0	34	1.2	0.6
Ile	635	3.7	93	1.9	148	5.0	6.2
Leu	1,119	6.6	255	5.3	175	5.9	4.3
Tyr	876	5.2	108	2.2	59	2.0	2.5
Phe	828	4.9	169	3.5	28	1.0	0.4
Lys	409	2.4	77	1.6	53	1.8	1.2
His	304	1.8	137	2.8	155	5.3	2.2
Arg	741	4.4	131	2.7	28	1.0	4.3
Pro	1,085	6.4	21	0.4	N.D.	0.0	3.1
Total	16,943	100.0	4,815	100.0	2,947	100.0	100.0

N.D.: not detected.

Table 2. Comparison of the NH₂-terminal sequences of the 48-, 34- and 31.5-kDa polypeptides with thermomycolin

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
31.5-kDa	G-	V-	(C)-	G-	G-	I-	(C)-	I-	R-	(C)-	(P)-	E-	F-	A-	(G)-	R-	(I)
34-kDa	A-	L-	V-	T-	Q-	P-	N-	A-	P-	T-	W-	G-	L-	G-	R-	V-	S
thermomycolin	A-	L-	V-	T-	Q-	(S)-	N-	A-	P-	(S)-	W-	G-	L-	G-	R-	I-	S
48-kDa	A-	E-	F-	K-	V-	F-	A-	W-	G-	L-	N-	D-	P-	T-	E-	G-	N

Amino acid residues in brackets are tentatively assigned.

Fig. 3. Heat stability of Ekase.

After being held at a designated temperature for 10 min, Ekase activity was measured using a casein as the substrate. Symbols: ● Ekase with 50 mM CaCl₂, ▲ Ekase without CaCl₂.

protein band was stained in the gel^{4,9)}. In the present study, a single protein component obtained by HPLC separation had Ekase activity. When SDS-PAGE was performed using purified

Ekase, 48-, 34- and 31.5-kDa polypeptides were stained in the gel. We confirmed by immunoblotting that the mAb reacted with all three components. These results indicate that Ekase

consists of three subunits. However, properties such as molecular weight substantially differ between species of dermatophytes. The estimated molecular weight of Ekase from *T. mentagrophytes* is 48-kDa¹⁾ or 34.8-kDa¹⁵⁾, and that from *T. rubrum* is 34.7-kDa¹⁶⁾ and 44-kDa¹⁷⁾, whereas 45-kDa¹⁸⁾ and 32.5-kDa¹⁹⁾ Ekase has also been isolated from *M. canis*. These differences in molecular weight may result from differences in the number of subunits.

Thermomycolin is produced by *Malbranchea pulchella* var *sulfurea* which belongs to the same genus as dermatophytes. It is a serine protease with an estimated molecular weight of 33-kDa as measured by SDS-PAGE²⁰⁾. Ekase is also classified as a serine protease. The 48-, 34- and 31.5-kDa polypeptides have high aspartic acid, glycine and alanine levels (Table 1)²⁰⁾, like thermomycolin. When the initial amino acid NH₂-terminal sequences of the 34-kDa polypeptide were compared with those of thermomycolin, 14 of 17 amino acid residues were identical (Table 2). Two of the three different residues of thermomycolin were serine. Gaucher and Stevenson²⁰⁾ concluded that serine is not unambiguously resolved by gas-liquid chromatography, so our identification of serine was tentative. Therefore, these two residues might still be identical with those of the 34-kDa polypeptide. Thermomycolin is stable over a broad range of pH (6.5-9.5), and is particularly thermostable in the presence of Ca²⁺²¹⁾. In the present study, purified Ekase incubated at 55°C with 50 mM CaCl₂ was quite stable. Our results suggest that the 34-kDa polypeptide of Ekase is homologous to thermomycolin. An identical immunological response of the enzyme from *M. canis* as well as from *M. gypsum*, *Trichophyton rubrum*, and *T. mentagrophytes* has been demonstrated by immuno-double-diffusion using an antibody against Ekase from *M. canis*¹⁸⁾. These data indicate a close genetic relationship between dermatophytes and *M. pulchella*. Further immunological and kinetic studies are required to confirm homology between Ekase and thermomycolin.

References

- 1) Yu RJ, Harmon SR, Blank F: Isolation and purification of an extracellular keratinase of *Trichophyton mentagrophytes*. *J Bact* **96**: 1435-1436, 1968.
- 2) Yu RJ, Harmon SF, Grappel SF, Blank F: Two-cell keratinase of *Trichophyton mentagrophytes*. *J Invest Dermatol* **56**: 27-32, 1971.
- 3) Takiuchi I, Sei Y, Takagi H, Negi M: Partial characterization of the extracellular keratinase from *Microsporum canis*. *Sabouraudia* **22**: 219-224, 1984.
- 4) Higuchi D, Takiuchi I, Negi M: The effect of keratinase on human epidermis, especially on stratum corneum. *Jpn J Dermatol* **91**: 119-125, 1981 (in Japanese).
- 5) Sei Y, Takiuchi I, Masutani M: Electron and immunoelectron microscopical studies on the extracellular proteinase (keratinase) of dermatophytes. In: GW Bailey (ed), proceedings Electron Microscopy Society of America, 14th annual meeting, pp 158-159, Claitor's Publishing Division, Louisiana, 1982.
- 6) Koga M, Sei Y, Higuchi D, Takiuchi I: Partial purification and the localization of keratinase in plantar horny layer infected with tinea pedis. *Jpn J Med Mycol* **27**: 107-112, 1986 (in Japanese).
- 7) Sei Y, Takiuchi I: Studies on the effect of keratinase on the keratinized structure, and localization of keratinase. *Jpn J Med Mycol* **23**: 308-313, 1982 (in Japanese).
- 8) Tazawa M, Higuchi D, Takiuchi I, Tsuchida K, Adachi K: Production of mouse monoclonal antibody to an extracellular proteinase (keratinase) from *Microsporum canis*. *Jpn J Med Mycol* **34**: 35-41, 1993 (in Japanese).
- 9) Takiuchi I, Higuchi D, Sei Y, Koga M: Isolation of an extracellular proteinase (keratinase) from *Microsporum canis*. *Sabouraudia* **20**: 281-288, 1982.
- 10) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with folin phenol reagent. *J Biol Chem* **193**: 265-275, 1951.
- 11) Russell WC, Patel G, Orcious B, Sharp I, Gardner PS: Monoclonal antibodies against adenovirus Type 5; preparation and preliminary characterization. *J Gen Virol* **56**: 393-408, 1981.
- 12) Tedder RS, Yano JL, Anderson NL: The production of monoclonal antibodies to rubella haemagglutinin and their use in antibody-capture assays for rubella-specific IgM. *J Hyg* **88**: 335-350, 1982.
- 13) Laemmli UK, Faver M: Maturation of the head bacteriophage T4, 1 DNA packing events. *J Mol Biol* **80**: 575-599, 1973.
- 14) Noval RJ, Nickerson WJ: Decomposition of native keratin by *Streptomyces fradiae*. *J Bact* **77**: 251-263, 1959.
- 15) Day WC, Tonic P, Stratam SL, Leeman U, Harmon SR: Isolation and properties of an extracellular protease of *Trichophyton granulosum*. *Biochim Biophys Acta* **167**: 597-606, 1968.
- 16) Sanyal AK, Das SK, Banerjee AB: Purification and partial characterization of an exocellular proteinase from *Trichophyton verrucosum*. *Sabouraudia* **23**: 139-144, 1985.
- 17) Asahi M, Lindquist R, Fukuyama K, Apodaca G, Epstein WB, Mckerrow JH: Purification and characterization of major extracellular proteinase from *Trichophyton rubrum*. *Biochem J* **232**: 139-144, 1985.
- 18) Takiuchi I, Higuchi D, Sei Y, Koga M:

- Immunological studies of an extracellular keratinase. *J Dermatol (Tokyo)* **10**: 327-330, 1983,
- 19) Sei Y, Ogura Y, Higuchi D, Takiuchi I: Biochemical and immunological studies on neutral proteinase, -Localization and extraction *in vivo*-. *Jpn J Med Mycol* **33**: 137-145, 1992 (in Japanese).
- 20) Gaucher GM, Stevenson KJ: Thermomycolin. *Methods Enzymol* **45**: 415-433, 1976.
- 21) Ong PS, Gaucher M: Production, purification and characterization of thermomycolase, the extracellular serine protease of the thermophilic fungus *Malbranchea pulchella* var *sulfurea*. *Can J Microbiol* **22**: 165-176, 1976.