Yonsei Medical Journal
Vol. 28, No. 2, 1987

Isolation, Purification and Characterization of Keratinolytic Proteinase from *Microsporum canis*

Kwang Hoon Lee[1], Kwang Kyun Park[2], Sung Hyun Park[1] and Jung Bock Lee[3]

A keratinolytic proteinase secreted by Microsporum canis *in a broth containing human hair 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 purified enzyme was electrophoretically homogeneous with a molecular weight of 33,000. The enzyme had an optimum pH of 8.0, 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. The keratinolytic activity was not affected by the addition of nonionic detergents, was activated by Mg^{2+}, but inhibited by Zn^{2+}. The purified enzyme was used to obtain guinea pig antiserum. The antiserum tested by double diffusion against the purified enzyme showed a single line of precipitation and completely neutralized the proteinase activity. This study reaffirms that the proteinase from* M. canis *may be a biochemical mechanism for the invasion of keratinized tissue, and could possibly play a role in the hypersensitivity reactions arising from superficial infections of this fungus.*

Key Words: *Microsporum canis,* keratinolytic proteinase

Dermatophytes are parasitic fungi which cause superficial infections called dermatophytosis in humans and animals. They usually invade and thrive only on the keratinized layers of the skin, nail and hair. The mechanism by which dermatophytes utilize keratin as a nitrogen source has not yet been clarified. Recent morphologic studies using electron and fluorescent microscopes have suggested that the keratin substance may be digested by chemical substances produced by dermatophytes (Mercer and Verma 1963; Verma 1966). Based on these observations, several groups of workers isolated and purified proteinases from different dermatophytes (Yu *et al.* 1968; Yu *et al.* 1971; Takiuchi and Higuchi 1977; Takiuchi *et al.* 1982; Sanyal *et al.* 1985).

Microsporum canis (M. canis), a zoophilic dermatophyte, is frequently transmitted by pets such as cats and dogs to man or through animal contact in rural areas. With the recent world-wide increase in the incidence of *M. canis* infection, it is now considered to be an important public health problem in view of its epidemiologic aspects (Kaplan 1967; Kelley and Mosier 1977). Recently, Takiuchi *et al.* (1982) reported the isolation of a proteinase from *M. canis* cultvated in media containing human hair as a source of nitrogen. In this paper, we reaffirm the purification of the keratinolytic proteinase (KPase) from *M. canis*, and report on some of its biochemical and immunological properties.

Received January 30, 1987
Accepted March 25, 1987

Departments of Dermatology[1] and Biochemistry[2], Yonsei University Wonju College of Medicine, Wonju, Korea.
Department of Dermatology[3], Yonsei University College of Medicine, Seoul, Korea.

This study was supported by the faculty research grant (1986) of Yonsei University Wonju College of Medicine.

Address reprint requests to Dr. K.H. Lee, Department of Dermatology, Yonsei University Wonju College of Medicine, Wonju, Korea.

MATERIALS AND METHODS

M. canis growth conditions and KPase isolation

M. canis, strain 850425, isolated and maintained at the Department of Dermatology, Yonsei University Wonju College of Medicine was grown in Sabouraud's dextrose broth. Mycelia were removed and homogenized in 28mM phosphate buffer, pH 7.8, with a Waring blender (Winsted HDW MFG. Co., USA). The culture medium for proteinase induction contained glucose, 0.5g; $MgSO_4 \cdot 7H_2O$, 0.6g; inositol, 0.05g; thiamine·HCl, 0.01g and pyridoxine·HCl, 0.01g in 1,000ml of 28mM phosphate buffer, pH 7.8. As a

nitrogen source, human scalp hairs were added to the culture media in a final concentration of 2.6g/L. After the homogenate was inoculated into the medium, the cultures were allowed to stand for 5 days at 27°C and then were shaken for 5 days. Control experiments were performed using the same medium in which 2.6g of peptone was substituted for the human hair. At the end of the growth period, the fungal mycelium and the residual hair were removed from the flask by filtration. The culture filtrates were retained for purification of the enzyme.

Enzyme assay

The keratinolytic activity was assayed by a modification of the method of Yu *et al.* (1968). The substrate, white guinea pig hair, was washed with 50% chloroform-methanol and air dried. Fifty mg of guinea pig hair were suspended in 28mM phosphate buffer, pH 7.8, to which enzyme material was added; the final volume was 7ml. A boiled enzyme solution was used in the same manner as a control. The reaction mixtures were incubated in a shaking water bath at 37°C for 2h, then filtered. An increase of 0.100 in the absorbance value at 280nm, indicating the presence of soluble proteins and amino acids, was taken as one unit of enzyme, and specific activity was expressed as enzyme units per mg protein. The protein content was estimated by the method of Lowry *et al.* (1951).

Purification of proteinase

The culture filtrate was passed through a diethylaminoethyl (DEAE)-Sephacel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column, equilibrated with 28mM phosphate buffer, pH 7.8, and the effluent adjusted to pH 6.1 was transferred onto a carboxymethyl (CM)-Sephadex C-50 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column equilibrated with the same buffer, pH 6.1. The enzyme was eluted from the CM-Sephadex C-50 with 0.1M NaCl in 28mM phosphate buffer, pH 6.1 and was concentrated to a volume of 2ml by ultrafiltration with a PM-10 membrane on an ultrafiltration cell (model 8200, Amicon Corp., USA). Two ml of the concentrate was fractionated at 4°C on a Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) column, 2.0×100cm, equilibrated with 0.1M NaCl in 28mM phosphate buffer, pH7.8. The column was eluted with the same buffer at a flow rate of 15ml/h, and 5ml fractions were collected. Fractions with enzyme activity were pooled, concentrated and dialyzed.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) was performed in a Tris-HCl buffer, pH 8.8 using a discontinuous gel consisting of 4% stacking gel and 10% separating gel by the method of Hahm *et al.* (1983). Before application to the gels, the samples were boiled for 2min in 0.25M Tris-HCl buffer, pH 6.8 containing 2% SDS, 5% 2-mercaptoethanol, 15% glycerol, and 0.024% bromophenol blue. Electrophoresis was performed at 4°C until the tracking dye reached the bottom of the gel slab. The gels were stained with 0.1% Coomassie brilliant blue R-250 in 12.5% trichloroacetic acid and 50% methanol for 18h at room temperature.

Determination of molecular weight

SDS-PAGE was performed to determine the molecular weight on a discontinuous gel consisting of 4% stacking gel and 10% separating gel after the protein was heated at 100°C with 2% SDS, 5% 2-mercaptoethanol. The molecular weight of the proteinase was calculated from a plot of the log of the molecular weight of different reference proteins, treated similarly as above, versus their respective mobility.

Evaluation of antigenic activity

The purified enzyme solution (2mg/ml) was emulsified with equal volumes of Freund's complete adjuvant. The emulsion, 0.5ml for each animal, was injected intradermally into the foot-pads and mid-back of the guinea pigs, and 0.1mg of the purified enzyme in 28mM phosphate buffer, pH 7.8 was given intramuscularly into the nape and buttock as a booster 3 times at 7-day intervals. Sera from the guinea pigs immunized with the proteinase were analyzed for precipitating antibodies to the proteinase by immunodiffusion analysis (Oka *et al.* 1984). For the estimation of inhibition of keratinolytic activity by the antiserum, reaction mixtures of 2-fold serial dilution of immunized serum and proteinase were incubated at 37°C for 30min, then centrifuged at 2,000 × g for 5min. Enzyme activity was measured in each of the supernatants. Dilutions of non-immunized guinea pig sera were used as a control.

RESULTS

Purification

Keratinolytic activity first appeared in *M. canis*

Table 1. Purification of proteinase from *M. canis*

Purification step	Total volume (ml)	Total enzyme activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Culture filtrate	950	1672	1817	0.92	0	100
DEAE-Sephacel Chromatography	950	1254	286	4.38	4.8	74.9
CM-Sephadex chromatography	250	1100	75	14.6	15.9	65.8
Sephadex G-75 gel filtration	75	516	4.2	122.8	133.5	30.8

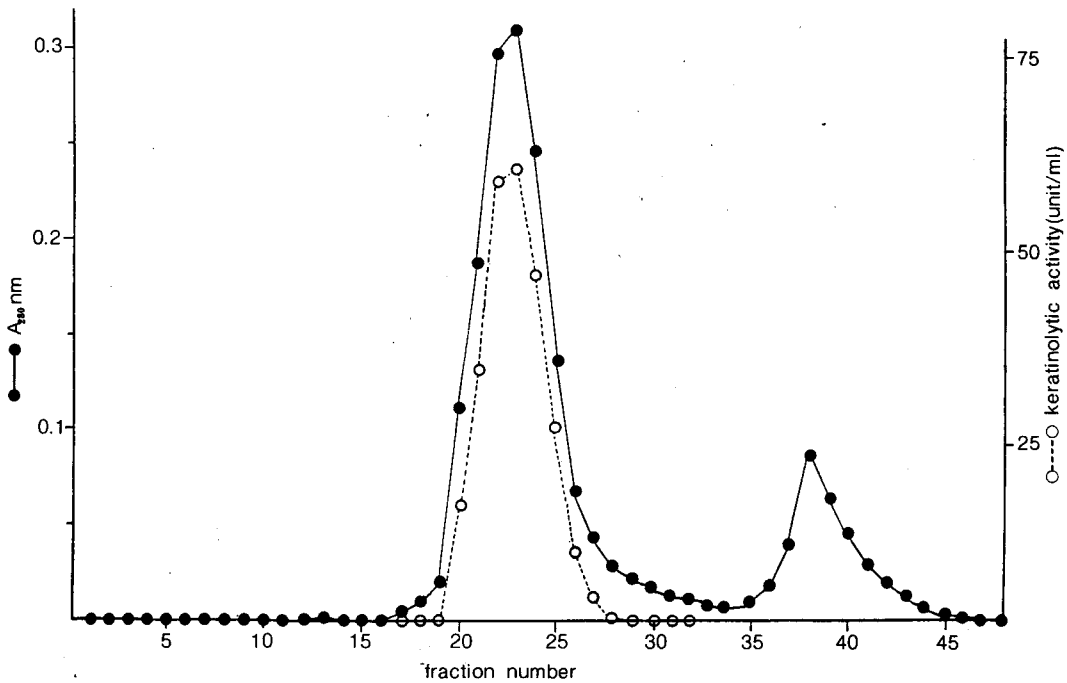

Fig. 1. *Chromatography of proteinase from M. canis on Sephadex G-75.*

culture filtrates of the medium with human hair on the 4th day of culture and reached a maximum on the 10th day. This activity was not demonstrable in filtrates of the control medium with peptone up to the 28th day of culture.

The purification of the extracellular KPase achieved after each step is summarized in Table 1. After DEAE-Sephacel and CM-Sephadex C-50 chromatography, the specific activity increased from 0.92 units/mg to 14.6 units/mg of protein. The ultimate preparation showed an overall 134-fold purification with a recovery of 30.8% of the original activity. Enzyme activity was observed in the first protein peak from the Sephadex G-75 column. The enzymatically active fractions (Fraction 20-28, Fig. 1) were combined and subjected to SDS-PAGE. The purified enzyme showed a single band in SDS-PAGE (Fig. 2).

Molecular weight of proteinase

The molecular weight was determined by SDS-PAGE which yielded a major protein band (Fig. 2)

Fig. 2. SDS-polyacrylamide gel electrophoretic homogeneity of the purified proteinase from M. canis.

corresponding to 33,000 by comparison with reference proteins (Fig. 3).

Chemical properties

The optimum pH for the keratinolytic activity of KPase was 8.0. Activity decreased sharply in the acidic pH range, and there was no detectable activity at pH 5.0 and below (Fig. 4). The reaction rate increased with temperature up to 35°C and pretreatment at temperatures higher than 45°C rapidly inactivated the enzyme (Fig. 5). Nonionic detergents such as Triton X and SDS had no effect on KPase activity at a final concentration of 0.1%. DMSO, an organic solvent, also had no effect on KPase activity at 10 mM.

The enzyme was activated by Mn^{2+}, Mg^{2+}, and Ca^{2+} but Zn^{2+} had an inhibitory effect on the keratinolytic activity at a concentration of 5mM (Table 2).

Antigenic activity

When the serum of the guinea pigs immunized with the purified proteinase was tested by a double-diffusion method against the purified proteinase from

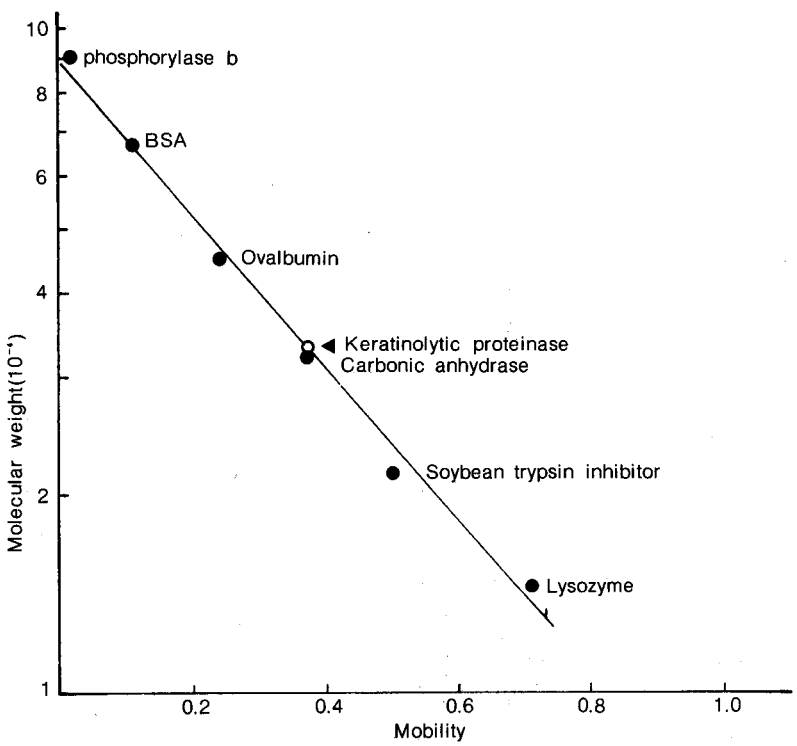

Fig. 3. Molecular weight of the proteinase from SDS-polyacrylamide gel electrophoresis.

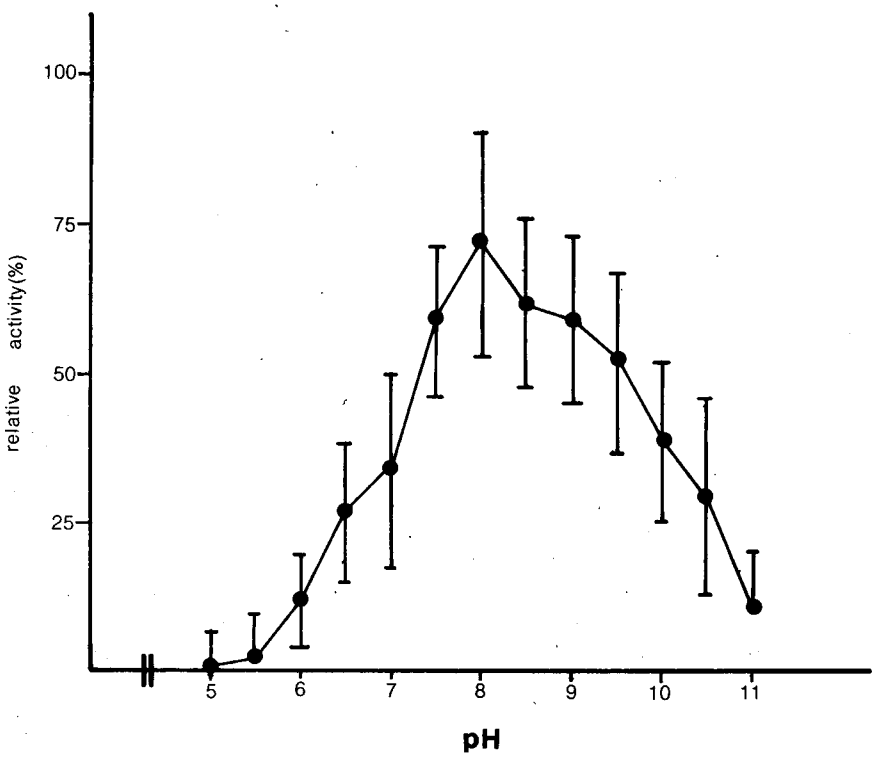

Fig. 4. *Effect of pH on the keratinolytic activity of proteinase from M. canis.*

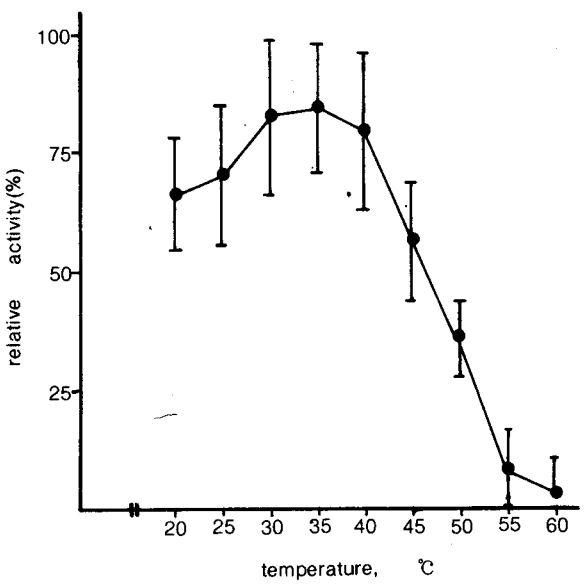

Fig. 5. *Effect of temperature on the keratinolytic activity of proteinase from M. canis.*

Table 2. Effect of metal ions on keratinolytic activity

Metal ion*	% Activity with control
Control	100
Mn^{2+}	151
Mg^{2+}	249
Ca^{2+}	148
Ba^{2+}	81
Zn^{2+}	11

*: the concentration of each metal ion was 5 mM.

the Sephadex G-75, a single line of precipitation was observed (Fig. 6). The serum also reacted with the enzyme preparation after DEAE-Sephacel chromatography but the precipitation lines were interconnected. No precipitation was observed in the same analysis with the non-immunized guinea pig serum. Enzyme activity was completely neutralized by the addition of anti-proteinase guinea pig serum, but not by the addition of that from non-immunized guinea pig (Fig. 7).

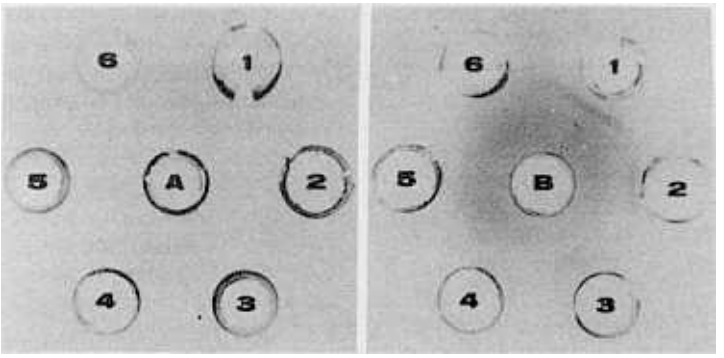

Fig. 6. Double immunodiffusion pattern of guinea pig antiserum and purified proteinase from M. canis.
Central wells: A: nonimmunized guinea pig serum, 15μl; B: immunized guinea pig serum, 15μl.
Peripheral wells: 1,2: purified proteinase, 8, 2μg; 3: enzyme preparation after DEAE-Sephacel chromatography, 10μg; 4: saline, 10μl; 5: 50mM phosphate buffer (pH 7.4), 10μl,; 6: distilled water, 10μl.

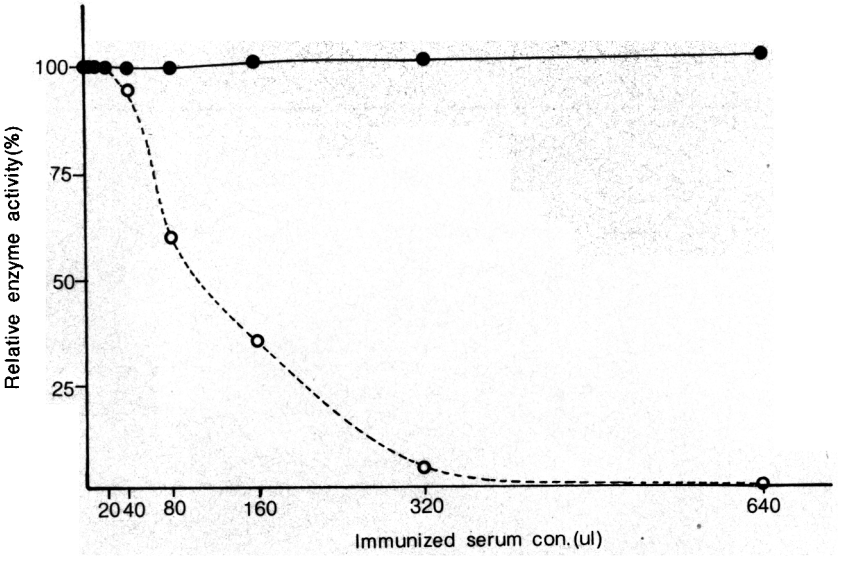

Fig. 7. Inhibition of enzyme activity by anti-proteinase guinea pig serum. Enzyme activity was inhibited completely by the addition of 640μl of immunized guinea pig serum (5mg/ml), while no inhibition was detected when nonimmunized guinea pig serum was added.
O---O : immunized guinea pig serum
●——● : nonimmunized guinea pig serum

DISCUSSION

Considerable efforts are presently devoted to the determination of the mechanism by which dermatophytes are able to invade and utilize keratinized tissue. There is evidence that some dermatophyte enzymes give rise to hydrolytic activity against keratin substance (Cruickshank and Trotter 1956; Chesters and Mathison 1963; English 1968; Baxter and Mann 1969). Yu *et al.* (1968) reported the isolation and purification of extracellular keratinases from *Trichophyton mentagrophytes (T. mentagrophytes)*. Recently, the keratinolytic proteinase from the culture filtrate of *M.*

gypseum was purified and characterized by Takiuchi and Higuchi (1977). Weary *et al.* (1965) reported that *M. canis* is capable of exerting a keratinolytic effect on ethylene oxide-sterilized wool obtained from sheep. O'Sullivan and Mathison (1971) presented evidence that the intracellular proteolytic system in *M. canis* is a multienzyme system, and that the extracellular protease found in the growth medium was induced by secretion not autolysis. Higuchi *et al.* (1981) demonstrated morphologically that the extracellular proteinase from *M. canis* is able to dissolve the cytoplasm of horny cells and also entire squamous cells. This was followed by the isolation and purification of *M. canis* proteinase from the culture filtrates of medium with human hair by the same group (Takiuchi *et al.* 1982).

In the present study, we purified a KPase from the human hair containing culture medium of *M. canis* to an apparent homogeneity. The enzyme was purified 134-fold to a specific activity of 123 units/mg by ion-exchange chromatography using DEAE-Sephacel, CM-Sephadex C-50, and Sephadex G-75 gel filtration. This contrasts with the results of Takiuchi *et al.* (1982) who achieved a 107-fold purification from the same source to a specific activity of 815.5 units/mg. It is noteworthy that the specific activities of the starting materials were different, ours was 0.92 and theirs 7.6. This and the large discrepancy in the final results are perhaps attributable to the possible difference in the strain of fungus.

The molecular weight of the single band measured by the SDS-PAGE method was 33,000. Contamination of this band by other protein(s), or, more seriously, other unseen protein(s) with KPase activity could not be ruled out at this time. However, the starting material is the filtrate of the culture medium, therefore not too many proteins are expected to be present, as would be the case with whole cells. Particularly O'Sullivan *et al.* ascertained that cell autolysis was minimal. Therefore, purification on the order of 100-fold may be sufficient to reach homogeneity.

Like Takiuchi *et al.* (1982), we also found that the culture filtrate from *M. canis* grown in the medium with human hair contained keratinolytic activity but the medium with peptone did not. This finding reaffirms that the production of this enzyme might be induced by the addition of the substrate, keratin. However, according to O'Sullivan *et al.*, the secretion of proteases by *M. canis* into the extracellular medium occured when the medium contained casein but not casein hydrolysate. Thus, it would be interesting to ascertain if the proteases induced by casein also contain the KPase induced by keratin, and if so, the common chemical or physical feature of these two substrates, keratin and casein.

The metal ion requirement of the enzyme in Table 2 suggests that the divalent metal ions Mg^{2+}, Ca^{2+}, and Mn^{2+} are activators while Ba^{2+} and Zn^{2+} are inhibitors. Specifically, Mg^{2+} is a strong activator (2.5 fold), and Zn^{2+} is a strong inhibitor (11%).

Grappel and Blank (1972) demonstrated the presence of circulating antibodies in the sera of guinea pigs immunized with keratinases from *T. mentagrophytes*. They suggested that the keratinases may not only be important for the penetration of dermatophytes, but also play a role in the hypersensitivity reactions associated with dermatophytosis. We attempted to clarify the immunogenicity of KPase by a double immunodiffusion and neutralization analysis. The reactivity of guinea pig antiserum was shown to be specific for the KPase of *M. canis*. These results indicate that KPase has an antigenicity, and may play a role in the immune responses of dermatophytosis. The biologic significance of KPase in human dermatophytosis remains to be determined by further investigation.

REFERENCES

Baxter M, Mann PR: Electron microscope studies of the invasion of human hair in vitro by three keratinophilic fungi. *Sabouraudia 7:33-37, 1969*

Chesters CGC, Mathison GE: The decomposition of wool keratin by Keratinomyces ajelloi. *Sabouraudia 2:225-237, 1963*

Cruickshank CND, Trotter MD: Separation of epidermis from dermis by filtrates of Trichophyton mentagrophytes. *Nature 177:1085-1086, 1956*

English MP: The developmental morphology of the perforating organs and eroding mycelium of dermatophytes. *Sabouraudia 6:218-227, 1968*

Grappel SF, Blank F: Role of keratinases in dermatophytosis. I. Immune responses of guinea pigs infected with Trichophyton mentagrophytes and guinea pigs immunized with keratinases. *Dermatologica 145:245-255, 1972*

Hahm KS, Tikkanen MJ, Dargar R, Cole TG, Schonfeld G: Limited proteolysis selectively destroys epitopes on apolipoprotein B in low density lipoproteins. *J Lipid Res 24:877-885, 1983*

Higuchi D, Takiuchi I, Negi M: The effect of keratinase on human epidermis, especially on stratum corneum. *Jap J Dermatol 91:119-125, 1981*

Kaplan W: Epidemiology and public health significance of ringworm in animals. *Arch Dermatol 96:404-408, 1967*

Kelley DC, Mosier JE: Public health aspects of mycotic diseases. *J Am Vet Med Ass 171:1168-1170, 1977*

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem 193:265-275, 1951*

Mercer EH, Verma BS: Hair digested by Trichophyton mentagrophytes. *Arch Dermatol 87:357-360, 1963*

Oka Y, Mottola C, Oppenheimer CL, Czech MP: Insulin activates the appearance of insulin-like growth factor II receptors on the adipocyte cell surface. *Proc Natl Acad Sci USA 81:4028-4032, 1984*

O'Sullivan J, Mathison GE: The localization and secretion of a proteolytic enzyme complex by the dermatophytic fungus Microsporum canis. *J Gen Microbiol 68:319-326, 1971*

Sanyal AK, Das SK, Banerjee AB: Purification and partial characterization of an exocellular proteinase from Trichophyton rubrum. *Sabouraudia 23:165-178, 1985*

Takiuchi I, Higuchi D: Isolation, purification and biochemical properties of keratinase elaborated from Microsporum gypseum. *Jap J Dermatol 87:305-309, 1977*

Takiuchi I, Higuchi D, Sei Y, Koga M: Isolation of an extracellular proteinase (keratinase) from Microsporum canis. *Sabouraudia 20:281-288, 1982*

Verma BS: The use of fluorescence microscopy in the study of in vitro hair penetration by ringworm fungi. *Br J Dermatol 78:222-224, 1966*

Weary PE, Canby CM, Cawley EP: Keratinolytic activity of Microsporum canis and Microsporum gypseum. *J Invest Dermatol 44:300-312, 1965*

Yu RJ, Harmon SR, Blank F: Isolation and purification of an extracellular keratinase of Trichophyton mentagrophytes. *J Bacteriol 96;1435-1436, 1968*

Yu RJ, Harmon SR, Grappel SF, Blank F: Two cell-bound keratinases of Trichophyton mentagrophytes. *J Invest Dermatol 56:27-32, 1971*