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Relationship Between Candida albicans Producing Proteinase (CAPP) and Its Environmental pH

– Comparison with a Case of Trichophyton mentagrophytes –

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Candida albicans produced a karatinolytic proteinase (KPase) or C. albicans *producing proteinase (CAPP), a proposed new term for this enzyme, and Trichophyton mentagrophytes also produced KPase when cultivated in liquid medium containing human stratum corneum (HSC) as the nitrogen source, but were unable to do so when cultivated in sabouraud dextrose broth. Purified KPase from the culture supernatants of* C. albicans *had a molecular weight of 42,000 and an optimun pH at 4.0. The KPase was found to belong to the carboxyl proteinases group and its activity was strongly inhibited by pepstatin. Both fungi were able to grow by secreting KPase which digested HSC for nutrients. KPase from both fungi had high activity in each optimum pH, such as weakly acidic pH on* C. albicans *and neutral pH on* T. mentagrophytes *to adapt their surrounding environment by changing the environmental pH into their own optimum pH.*

Key Words: *C. albicans, CAPP, T. mentagrophytes*

INTRODUCTION

In the course of superficial infections, *C. albicans* and *T. mentagrophytes* are able to invade and acquire nutritive substances from keratinized tissue which is insoluble and resistant to most proteolytic enzymes. Induction of proteolytic enzymes from various dermatophytes in vitro have been studied, and various properties of the enzymes have been characterized (Yu et al., 1968; Yu et al., 1969; Takiuchi et al., 1973; Sei et al., 1978; Higuchi and Takiuchi, 1980). Proteolysis of serum protein agar by *C. albicans* was initially reported by Staib (1965). Remold et al. (1968), Rüchel (1981) and Rüchel et al. (1982) have reported the characteristics of proteolytic enzymes from *C. albicans* cultivated in a medium containing albumin as a nitrogen source. Yu et al. (1968; 1969a; 1969b; 1971) isolated and purified both extracellular and cell-bound keratinolytic proteinase from *T. mentagrophytes.*

In order to clarify the role of proteolytic enzymes in superficial candidiasis, Hattori et al. (1984) observed the induction of keratinolytic proteinase (KPase)from *C. albicans* cultivated in a medium containing human stratum corneum (HSC) as a nitrogen source. Subsequently, Negi et al. (1984) purified KPase from a culture supernatant and clarified some of its properties. Recently, Tsuboi et al. (1985a; 1985b) revealed that *C. albicans* grows best with wealky acidic media, particularly at pH 4.0, when the pH of each media was fixed throughout the cultivation with the use of strong buffers.

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Throughout their series of investigations, they suggested that KPase may play an important role in the growth of *C. albicans* and that pepstatin, a carboxyl protease inhibitor, has the possibility of being used as a new type of antifungal agent.

The work presented here concerns the influences of various culture medium pH in *T. mentagrophytes* and *C. albicans* with their biochemical properties.

MATERIALS AND METHODS

Strains and cultivation method

A subcolony of *C. albicans,* NIH A-207was kindly provided by Dr. Fukazawa, Department of Microbiology, Medical university of Yamanashi. *C. albicans* and *T. mentagrophytes* were precultivated respectively in a 1% polypton-2% glucose broth at 27°C for three days in a 200ml flask containing 170 ml of seed medium. The liquid medium, sterilized through a 0.2μm membrane filter, had the following composition (g/liter); yeast carbon base 12g, inositol 0.05g, thiamine 0.01g, pyridoxine 0.01g. For use as a nitrogen source, HSC was obtained with a corn scraper from the plantar skin of healthy individuals and chopped into pieces smaller than 0.5 mm^3. After the minced HSC was repeatedly rinsed with distilled water, the insoluble protein was lyophilized and sterilized by ethylene oxide gas, and then it was added to each of the culture media to final concentration of 5 mg/ml. Inoculum size of fungi was adjusted to a concentration of 10^6 cells/ml using a hemacytometer. *T. mentagrophytes* that cannot be counted by hemacytometer was estimated by the turbidity of homogenized organism. Fungi were cultivated in a shaking water bath at 27°C for eight days. During the cultivation period, a cell count was taken every second day with a hemacytometer.

Enzyme assay

Proteolytic activity was assayed as follows:

0.1 ml of the enzyme solution was incubated at 37° for 60 min with 0.9 ml of 0.1 M sodium acetate buffer (pH 4.0) containing 5 mg of HSC as a substrate. The reaction was then terminated by the additon of 1 ml of 10% trichloroacetic acid (TCA) and the mixture centrifuged. The amount of proteolytic products in the supernatant were assayed according to the method of Lowry et al. (1951). One proteolytic unit (P.U.) was defined as an increase of 0.10 corrected absorbance value at 750 nm.

Purification of enzyme from C. albicans

The proteinase from the culture supernatants of *C. albicans* was purified by the method of Negi et al. (1984). Briefly, the culture supernatant were dialyzed against 10 mM sodium citrate buffer (pH 6.8). The solution, that not absorbed by CM-cellulose, was applied to DEAE-cellulose column and eluted by 0.2M sodium citrate buffer (pH 6.3). The elute was concentrated and chromatographed on a column of Sephacryl S-200.

Inhibitory effect of various kinds of proteinase inhibitor on the growth of C. albicans

According to Tsuboi et al. (1985b), the growth of *C. albicans* is the most prominently observed at pH 4.0 when HSC is used as the nitrogen source. Therefore the pH of the liquid medium used in this experiment was fixed at pH 4.0 using 0.1M citric acid-sodium phosphate buffer and 5 mg of HSC added as the nitrogen source. At the commencement of cultivation, various kinds of proteinase inhibitor such as pepstatin (10 μml), N-ethylmaleimide (10 μml), ethylene diamine tetraacetic acid (10 mM/ml), soy bean trypsin inhibitor (10μml) were added to the media to compare the inhibitory effect on the growth of *C. albicans.* With regard to pepstatin, solutions at lower concentration were also added to give the following concentrations in the media: 1.0, 0.1, and 0.01 μg/ml.

Influence of the culture medium pH on the growth of C. albicans and T. mentagrophytes

Two kinds of liquid culture medium, containing HSC as the nitrogen source, were prepared so as to examine the influence of the culture medium pH on the growth of both fungi. A pH of one medium was fixed and maintained throughout cultivation using 0.1M citric acid-sodium phosphate buffer (pH 3 to 7) and 0.1M maleic acid-tris buffer (pH 8.0). The other was not fixed, but the culture medium pH was adjusted by addition of 0.1M citric acid (pH 3 to 9). Measurements were made of the cell count and medium's pH every second day.

RESULTS

Biochemical properties of KPase from C. albicans

Table 1 shows some biochemical properties of KPase. The molecular weight of the enzyme was estimated to be 42,000 by SDS-PAGE and gel filtration. KPase had optimum pH at 4.0 and was inactive below 2.5 and above 6.0. The isoelectric point

Table 1. Some biochemical properties of KPase from *C. albicans*

1. Molecular weight	42,000
2. pH optimum	4.0 (inactive below 2.5 and above 6.0)
3. Isoelectric point (pI)	4.5
4. Effect of proteinase inhibitors	
a) Pepstatin (10-100μg/ml)	86-93% inhibition
b) Chymostatin (10-100μg/ml)	23-46% inhibition
c) α_2 -Macroglobulin (10-100μg/ml)	23-35% inhibition (pH 4.0) 47-95% inhibition (pH 6.8)
d) SBTI, NEM, EPNP, DAN, EDTA, EGTA	no effect
5. Substrates	Human Stratum Corneum (HSC), BSA, OVA, Hb, Casein

of the purified enzyme was 4.5. Pepstatin, an inhibitor of carboxyl proteinase, strongly inhibited enzyme activity, while chymostatin and α_2-macroglobulin also partially inhibited enzyme activity. However, soy bean trypsin inhibitor (SBTI), N-ethylmaleimide (NEM), 1, 2-eposy-3-(p-nitrophenoxy)-propane (EPNP), diazoacetylnorleucine-methylester (DAN), ethylene diamine tetraacetic acid (EDTA), and ethylene glycol-bis (β-aminoethylether) N, N, N′, N′-tetraacetic acid (EGTA) had no inhibitory effect on KPase. Inhibitory profiles suggested this enzyme to be carboxyl proteinase having cathepsin D-like action. With respect to substrate specificity, KPase was able to degrade bovine serum albumin (BSA), ovalbumin (OVA), hemoglobin (Hb) and Casein as well as HSC.

Inhibitory effect of various kinds of proteinase inhibitor on the growth of C. albicans

Although NEM, EDTA and SBTI didn't inhibit the cell growth of *C. albicans,* pepstatin, a carboxyl proteinase inhibitor, strongly inhibited the cell growth for eight days at a concentration of 10 μg/ml (Table 2). As pepstatin exhibited a stronger antifungal effect than that what we had expected. The inhibition at lower concentrations was examined. Pepstatin at a concentration of 1.0 and 0.1 μg/ml strongly inhibited cell growth of *C. albicans* and even at a very low concentration of 0.01 μg/ml was still able to inhibit growth until the sixth day.

Table 2. Inhibitory effect of various kinds of protease inhibitors on the growth of *C. albicans*

Inhibitors	X 10^6 Cells/ml(relative % growth compared with control)				
	Incubation time (days)				
	2	4	6	8	10
Control (no control)	16 (100%)	105 (100%)	400 (100%)	500 (100%)	370 (100%)
NEM (10 μg/ml)	11 (69%)	/	310 (78%)	270 (54%)	500 (135%)
EDTA (10 mM/ml)	12 (75%)	87 (83%)	520 (130%)	480 (96%)	450 (122%)
SBTI (10 μg/ml)	10 (63%)	/	480 (120%)	350 (79%)	400 (93%)
Pepstatin (10μg/ml)	6.8 (43%)	7.0 (6.7%)	7.2 (1.8%)	7.0 (1.4%)	6.9 (1.8%)

Inoculum size: 1.0 x 10^6 Cells/ml
/ : not examined

Table 3. Influence of culture medium pH on the growth of *C. albicans*

Culture medium pH	X 10^6 Cells/ml Incubation Time (days)			
3.0	4.4	27	92	160
4.0	8.7	150	130	230
5.0	3.6	8.7	16	30
6.0	2.2	1.1	2.0	1.4
7.0	2.1	0.9	1.1	1.4
8.0	1.5	1.1	0.8	1.7

Influence of the culture medium pH on the growth of C. albicans and T. mentagrophytes

Table 3 shows that growth of *C. albicans* was most prominently observed in weakly acidic media, particularly at pH 4.0, but was inhibited above pH 6.0, which was fixed and maintained throughout cultivation using buffer solution. When the pH of each media of *C. albicans* was not buffered but was adjusted by HCL or NaOH, the initial pHs varied according to their own environmental conditions. As shown in Fig.1, the pH of media initially at pH 5, 6, and 7 lowered gradually, while the pH of media initially at pH 3 became higher slightly. As the cultivation period progressed, all the varieties of pH

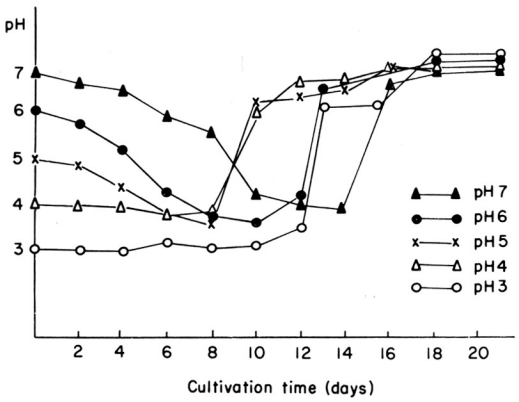

Fig. 1. Changes of medium pH during cultivation of *C. albicans*

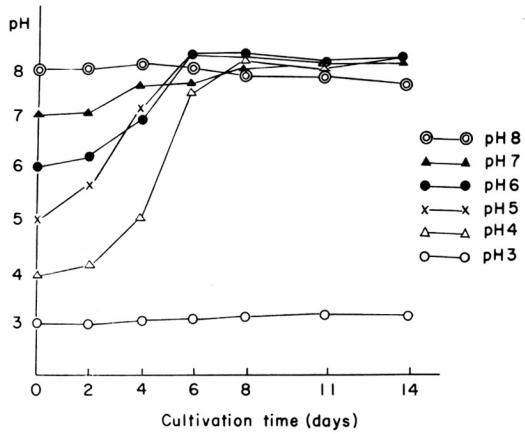

Fig. 2. Changes of medium pH during cultivation of *T.mentagrophytes*

ranges focused around pH 4 and then rapidly ascended to around pH 7. In order to make these relationships clear, a culture medium of pH 7 was chosen as an example. As the initial pH 7 decreased to around pH 4, a rapid increase of CAPP activity and growth of *C. albicans* was observed. However, as the stationary growth phase continued, the medium pH shifted again from pH 4 to around pH 7, and the activity of CAPP gradually decreased. In order to compare the data of *C. albicans* with that of *T. mentagrophytes* the pH of each media was not buffered. As the cultivation period progress, the initial pHs focused around pH 7 to pH 8 (Fig. 2).

DISCUSSION

When a superficial infection of yeast and dermatophyte occurs on the skin, it is of significance to determine how both fungi are able to invade and acquire nutritive substances from keratinized tissue. Remold et al. (1968) purified a proteolytic enzyme from a culture medium containing BSA as the nitrogen source. However, they did not mention whether this enzyme played an important role in superficial candidiasis. Negi et al. (1984) and Tsuboi et al. (1985b) demonstrated the induction of KPase in medium which contained HSC as the nitrogen source. However, KPase has never been obtained when cultivated in sabouraud dextrose broth. A possible explanation for this could be that as sabouraud dextrose broth already contains sufficient nutrients, the secretion of KPase is therefore not necessary. They proposed that KPase might have a very important role in helping both fungi to invade the stratum corneum and multiply within it. Subsequently we proceeded to try to purify the KPase from the culture supernatant of *C. albicans.*

The purified KPase of *C. albicans* had a molecular weight of 42,000 and optimum pH at 4.0. From the inhibitory profiles, this enzyme was though to be a carboxyl proteinase having cathepsin D-like action. Among the inhibitor prepared, only pepstatin, a specific inhibitor of KPase, was able to strongly inhibit cell growth at such a low concentration of 10 μg/ml (Table 2). This result suggests that pepstatin suppress the growth of *C. albicans* by inhibiting the activity of KPase. Thereby suggesting that KPase might be an essential pathogenic factor for the growth of *C. albicans.* This enzyme has a broad substrate specificity and the terms, "KPase" and "proteolytic enzyme", are confusable, therefore "*C. albicans* producing proteinse" (CAPP) might be a more suitable name.

To clarify the relationship between candidiasis and its environmental pH, firstly the pH of each media was strongly fixed using 0.1M citric acid-0.2M disodium phosphate buffer in HSC-containing medium and the growth of *C. albicans* was prominently observed in weakly acidic media, but was not observed in neutral media. CAPP has an optimum pH at 4.0 and its important role on the growth of *C. albicans* at a weakly acidic condition was confirmed by the fact that the growth of *C. albicans* was strongly inhibited by the addition of pepstatin, which is an inhibitor of CAPP. Secondarily,

when the pH of each media in *C. albicans* was not fixed with buffer solution, the initial pHs varied according to their own environment and *C. albicans* was able to change the environmental pH into a weakly acidic condition in order to get nutrients by secreting KPase. The pH of each media in *T. mentagrophytes* was adjusted by citric acid or sodium hydroxide and the initial pHs focused neutral pH during the progression of the cultivation period (Fig. 2).

Data revealed that when both fungi encountered the various environmental pHs, they have ability to adjust their optimum pH. Subsequently produced KPases enable both fungi to obtain soluble nutrients by digesting insoluble proteins under optimum pH condition.

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