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Influence of Culture Medium pH and Proteinase Inhibitors on Extracellular Proteinase Activity and Cell Growth of *Sporothrix schenckii*

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Sporothrix schenckii **produces two extracellular proteinases in albumin- or collagen-supplemented unbuffered liquid medium. Proteinase I had an optimal pH of 6.0, and its activity was strongly inhibited by chymostatin. Proteinase II had an optimal pH of 3.5, and its activity was strongly inhibited by pepstatin. Speculating that these two proteinases are key enzymes for fungal growth, we investigated the influences of culture medium pH and either or both of the proteinase inhibitors pepstatin and chymostatin on the cell growth of *S. schenckii*. In buffered medium at a pH of 3.5, an optimal pH for proteinase II, only proteinase II activities were observed, while at pH 6.0, an optimal pH for proteinase I, only proteinase I activities were observed. However, there was no cell growth inhibition. These results suggested that the regulation of the production of the two proteinases is dependent on environmental pH. The addition of pepstatin or chymostatin to the culture medium did not inhibit the cell growth of *S. schenckii*, but the addition of both inhibitors at a concentration of 10 μg/ml strongly inhibited growth. These results suggested that at least one of the two proteinases was expressed to allow fungal growth in albumin-supplemented media. The indirect fungistatic action of the proteinase inhibitors, which inhibit proteolysis, may be applied to a topical therapeutic agent in vivo.**

Sporotrichosis, a widely distributed fungal infection caused by *Sporothrix schenckii*, causes chronic granulomatous and ulcerative lesions (4, 10). However, the parasitic invasive factors which lead to the instigation of these lesions have not yet been clarified. Proteolysis of insoluble skin constituents is speculated to be an important biochemical factor.

Recently, we purified two extracellular proteinases from the yeast form of *S. schenckii* grown in albumin- or collagen-supplemented liquid medium (7). Proteinase I, a serine proteinase, had an optimal pH of 6.0, and its activity was strongly inhibited by chymostatin. Proteinase II, a carboxyl proteinase, had an optimal pH of 3.5, and its activity was strongly inhibited by pepstatin. Our previous investigations with *Candida albicans* (2, 3, 5, 6) suggested that these two proteinases may be expressed to allow fungal growth.

We investigated the influence of culture medium pH and the inhibitory effect of either or both of the proteinase inhibitors pepstatin and chymostatin on the cell growth of *S. schenckii* to clarify the role of proteinases in fungal infection.

Organism and cultivation method. A fresh clinical isolate of *S. schenckii*, the same strain used in our previous study (7), was obtained from a localized type of sporotrichosis and identified by standard morphological studies (4). Precultivation was carried out with 3.7% brain heart infusion liquid medium (Eiken, Tokyo, Japan) in a shaking water bath (80 cycles/min) at 27°C for 1 week. A yeast form of the organism was prepared to give 10^4 cells per ml in culture medium after washes with sterilized water. The liquid culture medium, which was sterilized with a GS membrane filter (0.22-μm pores; Millipore Corp., Bedford, Mass.), was prepared with the following contents in 1 liter of distilled water: 10 g of yeast carbon base (Difco Laboratories, Detroit, Mich.), 50 mg of inositol (Sigma Chemical Co., St. Louis, Mo.), 10 mg of thiamine (Sigma), 10 mg of pyridoxine (Sigma), and 2.5 g of bovine serum albumin (A 7030; Sigma). Liquid medium (70 ml) in 200-ml Erlenmeyer flasks was incubated in a shaking water bath (80 cycles/min) at 27°C for 10 days. Cell counts were done with a hemacytometer; cell counts and proteinase assays (see below) were performed on days 2, 4, 7, and 10 of cultivation. Each value used was the average of two samples. Reproducibility of the data was checked three times.

Proteinase assay. Culture filtrate was used for the proteinase assay with azocoll (Sigma) as a substrate (1). For the proteinase I assay, 0.2 ml of filtrate solution was incubated with 5 mg of azocoll and 10 μg of pepstatin (9) (Peptide Institute Inc., Tokyo, Japan) in 0.8 ml of 50 mM citric acid–100 mM disodium phosphate buffer (pH 6.0). For the proteinase II assay, 0.2 ml of filtrate solution was incubated with 5 mg of azocoll and 1 mM phenylmethylsulfonyl fluoride (Sigma) in 0.8 ml of 50 mM citric acid–100 mM disodium phosphate buffer (pH 3.5). Each sample was incubated for 1 h at 37°C, and the incubation was terminated by the addition of 2.0 ml of cold water and the transfer of the reaction tubes to ice water. After centrifugation at 1,500 × *g* for 15 min, dye released into the supernatant was measured at 520 nm with a spectrophotometer (model 100-60; Hitachi).

Influence of culture medium pH on extracellular proteinase production and cell growth of *S. schenckii*. To examine the influence of culture medium pH, three different media were prepared: (i) unbuffered medium (the same medium described above, with an initial pH of approximately 5.0), (ii) buffered medium at a pH of 6.0 (the optimal pH of proteinase I), and (iii) buffered medium at a pH of 3.5 (the optimal pH of proteinase II). The latter two media were buffered with 0.1 M citric acid–0.2 M disodium phosphate. Cell growth and protein activities were measured during the cultivation period.

Influence of proteinase inhibitors on extracellular proteinase

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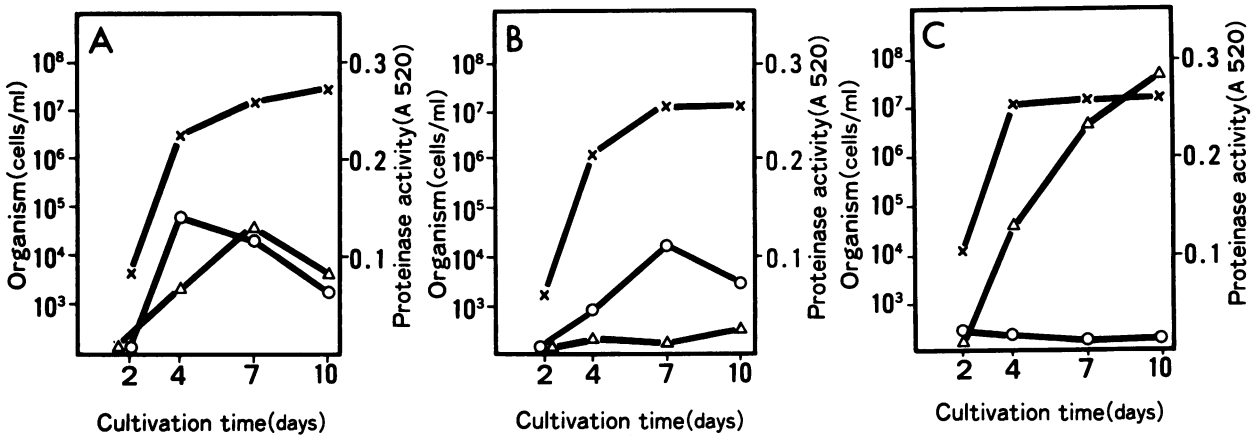

FIG. 1. Influence of culture medium pH on extracellular proteinase production and cell growth of *S. schenckii*. (A) Unbuffered medium; (B) buffered medium (pH 6.0); (C) buffered medium (pH 3.5). Symbols: ○, proteinase I activity; △, proteinase II activity; ×, culture density.

production and cell growth of *S. schenckii*. To examine the inhibitory effect of proteinase inhibitors, chymostatin (an inhibitor of proteinase I) or pepstatin (an inhibitor of proteinase II) or both were added to the culture medium at the commencement of cultivation. Stock solutions of pepstatin (9) (1 mg/ml) in 0.005 N sodium hydroxide or of chymostatin (8) (1 mg/ml; Peptide Institute) in 0.005 N acetic acid were added to give a concentration of 10 μg/ml in culture medium. Cultivation in medium without any inhibitors was carried out simultaneously as a control. Proteinase activities were measured in culture filtrates after 48 h of dialysis against distilled water to remove the effect of the inhibitors in the medium. Direct effects of pepstatin or chymostatin or both against the organism were examined by adding the inhibitor stock solutions to cultures in brain heart infusion liquid medium.

Influence of culture medium pH. In unbuffered medium, the activities of both proteinases were elevated on day 4 of cultivation (Fig. 1A). This was accompanied by prominent cell growth. The pH was approximately 5.0 until day 7. On day 10, after full fungal growth, the pH of the medium ascended to around 7.5, and the activities of proteinases I and II decreased. In buffered medium at an optimal pH for proteinase I (pH 6.0), proteinase I activities gradually increased to the same level as those in unbuffered medium; prominent cell growth was also observed (Fig. 1B). However, proteinase II activities were negligible during the cultivation period. Cultivation in buffered medium at an optimal pH for proteinase II (pH 3.5) yielded prominent proteinase II activities and cell growth (Fig. 1C). However, proteinase I activities were negligible during the cultivation period. The results obtained with buffered media suggested that a proteinase which has an optimal pH similar to the pH of the medium was expressed and correlated with cell growth.

Influence of proteinase inhibitors. Proteinase I activity (Fig. 2A) was detected in control (no inhibitor) and pepstatin-containing media but was barely detected in medium containing either chymostatin or both pepstatin and chymostatin. Proteinase II activity (Fig. 2B) was detected in control (no inhibitor) and chymostatin-containing media but was barely detected in medium containing either pepstatin or both pepstatin and chymostatin. Cell growth of *S. schenckii* (Fig. 2C) was inhibited to fewer than 10^5 cells per ml only in the medium containing both pepstatin and chymostatin.

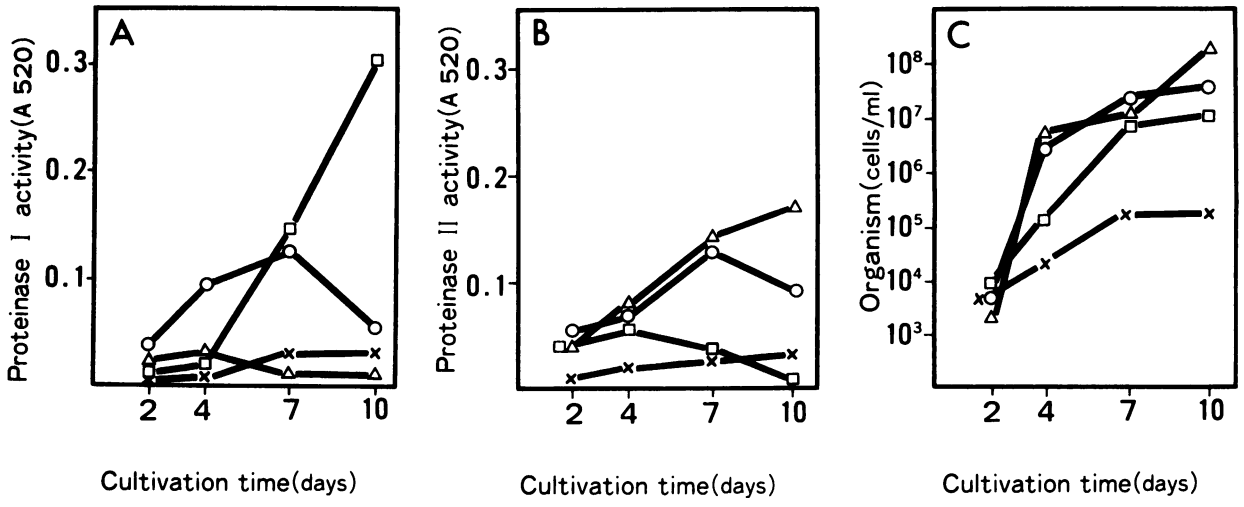

FIG. 2. Influence of proteinase inhibitors on extracellular proteinase production and cell growth of *S. schenckii*. (A) Proteinase I activity; (B) proteinase II activity; (C) culture density. Symbols: ○, no inhibitor (control); △, chymostatin (10 μg/ml); □, pepstatin (10 μg/ml); ×, pepstatin (10 μg/ml) plus chymostatin (10 μg/ml).

Medium containing either pepstatin or chymostatin alone did not inhibit cell growth. Fungal growth and microscopic morphology of the organism in brain heart infusion medium were not affected by the addition of pepstatin or chymostatin or both (not shown).

We have previously emphasized that an extracellular proteinase produced by *C. albicans* is an important invading factor in candidiasis (2, 3, 5, 6). *S. schenckii* produces two kinds of proteinases in albumin- or collagen-supplemented medium (7). Enzyme activities and cell growth were observed after cultivation for 1 week in these media. However, proteinase production was not observed in either brain heart infusion medium or Sabouraud medium in spite of rapid cell growth, probably because proteinase production is unnecessary in media which already supply sufficient aminopeptides for the organism. The natural specificity of the enzymes proved that insoluble proteins from skin, such as keratin, elastin, and collagen, were hydrolyzed, which suggests that the enzymes are able to break down skin constituents ahead of the organism invasion.

This in vitro study was attempted to confirm the biological role of these two proteinases from *S. schenckii*. Long-duration cultivation in albumin-supplemented medium was performed for up to 10 days to see the correlation between proteinase production, pH of the medium, and fungal growth. The results of the first experiment suggested that the ratio of proteinase I production to proteinase II production varied depending on the pH of the medium and that they each supplemented the activities of the other. However, prominent cell growth was observed in all three media. In the liquid medium with a pH of 3.5, proteinase II was overproduced, probably to supplement proteinase I activities. The second experiment suggested that at least one of the two proteinases was expressed to allow fungal growth in albumin-supplemented media. To inhibit fungal growth, the addition of both pepstatin and chymostatin was required. The enhancement of proteinase I activity in pepstatin-containing medium and of proteinase II activity in chymostatin-containing medium was probably due to supplemental production of the other proteinase. In the case of *C. albicans*, the addition of pepstatin was sufficient to inhibit cell growth, because *C. albicans* produces only one major extracellular proteinase (6). These inhibitory profiles suggest that extracellular proteinases are essential for fungal growth in nitrogen-restricted media and that the number of extracellular proteinases related to cell growth differs according to the fungus species.

Although the antifungal effect of pepstatin and chymostatin was an indirect fungistatic action caused by inhibition of the activities of proteinases, their nontoxic and selective action may lead to their use as new topical therapeutic agents. We are now investigating the effect of proteinase inhibitors, including pepstatin and chymostatin, on animal and human sporotrichosis.

LITERATURE CITED

1. **Chavira, R., Jr., T. J. Burnett, and J. H. Hageman.** 1984. Assaying proteinases with azocoll. Anal. Biochem. **136**:446–450.
2. **Hattori, M., K. Yoshiura, M. Negi, and H. Ogawa.** 1984. Keratinolytic proteinase produced by Candida albicans. Sabouraudia **22**:175–183.
3. **Negi, M., R. Tsuboi, T. Matsui, and H. Ogawa.** 1984. Isolation and characterization of proteinase from Candida albicans. J. Invest. Dermatol. **83**:32–36.
4. **Rippon, J. W.** 1982. Medical mycology: the pathogenic fungi and the pathogenic actinomycetes, p. 277–302. The W. B. Saunders Co., Philadelphia.
5. **Tsuboi, R., Y. Kurita, K. Iwahara, T. Hirotani, K. Matsuda, M. Negi, and H. Ogawa.** 1985. The biological role of keratinolytic proteinase (KPase) and its inhibitor on the growth of Candida albicans, p. 161–173. *In* H. Ogawa, G. S. Lazarus, and V. K. Hopsu-Havu (ed.), The biological role of proteinases and their inhibitors in skin. University of Tokyo Press, Tokyo.
6. **Tsuboi, R., Y. Kurita, M. Negi, and H. Ogawa.** 1985. A specific inhibitor of keratinolytic proteinase from Candida albicans could inhibit the cell growth of C. albicans. J. Invest. Dermatol. **85**:438–440.
7. **Tsuboi, R., T. Sanada, K. Takamori, and H. Ogawa.** 1987. Isolation and properties of extracellular proteinases from *Sporothrix schenckii*. J. Bacteriol. **169**:4104–4109.
8. **Umezawa, H., T. Aoyagi, H. Morishima, S. Kunimoto, M. Matsuzaki, M. Hamada, and T. Takeuchi.** 1970. Chymostatin, a new chymotrypsin inhibitor produced by actinomycetes. J. Antibiot. **23**:425–427.
9. **Umezawa, H., T. Aoyagi, H. Morishima, M. Matsuzaki, M. Hamada, and T. Takeuchi.** 1970. Pepstatin, a new pepsin inhibitor produced by actinomycetes. J. Antibiot. **23**:259–262.
10. **Urabe, H., and S. Honbo.** 1986. Sporotrichosis. Int. J. Dermatol. **25**:255–257.