

## Isolation and Characteristics of Collagenolytic Enzyme Produced by *Candida albicans*

HIDENORI KAMINISHI, YOSHISATO HAGIHARA,\* SACHIO HAYASHI, AND TAMAKI CHO  
Department of Oral Microbiology, Fukuoka Dental College, 700 Ta, Sawara-Ku, Fukuoka, Japan 814-01

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In media containing collagen as the nitrogen source, the pathogenic yeast *Candida albicans* secreted a collagenolytic enzyme. Purification of the enzyme from a culture filtrate was achieved by DEAE-Sephacel chromatography at pH 6.7. The molecular weight was found to be 46,000 by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the isoelectric point was at pH 4.2. The pH optimum lay between 3.5 and 4.0, and above pH 6.0, the enzyme underwent alkaline denaturation. The enzyme was heat labile, and a decrease in activity was found above 55°C. The enzyme activity was inhibited by the addition of urea, cysteine, and pepstatin. No other inhibitor among those tested had any effect. The *C. albicans* enzyme degraded both the native acid-soluble collagen and the insoluble dentinal collagen.

The pathogenic yeast *Candida albicans*, an opportunist found in the oral cavity and intestinal tract, causes serious candidosis and is the major cause of denture stomatitis (14). Remold et al. (10) and Rùchel (12) characterized an extracellular acid proteinase from *C. albicans*, and the relationship between the infectivity of mice and proteinase production was described.

It is well known that *C. albicans* can be found in dentinal caries (3). However, it is very difficult to explain the reason for its presence. We found that an enzyme which degrades human dentinal collagen was produced by *C. albicans*. It was thought that *C. albicans* may utilize the dentinal structure, especially collagen, for growth. In this paper we describe an experimental study on collagenolytic enzyme production by *C. albicans* and the characteristics of the enzyme.

### MATERIALS AND METHODS

**Organism.** *C. albicans* ATCC 1002 was used and was maintained on Sabouraud agar. The organism was cultured for 18 h at 37°C in glucose-peptone broth, collected, washed three times with a saline solution, and suspended in distilled water. To obtain a collagen-degrading enzyme, the cells were inoculated into a growth medium, which could produce an initial yeast concentration of  $10^5$  cells per ml.

**Collagen.** (i) **Human dentinal collagen.** Human dentinal collagen was prepared as follows. Human teeth without dental caries and stored at -20°C were used. The enamel and cementum of the teeth were mechanically removed with a diamond bar. Subsequently, the teeth were split open, and the surface of the root canal was scraped clean. To prevent irreversible denaturation of dentinal collagen, all manipulations were performed under cold running water. The fragmented dentine was then crushed and ground to a powder with an X-Press (Biotec, Sweden) and passed through a 100-mesh filter. The dentinal powder was then washed until there was no detectable ninhydrin substance. The powder was stored at -20°C until required. The ground dentine was demineralized for a period of 7 days at 4°C with a 3% EDTA

solution adjusted to a pH of 7.0 with 1 N NaOH. The EDTA solution was renewed twice daily. The dentinal collagen prepared by EDTA demineralization was washed free of EDTA with water and sterilized with ethylene oxide (1 kg/cm<sup>2</sup> at 50°C) after lyophilization. The dentinal collagen was suspended in growth medium or a buffer before use.

(ii) **Commercial collagen.** Azocoll (7) and acid-soluble collagen were purchased from Sigma Chemical Co., St. Louis, Mo. The Azocoll was suspended in buffer solutions at a concentration of 2 mg/ml. The acid-soluble collagen was dissolved in a 0.1 M acetate buffer (pH 4.0) at a concentration of 2 mg/ml, constantly stirred for 18 h at 4°C, and stored at 0°C. Before the experiment, the solution was centrifuged for 1 h at  $100,000 \times g$ , and the supernatant was used.

**Standard collagenase.** *Clostridium histolyticum* collagenase (EC 3.4.24.3) (Sigma type I) was used as a standard.

**Isolation and purification of enzyme.** The washed cells were cultured in yeast carbon base supplemented with 1% glucose and 0.2% human dentinal collagen as a nitrogen source for 16 days at 37°C. The culture was centrifuged for 10 min at  $2,000 \times g$ , and the supernatant was concentrated (protein content, 450 mg) approximately 10-fold by ultrafiltration with PM 10 Diaflo Membranes (Amicon Corp., Lexington, Mass.). Isolation and purification of the enzyme was achieved by using DEAE-Sephacel columns (2 by 30 cm). Proteins were eluted from the column with sodium citrate buffer (pH 6.7) with a linear increase in ionic strength ranging from 20 to 500 mM. Three milliliters of the fractions was collected at a flow rate of 30 ml/h. The absorbance of each fraction at 280 nm was determined against a citrate buffer blank, and representative fractions containing absorbing material were assayed for enzyme activity. The fractions containing activity were pooled, concentrated by ultrafiltration with PM 10 Diaflo Membranes (yield of enzyme, 2.4 mg), and stored in the presence of 5% glycerin at -80°C. Disc gel electrophoresis of the active fractions was performed by the method of Davis (2). Isoelectrophoresis and molecular weight determinations were done by using the techniques of Vesterberg (17) and Weber and Osborn (19), respectively. Protein concentration was determined by the method of Lowry et al. (5) with bovine serum albumin as a standard.

\* Corresponding author.

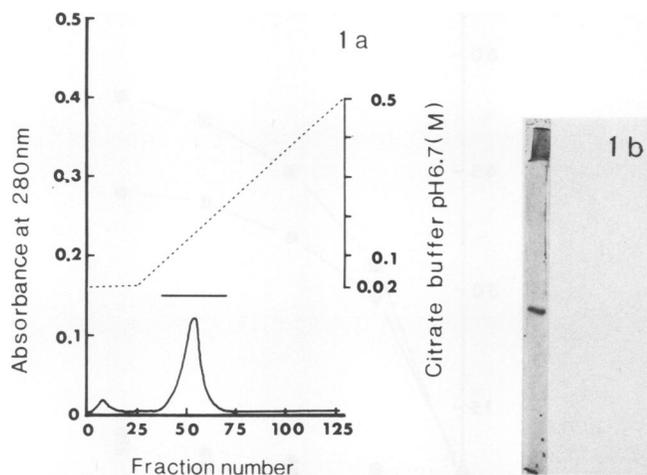


FIG. 1. (A) DEAE-Sephacel column chromatography of culture supernatant. Protein was eluted from the column with a linear increase of ionic strength of the sodium citrate buffer (pH 6.7). Protein concentration was measured at 280 nm for 3-ml fractions. The fractions containing activity (indicated by the bar) were pooled. (B) Disk gel electrophoresis on a 7.5% polyacrylamide gel of enzyme isolated from culture supernatant of *C. albicans* by DEAE-Sephacel column chromatography.

**Assay for hydrolysis of Azocoll.** Azocoll was suspended in 2 ml of 0.1 M acetate buffer (pH 4.0; 2 mg/ml), and 50  $\mu$ l of enzyme solution (25  $\mu$ g of protein per ml) was added. The reaction mixture was incubated for 18 h at 37°C. After incubation, the unhydrolyzed Azocoll was removed by centrifugation for 10 min at 2,000  $\times$  g, and the red solution was read at 540 nm in a spectrophotometer (model 200-20; Hitachi Inc., Japan).

**Assay for hydrolysis of collagen.** The assay for the hydrolysis of collagen was based on the determination of

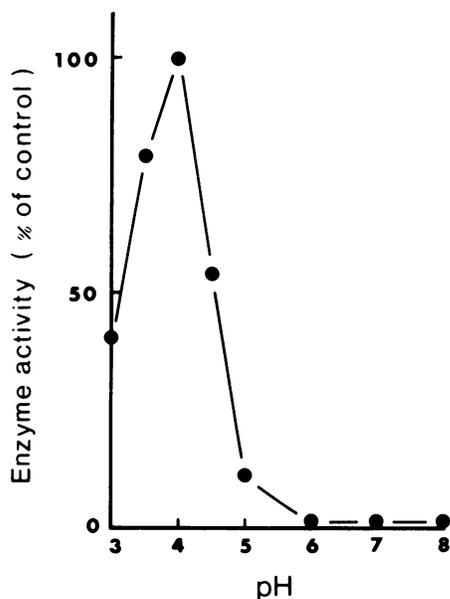


FIG. 2. Effect of pH on enzyme activity. The reaction mixture was prepared with buffers at the pHs described in the text. Enzyme activity was determined by the Azocoll assay (18 h at 37°C). The relative activity is expressed as a percentage of that in the control reaction mixture (pH 4.0).

hydroxyproline in the peptide released from the collagen. The reaction mixture contained 1 ml of collagen solution (dental collagen, 5 mg/ml, or acid-soluble collagen, 2 mg/ml) and 50  $\mu$ l of enzyme solution. After incubation for specified intervals at 37°C, an equal volume of 20% trichloroacetic acid was added to stop the reaction. The mixture was ultracentrifuged for 3 h at 150,000  $\times$  g, and the amount of hydroxyproline in the supernatant was determined by the method of Prockop and Udenfriend (9). Observation of enzymatic degradation of acid-soluble collagen was also performed by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Inhibition of enzyme activity.** The inhibitory effect of various substances on the activity of the isolated enzyme was investigated by using EDTA, cysteine, urea, 2-mercaptoethanol (2-ME), *p*-chloromercuribenzoate (PCMB), phenylmethylsulfonyl fluoride (PMSF), pepstatin, diazoacetyl norleucine methyl ester (DAN), and 1,2-epoxy-3-*p*-nitrophenoxypropane (EPNP). The reaction mixture, containing 50  $\mu$ l of the enzyme solution and 50  $\mu$ l of inhibitor solution, was kept at room temperature for 30 min. Activity was then measured and compared with that of the control by the Azocoll assay.

**pH optimum and pH denaturation.** The optimum pH for enzyme activity was determined by the Azocoll assay at pH 3.0 to 5.0 with a 0.1 M acetate buffer, at pH 6.0 to 7.0 with a 0.1 M phosphate buffer, and at pH 8.0 with a 0.1 M Tris hydrochloride buffer. The effect of pH on the denaturation of the enzyme was determined by the Azocoll assay at pH 4.0 after incubation of the enzyme for 3 h at 37°C in the same buffers (pH 3.0 to 8.0).

**Optimum temperature and heat stability.** The optimum temperature for enzyme activity was determined by the Azocoll assay at pH 4.0 in a water bath at temperatures ranging from 30 to 70°C. The heat stability of the enzyme was determined by the Azocoll assay at 37°C after heating the enzyme solution to temperatures of 40 to 100°C for 30 min in a 0.1 M acetate buffer (pH 4.0).

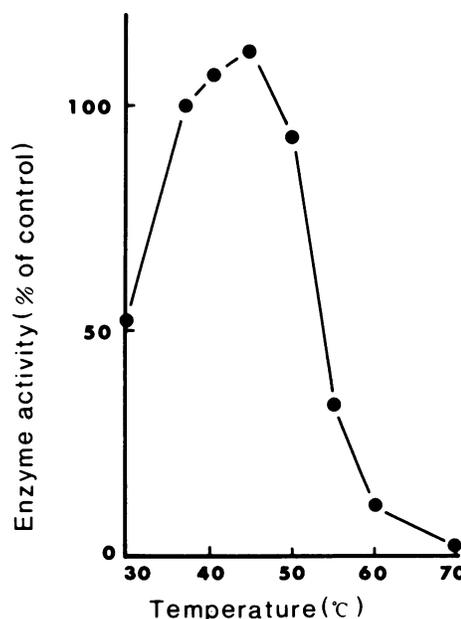


FIG. 3. Effect of temperature on enzyme activity as determined by Azocoll assay. The relative activity is expressed as a percentage of that in the control (37°C).

## RESULTS

The proteolytic activity of the *C. albicans* culture filtrate for both Azocoll and undenatured collagen was eluted by the citrate buffer between 0.1 and 0.2 M from the DEAE-Sephacel column (Fig. 1a). The fraction containing the collagenolytic activity appeared as a single protein band, as determined by disk gel electrophoresis (Fig. 1b). This preparation was used for further characterization studies.

The isoelectric point of the enzyme, as determined by analytical electrofocusing in a polyacrylamide gel, was at pH 4.2. The molecular weight, estimated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was 46,000.

The optimum pH for enzyme activity, measured by the hydrolysis of Azocoll, was 3.5 to 4.0 (Fig. 2). Enzyme activity began to decline above pH 4.5 and was not observed at pH 6.0 or higher, although the most stable pH for the enzyme was 6.0. Approximately 80% of the enzyme activity was lost at pH 7.5. The temperature optimum, as determined by the Azocoll assay, was 45°C. Only 10% of the enzyme activity remained at 60°C, and no activity was detected at 70°C (Fig. 3). When the enzyme was heated for 30 min at 40, 50, 55, 60, 70, and 100°C, a decrease in activity due to protein denaturation was observed at temperatures above 55°C.

Both the native acid-soluble collagen and the human dentinal collagen were hydrolyzed by the enzyme, as evidenced by the release of hydroxyproline into the hydrolysate (Fig. 4 and 5). Further, the enzyme caused a decrease in the amount of the  $\alpha$  and  $\beta$  components as compared with the amount in the control (heat-inactivated enzyme) and an

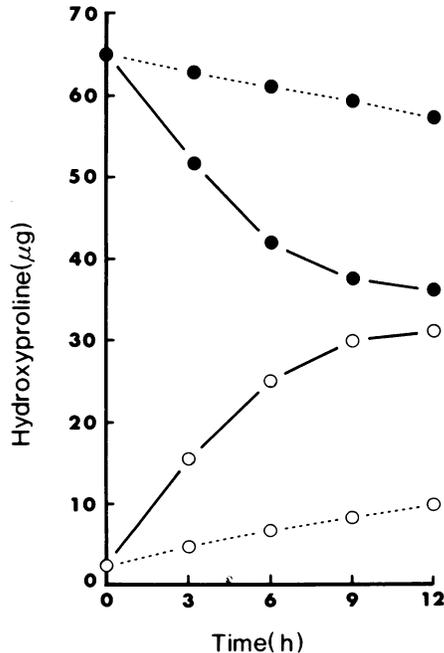


FIG. 4. Hydrolysis of acid-soluble collagen by *C. albicans* enzyme. The reaction mixture contained 1 ml of collagen solution and 50  $\mu$ l of enzyme solution (25  $\mu$ g of protein per ml). After the mixture was incubated at 37°C, an equal volume of 20% trichloroacetic acid was added. The mixture was centrifuged for 3 h at 150,000  $\times$  g, and the amounts of hydroxyproline in the supernatant (○) and the sediment (●) were determined separately by the method of Prockop and Udenfriend (9). Heated (100°C for 10 min) enzyme was used in reference assays (---).

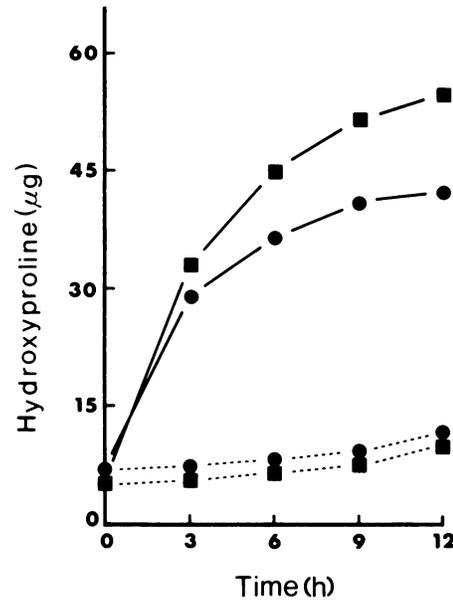


FIG. 5. Hydrolysis of dentinal collagen. The reaction mixture contained 1 ml of buffer solution (0.1 M acetate buffer [pH 4.0] for the *C. albicans* enzyme and 0.1 M Tris hydrochloride buffer [pH 7.5] for the *C. histolyticum* collagenase), 50  $\mu$ l of enzyme solution (●, *C. albicans* enzyme [25  $\mu$ g of protein per ml]; ■, *C. histolyticum* collagenase [25  $\mu$ g of protein per ml]), and 5 mg of dentinal collagen. After the mixture was incubated for specified intervals at 37°C, an equal volume of 20% trichloroacetic acid was added to stop the reaction. The mixture was centrifuged for 3 h at 150,000  $\times$  g, and the amount of hydroxyproline in the supernatant was determined by the method of Prockop and Udenfriend (9). To remove the soluble materials, the dentinal collagen was washed with each buffer and repeatedly centrifuged at 4°C for 18 h before the experiment. Heated (100°C for 10 min) enzyme was used in reference assays (---).

increase in molecular fragments lower than the  $\alpha$  chains (Fig. 6).

The inhibitory effects of various substances on enzyme activity are shown in Table 1. Pepstatin, urea, and cysteine inhibited enzyme activity. No other substances had any effect.

## DISCUSSION

In the work presented, a collagenolytic enzyme from the pathogenic yeast *C. albicans* was isolated, purified, and characterized. The enzyme, fractionated by DEAE-Sephacel column chromatography, showed activity against Azocoll and native collagen. The enzyme was inducible by native human dentinal collagen (insoluble) but was not obtained when the organism was cultured in a medium containing autoclaved denatured collagen (data not shown). This observation was reported previously by Nordwig and Jahn (8).

The human dentinal collagen used was thought to be native because it was not attacked by trypsin. The enzyme obtained from *C. albicans* is significantly different from the other collagenases which are produced by various microorganisms and which have been discussed in several reports (8, 11, 15, 16, 18). The pH optima of the collagenases of other microorganisms are as follows: *C. histolyticum*, 7.5 (15, 18); *Aspergillus oryzae*, 9 to 10 (8); *Pseudomonas aeruginosa*, 7.1 (16); and *Trichophyton schoenleinii*, 6.5 (11). In contrast,

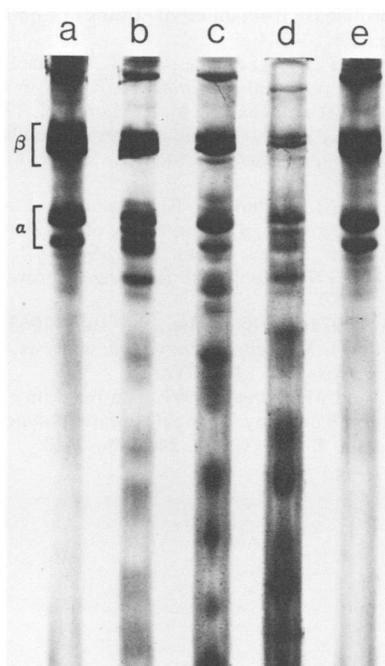


FIG. 6. Degradation of acid-soluble collagen by *C. albicans* enzyme. The reaction mixture contained 4 mg of collagen and 1.25  $\mu$ g of enzyme in 500  $\mu$ l of 10 mM acetate buffer (pH 4.0). The incubations were for 6 h (lanes a and b), 12 h (lane c), and 24 h (lanes d and e) at 37°C. Samples were run on a 5% polyacrylamide gel in a sodium dodecyl sulfate system. Lanes: a and e, control (collagen with heat-treated enzyme); b to d, collagen with intact enzyme.

the *C. albicans* collagenolytic enzyme was optimally active at pH 3.5 to 4.0 in an acetate buffer. Enzyme activity was not detected at pH 6.0, and incubation at a pH higher than 6.0 caused irreversible denaturation of the enzyme. Of the substances tested, only cysteine, urea, and pepstatin inhibited enzyme activity. Pepstatin, a group inhibitor of aspartic enzymes, strongly inhibited the enzyme activity, although the activity was not inhibited by EPNP or DAN, which are similar group inhibitors. Although EDTA is known to be an inhibitor of *C. histolyticum* collagenase (18), it did not affect the *C. albicans* enzyme. *C. albicans* proteinase induced by bovine serum albumin has been investigated (1, 5, 10, 12–14), but the proteinase was not reported to be collagenolytic. The alkaline denaturation pH (6.0), isoelectric point (pH 4.2), and molecular weight (46,000) of the enzyme induced by human dentinal collagen were different from those of the bovine serum albumin-induced enzyme reported by Remold et al. (10), although enzyme activity was affected by similar inhibitors. Further study to clarify the actual differences is necessary. The collagenolytic enzyme cathepsin B,L degrades the acid-soluble collagen restrictively, leading to the conversion of  $\beta$  and higher components, mainly to  $\alpha$  chains (4). However, the *C. albicans* enzyme nonspecifically degraded both the cross-linked  $\beta$  and non-cross-linked  $\alpha$  chains, which are components of the collagen. We also isolated a collagenolytic enzyme of *C. albicans* induced by insoluble bovine achilles tendon collagen (unpublished observation).

It is well known that *C. albicans* is frequently found in dentinal caries, and the relationship between *C. albicans* and dentinal caries has been discussed (3). It is proposed that *C. albicans* produces lactic acid by fermentation of carbohy-

TABLE 1. Influence of various substances on enzyme activity<sup>a</sup>

Inhibitor	Concn (mM)	Activity (%)
None		100
Pepstatin	1	0
	0.5	0
Urea	7 <sup>b</sup>	0
	5 <sup>b</sup>	20
Cysteine	50	20
	25	71
2-ME	50	90
	25	100
PCMB	10	95
	5	100
PMSF	10	117
	5	113
EDTA	1	100
	0.5	100
EPNP	10	100
	5	100
DAN	1 <sup>c</sup>	100
	0.5 <sup>c</sup>	100

<sup>a</sup> Enzyme activity was determined in the presence or absence of the substances, as described in the text (5 h at 14°C for DAN). Activity is expressed as a percentage of that in the control (untreated enzyme).

<sup>b</sup> Molar.

<sup>c</sup> Milligrams per milliliter.

drates and that this organic acid degrades the hydroxyapatite structure of teeth. However, the effect of microorganisms on the organic structure of the dentine, especially collagen, has not been clearly explained.

We demonstrated that *C. albicans* produces an extracellular, collagenolytic enzyme. Further studies are required to determine the role of this enzyme in dentinal caries.

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