Extracellular Protease from the Antarctic Yeast
*Candida humicola*

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The psychrotrophic, dimorphic yeast *Candida humicola*, isolated from Antarctic soil, secretes an acidic protease into the medium. The secretion of this protease by *C. humicola* was found to be dependent on the composition of the medium. In YPD or yeast nitrogen base medium containing either amino acids or ammonium sulfate as the nitrogen source, the activity of the protease in the medium was low (basal level). However, when yeast nitrogen base medium was depleted of amino acids or ammonium sulfate and supplemented with proteins, the activity of the enzyme increased. The secretion of the enzyme was greater during exponential growth at low temperatures than during growth at higher temperatures. The purified protease had a molecular mass of 36,000 Da and was inhibited by pepstatin, iodoacetamide, and sodium dodecyl sulfate. Despite the prevalent cold temperatures in Antarctica, this extracellular protease of the psychrotrophic yeast *C. humicola* was active at temperatures ranging from 0 to 45°C, with an optimum activity at 37°C.

In microbial ecosystems of Antarctica, psychrophilic and psychrotrophic organisms play a major role in the biodegradation of organic matter. These organisms are efficient not only in permanently cold areas but also in habitats which experience seasonal variation in temperature during late fall and spring. Therefore, it would be of interest to study the nature of extracellular enzymes such as proteases secreted by cold-adapted organisms. Earlier studies have indicated that yeasts belonging to the genera *Kluyveromyces*, *Endomycopsis*, *Cephalosporium*, *Aureobasidium*, *Saccharomyces*, *Rhodotorula*, and *Candida*, and most sporobolomyces and trichosporons secrete proteolytic enzymes (2). Many of these yeasts are probably also psychrotrophic (2), but the proteolytic enzymes secreted by them has been neither purified nor characterized.

During the course of our taxonomic studies on various psychrotrophic yeasts (15) and bacteria from Antarctica (19–23), we identified one dimorphic psychrotrophic yeast, *Candida humicola*, which secreted a protease into the culture medium. In an attempt to understand the induction, secretion, and properties of this protease from a cold-adapted yeast species, we characterized the secretory protease from the psychrotrophic yeast *C. humicola*.

**MATERIALS AND METHODS**

**Maintenance and growth of yeast culture.** *C. humicola*, collected from the Schirmacher Oasis, Antarctica, was maintained on YPD medium as previously described (15). YPD contained (per liter) 10 g of yeast extract, 10 g of peptone, and 20 g of glucose. Cultures were stored on YPD slopes and plates at 4°C and subcultured every month. The culture (MTCC Y6) has been deposited with the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India.

The production of protease by *C. humicola* was studied in both YPD and yeast nitrogen base (YNB) media with or without amino acids and ammonium sulfate. YNB and yeast carbon base (YCB) media were from Hi Media Laboratories, Bombay, India, and contained the ingredients described by Kreger-Van Rij (9). Exogenous nitrogen sources such as ammonium sulfate, Casamino Acids (1% [wt/vol]), yeast extract (1% [wt/vol]), bovine serum albumin (BSA; 0.1 mg/ml), casein (0.1 mg/ml), and gelatin (0.1 mg/ml) were added when required to YNB without amino acids and ammonium sulfate. The pH values of the media were adjusted with the following buffers of 50 mM final concentrations: sodium citrate buffer for pH 3 to 6; sodium phosphate buffer for pH 6 to 8; and Tris-HCl buffer for pH 8 and 9. For protease production, the cells were routinely grown at room temperature (22 ± 1°C). However, when required, they were grown at 4 and 15°C in a controlled environmental incubator (New Brunswick Scientific, Edison, N.J.) and at 30°C in a gyrotary water bath shaker (New Brunswick Scientific). Only batch cultures were grown in Erlenmeyer flasks. All media were sterilized by either autoclaving or the filter sterilization method. Growth was followed by measurement of the optical density at 600 nm (OD$_{600}$).

**Assay of protease activity.** Protease activity with casein as a substrate was determined by the modified method of Ramakrishna and Pandit (14). Briefly, 300 µl of the culture supernatant was added to 500 µl of 1% (wt/vol) casein (in sterile distilled water) and 200 µl of 50 mM sodium acetate buffer (pH 5.0) contained in an Eppendorf tube and incubated at the desired temperature for 2 h. The reaction was stopped by the addition of 500 µl of 20% (wt/vol) trichloroacetic acid; the resulting precipitate was allowed to settle for 30 min on ice and then was pelleted by centrifugation in a microcentrifuge (Kubota, Tokyo, Japan) for 15 min. Proteolytic activity was measured by determining the OD$_{280}$ of the supernatant. In blanks, trichloroacetic acid was added to the incubation mixture at time zero. The activity is expressed in units, 1 U being the activity required to generate an OD$_{280}$ of 0.01 in the supernatant described above per hour. However, when hemoglobin, BSA (1% [wt/vol]), and Azocoll (Sigma Chemical Co., St. Louis, Mo.) were used as the substrates, the protease assay method of Nelson and Young was used (13).

Protease activity on various other synthetic substrates was assayed by established procedures (3, 4).

**Purification of the protease.** The cell-free culture supernatant obtained after centrifugation was concentrated by Am-
TABLE 1. Effect of inhibitors on the activity of the acid protease secreted by *C. humicola*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc</th>
<th>Protease activity (µU of control)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>1 mM</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>82</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>83</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>0.001 mM</td>
<td>34.5</td>
</tr>
<tr>
<td></td>
<td>0.025 mM</td>
<td>0</td>
</tr>
<tr>
<td>TLCK</td>
<td>1 mM</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>52</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>5 mM</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>6</td>
</tr>
<tr>
<td>SDS</td>
<td>0.05% (wt/vol)</td>
<td>0</td>
</tr>
<tr>
<td>Soybean trypsin</td>
<td>5 µg/ml</td>
<td>100</td>
</tr>
</tbody>
</table>

* The control had 35 U of activity, measured as described in Materials and Methods.

icon filtration at 4°C and recentrifuged in the cold, and 0.5 ml of the clear supernatant was loaded onto a fast protein liquid chromatography (FPLC) Mono Q preparative column (Pharmacia). The column was washed with 15 mM sodium acetate buffer (pH 6.8) and then eluted with a gradient of 0 to 1 M NaCl in 15 mM sodium acetate (pH 5.0). The eluted fractions were monitored at 280 nm, and only the peak fractions were checked for protease activity on an X-ray film (6). Active fractions from various FPLC runs were pooled, concentrated by lyophilization, dialyzed against 10 mM sodium acetate buffer, and stored at −20°C.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli and Favre (10). Silver nitrate staining of proteins in polyacrylamide gels was done by the method of Wray et al. (27).

Substrate-impregnated gel electrophoresis (5) was carried out in 10% (wt/vol) native polyacrylamide gels impregnated with 0.2% (wt/vol) casein except that no SDS or β-mercaptoethanol was used.

**Inhibition of protease activity.** Inhibitors listed in Table 1 were obtained from Sigma and assessed for their effect on the activity of the protease secreted by *C. humicola*.

**Freeze-thawing and heat resistance characteristics.** The stability of the enzyme was studied by repeated freezing in liquid nitrogen and thawing slowly to room temperature (for 20 cycles). At the end of each cycle, an aliquot was assessed for protease activity. To ascertain the thermal stability of the enzyme, the enzyme was preincubated for various time periods (10 min, 30 min, 1 h, 2 h, and 4 h) at various temperatures (15, 22, 37, 45, 56, and 65°C) after which the activity was measured at 37°C with casein as the substrate. For pH stability, the enzyme was preincubated at the desired pH (pH 2, 3, 4, 5, 6, 7, 8 or 9) overnight and assayed for activity in 10 mM sodium acetate (pH 5).

**RESULTS**

**Protease production.** (i) **Effect of nitrogen source.** The growth of the psychrotrophic yeast *C. humicola* and its ability to produce the protease were monitored in various media with and without a nitrogen source (Fig. 1). In an unbuffered medium containing BSA as the sole nitrogen source, the activity of the protease was maximum and this maximum activity was observed throughout the exponential and the stationary phases. However, after 5 days, the protease activity in the medium began to decrease. When (NH₄)₂SO₄ and amino acids (Casamino Acids; Difco) were present in the medium, the protease activity was reduced dramatically and the activity level was similar to that observed in media containing BSA but no glucose. It is probable that this protease is ordinarily secreted at a minimum basal level and is induced in the presence of a protein. A change in the concentration of glucose (e.g., from 0.1 to 1% [wt/vol]) did not alter the level of protease in the medium. In an enriched medium (e.g., YPD), the activity of this protease was poor. In YCB medium containing either casein, BSA, or gelatin as a nitrogen source, it was observed that casein-containing medium induced more protease activity than BSA- or gelatin-containing medium did (data not shown).

When intact, dead *C. humicola* cells and bacterial cells (fixed in 70% [vol/vol] ethanol for 48 h) were used as a possible nitrogen source in YNB medium containing glucose but not containing ammonium sulfate or amino acids, no
protease activity was induced and, in addition, the growth of C. humicola was poor (maximum OD₆₀₀ unit observed, ca. 0.4). However, when bacteria were killed by boiling for 10 min and then used as a nitrogen source in this same medium, protease activity was induced and C. humicola growth reached the same level as it did in medium with 0.01% (wt/vol) BSA.

(ii) Effect of temperature and pH. An earlier study indicated that the optimum temperature of growth of the psychrotrophic yeast C. humicola was 22 ± 2°C (15) and that this yeast was unable to grow at 30°C when incubated in a poor nutrient medium (e.g., YNB plus 1% [wt/vol] glucose). When the extracellular medium from batch cultures grown at 4, 15, and 22°C in YNB (without amino acids or ammonium sulfate but with 0.1% [wt/vol] glucose) was assayed for proteolytic activity (Fig. 2a), the activity of the protease was found to increase with an increase in growth, reaching a plateau in the early stationary phase. Furthermore, the protease activity per unit number of cells (OD₆₀₀/OD₆₀₀) was higher at the two lower temperatures (4 and 15°C) than it was at the highest temperature (Fig. 2b). When analyzed by SDS-PAGE, the protein band corresponding to the protease activity (see below) also showed a higher intensity at the lower temperatures than it did at the highest temperature (Fig. 2b, inset).

Studies using radioactively labelled methionine also indicate that more of the protein was secreted at 4°C than at 22°C. In these experiments, C. humicola was grown in YNB medium (without amino acids and ammonium sulfate but with glucose and BSA) containing [³⁵S]methionine (50 μCi/ml of culture) for 48 h at 22°C, washed, and resuspended in fresh medium containing BSA and glucose and allowed to stand for another 30 min at 4 and 22°C. The cells (500-μl suspension) were then pelleted by centrifugation, and [³⁵S]methionine counts in the medium were determined. The count of [³⁵S]methionine-labelled proteins secreted by cells at 4°C (75 × 10⁶ cpm) was higher than that of cells maintained at room temperature (30 × 10⁶ cpm). From this time onwards, protease was the only protein secreted into the medium (see below).

When grown in a medium which was not buffered (YNB containing BSA and glucose), C. humicola changed the initial pH of the medium from 5.1 to 4.8 by the end of the stationary phase. In buffered media of various pH values, the secretion of the protease was detectable between pH 3 and 6 and maximum activity was observed in a medium of pH 4. The highest maximum growth was observed at pH 4 and 5 (OD₆₀₀, ~1.3 at the end of 72 h), and at pH 6, 7, and 8, the maximum growth obtained was lower (OD₆₀₀, 0.7, 0.5, and 0.5, respectively, after 72 h). At pH 3, growth was extremely slow and reached a maximum OD₆₀₀ of 0.65 after 7 days.

Purification of the protease. Because of its extracellular nature, the crude protease was obtained from the culture supernatant after the cells were pelleted by centrifugation. The changes at various time points in the protein profile of the supernatant of C. humicola grown in YNB medium containing BSA and glucose is shown in Fig. 3a. After mid-log phase, when the activity of the protease was at its highest, there was a single major band of protein (lanes 5 to 11) whose quantity was also at its highest as revealed by densitometer scanning of the gel. The activity dropped gradually, and the band corresponding to the protease ultimately disappeared from the supernatant (lanes 12 to 14). Culture supernatants obtained before 30 h of growth contained various degradation products of BSA, the substrate which was used as an inducer. Purification of the protease was achieved by concentration of the culture supernatant (after 2 days of growth at 22°C) by Amicon filtration and subsequent FPLC fractionation on a preparative Mono Q Column which yielded six distinct peaks (Fig. 3b). All of the protease activity was confined to peak 5. SDS-PAGE analysis of peak 5 indicated the presence of a single protein with a molecular weight of 36,000 (Fig. 3c).

Characterization of the protease. The protease was active at temperatures ranging from 0 to 45°C and exhibited optimum activity at 37°C (see below). The activity at 0°C was about 12 to 15% maximum activity. The enzyme exhibited activity over a broad range of pH values (pH 1 to 7). When hemoglobin and BSA were used as the substrates, the enzyme exhibited maximum activity at pH 1 to 2.2, and at all other pHs, the activity was lower, with no activity at pH 7.2. However, in the presence of casein as the substrate, the protease exhibited maximum activity at pH 1.0 and a second peak of activity between pH 5 and 7. These activities were independent of buffer ion effects as confirmed by the use of various overlapping buffer systems in the pH range of 0.7 to 7.6. The sensitivity of the protease to various inhibitors is shown in Table 1.
Substrate specificity. To ascertain the substrate specificity of the protease, its ability to hydrolyze a number of synthetic and native substrates was assessed. The substrates were obtained from Sigma and from Boehringer Mannheim (Mannheim, Germany). The protease hydrolyzed poly-L-Ala, poly-L-Ser, poly-L-Pro, and poly-L-2Glu and released some free amino acids after an extended period of hydrolysis, but it did not hydrolyze poly-L-Lys, poly-L-His, poly-L-Pro, poly-L-Tyr, and poly-L-Trp. The protease was also inactive on poly (Lys, Ala; 3:1), poly (Lys, Ser; 3:1), poly (Lys, Phe; 1:1), poly (Lys-Tyr) (4:1), poly (Lys-Trp) (4:1), poly (Arg-Ser) (3:1), poly (Arg-Tyr) (4:1), chromozyme x (N-methoxy carbonyl-nle-gly-ar-4-nitranilide acetate), chromozyme PL (tosyl-gly-pro-lys-4-nitranilide-acetate), carboxybenzoyl-L-phenylalananyl-L-leucyl-L-α-glutamyl-4-nitranilide, succinyl-L-phenylalanline-p-nitraniilide, α-N-benzoyl-L-arginine ethyl ester, α-N-benzoyl-DL-arginine-p-nitranilide, and p-tosyl-L-arginine methyl ester. However, it appeared to be more active on native proteins such as BSA, casein, gelatin, and melitin and generated peptides which could be detected on a gel (Fig. 3a shows breakdown peptides of BSA) or by high-pressure liquid chromatography (as detected for melitin [unpublished results]). All of these results indicate that the secretory protease is an endopeptidase.

DISCUSSION

Production of the protease. The secretion of the protease was proportional to the number of cells in the media (Fig. 2a), and the secretion of the protease per cell of C. humicola in the log phase at 4°C was higher than that at 22°C, the optimum temperature for the growth of the organism (Fig. 2b). Similar results were recently reported for an extracellular lipase from a psychrotrophic bacterium, Moraxella sp. (7). Possibly, the secretion of more protease at lower temperatures holds the key to the maximum utilization of protein substrates of the soil in the Antarctic environment.

The protease was secreted in substantial amounts only when an exogenous protein was available as the sole nitrogen source (Fig. 1). In the presence of an ammonium salt or during nitrogen starvation, the protease was produced at a minimum basal level. Although the presence of a protein in the medium induced the production of the protease, the presence of amino acids reduced its production. SDS-PAGE analysis confirmed this observation. Similar observations have been reported for Candida albicans (16, 17).

The addition of intact, dead bacterial and yeast cells did not induce the secretion of protease into the medium, whereas the addition of lysed bacteria obtained after boiling the culture for 10 min did, indicating that proteins released from the lysed bacteria were responsible for the induction of the protease. Pepstatin (25 μM) inhibited the growth of C. humicola in YNB medium containing BSA and glucose; there was also no protease activity in this medium. The addition of other nitrogen sources, such as Casamino Acids or ammonium sulfate, to this medium led to the resumption of normal growth (OD_{max} 1.2 to 1.5), but no protease activity was observed in the medium. The observation described above, besides demonstrating the susceptibility of the protease to pepstatin, also showed that the basal level of protease which was observed normally in the culture medium was not essential for growth of C. humicola.

Secretion of protease into YCB medium supplemented with various protein substrates (e.g., casein, BSA, and gelatin) indicated that the protease activity in the superna-
tant varied in the order casein > BSA > gelatin. It was also observed that casein was a better substrate than BSA for the protease; the reason for this preference is not clear.

Characterization of the protease. The apparent molecular mass of the protease by SDS-PAGE analysis was 36,000 Da (Fig. 3c), and in substrate-impregnated native gels, a distinct single activity band was detected (data not shown). However, in a substrate-impregnated SDS-polyacrylamide gel, no activity band was observed even after the removal of SDS; SDS probably irreversibly inactivated the protease. The protease was an acidic protease and was inhibited by pepstatin, as expected of an acidic protease. It was also inhibited by SDS (Table 1). The protease did not require any metal ions for its activity and hence was not inhibited by EDTA. It was also not inhibited by phenylmethylsulfonyl fluoride (PMSF) and soybean trypsin inhibitor but was partially inhibited by N-acetyl-L-lysine chloromethylketone (TLCK). Earlier studies have indicated that TLCK, an alkylating agent, reacts with serine, histidine, or cysteine residues at the active site of proteases and inhibits their activity (4). Since iodoacetamide also inhibited the protease, it is possible that some sulfhydryl groups are probably involved in the activity of this protease. It was shown earlier that the secretory acidic protease from Paecilomyces variotii is inhibited by thiol reagents (18). The protease was quite stable at the higher pH values at which it is inactive; for example, preincubation at pH 8 or 9 overnight at 4°C did not reduce the activity.

The protease exhibited optimum activity at 37°C. Considering the cold Antarctic temperature, it is unusual that this Antarctic psychrophilic yeast still retains this extracellular enzyme with a high temperature optimum. Earlier studies have indicated that psychrophilic bacteria that contaminate milk and frozen food produce thermostable enzymes (1, 24). Therefore, this Antarctic yeast, C. humicol a, might have evolved only recently from such psychrophilic organisms. The protease was stable to several freeze-thawing cycles, a phenomenon to be expected in the natural environment of Antarctica.

Acid proteases isolated from various fungal species are known to have very little side chain specificity, although a certain preference for hydrophobic residues could be demonstrated (16, 25). The protease from C. humicol a also showed a low side chain specificity as tested for melittin (data not shown). However, this protease preferentially cleaves some of the polyamino acids (see Results).

Properties of secretory proteases from yeasts and psychrophilic bacteria. As yet, no other extracellular protease to our knowledge has been characterized from psychrophilic or psychophilic yeasts but other extracellular proteases have been characterized from mesophilic C. albicans (16, 17), Candida lipolytica (26), Candida olea (13), Saccharomyces lipolytica (28), and Rhodotorula glutinis (8). All of these yeasts (except C. lipolytica which produced an alkaline serine protease) secrete acidic proteases ranging in molecular mass from 30 to 45 kDa and with a pH optimum varying from 2.5 to 3.9. The acid protease of C. humicol a has a similar molecular weight, well within the range of the molecular weights of these secretory acid proteases. However, its pH optimum (pH 1.0) seems to be much lower than those of the other yeasts. In contrast to the protease of the pathogenic yeast C. albicans, the protease of C. humicol a is more sensitive to SDS (0.1%) but more tolerant to heat (retains 40% of its activity after 4 h at 45°C compared to the total loss of activity observed within 10 min in the case of C. albicans). In comparison with the acid protease of C. olea, which showed 5 to 10% of its activity at 0°C, the protease of C. humicol a is marginally more active (12 to 15%) at 0°C. The protease of C. humicol a is also freeze-thaw resistant. Although this is probably the first report on an acidic secretory protease from a psychrophilic yeast, attempts have been made earlier to characterize such enzymes from psychrophilic bacteria. Secretory proteases have been detected thus far in psychrophilic Pseudomonas fluorescens, Xanthomonas maltophilia, Escherichia freundii, and Lactococcus lacti, but the enzymes were found to be either metalloproteases or alkaline serine proteases (1, 11, 12, 24). However, it is interesting to note that production of the proteases from these psychrophilic bacteria is increased at lower temperatures and the proteases exhibit significant activity at 0°C (16 to 33%), thus demonstrating a great degree of similarity with the protease of the psychrophilic yeast C. humicol a.

REFERENCES


