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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*, *Saccharomycopsis*, *Rhodotorula*, and *Candida*, and most sporobolomycetes 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 shaker (New Brunswick Scientific, Edison, N.J.) and at 30°C in a gyratory 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-

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TABLE 1. Effect of inhibitors on the activity of the acid protease secreted by *C. humicola*

Inhibitor	Concn	Protease activity (% of control)[a]
PMSF	1 mM	98
	10 mM	82
EDTA	1 mM	96
	10 mM	93
Pepstatin	0.001 mM	34.5
	0.025 mM	0
TLCK	1 mM	71
	5 mM	52
Iodoacetamide	5 mM	73
	10 mM	6
SDS	0.05% (wt/vol)	0
Soybean trypsin inhibitor	5 μg/ml	100

[a] 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

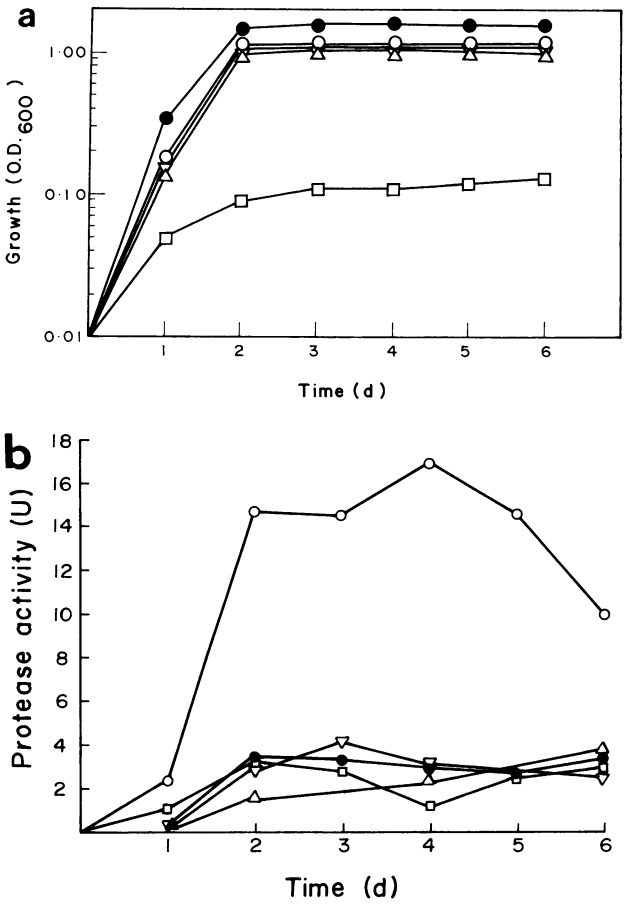

FIG. 1. Growth (a) and production (b) of extracellular protease by *C. humicola* grown in different media at 22°C. ○, YNB without amino acids or ammonium sulfate (YNB w/o) but containing glucose and BSA; ●, YNB w/o plus glucose, BSA, and Casamino Acids; □, YNB w/o plus BSA; △, YNB w/o plus glucose and ammonium sulfate (0.5% [wt/vol]); ▽, YNB w/o plus glucose, ammonium sulfate, and BSA. The final concentrations of YNB, BSA, and glucose in the media were 0.67, 0.01, and 0.1% (wt/vol), respectively.

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 $(NH_4)_2SO_4$ 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

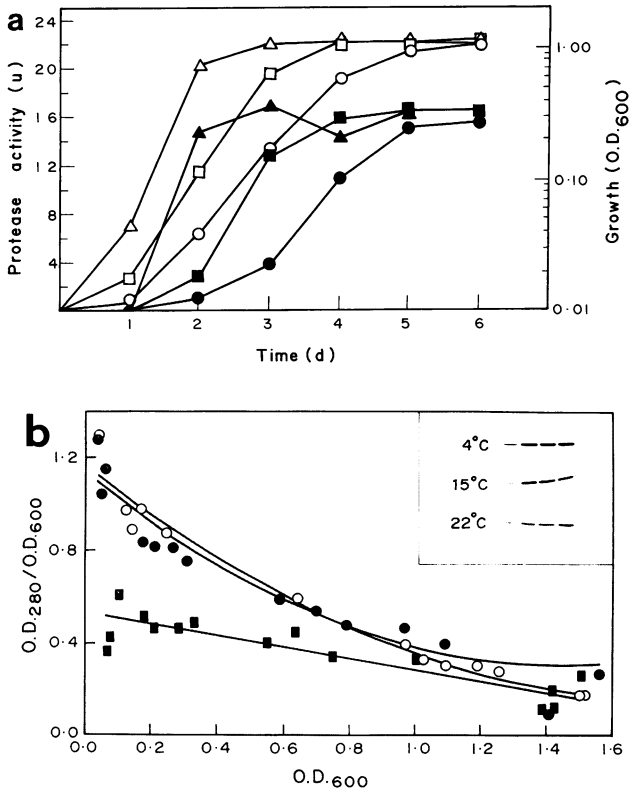

FIG. 2. Production of extracellular protease by *C. humicola* grown at various temperatures. (a) Growth at 4°C (○), 15°C (□), and 22°C (room temperature) (△). Protease activity at 4°C (●), 15°C (■), and 22°C (▲). The media contained YNB (0.67% [wt/vol]), BSA (0.01% [wt/vol]), and glucose (0.1% [wt/vol]). (b) Secretion of the protease, i.e., the ratio of OD_{280} (protease activity) to OD_{600} (index of number of cells), at 4°C (●), 15°C (○), and 22°C (□). The inset shows the band corresponding to the protease after SDS-PAGE of the culture medium supernatant obtained from cultures grown for different periods of time at 4, 15, and 22°C. The gel was stained with silver nitrate as described previously (27).

protease activity was induced and, in addition, the growth of *C. humicola* was poor (maximum OD_{600} 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 0.1% [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 (OD_{280}/OD_{600}) was higher at the two lower temperatures (4 and 15°C) during the log phase 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 [^{35}S]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 [^{35}S]methionine counts in the medium were determined. The count of [^{35}S]methionine-labelled proteins secreted by cells at 4°C (75×10^4 cpm) was higher than that of cells maintained at room temperature (30×10^4 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 (OD_{600}, ~1.3 at the end of 72 h), and at pH 6, 7, and 8, the maximum growth obtained was lower (OD_{600}, 0.7, 0.5, and 0.5, respectively, after 72 h). At pH 3, growth was extremely slow and reached a maximum OD_{600} 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 1.2, 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.

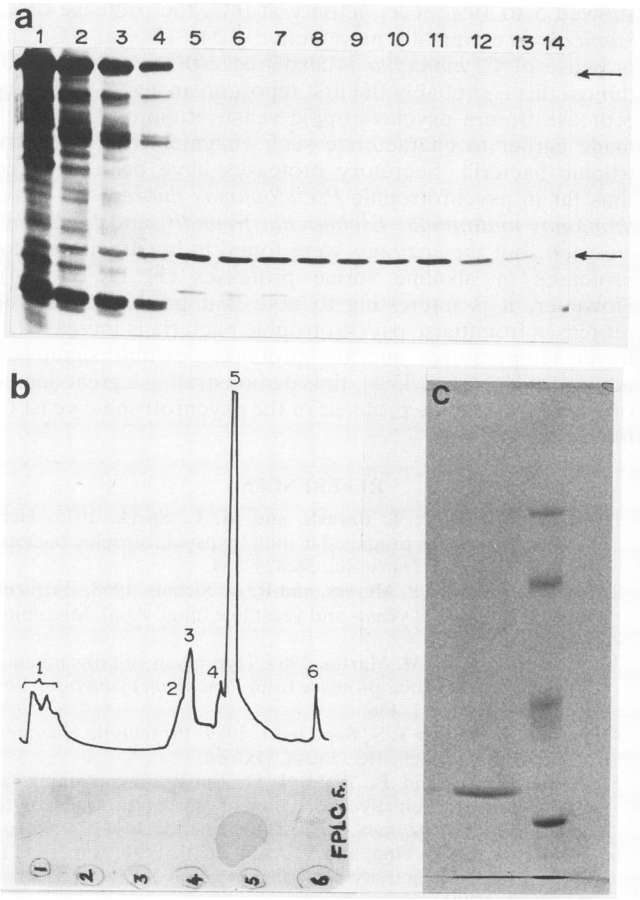

FIG. 3. (a) SDS-PAGE protein profile of the culture supernatant of *C. humicola* grown at 15°C. Lanes 1 through 14 indicate protein profiles of supernatants obtained at 9, 23, 26, 30, 47, 49, 51, 55, 58, 71, 78, 96, 120, and 140 h of growth. The corresponding protease activities in these supernatants were 3.4, 6.1, 8.4, 10.8, 19.3, 16.8, 16.2, 16.1, 16.1, 14.0, 12.6, 6.6, 4.8, and 0 U, respectively. The top arrow indicates a band corresponding to BSA (inducer), and the bottom arrow indicates the band corresponding to the protease. In early growth phase, BSA gives rise to various intermediate fragments of BSA (e.g., lanes 1 through 4). One hundred twenty microliters of culture supernatant was used per well. The gel was stained with silver nitrate (27). (b) FPLC profile of the culture supernatant resolved on a Mono Q column. The protease activity of fractions 1 to 6, shown below the FPLC profile, was assayed on a strip of X-ray film as described in Materials and Methods. (c) Lane 1, SDS-PAGE (7.5%) of the FPLC peak 5 (lane 1). Lane 2, molecular weight markers for (from top to bottom) phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000).

Stability of the protease. Thermal stability studies have indicated that the protease retained 100% of its activity when exposed for 2 h to 15, 22, or 37°C. However, when exposed to 45°C, it lost 25% of its activity within 2 h and was totally inactivated within 10 min at 56°C. Thus, the protease appeared to be heat labile at temperatures above 37°C. The protease was also found to be resistant to freeze-thaw. Even after 20 cycles of repeated freeze-thaw, the enzyme retained 100% of its activity.

The protease was stable at pHs ranging from 2 to 9 at 4°C, but on preincubation at pH 9 at room temperature overnight, it lost its activity. The protease was also active in the presence of 1.5 M NaCl.

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-Phe, and poly-L-Glu 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-arg-4-nitranilide acetate), chromozyme PL (tosyl-gly-pro-lys-4-nitranilide-acetate), carbobenzoxyl-L-phenylalanyl-L-leucyl-L-α-glutamyl-4-nitranilide, succinyl-L-phenylalanyline-*p*-nitranilide, 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 melittin 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 melittin [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_{600}, 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-α-*p*-tosyl-L-lysine chloromethyl ketone (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 varioti* 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 psychrotrophic yeast still retains this extracellular enzyme with a high temperature optimum. Earlier studies have indicated that psychrotrophs that contaminate milk and frozen food produce thermostable enzymes (1, 24). Therefore, this Antarctic yeast, *C. humicola*, might have evolved only recently from such psychrotrophic 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. humicola* also seems to have no 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 psychrotrophic bacteria. As yet, no other extracellular protease to our knowledge has been characterized from psychrotrophic or psychrophilic yeasts but other extracellular proteases have been characterized from mesophilic *C. albicans* (16, 17), *Candida lipolytica* (26), *Candida olea* (13), *Saccharomycopsis 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. humicola* has a similar molecular weight, well within the range of 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. humicola* 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. humicola* is marginally more active (12 to 15%) at 0°C. The protease of *C. humicola* is also freeze-thaw resistant. Although this is probably the first report on an acidic secretory protease from a psychrotrophic yeast, attempts have been made earlier to characterize such enzymes from psychrotrophic bacteria. Secretory proteases have been detected thus far in psychrotrophic *Pseudomonas fluorescens*, *Xanthomonas maltophila*, *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 psychrotrophic 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 psychrotrophic yeast *C. humicola*.

REFERENCES

1. **Adams, D. M., J. T. Barach, and M. L. Speck.** 1975. Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. J. Dairy Sci. **58:**828–834.
2. **Ahearn, D. G., S. P. Meyers, and R. A. Nichols.** 1968. Extracellular proteinases of yeasts and yeast like fungi. Appl. Microbiol. **16:**1370–1374.
3. **Ankel, H., and S. M. Martin.** 1964. Hydrolysis of polyaminoacids by an extracellular protease from *Pencillium cyaneo-fulvum*. Biochem. J. **91:**431–436.
4. **Beynon, R. J., and J. S. Bond (ed.).** 1989. Proteolytic enzymes: a practical approach. IRL Press, Oxford.
5. **Brown, T. L., and F. Wold.** 1982. Substrate-containing gel electrophoresis: sensitive detection of amylolytic, nucleolytic and proteolytic enzymes. Anal. Biochem. **122:**164–172.
6. **Cheung, A. L., P. Ying, and V. A. Fischetti.** 1991. A method to detect proteinase activity using unprocessed X-ray films. Anal. Biochem. **193:**20–23.
7. **Feller, G., M. Thiry, J. Arpigny, M. Mergeay, and C. Gerday.** 1990. Lipases from psychrotrophic Antarctic bacteria. FEMS Microbiol. Lett. **66:**239–244.
8. **Kamada, M., K. Ode, and S. Murao.** 1972. The purification of the extracellular acid protease of *Rhodotorula glutinis* K24 and its general properties. Agric. Biol. Chem. **36:**1095–1101.
9. **Kreger-Van Rij, N. J. W.** 1984. The yeasts: a taxonomic study. Elsevier/North Holland Publishing Co., Amsterdam.
10. **Laemmli, U. K., and M. Favre.** 1973. Phage T4 assembly. J. Mol. Biol. **80:**575–599.
11. **Margesin, R., and F. O. Schinner.** 1991. Characterization of a metalloprotease from psychrophilic *Xanthomonas maltophila*. FEMS Microbiol. Lett. **79:**257–262.
12. **Nakajima, M., K. Mizusawa, and F. Yoshida.** 1974. Purification and properties of an extracellular proteinase of psychrophilic *Escherichia freundii*. Eur. J. Biochem. **44:**87–96.
13. **Nelson, G., and T. W. Young.** 1987. Extracellular acid and alkaline proteases from *Candida olea*. J. Gen. Microbiol. **133:**1461–1469.
14. **Ramakrishna, T., and M. W. Pandit.** 1988. Self-association of a-chymotrypsin: effect of amino acids. J. Biosci. **13:**215–222.
15. **Ray, M. K., S. Shivaji, N. Shyamala Rao, and P. M. Bhargava.** 1989. Yeast strains from the Schirmacher Oasis, Antarctica. Polar Biol. **9:**305–309.
16. **Renold, H., H. Fasold, and F. Staib.** 1968. Purification and characterization of a proteolytic activity from *Candida albicans*. Biochim. Biophys. Acta **167:**399–406.
17. **Ross, I. K., F. Bernardis De, G. W. Emerson, A. Cassone, and P. A. Sullivan.** 1990. The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of a proteinase deficient mutant. J. Gen. Microbiol. **136:**687–694.
18. **Sawada, J.** 1964. Studies on the acid-protease of *Paecilomyces varioti* Bainier TPR-220. III. Some enzymic properties of the crystalline acid-protease. Agric. Biol. Chem. **28:**348–356.
19. **Shivaji, S., M. K. Ray, G. Seshu Kumar, G. S. N. Reddy, L.**

Saisree, and D. D. Wynn Williams. 1991. Identification of *Janthinobacterium lividum* from the soils of the islands of Scotia Ridge and from Antarctic Peninsula. Polar Biol. **11:**267–271.

20. **Shivaji, S., M. K. Ray, N. Shyamala Rao, L. Saisree, M. V. Jagannadham, G. Seshu Kumar, G. S. N. Reddy, and P. M. Bhargava.** 1992. *Sphingobacterium antarcticus* sp. nov., a psychrotrophic bacterium from the soils of Schirmacher Oasis, Antarctica. Int. J. Syst. Bacteriol. **42:**102–106.
21. **Shivaji, S., N. Shyamala Rao, L. Saisree, G. S. N. Reddy, G. Seshu Kumar, and P. M. Bhargava.** 1989. Isolates of Arthrobacter from the soils of Schirmacher Oasis, Antarctica. Polar Biol. **10:**225–229.
22. **Shivaji, S., N. Shyamala Rao, L. Saisree, V. Sheth, G. S. N. Reddy, and P. M. Bhargava.** 1988. Isolation and identification of *Micrococcus roseus* and *Planococcus* sp. from Schirmacher Oasis, Antarctica. J. Biosci. **13:**409–414.
23. **Shivaji, S., N. Shyamala Rao, L. Saisree, V. Sheth, G. S. N. Reddy, and P. M. Bhargava.** 1989. Isolation and identification of *Pseudomonas* sp. from Schirmacher Oasis, Antarctica. Appl. Environ. Microbiol. **55:**767–770.
24. **Stepanaik, L., P. F. Fox, and C. Daly.** 1982. Isolation and general characterization of a heat-stable proteinase from *Pseudomonas fluorescens* AFT36. Biochim. Biophys. Acta **717:**376–383.
25. **Tanaka, N., M. Takeuchi, and E. Ichishima.** 1977. Purification of an acid proteinase form *Aspergillus saitoi* and determination of peptide bond specificity. Biochim. Biophys. Acta **485:**406–416.
26. **Tobe, S., T. Takami, S. Ikeda, and K. Mitsuzi.** 1976. Production and some enzymatic properties of alkaline proteinase of *Candida lipolytica*. Agric. Biol. Chem. **40:**1087–1092.
27. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118:**197–203.
28. **Yamada, T., and O. M. Ogrydziak.** 1983. Extracellular acid proteases produced by *Saccharomycopsis lipolytica*. J. Bacteriol. **154:**23–31.