

Extracellular Acid and Alkaline Proteases from *Candida olea*

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(Received 16 December 1986; revised 13 February 1987)

Candida olea 148 secreted a single acid protease when cultured at acidic pH. In unbuffered medium, the culture pH eventually became alkaline and a single alkaline protease was produced. This was the only proteolytic enzyme produced when the organism was grown in buffered medium at alkaline pH. Both proteolytic enzymes were purified to homogeneity (as assessed by SDS-PAGE). The M_r of the acid protease was 30900, the isoelectric point 4.5; optimum activity against haemoglobin was at 42 °C and pH 3.3. This enzyme was inactivated at temperatures above 46 °C and was inhibited by pepstatin and diazoacetyl-norleucine methyl ester but was insensitive to inhibition by either 1,2-epoxy-3-(*p*-nitrophenoxy)-propane or compounds known to inhibit serine, thiol or metallo proteases. The acid protease contained 11% carbohydrate. The alkaline protease had an M_r of 23400 and an isoelectric point of 5.4. The activity of this enzyme using azocoll as substrate was optimal at 40 °C in the range pH 8.0-9.0. This enzyme was inactivated at temperatures above 42 °C and was inhibited by phenylmethylsulphonyl fluoride and irreversibly inactivated by EDTA. The enzyme was also partially inhibited by DTT but was insensitive to either pepstatin or *p*-chloromercuribenzoic acid.

INTRODUCTION

Several yeasts have been shown to secrete proteolytic enzymes (Ekland *et al.*, 1965; Ahearn *et al.*, 1968; Foda & El-Din, 1979). Strains most actively secreting such enzymes were found in the genera *Candida*, *Endomyces*, *Rhodotorula* and *Kluyveromyces*. The acid protease secreted by *Candida albicans* has been particularly well studied because of its possible role in the pathogenicity of the organism. This enzyme is a single polypeptide with an M_r of 45000 (Remold *et al.*, 1968; Ruchel, 1981). Antibodies to this protease have been used as diagnostic agents for the detection of candidosis in man (MacDonald & Odds, 1980).

Strains of *Saccharomyces lipolytica* secrete a number of different extracellular proteases active at acidic, neutral and alkaline pH. When cultured at acidic pH, strain CX161-1B produced three carboxyl proteases (Yamada & Ogrydziak, 1983). Production of each protease varied during growth, suggesting separate regulatory pathways for their synthesis. When cultured at neutral pH, the same strain produced virtually no carboxyl proteases but high levels of an alkaline serine protease were found (Ogrydziak & Scharf, 1982). Another strain grown at neutral or alkaline pH secreted an enzyme active only at neutral pH (Abdelal *et al.*, 1977).

Other yeast extracellular proteases examined in detail include those secreted by *Rhodotorula glutinis* and *Candida lipolytica*. The former strain produced an acid protease inhibited by *Streptomyces maniuaraensis* pepsin inhibitor and by diazoacetyl-norleucine methyl ester (DAN) (Kamada *et al.*, 1972). *C. lipolytica* secreted a single alkaline serine protease (Tobe *et al.*, 1976).

An uncharacterized extracellular protease prepared from the yeast *Candida olea* 148 was shown to have potential use in the brewing industry as a replacement for the plant thiol protease

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Abbreviations: DAN, diazoacetyl-norleucine methyl ester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)-propane; PCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethylsulphonyl fluoride.

papain, which is used to prevent the formation of chill-haze in beer (Nelson & Young, 1986). This paper describes the production, purification and characterization of the extracellular proteolytic enzymes present in the beer chill-proofing preparation.

METHODS

Maintenance and culture of yeast. *Candida olea* 148 (from the yeast collection of the Department of Food Science and Technology, University of California, Davis, USA) was maintained by regular subculture on medium comprising (w/v): yeast extract, 0.3%; malt extract, 0.3%; bacteriological peptone, 0.5%; glucose, 2.0%; and agar, 2.0%. Starter cultures were grown on medium without agar.

Medium for protease production. Starter cultures (10 ml) grown at 29 °C were inoculated into 1 litre of medium – 1% (w/v) glucose, 0.2% BSA and 0.17% Difco yeast-nitrogen-base without amino acids and ammonium sulphate – in a 2 litre Erlenmeyer flask. BSA and yeast-nitrogen-base were sterilized by filtration through 0.22 µm membranes and aseptically added to an aqueous glucose solution previously sterilized by autoclaving at 15 lbf in⁻² (103.5 kPa) for 15 min. For experiments requiring controlled values of pH in the range 2–8, citrate-phosphate buffers were used (prepared from 0.1 M-citric acid and 0.2 M disodium hydrogen phosphate, by mixing appropriate volumes to give 100 ml buffer in which the medium constituents were dissolved). For cultures at pH 9, Tris/HCl buffer at a final concentration in the medium of 0.1 M was used. The pH values of these cultures remained constant throughout fermentation. Cultures were incubated at 29 °C on a rotary shaker at 120 r.p.m. and were harvested by centrifugation (5000 g, 10 min) at 4 °C. Sodium azide was added to the supernatant to a final concentration of 0.02% before proceeding with enzyme purification.

Assay of acid protease. Proteolytic activity was estimated by measuring the hydrolysis of a standard haemoglobin substrate by a method based on that of Remold *et al.* (1968). Enzyme solution (0.5 ml) was added to 2.0 ml of acid denatured haemoglobin (1.0%, w/v, in 0.05 M-sodium citrate buffer pH 3.2) and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 5 ml 5.0% (w/v) trichloroacetic acid and the precipitate was removed by centrifugation. A sample of the supernatant (2.0 ml) was neutralized by the addition of 1.0 ml 0.5 M-NaOH and 0.8 ml of this solution was assayed for the presence of soluble peptides by the Lowry method using tyrosine as a standard. One unit (U) of enzyme activity was defined as that amount which released 1 µmol tyrosine equivalents (ml enzyme solution)⁻¹ min⁻¹. During column chromatography, proteolytic activity was located by measuring the absorbance of the un-neutralized supernatant at 280 nm.

Assay of alkaline protease. Azocoll (Sigma) was used as substrate and the method was based on that of Moore (1969). Enzyme solution (0.5 ml) was mixed with 2.5 ml 0.6% azocoll in 0.2 M Tris buffer pH 7.5. The mixture was incubated with shaking at 37 °C for 30 min and the reaction stopped by removing the insoluble azocoll by centrifugation. The absorbance of the supernatant was measured at 520 nm against a reagent blank prepared by removing the azocoll at time 0. The enzyme activity was expressed as change in A_{520} (ml of enzyme solution)⁻¹ min⁻¹.

Assay of protein. The Lowry method was used with BSA as the standard. Elution of proteinaceous material during column chromatography was monitored continuously at 280 nm using an Altex 150 UV monitor equipped with a 0.5 mm pathlength flow cell.

Assay of carbohydrate. Carbohydrate concentration was measured by the phenol/sulphuric acid method (Ashwell, 1966) with glucose as the standard.

Purification of proteolytic enzymes. Purification of the acid and alkaline proteases was based on the methods of Yamada & Ogrzydziak (1983) and Ogrzydziak & Scharf (1982) respectively. All enzyme purification steps were conducted at 4 °C. Cell free supernatants were concentrated through a Diaflo PM10 membrane (Amicon).

Acid protease. (a) *Gel permeation chromatography.* A 5 ml portion of concentrate was chromatographed on a 95 × 2.6 cm column of Sephadex G-75 equilibrated in 0.01 M-sodium citrate buffer pH 3.2. Fractions containing proteolytic activity were pooled.

(b) *Ion-exchange chromatography.* The pooled fractions containing proteolytic activity from gel-permeation chromatography were dialysed for 16 h against 0.01 M-sodium citrate buffer pH 6.0 and applied to 25 × 2.6 cm column of Whatman DE 52 ion-exchange cellulose equilibrated with the same buffer. The column was eluted with a 400 ml linear gradient from 0 to 1.0 M-NaCl in 0.01 M-sodium citrate buffer pH 6.0. Fractions containing proteolytic activity were pooled, dialysed for 16 h against distilled water and freeze-dried.

Alkaline protease (a) *Gel-permeation chromatography.* The same procedure was used as for the acid protease except that the column was equilibrated and eluted with 0.03 M-sodium phosphate buffer pH 6.8.

(b) *Ion-exchange chromatography.* The same procedure was used as for the acid protease except that the column was equilibrated with 0.01 M-Tris/HCl buffer pH 8.4 and eluted with a 400 ml linear gradient of 0–1.0 M-NaCl in the same buffer.

Polyacrylamide gel electrophoresis. Freeze-dried protease preparations were dissolved in sample buffer [0.0625 M-Tris/HCl, pH 6.8, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 6% (w/v) SDS] heated to 100 °C for 4 min, cooled to ambient temperature and clarified by centrifugation. Samples were subjected to electrophoresis

on a linear (5–20%, w/v) acrylamide gradient in 0.375 M-Tris/HCl, pH 8.8, according to the method described by Hames (1981). A 2.5% acrylamide stacking gel was used in 0.125 M Tris/HCl, pH 6.8. Gels were electrophoresed at 20 °C at 100 V during stacking and then at 180 V. Gel slabs were fixed in methanol/glacial acetic acid/water (43:7:50, by vol.) and stained with 0.2% Page Blue 83 (BDH). Destaining was achieved using absolute ethanol/glacial acetic acid/water (43:7:50 by vol.).

Isoelectric focussing. LKB Ampholine PAG plates, pH 3.0–9.5, were used. Gels were focussed on an LKB multiphor for 1.5 h. An LKB 2917 power source was used and focussing was conducted at 30 W, 1500 V and 50 mA. Samples were focussed alongside standards with known isoelectric points (pH 4.7–1.0, BDH). Gels were stained with Page Blue 83 or layered onto agar medium containing either precipitated casein – 0.375% casein, 3% (w/v) agar in 0.05 M-sodium citrate buffer pH 4.2 – or gelatine – 1% (w/v) gelatine, 3% agar in 0.3 M-Tris/HCl, pH 7.5 – to identify proteolytic activity.

pH and temperature characteristics. The pH optimum of each protease was determined using haemoglobin (acid protease) or azocoll (alkaline protease) as substrates. Haemoglobin (1%, w/v) was prepared in citrate-phosphate buffers of pH 2.2–8.0. For pH values above 8.0, Tris/HCl buffers were used. For values less than pH 2.0, the solution pH was adjusted with 3.0 M-HCl. Azocoll substrate was added to the same range of solutions for the assay of alkaline protease activity. Enzyme activity was expressed as a percentage of the maximum activity attained. Enzyme preparations were dialysed for 16 h at 4 °C against sodium phosphate buffer (0.1 M, pH 6.8) before assay. For determining the optimum temperature under the conditions of assay, assays were done at a range of temperatures from 0 to 60 °C.

Inhibition of protease activity. Stock inhibitor solutions were prepared, diluted with assay buffer and added to enzyme solution (1:1). Each inhibitor was added so that the final concentration would be either 10 mM or 1 mM, except for pepstatin A which was added at 100 µM, 10 µM, and 1 µM final concentration and *p*-chloromercuribenzoate (PCMB) where only 1 mM final concentration was used.

Stock solutions were prepared by dissolving phenylmethylsulphonyl fluoride (PMSF) in propan-2-ol, PCMB in dilute alkali (10 mM-NaOH), EDTA in water, pepstatin A in 50% (v/v) aqueous methanol, 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (EPNP) in methanol and dilute alkali (10 mM-NaOH), DAN in methanol and DTT in water. 4 mM- and 1 mM-cupric acetate was included in the 10 mM- and 1 mM-EPNP and DAN assays mixes respectively.

The enzyme/inhibitor mixes were incubated at 37 °C for 30 min and the enzyme activity was measured relative to an uninhibited control. Controls without inhibitor but containing solvents used to dissolve inhibitors were also included: enzyme activity was not affected. Activity was measured using either haemoglobin or azocoll as substrate as above. The EPNP-enzyme mixture required preincubation at 4 °C for 20 h before the 30 min incubation at 37 °C.

The action of various metal ions on protease activity was also recorded. Divalent cations (1 mM final concentration) were mixed with the enzyme, which was assayed as above.

In all studies of inhibition and effect of metal ions on enzyme activity, replicate experiments agreed within ± 3% of enzyme activity.

RESULTS AND DISCUSSION

Protease production

Growth and protease production by *C. olea* 148 were monitored for 14 d in culture in unbuffered medium containing BSA as the sole nitrogen source and glucose as carbon source (Fig. 1). The initial pH of the medium was close to pH 4.6 and after inoculation of *C. olea*, acid protease activity was detected throughout the growth phase as found previously in some *S. lipolytica* strains (Tobe *et al.*, 1976; Abdelal *et al.*, 1977; Ogrydziak *et al.*, 1977). Activity increased throughout the growth phase and early stationary phase to a maximum of 115 U (tyrosine) at 5 d incubation. At this point there was a dramatic reduction in acid protease activity such that only 7.8 U (tyrosine) of activity remained at day 7. No alkaline protease activity was detected during the growth and early stationary phases. However, on day 6 some alkaline protease activity was detected and this increased to a maximum of 3.5 U (A_{520}) after 9 d incubation (Fig. 1). Cell lysis was not detected but the sharp reduction in acid protease activity and the onset of alkaline protease activity was associated with a dramatic increase in pH from around pH 3.0 after 3 d incubation to pH 7.0 after 5 d (Fig. 1). The secretion of alkaline protease was directly related to the increasing pH of the medium. This was confirmed when *C. olea* 148 was grown in the same liquid medium, but where the pH was regulated between pH 2.0 and pH 9.0 (Fig. 2). In cultures above pH 8.0 no acid protease activity was detected and at pH

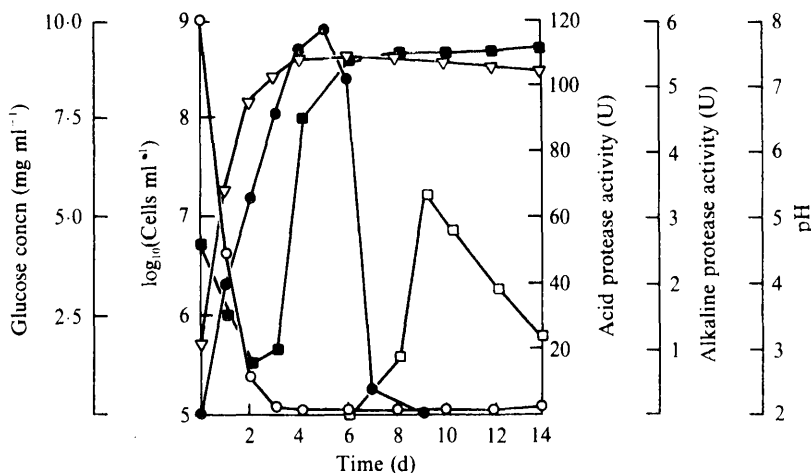


Fig. 1. Production of extracellular protease by *C. olea* 148 grown in unbuffered 1 litre shaken culture at 29 °C. ○, Glucose, mg ml⁻¹; ▽, log₁₀ (cell number ml⁻¹); ●, acid protease activity (μmol tyrosine equivalents released ml⁻¹ min⁻¹ from haemoglobin at pH 3.2); □, alkaline protease activity (change in A_{520} ml⁻¹ min⁻¹ on hydrolysis of azocoll at pH 7.5); ■, pH of culture.

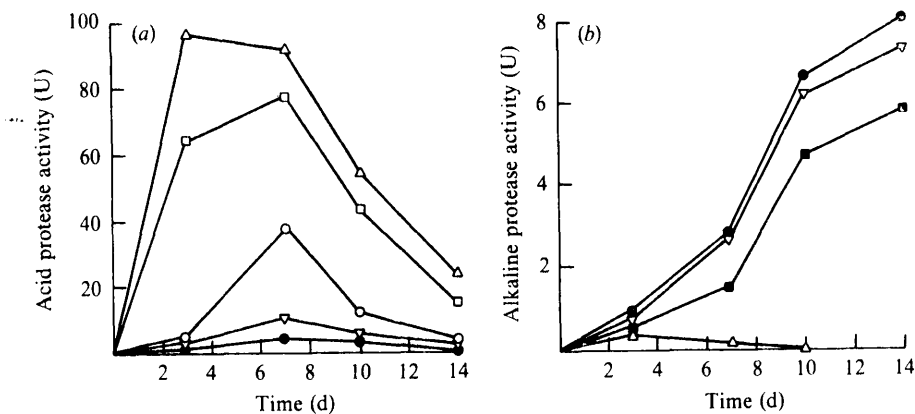


Fig. 2. Production of acid and alkaline extracellular proteases by *C. olea* 148 grown in buffered 1 litre shaken culture at 29 °C. (a) Acid protease activity (μmol tyrosine equivalents released ml⁻¹ min⁻¹ from haemoglobin at pH 3.2). (b) Alkaline protease activity (change in A_{520} ml⁻¹ min⁻¹ on hydrolysis of azocoll at pH 7.5). Culture buffered with citrate-phosphate pH 2 (○), pH 4 (□), pH 6 (△), pH 7 (▽), pH 8 (●). Culture buffered with 0.1 M-Tris/HCl pH 9 (■). No acid protease activity was detected at pH values > 8 and no alkaline protease activity was detected at pH < 6.

less than 6.0 no alkaline protease activity was detected. When pH was controlled, alkaline protease secretion in alkaline culture began as with acid culture, during the growth phase and increasing into early stationary phase. However, the alkaline protease secretion continued to increase well into stationary phase and above pH 7.0 reached a maximum of about 9 U (A_{520}) after 14 d. In the initial culture, with no pH control, the concentration of glucose was monitored and was found to be only 50% depleted after 14 h at which point the acid protease activity was 40 U (tyrosine). Therefore carbon depletion was not necessary for initiation of acid protease production (see Fig. 1).

Purification of the proteases

The protease activity produced by *C. olea* 148 was isolated from the culture supernatant obtained from 7 d growth in unbuffered medium and concentrated by ultrafiltration (Table 1).

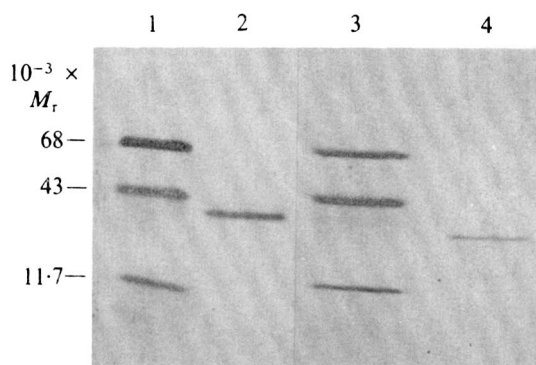


Fig. 3. SDS-PAGE of purified acid and alkaline proteases from *C. olea* 148. Lanes 1 and 3, BSA (68000), ovalbumin (43000), cytochrome *c* (11700) (5 μ g each); lane 2, acid protease preparation (10 μ g); lane 4, alkaline protease preparation (20 μ g).

Table 1. Purification of acid and alkaline extracellular proteases secreted by *C. olea* 148

	Volume (ml)	Total activity† (U)	Total protein* (mg)	Specific activity (U mg ⁻¹)	Recovery (%)
Acid protease					
Supernatant	1500	14850	600	25	100
Ultrafiltrate	5	14198	278	51	95.6
Sephadex G-75	114	13178	15.4	856	92.8
DEAE-cellulose	63	8962	2.4	3703	60.4
Alkaline protease					
Supernatant	1000	48	573	0.084	100
Ultrafiltrate	5	45.9	236	0.195	95.6
Sephadex G-75	66	38.4	14.5	2.64	80.0
DEAE-cellulose	38	25.1	4.4	5.64	52.3

* Protein concentration calculated using the Lowry method.

† Acid protease activity, haemoglobin hydrolysis at pH 3.2; 1 U = 1 μ mol tyrosine released ml⁻¹ min⁻¹. Alkaline protease activity, azocoll hydrolysis at pH 7.5; 1 U = change in A_{520} ml⁻¹ min⁻¹.

This material contains less than the maximum concentrations of acid and alkaline protease activity that could be achieved in culture at controlled pH (Fig. 2). However, it was the material containing both enzymes which was used to chill-proof beer (Nelson & Young, 1986) and which was selected for purification studies. Acid protease alone may be produced by culture at pH 4.6 and alkaline protease by culture at pH 8 (Fig. 2) and may be purified by the same procedures described here.

Gel filtration chromatography on a Sephadex G-75 column gave a single peak of acid protease activity eluting at 220–234 ml. The protease activity was determined using haemoglobin hydrolysis at pH 3.2. The fractions containing proteolytic activity were pooled and applied to a DEAE-cellulose column and a single peak of acid protease activity was eluted at 170–240 ml after applying a 400 ml linear gradient of 0–1.0 M-NaCl in 0.01 M-sodium citrate pH 6. A 148-fold purification was achieved and 60% of the total activity was recovered (Table 1). The active fractions were pooled, lyophilized and resolved on an SDS-polyacrylamide gel and found to be homogeneous (Fig. 3), the principal component had an M_r of 29500. This purified preparation was tested for alkaline protease activity using urea denatured haemoglobin (Laskowski, 1955) and azocoll. No activity was found.

Cell free supernatants from 7 d cultures (uncontrolled pH), however, did contain alkaline protease activity when assayed using azocoll as substrate. Such supernatants were concentrated and chromatographed on Sephadex-G75, alkaline protease activity eluted as a single component at 260–326 ml and the active fractions were pooled and subjected to ion exchange

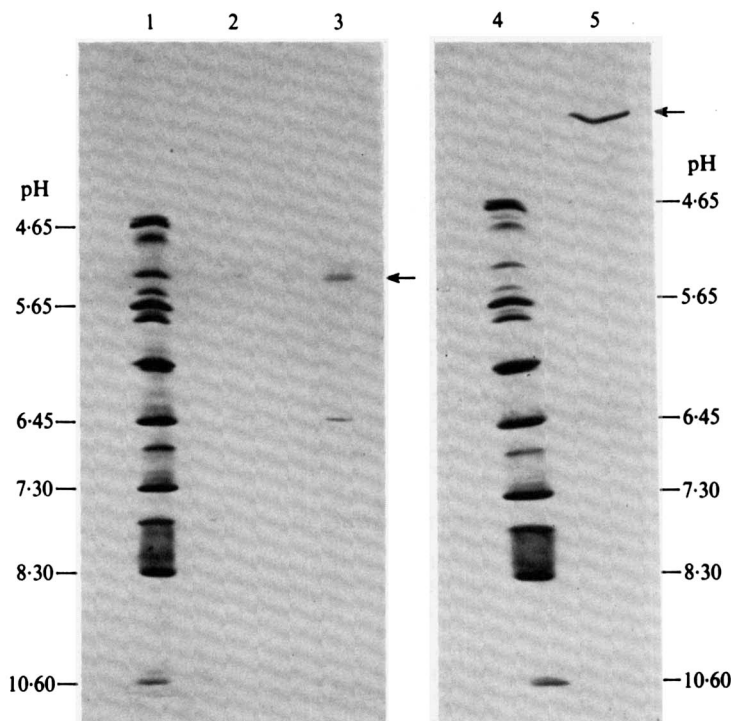


Fig. 4. Isoelectric focussing (pH 3.5–9.0) of extracellular acid and alkaline protease. Lanes 1 and 4, isoelectric point markers pI 4.7–10.6; lanes 2 and 3, alkaline protease (5 and 10 μ g respectively); lane 5, acid protease (10 μ g). Arrows identify components exhibiting proteolytic activity on medium containing gelatin. The pI values of the markers are shown.

chromatography. A single peak of activity was detected 200–240 ml after applying a 400 ml linear gradient of 0–0.15 M-NaCl in 0.01 M-Tris/HCl pH 8.4. A 67-fold purification was achieved and 52% of the total activity was recovered (Table 1). Analysis of this material by SDS-PAGE revealed a single component (Fig. 3) of M_r 24000.

Estimation of M_r by gel permeation chromatography

Native acid and alkaline proteases were chromatographed on Sephadex-G75 and their elution volumes compared with those of proteins of known M_r (BSA, 68000; ovalbumin, 43000; myoglobin, 17200; and cytochrome *c*, 11700). Four independent analyses were made for each enzyme and the estimated M_r values (mean \pm SD) for the acid and alkaline proteases were 30900 ± 380 and 23400 ± 680 . These values agree well with those estimated by SDS-PAGE (29500 and 24000 respectively) and indicate that both enzymes are monomers in the native state.

Carbohydrate content

Samples of both the acid and alkaline proteases when resolved on SDS-polyacrylamide gels were stained pink using perchloric acid and Schiff's reagent according to the method of Zaccharius *et al.* (1969) and are therefore judged to be glycoproteins. The carbohydrate content of pure samples of the alkaline and the acid protease, calculated using the phenol/sulphuric acid method described by Ashwell (1966), was 11% and 17% carbohydrate respectively.

Isoelectric point

The isoelectric points of the proteases were determined using crude enzyme samples and the proteolytic activity of the resolved protease bands was confirmed by laying the gels on casein or gelatin plates. The isoelectric point of the acid protease was < pH 4.65 and that of the alkaline protease was pH 5.4 (Fig. 4).

Table 2. Effect of inhibitors on the activity of the acid and alkaline proteases secreted by *C. olea* 148

Inhibitor	Concn* (mM)	Activity remaining (%)	
		Acid protease	Alkaline protease
Control†	—	100	100
PMSF	1	96	0
	10	96	0
PCMB	1	106	97
EDTA	1	117	3
	10	107	0
EDTA‡	1	—	96
	10	—	74
EDTA	1 (+1 mM-Ca ²⁺)	—	101
	10 (+1 mM-Ca ²⁺)	—	75
DAN	1 (+1 mM-Cu ²⁺)	91	—
	10 (+1 mM-Cu ²⁺)	58	—
EPNP	1 (+1 mM-Cu ²⁺)	104	—
	10 (+1 mM-Cu ²⁺)	107	—
Pepstatin	0.001	70	—
	0.01	56	—
	0.1	22	103
DTT	1	106	83
	10	102	34

* Concentration of inhibitor in enzyme/inhibitor preincubation mixture.

† The acid protease activity of the control was 25 μmol tyrosine released $\text{ml}^{-1} \text{min}^{-1}$ measured by haemoglobin hydrolysis at pH 3.2. The alkaline protease activity of the control (change in $A_{520} \text{ml}^{-1} \text{min}^{-1}$) was 1.12 using azocoll at pH 7.5.

‡ Measured after dialysis against buffer not containing EDTA.

pH and temperature characteristics

The pH stability was determined using haemoglobin hydrolysis at pH 3.2, for acid protease, and azocoll hydrolysis at pH 7.5, for alkaline protease, after preincubation for between 0 and 60 min at defined pH. The acid protease was unstable at pH 8.0 and pH 9.0, losing over 70% of activity after 10 min at pH 8.0 and 100% after 10 min at pH 9.0. The alkaline protease was unstable below pH 5.0. At pH 3.0 almost 90% of the activity was lost after 20 min incubation.

The temperature stability was analysed at pH 3.2 and pH 7.5 at temperatures between 30 and 60 °C. The acid protease lost a small degree of activity after 60 min at 30 °C but a sharp drop in activity was noted above 46 °C. At 60 °C almost 80% of activity was lost after 10 min while at 20 min no activity remained. Thus pasteurization of any preparation containing this enzyme would result in substantial inactivation. A similar pattern was obtained for the alkaline protease. It was unstable above 42 °C; 90% of the activity was lost after 10 min at 60 °C, and complete inactivation occurred after 20 min at 60 °C.

The acid protease was optimally active against acid denatured haemoglobin at pH 3.3 and 42 °C. The alkaline protease showed optimal activity against azocoll at a pH value between 8.0 and 9.0 and at 40 °C. The acid protease retained between 5 and 10% of its maximum activity at 0 °C. This may in part explain its usefulness as an enzyme for chill-proofing beer, a process usually conducted at a temperature close to 0 °C.

Inhibition of activity

The acid protease was inhibited by pepstatin and partially inhibited by DAN but not by EPNP or compounds known to inhibit serine, thiol or metallo proteases or the reducing agent DTT (Table 2). Therefore the enzyme is a carboxyl protease similar to pepsin, although the latter is inhibited completely by pepstatin (Umezawa, 1976), DAN (Rajagopalan *et al.*, 1966) and EPNP (Tang, 1971). The enzyme was unaffected by divalent cations. These observations

Table 3. *Effect of 1 mM divalent cations on the activity of the alkaline protease secreted by C. olea 148*

Divalent cation	Protease activity remaining (%)
Control*	100
Ca ²⁺	129
Mg ²⁺	103
Mn ²⁺	84
Hg ²⁺	2
Cu ²⁺	3
Ba ²⁺	58

* The activity of the control sample was 1.33 units measured by azocoll hydrolysis (pH 7.5).

suggest that the nature of the active site of the *C. olea* enzyme is similar to that of an enzyme from *Rhodotorula glutinis* (Liu *et al.*, 1973; Kamada *et al.*, 1972).

The alkaline protease was completely inhibited by the serine protease inhibitor PMSF and reversibly inhibited by EDTA (Table 2). It was largely unaffected by compounds known to inhibit thiol and carboxyl proteases. If after incubation of the protease with EDTA, the incubation mixture was dialysed overnight at 4 °C against assay buffer and assayed with azocoll without EDTA, little inhibition was found. Partial inhibition was found on incubation with DTT, which suggests that the protease is a serine protease with a disulphide linkage essential for activity. When assayed in the presence of 1 mM divalent cations (Table 3) inhibition was found with Hg²⁺ and Cu²⁺ ions, partial inhibition with Mn²⁺ and Ba²⁺, no effect with Mg²⁺ and some partial activation with Ca²⁺. As expected, excess calcium prevented inhibition of activity by EDTA. These inhibition characteristics are similar to those of *S. lipolytica* alkaline serine protease (Ogrydziak & Scharf, 1982). Indeed, *C. olea* 148 may well be a strain of *S. lipolytica* (Kreger-van Rij, 1984). In contrast, however, the *C. olea* 148 alkaline protease is sensitive to DTT, and the sensitivity to EDTA and the reactivation with Ca²⁺ suggest that metal ions have some role in maintaining the active conformation of the enzyme. The failure to demonstrate the ability of Ca²⁺ to stabilize the enzyme against changes in pH and temperature suggests that the role of Ca²⁺ is not in maintaining enzyme structure.

This work would not have been possible without the financial support of the Science and Engineering Research Council. Thanks are also due to Allied Breweries Ltd, Burton-on-Trent. T. W. Y. thanks Professor M. J. Lewis, of UC, Davis for permission to screen the yeast culture collection.

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