Journal of Applied Microbiology 2001, **91**, 916–921

Characterization of a broad pH range protease of *Candida caseinolytica*

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815/5/01: received 25 April 2001 and accepted 16 May 2001

M. POZA, T. DE MIGUEL, C. SIEIRO AND T. G. VILLA. 2001.

Aims: The study of a protease secreted by *Candida caseinolytica* for use in future industrial applications.

Methods and Results: Growth of *Candida caseinolytica* on a medium containing milk induced a rapid production of an extracellular enzyme able to hydrolyse casein. The crude extract was applied to both Sephacryl S-200 and DEAE-Biogel A columns, obtaining one peak of activity showing a molecular mass of ≈ 30 kDa and three active peaks, respectively. These four peaks showed the same biochemical parameters. In all cases, an extremely broad pH range of action was determined.

Conclusions: *Candida caseinolytica* secretes high levels of an extracellular protease when grown either in rotary shakers or in batch-fermenters.

Significance and Impact of the Study: The biochemical properties of this enzyme suggest its possible industrial application in the brewing industry, in the formulation of certain type of detergents and in the fur and leather industries, among others.

INTRODUCTION

Proteases are the single class of enzymes that occupy a pivotal position with respect to their applications in both the physiological and commercial fields. Their vast diversity and specific range of action have attracted the attention of biotechnologists worldwide. Although they are widely distributed in nature, micro-organisms are the preferred source of these enzymes in fermentation bioprocesses because of their fast growth rate and also because they can be genetically engineered to generate new enzymes with desirable abilities or simply for enzyme overproduction (North 1982; Mala Rao *et al*. 1998). Strong proteolytic activity is relatively rare in yeast. However, several authors have found some strong activities in different yeast species (Ogrydziak 1993). The screening of a large number of microorganisms with industrial applications in milk treatment was boosted due to the high nutritional value of milk and many studies have been carried out to search for activities of interest. Different caseinolytic yeasts have been found, including some species of *Kluyveromyces* and *Candida*, such as *C. punicea*, *C. lipolytica*, *C. aquatica* and *C. curiosa* (Ahearn *et al*. 1968). *Candida caseinolytica* was first isolated during a screening of more than 5000 microorganisms from necrotic tissues of several *Opuntia* and *Stenocereus* cactus species growing in a restricted region of the North American Sonoran Desert and a few other localities in Baja California, Mexico. In fact, this yeast is the only cactus-specific species that exhibits strong extracellular caseinolytic activity and it also differs from other species of the genus in both morphological and physiological properties (Phaff *et al*. 1994). In the present work we report on the isolation and the basic biochemical properties of the enzymes involved in the degradation of casein produced by *C. caseinolytica* strain UCD-FST 83–438·3.

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MATERIALS AND METHODS

Strains, media and culture conditions

The yeast used in this work was *Candida caseinolytica*, type strain UCD-FST 83–438·3. Milk medium (MM) containing beef extract 0·3%, bactotryptone 0·5%, glucose 0·1%, agar

1·7% and skim milk powder (Nestlé, S.A.) 2·4% was prepared for the enzyme extraction (medium described by Ahearn *et al*. 1968 and modified by Phaff *et al*. 1994). Optimization of enzyme production was accomplished by supplementing the previous medium and YNB (w/o amino acids) medium enriched with skim milk powder (2·4%) with each of the following sugars; glucose, fructose, galactose, sorbose, trehalose and xylose at concentrations ranging from 0·05 to 5% (w/v). YNB supplemented with the previously cited sugars and without milk and YNB supplemented only with milk were used as controls. In all cases, plates were incubated at two different temperatures, 25°C or 30°C, in order to determine the best one for protease production. For enzyme characterization the yeast was inoculated in 1-l flasks containing 250 ml of the different media and after 4 d the cultures were harvested, centrifuged at 7000 *g* for 15 min at 4°C, and the supernatant filtered through 0·22 μm filter membranes and finally 10-fold ultrafiltered with Amicon devices using PM-10 membranes and dialysed overnight against 0·1 mol l^{-1} potassium phosphate buffer (pH 7·5) at 4°C with gentle stirring. Fermentation of *C. caseinolytica* was accomplished in a Biostat C (Braun Biotech International) fermenter of 30-l capacity using 20 l of MM medium, sterilized *in situ* according to the manufacturer's instructions at 121°C for 35 min with gentle stirring. After sterilization, 3 l of *C. caseinolytica* culture, grown in shake flasks, were added, obtaining an initial cellular density of $1{\cdot}3 \times 10^8$ cells ml^{-1}. Fermentation was started with an airflow of 10 l min^{-1}, at a pH value of 6·45 and a stirring speed of 200 r.p.m. Addition of the antifoam was controlled automatically. Samples were removed from the vessel over 48 h in order to analyse the following parameters: pH, cell density and enzymatic units, as well as the fermentation parameters; temperature, stirring and air flow. Temperature was kept at 30°C and stirring at 200 r.p.m. The airflow was increased from 10 l min^{-1}–20 l min^{-1} during the process because of the increase of the biological oxygen demand. The fermentation was stopped and the vessel emptied using a continuous flow centrifuge at 7000 *g* for 20 min at 4°C. The supernatant was filtered through a 20-μm membrane and concentrated to 420 ml using 4 Prep/Scale TM-TFF cartridges (Millipore).

Protease assays

Caseinolytic activity measurements in test tubes were carried out according to Kunitz's (1947) method in which casein is employed as the enzymatic substrate. Standard reaction mixtures were incubated for varying times at 37°C. Readings at 280 nm were referred to a tyrosine standard, defining 1 enzymatic unit as the amount of enzyme that releases 1 mmol of tyrosine at 37°C in 30 min. Protease activity on Petri dishes was tested by the cup plate assay, using a gel comprising 1·5% agarose and 2·5% skim powder milk in 0·5 mol l^{-1} Tris-HCl (pH 7·5). After solidification, 1·5-mm wells were made, filled with the enzyme solutions and incubated at 37°C. Protease activity was visualized as clear haloes surrounding the wells. When necessary, casein was replaced by either bovine serum or egg albumin. Protein determinations were performed according to Lowry's method (1951). Kinetic parameters such as K_m and V_{max} were calculated using Lineweaver–Burk (1934) plots and the different K_i values for bromosuccinimide (NBS), *p*-hydroxymercuric-benzoate (PHMB), *N*-ethylmaleimide (NEM), guanidine hydrochloride (Gnd-HCl), *N*-acetylimidazol (NAI), phenyl-methane-sulphonyl-fluorhydric (PMSF), phenylglyoxal (PG) and *L*-1-chloro-3-tosylamide-4-phenyl-2-butanone (CTPB), according to the method of Dixon and Webb (1979). Optimal temperature was tested in the 10–50°C range, and optimal pH was determined in a range from 2 to 12. Results were obtained in duplicate following both Kunitz's and the cup-plate methods.

Cellular location of the enzyme

The strain was grown for two days in MM (2 l) at 30°C, 200 r.p.m. until enzyme activity was detected in the culture medium. Then, cells were harvested and divided into two identical samples according to Villa *et al*. (1975). One sample was centrifuged, resuspended in 30 ml of 0·05 mol l^{-1} Tris-HCl buffer (pH 7·5) and broken down (3 min) in a CO_2-cooled Braun MSK cell homogenizer at 4000 r.p.m. with 0·45-mm glass beads. Following this, cellular debris was removed by centrifugation (3000 *g*) and the enzyme was evaluated in the supernatant. Cells from the other sample were resuspended in 0·1 mol l^{-1} HCl and incubated at 18°C for 1 h. After this, cells were spun down again and resuspended in the same buffer and homogenized as before. Glyceraldehyde-3-phosphate dehydrogenase was used as the intracellular marker in both HCl-treated and non-treated samples (Gacto 1973).

Gel exclusion and anion exchange chromatography

Concentrated supernatants (4 ml) were applied to both a Sephacryl S-200 column (LKB 80 × 3·5 cm) equilibrated with 0·05 mol l^{-1} Tris-HCI buffer, pH 7·5, and a DEAE-Biogel A (LKB 38 × 3·5 cm) column, also equilibrated with the same buffer. Chromatographies were carried out with the same buffer and 4-ml fractions were collected at a flow rate of 0·5 ml min^{-1}. The absorbance of the samples was measured at 280 nm. The DEAE-Biogel A column was then eluted with a sodium chloride gradient (0–1 mol l^{-1}) prepared in the same buffer. Bovine serum albumin

(67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and lysozyme (14 kDa) were used as molecular weight standards. The molecular weight of the enzyme was estimated by the method described by Whitaker (1963).

End product analysis

The proteolysis end product was evaluated with 1000 enzymatic units (10 ml) and 2% casein supplemented with 0·02% sodium azide for 5 days at 37°C. The reaction mixture was then ultrafiltered through PM-10 membranes and eluted in a Biogel P4 column (120 × 2 cm) equilibrated in the same buffer as before and calibrated with peptide antibiotics of known molecular weights as markers; nisin (3·35 kDa) and bacitracin (1·43 kDa). Fractions of 1·5 ml were collected and analysed spectrometrically by UV absorption (280 nm) and Lowry's method.

Isoelectrofocusing

Isoelectrofocusing was performed on an LKB Ampholine 8100 (110 ml) column refrigerated at 4°C. The gradient was made up with glycerol (40–2% v/v) and the ampholines, pH 3–10, were obtained from Pharmacia Fine Chemicals (Sweden). The samples were applied in the central part of the column as described by Vesterberg (1972). The focusing was completed in two days at 500 V.

Fractions of 3 ml were collected at a flow rate of 1·5 ml min^{-1}, monitoring UV absorbance at 280 nm, and the pH values and protease activities as defined previously.

RESULTS

Enzyme production and effect of the carbon source

Different carbon sources were tested via halo production for their ability to support protease production in *C. caseinolytica*. All the sugars tested supported growth. As shown in Table 1, glucose, fructose, galactose, sorbose and xylose at 0·1% (w/v) and trehalose at 0·05% (w/v) proved to be the best carbon sources for protease production of those investigated. In all cases enzymatic activity was better at 30°C.

A typical one-step growth and enzyme production when *C. caseinolytica* was grown asynchronously in MM medium are shown in Fig. 1a. Cells entered stationary phase after 35 h of growth (2×10^9 cells ml^{-1}) and enzyme formation started 20 h before. The estimated generation time for this yeast under these conditions was 4 h. Enzyme production in shake flasks required high aeration (200 r.p.m.) rates, being inhibited under anaerobic conditions. When growth was carried out in the fermenter (in MM medium), the strain exhibited a similar growth pattern to that obtained when growing *C. caseinolytica* in the same medium in shake flasks, although in this case the maximum production of the enzyme appeared about 10 h beforehand (Fig. 1c). The variations in pH values during the fermentation process are shown in Fig. 1b. After processing the 23 l of culture, 420 ml of an enzymatic concentrate were obtained showing 15-fold higher activity; 48·57 U $(mg\ prot)^{-1}$ than that obtained from the non-concentrated supernatant; 3·85 U $(mg\ prot)^{-1}$.

No activity was detected at either the periplasmic or cytosolic level and only the culture supernatants were active against casein. The crude extract did not show proteolytic activity against egg or bovine albumins.

Basic enzyme physico-chemical parameters

A concentrated enzyme sample was applied to a Sephacryl S-200 column in order to study certain basic physicochemical parameters of a partially purified enzyme. The concentrated crude extract eluted as a single enzyme (Fig. 2a) with a molecular mass of ≈ 30 kDa (Fig. 2b). This partially purified enzyme exhibited its maximum proteolytic potential between pH values of 4·5–11 and it was even partially active

Table 1 Effect of carbon sources on halo diameters after growing *Candida caseinolytica* type strain UCD-FST 83–438·3 for 8 days in two different media

Carbon source	Glucose			Fructose			Galactose			Sorbose			Trehalose			Xylose		
(% w/v)	0·1	1	5	0·1	1	5	0·1	1	5	0·1	1	5	0·05	0·5	2·5	0·1	1	5
Milk-enriched YNB medium																		
25°C	+	+	–	+	+	–	+	+	+	+	–	–	+	–	–	+	–	–
30°C	+	+	–	+ +	+	+	+	+	+	+	+	–	+	–	–	+	–	–
MM																		
25°C	+ +	+	–	+ +	+ +	+	+ +	+ +	+	+ +	–	–	+ +	+	–	+	–	–
30°C	+ + +	+	–	+ + +	+ +	+	+ +	+ +	+	+ +	+	–	+ + +	+ +	+	+ +	–	–

Absence of halo (–), 0–1 cm (+), 1–2 cm (+ +), > 2 cm (+ + +).

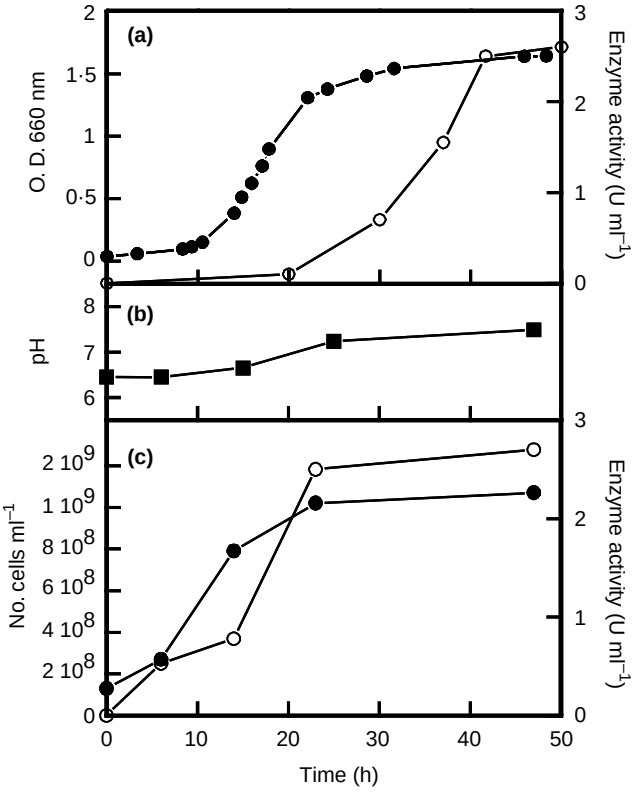

Fig. 1 ● Growth of *Candida caseinolytica* (UCD-FST 83–438·3) in MM medium and ○ its protease production; (a) in shake flasks and (c) in a Biostat C (Braun Biotech International) fermenter. (b) Squares show pH variations during fermentation

at pH 12. Regarding temperature, the enzyme showed detectable activity between 10 and 55°C, the maximum being reached at 37°C. The Michaelis constant value (K_m) as well as the V_{max} were calculated from Lineweaver–Burk regression lines and proved to be 0 · 05 mg ml^{-1} and 5 μg (ml min^{-1})$^{-1}$, respectively, for the semipurified protease. The enzyme exhibited an isoelectric point of 4·7. When the crude enzymatic solution was applied to the DEAE-Biogel A column, the activity was resolved in three different fractions (Fig. 3). Fractions I and II did not bind the matrix under the conditions reported here, whereas fraction III was eluted from the columns after the sodium chloride gradient had been applied (at ≈ 0·45 mol l^{-1}). The three active fractions showed the same broad pH range of action, the same optimum for temperature and the same K_m and isoelectric point values as reported immediately above. When the three fractions from anion exchange chromatography were applied to a Sephacryl S-200 column separately, the same molecular mass was obtained for all of them. The semipurified enzyme from Sephacryl S-200 hydrolysed casein (see Fig. 4), giving a mixture of small amounts of aminoacids (peak 3) and two clearly defined peptides (peaks 1 and 2). Peak 1 eluted at a similar point as bacitracin (1·43 kDa). The three active fractions from DEAE-Biogel A chromatography also originated a similar peptide pattern of casein degradation. All the peaks with caseinolytic activity obtained from both chromatographic assays were inhibited by PHMB, NEM and PMSF, with K_i values of 0·2 mmol l^{-1}, 2·7 mmol l^{-1} and 0·04 mmol l^{-1}, respectively, and were also inhibited by different cations such as Ba^{+2}, Fe^{+2}, Mg^{+2}, Hg^{+2}, Zn^{+2} and

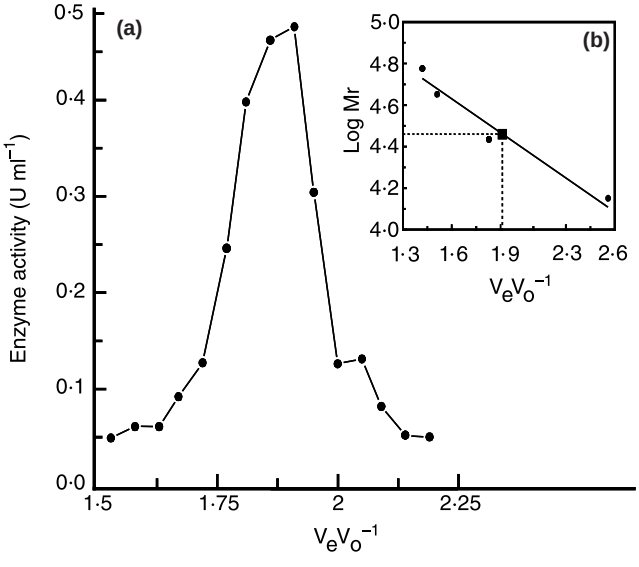

Fig. 2 (a) Gel filtration chromatography over Sephacryl S-200 of a concentrated supernatant medium of *Candida caseinolytica*.
(b) Molecular weight determination according to Whitaker's method. ● Molecular weight markers and ■ the enzyme

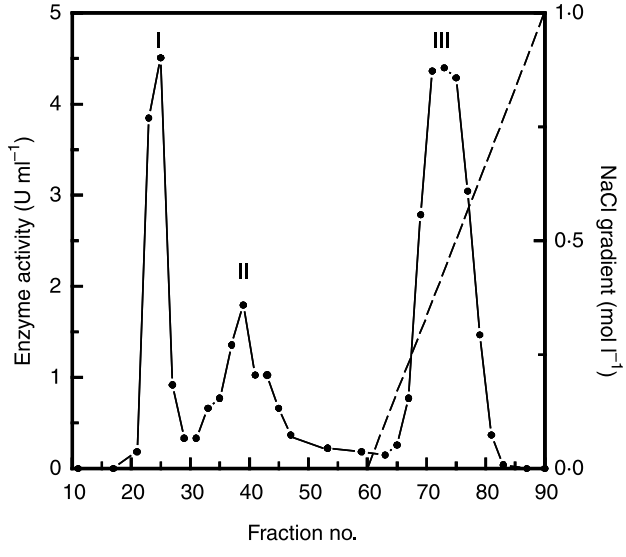

Fig. 3 Anion exchange chromatography on DEAE-Biogel A. ● Enzyme activity and (– –) NaCl gradient

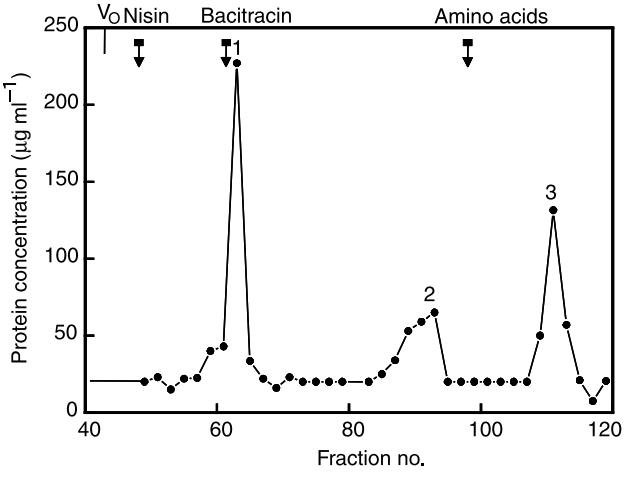

Fig. 4 Biogel P4 chromatography of proteolytic products originated by the protease of *Candida caseinolytica*. Squares show the elution of different peptide markers and amino acids

Ca^{+2}, full inhibitions being seen at concentrations higher than 50 mmol l^{-1}.

DISCUSSION

C. caseinolytica shows strong proteolytic activity against casein, this being a rare character among yeasts (Phaff *et al.* 1994). The enzyme is formed in the presence of casein in the growing medium; other proteins such as egg or bovine albumins do not induce caseinolytic enzymes. This type of specific induction also occurs in another yeast species (Federici 1985). Typical carbon sources such as glucose, fructose, xylose, galactose, sorbosa or trehalose at low concentrations (0·05–1% w/v) allow growth and enzyme synthesis up to maximum values. Higher values of glucose, sorbose and xylose (more than 1% w/v), however, resulted in catabolite repression of the enzyme activity. The crude enzymatic preparation was resolved into three different fractions with caseinolytic activity by means of anion exchange chromatography but only one active peak was observed when elution was performed by gel exclusion chromatography. Since all the physico-chemical parameters evaluated here (i.e. molecular mass, optimum pH and temperature, isoelectric point, K_m, K_i of different inhibitors, proteolytic products analysis) were similar in all cases, we propose the existence of only one enzyme. Its binding to casein while proteolysing would generate enzyme molecules with different overall charge, this being the reason for their separation in the DEAE-Biogel A column. The profile obtained from casein degradation revealed the endoproteolytic character of the enzyme, since the peptide pattern analysed showed small amounts of aminoacids. The inhibition produced by PHMB, NEM and PMSF suggests that the active site of the enzyme must be rich in serine and cysteine amino acids. When the strain was grown in the fermenter the enzyme was efficiently secreted into the culture medium. High levels of secreted broad pH range protease were detected, the maximum of production being reached even earlier than when the strain was grown in shake flasks. The fact that the enzyme is able to continue with digestion at a high range of pH values (from 4·5 to 11) leads us suggest this particular protease for use in the brewing industry and it could even eventually find biotechnological applications in the elaboration of certain type of detergents (low temperature) or in the fur and leather industries due to its ability to proteolyse at extreme pH values (even up to 12), as has been reported in *Yarrowia lipolytica* or *C. olea* (Nelson and Young 1987; Matoba *et al.* 1988). This rare property of the broad pH range may be related to the natural habitat of this yeast, i.e. in cactus tissues. As determined by Phaff *et al.* (1994), the pH values of cactus tissue when necrosis starts are at first relatively low (≈ 4·5) this increasing up to 8·5 as necrosis proceeds. Then, bacteria disappear altogether, yeast microbiota being the only micro-organisms that can be isolated. The broad pH range of action of this protease would represent an advantage for yeasts colonization.

ACKNOWLEDGEMENTS

This work was supported by the Fundación Ramón Areces of Madrid, Spain and a FEDER project (1FD97–2156) and PESCANOVA, S.A.

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