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Production and Characterization of Recombinant Cyprosin B in *Saccharomyces cerevisiae* (W303-1A) Strain

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The *Saccharomyces cerevisiae* W303-1A strain transformed with a centromeric plasmid containing *CYPRO11*, which codifies the aspartic protease cyprosin B, was grown in a 3 *l* bioreactor under aerobic conditions. Expression of cyprosin B is directly dependent on the concentration of galactose used as the inducer and carbon source in 1% yeast extract, 2% bactopeptone, and 4% galactose in culture medium. For 4% of galactose, 209 mg·*l*$^{-1}$ total protein, and 1036 U·ml^{-1} recombinant cyprosin B activity were obtained from 6.1 g dcw·*l*$^{-1}$ biomass. The recombinant cyprosin B, purified by two consecutive anion-exchange chromatographies (diethyl amino-ethyl [DEAE]-Sepharose and Q-Sepharose XK-16 columns), shows a specific activity of 62×10^3 U·mg^{-1}, corresponding to a purification degree of 12.5-fold and a recovery yield of 25.6% relative to that in fermentation broth. The proteolytic activity of recombinant cyprosin B is optimal at 42°C and pH 4.5. The recombinant cyprosin B activity is 95% inhibited by pepstatin A, which confirms its aspartic protease nature. The pure recombinant cyprosin B is composed of two subunits, one with 14 and the other with 32 kDa. It exhibits clotting activity, similar to that of the natural enzyme from *Cynara cardunculus* flowers. The results reported here show that recombinant cyprosin B, the first clotting protease of plant origin produced in a bioreactor, can now be produced in large scale and may constitute a new and efficient alternative to enzymes of animal or fungal origin that are widely used in cheese making.

[**Key words:** *Cynara cardunculus*, cyprosin B, aspartic protease, fermentation, milk clotting, *Saccharomyces cerevisiae*]

The *Saccharomyces cerevisiae* W303-1A strain was transformed with the pCAF23 vector containing *CYPRO11* (Calixto, F. *et al.*, International Patent WO 0075283, 2002), which codifies cyprosin B, an aspartic protease present in pistils of *Cynara cardunculus* (1). The flowers of this species are traditionally used in Portugal and Spain for cow, goat and ewe cheese making, such as Serpa and Serra cheeses, which are unique in Portugal (2). The milk-clotting activity of this enzyme is due to the presence of three aspartic proteases, initially named cynarases 1, 2 and 3 (cyprosin B corresponding to cynarase 3), which have been purified and partially characterized (3). Cyprosins are heterodimeric enzymes with a molecular weight of 49 kDa. They are formed by a large (32.0–34.0 kDa) subunit and a small subunit (14.0–18.0 kDa) containing high mannose-type glycosylations (4, 5). Isoelectric focusing revealed a microheterogeneity of the apparently pure cyprosins from *C. cardunculus* flowers with an isoelectric point of 4.0 (4, 5). Similarly to other aspartic proteases, cyprosins preferentially cleave peptide bonds between hydrophobic amino acid residues and are strongly inhibited by pepstatin A (6). The enzymatic proteolysis of milk by plant aspartic proteases and by other aspartic proteases occurs by the hydrolysis of the phenylalanine-methionine bond of bovine K-casein, specifically between the amino acids Phe^{105}-Met^{106} (7–9). Two other aspartic proteases (cardosins A and B) have been isolated and purified from *C. cardunculus* flowers (10–12). The proteolytic activity of cardosin B is higher than that of cardosin A (13).

Clotting enzymes have been found in animals, plants, and microorganisms. Genetically engineered chymosin has been produced and generally recognized as safe (GRAS) by the FDA in 1989. Currently, it is estimated that 50% of the marketed chymosin is produced by biotechnological processes and about 70% of domestic cheese in USA is produced using bioengineered chymosin.

The aim of this work was to produce recombinant cyprosin B, a milk-clotting enzyme of plant origin, using the transformed *S. cerevisiae* W303-1A strain in a bench bioreactor, by monitoring strain behavior in terms of plasmid stability and cell viability as well as nutrient uptake and metabolite production during culture. The expression, secretion, and

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purification of recombinant cyprosin B were also evaluated and, finally, the biochemical characterization of the pure recombinant enzyme was performed in terms of optimum temperature and pH, protease activity inhibition, structural molecular weight, immunolocalization, thermal stability, and milk-clotting properties.

MATERIALS AND METHODS

Production and purification of cyprosin B

Saccharomyces cerevisiae strain The *Saccharomyces cerevisiae* W303-1A strain (*MATα leu2-3*, *112 ura3-1 trp1-1 his3-11*, *15 ade2-1*) was transformed with the centromeric plasmid pCAF23 containing *CYPRO11*, which codifies cyprosin B (Calixto, F. *et al.*, International Patent WO 0075283, 2002). The transformed yeast strain was maintained at −80°C in 50% glycerol (v/v).

Inoculum and enzyme production media YNB medium (0.67% yeast nitrogen base and 2% glucose) supplemented with auxotrophic amino acids (50 mg·l^{-1} L-histidine; 20 mg·l^{-1} L-adenine; 70 mg·l^{-1} L-tryptophan, and 20 mg·l^{-1} uracil) and sterilized by microfiltration was used to start *S. cerevisiae* W303-1A culture (14). The strain was grown for 16 h in 25-ml flasks containing 5 ml of YNB medium maintained in an orbital shaker (Sanyo, Tokyo) (200 rpm) at 30°C. Afterwards, 5 ml of this preinoculum were transferred into 500-ml flasks containing 100 ml of YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose). The inoculum was grown for 24 h in an orbital shaker (200 rpm) at 30°C and then transferred into the bioreactor containing the expression medium YPGal (1% yeast extract, 2% bactopeptone, and 4% galactose).

Enzyme production in bioreactor Fermentation was carried out in a 3 *l* bioreactor (HT-Infors, Bottmingen, Switzerland) with a working volume of 2 *l* at pH 5.5 and 30.0±0.1°C. YPGal medium and the reaction vessel were sterilized at 121°C for 20 min. The fermenter was inoculated at 5% (v/v) ratio. Oxygen tension was measured as the percentage of dissolved oxygen in the culture medium in relation to air saturation using an oxygen electrode (Ingold, Giessen, Germany). The electrode was calibrated according to Cooper *et al.* (15). Zero percent of dissolved oxygen was calibrated with the sensor immersed in a sulfite solution before sterilization of the bioreactor vessel and culture medium. We realized 100% dissolved oxygen at a flow rate of 2 $l \cdot min^{-1}$ (500 rpm), at 30°C before inoculation. During fermentation, air was supplied at a constant flow rate of 2 $l \cdot min^{-1}$ (equivalent to 1 VVM). Mechanical agitation, produced by two Rushton turbines, was controlled automatically by setting the percentage of dissolved oxygen in the culture medium at 55%. Samples were collected under aseptic conditions to control fermentation performance and monitor medium composition, biomass, protein concentration, and enzymatic activity.

Enzyme recovery and concentration Fermentation broth was centrifuged at 8500×*g* (10 min) at 4°C (Heraeus, Hesse, Germany) for cell separation. Protein was precipitated by gradually adding ammonium sulfate to the supernatant (100 ml) to obtain 80% saturation (16). The precipitate was collected by centrifugation at 18,000×*g* (10 min) at 4°C and redissolved in 20 ml of 50 mM Tris–HCl containing 10 mM EDTA (pH 8.3 TE buffer). The extract was dialyzed overnight against TE buffer at 4°C.

Enzyme purification Enzyme purification involved loading the dialyzed enzyme extract (35 ml) into the loop and the transfer of the extract to an anionic exchange DEAE-Sepharose column (Pharmacia, Uppsala, Sweden) previously equilibrated with TE buffer and washed with the same buffer (90 ml), at a flow rate of 1 ml·min^{-1}. Elution was performed using three gradients of 1 M NaCl in TE buffer: 0% to 50% (10 ml), 50% to 100% (60 ml), and 100% (10 ml) at a flow rate of 1 ml·min^{-1}. The pool of fractions (15 ml) with highest enzymatic activity was dialyzed, loaded into a second anionic exchange Q-Sepharose column (Pharmacia) previously equilibrated with TE buffer and washed with the same buffer (50 ml). The elution was carried out with three gradients of 1 M NaCl in TE buffer: 0–50% (20 ml), 50–100% (40 ml), and 100% (10 ml). The chromatographic steps were monitored using on FPLC system (Pharmacia).

Analytical methods

Biomass Biomass concentration was determined using a spectrophotometer (Shimadzu UV-1603; Shimadzu, Kyoto) at an optical density of $OD_{600\,nm}$. Cells were diluted in 0.8% NaCl solution and absorbance values were converted to dry cell weight using a standard curve based on the weight of cell samples dried at 80°C until constant weight.

Protein concentration Protein concentration was determined according to the Bradford method, using Coomassie reagent (Bio-Rad Laboratories, Hercules, CA, USA) (17). Bovine serum albumin (BSA) (Sigma, Munich, Germany) was used as a standard. Absorbance was measured at 595 nm using a spectrophotometer.

Enzymatic activity Enzymatic activity was evaluated according to Heimgartner *et al.* (3) using flourescein isothiocyanate-labelled K-casein (FITC-casein) as the substrate, prepared according to the Twining method (18). One unit of proteolytic activity was defined as the amount of enzyme that increases one unit of emitted fluorescence at 525 nm (excitation at 495 nm) after 60 min of hydrolysis at 37°C (3). Fluorescence intensity was measured using a spectrofluorimeter (Shimadzu RF-1501; Shimadzu).

Cell viability and plasmid stability Cell viability was estimated by the methylene blue staining method and direct cell counting using a Neubauer chamber. Plasmid stability was estimated by comparing the number of colonies grown in selective solid medium (YNB supplemented with auxotrophics amino acids and 2% bacto-agar) and the number of colonies grown in non selective solid medium (YNB supplemented with auxotrophics amino acids complemented by 40 mg·l^{-1} L-leucine and 2% Bacto-agar).

Efficiency of protein secretion The pellet of biomass collected after centrifugation at 8500×*g* (5 min) was ressuspended and washed twice with TE buffer. Cells were disrupted by vigorous mixing in TE buffer with 50% (v/v) 500-μm glass beads (Sigma) for 5 min with (1 min) intervals in ice. Cell debris were removed by centrifugation at 8500×*g* (15 min) at 4°C. Secretion efficiency was evaluated by calculating the ratio between extracellular and intracellular enzymatic activities.

HPLC Fermentation was monitored by analyzing nutrients (galactose and glucose) and metabolites (ethanol and succinate) using on HPLC column Aminex (Bio-Rad Laboratories) connected to an HPLC system (Merck, Frankfurt, Germany), operating at 50°C. Elution was carried out using 3.5 mM sulfuric acid solution at a flow rate of 0.6 ml·min^{-1}. Thirty microliters of each sample and standards were injected, and elution was carried out for 15 min.

Biochemical characterization of cyprosin B

Optimum pH and temperature The optimum pH was determined by dilution of 10 μl of pure recombinant cyprosin B in 30 μl of 200 mM sodium citrate buffer at pHs values of: 2.6, 3.1, 3.7, 4.1, 4.5, 5.1 and 5.5 and in 200 mM sodium phosphate buffer at pHs values of 6.0, 6.4, 7.0, and 7.5. For pH values of 8.0 and 8.3, 200 mM TE buffer was used. The optimum temperature was determined by analysis of enzymatic activity in 200 mM sodium citrate buffer (pH 5.1) at 25°C, 37°C, 42°C, 47°C, 52°C, 60°C, and 70°C.

Thermal stability of the enzyme The pure recombinant cyprosin B was maintained at 4°C in different solutions: 50 mM TE buffer (pH 8.3), 50 mM NaCl, 50 mM ammonium sulphate, 50 mM sodium citrate buffer (pH 5.1) and the resulting pool of elution fractions with pure enzyme (NaCl and TE buffer, 50 mM, pH 8.3). Enzymatic activity was evaluated 1, 3, 5, 10 and 24 h after incubation.