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Purification and characterisation of Proteases A and D from *Debaryomyces hansenii*

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ABSTRACT

The proteases A (PrA; EC. 3.4.23.25) and D (PrD; EC. 3.4.24.37) of *Debaryomyces hansenii* CECT 12487 were characterised after their isolation by fractionation with protamine sulfate followed by three chromatographic separations, which included two anion exchange and one gel filtration chromatographic steps. The whole procedures for PrA and PrD resulted in 1349 and 2560 purification-fold with a recovery yield of 1.4 and 1.3%, respectively.

PrA was active at acidic–neutral pH with an optimum pH between 5.0 and 6.0. PrD was active at neutral–basic pH with an optimum pH between 7.0 and 8.0. The molecular mass of the native PrA was 55 kDa and (being) 42 kDa in denaturing conditions. Polyclonal-antibodies raised against PrA from *Saccharomyces cerevisiae* cross-reacted with the corresponding PrA from *D. hansenii*. PrD showed a native molecular mass of 68 kDa and 65 kDa in denaturing conditions. PrA was an aspartic protease effectively inhibited by pesptatin A while PrD was classified as a metallo protease inhibited by 1,10-phenantroline and affected by some divalent cations such as zinc, cadmium and magnesium. The homology of the PrA to the lisosomal cathepsin D suggests its possible participation in the ripening of fermented meat products.

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1. Introduction

Debaryomyces hansenii is a hemiascomycetous yeast which has attracted scientific interest mostly because of its halotolerance. Therefore, most of the latest studies on *D. hansenii* deal with the mechanisms that confers salt tolerance to this yeast and with the analysis of its genome (Lépingle et al., 2000, Sherman et al., 2004, Thomé, 2004). In addition, the interest in this specie has also been increased as related to its physiologic and biochemical traits with an impact in industrial fermentations (Nobre et al., 1999, Strauss et al., 2001, Bolumar et al., 2003a,b). *D. hansenii* is often found in protein-rich fermented products such as cheese and sausage. The ability of this yeast to contribute to the flavor development in these products has been the main objective of recent studies (Cook, 1995, Olensen, and Stahnke, 2000, Encinas et al., 2000, Van Den Tempel and Jakobsen, 2000, Petersen et al., 2002, Martin et al., 2003, Bintsis et al., 2003). However, there are very few biochemical data related to the identification of the enzymes behind these effects (Besançon et al., 1995, Durá et al., 2002, Bolumar et al., 2003a,b, 2005).

Within the pool of biochemical reactions that are involved in flavor development, proteolysis plays an outstanding role. The comprehension of the proteolytic systems of meaty microorganism is a key factor for the control of those industrial processes (Toldrá, 2002). So far, most of the studies on the proteolytic system of meaty microorganisms have been carried out in lactobacilli and, specially, in *L. sakei* (Sanz and Toldrá, 2002). However, the metabolic activities of yeasts adapted to the meat ecosystem are still poorly understood (Toldrá et al., 2001).

Basically, the proteolytic system from *Saccharomyces cerevisiae* consists of three major protease groups: the vacuolar proteases, the cytosolic proteosome and the proteases involved in the secretory pathway (Jones, 1991a,b). The vacuolar proteases constitute the most numerous pool integrated by two major endopeptidases (PrA and PrB) and several exopeptidases (carboxy- and aminopeptidases; (Klionsky et al., 1990). Proteolytic enzymes are involved in several essential biological processes for the cell such as protein turnover, aberrant protein degradation, correct protein processing and adaptation to environmental conditions of reduced availability or high demand for nitrogen (Achstetter and Wolf, 1985; Jones, 1991a,b; 2002; Jones et al., 1997; Klionsky et al., 1990; Strauss et al., 2001).

At present, there is wide information on the proteolytic pathways that are functional in *S. cerevisiae*. In contrast, studies on the proteolytic system of *D. hansenii* are still limited although it could have several implications in its physiology and in flavor development in food fermentations. To date, the following peptidases have been characterised in *D. hansenii:* two exopeptidases, an arginyl amino-peptidase that is homologous to the ApY of *S. cerevisiae* (Bolumar et al., 2003b) and a prolyl aminopeptidase with no homology in *S. cerevisiae* (Bolumar et al., 2003a), and just one endoprotease, the protease B (PrB) that it is homologous to the PrB from *S. cerevisiae* (Bolumar et al., 2005). The second major vacuolar protease, PrA, was just detected in a previous study, being the aim of the present work.

With regard to fermented sausages, Molly et al. (1997) pointed out that the acid aspartyl protease, cathepsin D, was the responsible

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protease for the initial protein degradation in fermented sausage, being the PrA its homologous in *S. cerevisiae*. Therefore, it was interesting to identify and characterise this protease in a meaty microorganism as *D. hansenii*.

So, the aim of this work was to characterise the PrA and simultaneously the PrD. These biochemical data contribute to the knowledge about the endoproteolytic system of *D. hansenii* Comparisons of the purified enzymes with their counterparts from *S. cerevisiae* as well as discussions about its possible implication especially for the PrA during meat fermentation are included.

2. Materials and methods

2.1. Yeast strains and growth conditions

D. hansenii CECT 12487 was isolated from the natural microflora of fermented sausages and selected as possible starter culture on the basis of its physiological and biochemical properties and its ability to compete during sausage manufacturing processes (Santos-Mendoza, 2000, Santos et al., 2001). It was routinely grown in malt extract agar or broth (Scharlau, Barcelona, Spain) at 27 °C, for 48–72 h and, then, stored at 4 °C or at −80 °C in 15% glycerol. For purification purposes, the microorganism was grown in 1.17% (w/v) Yeast Carbon Base (Difco, Detroit, USA) plus 0.1% (w/v) urea as nitrogen source. 120 mL of this medium was inoculated and incubated at 27 °C, for 2 days, in an orbital incubator at 110 rpm. This pre-culture was used to inoculate 400 mL fresh medium, which was incubated in the same conditions for 5 days and, finally, used for enzyme purification. *S. cerevisiae* ATCC 18824 was used as positive control for immunodetection of PrA by Western analysis, as described below. The strain was routinely grown in the same conditions as those used for *D. hansenii*.

2.2. Preparation of cell extract

Cells were harvested at 4080 ×*g* for 10 min, at 4 °C, washed with 20 mM sodium phosphate, pH 7.5, and then resuspended in an equivalent volume of the same buffer. This cell-suspension was used immediately or frozen with liquid nitrogen and stored at −80 °C till use. Cell disruption was carried out in a bead beater (Biospec Products, Washington, N.C., USA). An equivalent volume of glass beads (0.5 mm diameter, Sigma, St. Louis, MO, USA) was added to the cell-suspension and then four shakings for 30 s were applied, with 2 min intervals on ice. Afterwards, non-broken cells and debris were separated by two centrifugation steps (14,500 ×*g*, 15 min and 27,000 ×*g*, 20 min, 4 °C) and the supernatant constituted the cell extract used for purification.

2.3. Enzyme standard assay

Activity was measured by adding 100 μL of enzyme to 70 μL of McIlvaine buffer (0.1 M citric acid, 0.2 M disodium phosphate), pH 7.5, containing 0.21 mM *N*-Succinyl-Leucine-Tyrosine-7-amido-4-methyl-coumarin (*N*-Succinyl-Leu-Tyr-AMC; Sigma, St. Louis, MO, USA). The reaction mixture was incubated at 37 °C, for 10 min. Fluorescence was measured in a multiscan fluorometer (Fluoroskan II, Labsystem, Finland) using excitation and emission wavelengths of 355 and 460 nm, respectively. Three replicates were measured for each experimental point. One unit of enzyme activity (U) was defined as the release of 1 nmol of substrate per hour at 37 °C.

Table 1
Purification of protease A (PrA) from *Debaryomyces hansenii*

Purification step	Protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell extract	541.280	384.5	0.7	100.0	1.0
Resuspended pellet from protamine fractionation	29.920	451.7	15.1	117.5	21.3
Weak anion exchange chromatography	6.040	117.2	19.4	30.5	27.3
Strong anion exchange chromatography	0.772	38.8	50.3	10.1	70.8
Gel filtration chromatography	0.006	5.4	958.3	1.4	1349.2

[a] U = nmol AMC released/h.

Table 2
Purification of protease D (PrD) from *Debaryomyces hansenii*

Purification step	Protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell extract	541.280	384.5	0.7	100.0	1.0
Resuspended pellet from protamine fractionation	29.920	451.7	15.1	117.5	21.3
Weak anion exchange chromatography	6.040	117.2	19.4	30.5	27.3
Strong anion exchange chromatography	0.420	33.2	79.1	8.6	111.4
Gel filtration chromatography	0.003	5.1	1818.7	1.3	2560.5

[a] U = nmol AMC released/h.

2.4. Enzyme purification

2.4.1. Protamine sulfate fractionation

Protamine sulphate fractionation was used as described elsewhere (Bolumar et al., 2003a,b, 2005). A concentration of 0.03 mg protamine sulfate/mg protein was slowly added to the cell extract at 5 °C keeping it under stirring for 20 min. Afterwards, the solution was centrifuged (14,500 ×*g* for 11 min) and then 0.08 mg protamine sulfate/mg protein were added again to the new supernatant, as described above. The solution was centrifuged (23,500 ×*g* for 11 min) and the pellet was finally resuspended in 0.2 M sodium phosphate, pH 7.5. After 5 min of resting, 3.5 μL of 1% (w/v) salmon DNA per mg of protein were added. Then, the solution was centrifuged (27,000 ×*g* for 11 min) and the supernatant was submitted to the following purification step.

2.4.2. Weak anion exchange chromatography

The supernatant was injected into a HiPrep™ 16/10 DEAE column (Amershan Pharmacia Biotech AB, Uppsala, Sweden). The column was initially equilibrated with 25 mM Tris–HCl, pH 6.5, containing 150 mM NaCl. The running conditions were as follows: a washing step was initially applied with the equilibration buffer till the non-retained proteins were removed as indicated by a decrease in absorbance and, then, a gradient from 150 to 500 mM NaCl in the same buffer for 45 min. The flow rate was 4 mL/min and fractions of 4 mL were collected.

Fractions number 13 to fraction number 27 with maximum activity were pooled and concentrated using a filter device biomax 10 K NMWL membrane (Millipore Corporation, Bedford, MA, USA).

2.4.3. Strong anion exchange chromatography

Concentrated fractions were injected into a Resource™ Q column (1 mL, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Proteins were eluted by applying an initial isocratic step in 20 mM MES, pH 6.1, for 5 min, followed by a linear gradient from 250 mM to 360 mM NaCl in the same buffer, in the next 25 min. The flow rate was 1 mL/min and fractions of 1 mL were collected. Fraction numbers 6 to 9 and fraction numbers 13 to 16 containing the maximum activity against *N*-Succinyl-Leu-Tyr-AMC were collected separately, concentrated using a filter device biomax 10 K NMWL membrane (Millipore Corporation, Bedford, MA, USA) and submitted to gel filtration.

2.4.4. Gel filtration chromatography

Sephacryl S-200 HR column (100 by 1.5 cm; Amershan Pharmacia Biotech AB, Uppsala, Sweden) and 50 mM phosphate pH 7.0 as running buffer were used. The column was run at a flow rate of 10 mL/h. Fractions of 3 mL were collected and assayed for *N*-Succinyl-Leu-Tyr-AMC activity. The two fractions containing maximum activity were employed for characterisation studies.

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