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# Protease (PrA and PrB) and prolyl and arginyl aminopeptidase activities from *Debaryomyces hansenii* as a function of growth phase and nutrient sources

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**Abstract**

The effects of nutrient sources and growth phase of *Debaryomyces hansenii* on the protease (PrA and PrB) and aminopeptidase (prolyl-[PAP] and arginyl-[AAP] aminopeptidases) activities were investigated. These activities were also monitored during growth on a whole sarcoplasmic muscle protein extract (WSPE) and on an equivalent medium but free of compounds under 10 kDa (SPE>10 kDa). The levels of specific protease and aminopeptidase activities were higher when cells were grown in urea and dipeptides than when grown in either ammonium or free amino acids as nitrogen sources. The level of each aminopeptidase (PAP or AAP) activity was preferentially induced by its own substrate (ProLeu or LysAla), suggesting a role in the utilization of exogenous peptides. Higher specific activities for all proteolytic enzymes were detected when using acetate as carbon source. The time course experiments carried out on urea or sarcoplasmic protein-containing media revealed an increase in all activities during transition and advanced stages of stationary phase of growth. In muscle protein extracts, the absence of low molecular mass nutrients (SPE>10 kDa) initially induced the production of PrA, PrB, and AAP activities, possibly involved in the breakdown of muscle oligopeptides.


*Keywords:* *Debaryomyces hansenii*; Yeast; Proteases; Peptidases; Nitrogen regulation

## 1. Introduction

Nowadays, the interest in non-*Saccharomyces* yeasts has been increased as related to its physiology, biochemistry and genetic aspects with impact on industrial fermentations (Nobre et al., 1999; Lépingle et al., 2000; Strauss et al., 2001; Bolumar et al., 2003a; Sherman et al., 2004). Such yeasts are involved in a wide variety of food fermentation processes such as baking, brewing, and cheese and sausages making (Boekhout and Robert, 2003). *Debaryomyces hansenii* is a halophile yeast found in shallow seawaters (Gonzalez-Hernandez et al., 2004) but also in salty food products like meat and dairy products which are highly valued (Cook, 1995; Encinas et al., 2000; Van Den Tempel and Jakobsen, 2000; Petersen et al., 2002; Bintsis et al., 2003).

Nitrogen metabolism constitutes one of the most important biochemical pathways involved in yeast physiology, with deep repercussions in the processing of fermented foodstuffs. Indeed, proteases are partially responsible for cell survival through physiological adaptations but also have an indirect effect on flavor development due to the activation of different metabolic routes (Jones et al., 1997).

In *Saccharomyces cerevisiae*, the expression of nitrogen catabolic genes is controlled in response to the quality of the available nitrogen source. The presence of easily metabolized nitrogen sources (ammonia, glutamine, asparagine and so on) represses the expression of genes encoding transporters and catabolic enzymes (proteases) necessary for the uptake and utilization of poor nitrogen sources (Winderickx et al., 2003). This phenomenon is known as nitrogen catabolite repression (NCR) (Hofman-Bang, 1999). On the other hand, when cells are transferred to a glucose-based medium that lacks a nitrogen source, the rate of protein degradation markedly increases by the higher production of proteolytic activities (Jones, 1991). Neither genetic nor physiological information related to nitrogen regulation is available for *D. hansenii.* In addition, the regulation of nitrogen metabolism in *D. hansenii* could affect the proteolytic chain during food fermentation and

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ripening of protein-rich materials (Kumura et al., 2002). Proteolysis leads to an increase of small peptides and free amino acids, that could further react and generate volatile aroma compounds, altogether affecting sensory perception. Furthermore, the generated peptides and free amino acids have an influence on the microbial physiology contributing to regulate the microbial succession and the whole fermentation process (Toldrá et al., 2001). For these reasons, it will be of interest to know how proteolytic enzymes are regulated in *D. hansenii*, a typical species in meat fermentation, as a function of environmental factors, especially those closer to the meat ecosystem.

Recently, our group has carried out the purification and characterization of the most important proteases from *D. hansenii*. These are the aspartic endoprotease A (PrA) (Bolumar et al., 2005) and the serine endoprotease B (PrB) (Bolumar et al., in press) and a prolyl aminopeptidase (PAP) (Bolumar et al., 2003a) and an arginyl aminopeptidase (AAP) (Bolumar et al., 2003b). In addition, there are two endogenous intracellular inhibitors $I^A$ and $I^B$ for PrA and PrB, respectively which both are inactivated after an overnight treatment at acid pH as happens for *S. cerevisiae* (Magni et al., 1986; Slaughter and Nomura, 1992; Tanimizu and Hayashi, 1996; Van Den Hazel et al., 1996; Bolumar et al., 2005).

Thus, the aim of this work is to study the production of PrA, PrB, PAP and AAP activities in *D. hansenii* growing under different nutrient sources, during different growth phases and using sarcoplasmic proteins as meat substrate. This is an initial approach for a better knowledge of the regulation of nitrogen catabolic enzymes in *D. hansenii* and its possible implications in meat fermentation.

## 2. Materials and methods

### 2.1. Yeast strain and culture conditions

The studied strain was *D. hansenii* CECT 12487 which was isolated from the natural micro flora 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). It was routinely grown in malt extract agar (Scharlau, Barcelona, Spain) at 31 °C, for 48–72 h and, then, stored at 4 °C or at −80 °C in 15% glycerol. The strain was grown on YPD broth at 37 °C for 48 h. Then, cells were harvested by centrifugation at 5000×*g* at 4 °C for 10 min and washed in 20 mM sodium phosphate buffer, pH 7.5. The final pellet was resuspended in sterile water and used to inoculate all the different media at initial optical density (O.D) of 0.01 at 600 nm. Flasks containing media of different composition were incubated without shaking to generate a microaerophilic environment close to that found in the sausage, at 31 °C for a maximum period of time of 14 days. Chloranphenicol (Boehringer Mannheim Gmbh, Heidelberg, Germany) was added to the medium at 0.01 g/100 mL to avoid contamination. Two independent experiments were done for each particular case. The growth of the different cultures was monitored by measuring the absorbance at 600 nm.

#### 2.1.1. Studies of the effects of different nitrogen and carbon sources

In order to assess the effect of different nitrogen and carbon sources, yeast carbon base and yeast nitrogen base (Difco Laboratories, Detroit, USA) were used. Yeast carbon base was supplemented at 0.5 g/100 mL with the following nitrogen sources: ammonium sulfate, urea, proline, ProLeu, LysAla, ProLeu plus an amino acid mixture (1 g/mL), and LysAla plus an amino acid mixture (1 g/mL). The composition of the amino acid mixture was according to a complete media for *S. cerevisiae* (E.W. Jones, Carnegie Mellon University, Pittsburgh, USA, personal communication) and included in mg/L; 530 arginine, 530 histidine, 490 leucine, 500 lysine, 510 methionine, 500 serine, 480 phenylalanine, 510 tryptophan, 350 threonine and 530 tyrosine. Ammonium sulfate and urea were purchased from Panreac (Barcelona, Spain), proline from Scharlau (Barcelona, Spain) and the dipeptides and free amino acids from Sigma (St. Louis, MO, USA).

Yeast nitrogen base was supplemented at 1 g/100 mL with the following carbon sources: glucose (Panreac, Barcelona, Spain), galactose (Sigma, St. Louis, MO, USA) and acetate (Scharlau, Barcelona, Spain). All the components were freshly prepared and sterilized by filtration through a 0.22 μm pore-size membrane (Millipore, Bedford, MA, USA). Samples were taken in the exponential phase of growth for proteolytic activity assays.

#### 2.1.2. Studies of the effects of the phase of growth in media with different nitrogen sources

To study the levels of proteolytic activities during different phases of growth, the strain was grown on yeast carbon base supplemented at 0.5 g/100 mL with urea or sarcoplasmic muscle protein as nitrogen sources. Sarcoplasmic proteins were extracted from pork *Longissimus dorsi* muscles as previously described (Santos et al., 2001). In the case of sarcoplasmic proteins based medium, two different media were prepared: in the first one a whole sarcoplasmic protein extract (WSPE) was added and in the second a sarcoplasmic protein extract in which the components under 10 kDa were eliminated by ultrafiltration (SPE>10 kDa) by using Amicon Centrifugal Filter Devices of 10 kDa cut off (Millipore, Bedford, MA, USA). The protein content of the sarcoplasmic extract was around 0.68 g/100 mL. Samples were taken every 2 days (2, 4, 6, 8, 10, 12, 14 days) during a total incubation period of 14 days and used for activity assays.

### 2.2. Preparation of cell extracts

Cells were harvested at 8500×*g* for 10 min, at 4 °C, washed in 20 mM sodium phosphate, pH 7.5, and then suspended in a volume of buffer to reach a final O.D. of 25 at 600 nm. This cell suspension was immediately frozen with liquid nitrogen and stored at −80 °C until use. Cell disruption was carried out in a Mini Bead-Beater (Biospec Products, Washington, DC, USA). A volume of 0.750 mL glass beads (0.5 mm diameter, Sigma, St. Louis, MO, USA) was added to the same volume of cell suspension. This mixture was submitted to four shakings