ENZYME ACTIVITY FOR THE GENERATION AND METABOLISM OF FREE AMINO ACIDS IN *Debaryomyces* spp.

Durá, M.A., Bolumar, T., Sanz, Y., Flores, M., Aristoy, M.C., Toldrá, F.
Instituto de Agroquímica y Tecnología de Alimentos (C.S.I.C.), Apt. 73, 46100 Burjassot (Valencia), Spain

Background

Proteolysis phenomena is an important factor contributing to flavour development during the processing of dry-fermented sausages. The roles of muscle and microbial proteases from lactic acid bacteria, *Micrococcaceae* and some moulds have been extensively reported but little information on the contribution of yeasts is available yet. However, the function of yeast proteases in the proteolytic pathway can be also important as a conditioning step for the further activity of amino acid converting enzymes from these organisms.

Free amino acids generated as a result of the proteolysis phenomena during the curing process of meat products (DeMasi et al., 1990) can be degraded later (Molly et al., 1997), contributing in this way to an increase in the ammonium and pH levels (Demeyer, 1992). Some researchers have indicated that yeasts favour flavour development in dry fermented sausages, specially because they produce an increase in the ammonium content and reduce the lactic and acetic acid amounts, with the concomitant suppression of the sour taste (Gehlen et al., 1991; Larpent-Gourgaud et al., 1993; Cook, 1995). The amino acids accumulated by yeasts are degraded to ammonium and/or glutamate (Henschke and Jiranek, 1993). This degradation is partly due to deamidating or deaminating reactions. Therefore, the activities of both metabolic routs (proteolysis and amino acid catabolism) in the yeast predominant in sausages, *Debaryomyces* spp., could be critical to modulate flavour development in dry-fermented sausages.

Objectives

The objective is to study the enzymes involved in the generation of free amino acids and their metabolism with further generation of ammonia and subsequent rise in pH. The final goal is the development of starter cultures with strains endowed with adequate enzymatic properties that allow the control of the excessive acidification and sour taste in dry-fermented sausages.

Methods

Proteolytic activity. Four strains of the species *Debaryomyces hansenii*, two isolated in our laboratory CECT 12487 and 12488 and two commercial ones C3 and C4, were screened for proteolytic activities. The yeasts were grown in a liquid medium composed by 13 g/ L meat extract and 5 g/ L NaCl, simulating the sausage environment, at 27 ºC for 3 days. Cells were harvested by centrifugation (3000 g, 10 minutes, 5 ºC) and then broken using a bead beater (Biospec Products, USA) to obtain cell free extracts. The activities to be determined were chosen based on previous studies of amino acid release in meat extracts (Santos et al., 2001) and own data (unpublished). The endoproteolytic activity was assayed against two synthetic substrates, 0.4 % (w/v) FITC (fluorescein isothiococyanate) casein in McIlvaine´s buffer (0.1 M citric acid and 0.2 M disodium phosphate) pH 5.5 and in 0.2 M Tris-HCl pH 9, and against 0.1 mM N-Succinyl LeuTyr-AMC in 0.2 M Tris-HCl, pH 8.0. The reaction mixture for all endoproteolytic activities consisted of 100 µL of enzymatic extract plus 70 µL of each substrate solution. The aminopeptidase activities were assayed against four substrates, Alanine-, Proline-, Leucine- and Arginine-AMC (AMC=7-amino-4-methylcoumarin) derivates, at 0.1 mM, in McIlvaine´s buffer at pH 8.0, 5.0, 6.5 and 6.5, respectively. The reaction was carried out adding 50 µL of enzymatic extract to 250 µL of substrate solution. The carboxypeptidase activity was assayed against 0.25 mM N-Benzoyl L-Tyr pNA (p-nitroanilide) in 0.2 M Tris-HCl, pH 6.5. In this case, 60 µL of enzymatic extract were added to 200 µL of substrate solution. The hydrolysis was monitored fluorimetrically for the FITC and AMC derivates at the excitation wavelengths of 485 and 355 nm and emission wavelength of 538 and 460 nm, respectively. The hydrolysis of colorimetric substrates (pNA derivatives) was determined by measuring the absorbance at a wavelength of 405 nm. The protein content was determined using the bicinchoninic acid method and bovine serum albumin as standard (Smith et al., 1985).

Deamidase and deaminase activity. These activities were studied in *Debaryomyces* spp. CECT 11815, which was originally isolated from dry-fermented sausages. The yeast was grown in 250 mL Erlenmeyer flasks containing 100 mL of the following medium (in g/L): malt extract, 3; yeast extract, 3; calcium lactate, 10; tryptone, 5, pH 6.7), at 27 ºC without shaking. Cells were harvested by centrifugation (7000g x 10 min), washed and suspended in the corresponding reaction buffer, described below. For measuring enzymatic activity, cell suspensions were incubated with the amino acid substrate in the reaction buffer, at 27 ºC for 3 hours. Simultaneously a blank, with the same composition but without incubation, was assayed for each substrate. The reaction buffers were: 50 mM Tris-HCl, pH 7.5, for assays with L-Gln and L-Asn, 100 mM potassium phosphate, pH 8.0, containing 0.05 mM pyridoxal 5-phosphate, for assays with L-Thr and L-Ser, 50 mM potassium phosphate, pH 7.5, containing 0.8 mM magnesium chloride, for assays with L-Asp, and 100 mM Tris-HCl, pH 8.8, containing 1 mM NAD^+, for assays with L-Glu. The amino acids were added to the reaction mixtures at the following concentrations: 10 mM L-Gln and L-Asn, 90 mM L-Thr and L-Ser, 30 mM L-Asp, and 60 mM L-Glu. The enzymatic reactions were stopped by adding perchloric acid to the incubated mixtures up to 0.6 M final concentration. Samples were neutralised with KOH and water was added up to a known final volume. Samples were kept at 4 ºC for 20 min and, then, filtered through a Millex AP 20 prefilter (Millipore). The ammonia present in the filtrate was analysed by the method of Bergmeyer and Beutler (1985), measuring the final absorbance in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech).

Results and discussion

The four *D. hansenii* strains, CECT12488, CECT12487, C3 and C4, showed both endo- and exoproteolytic activity (see table 1). The endoproteolytic activities were similar for all the strains but the strain CECT 12488 displayed slightly higher specific activity than the rest (Santos et al., 2001). In every case, the exoproteolytic activities of both amino- and carboxypeptidases were markedly higher than endoproteolytic activities. All tested strains showed the highest activities against Pro-, Leu- and Arg-AMC and the lowest against Ala-AMC, suggesting that their proteolytic system has the same composition. However, the intensity of these aminopeptidase activities was clearly different amongst strains and specially high in CECT 12487. The carboxypeptidase activity was higher in the strains CECT 12487 and 12488 but very poor in the commercial ones. These results empathize the importance of detailed metabolic studies for strain selection.

In summary, *D. hansenii*CECT 12487 showed the most active and complete proteolytic system, specially in relation to aminopeptidases whose purification and characterization is under investigation due to their possible roles in the generation of free amino acids that enhance sausage flavour.

The metabolic activity of *Debaryomyces* spp. on several amino acids (L-Gln, L-Asn, L-Thr, L-Ser, L-Glu and L-Asp) was determined at different growth phases. This strain was able to generate ammonia from all the assayed amino acids (Figures 1 and 2). So, it is possible that

Debaryomyces spp. produces deaminase enzymes acting on L-Thr, L-Ser, L-Glu and L-Asp and deamidase enzymes acting on L-Asn and L-Gln. Moreover, the development of these activities depends on the growth phase, especially in the case of activities on L-Thr, L-Glu and L-Gln. In consequence, the enzymes responsible for these activities are probably generated, above a basal level, in response to changes in the composition of medium. A detailed biochemical study of some of these enzymes is also being carried out to get a better knowledge of their possible function in flavour development in dry-fermented sausages.

Conclusions

The results from the screening of enzyme activities of different *Debaryomyces* strains for the generation of free amino acids and their metabolism reveal important differences in the intensity and potential function of those strains to complete the proteolytic chain to free amino acids and further production of ammonium resulting in pH increases. The coordinate function of these enzymes may contribute to modulate other enzyme activities, reduce acidification and, as a whole, improve flavour. Further studies on the biochemistry of these enzymes and their application will allow the design of mixtures of yeast starter cultures with improved properties.

Pertinent literature

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Table 1. Comparison of the endo- and exoproteolytic activity (μmol hydrolysed/ mg protein hour) of four strains of *Debaryomyces hansenii.*

	pH	CECT 12488	CECT12487	C3*	C4*
FITC-casein	5.5	8.3	7.8	7.8	6.4
FITC-casein	9.0	1.8	1.2	1.1	1.1
N-SuccinylLeuTyr-AMC	8.0	6.2	3.4	4.0	2.8
Alanine-AMC	8.0	132.5	96.6	132.2	58.5
Proline-AMC	5.0	196.1	235.6	231.0	147.5
Leucine-AMC	6.5	180.1	356.0	233.7	147.5
Arginine-AMC	6.5	191.1	360.4	227.2	138.0
N-BenzoylL-Tyr-pNA	6.5	46.6	61.9	10.0	0.0

*C3 and C4 are commercial yeast strains.

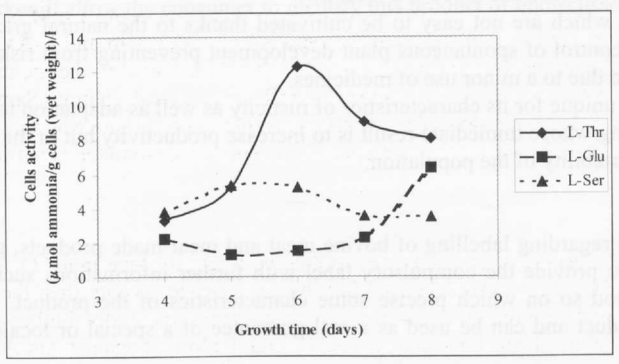

Figure 1. Ammonia generation by *Debaryomyces* spp. in the presence of either L-Threonine, L-Glutamic acid or L-Serine

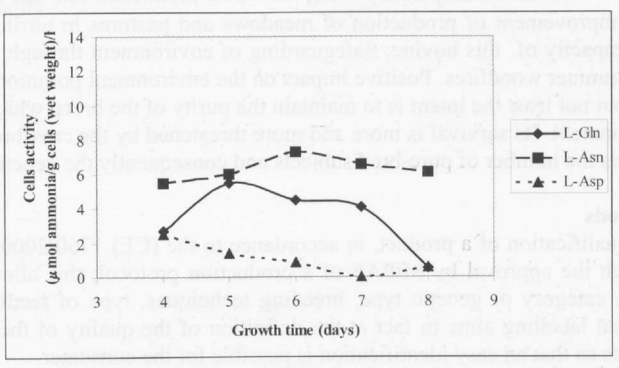

Figure 2. Ammonia generation by *Debaryomyces* spp. cells in the presence of either L-Glutamine, L-Asparagine or L-Aspartic acid