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Hydrolysis of pork muscle sarcoplasmic proteins by *Debaryomyces hansenii*

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Abstract

Strains of *Debaryomyces hansenii* originally isolated from sausages were screened for proteinase and aminopeptidase activity towards synthetic substrates. On the basis of these results, *D. hansenii* CT12487 was selected for further assays. The activities of the whole cells (WC), cell-free extracts (CFE) and a combination of both from the selected strain on pork muscle sarcoplasmic protein extracts were determined by protein, peptide and free amino acid analyses. There was a pronounced hydrolysis of protein bands of 110 kDa and 27–64 kDa regardless the incorporation of WC, CFE or a combination of both. The proteolytic activity also resulted in the generation of polar and non-polar peptides showing noticeable differences depending on the addition of WC or CFE. Whole cells generated greater amounts of free amino acids than the cell-free extracts.

Keywords: Hydrolysis; *Debaryomyces hansenii*; Sausages

1. Introduction

Dry-fermented sausages constitute a typical Mediterranean food product. Its quality depends on many factors such as proteolysis, which is related to the final flavor and texture, one of the most important. However, the knowledge of the role of the enzymatic systems involved in proteolysis during the fermentation and ripening of meat products is still limited. It has been suggested that endogenous cathepsin D initiates protein degradation followed by the action of microbial peptidases that further degrades the protein fragments initially generated (Molly et al., 1997). Thus, the implication of microorganisms in these biochemical changes depends on the proteolytic capacity of the strains used as starter cultures. In this sense, the action of the proteolytic system of different lactobacillus such as *Lactobacillus sake*, *L. curvatus*, *L. plantarum* and *L.*

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casei on both sarcoplasmic and myofibrillar proteins have been recently studied (Fadda et al., 1999a,b; Sanz et al., 1999a,b).

In addition to lactic acid bacteria, yeast may also proliferate during the fermentation and ripening of sausages, thereby contributing to the final quality of the product. *Debaryomyces hansenii* is the most frequent yeast isolated from dry-fermented sausages (Cook, 1995; Santos-Mendonça, 2000) and it is commercialized as starter culture. The lipolytic and proteolytic activity of yeast has been considered important for the quality of fermented meats. However, little is known presently about the proteolytic system of *D. hansenii* while it has been deeply studied in *Saccharomyces cerevisiae* (Lurton et al., 1989; Jones, 1991; Slaughter and Nomura, 1992) where several proteinases and exopeptidases have been identified.

The objective of this work is to study the activity of the proteolytic system of *D. hansenii* on pork muscle sarcoplasmic proteins and synthetic substrates in order to better understand its impact during meat fermentation and provide additional information for evaluating its suitability as a starter culture for sausages.

2. Materials and methods

2.1. Yeast strains and growth conditions

The strains of *D. hansenii* CECT 12487 and CECT 12488, previously isolated from sausages (Santos-Mendonça, 2000), and the commercial starter culture PRISCA (Texel, Dangé Saint Romain, France) were used. Yeast counts were determined in Yeast Malt agar (Adsa-Micro, Barcelona, Spain) after incubation at 27°C, for 48 h. The growth medium used when the proteolytic activity of the strains was tested consisted of 13 g/l meat extract (Merck, Darmstadt, Germany) and 5 g/l NaCl, pH 6.5. The strains were grown aerobically at 27°C, for 48 h to a final level of 10^8 cfu/g. Cells were harvested by centrifugation ($6000 \times g$, 5 min, 4°C), washed in 50 mM sodium phosphate, pH 6.5, and re-suspended in the same buffer (10% initial volume). This sample was designated whole cell suspension (WC). Cell disruption was carried out in a Mini Bead-Beater (Biospec Products, USA) using an equivalent volume of glass beads (0.5 mm diameter, Sigma, St. Louis, MO, USA). Cells were submitted to three shakings for 45 s each, with 15-s intervals on ice. Glass beads, non-broken cells and debris were separated by centrifugation ($10\,000 \times g$, 15 min, 4°C) and the supernatant was designated cell-free extract (CFE).

2.2. Proteinase and aminopeptidase activity on synthetic substrates

The proteinase and aminopeptidase activity of each fraction was assayed against synthetic substrates. The proteinase activity was assayed against casein-fluorescein isothiocyanate (FITC) Type II (Sigma) following the procedure described by Twining (1984) with slight modifications (Sanz et al., 1999a,b). One hundred microliter of the enzymatic sample was added to 70 μl of 0.4% (w/v) casein-FITC in 50 mM sodium phosphate buffer (pH 6.5) containing 2 mM $CaCl_2$ and incubated at 37°C for 60 min. Fluorescence was measured in a Fluoroskan fluorometer (Labsystems, Finland) at 485 and 538 nm as excitation and emission wavelengths, respectively. The aminopeptidase activity was determined against several aminoacyl-7amido-4-methyl coumarin (AMC) derivatives: L-Ala, L-Lys-, L-Ser-, L-Phe-, L-Arg-, L-Gly-, L-Leu-, L-Tyr-, L-Met-AMC (Sigma) and L-Glu-1-4-*p*-nitroanilide (*p*-NA) (Fluka Biochemika, Buchs, Switzerland) as previously described (Sanz and Toldrá, 1997a,b). Fifty microliters of enzymatic sample was mixed with 250 μl of either 0.12 mM AMC substrate or 1 mM *p*-NA substrate in 50 mM sodium phosphate buffer (pH 6.5) and incubated at 37°C for 60 min. AMC fluorescence was measured at 355 and 460 nm as excitation and emission wavelengths, respectively. Absorbance produced by *p*-NA was measured at 405 nm in a spectrophotometer.

In all cases, assays were performed in triplicate. One unit of activity (U) was defined as the amount of enzyme hydrolyzing 1 μmol of substrate per hour at 37°C. The proteinase and aminopeptidase activities were expressed as units (U) per milligram of protein.

Table 1
Proteinase and aminopeptidase activities[a] of *D. hansenii* on synthetic substrates

Substrate	Strains		
	CECT 12487	CECT 12488	Commercial starter
Casein-FICT	7.65 ± 0.65	18.89 ± 1.07	19.75 ± 1.36
Ala-AMC	43.09 ± 2.73	22.66 ± 2.13	26.45 ± 2.69
Lys-AMC	179.13 ± 15.43	131.03 ± 8.26	136.92 ± 8.15
Ser-AMC	1.78 ± 0.48	nd[b]	nd
Phe-AMC	42.14 ± 2.30	30.35 ± 1.14	36.51 ± 1.95
Arg-AMC	192.67 ± 12.00	89.44 ± 6.34	110.40 ± 10.01
Gly-AMC	2.16 ± 0.19	nd	nd
Leu-AMC	138.70 ± 10.75	81.70 ± 3.41	115.78 ± 10.70
Tyr-AMC	18.17 ± 1.62	8.87 ± 0.62	11.76 ± 1.31
Met-AMC	120.16 ± 5.30	59.10 ± 3.56	82.59 ± 7.62
Glu-*p*-NA	7.95 ± 0.72	9.37 ± 1.39	11.25 ± 2.04

[a] Proteinase and aminopeptidase activities, expressed as *U*. 10^3 per mg protein, were determined in CFE. The values are the mean of three independent assays ± SD.
[b] Nondetected.

2.3. Protein concentration

The concentration of protein was determined by the bicinchoninic acid method, using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Bovine serum albumin was used as the standard.

2.4. Proteolytic activity on muscle protein extract

2.4.1. Extraction of sarcoplasmic muscle proteins

Sarcoplasmic proteins were extracted from *Longissimus dorsi* muscles with 10 volumes of 2 mM sodium phosphate buffer, pH 6.5, as described elsewhere (Molina and Toldrá, 1992, Sanz et al., 1999a,b). The extract was filtered sterilized through a 0.22-μm pore-size membrane (Millipore, Bedford, MA, USA). The protein content of the sarcoplasmic extract was around 4.2 mg/ml.

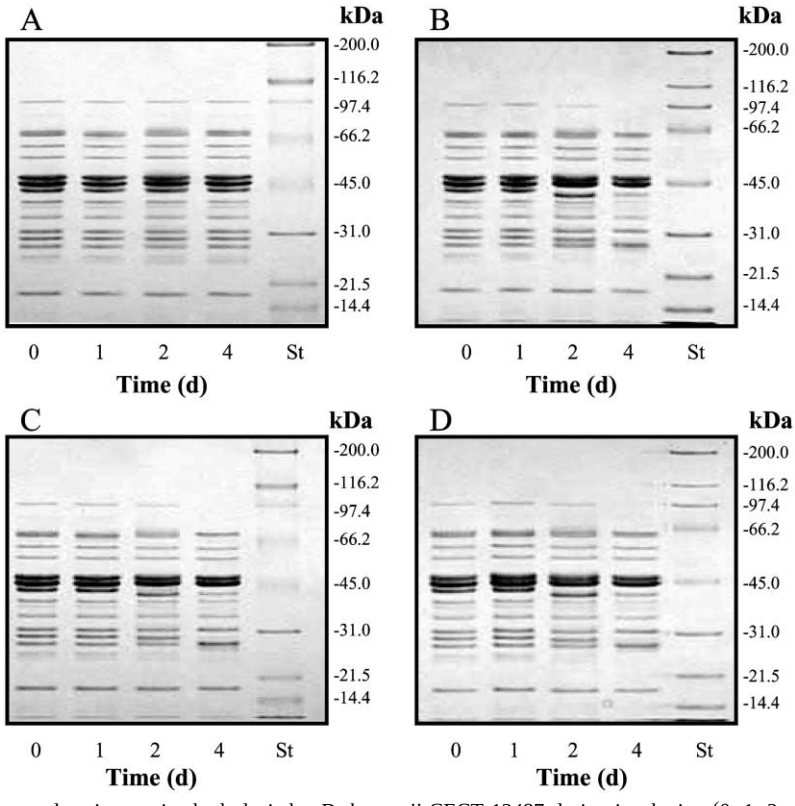


Fig. 1. SDS–PAGE of sarcoplasmic proteins hydrolysis by *D. hansenii* CECT 12487 during incubation (0, 1, 2 and 4 days) at 27°C. (A) Control, (B) whole cells, (C) cell-free extract and (D) whole cells + cell-free extract. St lane: standard proteins.

2.4.2. Enzymatic mixtures

Three independent assays were carried out using as an enzymatic source either whole cells (WC), cell-free extracts (CFE) or a combination (1:1) of both (WC + CFE). The enzymatic mixture consisted of 150 ml of sterile sarcoplasmic protein extract + 2.5 ml of the corresponding enzymatic sample. A control protein extract without the addition of any enzymatic sample was assayed simultaneously. The mixtures were incubated at 27°C. Samples were taken at different times during the incubation period (0, 24, 48 and 96 h) for further analyses.

2.4.3. Yeast counts and pH measurements

Yeast counts were determined in the control and each protein extract upon addition of WC or CFE during incubation period (0, 24, 48 and 96 h) on Yeast Malt agar as described above. The pH of the reaction mixtures was periodically monitored.

2.4.4. Gel electrophoresis

The hydrolysis of sarcoplasmic proteins in the extracts was determined by sodium dodecyl sulphate gel electrophoresis (SDS–PAGE) analysis (Laemmli, 1970) with 3% stacking and 12% resolving polyacrylamide gels. Broad range molecular weight standards were ran simultaneously (Bio-Rad, Hercules, CA, USA). The standard proteins were myosin (200 kDa), β-galactosidase (116.5 kDa), phosphorilase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhidrase (31.0 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Proteins were visualised by staining with Coomassie Brilliant Blue R-250.

2.4.5. Peptide analysis

The evolution of the peptide contents in protein sarcoplasmic extract was analyzed in a 1050 Hewlett-Packard liquid chromatograph (Palo Alto, CA, USA), equipped with a diode array detector and an automatic injector. Three milliliters of each sample was de-proteinized with 7.5 ml of acetonitrile. After 60 min in the freezer, the sample was centrifuged and the supernatant concentrated by vacuum evaporation. Samples were re-suspended in 200 μl of solvent A, 0.1% (v/v) trifluoroacetic acid (TFA) in MilliQ water, before analyzed. Samples of 20 μl were applied onto a Waters Symmetry C18 (4.6 × 250 mm) column (Waters, Milford, MA, USA). The eluate system consisted of solvent A, described above, and solvent B, acetonitrile/water/TFA, 60:40:0.085% (v/v). The elution was performed as follows: a 5-min isocratic step with 1% of solvent B followed by a 20-min linear gradient to 100% of solvent B, at a flow rate of 0.9 ml/min and 40°C. Peptides were detected at 214 nm. Spectral data from 200 to 350 nm were acquired for all peaks along the chromatogram to detect the presence of nucleotides and nucleosides.

2.4.6. Amino acid and natural dipeptide analysis

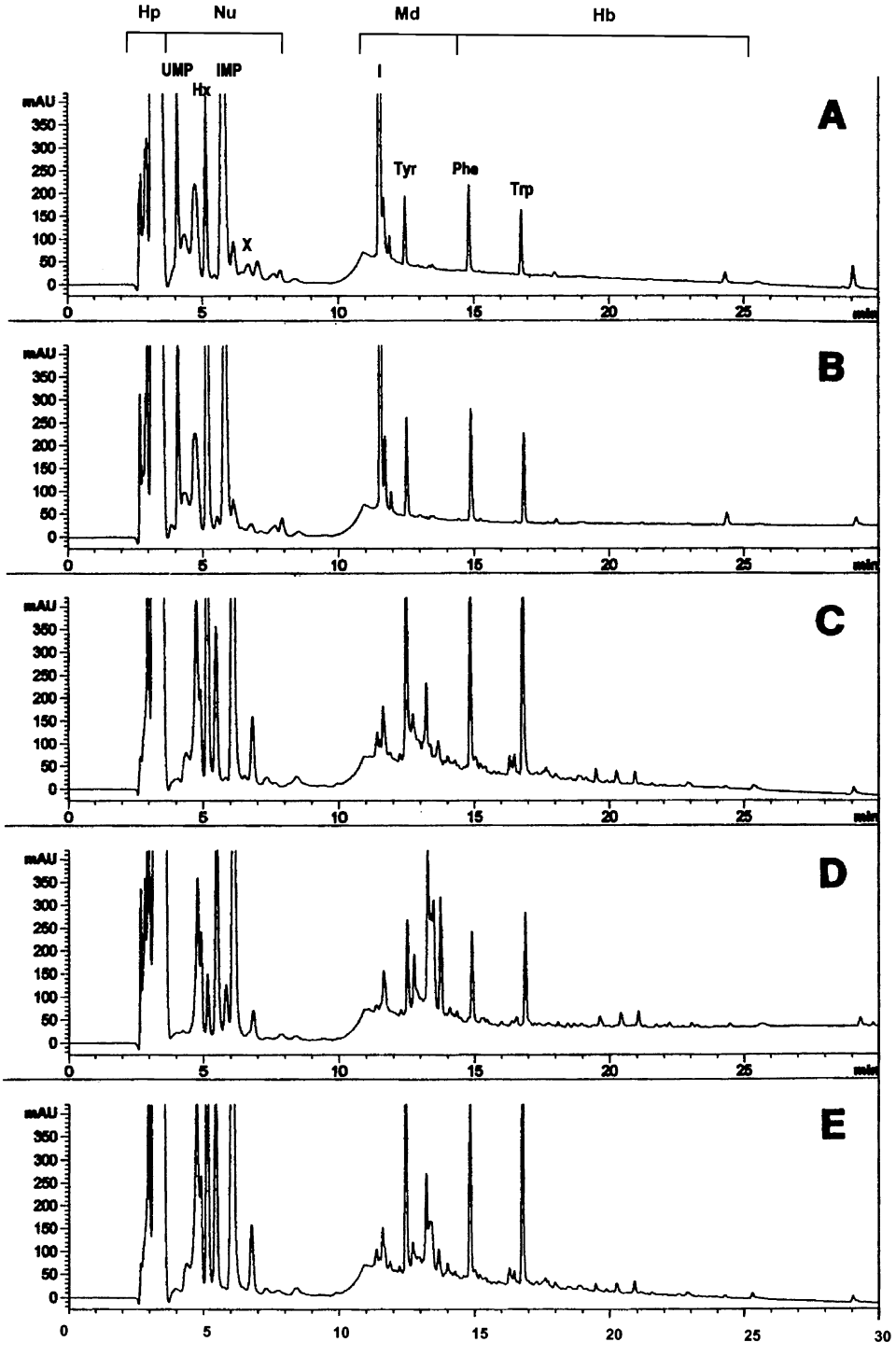
The changes in free amino acids and natural dipeptides content in muscle extracts were also monitored. Duplicate samples of 500 + 50 μl of an internal standard (0.325 mg/ml hydroxyproline) were de-proteinized with 1375 μl of acetonitrile. The supernatant (200 μl) was derivatized to their phenylthiocarbamyl derivatives according to the method of Bidlingmeyer et al. (1987). The derivatized amino acids were analyzed by reverse-phase HPLC in a Waters Nova Pack C18 (3.9 × 300 mm) column (Waters) according to the method of Flores et al. (1997) and final concentrations expressed as microgram per 500 μl of extract.

3. Results and discussion

3.1. Screening for proteinase and aminopeptidase activity

Maximal proteinase and aminopeptidase activities were detected in CFE from the strains of *D. hansenii*

Fig. 2. Reverse-phase HPLC patterns of soluble peptides contained in sarcoplasmic protein extracts treated with *D. hansenii* CECT 12487 at 0 and 4 days of incubation at 27°C. (A) Control at 0 h, (B) control at 4 days, (C) whole cells at 4 days, (D) cell-free extract at 4 days and (E) whole cells + cell-free extract at 4 days. Hydrophilic (Hp), Nucleotide (Nu), Medium polarity (Md) and Hydrophobic (Hb) regions indicated on top of the figure. Identified peaks: Uridin monophosphate (UMP), Hypoxanthine (Hx), inosin monophosphate (IMP), Xanthine (X), Inosine (I), Tyrosine, (Tyr), Phenylalanine (Phe) and Tryptophane (Trp).


Hp
Nu
Md
Hb
UMP
IMP
Hx
I
X
Tyr
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Trp
mAU
A
B
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min

against synthetic substrates (Table 1). The three strains, especially the CECT 12488 strain and the commercial starter, showed a high proteinase activity against the FITC-labeled casein. The aminopeptidase activity was also very high. The CECT 12487 strain showed the highest activity and widest specificity. The amino acids hydrolized at higher rates were lysine, arginine, leucine and methionine. Alanine and phenylalanine were hydrolyzed at intermediate rate. *D. hansenii var. hansenii* CECT 12487 was selected based on its high aminopeptidase activity for further assays with sarcoplasmic proteins.

3.2. Proteolysis on sarcoplasmic protein extracts

3.2.1. pH evolution

The initial pH of the homogenates was 5.8 and remained constant for the whole incubation period of controls. In the trials, the pH of the homogenate increased after 48 h of incubation, reaching final pH values of 6.6–6.7 for extracts containing WC and WC + CFE and as high as 7.4 for those containing CFE (results not shown).

3.2.2. SDS–PAGE analysis

The protein profiles resulting from the hydrolysis of muscle sarcoplasmic proteins by *D. hansenii* CECT 12487 are shown in Fig. 1. No major proteolytic changes were observed in the control samples (Fig. 1A) confirming previous results (Fadda et al., 1999a,b), so that a significant contribution from endogenous muscle proteinases could be discarded. The incubation of sarcoplasmic proteins either with the intact whole cell suspension (WC, see Fig. 1B), the cell-free extract (CFE, see Fig. 1C) or the combination of both (see Fig. 1D) resulted in similar protein patterns. A severe degradation of protein bands of 101 kDa and the appearance of a fragment at 42.5 were detected. In addition, a partial hydrolysis of protein bands at approximately 64, 44, 40, 34.4 and 29.5 kDa was observed (Fig. 1B, C and D). These results confirm the ability of *D. hansenii* CECT 12487 to use muscle sarcoplasmic proteins as substrates.

3.2.3. Peptide analysis

Peptide reverse-phase HPLC chromatograms resulting from the proteolytic activity of *D. hansenii* CECT 12487 on sarcoplasmic proteins are shown in Fig. 2. As a general comment, some peaks corresponding to non-peptide compounds were easily identified based on their respective retention times and spectral characteristics. These compounds are indicated in the figure. The peaks eluted in the hydrophilic region corresponded to polar amino acids and very polar peptides such as the dipeptides carnosine and anserine. Those peaks eluted in the Nu region (4 and 8 min) were nucleotide derivatives that could mask some peptides and free amino acids. Other late-eluting peaks would be peptides distributed along the chromatogram as a function of their polarities (medium polarity and hydrophobic regions) except some peaks around 11.5 that would correspond to nucleosides as inosine. Control samples containing only the sarcoplasmic protein extract showed minor changes after 96 h of incubation (Fig. 2B vs. A). In fact, only a small increase in the free amino acids tyrosine, phenylalanine and tryptophane, and in the most hydrophobic region was observed. When whole cells were inoculated to the sarcoplasmic protein extract, there were very drastic changes after 96 h of incubation (Fig. 2C) in the nucleotide derivative profile (Nu region and at 11.5 min) presumably due to a strong yeast nucleotide metabolism. Noticeable changes in the peptide profile were observed in the peaks eluting in the hydrophilic region and in those eluted between 11 and 12 min. Several peptides appeared along the chromatogram eluting in the medium and hydrophobic regions as well as a high increase in the amino acids tyrosine, phenylalanine and tryptophane. When only CFE was added (Fig. 2D), and after 96 h of incubation, a different quantitative and qualitative peptide profile was obtained in comparison to the action of WC. So, there was a bigger generation of the most polar peaks (polar small peptides or polar amino acids) and peptide peaks eluting in the medium region, but a lower amount of peptides eluting in the hydrophobic region and of the amino acids tyrosine, phenylalanine and tryptophane (see Fig. 2D vs. C). The inoculation of both, WC and CFE together (Fig. 2E), resulted in an intermediate profile, more similar to that obtained after the addition of WC (Fig. 2C).

3.2.4. Amino acid analysis

The generation of free amino acids and natural dipeptides in the homogenates due to the activity of

D. hansenii CECT 12487 is shown in Table 2. Increases bigger than 30% of almost all amino acids were observed in the controls incubated at 27°C for 96 h. The incubation of the sarcoplasmic extract with WC contributed to a higher generation of nearly all amino acids. Different changes occurred when the sarcoplasmic extract was incubated with the CFE. A net generation of amino acids due to the presence of the enzymes from the CFE was only obtained for aspartic acid, histidine and, in a minor scale, for isoleucine and leucine. In general, free amino acids and dipeptides were degraded by the enzymes present in the CFE. The combination of both WC and CFE produced a very similar effect than in the case of adding only the WC but, in general, with lower intensity.

The activity of yeast peptidases, which have optimum pH in the range 7–8 (Lurton et al., 1989), could be favoured by the pH increase till 6.6–6.7 caused by the addition of WC and WC + CFE and 7.4 by CFE, observed during the incubation of the homogenates. This increase, approaching the optimal pH of the peptidases from yeast characterized to date, and the previous action of endoproteases promoted their activity especially in the last 2 days of incubation.

In summary, this study constitutes an initial approach to the proteolytic activities of *D. hansenii*, a yeast present in dry-fermented sausages. This yeast has shown good potential for the hydrolysis of sarcoplasmic proteins and the generation of several polar and non-polar peptides and free amino acids. Further research is needed for a better comprehension on the specific role of these enzymes and how they interact with bacterial and muscle proteolytic activities during meat fermentation.

Table 2
Net increments (Δ, expressed as μg in 500 μl of extract) and relative increase respect to the initial concentrations (RI in %) in free amino acids and natural dipeptides after 4 days of incubation at 27°C with *D. hansenii* CECT 12487

Amino acid	Control		WC[a]		CFE[b]		WC + CFE	
	Δ	RI	Δ	RI	Δ	RI	Δ	RI
Aspartic acid	0.03	10.8	0.18	50.3	0.52	165.8	0.18	52.9
Glutamic acid	3.02	98.8	10.90	320.2	−0.65	−18.6	9.29	265.4
Serine	1.20	67.1	−1.70	−89.9	−1.16	−69.6	−1.54	−87.5
Asparagine	0.32	23.6	−1.04	−100.0	−0.93	−72.8	−1.16	−100.0
Glycine	1.21	29.7	2.48	57.5	−0.87	−20.6	2.00	47.0
Glutamine	9.14	15.8	−59.80	−98.2	−55.57	−98.0	−58.44	−98.2
β-Alanine	0.26	21.7	0.05	3.8	1.17	99.0	0.08	7.0
Histidine	0.78	64.6	2.37	195.0	3.34	309.6	2.02	166.0
α-ABA	0.25	26.3	−0.06	−6.3	−0.05	−9.9	0.62	130.8
Threonine	1.94	37.9	−0.31	−3.9	−0.23	−4.9	1.19	22.0
Alanine	3.66	43.7	3.64	40.4	−5.09	−60.0	3.52	40.4
Arginine	2.75	63.7	−3.01	−60.2	−5.02	−100.0	−3.16	−61.7
Proline	1.28	67.9	1.90	92.2	−1.19	−59.0	1.66	81.2
Tyrosine	0.83	68.3	4.74	348.8	0.82	65.1	3.13	232.5
Valine	1.53	63.9	6.59	236.5	1.71	72.4	4.48	164.8
Methionine	0.52	60.0	1.59	152.8	0.30	41.9	1.01	92.4
Isoleucine	0.53	38.1	5.91	395.1	1.66	131.0	3.81	268.5
Leucine	0.99	48.3	8.40	390.1	2.04	96.5	5.94	273.4
Phenylalanine	0.67	42.9	3.79	220.8	0.18	11.2	2.36	139.6
Lysine	1.46	76.7	5.46	261.7	1.36	67.3	5.48	261.1
Taurine	1.71	17.6	2.02	18.6	1.56	15.6	2.41	23.9
Carnosine	34.49	17.8	28.54	13.7	−7.46	−3.7	34.06	16.9
Anserine	0.93	17.2	1.14	19.02	−0.63	−10.6	1.18	19.9

[a] WC = Whole cells.
[b] CFE = cell free extract; α-ABA = α-aminobutiric acid.

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References

Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L., Forst, B.A., 1987. A new, rapid, high sensitivity analysis of amino acids in food type samples. J. Assoc. Off. Anal. Chem. 70, 241–247.

Cook, P.E., 1995. Fungal ripened meats and meat products. In: Campbell-Plott, G., Cook, P.E. (Eds.), Fermented Meats. Chapman & Hall, Glasgow, UK, pp. 110–129.

Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldrá, F., 1999a. Hydrolysis of pork muscle sarcoplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. Appl. Environ. Microbiol. 65, 578–584.

Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldrá, F., 1999b. Characterization of muscle sarcoplasmic and myofibrillar protein hydrolysis caused by *Lactobacillus plantarum*. Appl. Environ. Microbiol. 65, 3540–3546.

Flores, M., Aristoy, M.-C., Spanier, A., Toldrá, F., 1997. Non volatile component effects on quality of Serrano dry cured ham as related to processing time. J. Food Sci. 62, 1235–1239.

Jones, E.W., 1991. Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. 266, 7963–7966.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lurton, L., Segain, J.P., Feuillat, M., 1989. Proteolysis during the autolysis of yeasts under acidic conditions. Sci. Aliment. 9, 111–124.

Molina, I., Toldrá, F., 1992. Detection of proteolytic activity in microorganisms isolated from dry cured ham. J. Food Sci. 61, 1308–1310.

Molly, K., Demeyer, D., Johansson, G., Raemaekers, M., Ghistelink, M., Geenen, I., 1997. The importance of meat enzymes in ripening and flavour generation in dry fermented sausages. First results of a European project. Food Chem. 59, 539–545.

Santos-Mendonça, R.C. 2000. Aislamiento, selección y caracterización de levaduras de embutidos con vistas a su utilización como coadyuvante en el proceso de curado. PhD thesis. Universidad de Valencia, Spain.

Sanz, Y., Toldrá, F., 1997a. Purification and characterization of an aminopeptidase from *Lactobacillus sake*. J. Agric. Food Chem. 45, 1552–1558.

Sanz, Y., Toldrá, F. 1997b. Activities of aminopeptidases from *Lactobacillus sake* in models of curing ingredients and processing conditions for dry sausage. J. Food Sci. 62, 1211–1213, 1234.

Sanz, Y., Fadda, S., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldrá, F., 1999a. Muscle myofibrillar proteins as substrates for *Lactobacillus curvatus* and *Lactobacillus sake*. Int. J. Food Microbiol. 53, 115–125.

Sanz, Y., Fadda, S., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldrá, F., 1999b. Hydrolytic action of *Lactobacillus casei* CRL 705 on pork muscle sarcoplasmic and myofibrillar proteins. J. Agric. Food Chem. 47, 3441–3448.

Slaughter, C., Nomura, T., 1992. Activity of the vacuolar proteases of yeast and the significance of the cytosolic protease inhibitors during the post-fermentation decline phase. J. Inst. Brew. 98, 335–338.

Twining, S.S., 1984. Fluorescein isothiocyanate-labelled casein assay for proteolytic enzymes. Anal. Biochem. 143, 30–34.