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# Hydrolytic Action of *Lactobacillus casei* CRL 705 on Pork Muscle Sarcoplasmic and Myofibrillar Proteins

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*Lactobacillus casei* CRL 705 was screened, among other meat isolates, for its proteinase and aminopeptidase activities toward synthetic substrates and, according to that, selected for specific assays on muscle proteins. The hydrolytic effects of whole cells, cell free extracts (CFE), and the combination of both on muscle sarcoplasmic and myofibrillar protein extracts was evaluated by SDS–PAGE and reverse phase HPLC analyses. The proteinase activity of whole cells caused the degradation of a great number of sarcoplasmic protein bands. A partial hydrolysis was also associated with CFE that when combined with whole cells showed an important additional degradation. Peptide profiles from sarcoplasmic protein extracts were greatly modified regardless of the addition of whole cells or CFE, although their combination intensified these changes. The generation of free amino acids was remarkable when whole cells and CFE were incorporated together to sarcoplasmic protein extracts.

**Keywords:** *Muscle proteins; proteases; peptidases; lactobacilli; sausages*

## INTRODUCTION

The biochemical pathway of proteolysis during the processing of cured-meat products is of paramount importance for the development of their characteristic flavors. The generated small peptides and free amino acids constitute important nonvolatile compounds with an impact in taste (Verplaetse, 1994; Toldrá and Flores, 1998). Free amino acids are involved in further enzymatic and chemical reactions constituting also the origin of other aroma volatile compounds (Maga, 1982; Shahidi et al., 1986). The enzymology of dry fermented sausages is quite complex due to the coexistence of enzymes from both endogenous and microbial origin. Muscle proteinases appear to be the main enzymes responsible for the initial breakdown of sarcoplasmic and myofibrillar proteins, such as myosin and actin, which are substrates for endogenous cathepsins (Molly et al., 1997). Nevertheless, the complete hydrolysis of oligopeptides is achieved by the activities of both endogenous and microbial peptidases (Toldrá and Verplaetse, 1995; Molly et al., 1997).

In recent years, the proteolytic system of lactobacilli involved in meat fermentation is becoming the focus of an increasing number of studies due to the technological roles of these organisms (Montel et al., 1995; Sanz and Toldrá, 1997a–c; Sanz et al., 1998; Sanz and Toldrá, 1998a,b). However, the system of dairy lactic acid bacteria has been explored in much more detail. Thus, most of the proteases from *Lactobacillus helveticus* and *Lactobacillus delbrueckii* have been purified and characterized (Kunji et al., 1996). In contrast, *Lactobacillus casei*, involved in the production of not only dairy but also meat products, has been the objective of fewer studies and confined to dairy strains (Kojic et al., 1991; Arora and Lee, 1992; Naes and Nissen-Meyer, 1992; Habibi-Najafi and Lee, 1994; Fernandez-Espla and Martin-Hernandez, 1997; Fernandez-Espla et al., 1997a,b). The bacteriocinogenic strain *L. casei* CRL 705, originally isolated from sausages, is being submitted to a thorough investigation for its potential as a starter culture in sausage production (Vignolo et al., 1988, 1995). Moreover, little is known about the proteolytic enzymes of this or other meat isolates belonging to the quoted species and their activity against muscle proteins and derived oligopeptides.

Thus, the main objective of this work is to study the effects of whole cells, cell-free extracts (CFE), and the combination of both from *L. casei* CRL 705 on muscle sarcoplasmic and myofibrillar proteins as an attempt to gain better knowledge of their hydrolytic ability and the nature of the generated products in relation to cured-meat flavor.

## EXPERIMENTAL PROCEDURES

**Bacterial Strains and Culture Conditions.** The strains of *L. casei* CRL 704, CRL 705, and CRL 678, originally isolated from sausages, were used for proteolytic assays. All strains were routinely grown in MRS broth (Merck, Darmstadt, Germany) at 30 °C, for 24 h and then maintained at either 4 or −80 °C in 15% (v/v) glycerol. For enzymatic assays cultures were 1% (v/v) inoculated with the microorganism, previously subcultured twice, and incubated for 16 h at 30 °C.

**Preparation of Cell Suspensions and Extracts.** The proteinase activity against casein–fluorescein isothiocyanate

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(FITC) was assayed in whole-cell suspensions. Cells were harvested by centrifugation (10000*g* for 20 min at 4 °C), washed twice in 0.085% (w/v) NaCl, containing 20 mM $CaCl_2$, and resuspended in 2% initial volume of 50 mM Tris-HCl, pH 6.5. The optical density of cell suspensions was determined at 660 nm, and the corresponding dry weight was deduced from a calibration curve.

The aminopeptidase activity was assayed using CFE obtained by a modification of the procedure described by Sanz and Toldrá (1997b). Cells were collected as stated above, washed twice in 20 mM phosphate buffer, pH 7.0, and resuspended in the same buffer (10% of initial volume) supplemented with 0.6 M sucrose and 5 mM $MgCl_2$, containing also 1 mg/mL lysozyme (Sigma, St. Louis, MO). After incubation at 30 °C for 1 h, the cell-wall fraction was removed by centrifugation (15000*g* for 20 min at 4 °C). The pellet was washed in 20 mM phosphate buffer, pH 7.0, resuspended in the same buffer, and sonicated for 15 min. Cell debris was removed by centrifugation (20000*g* for 20 min at 4 °C), and the supernatant constituted the CFE.

**Assay of Proteinase and Aminopeptidase Activities toward Synthetic Substrates.** Proteinase activity was determined using casein-FITC type II (Sigma) as substrate, by a modification of the procedure described by Twining (1984). The reaction mixture, consisting of 70 $\mu$L of 50 mM Tris-HCl, pH 6.5, containing 0.4% casein-FITC and 20 mM $CaCl_2$, and 100 $\mu$L of whole-cell suspension, was incubated at 37 °C for 1 h. The resulting fluorescence was measured in a multiscan fluorometer (Fluoroskan II, Labsystems) at 485 and 538 nm as excitation and emission wavelengths, respectively. One unit of activity was defined as the amount of enzyme hydrolyzing 1 $\mu$mol of substrate per hour at 37 °C. Proteinase activity was expressed as units per milligram of dry weight.

Aminopeptidase activity was measured against several aminoacyl-7-amido-4-methylcoumarin (AMC) derivatives (L-Ala, L-Lys-, L-Ser-, L-Met, L-Phe-, L-Val-, L-Arg-, L-Gly-, L-Leu-, L-Tyr-, L-Pro-, l-Pyr-AMC, from Sigma) and L-Glu-1−4-*p*-nitroanilide (pNA) (Fluka Biochemika, Buchs, Switzerland) according to the method of Sanz and Toldrá (1997a,b). Every reaction mixture was incubated at 37 °C for 15 min except for the chromogenic substrate, which was incubated for 1 h. One unit of activity was defined as described above, and aminopeptidase activity was expressed as units per milligram of protein. Assays were made per quadruplicate with four samples and controls measured for each experimental point.

**Determination of Protein Concentration.** The protein concentration was determined according to the bicinchoninic acid method, using the BCA protein assay reagent (Pierce, Rockford, IL). Bovine serum albumin was used as the standard.

**Activity on Muscle Protein Extracts.** *Extraction of Muscle Proteins.* Sarcoplasmic proteins were extracted according to the method described by Molina and Toldrá (1992) but using 20 mM phosphate buffer, pH 6.5, for homogenization. The final extract was filter sterilized through a 0.22 $\mu$m membrane (Millipore, Bedford, MA). The protein content of the sarcoplasmic extract was 1.80 mg/mL. To prepare the myofibrillar extract the pellet resulting from the sarcoplasmic protein extraction was resuspended in 100 mL of 0.03 N phosphate buffer, pH 6.5, previously sterilized, and homogenized for 4 min in a Stomacher 400 blender (London, U.K.). After centrifugation (10000*g* for 20 min at 4 °C), the pellet was washed three times in the same buffer to remove muscle proteinases. The resulting pellet was weighed, resuspended in 9 volumes of 0.1 N phosphate buffer with 0.7 M KI, pH 6.5, containing 0.02% sodium azide, and homogenized for 8 min in a Stomacher 400 blender. After the last centrifugation (10000*g* for 20 min at 4 °C), the supernatant obtained was diluted 10 times in the same buffer for enzymatic assays to prevent the possible inhibition of bacterial proteases by KI. The protein content of this myofibrillar extract was 0.75 mg/mL. In both cases, the sterility of the extract was confirmed by determining the absence of bacterial growth in plate count agar (Merck) as described below.

*Enzymatic Mixtures.* Three independent assays were carried out for each protein extract (sarcoplasmic and myofibrillar) using as enzymatic sample either whole-cell suspensions, CFE, or a combination (1:1) of both. The reaction mixture consisted of 6 mL of whole-cell suspension or CFE aseptically added to 30 mL of protein extract. For the combined incorporation of whole-cell suspensions and CFE, these were obtained as previously described but in half final volume (3 mL), mixed and, then, aseptically added to the protein extracts. The mixtures were incubated at 37 °C in a shaken water bath. Samples were taken initially and after 96 h of incubation for further analyses. In every case, control samples without the addition of any bacterial enzyme were analyzed simultaneously.

*Bacterial Count and pH Measurement.* Bacterial counts were determined on plate count agar (Merck) and MRS agar (Merck) after incubation at 30 °C for 48 h. The pH values of the reaction mixtures were monitored by using a Crison 2001 pH-meter (Crison Instrument S.A., Barcelona, Spain).

*Gel Electrophoresis.* The hydrolysis of muscle proteins was monitored by sodium dodecyl sulfate gel electrophoresis (SDS−PAGE) analysis (Laemmli, 1970) using 12 and 10% polyacrylamide gels for sarcoplasmic and myofibrillar proteins, respectively. The proteins used as standards were myosin (200.0 kDa), $\beta$-galactosidase (116.5 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa) from Bio-Rad. Proteins were visualized by Coomassie Brilliant Blue R-250 staining.

*Peptide Analyses.* The evolution of the peptide contents in protein extracts was analyzed in a 1050 Hewlett-Packard liquid chromatograph (Palo Alto, CA), equipped with a multiwavelength UV detector and an automatic injector. Two milliliters of each sample was deproteinized with 5 mL of acetonitrile. The supernatant was concentrated by evaporation to dryness and resuspended in 200 $\mu$L of solvent A, 0.1% (v/v) trifluoroacetic acid (TFA) in MilliQ water. Samples of 15 $\mu$L were applied onto a Waters Symmetry C18 (4.6 mm inside diameter by 250 mm) column (Waters Corp., Milford, MA). 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: an isocratic phase in 1% solvent B for 5 min and, then, a linear gradient from 1 to 100% solvent B for 20 min, at a flow rate of 0.9 mL/min and 40 °C. Peptides were detected at 214 nm.

*Amino Acid and Natural Dipeptide Analyses.* The changes in free amino acids and natural dipeptide contents in muscle extracts were also monitored. Samples of 500 $\mu$L plus 50 $\mu$L of an internal standard (0.325 mg/mL hydroxyproline) were deproteinized with 1375 $\mu$L of acetonitrile. The supernatant (200 $\mu$L) was derivatized to its phenylthiocarbamyl derivatives according to the method of Bidlingmeyer et al. (1987). The derivatized amino acids were analyzed by reverse-phase HPLC according to the method of Aristoy and Toldrá (1991).

## RESULTS

**Proteolytic Activity toward Synthetic Substrates.** The general proteinase and aminopeptidase activities showed by whole cells and CFE from *L. casei* strains are reflected in Table 1. Casein-FITC was hydrolyzed to different extents by the assayed strains. In every case, the aminopeptidase activities from CFE displayed broad substrate specificity except for pyroglutamic acid, which was not released (data not shown). Substrates containing alanine and leucine as N-terminal residues were preferentially hydrolyzed except for *L. casei* CRL 678, which released basic amino acids at higher rates. *L. casei* CRL 705 was selected for further assays on muscle proteins on the basis of its higher proteinase activity and intermediate levels of aminopeptidase activity.

**Table 1. Proteinase[a] and Aminopeptidase[b] Activities of Strains of *L. casei* toward Synthetic Substrates[c]**

| | strain of *L. casei* | | |
|---|---|---|---|
| substrate | CRL704 | CRL705 | CRL678 |
| casein-FITC | 0.019 ± 0.001 | 0.033 ± 0.001 | 0.028 ± 0.006 |
| Ala-AMC | 59.59 ± 11.14 | 15.15 ± 1.45 | 3.30 ± 0.31 |
| Lys-AMC | 0.01 ± 0.00 | 1.71 ± 0.15 | 7.03 ± 0.24 |
| Ser-AMC | 0.37 ± 0.04 | 0.22 ± 0.04 | 0.12 ± 0.00 |
| Met-AMC | 15.23 ± 1.64 | 6.88 ± 0.10 | 3.31 ± 1.74 |
| Phe-AMC | 0.29 ± 0.07 | 0.90 ± 0.11 | 1.10 ± 0.01 |
| Val-AMC | 14.54 ± 0.86 | 6.49 ± 0.30 | 1.50 ± 0.29 |
| Arg-AMC | 0.05 ± 0.01 | 2.27 ± 0.15 | 12.81 ± 4.90 |
| Gly-AMC | 0.26 ± 0.03 | 0.15 ± 0.01 | 0.05 ± 0.01 |
| Leu-AMC | 50.05 ± 13.8 | 14.37 ± 1.99 | 6.236 ± 0.66 |
| Tyr-AMC | 0.03 ± 0.01 | 0.50 ± 0.08 | 0.625 ± 0.11 |
| Pro-AMC | 1.323 ± 0.21 | 0.11 ± 0.01 | 0.02 ± 0.02 |
| Glu-pNA | 1.04 ± 0.20 | 0.10 ± 0.01 | 1.84 ± 0.05 |

[a] Proteinase activity toward casein-FITC expressed as units per mg dry wt$^{-1}$. [b] Aminopeptidase activity toward amino acid-AMC- and -pNA expressed as units per mg of protein$^{-1}$. [c] The values shown are the means of four experiments ± SEM.

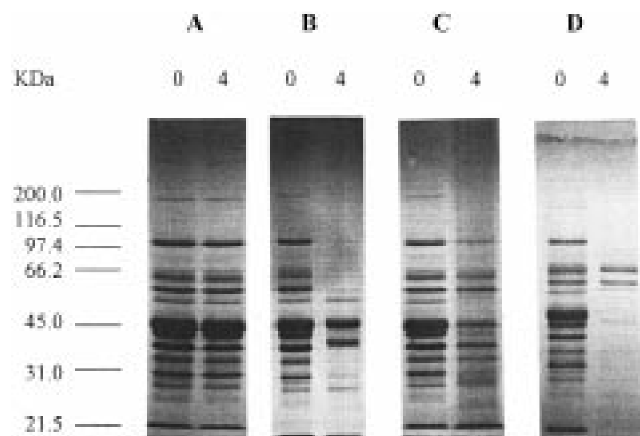


**Figure 1.** SDS−PAGE of sarcoplasmic protein hydrolysis by *L. casei* CRL 705: (lane A) control samples with no addition of microbial enzymes; (lane B) samples containing whole cells at 0 and 96 h of incubation, respectively; (lane C) samples containing CFE at 0 and 96 h of incubation, respectively; (lane D) samples containing both whole cells and CFE at 0 and 96 h of incubation, respectively.

**Bacterial Counts and pH Evolution in Muscle Protein Mixtures.** Whole cells inoculated in both sarcoplasmic and myofibrillar protein extracts reached initial levels of $4.5 \times 10^8$ colony forming units (CFU)/mL, which declined to $2.0 \times 10^2$ CFU/mL or undetectable levels, respectively, after 96 h of incubation. No bacterial growth was found when only CFE were added to the protein extracts. Whole cells incorporated together with CFE reached the same initial levels ($4.5 \times 10^8$ CFU/mL) in both protein extracts. In sarcoplasmic extracts the viability almost remained invariable, with final counts of $2.4 \times 10^7$ CFU/mL detected. In the case of myofibrillar extracts, bacterial counts were not detected following 96 h of incubation. The pH values always remained in the range of 6.5−7.0.

**Electrophoretic Analyses.** The protein profiles resulting from the hydrolysis of muscle sarcoplasmic proteins by *L. casei* CRL 705 are shown in Figure 1. In control samples, lacking any bacterial enzyme, proteolytic changes were undetectable (Figure 1, lane A). The activity of whole cells drastically hydrolyzed protein bands with sizes of about 200, 99−50, and 45−31 kDa (Figure 1, lane B). The addition of CFE also caused degradation or reduction in the intensity of protein bands in the whole molecular mass range (Figure 1, lane C). The additive effects following the incorporation of both whole cells and CFE resulted in a stronger protein hydrolysis (Figure 1, lane D). The protein profiles corresponding to myofibrillar protein extracts are shown in Figure 2. In control samples the activity of endogenous proteinases was responsible for the degradation of protein bands such as myosin and actin (Figure 2, lane A). The hydrolytic effects of *L. casei* CRL 705 on these proteins were not clearly identified by electrophoresis. The protein pattern obtained when only CFE were incorporated was similar to that obtained in control samples (data not shown). When whole cells were inoculated, myosin and actin were partially hydrolyzed while other faint bands of intermediate molecular mass appeared (Figure 2, lane B). These effects were intensified when using both whole cells and CFE as enzymes (Figure 2, lane C).

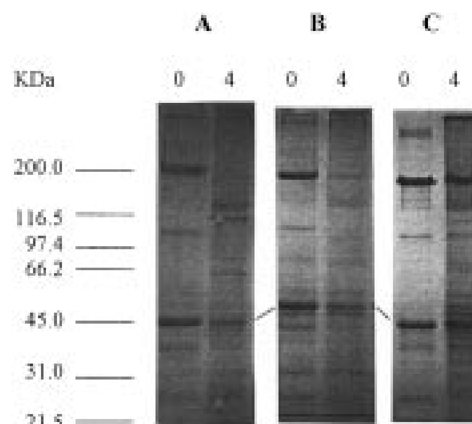


**Figure 2.** SDS−PAGE of myofibrillar protein hydrolysis by *L. casei* CRL 705: (lane A) control samples with no addition of microbial enzymes; (lane B) samples containing whole cells at 0 and 96 h of incubation, respectively; (lane C) samples containing both whole cells and CFE at 0 and 96 h of incubation, respectively.

**Peptide Analyses.** Peptide chromatograms resulting from the proteolytic activity of *L. casei* CRL 705 on sarcoplasmic and myofibrillar proteins are shown in Figures 3 and 4, respectively. In control samples of sarcoplasmic protein extracts, the intensity of peptide peaks eluting at 13, 15.5, 17.5, and 25 min increased while those eluting at 11−12 min were hydrolyzed after 96 h of incubation (Figure 3A,B). When whole cells were inoculated, there was a decrease in the intensity of peptide peaks eluting at 11.5, 13, 15.5, 17.5, and 30 min, although other small peaks appeared along the chromatogram (Figure 3C,D). When only CFE were added, the peptide profile showed some of the changes mentioned for control samples, such as the increase in intensity of peptides peaks eluting at 13, 15.5, and 17.5, although the chromatogram also appeared to be full of many other tiny peaks (Figure 3E,F). The changes described above were highly intensified by the incorporation of both enzymatic sources (Figure 3G,H). The peptide profile of control samples of myofibrillar protein extracts showed minor changes after the incubation period (Figure 4A,B). The addition of whole cells generated some peptide peaks eluting between 11 and 16 min (Figure 4C,D). Similar but less important changes resulted from the activity of CFE (Figure 4E,F). When whole cells and CFE were incorporated together, the profile became crowded with peaks in the same range (Figure 4G,H).

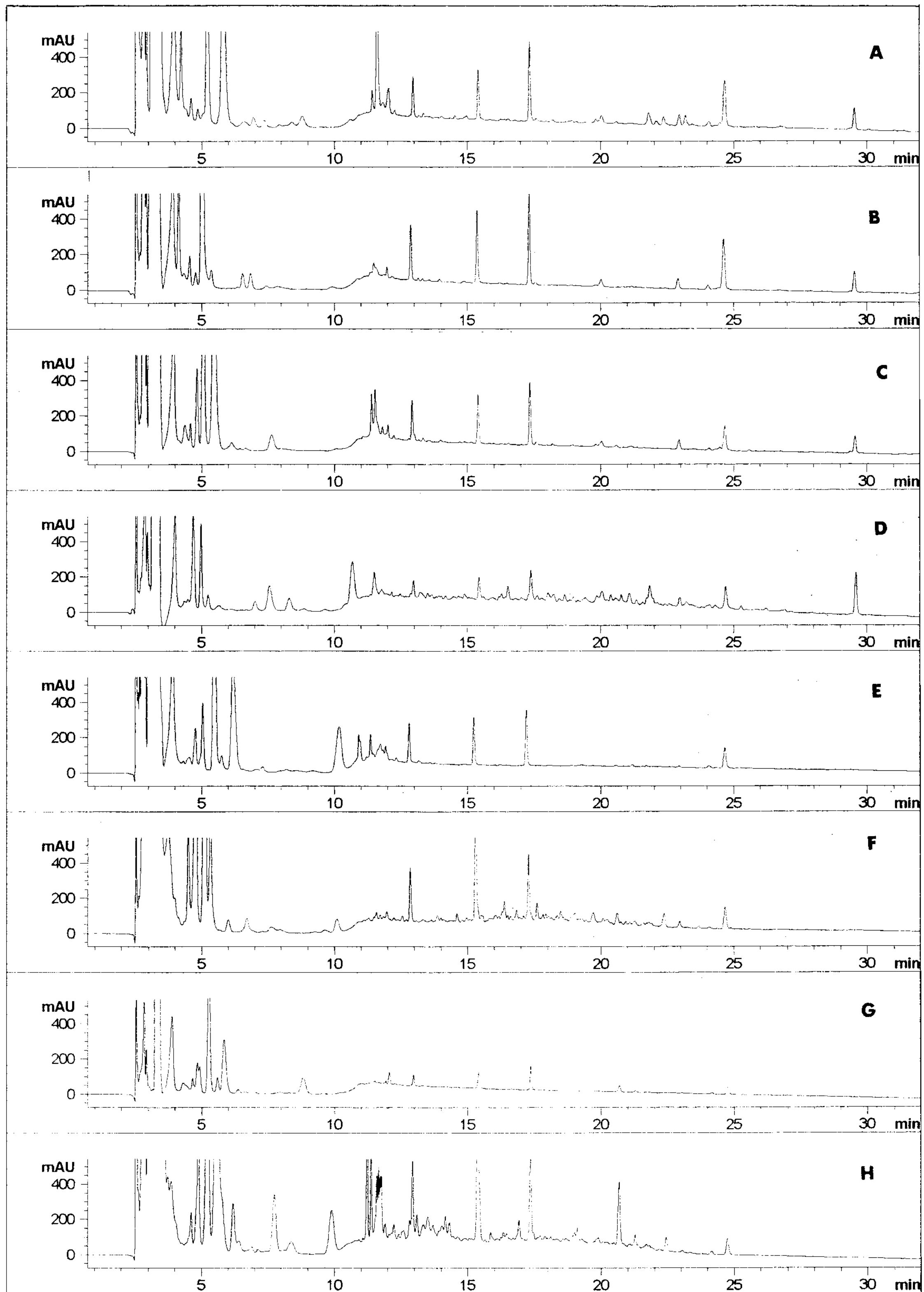


**Figure 3.** Reverse-phase HPLC patterns of soluble peptides contained in sarcoplasmic protein extracts treated with *L. casei* CRL 705 at 0 and 96 h of incubation: control samples (A and B), samples containing whole cells (C and D), samples containing CFE (E and F), and samples containing whole cells plus CFE (G and H).

**Amino Acid Analyses.** The generation of free amino acids and natural dipeptides in meat protein extracts by the activity of *L. casei* CRL 705 is shown in Table 2. In sarcoplasmic protein extracts, the inoculation of whole cells caused a decrease in the levels of almost every amino acid, especially glutamine and alanine, thereby, resulting in a negative total increment (−55.87 mg/100 mL). Whole cells contributed to the generation of only threonine, carnosine, and taurine. The activities of CFE were involved in the generation of free amino acids such as glutamic acid, histidine, and arginine. Moreover, levels of glutamine, alanine, and carnosine were reduced. CFE caused a total increase in free amino acid content of 64.66 versus −7.64 mg/100 mL of the

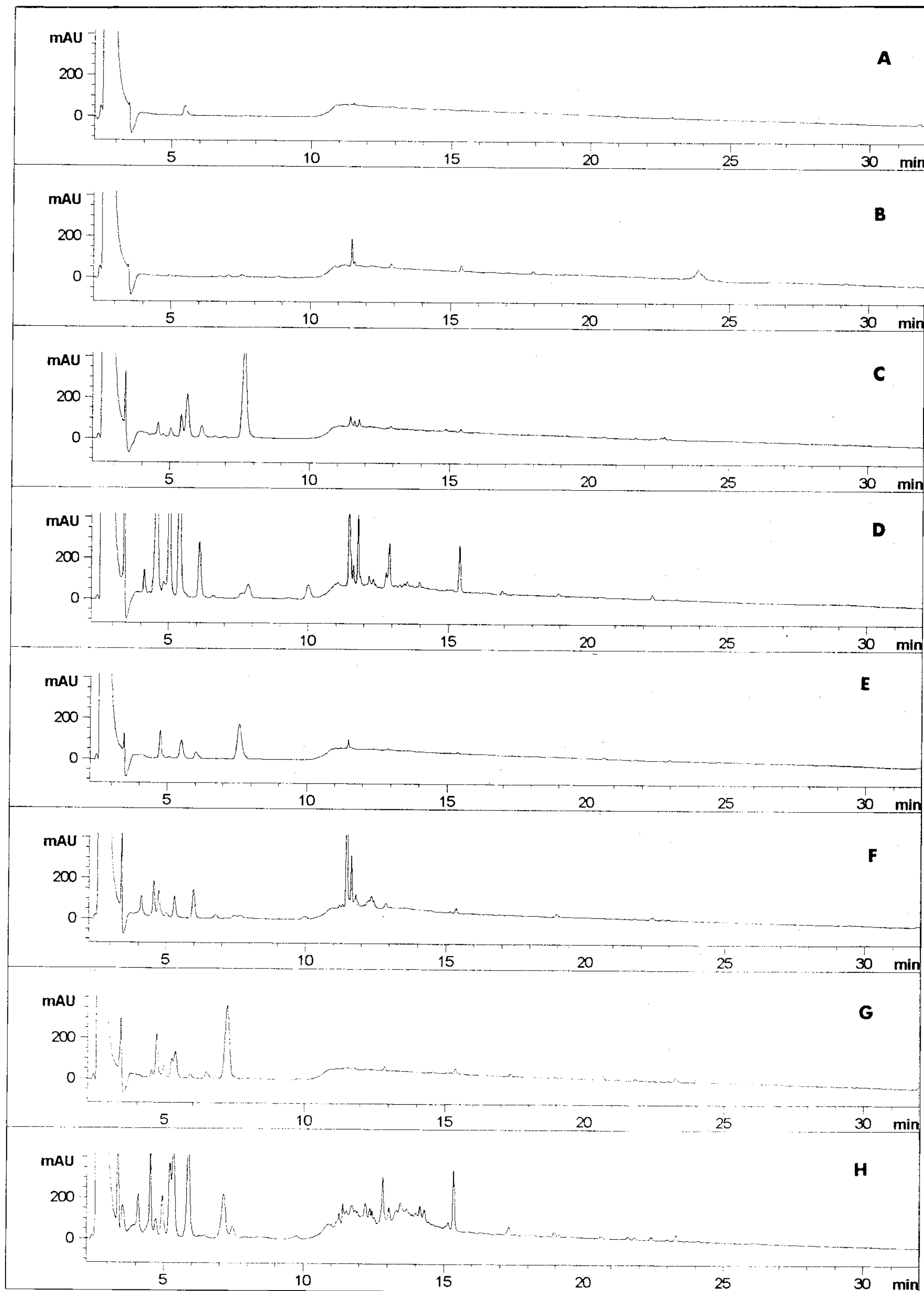


**Figure 4.** Reverse-phase HPLC patterns of soluble peptides contained in myofibrillar protein extracts treated with *L. casei* CRL 705 at 0 and 96 h of incubation: control samples (A and B), samples containing whole cells (C and D), samples containing CFE (E and F), and samples containing whole cells plus CFE (G and H).

control. The combination of both whole cells and CFE intensified the quoted increase (80.10 mg/100 mL). When compared to the control samples, glutamic acid, $\gamma$-aminobutyric acid, alanine, and leucine were the compounds mainly released, but also others such as glutamine decreased.

In myofibrillar protein extracts, there was a general but slight increase in free amino acid and natural dipeptide contents upon the inoculation of whole cells, CFE, and a combination of both. These increases were always lower than those of control samples, resulting in total amounts of 57.80, 15.69, and 63.38 mg/100 mL,

**Table 2. Net Free Amino Acid and Natural Dipeptide Content[a] after Incubation at 37 °C of Sarcoplasmic and Myofibrillar Extracts Containing Whole Cells, Cell-Free Extracts (CFE), and Combination of Both**

| amino acid | sarcoplasmic protein extract | | | | myofibrillar protein extract | | | |
|---|---|---|---|---|---|---|---|---|
| | control | whole cells | CFE | cells + CFE | control | whole cells | CFE | cells + CFE |
| Asp | 6.41 | −0.19 | −0.87 | 0.41 | 0.6 | 0 | 0.71 | −1.43 |
| Glu | −22.89 | −5.24 | 31.49 | 3.78 | 0.19 | −1.06 | 0.48 | 1.7 |
| OHPro | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.3 |
| Ser | 2.26 | −2.5 | −0.37 | 2.53 | −0.26 | 1.91 | 0.51 | 1.79 |
| Asn | 0.38 | −1.04 | −1.12 | 2.77 | −0.12 | 0 | 0.31 | 1.25 |
| Gly | 2.75 | −6.15 | 3.97 | 8 | −0.06 | 1.75 | 0.57 | 2.29 |
| Gln | −17.97 | −55.76 | −98.1 | −59.79 | 0 | 0 | 0.37 | 1.22 |
| bAla | −0.04 | −1.77 | 6.26 | 0.28 | 0 | 0 | 0 | 0 |
| His | 2.34 | −1.66 | 18.08 | 2.4 | 0 | 0 | 0.51 | 1.05 |
| GABA | 1.06 | −0.5 | 1.58 | 34.74 | 0 | 0 | 0.46 | −0.48 |
| Thr | 5.39 | 24.39 | −5.23 | 3.03 | 7.26 | 2.44 | 0.44 | 2.52 |
| Ala | 7.07 | −12.65 | −15.14 | 31.59 | 57.97 | 32.66 | 3.22 | 21.58 |
| Arg | 0.57 | −4.35 | 155.78 | 3.81 | 11.83 | 4.31 | 1.59 | 4.32 |
| Pro | 2.93 | −4.67 | −3.18 | 3.77 | 7.42 | −1.75 | 2.7 | 0.79 |
| Tyr | 0.81 | −0.81 | 0.6 | 2.64 | 5.76 | 2.53 | −0.5 | 1.37 |
| Val | 2.11 | −2.35 | −2.77 | 4.54 | 9.44 | 3.12 | 0.59 | 2.93 |
| Met | 1.06 | −1.16 | 4.97 | 1.11 | 3.29 | 0 | 0.2 | 1.26 |
| Ile | 1.13 | −1.15 | 0.26 | 3.38 | 5.7 | 2.16 | 0.49 | 2.9 |
| Leu | 2.83 | −1.99 | 4.63 | 17.37 | 8.78 | 2.64 | 0.61 | 4.59 |
| Phe | 1.46 | −1.49 | 3.39 | 7.59 | 6.5 | 1.02 | 0.22 | 1.44 |
| Trp | 0.27 | −0.65 | −0.88 | 1.34 | 2.08 | 0 | 0 | 0 |
| Orn | 0.63 | −0.4 | −0.56 | 0.14 | 2.03 | 0 | 0 | −0.19 |
| Lys | 2.9 | −3.46 | 0.97 | 4.25 | 11.17 | 5.87 | 2.37 | 5.9 |
| anserine | 0.31 | −0.89 | −7.57 | −0.4 | 63.73 | 0 | 0 | 4.71 |
| carnosine | −11.62 | 20.67 | −162.26 | −3.29 | −0.06 | 0 | −0.41 | 0.27 |
| taurine | 0.52 | 9.9 | 1.4 | 4.11 | 0.18 | 0 | 0.24 | 0.29 |

[a] Expressed as 100 mg/mL of extract.

respectively, versus 203.4 mg/100 mL. In every case, the compounds depleted in higher proportion were alanine and anserine if compared to the control values.

## DISCUSSION

A biochemical approach using proteolytic enzymes accessible as whole cells, CFE, and a combination of both from *L. casei* CRL 705 has been devised to get a better understanding of their different contributions to the proteolytic events in meat systems. Electrophoretic analyses undoubtedly demonstrated the suitability of sarcoplasmic proteins as substrates for proteinases from whole cells of *L. casei* CRL 705. The strongest hydrolytic activity seemed to be extracellularly located when compared to the degradation caused by the addition of exclusively CFE. This is in full agreement with the existence of a single cell-wall-associated proteinase in most dairy lactic acid bacteria, which also initiates the hydrolysis of caseins (Kunji et al., 1996). In fact, such an enzyme has been previously characterized from two dairy strains of *L. casei* (Kojic et al., 1991; Holck and Naes, 1992; Naes and Nissen-Meyer, 1992). However, the quoted activity did not fully account for the proteolysis observed and other endopeptidases could contribute to the whole hydrolysis of sarcoplasmic proteins. Also, other authors have found endopeptidase activity in CFE, although the bulk of the cell proteinase activity is wall-associated (Hegazi and Abo-Elnaga, 1987). Muscle aspartic and cysteine proteinases have been regarded as the enzymes mainly implicated in the initial hydrolysis of myofibrillar proteins (myosin and actin) and the generation of smaller fragments such as those of 122, 38, 29, and 13 kDa (Molly et al., 1997). In our extracts, residual endogenous activity could not be completely excluded and was responsible for the degradation of myosin and actin. Moreover, a minor implication of bacterial proteinases could not be discarded either.

The peptide profiles of sarcoplasmic protein extracts were greatly modified by the activities of *L. casei* CRL 705 regardless of the use of whole cells or CFE. The simultaneous addition of both enzymatic sources strengthened the effects mentioned above. In either case, the hydrolysis of myofibrillar proteins led to the generation of mainly hydrophilic peptides, which increased under the effects of both whole cells and CFE. It is worth noting that hydrophilic peptides are those correlated to desirable cured-meat flavors, whereas those constituted by hydrophobic residues are associated with bitterness (Aristoy and Toldrá, 1995).

In milk, a set of intracellular exopeptidases cooperates in the complete hydrolysis of a great variety of casein-derived peptides into free amino acids (Kunji et al., 1996). The components of the exoproteolytic system of dairy lactobacilli such as *L. helveticus* and *L. delbrueckii* have been thoroughly investigated, whereas only some peptidases from dairy strains of *L. casei* have been purified and characterized (Arora and Lee, 1992; Habibi-Najafi and Lee, 1994; Fernandez-Espla and Martin-Hernandez, 1997; Fernandez-Espla et al., 1997a,b). As expected, in sarcoplasmic protein extracts the activities of CFE were mainly involved in amino acid generation because there was a clear increase in their total content. In contrast, the activities of CFE released lower amounts of amino acids than those of whole cells from myofibrillar proteins. It is likely that the absence of substrates generated by extracellular proteinase activity associated with whole cells limited the activity of other peptidases. Indeed, the formation of amino acids in cheese slurries was mainly explained by the action of intracellular enzymes, although the activity of the cell-envelope proteinase constituted a rate-limiting factor for amino acid generation (Fernandez de Palencia et al., 1997). This is also the reason the strain *L. casei* CRL 705 was selected instead of *L. casei* CRL 704, which showed higher aminopeptidase but quite lower proteinase activities against synthetic substrates (see Table 1). In

sarcoplasmic extracts containing both whole cells and CFE, the generation of glutamic acid and the decrease in glutamin could be the result of their interconversion (Chopin, 1993). In general, the amino acid generation was promoted and the compounds that experienced the highest increases, such as alanine and leucine, were also those hydrolyzed at higher rates from synthetic substrates (see Table 1). Amino acids such as glutamic acid and alanine are important for flavor enhancer properties or sweet taste, respectively, and others such as leucine as precursors of other aroma volatile compounds (Kato et al., 1994; Montel et al., 1992; Henriksen and Stahnke, 1997).

Lactic acid bacteria have multiple amino acid auxotrophies and depend on their proteolytic system to obtain the amino acids required for optimal growth (Martin-Hernandez et al., 1994; Kunji et al., 1996). In meat systems, sarcoplasmic proteins may also contribute to amino acid supply for *L. casei* CRL 705 according to the general decrease in total free amino acid contents observed upon whole cell inoculation. This belief is confirmed when whole cells were provided with CFE as an extra source of enzymes because then the viability was kept almost constant. Nevertheless, this can be the result of both the suitability of these proteins as substrates and the presence of other soluble compounds essential for their survival in sarcoplasmic protein extracts. In sausages, Blom et al. (1996) also found stimulation of cell growth and fermentation rates when a purified proteinase was incorporated to accelerate their production.

In summary, the potential contribution of *L. casei* CRL 705 to the breakdown of sarcoplasmic and myofibrillar proteins has been demonstrated. The activities of whole cells together with CFE were required to intensify all of the proteolytic events. The generation of mainly hydrophilic peptides as well as free amino acids from myofibrillar and sarcoplasmic proteins, respectively, must be studied in more detail for their possible contribution to the development of desirable flavor in cured-meat products.

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