Effect of curing conditions and *Lactobacillus casei* CRL705 on the hydrolysis of meat proteins

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S. FADDA, G. VIGNOLO, M.-C. ARISTOY, G. OLIVER AND F. TOLDRÁ. 2000.

Aims: The effect of the common curing conditions used during the manufacture of dry fermented sausage on the proteolytic activity of *Lactobacillus casei* CRL705 against meat proteins was investigated.

Methods and Results: Hydrolysis of pork muscle sarcoplasmic and myofibrillar proteins was evaluated by SDS–PAGE and reverse phase-HPLC analysis. Ascorbic acid exerted a stimulatory effect on both sarcoplasmic and myofibrillar protein breakdown by *Lact. casei* CRL705 with the release of hydrophilic peptides and free amino acids, while NaCl and $NaNO_2$ mainly stimulated myofibrillar degradation.

Conclusions: Even when processing temperature (25°C) did not positively affect bacterial protein hydrolysis, the presence of curing salts accounted for a remarkable increase in the non-volatile components that constitute taste-active compounds that strongly influence the final flavour of the product.

Significance and Impact of the Study: To predict the suitability of *Lact. casei* CRL705 and its proteolytic enzymes as a starter culture for the dry processing of dry fermented sausages.

INTRODUCTION

The enzymology of dry fermented sausages is a complex phenomenon due to the co-existence of muscle and microbial enzymes. The proteolysis that occurs during the ripening of meat products is considered to be a result of the action of endogenous meat enzymes such as cathepsins, as well as of bacterial proteases (Díaz *et al.* 1997). Muscle proteinases appear to be 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). The generated peptides and free amino acids and the products of the further enzymatic and chemical reactions in which they are all involved constitute important volatile and non-volatile compounds with a strong impact on flavour (Demeyer *et al.* 1986; Johansson *et al.* 1994). 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 has become the focus of an increasing number of studies due to the technological role of these organisms (Fadda *et al.* 1999a, 1999b; Montel *et al.* 1995; Sanz and Toldrá 1997a, 1997b; 1998a, 1998b). The bacteriocinogenic strain *Lactobacillus casei* CRL705, originally isolated from sausages, is being subjected to a thorough investigation for its potential as a starter culture in dry cured sausage production (Palacios *et al.* 1999; Vignolo *et al.* 1986, 1993, 1996). Recently, the effect of this strain on the proteolysis of muscle proteins was reported by Sanz *et al.* 1999, who suggested its potential contribution to the degradation of sarcoplasmic and myofibrillar proteins together with its particular ability to hydrolyse hydrophobic peptides responsible for bitter flavours. Nevertheless, the extent of proteolysis during the ripening of dry fermented sausages varies with factors including the quality of meat, the initial microflora and the prevailing processing conditions. The effect of different levels of curing ingredients and process parameters on muscle proteases and peptidases has been reported (Toldrá *et al.* 1992a, 1992b; 1993a, 1993b;

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Flores *et al.* 1997; Fadda *et al.* 1999c). However, there have been very few reports on the effects of curing agents on the proteolytic activity of starter bacteria. Sanz and Toldrá (1997b) examined the effect of curing additives and processing parameters on the activity of aminopeptidases from *Lact. sake*. The objective of the present work is to evaluate the effect of curing agents and processing parameters on the proteolytic activity of *Lact. casei* CRL705 against muscle proteins and to use this information to predict the suitability of this strain and its proteolytic enzymes as a starter culture for the processing of dry fermented sausages.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Lact. casei CRL705, isolated originally from sausages, was used for proteolytic assays. The strain was grown routinely in MRS broth (Merck, Darmstad, Germany) at 30°C for 24 h and then maintained at either 4°C or − 80°C in 15% (v/v) glycerol. The growth media for enzymatic assays was inoculated 1% (v/v) with the microorganism, previously subcultured twice and incubated for 16 h at 30°C.

Preparation of cell suspensions

The proteinase activity against muscle proteins was assayed in whole-cell suspensions. Cells were harvested by centrifugation (10 000 *g* for 20 min at 4°C), washed twice in 20 mmol l^{-1} phosphate buffer, pH 7·0, and resuspended in the same buffer (20% of the initial volume).

Activity on pork muscle protein extracts

Extraction of muscle proteins. Sarcoplasmic proteins were extracted according to the method described by Molina and Toldrá (1992) but using 20 mmol l^{-1} phosphate buffer, pH 6·5, for homogenization. The final extract was filter sterilized through a 0·22-μm membrane (Millipore, Bedford, MA, USA). The protein content of the sarcoplasmic extract was 1·80 mg l^{-1}. To prepare the myofibrillar extract, the pellet resulting from the sarcoplasmic protein extraction was resuspended in 100 ml of 0·01 mol l^{-1} phosphate buffer, pH 6·5, previously sterilized, and homogenized for 4 min in a Stomacher 400 blender (London, UK). After centrifugation at 10 000 *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 weighted, resuspended in 9 vols of 0·03 mol l^{-1} phosphate buffer with 0·7 mol l^{-1} KI, pH 6·5 containing 0·02% sodium azide and homogenized for 8 min in a Stomacher. After the last centrifugation (10 000 *g* for 20 min at 4°C), the resulting supernatant was diluted 10

times in the 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 l^{-1}. In both extractions, sterility was confirmed by determining the absence of bacterial growth in plate count agar (Merck, Darmstad, Germany).

Incubation conditions. Five independent assays corresponding to each treatment (one control and four with curing salts) were carried out for each protein extract (sarcoplasmic and myofibrillar) using *Lact. casei* CRL705 cell suspensions, obtained as stated above, as an enzymatic source. The reaction mixture consisted of 6 ml of whole-cell suspension added to 30 ml of protein extract supplemented with the required additive. The mixtures were incubated at 25°C (the temperature usually employed during the fermentation period) in a shaken water bath. Samples were taken initially and after 96 h of incubation for further analyses. Control samples without the addition of bacterial enzymes or curing additives were also included.

Curing agents. The curing additives concentrations used in this study were those reported to produce the most significant effect on muscle protein hydrolysis in aseptic conditions (Fadda *et al.* 1996). Curing salts were added individually to each experimental system (sarcoplasmic and myofibrillar) at final concentrations of 3% NaCl, 200 mg l^{-1} NaNO$_2$ and 100 mg l^{-1} ascorbic acid. To study the total effect, all additives (3% NaCl, 200 mg l^{-1} NaNO$_2$ and 100 mg l^{-1} ascorbic acid) were added to the sarcoplasmic and myofibrillar systems (hereafter referred to as all additives) simultaneously. NaCl was sterilized by autoclaving for 15 min at 121°C; stock solutions of NaNO$_2$ and ascorbic acid were filter sterilized.

SDS-PAGE. The hydrolysis of muscle proteins was monitored by 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), β-galactosidase (116·3 kDa), phosphorylase B (97·4 kDa), serum albumin (66·2 kDa), ovalbumin (45·0 kDa), carbonic anhydrase (31·0 kDa), trypsin inhibitor (21·5 kDa), lysozyme (14·4 kDa) and aprotinin (6·5 kDa) from Bio-Rad (Richmond, CA, USA). Proteins were visualized by Coomassie Brilliant Blue R-250 staining.

Peptide analyses. The evolution of peptide profiles in protein extracts was analysed in a 1050 Hewlett Packard liquid chromatograph (Palo Alto, CA, USA), equipped with a variable UV detector and an automatic injector. Two ml of each sample were deproteinized with 5 ml of acetonitrile. The supernatant was concentrated by evaporation to dryness and resuspended in 200 μl of 0·1% (v/v) trifluoroacetic acid (TFA) in MilliQ water (solvent A). Samples of 15 μl were applied onto a Waters Symmetry C18 (4·6 mm inside diameter × 250 mm) column (Waters Corporation, Mildford, MA, USA). The eluate system consisted of solvent A,

described above, and acetonitrile–water–TFA
60 : 40 : 0·085% (v/v) (solvent B).

The elution was performed as follows: an isocratic step in
1% solvent B for 5 min followed by a linear gradient from
1 to 100% solvent B for 20 min, at a flow rate of
0·9 ml min^{-1} and 40°C. Peptides were detected at 214 nm.
Amino acid and natural dipeptide analyses. The changes in
free amino acids and natural dipeptides content in muscle
extracts were also monitored. Samples of 500 μl plus 50 μl
of an internal standard (0·325 mg ml^{-1} hydroxyproline)
were deproteinized with 1375 μl of acetonitrile. The super-
natant (200 μl) was derivatized to its phenylthiocarbamyl
derivatives according to the method of Bidlingmeyer *et al.*
(1987). The derivatized amino acids were analysed by
reverse-phase HPLC according to the method of Aristoy
and Toldrá (1991).

Reproducibility

Each experiment was performed twice as independent assays;
the values are the mean of three replicates for each sample.

RESULTS

Protein breakdown

Sarcoplasmic and myofibrillar protein degradation patterns
analysed by SDS-PAGE are shown in Figs 1 and 2,
respectively. In control samples, with no bacterial enzymes,

proteolytic changes in sarcoplasmic extracts after 96 h were
undetectable (Fig. 1, lanes 1 and 2) at the studied tempera-
ture (25°C). The activity of *Lact. casei* CRL705 in absence of
additives (lane 3) resulted in the disappearance and/or
decrease in intensity of protein bands at approximately 75
and 20 kDa in contrast with the same experiment run at
37°C (lane 4). At this temperature *Lact. casei* CRL705 cells
drastically hydrolysed protein bands. Even though at 25°C
sarcoplasmic proteins were not extensively degraded, the
presence of ascorbic acid seemed to favour the proteolytic
system of *Lact. casei*, this effect being responsible for the
appearence of two new bands of approximately 100 kDa and
25 kDa (Fig. 1, lane 6) (arrows). The addition of NaCl and
NaNO$_2$ to sarcoplasmic extract did not reflect major
proteolytic changes (data not shown). The protein profile
corresponding to all additives (lane 7) was similar to that
obtained when only ascorbic acid was present indicating that
this curing additive was the main sarcoplasmic proteolysis
inducing component in the all-additives mixture. With
respect to the degradation of myofibrillar proteins, no
proteolytic activity of endogenous origin was observed at
25°C after 96 h of incubation (Fig. 2, lanes 1 and 2). The
hydrolytic effect of *Lact. casei* CRL705 in the absence of
curing salts was observed to be only slightly discernible (lane
3). In the presence of NaCl, lacking any bacterial enzyme,
proteolytic changes were undetectable (lane 4), while the
effect following the addition of both *Lact. casei* CRL705 and
NaCl resulted in a stronger protein hydrolysis with the
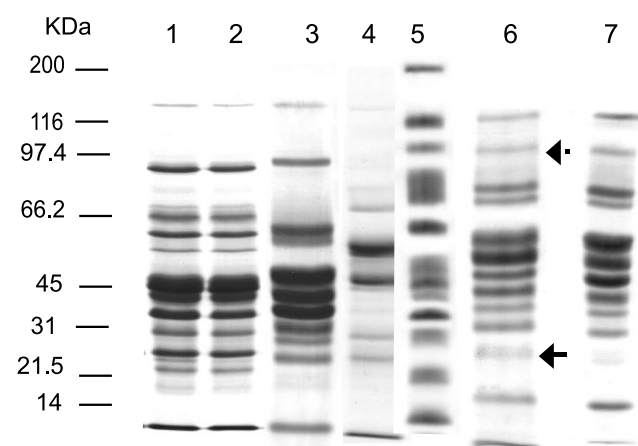generation of two new bands between 70 and 75 kDa (arrow)

Fig. 1 SDS-PAGE of sarcoplasmic proteins hydrolysis by *Lact. casei*
CRL705 subjected to different curing conditions after 96 h of
incubation. Lane 1: control at 0 h (non-inoculated and without
additives); lane 2: control at 96 h; lane 3: *Lact. casei* CRL705 without
additives at 96 h and 25°C; lane 4: *Lact. casei* CRL705 without
additives at 96 h and 37°C; lane 5: molecular weight markers; lane 6:
Lact. casei CRL705 + ascorbic acid and lane 7: *Lact. casei*
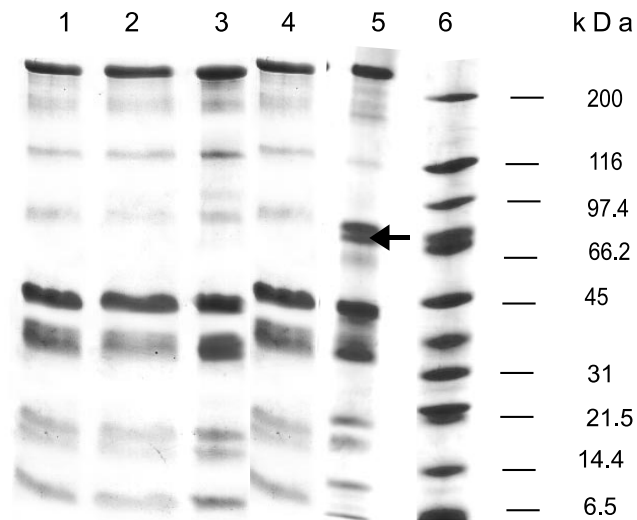CRL705 + all additives

Fig. 2 SDS-PAGE of myofibrillar proteins hydrolysis by *Lact. casei*
CRL705 subjected to different curing conditions after 96 h of
incubation. Lane 1: control at 0 h (non-inoculated and without
additives); lane 2: control at 96 h; lane 3: *Lact. casei* CRL705 without
additives at 96 h and 25°C; lane 4: NaCl (non-inoculated) at 96 h; lane
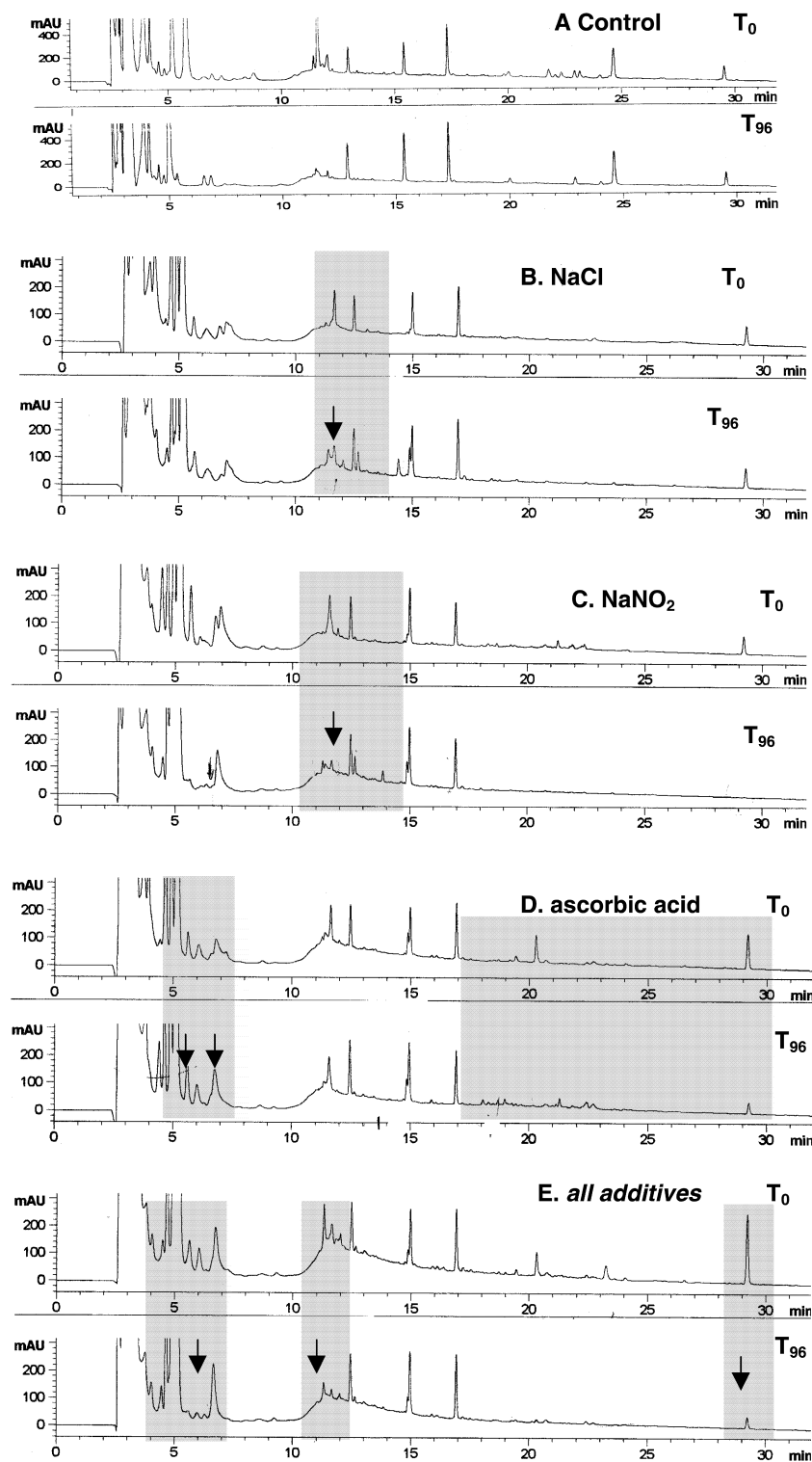5: *Lact. casei* CRL705 + NaCl; lane 6: molecular weight markers

Fig. 3 Reverse-phase HPLC patterns of soluble peptides contained in sarcoplasmic protein extracts inoculated with *Lact. casei* CRL705 at 0 and 96 h of incubation. Effect of curing conditions. (a) *Lact. casei* CRL705 without additives; (b) *Lact. casei* CRL705 + NaCl; (c) *Lact. casei* CRL705 + NaNO$_2$; (d) *Lact. casei* CRL705 + ascorbic acid; (e) *Lact. casei* CRL705 + all additives

(lane 5). Low or even no activity was recorded when the effect of sodium nitrite and ascorbic acid on the hydrolytic activity of *Lact. casei* CRL705 against myofibrillar proteins was studied (data not shown).

Peptide patterns

Peptide chromatograms resulting from sarcoplasmic and myofibrillar protein extracts incubated with or without curing
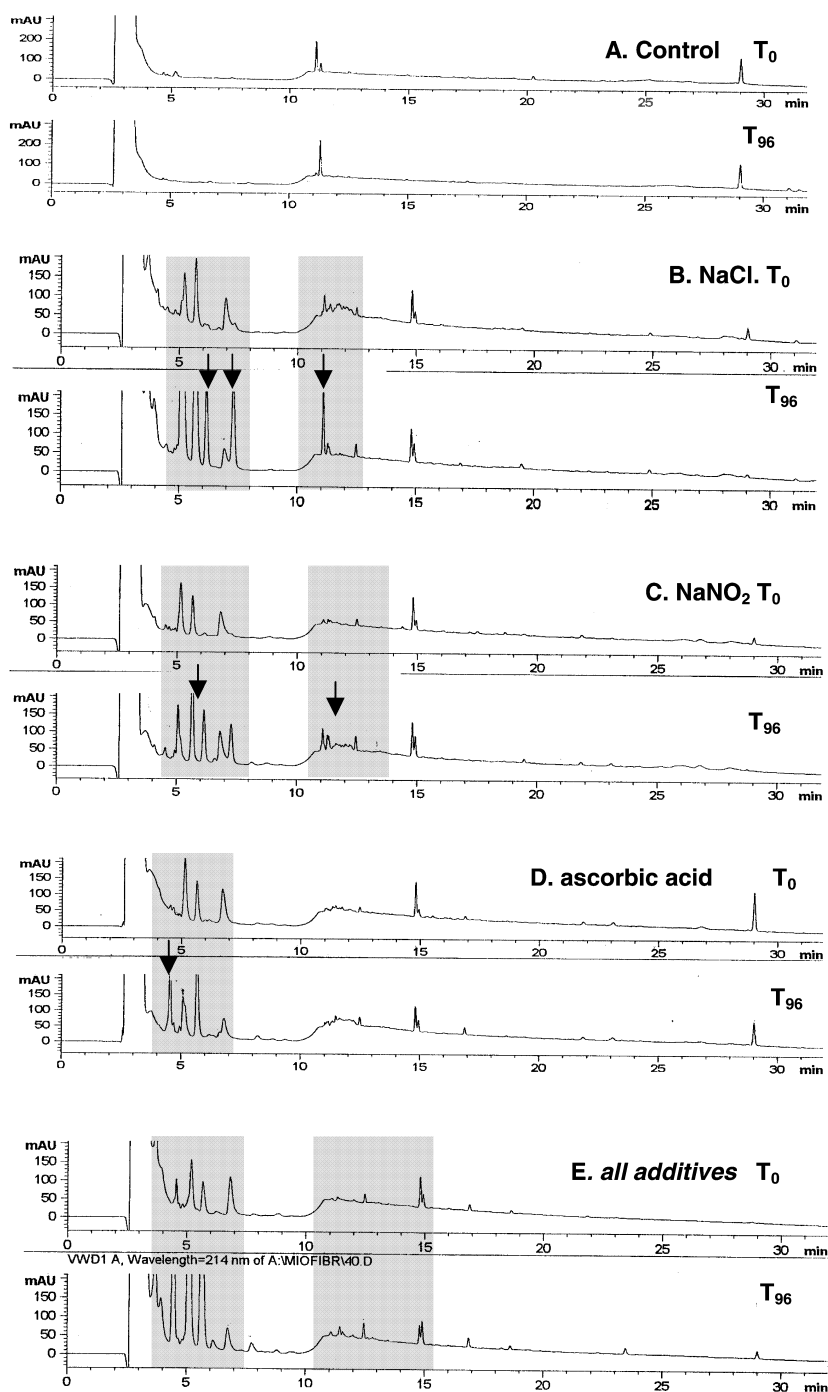
Fig. 4 Reverse-phase HPLC patterns of soluble peptides contained in myofibrillar protein extracts inoculated with *Lact. casei* CRL 705 at 0 h and 96 h of incubation. Effect of curing conditions. (a) *Lact. casei* without additives; (b) *Lact. casei* + NaCl; (c) *Lact. casei* + NaNO2; (d) *Lact. casei* + ascorbic acid and (e) *Lact. casei* + all additives

additives and inoculated with *Lact. casei* CRL705 are shown in Figs 3 and 4, respectively. In non-inoculated samples (data not shown), NaCl, NaNO2 and all additives were observed to induce the hydrolysis of either hydrophilic and hydrophobic peaks while the presence of ascorbic acid produced a positive effect on peptide generation. When *Lact. casei* CRL705 was inoculated (Fig. 3) there was a decrease in peak areas eluting between 11 and 12 min in the presence of NaCl and NaNO2

and two between 20 and 30 min in the presence of ascorbic acid (Fig. 3b,c,d), indicating that microbial peptidolytic activity was favoured by curing salts. In addition, ascorbic acid was observed to produce an increase in the hydrophilic peak area eluting at 5–7 min in concordance with SDS-PAGE analysis. When all additives together with *Lact. casei* CRL705 were present in the sarcoplasmic protein extract the peptide profile showed a decrease in peaks eluting at 6, 11 and

29 min when compared with the pattern in absence of the microorganism (data not shown). The peptide profiles of inoculated samples of myofibrillar protein extracts are shown in Fig. 4. Uninoculated samples showed only minor changes after the addition of curing additives (data not shown), while peptide maps resulting from the proteolytic action of *Lact. casei* CRL705 indicated a generation of new hydrophylic peaks at the end of the incubation time. A stimulation of curing additives on the *Lact. casei* enzymatic system was produced, this effect being observed in the peaks that eluted between 4 and 15 min in the presence of NaCl, NaNO$_2$ and ascorbic acid (Fig. 4b,c,d).

Free amino acid content

Total free amino acid content after incubation at 25°C for 96 h of sarcoplasmic and myofibrillar protein extracts inoculated with *Lact. casei* CRL705 in the presence of curing salts are shown in Table 1. In sarcoplasmic extracts, bacterial presence caused a decrease in the level of almost all amino acids, which would indicate their consumption by *Lact. casei*. Ascorbic acid and all additives combined with *Lact. casei* caused a total increase of free amino acid content of 44·02 and 6·18 mg 100 ml^{-1} against − 213·04 and − 23·41 mg 100 ml^{-1} in the controls without *Lact. casei*, repectively. In myofibrillar protein extracts, there was a general increase in net free amino acid content with or without *Lact. casei* CRL705, this effect being stimulated by the addition of NaCl (54·24 mg 100 ml^{-1}) and ascorbic acid (51·70 mg 100 ml^{-1}) in the presence of microbial enzymes. When all additives were assayed the highest amino acid accumulation was recorded, 153·48 and 102·22 mg 100 ml^{-1} for the control and *Lact. casei*, respectively.

Table 2 shows the variation of each amino acid and dipeptide after incubation at 25°C of sarcoplasmic extracts inoculated with *Lact. casei* CRL705. Increases in carnosine and threonine were observed for the controls without curing salts or inoculum while, when the *Lactobacillus* strain was added, a different amino acid profile was obtained with

increases in the content of tyrosine, leucine, histidine and β-alanine. When compared with the control samples, the effect of curing salts on inoculated sarcoplasmic extracts was found to affect the values of free amino acids, decreasing their concentration (glutamic acid and alanine) while increasing that of others such as β-alanine and leucine. The presence of NaCl in particular seemed to induce *Lact. casei* CRL705 metabolism to also release tryptophan and isoleucine. When NaNO$_2$ was assayed, the release of histidine after 96 h was observed when compared with the control, while in the presence of ascorbic acid the generation of phenylalanine, threonine and isoleucine by the *Lactobacillus* strain was also detected. Ascorbic acid, as stated in SDS-PAGE analysis, was the additive producing the strongest effect on the bacterial proteolytic system. The simultaneous addition of all additives and *Lact. casei* CRL705 promoted the accumulation of a high concentration of the dipeptide carnosine (106·48 mg 100 ml^{-1}) as well as histidine in the sarcoplasmic protein extract while, in uninoculated control samples, increases in glutamic acid, alanine, serine, tyrosine, threonine and tryptophan were recorded.

The amino acid concentrations obtained when myofibrillar proteins were used as a substrate is shown in Table 3. A stimulatory effect was observed in the presence of *Lact. casei* CRL705, this effect being higher when all additives or ascorbic acid were added to the protein extract. Indeed, curing salts could play an important role on the peptidolysis carried out by microbial enzymes. On the whole, amino acid generation was promoted, the compounds that showed the highest increases being glutamic acid, arginine, lysine and carnosine. NaCl and ascorbic acid also stimulated the release of threonine while NaNO$_2$ and all additives caused a decrease in this amino acid concentration of 50% and 32%, respectively.

DISCUSSION

The proteolytic activity of *Lact. casei* CRL705 responsible for the initial breakdown of soluble sarcoplasmic proteins

Table 1 rp-HPLC determination of the total amino acid content of sarcoplasmic and myofibrillar extracts after incubation with *Lact. casei* CRL705 and curing additives for 96 h at 25°C

		Controls without additives		NaCl		NaNO$_2$		Ascorbic acid		All additives*	
		Non-inoculated	*Lact. casei*	Control†	*Lact. casei*	Control	*Lact. casei*	Control	*Lact. casei*	Control	*Lact. casei*
	T$_0$	ΔT	ΔT	ΔT	ΔT	ΔT	ΔT	ΔT	ΔT	ΔT	ΔT
Sarcoplasmic	385·81‡	−20·27§	−67·40	−108·84	−48·07	−146·37	−81·48	−213·04	44·02	−23·41	6·18
Myofibrillar	14·58	1·37	5·75	4·00	54·24	6·04	33·60	−1·44	51·70	153·48	102·22

*(NaCl + NaNO$_2$ + ascorbic acid); T$_0$: 0 h of incubation; ΔT = (amino acid content at 96 h – aminoacid content at 0 h of incubation); †control: without *Lact. casei* CRL705; ‡expressed as miligrams of amino acid per 100 ml of meat protein extract (mg 100 ml^{-1}); §net change occurred after 96 h of incubation (T$_{96}$-T$_0$). −: negative number; indicating a net consum or metabolization of amino acid after 96 h of incubation; these results are the mean of three rp-HPLC determinations.

Table 2 Evolution of free amino acid and natural dipeptide content after incubation at 25°C for 96 h of sarcoplasmic extract with *Lact. casei* CRL705 in the presence of curing additives

		Amino acid or dipeptide content* in the protein extract with the indicated addition									
		Controls without additives		NaCl		NaNO$_2$		Ascorbic acid		All additives†	
Amino acid or dipeptide	T$_0$	Non-inoculated ΔT	*Lact. casei* ΔT	Control‡ ΔT	*Lact. casei* ΔT	Control ΔT	*Lact. casei* ΔT	Control ΔT	*Lact. casei* ΔT	Control ΔT	*Lact. casei* ΔT
Glutamic acid	39·32	− 0·74§	− 35·07	32·06	− 32·55	− 21·27	− 34·33	− 37·32	− 19·55	43·82	− 30·84
Alanine	19·90	− 15·85	− 19·90	62·88	− 17·08	71·27	− 18·65	46·95	− 15·75	49·80	− 18·78
β-Alanine	2·96	− 2·96	4·41	2·23	5·18	− 0·31	4·39	− 1·22	4·33	2·41	5·71
Anserine	15·92	− 15·92	− 15·92	− 15·92	− 1·90	− 0·46	0·28	− 5·74	1·75	− 15·92	− 15·92
Asparagine	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
Carnosine	170·87	9·76	− 33·04	− 170·87	− 2·23	− 170·87	− 9·71	− 170·87	42·79	− 170·87	106·48
Glutamine	6·70	0·00	− 6·70	0·05	− 6·70	2·78	− 6·70	− 0·09	− 6·70	− 6·70	1·65
Glycine	6·72	0·14	1·26	0·29	0·67	2·12	1·18	0·17	1·84	0·88	− 6·70
Histidine	0·00	0·00	4·99	0·00	0·00	0·00	6·95	0·00	0·00	0·00	13·22
Isoleucine	3·96	− 0·96	2·99	− 3·96	3·39	− 3·96	1·32	− 3·96	4·98	− 3·96	− 1·46
Leucine	7·03	− 1·03	11·93	− 7·03	8·07	− 7·03	3·50	− 7·03	12·75	− 7·03	− 1·29
Methionine	3·93	− 3·93	0·73	− 3·93	− 0·13	− 0·68	− 0·76	− 1·77	1·12	− 3·93	− 2·07
Phenylalanine	11·20	− 3·04	1·08	2·86	− 11·20	− 0·94	0·39	− 4·56	10·74	4·31	− 4·82
Serine	0·00	0·00	2·41	0·00	2·87	3·10	1·08	4·99	0·00	8·14	1·13
Taurine	40·62	− 19·39	− 1·09	− 10·65	− 6·62	− 5·98	− 6·87	− 15·93	− 1·84	− 10·28	− 14·93
Tyrosine	5·77	− 5·77	17·38	2·39	− 1·11	0·08	− 1·60	− 0·61	2·30	59·24	− 2·69
Threonine	24·65	38·25	− 9·97	− 0·73	− 1·87	− 11·31	− 14·41	− 12·57	10·12	17·37	− 17·56
Tryptophan	6·64	− 0·94	− 1·56	2·41	13·22	− 1·43	− 6·64	− 0·58	− 6·64	9·97	− 6·64
Valine	6·64	2·10	0·60	− 0·92	− 0·09	− 1·48	− 0·91	− 2·97	1·76	− 0·66	− 2·79

*Expressed as miligrams of amino acid per 100 ml of meat protein extract (mg 100 ml^{-1}); † (NaCl + NaNO$_2$ + ascorbic acid); T$_0$: 0 h of incubation; ΔT = (amino acid content at 96 h; – amino acid content at 0 h of incubation), ‡control: without *Lact. casei* CRL705; §net change occurred after 96 h of incubation (T$_{96}$-T$_0$); –: negative number; indicating a net consum or metabolization of amino acid after 96 h of incubation; these results are the mean of three rp-HPLC determinations.

has been attributed mainly to the temperature. Electrophoretic analysis revealed increases in sarcoplasmic meat proteins hydrolysis at 37°C, compared with 25°C when *Lact. casei* was incubated in absence of curing additives. These results are in accordance with previous work where, when *Lact. casei* CRL705 was incubated at 37°C in similar conditions, a pronounced hydrolytic activity was observed (Sanz *et al.* 1999). Wardlaw *et al.* (1973) and Martín *et al.* (1998) also stated that temperature is the main factor involved in the activity of muscle and microbial proteinases. When the effect of curing additives were studied at the processing temperature of 25°C, ascorbic acid was observed to exert a stimulation of sarcoplasmic hydrolysis. Due to its reducing nature, this additive might stimulate certain cystein bacterial proteinases that require a reduced environment to act. This effect would agree with the results of a previous study (Fadda *et al.* 1996), where no detectable changes were observed with ascorbic acid in aseptic conditions. SDS-PAGE from myofibrillar protein extract suggests that sodium chloride would greatly stimulate bacterial proteolytic system in contrast with endogenous enzymes. However, Toldrá *et al.* (1992b) reported NaCl as

an inhibitor of muscle cathepsins; Astiasarán *et al.* (1990) and Toldrá and Etherington (1988) reported the positive effect of sodium chloride and other curing salts on the solubilization of myofibrillar proteins as well as on the stabilization of the muscle proteolytic enzymes under study.

The peptide profiles of sarcoplasmic protein extracts showed an enhanced peptidolytic action of *Lact. casei* CRL705 with a preferential hydrolysis of hydrophobic peptides in the presence of ascorbic acid and all additives. On the other hand, the hydrolysis of myofibrilar proteins led to the generation of mainly hydrophilic peptides when compared with sarcoplasmic proteins. It is worth noting that hydrophilic peptides are those correlated with desirable cured-meat flavours, whereas those peptides constituted by hydrophobic residues are associated with bitterness (Aristoy and Toldrá 1995). As a whole, the hydrophilic nature of most of the generated peptides indicates the potential contribution of *Lact. casei* CRL705 to the development of a desirable cured-meat taste.

Lactic acid bacteria have multiple amino acid auxotrophies and depend on their proteolytic system to obtain the amino acids required for optimal growth (Martín-Hernan-

Table 3 Evolution of free amino acid and natural dipeptide content after incubation at 25 °C for 96 h of myofibrillar extract with *Lact. casei* CRL705 in the presence of curing additives

Amino acid or dipeptide	Amino acid or dipeptide content* in the protein extract with the indicated addition										
		Controls without additives		NaCl		NaNO$_2$		Ascorbic acid		All additives†	
	T$_0$	Non-inoculated ΔT	*Lact. casei* ΔT	Control‡ ΔT	*Lact. casei* ΔT	Control ΔT	*Lact. casei* ΔT	Control ΔT	*Lact. casei* ΔT	Control ΔT	*Lact. casei* ΔT
Aspartic acid	0·00	0·00§	0·00	0·00	2·84	0·00	2·13	0·00	0·95	0·00	0·00
Glutamic acid	0·62	2·30	1·93	0·10	15·36	1·76	6·77	1·65	12·62	7·09	51·20
Alanine	3·82	− 3·13	1·45	− 3·04	− 2·32	− 2·78	− 2·96	− 3·26	− 2·65	5·67	2·37
Arginine	1·31	− 1·31	2·80	− 1·31	5·54	− 1·31	2·94	0·13	4·78	− 1·31	9·15
Carnosine	8·56	− 1·87	− 5·80	− 2·23	8·91	− 4·15	7·60	− 6·59	6·03	57·54	10·62
Leucine	0·00	0·00	0·83	0·00	2·39	0·00	0·43	0·00	2·95	0·00	0·00
Lysine	0·00	0·81	4·17	0·00	1·07	0·00	0·58	0·51	6·39	9·23	4·48
Methionine	0·00	0·00	0·00	0·46	2·38	0·00	1·33	0·00	1·31	0·00	1·31
Ornithine	0·00	0·00	0·00	0·00	0·00	0·00	1·11	0·00	1·89	0·00	0·00
Serine	0·00	0·00	0·00	2·31	1·95	0·54	1·44	0·00	0·00	0·00	0·00
Taurine	0·27	− 0·27	0·36	0·48	− 0·27	0·53	− 0·27	0·26	− 0·27	9·99	− 0·27
Threonine	0·00	4·83	0·00	5·88	13·12	10·97	5·59	5·87	9·66	65·27	20·93
Valine	0·00	0·00	0·00	0·00	0·00	0·00	3·37	0·00	3·69	0·00	0·00

*Expressed as miligrams of amino acid per 100 ml of meat protein extrac (mg 100 ml^{-1}); †(NaCl + NaNO$_2$ + ascorbic acid); T$_0$: 0 h of incubation; ΔT = (amino acid content at 96 h – amino acid content at 0 h of incubation), ‡control: without *Lact. casei* CRL705; §net change occurred after 96 h of incubation (T$_{96}$-T$_0$); –: negative number; indicating a net consum or metabolization of amino acid after 96 h of incubation; these results are the mean of three rp-HPLC determinations.

dez *et al.* 1994; Kunji *et al.* 1996). In meat systems, sarcoplasmic proteins may also contribute to the amino acid supply for *Lact. casei* CRL705 according to the general decrease in total free amino acid contents observed upon inoculation. Nevertheless, other soluble compounds essential for bacterial survival must be present in sarcoplasmic protein extracts, as indicated by the total increase of free amino acid content in the presence of ascorbic acid and all additives. Conversely, in myofibrillar protein extracts and in the presence of all additives a high amino acid accumulation at the end of the incubation period was recorded. When the variation of each amino acid and dipeptide at 25°C was analysed the results were in agreement with those of Sanz and Toldrá (1997b), who reported that NaCl did not completely inhibit the activity of microbial aminopeptidases involved in the release of free amino acids. The proteolytic activity of *Lact. casei* CRL705 against sarcoplasmic proteins was enhanced in the presence of ascorbic acid as stated in SDS–PAGE analysis. Even though Toldrá *et al.* (1993b) and Sanz and Toldrá (1997b) did not find a direct stimulatory effect of ascorbic acid on microbial aminopeptidases by using synthetic substrates, in natural substrates other mechanisms such as the inhibition of enzymes connected with the degradation/assimilation of amino acids by micro–organisms would be at work. On the other hand, ascorbic acid was shown to exert a possitive effect on the depletion of most amino acids in samples with no bacterial inoculation,

suggesting that muscle catabolic amino acid pathways would be favoured by this additive, this phenomenon being a consequence of the further action of transaminases, dehy-drogenases, aminotransferases and oxidases also present in the muscle. The results obtained when *Lact. casei* and all additives were simultaneously present in sarcoplasmic extracts allow us to suggest the possible existence of a stimulatory coaction of the combined additives on the muscle enzymes involved in the peptide metabolism. There are no reports about this complex effect except for those concerning processed products such as dry sausages, but the lack of controls with individual additives did not permit an adequate comparison (Wardlaw *et al.* 1973; García de Fernando and Fox 1991; Berdagué *et al.* 1993). In myofibr-illar extracts, again the amino acid generation was promoted mainly in presence of ascorbic acid and all additives. Amino acids such as glutamic acid and alanine are important for their flavour-enhancing properties and for their sweet taste, respectively, while others such as leucine are significant as precursors of volatile aroma compounds, such as 2-methyl propanal and 3-methyl butanal (Henriksen and Stahnke 1997; Montel *et al.* 1992; Kato *et al.* 1994).

In brief, the potential contribution of curing conditions in the generation of hydrophilic peptides and free amino acids by the proteolytic acitivity of *Lact. casei* CRL705 has been demostrated. Even the processing temperature used during dry cured fermentation (25°C) did not favour meat protein

breakdown, ascorbic acid and the combination of all additives stimulated amino acid release. Further studies to confirm these results in a sausage model system are necessary, as well as an analysis in terms of taste and odour by a sensory evaluation panel.

ACKNOWLEDGEMENTS

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