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Free amino acids and proteolysis involved in 'salchichon' processing

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Abstract

Changes in free amino acid and nitrogen fraction contents involved in salchichón manufactured by industrial processing at different times of curing process were analysed. An increase in the free amino acid concentration and non protein fraction was observed during the curing process, specially between the fermentation stage and the second week of drying while soluble protein nitrogen decreased. The predominant amino acids in the initial mix of salchichon were Glutamic acid, Cysteine, Carnosine, Alanine and Taurine summing up the 73.7% of the total. During the last two weeks of drying, the concentration of some of the free amino acids went on increasing (Aspartic acid, Glutamic acid, Serine, Asparragine, Glycine, Taurine, Threonine, Alanine, Carnosine, Tryptophan and Lysine) but some others decreased significantly (Proline, Tyrosine, Valine, Methionine, Cysteine, Isoleucine and Leucine). The curing stage of salchichon might be estimated approximately based on the concentration of Aspartic acid, Glutamic acid, Serine, Glycine, Asparragine, Threonine and the total free amino acid content amino acids. The majority of amino acids that showed the greatest concentration in the final product were recorded as having 'bitter' flavour characteristics.

Keywords: Free amino acids; Nitrogen fractions; Curing process; Dry sausage

1. Introduction

Salchichon is a kind of dry cured sausage made in the Mediterranean countries. It is a medium-humidity meat product with good conservation capacity at room temperature. Its formulation is characterised by the presence of black pepper and the absence of garlic and paprika.

Some of the typical flavour characteristics of dry fermented sausages are related to enzymatic activities that alter the nitrogen constituents of muscle tissues during curing (Klement, Cassens & Fennema, 1973; Dierick, Vandekerckhove & Demeyer, 1974; Dineva, Nestorov, Krastev & Brankova, 1984; De Masi, 1990; Garcia de Fernando & Fox, 1991). These enzymes can be microbial and muscle proteases which are involved in the generation of small peptides and amino acids (De Masi, 1990). Aminopeptidases degrate peptides by removing single amino acid residues sequentially from the N-terminus and are responsible for amino acid generation during the processing of pork meat and contribute to flavor development (Mc Donald & Barret, 1986; Toldrá et al., 1992). Therefore, the activity of these enzymes could be strongly involved in the quality of the final product. The wide diversity of product fermentations due to metabolic reaction of the meat microflora (with or without starter culture addition), temperatures and periods of ripening, smoked or air-dried, differences in the kinetics and the extend of the process could explain results for specific free amino acid changes that vary considerably between similar studies (Reuter, 1971; Dierick et al., 1974; De Masi, 1990).

Free amino acids are highly correlated with flavour development in cured products (Mc Lain, Blumer, Graig & Stelel, 1968; Córdoba et al., 1994). Sweet taste is related to D-triptophane, Phenil Alanine, Histidine, Tyrosine, Leucine, Glycine and L-alanine; bitter taste with Valine; Methionine is related to sea flavour and the presence of Glutamic acid is associated with characteristic meaty flavour (Kato, 1989). It is of particular interest to take into account the evolution of free amino acids during curing of fermented sausages due to their role in sausage flavour development and their use as quantitative indicators of proteolytic activity (Mc Lain

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et al., 1968; Kato et al., 1991). The purpose of this study was to determine N fractions and free amino acid concentration in salchichon manufactured by industrial process at different times of the curing process.

2. Material and methods

2.1. Materials

In this study salchichon manufactured in a local factory were studied. The ingredients were: lean pork (60%), beef meat (20%), pork back fat (20%), common salt (20 g/Kg), sugar (45 g/Kg), nitrate (0.2 g/Kg), nitrite (0.15 g/Kg), polyphosphates (sodium pyrophosphate and potassium metaphosphate) (0.2 g/Kg), sodium ascorbate (0.3 g/Kg), black pepper (4 g/Kg) and artificial colouring: azorubin (E122) (100 ppm) and cochineal red (E124) (40 ppm). A commercial frozen dried mixture of *Pediococcus pentosaceus* and *Micrococcus varians* (phh®) was used as starter culture (0.1 g/Kg batter).

Sausages were manufactured as follows: frozen pieces of pork and beef were mixed with salts, spices and starter culture in a cutter. Frozen pork fat was added after being minced in a 3 mm diameter mincer. The mixture was vacuum minced and stored at 4°C for 20 h. It was then stuffed in a 60 mm diameter sausage cellulose casing, fermented for 3 days (25°C, 90% RH) and dried for 4 weeks (15–18°C, 75–80% RH).

2.2. Methods

2.2.1. General parameters

Moisture (ISO 1442-1973), fat (ISO 1443-1973), ash contents (ISO R936-1978), water activity (a_w) using an Aqualab CX2 instrument and pH (ISO R2917-1974) with an Orion Research potentiometer for solid samples were determined in duplicate for each sample. Although complete compositional data are not given in this report, the data were use to confirm that the typical composition was obtained at each phase of processing.

2.2.2. Nitrogen fractions determination

Fraction soluble in phosphate buffer (0.1N and ionic strength of 1.3) that corresponds to sarcoplasmic and myofibrillar fractions or *soluble nitrogen*; fraction insoluble in NaOH (0.1N) or *insoluble nitrogen*; fraction soluble in tricloroacetic acid (20%) or *non protein nitrogen* (NPN) and *total nitrogen*, were separated according to Helander's method (1957) modified by Bello, Larralde and Saenz de Buruaga (1974) and then determined using the Kjeldahl method (ISO R 937-1981).

2.2.3. Free amino acids analysis

The preparation of amino acid extracts was made according to the method described by Aristoy and Toldrá (1991). 4 g of salchichon were diluted (1:5) (w/v) with 0.1N HCl, and homogenised in a stomacher (LABBLENDER, 400). 20 ml of Methionine sulfone was added to the mix. Supernatant was filtered and deproteinized by adding 2.5 volumes of acetonitrile. Deproteinized samples were centrifuged at 15 000 rpm for 5 min and vacuum-dried. Dryed samples received 20 ml of amino acid elution solution (6:2:2 ratio of H_2O:triethylamine:methanol) and were freeze-dried again. Dried samples then received 20 ml of derivatization solution (7:1:1:1 ratio of methanol:triethylamine:phenylisothiocyanate:H_2O) and were incubated for 20 min at room temperature before vacuum-drying. Samples were resuspended in a phosphate buffer containing 5% acetonitrile and were filtered through a 0.45 μm membrane. Samples were analysed on a Waters HPLC system (Millipore/Waters, Mississauga, ON) consisting of two 6000A pumps, a 440 Fixed-Wavelength Absorbency Detector set at 254 nm, a Temperature control module, a 710B WISP™ Sample Processor and a 840 Data and Chromatographic Control Station. The column used was a Waters Pico-tag™ C18 reverse phase column maintained at 46°C. Peak identification and quantification were accomplished by determining retention times and recoveries of free amino acids standards (Sigma Chemical, St Louis, MO).

A gradient with two solvents was used: (a) 70 mM sodium acetate adjusted to pH 6.55 with acetic acid and added with 5% acetonitrile and (b) 45% acetonitrile 40% water and 15% methanol.

Four sausages from each batch were analysed at different manufacturing stages: after mincing, after fermentation, half-way through the drying period (second week of drying) and in the final product (fourth week of drying). Each sample analysis was carried out in duplicate.

2.2.4. Statistical analysis

One-way analysis of variance and a multiple comparison test of Tukey were applied to the data. A multivariate factor analysis (principal components analysis for factor extraction and varimax rotation) was also used to study the contribution of the studied amino acids to the differentiation among the phases of the sausage curing process and among the three commercial brands. There was also applied a regression analysis between the free amino acid content and the curing time. All analysis were performed using the SPSS 6.1.2 (1995) statistical program.

3. Results and discussion

The total, soluble and insoluble nitrogen and the NPN content at each phase of the curing process are shown in Table 1. No changes ($p > 0.05$) were observed

Table 1
Evolution of the different nitrogen fraction contents[A] during the curing process of salchichon[B]

	Minced mix	Fermentation	Drying	Final product	Signification
Total nitrogen	5160[b]	5230[b]	5370[c]	5490[c]	**
Soluble nitrogen	3570[b]	3310[b]	2590[c]	2020[d]	**
Insoluble nitrogen	365[b]	485[bc]	551[c]	583[c]	*
Non protein nitrogen	485[b]	772[c]	910[d]	876[d]	**

[A] Different nitrogen fraction contents calculated as mg/100 g dry matter.
[B] [b,c,d:] means in the same row bearing a common superscript are not significantly different ($p > 0.05$).; * $p < 0.001$; ** $p < 0.05$. The same superscript means no significant differences ($p > 0.05$).

in the total and insoluble nitrogen content during fermentation but upon drying, the total and insoluble nitrogen content increased significantly ($p < 0.05$). This fact could be the result of small losses in liquefied surface fat and moisture that migrate to the surface during drying (De Masi, 1990). Percentage of soluble nitrogen showed a slight but not significant decrease ($p > 0.05$) during fermentation, and it decreased throughout the rest of the ripening process, this behaviour might explain the increase of the NPN fraction due to proteolysis (Garcia de Fernando & Fox, 1991).

The NPN concentration obtained by extraction with tricloroacetic acid in the initial mix of the salchichon (485 mg/100 g dry sample) was slightly lower than the initial NPN concentration obtained by other authors when employing solutions of sulfosalicylic acid solution (602–605 mg/100 g dry sample, De Masi, 1990), and by extraction with 0.6 $HClO_4$ solution (537–633 mg/100 g dry sample, Dierick et al., 1974). In this study, the NPN concentration increased significantly during fermentation (Table 1). It also increased during the first two weeks of drying ($p < 0.05$) making in evidence that the proteolytic enzyme activity that began in the fermentation stage at 25°C went on during drying. Dierick et al. (1974) reported that the NPN concentration increased about 30–44% during the initial day of ripening of cured sausages at approximately 22°C. Similar effects on NPN changes were described by De Masi (1990) in accelerated processes for dry sausages. There was a slight but in significant ($p > 0.05$) decrease in the NPN content of salchichon during the third and fourth weeks of ripening at 15–18°C. The results of our work are in agreement with those reported by Wardlaw, Skelley, Johnson and Acton (1973) for summer sausage prepared by an accelerated process. The NPN fraction represented 9.40% of the total nitrogen in the initial mix and increased to 14.78% at the fermentation phase and to 16.94% during the first two weeks of drying. In the final product, it represented the 15.95% of the total nitrogen. Similar changes on NPN fraction were found by Wardlaw et al. (1973) that observed an increase from 10.5% of total nitrogen as NPN in the initial mix to 14.8% and 17.2% of total N after 10 and 60 days of ripening, respectively. De Masi (1990) found that the NPN fraction in initial and fermented mixes was approximately 9–11% of total nitrogen and increased to 13–14% of total nitrogen in heated and dried sausages. These differences could be explained by the differences in the kinetics and the extent of the process in different meat products due to the wide, diversity of product formulations, temperatures, periods of ripening etc.

The chromatography method used in this research for the analysis of amino acids permitted the identification of 22 compounds in salchichon: Aspartic acid, Glutamic acid, Serine, Asparragine, Glycine, Taurine, Threonine, Alanine, Carnosine, Proline, 3-methil-Histidine, alpha amino butyric, Tyrosine, Valine, Methionine, Cysteine, Isoleucine, Leucine, Phenylalanine, Tryptophan, Ornitine and Lysine. Their concentrations at each phase of salchichon curing process are presented in Table 2. There was an increase in the total amino acid content along the curing process ($p < 0.001$). Mateo, Dominguez, Aguirrezábal and Zumalacárregui (1996) also observed an increase in the total free amino acid content during the ripening of chorizo, being the total free amino acid content in the initial mix similar to the results reported in the present work. However, a higher amount of total free amino acids was found in the final product in the present work. The steepest change occurred during fermentation and the first two weeks of drying indicating that the highest enzymatic activity took place in these stages. Verplaetse, DeBosschere and Demeyer (1989) pointed out that the pH values of 4.5–5.0 and the temperature of ripening of 15–20°C were optimal for the activity of proteolytic enzymes. In some studies direct evidence of proteolysis produced by cathepsins was obtained (Verplaetse et al., 1989; Garcia de Fernando & Fox, 1991). Some authors have described an increase of aminopeptidase activities during the first steps of ripening (Sagarra & Garcia-Regueiro, 1998). These authors found no differences in the evolution of aminopeptidase activities determined during the ripening of fermented sausage between the sausages with the individual starter cultures (*Staphylococcus carnosus* LTH 2102, *S. xylosus* CTC 3037 and *S. xylosus* CTC 3050) and the control samples (without starter culture). Muscle aminopeptidases except leucyl aminopeptidase may contribute to the generation of free amino acids specially during the

Table 2
Evolution of free amino acid concentration during the curing process of salchichon[A]

	Minced mix	Fermentation	Drying	Final product	Signification
Aspartic acid	6.32[a]	8.53[a]	16.70[b]	22.97[c]	**
Glutamic acid	274.51[a]	393.90[b]	430.13[b]	586.85[c]	**
Serine	8.27[a]	13.02[a]	16.24[a]	45.41[b]	**
Asparragine	ND	4.56[a]	6.28[a]	66.80[b]	**
Glycine	18.16[a]	25.99[a]	41.16[b]	69.59[c]	**
Taurine	43.50[a]	20.09[a]	15.08[a]	81.92[b]	**
Threonine	1.65[a]	11.07[b]	12.07[b]	34.11[c]	**
Alanine	48.10[a]	148.06[b]	190.18[c]	216.36[B]	**
Carnosine	63.18[a]	13.95[a]	13.18[a]	206.26[b]	**
Proline	10.00[a]	55.23[b]	68.80[c]	53.50[b]	**
3-methil-histidine	ND	ND	26.01[a]	23.60[a]	**
Alpha amino butyric	ND	4.29[a]	20.30[b]	13.55[b]	**
Tyrosine	18.50[a]	39.81[b]	71.10[c]	38.14[b]	**
Valine	22.38[a]	125.58[b]	198.80[c]	158.46[B]	**
Methionine	9.65[a]	43.29[b]	95.07[c]	60.12[B]	**
Cysteine	88.09[ab]	67.37[a]	133.97[b]	45.54[a]	**
Isoleucine	18.84[a]	97.31[b]	169.59[c]	132.66[b]	**
Leucine	39.43[a]	221.47[b]	392.44[c]	305.41[B]	**
Phenylalanine	21.46[a]	21.30[a]	57.76[b]	45.49[b]	**
Tryptophan	5.76	25.04	37.47	43.59	ns
Ornitine	ND	ND	ND	11.99	
Lysine	4.43[a]	6.29[a]	9.20[a]	28.37[b]	*
Total free amino acids (mg/g dry matter)	7.02[a]	13.46[b]	20.21[c]	22.91[B]	**

[A] [a,b,c:] means in the same row bearing a common superscript are not significantly different ($p > 0.05$).; * $p < 0.001$; ** $p < 0.05$. The same superscript means no significant differences ($p > 0.05$).
[B] Free amino acid concentration calculated as mg/100 g dry matter.

fermentation stage where pH is not so low and temperature is around 25°C (Flores, Sanz & Toldrá, 1998). However, other studies using different bacteria added to the initial sausage mix pointed out the important role of microorganisms such as micrococci (Sajber et al., 1971) and lactobacilli (Reuter et al., 1968) or pediococci (De Masi, 1990) in the increase in NPN in sausages.

The predominant amino acids in the initial mix of salchichon were Glutamic acid, Cysteine, Carnosine, Alanine and Taurine, (concentrations greater than 40 mg/100 g dry matter), summing up the 73.7% of the total. Mateo et al. (1996) found in chorizo that the majority of free amino acids present in the initial sausage mix were Glutamic acid, Taurine, Alanine and Lysine, with average concentrations ranging between 75–200 mg/100 g of dry matter. Our results showed lower Taurine, Lysine and Alanine contents and higher Glutamic acid concentration.

The behaviour of the amino acids was different during the last two weeks of drying. The concentration of some of them went on increasing (Aspartic acid, Glutamic acid, Serine, Asparragine, Glycine, Taurine, Threonine, Alanine, Carnosine, Tryptophan and Lysine) but some others decreased significantly during the last 15 days (Proline, Tyrosine, Valine, Methionine, Cysteine, Isoleucine and Leucine). This decrease might indicate that the degradation of these compounds into volatile compounds (Berdagué, Monteil, Montel & Talon, 1993; Waade & Stahnke, 1997) was more active than their synthesis during this period. Levels of the amino acids Glutamic acid, Taurine, Alanine, Carnosine, Valine, Isoleucine and Leucine made up 73.69% of the total free amino acid content in the final product. Dierick et al. (1974) reported that Glutamic acid, Leucine and Alanine summed up the higher percentage of the amino acids of the NPN fraction in the initial mix and in the sausages fermented with starter culture of 'Duplofermen' (a mixture of lactobacilli and micrococi). The concentration of most of the free amino acids shown in Table 2 were higher than the concentrations reported by Langner (1969), Dierick et al. (1974) and De Masi, (1990). Although a gentle slope of aminopeptidase activities was detected from seventh day to the end of ripening of fermented sausages by Sagarra and Garcia-Regueiro (1998), we found a slight increase in free amino acids in the last two weeks of drying.

Carnosine and Taurine which had non protein origin, represented 15.19% of the total amino acid content in the initial mix. Carnosine, a dipeptide of Alanine and Histidine, is known to be present in typical post rigor red meat tissues (De Masi, 1990). Both of them decreased during fermentation and the first two weeks of drying and increased significantly in the final product. De Masi (1990) reported that Taurine represented 48% of the total amino acid concentration in the initial mix of fermented and non fermented sausages. Glutamine

Table 3
Results of linear regression between some free amino acids and curing process time of salchichon. amino acid (mg/100 g dry matter) $= a + b \times$ time (days)

Amino acid	a	b	R^2 [c]
Aspartic acid	8.98	0.47	0.89
Glutamic acid	430	7.57	0.78
Serine	6.32	1.08	0.87
Glycine	20.45	1.45	0.95
Anserine	7.32	2.03	0.83
Threonine	6.06	0.79	0.89
Total free amino acids[d]	12.87	0.38	0.75

[c] R^2 = determination coefficient.
[d] Total free amino acid concentration calculated as mg/g dry matter.

was not found in this work, unlike the results from De Masi (1990) and Mateo et al. (1996). The losses or absence of Glutamine were likely due to (a) Cysteine metabolized to sulfate and piruvate or converted to Taurine which experimented a high increase in the final product and (b) Glutamine converted to glutamic acid and NH_3, (De Masi, 1990). In the studied salchichon, no other amino acids (β-Alanine and Cystathionine) or dipeptids (Anserine) of non protein origin were observed either.

Some amino acids might be used as indicators of the changes that occur in meat proteins. Thus, 3-methil-histidine is located mainly in myofibrillar proteins, especially myosin and actin, and it can reach 56 mg/g N in pig muscle (Rangeley & Lawrie, 1976). Thus, the presence of 3-methil-histidine in the drying phase, joined with the decrease in soluble nitrogen found in this work might be related to the degradation of the myofibrillar proteins during drying. Besides, there was a high positive correlation ($R^2 > 0.75$) between the concentration of Aspartic acid, Glutamic acid, Serine, Glycine,

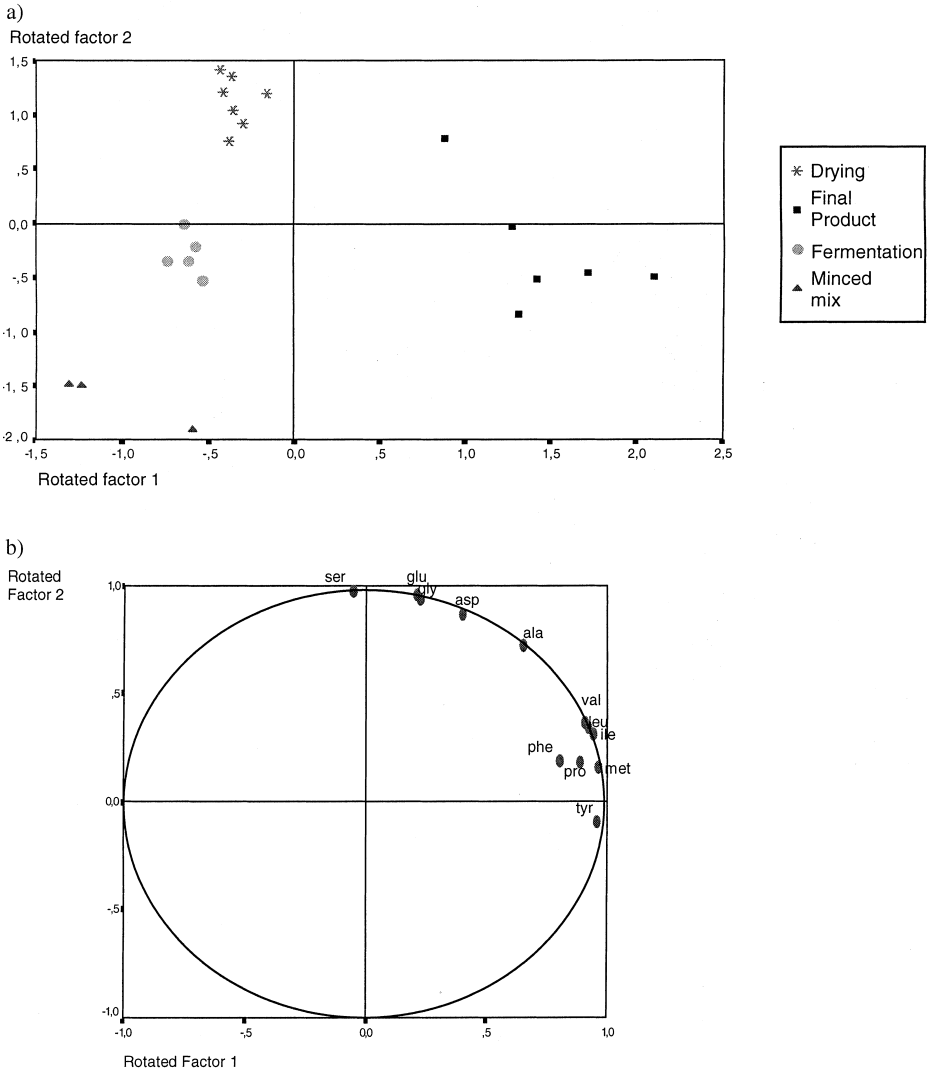

Fig. 1. Principal component analysis of the free amino acids: plot of the first two principal components (a) Location of the individuals (salchichon in each stage) and (b) Location of the variables in the multivariate space (▲ minced mix; • fermentation; * drying; ■ final product).

Asparragine, Threonine and the total free amino acid content with the curing time. Table 3 sets out the regression equations and the correlation coefficient (R^2) for those variables most highly correlated with curing time. Thus, the curing stage might be estimated approximately based on the concentration of these amino acids.

3.1. Principal component analysis

When principal component analysis was applied to the data, the first two principal factors accounted for 92.0% of the total variance. Fig. 1 represents the plot of the samples in the plane defined by these two principal rotated factors. Proline, Tyrosine, Valine, Methionine, Isoleucine and Leucine were highly correlated with the first factor which explained 68.7% of the total variance. These amino acids were the responsible for the differences found among the first curing stages of salchichon. Glutamic acid, Aspartic acid, Threonine, Serine, Glycine and Alanine contributed more strongly to the second factor, and thus to set differences between the final product and the rest of the stages of the process.

The proteolytic phenomena referred to above are quoted by many authors as being of importance in flavour development (Langner, 1969; Demeyer et al., 1974; Verplaetse et al., 1989), but there is not any published confirmation of this conclusion and it must be assumed that it is based on the known sensory properties of amino acids and peptides and their substained increases in concentration during ripening. However, the majority of amino acids stated above as showing the greatest concentration increases were recorded as having 'bitter' flavour characteristics (Kato, Rhue & Nishimura, 1989).

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