Addition of a Neutral Proteinase from *Bacillus subtilis* (Neutrase) Together with a Starter to a Dry Fermented Sausage Elaboration and Its Effect on the Amino Acid Profiles and the Flavor Development

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Amino acid profiles from the peptide and free amino acid fractions were studied during the ripening of a traditional fermented sausage (chorizo) when a proteinase from *Bacillus subtilis* (Neutrase) was added. Total increase in free amino acids (FAA) was similar in both products, but its evolution was different. A slight decrease of some amino acids (Asp, Thr, Ala, Met, and Lys) was found in the product with Neutrase between the third and ninth days against the continuous increment observed in the control along the ripening. Total increase of the peptide fraction amino acids during the elaboration was more intense with enzyme. Both products showed a significant decrease of this fraction in the last week, which was higher in the product with Neutrase, which showed the highest increase in the FAA at that period. Sensory analysis showed a slight improvement on the overall acceptability significantly correlated with some flavor parameters.

Keywords: *Fermented sausages; exogenous proteases; amino acids profiles*

INTRODUCTION

García de Fernando and Fox (1991) suggested that an understanding of the type and extent of proteolysis can help to achieve better quality sausages because the nature and concentration of protein degradation products, ranging from peptides to the products of amino acid catabolism, determine, together with other factors, the flavor and texture of fermented sausages.

Berger et al. (1990) in an Italian fermented sausage found no N-containing volatile substances among the 68 compounds identified, suggesting that the degree of proteolysis had no effect on odor properties. However, Berdagué et al. (1993), through the application of GC−MS to the analysis of volatile compounds in dry sausages, found that 7% of these substances were nitrogen compounds. Demeyer (1992) suggested that proteolysis and subsequent amino acid fermentation may be an important process during dry sausage ripening, which interacts with carbohydrate fermentation and determines flavor as well as safety characteristics of the final product (this latter aspect is related to the variable fermentation of amino acids during ripening). Furthermore, almost all free amino acids and/or some peptides have some sweetness, bitterness, sourness, saltiness, and umami (or beefy) and are very important as taste substances in foods (Kato et al., 1989).

All of this suggests that the changes induced in protein degradation by application of different technologies, such as the use of exogenous enzymes, could lead to changes in the intensity, nature, or formation rate of their metabolic products, which could accelerate or change the aroma of dry fermented sausages. Naes et al. (1994) found that the addition of a proteinase isolated from *Lactobacillus paracasei* ssp. *paracasei* NCDO151 to a sausage mixture caused a significant increase in flavor intensity. In contrast, Diaz et al. (1996) observed that the addition of Papain to dry fermented sausage caused no improvement of the flavor.

The aim of this study was to examine the changes that took place in the amino acid profiles of both the peptide and the free amino acid fractions during ripening of a Spanish fermented sausage (chorizo) and to analyze the differences when a proteinase from *Bacillus subtilis* was added to a traditional formulation. Also the effect over the flavor development of the product was sensorially analyzed.

MATERIAL AND METHODS

Two types of sausages were manufactured in a pilot plant: a sausage without protease (control) and another one with protease (Neutrase). The same starter culture was added to both types of sausage: a mixture of *Lactobacillus plantarum* L115 (10%) + *Staphylococcus carnosus* M72 (90%) from Lacto-Labo (TEXEL). The amount added was 10^6−10^7 cfu/g. The protease employed was a *B. subtilis* metalloproteinase from Novo Nordisk, and 10^{-5} AU/g (AU = Anson units; 1 AU = amount of enzyme that digests hemoglobin at an initial rate such that there is liberated per minute an amount of TCA-soluble product which gives the same color with phenol reagent as 1 mequiv of Tyr) was added in accordance with previous research (Zapelena et al., 1995). The enzyme and the starter culture were added at the same time as the other ingredients. The two types of sausage were elaborated with a standard formulation and fermented in a laboratory ripening cabinet (Kowell Model CC-I) according to Zalacain et al. (1996).

A. HPLC Analysis. Free amino acids, amino acids from peptides, and total amino acids were identified and quantified by reverse-phase high-performance liquid chromatography, prior precolumn derivatization with phenyl isothiocyanate. Free amino acids and amino acids from peptides were analyzed at four stages (kneading, 72 h, 7 days, and 15 days). Total amino acids were determined only in the final product.

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The extraction of free amino acids was carried out with citrate buffer, and TCA was added to precipitate the proteins (Massi, 1963).

Amino acids from peptides were obtained from the NPN fraction by hydrolysis with 6 N HCl (110 °C/22 h). After the chromatographic analysis, the amount of each amino acid was corrected by the respective amounts of free amino acids.

To analyze the profile of total amino acids, the fat was removed with petroleum ether (Soxhlet method) and the samples were subsequently subjected to acid hydrolysis with 6 N HCl (110 °C/22 h). The hydrochloric acid was then evaporated and the hydrolyzed samples dissolved in 0.1 N HCl.

Phenylthiocarbamil derivatives of the different amino acid types were obtained according to Yang and Sepulveda (1985). They were injected into a Perkin-Elmer (PE Nelson) high-performance liquid chromatograph equipped with a Rheodyne manual injector with a 20-μL loop, a series 200 LC pump quaternary version, and a diode array detector operating at 255 nm. A column Nova Pack C18 (3.9 × 300 mm) from Waters was employed. The pump and the detector were connected to a series 600 link, and the conditions were controlled by a Turbochrom Navigator program (PE Nelson). Resolution of the peaks was accomplished by using a gradient elution with the mobile phase in pump A consisting of 2.5% acetonitrile in pH 6.4 and 70 mM sodium acetate 3-hydrate buffer, and that in pump B of acetonitrile−water (3:2). The analysis was carried out at 46 °C, and the flow rate was 1.0 mL/min. The mobile phase began at 0% B and increased to 46% B over 36 min, then further increased to 100% B over 1 min, and finally held at this ratio for 5 min. At the end of the isocratic period the percentage of B was lowered to 0%. Amino acids were identified by comparison of their retention times with those of standards from Sigma and were quantified by the internal standard method with L-norleucine (Sigma). Arginine could never be quantified in the free amino acids as its peak sometimes overlapped with that of carnosine. Thus, it was excluded from the total amount of amino acids in both FAA and peptide fractions.

B. Sensory Analysis. Quantitative descriptive analysis (QDA) was applied to evaluate flavor parameters of the fermented sausages. A total of 12 people were selected with a triangle test according to Cross et al. (1978). Selected judges were trained during three 1-h sessions with this type of product. Four samples (two pieces × two batches) of product with Neutrase were analyzed by each judge by comparison with a control sausage that was taken as a reference value (five points for all parameters). Samples were scored on a 1−9-point scale for odor intensity, pleasant odor, saltiness, astringency, pleasant taste, and overall acceptability.

Odor intensity was the degree of odor (1 = no odor; 9 = very intense odor). Pleasant odor and pleasant taste were hedonic parameters that evaluated the degree of pleasure of odor and taste (1 = disagreeable; 9 = very pleasure). Saltiness was the amount of salt perceived during chewing (1 = not salty; 9 = very salty). Spiciness was the degree of spicy taste perceived during chewing (1 = not spicy; 9 = very spicy). Astringency was the degree of astringency perceived after swallowing (1 = no astringency; 9 = very astringent). Overall acceptability was a parameter which evaluated the degree of acceptability of the sausage (1 = not acceptable; 9 = very acceptable).

C. Data Analysis. Data analysis were carried out with the Statgraphics STSC program. Shown results are the means obtained from four sausages of each type of product.

Analysis of variance was used to study significant differences ($p < 0.05$) for each amino acid (free and from peptides) at different phases in the same type of sausage.

Sensory data were subjected to correlation analysis (multivariate method) to determine possible statistical relationships between overall acceptability and the flavor parameters.

RESULTS AND DISCUSSION

The evolutions of the profile of free amino acids (FAA) in both types of sausage, with and without enzyme, during the ripening are shown in Tables 1 and 2. By comparing the results obtained for the control sausages (without enzyme) with those obtained for similar sausages by other authors (Dierick et al., 1974; DeMasi et al., 1990; Diaz et al., 1993), many differences can be found. This variability in specific amino acid changes during fermentation between similar studies was reported by DeMasi et al. (1990). Vandekerckhove (1977) pointed out that the extensive variation in the amine composition and concentration produced by free amino acid decarboxylation may be due to sausage fermentation conditions and/or the activities of the bacteria used in fermentation. In our study, the conditions were the same for both products.

Total increase in FAA during elaboration was quite similar in both types of products: 611.59 and 602.83 mg of FAA/100 g of dry matter (gdm) for control and modified products, respectively. Satterlee and Lillard (1967) found in Genoa sausages an increase in free amino acid content of 226 mg/100 gdm in the raw product to 1800−2800 mg/100 gdm in the finished products. Diaz et al. (1993) in traditional dry fermented sausages found 743 mg/100 gdm in the mixture and 1026, 1992, and 2584 mg/100 gdm at the end of ripening (26 days) in products without enzyme, with 600 and 6000 units of Pronase E, respectively.

Table 1. Free Amino Acids (mg/100 g of dry matter) in the Control Sausage[a]

	mixing	3 days	9 days	15 days
Asp	tr[a]	tr[a]	tr[a]	0.07[b]
Glu	tr[a]	13.63[b]	30.74[c]	50.16[d]
His	10.20[a]	27.17[b]	40.88[c]	46.06[c]
Lys	17.29[a]	46.57[b]	71.12[c]	90.10[d]
Arg	NI	NI	NI	180.20
Thr	40.98[a]	59.81[b]	29.06[a]	32.50[a]
Ser	tr[a]	8.36[b]	12.99[b]	28.78[c]
Gly	32.30[ab]	38.41[b]	48.77[c]	30.08[a]
Ala	15.53[a]	42.22[b]	83.47[c]	111.34[d]
Met	3.11[a]	24.95[b]	32.92[c]	37.58[c]
Val	0.15[a]	32.69[b]	51.24[c]	63.06[d]
Ile	tr[a]	32.58[b]	44.70[c]	50.34[c]
Leu	8.92[a]	41.81[b]	56.92[c]	65.32[d]
Tyr	3.89[a]	32.87[b]	39.18[c]	45.00[d]
Phe	3.91[a]	49.35[b]	66.56[c]	75.96[c]
Pro	50.60[a]	52.83[ab]	64.18[ab]	72.12[b]
total[b]	186.88[a]	503.25[b]	672.73[c]	798.47[d]

[a] Within a file, different letters denote significant differences ($p < 0.05$) between the times of ripening. [b] Without Arg.

Table 2. Free Amino Acids (mg/100 g of dry matter) in the Sausage with Neutrase[a]

	mixing	3 days	9 days	15 days
Asp	tr[a]	3.63[c]	3.06[b]	13.10[d]
Glu	tr[a]	25.69[b]	25.52[b]	47.20[c]
His	10.38[a]	30.91[b]	38.27[c]	48.22[d]
Lys	43.88[a]	82.31[c]	69.90[b]	86.38[c]
Arg	NI	320.99	201.59	184.22
Thr	33.99[a]	42.12[c]	33.28[a]	36.70[b]
Ser	tr[a]	tr[a]	23.10[b]	25.44[c]
Gly	26.59[b]	21.15[a]	22.12[a]	26.30[b]
Ala	16.61[a]	96.09[c]	73.87[b]	87.34[c]
Met	3.80[a]	40.95[c]	37.59[b]	42.54[c]
Val	0.99[a]	48.47[b]	51.10[b]	69.44[c]
Ile	12.65[a]	41.21[b]	42.17[b]	57.54[c]
Leu	13.70[a]	57.50[b]	54.74[b]	71.46[c]
Tyr	3.69[a]	43.38[bc]	41.51[b]	46.04[c]
Phe	4.71[a]	62.56[b]	58.28[b]	80.26[c]
Pro	49.54[a]	75.12[b]	68.50[b]	85.40[c]
total[b]	220.53[a]	671.09[c]	643.01[b]	823.36[d]

[a] Within a file, different letters denote significant differences ($p < 0.05$) between the times of ripening. [b] Without Arg.

Table 3. Amino Acids from Peptides (PAA) (mg/100 g of dry matter) in the Control Sausage[a]

	mixing	3 days	9 days	15 days
Asp	126.45^{a}	170.02^{b}	198.29^{b}	181.62^{b}
Glu	294.70^{a}	365.77^{b}	453.09^{c}	430.79^{c}
His	573.87^{c}	530.83^{b}	536.76^{b}	429.58^{a}
Lys	67.55^{a}	115.36^{b}	128.88^{b}	130.24^{b}
Arg	399.22	417.17	418.68	211.57
Thr			3.75^{a}	13.82^{b}
Ser	13.19^{a}	45.99^{c}	17.49^{ab}	25.96^{b}
Gly	132.52^{a}	148.99^{ab}	163.95^{b}	167.69^{b}
Ala	89.08^{a}	139.07^{b}	139.53^{b}	112.87^{a}
Met	2.90^{a}	18.84^{c}	13.82^{b}	12.50^{b}
Val	31.26^{a}	40.47^{ab}	56.48^{c}	44.92^{bc}
Ile	21.66^{a}	20.97^{a}	30.27^{a}	21.12^{a}
Leu	19.75^{a}	27.92^{ab}	37.78^{b}	22.57^{a}
Tyr	1.92^{a}	11.63^{b}	4.82^{a}	
Phe	21.24^{b}	17.18^{b}	22.07^{b}	8.38^{a}
Pro		68.14^{b}	78.37^{c}	85.02^{c}
total[b]	1396.09^{a}	1721.18^{b}	1885.35^{c}	1687.08^{b}

[a] Within a file, different letters denote significant differences ($p < 0.05$) between the times of ripening. [b] Without Arg.

Table 4. Amino Acids from Peptides (PAA) (mg/100 g of dry matter) in the Sausage with Neutrase[a]

	mixing	3 days	9 days	15 days
Asp	74.07^{a}	151.13^{b}	205.74^{d}	182.99^{c}
Glu	223.56^{a}	364.21^{b}	507.75^{c}	455.71^{c}
His	550.99^{a}	555.75^{a}	678.83^{c}	608.63^{b}
Lys	19.50^{a}	105.53^{b}	200.24^{c}	186.82^{c}
Arg	NI	44.84	169.63	149.03
Thr		16.88^{b}	50.87^{c}	43.73^{c}
Ser	22.18^{a}	74.16^{b}	90.11^{b}	85.39^{b}
Gly	77.47^{a}	122.24^{b}	166.83^{d}	143.40^{c}
Ala	70.23^{a}	75.88^{a}	167.97^{b}	155.41^{b}
Met			10.95^{b}	11.33^{b}
Val	42.46^{a}	33.96^{a}	71.24^{c}	56.96^{b}
Ile	5.10^{a}	14.91^{a}	45.51^{c}	27.96^{b}
Leu	13.90^{a}	13.45^{a}	53.89^{c}	33.96^{b}
Tyr				
Phe	18.29^{b}		34.88^{c}	32.59^{c}
Pro		34.00^{b}	81.85^{d}	60.69^{c}
total[b]	1117.75^{a}	1562.10^{b}	2366.66^{d}	2085.57^{c}

[a] Within a file, different letters denote significant differences ($p < 0.05$) between the times of ripening. [b] Without Arg.

Although in our study the use of Neutrase did not imply a different increase in total FAA fraction at the end of ripening, some changes during the evolution are worthy of mention. The Neutrase addition gave rise to a higher increase of total FAA during the first 3 days (fermentation) (+450.56 and +316.37 mg/100 gdm of increment in the products with and without Neutrase, respectively). This excess of amino acids could lead to a higher intensity of the different reactions suffered by the amino acids, giving rise to different products such as amines and volatile compounds.

In contrast to the continuous increase in the FAA content observed in the control sausage, there was a slight decrease in the product with Neutrase between the third and the ninth days owing to the decrease of some amino acids (Asp, Thr, Ala, Met, and Lys). During the last week a higher increase was observed again in products with Neutrase (+180.35 and 125.74 mg/100 gdm of increment in the products with and without Neutrase, respectively). It seems that the proteolytic activity during the last week overcomes the intensity of the amino acid degradation reactions, giving rise to similar amounts of total FAA in both types of products at the end of the ripening. Diaz et al. (1993) observed a clear decrease of some FAA (Tyr, Val, Met, Ile, Leu, and Phe) in the last period of the ripening which was consistent with the increase found in total volatile nitrogen. They suggested that Pronase E would supply sufficient substrate for the deamination of these FAA which occurred during ripening as a result of microbial activity.

Tables 3 and 4 show the peptide amino acid profile. No data on the amino acids from peptides with which to compare our results have been found in the bibliography consulted. The increase in total amino acids from peptides was significant over the ripening in both types of products, but the increment was much higher in the product with Neutrase (+967.82 mg/100 gdm) than in the control (+290.99 mg/10 gdm). Although levels of peptide amino acid fraction were lower at the first stage in the product with Neutrase, they increased with more

Table 5. Control: Amino Acid Profile in the End Product

	total amino acid (mg/100 gdm)	free amino acids (%)	amino acids from peptides (%)
Asp	4277.66	0.00	4.25
Glu	7028.1	0.71	6.13
His	1464.2	3.15	29.34
Lys	3617.5	2.49	3.60
Arg	3146.1	5.73	6.73
Thr	1835.2	1.77	0.75
Ser	1552.7	1.85	1.67
Gly	2479.5	1.21	6.76
Ala	2737.5	4.07	4.12
Met	1268.1	2.97	0.99
Val	2439.0	2.59	1.84
Ile	2247.6	2.24	0.94
Leu	2517.1	2.59	0.90
Tyr	74.4	60.48	
Phe	1893.5	4.01	0.44
Pro	2088.1	3.45	4.07
HOPro	844.5		

Table 6. Neutrase: Amino Acid Profile in the End Product

	total amino acid (mg/100 gdm)	free amino acids (%)	amino acids from peptides (%)
Asp	3722.4	0.35	4.92
Glu	6169.7	0.77	7.39
His	1120.0	4.30	54.34
Lys	2549.0	3.39	7.33
Arg	2795.2	6.59	5.33
Thr	1611.4	2.28	2.71
Ser	1464.7	1.74	5.83
Gly	2540.5	1.04	5.65
Ala	2501.3	3.49	6.21
Met	982.4	4.33	1.15
Val	1967.3	3.53	2.90
Ile	1683.5	3.42	1.66
Leu	1952.8	3.66	1.74
Tyr	65.9	69.86	
Phe	1321.2	6.07	2.47
Pro	1833.6	4.66	3.31
HOPro	1247.6		

Table 7. Multivariate Correlation between Sensory Parameters[a]

	odor intensity	pleasant odor	saltiness	spiciness	astringency	pleasant taste
overall acceptability	$r = 0.1885$	$r = 0.3468$	$r = -0.0064$	$r = 0.2349$	$r = 0.3007$	$r = 0.9453$
	$p = 0.1641^{n.s.}$	$p = 0.0088^{**}$	$p = 0.9626^{n.s.}$	$p = 0.0813^{n.s.}$	$p = 0.0243^{*}$	$p = 0.0000^{***}$

[a] n.s., not significant, $p > 0.005$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

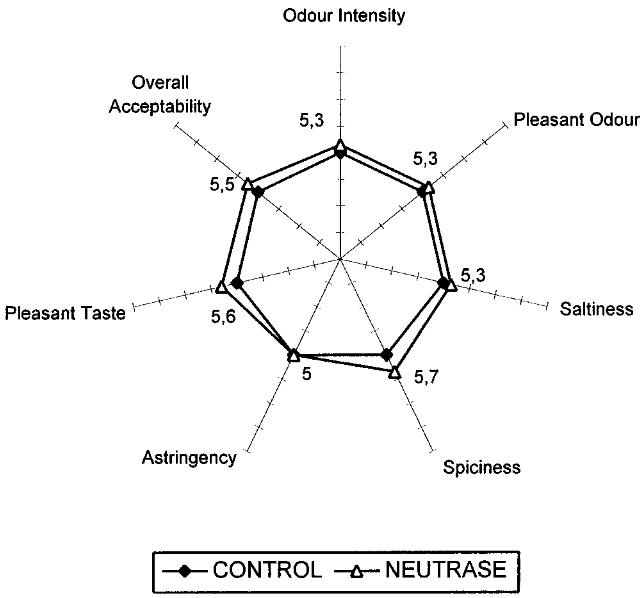

Figure 1. Sensory analysis: QDA graphics of flavor parameters.

intensity during the ripening reaching higher values in the final product.

The increase of this fraction during the first 9 days of ripening was higher in the product with Neutrase (+1248.91 mg/100 gdm) than in the control (+489.26 mg/100 gdm) due to the highest increment for every amino acid including His, Ser, Ile, Tyr, and Phe which did not increase significantly in the control.

Both products showed a significant decrease in the peptide amino acids in the last week. The highest decrease in the product with Neutrase (−281.09 mg/100 gdm against −198.27 mg/100 gdm in the control) would explain the highest increase observed in the free amino acids at that period (+180.35 mg/100 mg/100 gdm against +125.74 mg/100 gdm in the control).

Tables 5 and 6 show the total amino acid profile in the final products and the percentages of each amino acid, both those found as free amino acid and those from peptides. The profiles of total amino acid were similar in both sausages with the acid amino acids (Asp and Glu) being predominant. Of the 17 amino acids analyzed, 12 increased their presence in peptides, the most affected being His, Ser, and Lys. In the amino acid fraction, increases were found in 13 amino acids, but the intensity of the increment was much lower.

All of these differences in free amino acids and peptides could have an influence on sausage taste and odor. A sensorial analysis of flavor parameters was carried out comparing the modified sausage with the control (Figure 1). The synergistic effect that FAA can show in a food, even when levels are lower than their threshold values (Kato et al., 1989), could explain the higher scores obtained by the sausages with Neutrase for spiciness. The sausage with Neutrase scored slightly better for pleasant odor and taste and overall acceptability. Although these differences probably would not be detected by the ordinary consumer.

Table 7 shows a multivariate correlation between overall acceptability and the analyzed flavor parameters. Results showed that product acceptability was more related to pleasant taste ($p < 0.001$) than pleasant odor ($p < 0.01$). Then the slight improvement in the sensory quality of product with Neutrase could be related with its different amino acid and peptide profiles.

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