Effect of the Addition of a Neutral Proteinase from *Bacillus subtilis* (Neutrase) on Nitrogen Fractions and Texture of Spanish Fermented Sausage

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The effect of a metalloproteinase from *Bacillus subtilis* (Neutrase from Novo Nordisk) on the evolution of some nitrogen fractions during the ripening of a traditional dry fermented sausage (chorizo) was studied. The use of Neutrase especially affected myofibrillar proteins, giving rise to a higher loss of solubility than that produced in the sarcoplasmic proteins. The lowest soluble myofibrillar nitrogen of the products with Neutrase did not decrease the water-holding capacity. Sausages with Neutrase showed an increase in the nonprotein nitrogen fraction higher than that for sausages without enzyme. The increases in free α-NH$_2$ nitrogen during the ripening were very similar for both types of sausage. In the peptide fraction the α-NH$_2$ nitrogen increase was much higher in the product with Neutrase (78.29%) than in the product without enzyme (20.93%). Sensory analysis showed some slight differences in the texture parameters which were not correlated to product acceptability.

Keywords: *Fermented sausages; exogenous proteases; nitrogen fractions*

INTRODUCTION

The accelerated ripening of dry fermented sausages is an interesting aim of the meat industry. One of the methods available to accelerate ripening, which is being used in the dairy industry, is the use of exogenous enzymes. During the ripening many reactions affecting protein and fat take place. The use of exogenous proteinases and lipases could accelerate these reactions, giving rise to a faster development of the characteristics of the final product. In this way, lipases from different sources have been assayed (Fernández et al., 1991, 1995a,b; Zalacain et al., 1995, 1996) and the changes brought about by their use have been analyzed. Also, the use of some exogenous proteinases in dry fermented sausages is being investigated (Díaz et al., 1992, 1993, 1996; Naes et al., 1995).

The functional properties of proteins, such as water-holding capacity, fat-binding capacity, solubility, viscosity and gelation, are very important in relation to the textural attributes of the product. Moreover, nitrogen compounds could play an important role in the flavor (Demeyer, 1992). Induced changes in the proteolysis by application of different technologies could have an important effect on the sensorial properties of the product. Proteolysis has been mainly attributed to the Micrococcaceae (Cantoni et al., 1975; Selgas et al., 1986; DeMasi et al., 1990), but some endogenous meat enzymes could also be involved (Pezacki and Pezacka, 1986). Toldrá (1992) observed that cathepsins B, L, and D showed some activity during the mixing and fermentation stages, but during drying only cathepsin L remained quite active. Demeyer (1992) suggested that endogenous muscle proteases (cathepsins) contribute significantly to nonprotein nitrogen (NPN) formation during dry sausage metabolism.

Neutrase is a metalloproteinase from Novo Nordisk A/S and is produced by a selected strain of *Bacillus*

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subtilis. It is employed in brewing and baking and to improve animal and vegetable proteins. The optimum working conditions of Neutrase are 45–55 °C and pH 5.5–5.7, similar to the pH of drying fermented sausages. This enzyme was used in a previous work (Zapelena et al., 1997), and differences in the free amino acid and peptide compositions that seemed to be related with some flavor parameters were observed.

The objective of the present work was to study the effect of a selected dose of Neutrase on the evolution of some nitrogen fractions during the ripening of a dry fermented sausage (chorizo) using a traditional formulation and technology, including the use of a starter culture added at the same time. Also, the influence of those changes on the textural properties of the product was analyzed.

MATERIALS AND METHODS

Dry fermented sausages were elaborated in a pilot plant with a *B. subtilis* metalloproteinase from Novo Nordisk. Doses of 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 sausages were made with a standard formulation of 70% lean pork meat and 30% pork back fat. Other ingredients were added as follows: NaCl, 30 g/kg of mixture; dextrin, 15 g/kg, lactose, 20 g/kg; dextrose, 3 g/kg; polyphosphates, 2 g/kg; sodium ascorbate, 0.5 g/kg; NaNO$_2$, 0.2 g/kg; red pepper, 20 g/kg; cayenne pepper, 0.5 g/kg; garlic, 6 g/kg; Ponceau 4R, 0.15 g/kg; and oregano, 1 g/kg. Lean pork meat and fat back pork were minced in a cutter. Subsequently, all ingredients were mixed in a vacuum kneading machine and the mixture was stuffed into an artificial casing (60 mm diameter). A mixture of *Lactobacillus plantarum* L115 (10%) plus *Staphylococcus carnosus* M72 (90%) from Lacto-Labo (TEXEL) was also added as starter culture. The enzyme and the starter culture were added at the same time as the other ingredients. A portion of the mixture without the enzyme was used to elaborate the control sausages at the same time. The sausages were fermented in a laboratory ripening cabinet (Kowel Model CC-I) programmed to give the following conditions: constant temperature of 22 °C and relative humidity

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of 85% for 72 h, after which time the sausages were transferred to a drying chamber at 15 °C and relative humidity of 77% until the end of the ripening (15 days).

Analytical Methods. *A. General Parameters.* pH was determined using the potentiometer Orion Research Microprocessor Ionalyzer-901 using needle electrodes for solid samples (ISO, 1974). Moisture was determined according to the AOAC method (AOAC, 1984). Total nitrogen was analyzed according to the Kjeldahl method (AOAC, 1990). Water activity was measured with an EEJA-3 Novasina apparatus.

B. Fractionation of Sausage Nitrogen. The following nitrogen fractions were determined: sarcoplasmic protein N, myofibrillar protein N, insoluble protein N, and nonprotein N (NPN), according to the method described by Astiasarán et al. (1990). Each was reported as a percentage of total N.

With regard to the NPN fraction, two more determinations have been carried out: free α-NH$_2$-N and peptides. Total free α-NH$_2$-N was analyzed using a ninhydrin colorimetric method previous protein precipitation with TCA and tyrosine as the standard (Massi, 1963). The determination of total peptide N was obtained as free α-NH$_2$-N prior to acid hydrolysis of NPN fraction with 6 N HCl (22 h) and correction for free total α-NH$_2$-N.

C. Microbial Analysis. Ten grams of sausage was homogenized into 90 mL of peptone water (under sterile conditions for 2 min with a Stomacher). Then from this suspension decimal dilutions in peptone water were prepared and spread on the corresponding plates.

De Man Rogosa and Sharpe Agar No. 1 (MRS, Oxoid) for lactic acid bacteria (30 °C/72 h) in an anaerobic jar with a CO$_2$-enriched atmosphere (Gaspack, BBL) was used.

D. Sensory Analysis. Quantitative descriptive analysis (QDA) was carried out according to the method of Zapelena et al. (1997). Sausage with Neutrase was compared with the control sausage, which was taken as reference value (five points for all parameters). In this case, the selected parameters were fat-binding capacity (degree of binding between lean and fat), cohesiveness (degree to which mass holds together during chewing), hardness (amount of chews required to prepare sample for swallowing), and overall acceptability (the degree of acceptability of the sausage). Samples were scored on a 1–9 point scale (1 = very low fat-binding capacity and cohesiveness, very soft, and not acceptable; 9 = very high fat-binding capacity and cohesiveness, very hard, and very acceptable).

E. Data Analysis. Data analysis was carried out with Statgraphics STSC Inc. program (version 4.0). This is a registered trademark of Statistical Graphics Corp.

Analysis of variance (ANOVA) was used to study significant differences ($p < 0.05$) for water activity, moisture, and every nitrogen fraction at different phases in the same type of sausage. Student's *t*-test was used to determine whether there were any significant differences between the parameter at each phase in the two types of dry fermented sausages analyzed.

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

RESULTS AND DISCUSSION

The use of a proteinase is expected to increase the proteolysis process affecting proteins during the ripening. Previous to this work, an assay to test the activity of the enzyme Neutrase in the same conditions of the elaboration was carried out (Zapelena et al., 1995). Different doses were assayed, some of them (1 × 10^{-3} and 3.13 × 10^{-4} AU/g) giving defects in texture (too soft products). A dose of 1 × 10^{-5} was chosen.

The influence of this dose on some nitrogen fraction evolutions was analyzed in the present work. Table 1 shows the evolution of soluble protein nitrogen fractions in the control sausages and sausages with enzyme. Solubility of proteins, both sarcoplasmic and myofibrillar, showed a different evolution when the enzyme was

Table 1. Soluble Protein Nitrogen Fractions in the Control and in the Sausage with Neutrase (Milligrams of N per 100 mg of Total N): Myofibrillar Nitrogen and Sarcoplasmic Nitrogen[a]

	myofibrillar N			sarcoplasmic N		
	control	Neutrase	LS	control	Neutrase	LS
0 days	40.02[a]	40.02[a]		22.11[a]	22.11[a]	
3 days	35.32[b]	38.91[a]	**	17.04[c]	21.44[a]	**
9 days	33.93[b]	33.78[b]	NS	19.41[b]	20.12[ab]	NS
15 days	33.90[b]	27.10[c]	***	15.75[c]	17.88[b]	***

[a] LS, level of significance between types of sausage in each parameter at same time of ripening: NS, not significant $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Within a column, different letters denote significant differences ($p < 0.05$) between the times of ripening.

Table 2. Water Activity and Moisture in the Control Sausage and in the Sausage with Neutrase[a]

	A_w			moisture (g/100 g)		
	control	Neutrase	LS	control	Neutrase	LS
0 days	0.965[a]	0.965[a]		53.53[a]	53.53[a]	
3 days	0.944[b]	0.951[b]	NS	46.96[b]	48.06[b]	NS
9 days	0.906[c]	0.922[c]	**	38.20[c]	41.19[c]	***
15 days	0.865[d]	0.911[d]	**	32.03[d]	38.58[d]	***

[a] LS, level of significance between types of sausage in each parameter at same time of ripening: NS, not significant $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Within a column, different letters denote significant differences ($p < 0.05$) between the times of ripening.

employed. During the first 3 days the loss of solubility of both fractions was significant in the control products but not in the products with enzyme. However, from that stage the loss of solubility was much more intense in products with Neutrase, affecting especially the myofibrillar fraction. The decreases after 15 days of ripening were 28.77 and 19.13% for the sarcoplasmic fraction and 15.29 and 32.28% for the myofibrillar fraction, in the control and Neutrase-added products, respectively. Astiasarán et al. (1990) found similar losses of solubility (about 63%) in both fractions in a similar dry fermented sausage (chorizo) after 4 weeks of ripening.

As a consequence of the different evolutions found, the finished products show a significantly lower amount of myofibrillar nitrogen ($p < 0.001$) and a significantly higher amount of sarcoplasmic nitrogen ($p < 0.001$). Naes et al. (1995), adding a proteinase isolated from *Lactobacillus paracasei* ssp. *paracasei* NCDO151 to accelerate the ripening of dry sausages, detected that the proteinase activity was apparently restricted to the degradation of sarcoplasmic proteins and myofibrillar degradation products (water soluble proteins) rather than of myofibrillar proteins. It seems that the effect of the use of different proteinases on the evolution of soluble protein nitrogen fractions in dry fermented sausage elaboration depends on the employed enzyme.

The lowest soluble myofibrillar nitrogen of the products with Neutrase did not decrease the water-holding capacity. Data for moisture and water activity (Table 2) show that they were even statistically higher in the product with enzyme.

Loss of solubility depends on pH changes (Klement et al., 1974). The acidification process was similar in both types of products, reaching values of 5.21 and 5.11 in control and Neutrase-added products, respectively (Figure 1), which were not significantly different ($p > 0.05$). Díaz et al. (1993) reported a higher pH in the sausages with 6000P of Pronase E added and explained these findings by a higher amount of base nitrogen
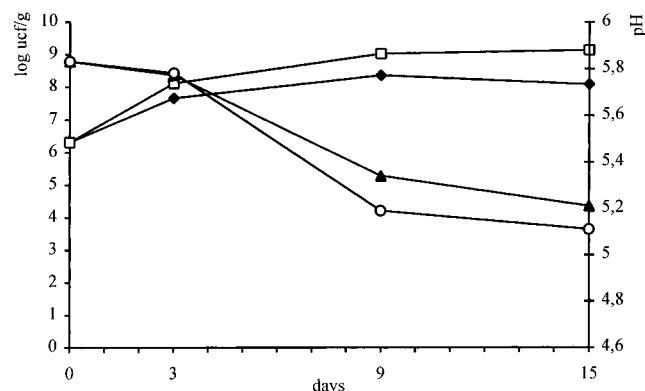
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Figure 1. Evolution of pH and acid lactic bacteria counts during ripening: acid bacteria of control sausage (◆) and of sausage with Neutrase (□); pH of control sausage (▲) and of sausage with Neutrase (○).

Table 3. Evolution of Insoluble and Nonprotein Nitrogen Fractions (Milligrams of N per 100 mg of Total N) in the Control and in the Sausage with Neutrase[a]

	insoluble N			nonprotein N		
	control	Neutrase	LS	control	Neutrase	LS
0 days	6.12[a]	6.12[a]		15.88[a]	15.88[a]	
3 days	14.05[b]	8.34[b]	***	16.68[ab]	16.40[a]	NS
9 days	14.85[b]	12.36[c]	**	17.89[b]	18.60[b]	NS
15 days	19.41[c]	17.55[d]	**	17.29[b]	19.88[b]	***

[a] LS, level of significance between types of sausage in each parameter at same time of ripening: NS, not significant $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Within a column, different letters denote significant differences ($p < 0.05$) between the times of ripening.

compounds in these sausages compared to the control sausages. Naes et al. (1995) found an increased *d*-lactic acid production and a rapid and pronounced pH drop in sausages with NCDO151 proteinase, which were interpreted as a stimulation of the starter culture. The similar acidification found in our study agrees with the similar counts of *Lactobacillus* (Figure 1). Likewise, Díaz et al. (1992, 1993) found no increases in microbiological counts. Observed differences in the pH evolution could be due to the different employed proteases.

The loss of solubility should be reflected by the insoluble fraction (IN). The increase of IN (Table 3) during the first 3 days was significant in both types of products, but the increment was lower in the product with Neutrase ($p < 0.001$). Differences between control and Neutrase products were maintained during all of the ripening, giving rise to a significantly lower amount of IN in products with Neutrase ($p < 0.01$). These differences did not explain the differences observed in the evolution of the soluble nitrogen fractions.

Increases in the NPN fraction during ripening have been found in many studies (Dierick et al., 1974; Garriga et al., 1986; Dominguez, 1988; García de Fernando and Fox, 1991; Astiasarán et al., 1990; DeMasi et al., 1990). Sausages with Neutrase showed an increase in the NPN fraction (25.19%) higher than that for sausages without enzyme (8.89%). The significantly lower amount of myofibrillar protein solubility observed in the sausage with Neutrase at the end of the fermentation could be related with the highest amount of NPN fraction observed in that product.

In a previous work the amino acid profiles from the peptide and free amino acid fractions by HPLC in similar products were analyzed. The total increases in free amino acids were similar in both products, but their evolutions were different. Also, the total increase of the

Table 4. Percentage of Free α-NH₂ Nitrogen and α-NH₂-N from Peptides Referred to the Total Nitrogen Content (Milligrams of N per 100 mg of Total N)[a]

	free α-NH$_2$-N			α-NH$_2$-N from peptides		
	control	Neutrase	LS	control	Neutrase	LS
0 days	2.81[a]	2.81[a]		2.58[a]	2.58[a]	
3 days	3.03[a]	3.89[b]	*	2.91[ab]	4.31[b]	***
9 days	3.93[b]	4.28[b]	NS	3.32[b]	5.54[c]	**
15 days	4.96[c]	4.91[c]	NS	3.12[ab]	4.60[b]	***

[a] LS, level of significance between types of sausage in each parameter at same time of ripening: NS, not significant $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Within a column, different letters denote significant differences ($p < 0.05$) between the times of ripening.

Table 5. Multivariate Correlation between Parameters

	fat-binding capacity	hardness	cohesiveness
overall acceptability	$r = 0.1513$, $p = 0.2657$NS	$r = 0.1369$, $p = 0.3143$NS	$r = 0.1626$, $p = 0.2313$NS

amino acids from the peptide fraction during the elaboration was found to be more intense with Neutrase (Zapelena et al., 1997).

In this work, the analysis of the fractions of NPN owing to free α-NH$_2$-N and to α-NH$_2$-N from peptides gave quite similar results (Table 4). The increases in free α-NH$_2$-N during the ripening were very similar for both types of sausages with no significant differences in its amounts at 9 and 15 days of ripening. However, differences were found in the peptide fraction from the first 3 days. The α-NH$_2$-N from peptides was significantly higher in the products with enzyme than in the control at every analyzed phase. The highest increase in peptide nitrogen fractions in the product with enzyme (78.29% of increment in contrast with the 20.93% in the control) should be due to the fact that Neutrase is an endopeptidase which hydrolyzes the interior peptide bonds of protein molecules. Furthermore, the greater increase in peptides that took place in the first stage in the sausage with Neutrase would have enhanced the exopeptidase activity during fermentation, giving rise to the highest free α-NH$_2$-N found at the third day.

The decrease observed in the α-NH$_2$-N from peptides in the last week (which is statistically significant in the product with Neutrase) agrees with the more important exopeptidase activity in the later drying period pointed out by Dierick et al. (1974).

The overall acceptability of a food is determined, among other properties, by textural characteristics which could be affected by the processes suffered by proteins. Díaz et al. (1993, 1996) found with the addition of Pronase E and papain at high doses a remarkable softening of dry fermented sausages. Naes et al. (1995), adding a proteinase isolated from *L. paracasei* ssp. *paracasei* NCDO151 to sausage mixtures, found a significant increase in hardness. The sensory analysis of our products showed some slight differences in the texture parameters, which probably will not be detected by the ordinary consumer (Figure 2). Furthermore, multivariate correlation (Table 5) showed that these parameters were not correlated to product acceptability, which has an even higher score in the product with enzyme. This seems to indicate that the slight changes observed in the sensory evaluation of hardness, cohesiveness, and fat-binding capacity are not related to product acceptability.

All of this suggests that although the addition of 10^{-5} AU of Neutrase/g of mixture has a significant proteolytic effect, the textural characteristics of sausages are not

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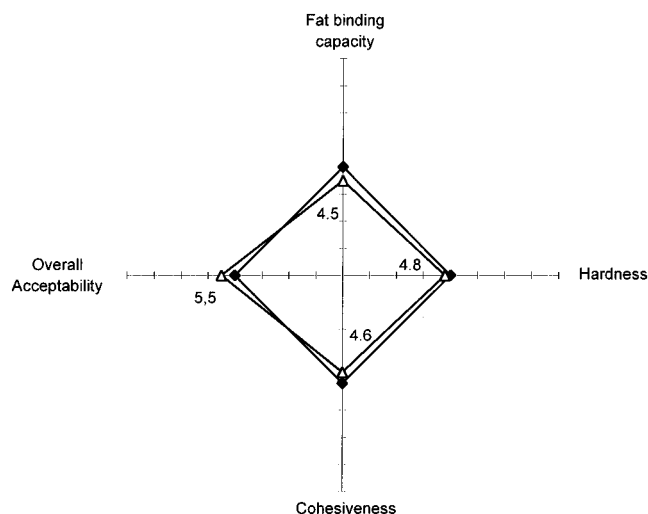*J. Agric. Food Chem.,* Vol. 45, No. 7, 1997 **2801**

Figure 2. Sensory analysis: QDA graphics of texture parameters: control (◆); Neutrase (△).

affected. This fact shows that it can be possible to produce a proteolysis increase by the addition of proteases to dry fermented sausages with an improvement of some of its sensorial properties and without excessive softening of these types of products.

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