Application of Lipozyme 10,000 L (from *Rhizomucor miehei*) in Dry Fermented Sausage Technology: Study in a Pilot Plant and at the Industrial Level

Izaskun Zalacain, M. Jose Zapelena, M. Paz De Peña, Iciar Astiasarán,* and José Bello

Departamento de Bromatología, Tecnología de alimentos y Toxicología, Facultad de Farmacia, 31008 Pamplona, Spain

A dose of 0.072 LU of lipozyme 10,000 L (lipase from *Rhizomucor miehei*)/g of sausage mixture was selected from three doses tested (0.072, 0.100, and 0.144 LU/g). The addition of this amount of lipase to fermented sausage formulation was studied in a pilot plant (15 days of ripening in artificial conditions) and at the industrial level (natural drying during 1 month for control sausages and 3 weeks for sausages with enzyme). The results showed no problems in the technological process (drying and acidification processes) owing to the lipase addition. No rancidity problems were found. The use of the lipase increased the release of all free fatty acids in both studies. The production of short chain fatty acids was also increased except for acetic and propionic acids at the industrial level. The sensory profiles of the two industrial sausages were quite similar in spite of the different ripening times.

Keywords: *Fermented sausages; exogenous lipases; accelerated ripening; lipid fraction*

INTRODUCTION

There are few papers in the literature on the use of enzymes, proteases, and lipases in the manufacture of dry fermented sausages in contrast with other dry fermented products, such as cheeses. Naes et al. (1992, 1993, 1995) investigated accelerated ripening of dry sausage by adding two concentrations of proteinase isolated from *Lactobacillus paracasei* subsp. *paracasei* to sausage mixtures. They concluded that the addition of proteinase produced earlier changes in the fermentation and ripening period than in the control, thereby indicating an acceleration of maturation.

Another research team investigated the effect of the addition of aspartyl proteinase from *Aspergillus oryzae* (Díaz et al., 1992) and *Streptomyces griseus* protease (Díaz et al., 1993). With regard to the use of lipases, they studied the addition of pancreatic lipase (Fernández et al., 1991, 1995a,b) and observed that the greater the lipase added the higher the lipolysis produced. Thus, the lipase produced a greater release of all free fatty acids (FFA) and an increase of the carbonyl content as compared with the control sausages.

We have studied two lipases: a chemical use lipase (*Candida cylindracea* lipase; Sigma Chemical Co.) (Zalacain et al., 1995, 1996) and a food lipase (*Rhizomucor miehei* lipase, palatase M 200 L; Novo Nordisk) (Zalacain et al., 1997). We observed differences in the release of the different free fatty acids by different lipases owing to their specificity. We also observed that juiciness and taste were slightly better in the sausages with palatase than in those without it, although these differences were not reflected in the overall acceptability. These results highlight the necessity of testing lipases from other sources with the aim of selecting the most adequate lipase for this application.

The purpose of this study has been to study the use of a *R. miehei* lipase (lipozyme 10,000 L; Novo Nordisk) in dry fermented sausage manufacture. This is a food lipase like palatase M 200 L (Novo Nordisk) but with higher activity (10 000 versus 200 LU/g). The work included the selection of the most suitable dose and the application of the enzyme in a pilot plant and at the industrial level.

* To whom correspondence should be addressed (phone, 34-48-105600; fax, 34-48-105649; e-mail, iastiasa@mail2.cti.unav.es).

MATERIALS AND METHODS

Pilot Plant. For the study of selection of the more suitable lipase dose, four different types of sausages were elaborated in a pilot plant. One of them was the control sausage without enzyme, and the others were elaborated with different amounts of lipase: 0.072, 0.100, and 0.144 LU/g. The same raw material was employed for both types of sausages which were elaborated consecutively in a pilot plant. The formulation was 75% lean pork meat and 25% pork back fat. The starter culture added was a mixture of *Lactobacillus plantarum* L 115 (10%) + *Staphylococcus carnosus* M 72 (90%) (Lacto-Labo, TEXEL, Madrid). The amount was 10^6–10^7 cfu/g of mixture.

Other ingredients were added as follows: NaCl, 30 g/kg of mixture; dextrin, 15 g/kg; lactose, 20 g/kg; red pepper, 20 g/kg; cayenne pepper, 0.5 g/kg; garlic, 6 g/kg; ponceau 4R, 0.15 g/kg; oregano, 1 g/kg. Lean pork meat and pork back fat were minced in a cutter and subsequently mixed with the other ingredients in a vacuum kneading machine, and the mixture was stuffed into artificial casings (60 mm diameter). The sausages were fermented in a laboratory ripening cabinet (Kowel Model CC-I) at 22 °C and 85% RH for 72 h, after which the sausages were transferred to a drying chamber at 15 °C and 77% RH until the end of ripening (15 days). Samples were taken on days 3, 7, 10, and 14, and acidity values were measured.

The second part of the work was the study in a pilot plant with the selected dose of lipase. A control sausage and a sausage with lipase were elaborated with the same formulation and ripening conditions as before. Samples were taken on day 15 (finished product).

Industrial Level. In this part of the work two types of sausages were made in a local industry. The formulation was 80% lean pork meat and 20% pork back fat. The starter culture added was a mixture of *Pediococcus pentosaceus* (50%) + *Micrococcus* sp. (50%). The additives were red pepper, 6.75 g/kg; garlic, 2.15 g/kg; NaCl, 20.98 g/kg; carbohydrates, 42.34 g/kg; sodium caseinate, 6.75 g/kg; polyphosphates, 1.76 g/kg; E-124, 1.37 g/kg; and curing salt, 3.80 g/kg.

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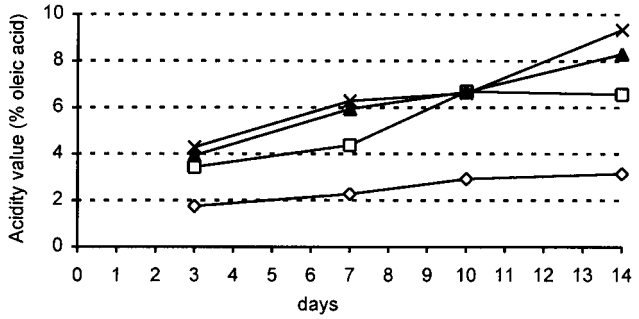

Figure 1. Selection of lipozyme dose: (◊) control, (□) 0.072 LU/g lipozyme, (▲) 0.100 LU/g lipozyme, and (×) 0.144 LU/g lipozyme.

A part of the lean pork meat was minced in a mincer with a 12 mm plate. The rest was minced with a 25 mm plate. The other ingredients were mixed in a beater with the water, and a pulp was made. All ingredients were mixed in a vacuum kneading machine, and the mixture was stuffed into "fibran" casings (70 mm diameter). The sausages were ripened in a natural drying place. Two small boilers with charcoal were placed in the drying place and went on combustion for 12 h. The boilers were changed 10 times during 5 days. From the sixth day the drying was regulated by opening and closing the windows. The sausages were ripened for 32 days (control sausage) and 25 days (lipozyme-added sausage).

Analytical Methods. *Chemical Analysis.* pH was determined with a pH meter micropH 2000 with a needle electrode (CRISON Instrument SA, Barcelona). Moisture was determined using method ISO R-1442 (ISO, 1973). Water activity was determined using a Novasina Model 5803 meter.

Qualitative extraction of fat was made according to Bligh and Dyer (1959). Acidity value (g of oleic acid/100 g of fat) was determined using method ISO 1740 (ISO, 1980). Peroxide value was determined using method ISO 3960 (ISO, 1977). Carbonyl compounds were determined according to Henick et al. (1954). Thiobarbituric acid value (TBA number) was determined according to Tarladgis et al. (1960, 1964) with the modifications of Pikul et al. (1989).

Free fatty acids (FFA) were recovered by shaking the lipid extract with an anion exchange resin (Amberlyst A-26, Sigma Chemical Co.) (Needs et al., 1983). The resin-bound FFAs were methylated directly and the individual acids quantified using as internal standard heptadecanoic acid (Sigma Chemical Co.), by gas chromatography. Determination was carried out using a Perkin Elmer Model Sigma-300/dual FID (oven temperature 185 °C; detector temperature, 240 °C; injector temperature, 250 °C). The fatty acids were identified and quantified using appropriate standards (Sigma Chemical Co.).

Short and intermediate chain fatty acids were isolated and separated using the method described by Duda et al. (1981). The dry sodium salts were dissolved in 3 mL of dichloromethane (Merck), and the resulting solution was acidified by the addition of 0.1 mL of phosphoric acid. Quantitative determination was carried out using a Perkin Elmer autosystem gas chromatograph with FID and Nukol capillary column (Ceccon, 1991) (oven temperature, 172 °C; detector temperature 220 °C; injector temperature, 220 °C). The quantitative analysis were carried out using crotonic acid as internal standard. Propionic, butyric, isovaleric, caproic, caprilic, pelargonic, and crotonic acids were purchased from Merck KGaA (Darmstadt, Germany), and acetic acid was from Panreac Quimica SA (Barcelona). All were standards for gas chromatography.

Sensory Analysis. Quantitative descriptive analysis (QDA) was carried out on the four types of sausages (two from pilot plant and two from industrial level) as described by Zook and Pearce (1988). The panel was composed of eight judges previously selected by triangle test and trained. Odor intensity, juiciness, acidity, rancidity, spiciness, pleasant taste, and overall acceptability were evaluated on a 1−9 scale on a hedonic scale.

Statistical Analysis. The values shown in the tables are the mean of eight determinations obtained from four batches of each type of sausage and their standard errors. Student's *t*-test was used to determine whether there were differences between the two types of sausages: control without lipase and lipozyme-added sausages.

RESULTS AND DISCUSSION

The lipase dose selection studies showed an increase in acidity values during the ripening in all sausages (Figure 1) and also that the greater the dose added, the higher the acidity value. These results displayed a greater activity of this lipase than palatase (Zalacain et al., 1997): at a similar dose the acidity value was higher. In the study with palatase, the dose selected was 0.100 LU/g of sausage, which produced an acidity value of 5.36 g of oleic acid/100 g of fat. At the same dose lipozyme produced 8.30 g of oleic acid/100 g of fat, which was considered excessive for this type of sausage. The dose of 0.072 LU/g of sausage which gave rise to 6.57 g of oleic acid/100 g of fat was selected.

With the selected dose two studies were carried out, one in a pilot plant and the other at the industrial level, with comparisons being made in both cases with control products (without enzyme). No significant differences were found for moisture and water activity between sausages with and without enzyme elaborated in the pilot plant as a consequence of the similar drying process (Table 1). In contrast, in the industrial products these parameters showed higher values in the sausages with enzyme than in the control due to the shorter ripening period. The acidification process was controlled by pH measurement. Although some significant differences were found between the two types of sausages at both pilot plant and industrial level all the values could be considered as normal for this type of product (Lois et al., 1987; Coventry and Hickey, 1991; Montel et al., 1993; Ibáñez et al., 1995).

The effectiveness of lipozyme was observed through the results obtained in the analysis of FFA (Table 2). For sausages elaborated in the pilot plant, a greater release of all FFA was found in sausages elaborated with lipase than in the controls. When palatase was used (Zalacain et al., 1997) only palmitic, palmitoleic, stearic, and oleic acids were significantly higher in the products with enzyme than in their respective controls. Furthermore, the rate of increase of these acids was lower than when lipozyme was used. In fact, the amount of these acids present increased 1.37, 1.61, 1.08, and 1.20 times, respectively, whereas in sausages elaborated with lipozyme the acid content increased 1.54, 1.74, 1.62, and 1.73 times, respectively. At the industrial level the sausages with lipase also showed

Table 1. Acidification and Drying Processes[a]

	pilot plant			industrial level		
	control, 15 days	lipozyme, 15 days	LS	control, 32 days	lipozyme, 25 days	LS
water activity	0.92 ± 0.01	0.91 ± 0.01	ns	0.91 ± 0.00	0.93 ± 0.00	**
moisture (%)	39.94 ± 0.46	40.72 ± 0.44	ns	43.52 ± 0.42	48.30 ± 0.24	***
pH	4.76 ± 0.05	5.02 ± 0.04	**	4.84 ± 0.03	4.73 ± 0.03	*

[a] LS, level of significance; ns, not significant ($p > 0.05$); *$p < 0.05$;** $p < 0.01$; ***$p < 0.001$.

Table 2. Lipolysis Process: Free Fatty Acids (mg/100 g of Fat)[a]

	pilot plant			industrial level		
	control, 15 days	lipozyme, 15 days	LS	control, 32 days	lipozyme, 25 days	LS
myristic acid	44.51 ± 2.15	91.38 ± 4.03	***	38.53 ± 3.39	73.46 ± 6.55	**
palmitic acid	424.99 ± 13.35	653.04 ± 38.99	***	441.59 ± 37.94	761.68 ± 38.36	**
palmitoleic acid	85.29 ± 1.18	149.53 ± 7.25	***	55.85 ± 3.82	136.29 ± 7.94	***
stearic acid	218.34 ± 3.09	353.48 ± 20.81	***	218.11 ± 16.26	367.64 ± 15.36	***
oleic acid	1183.80 ± 32.47	2048.72 ± 21.16	***	1005.85 ± 50.82	1917.87 ± 14.51	***
linoleic acid	249.07 ± 6.56	349.26 ± 22.02	**	764.72 ± 61.27	1361.98 ± 13.17	***
linolenic acid	50.29 ± 3.12	74.42 ± 4.95	**	48.03 ± 4.08	88.81 ± 1.01	***

[a] LS, level of significance; ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 3. Oxidation Process[a]

	pilot plant			industrial level		
	control, 15 days	lipozyme, 15 days	LS	control, 32 days	lipozyme, 25 days	LS
TBA number (MA/kg of dry matter)	0.50 ± 0.03	0.49 ± 0.04	ns	1.63 ± 0.15	1.78 ± 0.07	ns
peroxide value (mequiv of O_2/kg of grasa)	4.19 ± 0.14	3.71 ± 0.13	*	19.45 ± 0.81	14.49 ± 0.58	**
carbonyl compounds (μmol/g fat)	19.92 ± 0.16	19.28 ± 0.37	ns	31.18 ± 0.54	38.36 ± 0.86	***

[a] LS, level of significance; ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 4. Short and Intermediate Chain Fatty Acids (mg/100 g of Dry Matter)[a]

	pilot plant			industrial level		
	control, 15 days	lipozyme, 15 days	LS	control, 32 days	lipozyme, 25 days	LS
acetic acid	53.69 ± 1.27	88.60 ± 1.70	***	236.84 ± 9.01	207.30 ± 13.78	ns
propionic acid	4.41 ± 0.01	4.55 ± 0.01	***	7.39 ± 0.10	7.21 ± 0.04	ns
butyric acid	4.85 ± 0.03	5.18 ± 0.01	***	6.04 ± 0.02	6.85 ± 0.06	***
isovaleric acid	4.33 ± 0.01	6.02 ± 0.01	***	ND	4.41 ± 0.11	
caproic acid	4.87 ± 0.03	4.90 ± 0.02	ns	4.15 ± 0.08	4.76 ± 0.02	***
caprylic acid	4.87 ± 0.03	5.02 ± 0.02	**	5.54 ± 0.01	6.50 ± 0.12	***
pelargonic acid	ND	ND		75.16 ± 3.93	84.67 ± 2.76	ns

[a] LS, level of significance; ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ND, not detected.

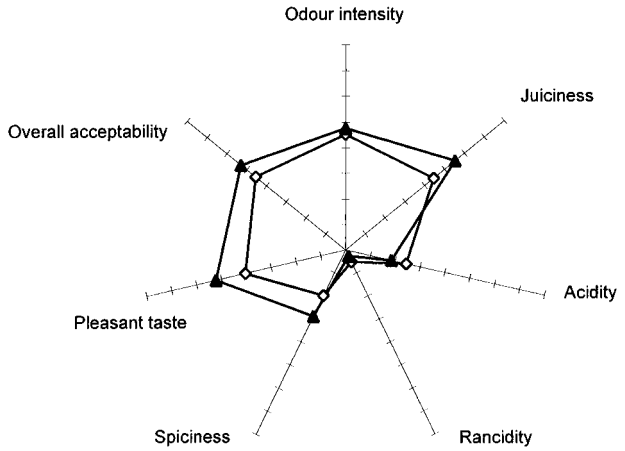

Figure 2. Sensory evaluation, pilot plant, QDA (scale 1−9): (◇) control and (▲) lipozyme.

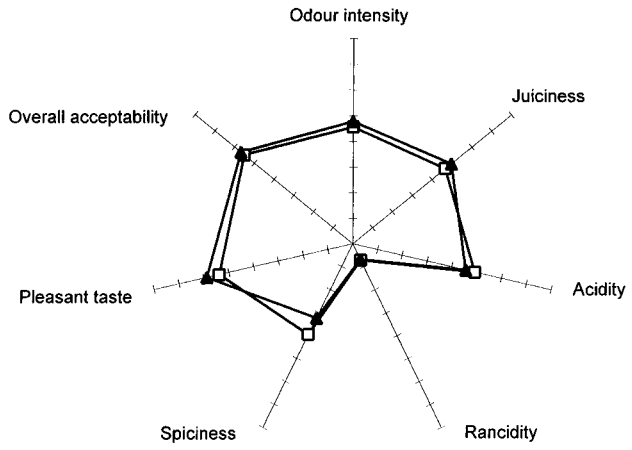

Figure 3. Sensory evaluation, industrial level, QDA (scale 1−9): (□) control (32 days) and (▲) lipozyme (25 days).

the greatest quantities for all FFA in spite of their shorter ripening time.

These results would corroborate the theory that lipozyme in the conditions of sausage ripening is more active than palatase and thereby would be more appropriate for this application.

As in previous research (Zalacain et al., 1995, 1996, 1997) the measurement of the oxidation showed no increment of this process with the use of the lipase (Table 3). The scores obtained for rancid taste in the sensorial evaluation of the sausages corroborated this fact (Figures 2 and 3). It must be noted that the oxidative processes were more intense in sausages elaborated at the industrial level than in those elaborated in the pilot plant. This could be due to the different formulation, ripening time, or technological process.

Short and intermediate chain fatty acids are compounds produced by the degradation of FFA, and they influence the odor and taste of the fermented sausages. The increased lipolysis induced by lipases could increase the synthesis rate of these substances and subsequently the sensorial properties of the final products. In the pilot plant, the addition of lipozyme produced an increment of all short chain fatty acids, with particularly significant increases in acetic, propionic, butyric, and isovaleric acids (Table 4). Working with *C. cylindracea* lipase, Zalacain et al. (1995, 1996) observed higher amounts of these acids in the sausage with lipase, indicating an influence of the lipolytic process on the formation of volatile fatty acids. At the industrial level, no differences in acetic and propionic acids were observed between the two types of sausages, probably because the activity of the enzyme was compensated for by the longer ripening time of the industrial sausage.

Acetic acid content was much higher in sausages elaborated at the industrial level than in the pilot plant, probably because of their longer ripening time. In the literature very different values of acetic acid content have been observed as follows: 48 and 58 mg/100 g of product and 62 and 240 mg/100 g of dm (Montel et al., 1993; Halvarson, 1973; Ibáñez et al., 1995; Domínguez and Zumalacárregui, 1991, respectively).

Butyric, caproic, and caprylic acids were in general significantly greater in the sausage with lipase. This fact could be due to the addition of the lipase and also, in the case of products elaborated at the industrial level, to the different ripening time. Effectively there is evidence indicating that these acids decrease during the ripening (Zapelena et al., 1993).

Pelargonic acid, which can be produced by the oxidation of longer chain fatty acids, was found in very high amounts in both types of sausages elaborated at the industrial level. In other commercial sausages similar amounts of pelargonic acid have been observed (Zapelena et al., 1993, 1994).

Although many compounds are responsible for the aroma of sausages, differences observed in the quantities of the short chain fatty acids are probably related to some of the differences found in the sensory evaluation. Figures 2 and 3 show the typical spider web graph of the quantitative descriptive analysis obtained for both types of products elaborated in the pilot plant and at the industrial level, respectively. In the pilot plant, significantly higher scores ($p < 0.01$) were found for juiciness, spiciness, pleasant taste, and overall acceptability. The higher scores on pleasant taste and overall acceptability in sausages with lipozyme are worthy of mention. Differences in pleasant taste but not in overall acceptability have been seen when using palatase (Zalacain et al., 1997).

The sensory profiles of the two industrial sausages were quite similar in spite of the different ripening times. Only the pleasant taste showed significantly better scores ($p < 0.05$) in the lipase sausages. The industrial sausages presented more acidity and spiciness than the pilot plant sausages, probably because of the higher amounts of short chain fatty acids, mainly acetic acid, which can cause these tastes (Girard and Bucharles, 1991).

In summary, these results show that the use of this lipase may have advantages by improving the sensory quality and, regarding lipolytic processes, reducing ripening time. Furthermore, no negative effects on the technological process were observed.

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