EFFECT OF THE ADDITION OF THE ASPARTYL PROTEINASE FROM *Aspergillus oryzae* ON DRY FERMENTED SAUSAGE PROTEOLYSIS DURING RIPENING

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SUMMARY: The effect of the addition of the aspartyl proteinase from *Aspergillus oryzae* on the proteolysis of dry fermented sausages ("salchichón") has been investigated. All batches, with or without (control) proteinase, showed a similar pattern in the microbial, pH and moisture changes during ripening, which coincided with those reported for other conventional dry fermented sausages. The selected nitrogen fractions (water soluble, non protein, 5% phosphotungstic acid soluble, 5% sulfosalicylic acid soluble and total volatile basic nitrogens) reached higher values in proteinase treated sausages. Organoleptically, the sausages manufactured with proteinase were low scored due to a remarkable softening.

INTRODUCTION: Dry fermented sausage manufacturing consists of three phases: formulation, fermentation and ripening. Several changes occur during fermentation and ripening processes, in which are included the formation of nitric oxide by the nitrate and nitrite reduction, the decrease of the pH via glicolysis, lipolysis, proteolysis and a partial dehydration. All these phenomena lead to the typical organoleptic properties.

Protein breakdown has an obvious role in determining the texture, the background flavour and the availability of flavour precursors in all matured dry sausage varieties. Proteolysis occurring during ripening yields polypeptides, peptides, free amino acids, etc. The reactions involved in the generation of these compounds are catalyzed by endogenous enzymes, such as cathepsines and trypsine-like proteinases (Pezacki & Pezacka, 1986) and other proteinases produced by microorganisms involved in the ripening process, mainly those of *Micrococcaceae* (Selgas *et al.*, 1986), at which have been attributed a major role in the proteolysis phenomena (Cantoni *et al.*, 1975).

The ripened products manufacturing involves a cost of storing until they reach a suitable matured state. Several attempts has been made in cheeses to shorten the ripening period. One of the approaches used has been the proteinase addition to the curd for increasing proteolysis. One of the proteinase added has been the aspartyl acid proteinase from *Aspergillus oryzae* (Law, 1984). Dry fermented sausages seem to be an appropriate substrate to apply this method. Recently, Díaz *et al.* (1992) have applied this approach to a Spanish salami-like dry fermented sausage ("salchichón") by using the pronase E from *Streptomyces griseus* . They concluded that the greater the pronase E added the higher were the values reached for all nitrogen fractions studied and, organolleptically, the sausage manufactured with 600 units (see below) of pronase E were well qualified by panelists. In the present work, the aspartyl acid proteinase from *Aspergillus oryzae* has been assayed with the same aim.

MATERIAL AND METHODS: Dry fermented sausages were manufactured in an experimental plant of a local factory. The composition of sausages was (% w/w): pork (56), beef (12), lard (25), dextrose (0.8), lactose (1.0), dextrine (1.8), salt (2.5), glutamate (0.25), nitrates (0.0085), nitrites (0.0065), black pepper (0.14), and ascorbate (0.046). Ingredients were mixed in a cutter with particle size reduction to about 3 mm. Sausage mixture was divided in three batches (2 kg each). Aspartyl proteinase was added to two batches at the concentration of 800 and 4500 enzyme units, named as 800A and 4500A, respectively. One unit represented the amount of enzyme that produced an increase of 1 unit in the absorbance at 440 nm per hour, using azocasein (Sigma) as substrate (0.8% in TRIS buffer 0.2M, pH 6.5). The third batch was the control, in which no proteinase was added.

Enzyme (dissolved in about 200 ml of distilled water) was mixed with the ingredients mixture in a kneading machine, and then the paste was stuffed into artificial casings (3 cm diameter). The total weights of experimental sausages were about 2 Kg divided in portions of about 200 g. Sausages were ripened in a laboratory ripening cabinet (KOWEL Mod CC-3-1) programmed to give the following conditions: the temperature was maintained at 22°C for 24 h and the relative humidity (RH) at 90 % for 12 h. After these periods, the temperature and the RH were gradually decreased up to 12°C and 75% respectively. These values were reached at the fourth day and maintained until the end of the experience (14 days). Samples (about 200 g) of each batch were taken at different times during ripening.

Total viable counts were determined on Plate Count Agar (Oxoid) and *Micrococcaceae* on Mannitol Salt Agar (Oxoid), both incubated at 32°C for 2 days. Lactic acid bacteria were enumerated on double layer MRS agar (Oxoid) at pH 5.6 and incubated for 4 days at 32°C.

The pH was assayed inserting the electrode into the sausage sample and dry matter by drying at 100°C to constant weight. Water activity (Aw) was measured in a Decagon CX-1 (Decagon Devices Inc. Pullman, Washington. U.S.A.).

For nitrogen fraction determinations, a portion of sausages (30 g) was homogenized with distilled water in a Polytron Mod PTA20TS up to a final volume of 350 ml and then centrifuged (6500 x g for 6 min) in a Sorvall RC5B centrifuge. The resulting pellet was reextracted with an additional volume of water (100 ml) and recentrifuged under the same conditions. Both supernatants were combined and the volume recorded. This fraction was the water soluble nitrogen (WSN). Non protein (NPN), phosphotungstic acid soluble (PTN) and sulfosalicylic acid soluble (SSN) nitrogens were obtained from WSN. An aliquot (20-50 ml) of WSN was mixed with the same volume of 25% trichloroacetic, 10% phosphotungstic or 10% sulfosalicylic acid solutions, respectively. Mixtures were left at 4°C for 30 min (NPN and PTN) or 17 hours (SSN). Insoluble materials were removed by filtration through Whatman paper No 2. Total nitrogen and nitrogen of WSN, NPN and PTN were determined by the Kjeldahl method in a Büchi digestor (mod. 425) and distillation (mod. 315) units. The SSN was considered as α-amine nitrogen and measured with the ninhydrin reagent as described by Clark (1966). Other aliquot of WSN was used for the total volatile basic nitrogen (TVBN) determination, which was carried out by the Conway microdiffusion technique (Pearson, 1973).

At the end of the ripening, samples of the three batches were assessed by a panel composed by, at least, 18 members. Triangle test was made according to the I.S.O. (TC 34/SC 12 Regulation). Samples were also examined by panelists to judge the colour, appearance, texture and flavour according to a hedonic scale from 1 (very bad) to 10 (very good).

RESULTS AND DISCUSSION:

1. Microbial flora: As in the case of pronase E treated sausages (Díaz *et al.*, 1992), changes during ripening were similar in all batches and also similar to those reported by other authors in different kind of dry sausages (Liepe, 1982; Lücke, 1984; Selgas *et al.*, 1988).

2. pH: All three batches showed a similar pattern following the common profile of conventional dry fermented sausages. It decreased sharply during fermentation (from 5.9 to 5.1 in three days) and stabilized thereafter until the end of ripening.

3. Water activity (Aw)**:** The changes in Aw also followed the typical trend in these products. Aw linearly decreased from 0.97 to 0.90 for control and 800A batches and to 0.88 for 4500A batch.

4. Nitrogen fractions: Figure 1 shows the changes in WSN, NPN and PTN during ripening. In the control batch, these fractions remained roughly constant during fermentation and ripening, while a strong increase during fermentation was observed in the proteinase added batches, to become stabilized during the remaining ripening time. The levels and changes during ripening of NPN of the control batch were, in general terms, similar to those reported for Spanish (Lois *et al.*,1987), other European (Dierick *et al.*, 1974) and American (DeMasi *et al.*, 1990) dry fermented sausages. As expected, the levels of nitrogen in all fractions were higher in the 4500A batch than those found in the batch added of 800 units of enzyme. In control batch, SSN and TVBN (fig. 2) showed a similar pattern to those observed for WSN, NPN and PTN. However, in proteinase added batches, a consistent increase in SSN and TVBN fractions was observed, being the values higher in the 4500A batch.

The greater increase in the nitrogen fractions during fermentation is obviously due to the activity of aspartyl proteinase. The deacceleration of protein breakdown in the further stages of ripening was similar to those observed in the experiences, in which pronase E was used (Díaz *et al.*, 1992). In this work, the proteolysis deacceleration was attributed to several factors, i.e.the different temperature, conditions used during fermentation and ripening, the decrease of the pH and, probably, an inhibition of the enzyme activity by the accumulation of the resulting products from proteolysis during fermentation and ripening phases. However, aspartyl proteinase has its optimum pH at values between 3 and 5 (Belitz and Grosch, 1987). Therefore, the low pH is not an inhibition factor of the aspartyl proteinase activity. In this case, the other two factors involved may be sufficient to explain the enzyme inhibition. Indeed, the inhibition of the enzyme activity due to the accumulation of hydrolytic products is in agreement with the fact that in spite of the different characteristics (mainly the pH, i.e. the optimum of pronase E is neutral and that of aspartyl proteinase is acid) of both proteinases, the amounts of degradative nitrogen products were, in general terms, similar in the experiences performed by Diaz *et al.* (1992) and in the present work, when similar enzyme concentrations were used. SSN and TVBN were the only fractions that reached higher values in aspartyl proteinase treated sausages than in pronase E ones. This circumstance could explain the lower Aw found in the 4500A batch than those of the control and 800A batches.

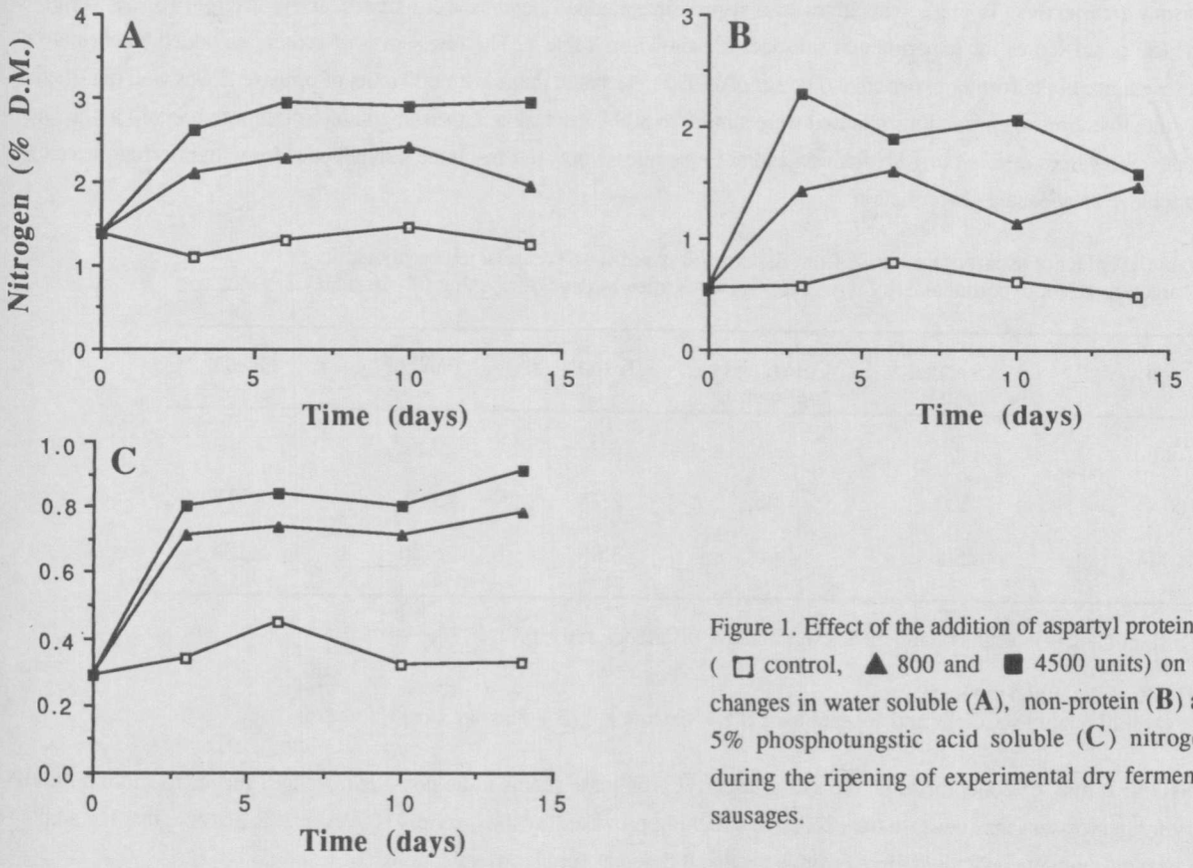

Figure 1. Effect of the addition of aspartyl proteinase (□ control, ▲ 800 and ■ 4500 units) on the changes in water soluble (**A**), non-protein (**B**) and 5% phosphotungstic acid soluble (**C**) nitrogens during the ripening of experimental dry fermented sausages.

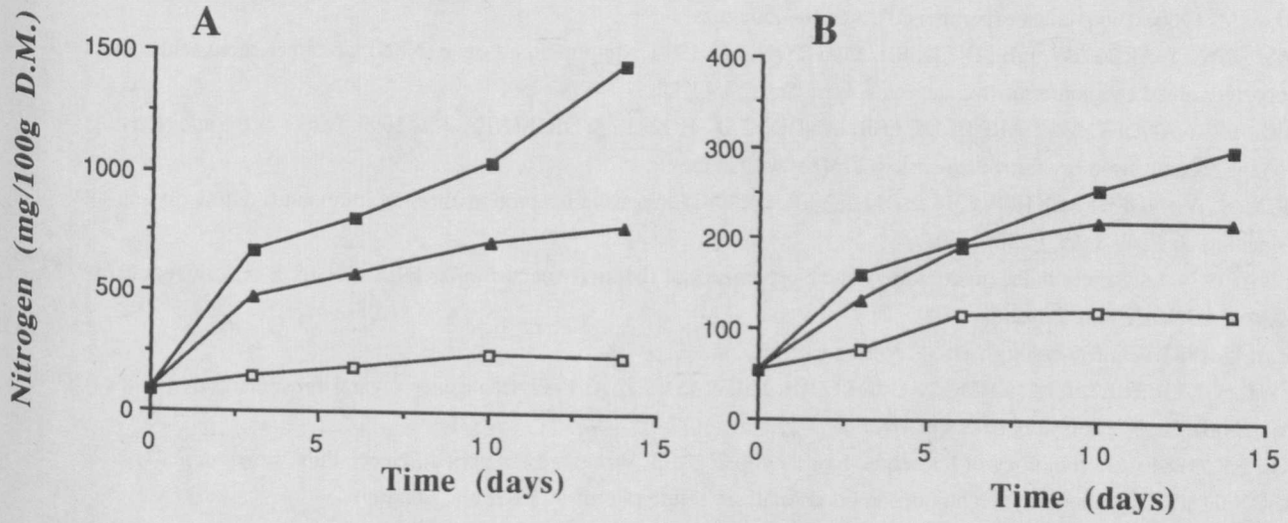

Figure 2. Effect of the addition of aspartyl proteinase (□ control, ▲ 800 and ■ 4500 units) on the changes in 5% sulfosalicylic acid soluble (**A**) and total volatile basic (**B**) nitrogens during the ripening of experimental dry fermented sausages.

5. Sensory properties: Triangle tests showed that proteinase added batches were significantly different ($p < 0.01$) to control batch. Mean taste panel scores for experimental sausages are shown in Table 1. The low scores of proteinase added batches are mainly related to its softening. In the former experience (Díaz *et al.*, 1992), the batch made with 600 units of pronase E was well qualified (8.18), although the amounts of nitrogen fractions released were similar to 800A batch. Then, the remarkable softening observed in the latter may be related to the high values reached by SSN fraction and/or to the release of water insoluble polypeptides from myofibrillar proteins which are unable to achieve an adequate gel formation.

Table 1. Effect of aspartyl proteinase from *Aspergillus oryzae* (AS) on selected organoleptic characteristics of experimental dry fermented sausages after 14 days of ripening (0 - 10 scale)

Batch	AS added (units)*	Colour and appearance	Texture	Flavour	Overall quality**
Control	0	7.4a	6.2a	7.8a	7.36a
800A	800	4.0b	3.2b	5.9b	5.11b
4500A	4500	4.0b	3.0b	6.2b	5.18b

Different letters in each column means significative difference at $p < 0.01$

*For unit definition see text
**Overall quality = Colour and appearance x 0.1 + Texture x 0.25 + Flavour x 0.65

CONCLUSION: It may be concluded that the use of aspartyl proteinase seems to be non-adequate as a means to shorten the ripening period of dry fermented sausages because the exccesive softening provoked by the enzyme. However, it is possible that the addition of a much lower enzyme amount could yield more positive results. It deserves further investigations.

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