CURING AGENTS AS REGULATORS OF MUSCLE AND MICROBIAL AMINOPEPTIDASE ACTIVITY IN DRY FERMENTED SAUSAGES.

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Introduction.

Dry fermented sausages are characterised by a distinctive flavour resulting from enzymatic action. These enzymes are microbial and muscle proteases which are involved in the generation of small peptides and amino acids (DeMasi et al., 1990). Aminopeptidases are proteolytic enzymes that degrade peptides into single amino acids sequentially from the N-terminal side (McDonald and Barret, 1986). The concentration and composition of amino acids and peptide compounds in sausages have been correlated with flavour development (Verplaetse, 1994). The extent of proteolysis during ripening of dry-fermented sausages is affected by the processing conditions. Our objective was to study the effect of different curing agents and process parameters on muscle and microbial (*Lactobacillus sake*) aminopeptidases to elucidate their contribution in the generation of free amino acids during the processing of dry-fermented sausages.

Materials and Methods.

Bacterial aminopeptidases. *Lactobacillus sake* IATA 115 (CECT 4808) was isolated from dry fermented sausages. The cell-free extract was obtained as described by Sanz and Toldrá (1997) and used for the enzyme purification. The cell free extract was subjected to ammonium sulphate fractionation (50 to 80 %) and anion exchange chromatography (Resource Q anion exchange column) where four aminopeptidases, API, APII, APIII and APIV, with different specificity were isolated (Sanz and Toldrá, 1997).

Muscle aminopeptidases. Preparation of muscle enzyme extract was done by homogenising 5 g of muscle *biceps femoris* in 25 ml of 50 mM phosphate buffer pH 7.5 with 5 mM EGTA. The muscle extract was used for enzyme purification of alanyl (AAP), arginyl (RAP), leucyl (LAP) and pyroglutamyl (PGAP) aminopeptidases as described by Flores et al., (1993) and (1996).

Assay of aminopeptidase activity. Aminopeptidases were measured by fluorometric assays using aminoacyl-7-amido-4-methyl coumarin as substrates (aa-AMC). AAP was assayed by using 0.1 mM Ala-AMC, RAP with 0.1 mM Arg-AMC, LAP with 0.25 mM Leu-AMC and PGAP with 0.1 mM pyroglutamic-AMC (Flores et al., 1993, 1996). API was measured with 0.1 mM Leu-AMC, APII with 0.1 mM Arg-AMC, APIII with 0.1 mM Leu-AMC and APIV with 0.1 mM Arg-AMC (Sanz and Toldrá, 1997). The reaction mixture was incubated at 37°C for 10 min (microbial) and 15 min (muscle aminopeptidases). The fluorescence was measured at 355 nm and 460 nm as excitation and emission wavelength, respectively, in a Fluoroskan II fluorophotometer (Labsystems, Finland). Four measurements were made for each experimental point.

Effect of curing agents and process parameters. Curing agents were individually added to the respective reaction buffers at different final concentrations (20-40 g/L NaCl, 5-20 g/L glucose, 100-300 mg/L $NaNO_3$, 100-500 mg/L ascorbic acid). The effect of different pH (5.8, 5.2 and 5.0) were measured in 10 mM Na_2HPO_4-5 mM citric acid buffer. Activity at different temperatures (37, 25 and 15°C) was measured in their respective reaction buffers.

Results and Discussion.

The characteristics of porcine muscle and microbial (from *Lactobacillus sake*) aminopeptidases are shown in Table 1. The most relevant aminopeptidase in porcine muscle was AAP due to its broad substrate specificity. RAP had a substrate specificity against basic aminoacyl bonds. LAP had an optimal alkaline pH being of less importance in meat products and PGAP had a high specificity against pyroglutamic acid. Microbial aminopeptidases such as API showed broad substrate specificity, APII hydrolysed basic amino acids as APIII and APIV but, these enzymes showed extreme pH, acid and basic, respectively.

Table 1. Muscle and microbial aminopeptidase properties.

	Aminoacyl-AMC Activity expressed as a percentage								Optimum	
	Ala-	Arg-	Leu-	Lys-	Met-	Tyr-	Phe-	p-Glu-	T°C	pH
AAP	100 (0.052)*	64 (0.009)	98 (0.009)	130 (0.026)	124 (0.029)	11	210 (0.043)	nh**	50°	6.5
RAP	3	100 (0.050)	nh	42 (0.105)	2	nh	8	nh	37°	6.5
LAP	nh	nh	100 (0.077)	nh	50	-	-	nh	37°	8.5
PGAP	nh	nh	nh	nh	nh	nh	nh	100 (0.036)	37°	7.5
API	93	nh	100 (0.091)	nh	40	nh	nh	nh	37°	7.5
APII	-	100	-	41	-	12 (0.096)	23	nh	37°	7.0
APIII	-	100 (0.0084)	-	8	-	-	-	-	37°	4.5
APIV	-	100 (0.0071)	-	2	-	-	-	-	25°	8.0

*The value in brackets represents the Km (in mM). **nh, not hydrolysed.

Sodium chloride is usually added at 2.5-3% to raw sausages contributing to taste, solubility of proteins and microbial selection (Leistner, 1995). The presence of NaCl exerted the strongest effect on muscle and microbial amniopeptidases (Fig 1). So, API, AAP and PGAP were strongly inhibited. However, APII and APIV were stimulated at 20g/L of NaCl although RAP was strongly activated at all the assayed concentrations. Nitrate and nitrite are used to promote cured colour and aroma formation as well as to inhibit undesirable microflora. The presence of nitrate did not inhibit the aminopeptidases, except APIII, and slightly affected APIV (Fig 2). Ascorbic acid is used as adjunct to reduce residual nitrite levels and prevent formation of nitrosamines (Izumi and Cassens, 1989). The ascorbic acid produced an inhibition of LAP, API and APIII (Fig 3). Carbohydrates are often added to enhance lactic acid bacteria growth ensuring proper acidification of the product. The effect of glucose resulted in a noticeable activation of LAP and inhibition of APIII (Fig 4). Process parameters such as pH and temperature have a decisive influence on proteolytic activity (Toldrá et al., 1992). Low pH is a crucial factor in dry fermented sausages. Muscle and microbial aminopeptidases were inhibited at low pH except APIV

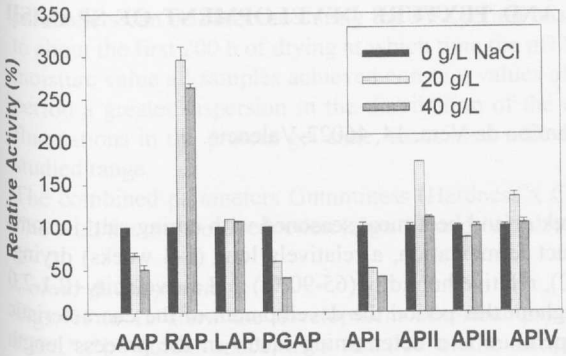

Figure 1. Effect of sodium chloride on aminopeptidases

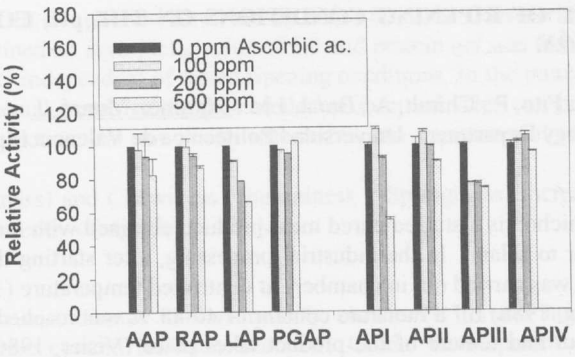

Figure 3. Effect of ascorbic acid on aminopeptidases

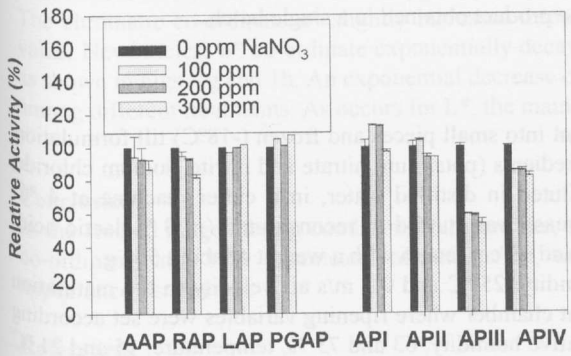

Figure 2. Effect of sodium nitrate on aminopeptidases

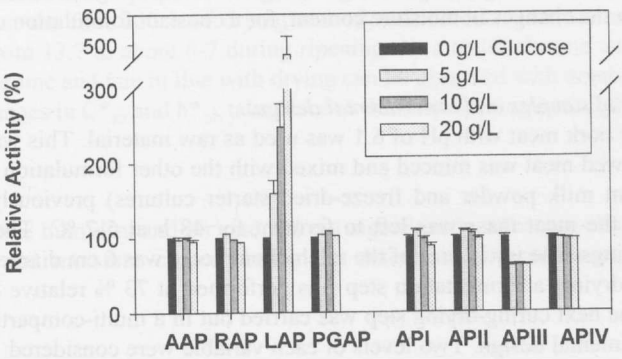

Figure 4. Effect of glucose on aminopeptidases

(Fig 5). Fermentation at 25°C and drying at 15°C diminished all muscle and microbial activities, although still active at 15°C (Fig 6).

Conclusion

Muscle aminopeptidases, except LAP, may contribute to the generation of free amino acids specially during the fermentation stage where pH is not so low yet and temperature around 25°C. Microbial aminopeptidase APIII may be negligible in dry fermented sausages, while API is reduced due to pH. APII and APIV, which are chloride enhanced, may be considerable in the whole process.

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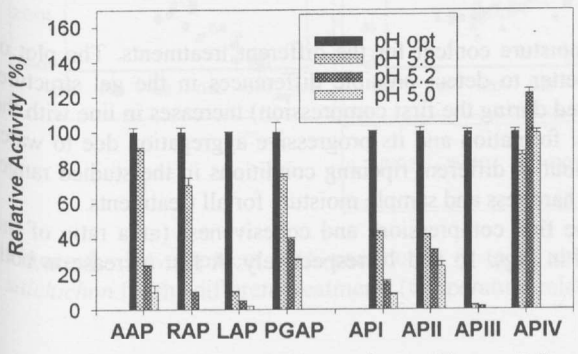

Figure 5. Effect of pH on aminopeptidase activity

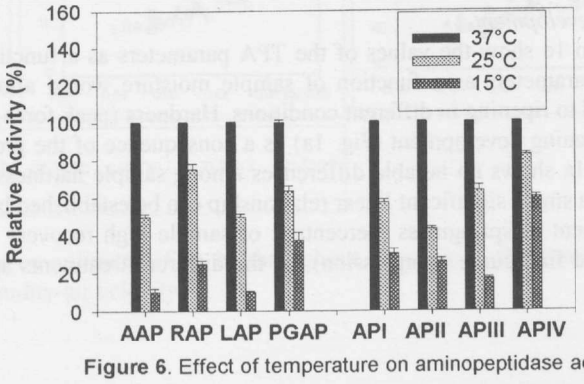

Figure 6. Effect of temperature on aminopeptidase activity