Feedback Inhibition of Porcine Muscle Alanyl and Arginyl Aminopeptidases in Cured Meat Products

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Alanyl and arginyl aminopeptidases from porcine skeletal muscle were purified, and the effect of free amino acids on their activities was studied. Both aminopeptidases were inhibited by free amino acids via the competitive mode and were more strongly inhibited in the pure form than when they were in the meat extract. Arginyl aminopeptidase (RAP)was less susceptible to inhibition by free amino acids than alanyl aminopeptidase (AAP), as shown especially by the strong inhibitory effect of hydrophobic amino acids on AAP activity in the meat extract. The free amino acid concentration present in meat products such as dry-cured loin and dry-cured ham produced a strong inhibitory effect on AAP and RAP activity. The concentration of free amino acids in cooked ham mainly affected the activity of AAP. These facts can explain the differences obtained in free amino acid concentration depending on the kind of process or product.

Keywords: *Aminopeptidase; muscle exopeptidases; peptidase inhibitor; amino acid*

INTRODUCTION

A sensible increment in the concentration of free amino acids in post-mortem muscle has been observed during meat aging (Okitani et al., 1981; Nishimura et al., 1988a, 1990) and in meat products such as dry-cured and cooked hams (Aristoy and Toldrá, 1991; Buscailhon et al., 1994; Toldrá and Flores, 1998). The contribution of peptides and amino acids to the improvement of meat taste has been reported at different cooking temperatures (Spanier et al., 1988; Spanier and Miller, 1993). Also, the contribution of free amino acids accumulated during dry-cured processing is relevant for the development of the specific dry-cured flavor (Flores et al., 1997a; Toldrá and Flores, 1998). These amino acids are of great importance not only because of their specific tastes (Nishimura and Kato, 1988) but also for their involvement in further degradation reactions that generate volatile compounds (Shahidi et al., 1986).

The accumulation of free amino acids in meat products is produced by the action of exoproteolytic enzymes such as aminopeptidases (Nishimura et al., 1988b; Toldrá et al., 1995), which are responsible for the hydrolysis of amino acids from the N terminus of peptides and proteins (McDonald and Barret, 1986). In meat products, aminopeptidases have been detected even after >8 months of processing (Toldrá et al., 1992), although they are controlled (activated or inhibited) by curing agents (Flores et al., 1997b; Toldrá et al., 1997).

Aminopeptidases have been found in the cytosolic fraction of skeletal muscle and in other tissues (Lauffart and Mantle, 1988; Hiroi et al., 1992; Ishiura et al., 1987). Four aminopeptidases have been distinguished in skeletal muscle. The one found in the highest amount is alanyl aminopeptidase (AAP), which accounts for as much as 83% of the total aminopeptidase activity and has a broad substrate specificity toward aromatic, aliphatic, and basic aminoacyl bonds (Mantle et al., 1983; Flores et al., 1996; Toldrá et al., 1995). This enzyme has also been named cytosol aminopeptidase III, thiol-activated aminopeptidase, and puromicin sensitive aminopeptidase (McDonald and Barret, 1986). The second one, arginyl aminopeptidase (RAP) or aminopeptidase B, accounts for 11% of the total aminopeptidase activity in muscle and has specificity toward basic aminoacyl bonds (Flores et al., 1993). Other aminopeptidases found, although in a lower proportion, in skeletal muscle are leucyl and pyroglutamyl aminopeptidases (Toldrá et al., 1995).

Aminopeptidases have been subjected to inhibition by free amino acids (McDonald and Barret, 1986; Toldrá et al., 1996), this function being the control of cellular protein turnover (Mantle, 1992). Therefore, the levels of free amino acids within the meat and meat products may regulate the aminopeptidase activity.

The action of muscle exopeptidases is very important for meat quality because these enzymes are involved in the post-mortem breakdown of protein and peptides to free amino acids. Our objective was to study the effect of free amino acids on the activity of the main muscle aminopeptidases (arginyl and alanyl aminopeptidases) and how these enzymes may contribute to the generation of free amino acids in aged meat and cured meat products.

MATERIALS AND METHODS

Raw and Cured Meat Products. Six carcasses from 6-month-old pigs were selected, and their biceps femoris (BF) and longissimus dorsi (LD) muscles were removed for the study. Raw hams ($n = 6$) were aged for 7 days at 4 °C. Spanish dry-cured hams ($n = 6$) with 12 months of total ripening time, cooked hams ($n = 3$), and dry-cured loins ($n = 6$) with 1.5 months of ripening time were obtained from a local factory.

Preparation of Muscle Extracts. Ten grams of muscle biceps femoris, free of visible fat and connective tissue, was homogenized in 50 mL of 50 mM phosphate buffer containing 5 mM EGTA, pH 7.5, using a Polytron homogenizer (3 × 10 s

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strokes at 27 000 rpm with cooling in ice) (Lauffart and Mantle, 1988). The homogenate was centrifuged at 10000*g* for 20 min at 4 °C, and then the supernatant was filtered through glass wool and collected for enzyme purification. These extracts were also used for direct measurement of enzyme activity.

Purification of Muscle Aminopeptidases. Aminopeptidases were purified as previously described (Flores et al., 1993, 1996). The purification briefly consisted of an ammonium sulfate precipitation (40−60%) and HPLC anion-exchange column separation. The HPLC separation was done on a 1050 Hewlett-Packard liquid chromatograph (titanium) system (Palo Alto, CA) equipped with a variable wavelength UV detector (280 nm). The column PL-1000 SAX (50 × 5 mm, 8 μm, from Hewlett-Packard) was previously equilibrated with 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM sodium chloride, 0.1% (v/v) β-mercaptoethanol, and 0.02% (w/v) sodium azide. The column was eluted at 0.5 mL/min with a linear salt gradient (100−400 mM NaCl) for 20 min. Fractions were collected and assayed for AAP and RAP activity. RAP and AAP eluted at 180 and 310 mM NaCl, respectively.

Aminopeptidase Activity. AAP was measured using 100 mM phosphate buffer, pH 6.5, containing 2 mM β-mercaptoethanol and 0.1 mM L-alanyl-7-amido-4-methylcoumarin (Ala-AMC) (Sigma Chemical Co., St. Louis, MO) as substrate (Flores et al., 1996). RAP was assayed in a reaction buffer consisting of 50 mM phosphate buffer, pH 6.5, containing 200 mM NaCl and 0.1 mM Arg-AMC as substrate (Flores et al., 1993). The generated fluorescence was measured after 15 min of incubation at 37 °C at excitation and emission wavelengths of 355 and 460 nm, respectively, in a thermostated plate reader Fluoroskan II (Labsystems, Finland). One unit (U) of enzyme activity was defined as the release of 1 μmol of 7-amido-4-methylcoumarin/h at 37 °C.

Amino Acid Inhibition and Kinetics. The inhibitory effect of free amino acids on RAP and AAP, directly obtained from the muscle extract or purified, was studied using their respective specific buffers. The amino acids were added to the buffer in increased concentrations, and the remaining activity after incubation for 15 min at 37° was measured. The inhibitory effect was expressed as the amino acid concentration that inhibits 50% of the enzyme activity (IC_{50}). The type of inhibition produced by amino acids on the purified aminopeptidases (arginine and lysine on RAP and arginine and methionine on AAP) was studied using their specific buffers and substrate (Arg-AMC and Ala-AMC) concentrations in the range 0.025−0.2 mM.

Amino Acid Content Analysis. Raw meat and cured products were diluted (1:4) in 0.1 M HCl, homogenized in a stomacher during 8 min at 4 °C, and deproteinized with 2.5 volumes of acetonitrile (Aristoy and Toldrá, 1991). The deproteinized samples were derivatized with phenyl isothiocyanate according to the method of Bidlingmeyer et al. (1987). The derivatized amino acids were analyzed by reversed-phase HPLC in a Waters (Milford, MA) Nova-Pak C18 column (300 × 3.9 mm) at 52 °C and monitored at 254 nm. Hydroxyproline was added as internal standard before derivatization.

Effect of Free Amino Acids Content of Raw Meat and Cured Meat Products on Aminopeptidase Activity. The effect of amino acid concentration on aminopeptidase activity was studied in 100 mM phosphate buffer containing the pool of free amino acids at the concentrations given in Table 3 for each raw and cured meat product, assuming that these amino acids are present in soluble form in the cytosol. The activity was assayed in this buffer containing the specific substrate for each aminopeptidase (Arg-AMC and Ala-AMC) at different pH values depending on the product: pH 5.8 for raw meat, pH 6.0 for dry-cured loin, pH 6.2 for dry-cured ham, and pH 6.5 for cooked ham. The effect of free amino acids was studied on aminopeptidases directly assayed in the muscle extract. The activity of RAP was measured in the presence of 0.25 mM puromycin to inhibit the interference of AAP activity on Arg-AMC.

In all cases, four measures were made for each experimental point and the initial fluorescence at time zero was used as blank. Three replicates were tested for each meat or meat product, and the activity was expressed as a percentage of the control without amino acid concentration in the reaction buffer and at the respective specific pH. Vertical bars in the figures represent the means ± standard error of the mean (sem), and the bars were not drawn when the interval was too small.

Table 1. Inhibitory Effect of Free Amino Acids on AAP Assayed Directly in the Muscle Extract and after Purification

amino acid	IC_{50} AAP purified (mM)	IC_{50} AAP extract (mM)	amino acid	IC_{50} AAP purified (mM)	IC_{50} AAP extract (mM)
Trp	0.4	0.4	His	31.8	–[a]
Phe	1.2	1.4	Thr	43.4	–
Leu	1.6	2.3	Ala	76.6	–
Arg	2.8	4.7	Ser	250	–
Ile	4.4	9.8	Glu	>250	–
Lys	6.7	13.4	Asp	>250	–
Met	6.8	10.4	Pro	>250	–
Val	9.4	18.4	Tau	>250	–

[a] Not determined.

Table 2. Inhibitory Effect of Free Amino Acids on RAP Assayed Directly in the Muscle Extract and after Purification

amino acid	IC_{50} RAP purified (mM)	IC_{50} RAP extract (mM)	amino acid	IC_{50} RAP purified (mM)	IC_{50} RAP extract (mM)
Arg	1.14	19.6	Phe	14.40	78.9
Lys	5.40	57.0	Ala	18.40	>100
Ile	4.50	41.5	Orn	21.80	>100

RESULTS

The study of inhibition of aminopeptidase activity by free amino acids was evaluated and the results are shown in Tables 1 and 2 for AAP and RAP, respectively. The data show the concentration of individual amino acids required to inhibit 50% of the initial enzyme activity (IC_{50}). The susceptibility of both aminopeptidases from porcine muscle to inhibition by free amino acids varies considerably and depends on the purified status of these aminopeptidases. Therefore, AAP, which has a broad substrate specificity (Flores et al., 1996), is inhibited by a broad range of amino acids (Table 1), whereas RAP is mainly inhibited by arginine, lysine, isoleucine, and phenylalanine (Table 2). The susceptibility to amino acid inhibition is parallel to the hydrolysis of aminoacyl-AMC substrates by RAP as observed by the specificity of RAP against basic aminoacyl bonds (Flores et al., 1993) and its inhibition by them. However, this is not the case of AAP inhibition in view of the poor inhibitory effect of alanine that is highly hydrolyzed as a substrate by AAP and the high inhibition by arginine that is not one of the main substrates hydrolyzed.

Both enzymes, AAP and RAP, are more strongly inhibited in the pure form than when they are in the meat extract. The concentration of amino acids needed to produce 50% inhibition on RAP in the meat extract is >10 times higher than that needed with the purified RAP (Table 2). On the other hand, AAP is very susceptible to tryptophan and phenylalanine, because the same concentrations inhibit the enzyme, in the meat extract or purified. Meanwhile, the concentration of amino acids such as leucine, arginine, isoleucine, lysine, methionine, and valine required to inhibit 50% of the AAP activity in the meat extract is double that with the purified AAP (Table 1).

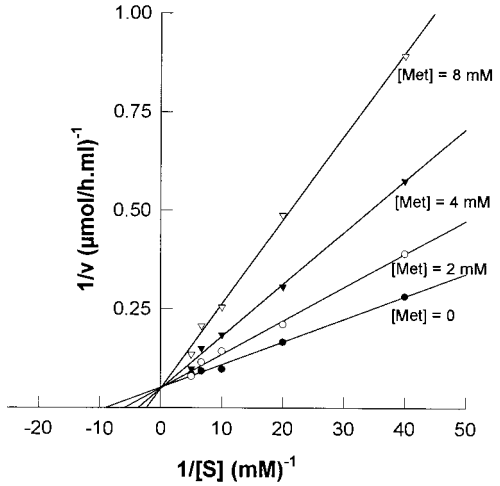

Figure 1. Inhibition of AAP activity by methionine (2, 4, and 8 mM) when assayed against Ala-AMC (0.025−0.2 mM) as substrate at 37 °C.

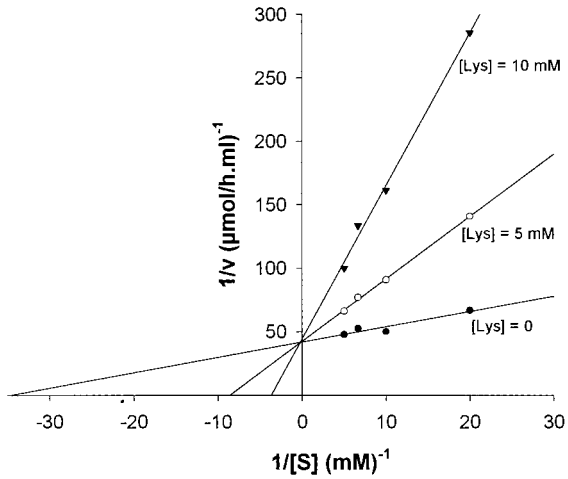

Figure 2. Inhibition of RAP activity by lysine (5 and 10 mM) when assayed against Arg-AMC (0.025−0.2 mM) as substrate at 37 °C.

To elucidate the mechanism of aminopeptidase inhibition by free amino acids, we studied the inhibition mode of several amino acids by using the method of Lineweaver and Burk (1934). Figures 1 and 2 show the inhibition of AAP by methionine (K_i = 2.67 mM) and RAP by lysine (K_i = 1.36 mM), respectively. Both enzymes are inhibited via the competitive mode. Thus, amino acids inhibit the enzyme activity by binding at the same site occupied by the substrate. Another amino acid such as arginine inhibits RAP (K_i = 0.27 mM) and AAP (K_i = 1.78 mM) by competitive mode (data not shown).

The free amino acid content of raw and prepared meat products was determined to predict the possible inhibitory or regulatory effect of these amino acids on the aminopeptidase activities. As shown in Table 3, there is a slight variation in the content of free amino acids in the muscles biceps femoris and longissimus dorsi from raw meat. The content of glutamic acid and alanine is low in longissimus dorsi. On the other hand, an increase in almost all of the amino acids, except glutamine, is observed during the aging of biceps femoris muscle. The meat product with the highest free amino acid concentration is dry-cured ham, followed by dry-cured loin and cooked ham. The high temperature of cooking during the processing of cooked ham affects the content of lysine, methionine, and tryptophan. Cooked ham is characterized by a high content in alanine, glycine, and glutamic acid. The long processing time of dry-cured ham is characterized by an extremely high content of lysine, glutamic acid, alanine, leucine, valine, and glycine. The processing of dry-cured loin is similar to the initial period of processing of dry-cured ham and because of the shorter time (1.5 versus 12 months) gives also important increments in glutamic acid, glycine, alanine, valine, and leucine.

The effect of the concentration of free amino acids in raw and prepared meat products on the aminopeptidase activity is studied in vitro and at the specific pH typical of each product. The remaining activity of AAP and RAP in each buffer containing the amino acid concentration and pH typical for each product is shown in Figure 3. The free amino acid concentrations found in raw meat and during meat aging do not affect the RAP activity, as 100% activity remained when we compared with its activity at pH 5.8 without any free amino acids present in the buffer. A slight reduction in RAP activity is observed when the simulation of cooked ham is compared with the control at pH 6.5. A higher inhibitory effect (~80%) of RAP activity is found for dry-cured loin in comparison with the control activity at pH 6.0 and almost complete inhibition in the case of dry-cured ham. On the other hand, AAP is more susceptible to the free amino acid concentration. As shown in Figure 3, AAP is affected by the free amino acid concentrations found in raw and aged meat, as only 60% of AAP activity remains when compared with the control activity at pH 5.8. In the simulation of cooked ham, only 50% of activity is detected when compared to the control activity at pH 6.5, and no AAP activity is found in the buffers simulating the free amino acid concentration in dry-cured ham and loin. The pH also has an important effect on the activity of both enzymes. AAP and RAP have an optimum pH at 6.5 (Flores et al., 1993, 1996) as shown in Figure 4, but the effect of pH is more pronounced in RAP than in AAP.

DISCUSSION

The development of characteristic meat flavor is partly due to the contribution of free amino acids not only for their specific tastes (Nishimura and Kato, 1988) but also for their involvement in further degradation reactions generating volatile compounds (Shahidi et al., 1986). Thus, the generation of these free amino acids is of great importance in the study of the mechanism of intracellular protein degradation in muscle and, therefore, the development of meat flavor in post-mortem meat.

The physiological relevance of aminopeptidase inhibition by free amino acids is not clear, because the relative intracellular distribution of amino acids and aminopeptidases in the cytoplasm is unknown (Toldrá et al., 1996). The increment of free amino acids produced during the processing of meat products can reach levels that will regulate the aminopeptidase activity.

Numerous changes occur in skeletal muscle during the processing of meat products. The main changes consist in the fragmentation of myofibrils, and also some proteolytic degradation is experienced by sarcoplasmic proteins, although they do not contribute to increased tenderness (Toldrá et al., 1995). The muscle enzyme system involved in the proteolytic events are muscle proteinases that will generate substrates appropriate to the action of aminopeptidases.

Table 3. Free Amino Acid Contents (Millimoles per Kilogram of Muscle) in Raw Meat and Cured Meat Products

	raw biceps femoris		raw longissimus dorsi		aged biceps femoris		dry-cured loin		dry-cured ham		cooked ham	
	mean	sem	mean	sem	mean	sem	mean	sem	mean	sem	mean	sem
Asp	0.06	0.02	0.03	0.01	0.16	0.01	1.45	0.45	27.77	1.63	1.34	0.24
Glu	2.26	0.39	0.16	0.06	0.70	0.13	17.90	1.21	48.08	3.94	3.27	0.81
Ser	0.45	0.08	0.23	0.05	0.49	0.09	6.44	1.25	30.37	2.00	1.19	0.44
Asn	0.13	0.02	0.08	0.02	0.14	0.01	1.23	0.38	5.30	1.73	0.20	0.04
Gly	1.57	0.23	0.99	0.18	1.62	0.42	7.95	1.30	43.59	1.60	2.42	0.37
Gln	2.59	0.72	3.04	0.42	1.67	0.14	1.20	0.27	0.85	0.23	1.03	1.10
His	0.28	0.04	0.21	0.02	0.31	0.10	1.75	0.45	15.75	1.22	0.80	0.01
Thr	0.36	0.07	0.28	0.04	0.41	0.03	4.37	0.82	23.06	3.59	0.90	0.18
Ala	3.23	0.71	1.59	0.19	3.13	0.63	14.95	2.06	57.89	3.80	3.84	1.40
Arg	0.35	0.07	0.33	0.04	0.26	0.12	1.81	0.54	24.06	1.89	0.92	0.13
Pro	0.43	0.08	0.29	0.03	0.35	0.04	4.35	0.53	26.18	0.45	0.75	0.14
Tyr	0.18	0.02	0.13	0.01	0.22	0.04	2.23	0.74	9.26	2.17	0.47	0.08
Val	0.45	0.08	0.28	0.02	0.56	0.08	7.73	1.62	33.15	1.20	0.77	0.09
Met	0.08	0.05	0.07	0.01	0.20	0.04	3.36	0.91	11.01	0.98	0.34	0.01
Ile	0.25	0.04	0.13	0.01	0.25	0.03	5.88	1.63	22.42	1.35	0.52	0.14
Leu	0.41	0.07	0.22	0.03	0.38	0.06	10.72	3.32	35.25	2.13	0.60	0.14
Phe	0.21	0.03	0.10	0.01	0.24	0.03	4.87	1.77	15.80	0.74	0.47	0.17
Trp	0.05	0.01	0.02	0.01	0.09	0.01	0.81	0.32	2.89	0.17	0.01	0.02
Orn	0.12	0.05	0.09	0.04	0.15	0.06	0.87	0.52	2.72	1.68	0.22	0.01
Lys	0.37	0.06	0.20	0.04	0.41	0.12	6.20	1.96	54.85	2.00	0.98	0.17

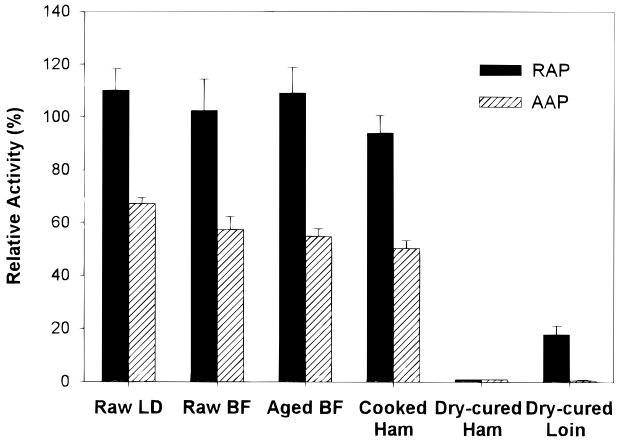

Figure 3. Activity of AAP and RAP in a buffer system resembling the free amino acid concentrations and pH usual in raw meat and cured meat products. Activity is expressed as a percentage of the activity found without any amino acid in the reaction buffer and at the respective pH for each meat product.

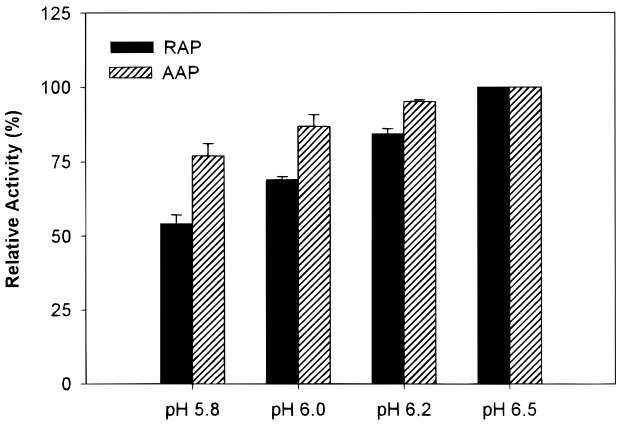

Figure 4. Effect of pH on the activity of AAP and RAP. Activity is expressed as a percentage of the activity found in their respective buffer systems at pH 6.5.

The inhibition of AAP activity from porcine skeletal muscle by free amino acids, especially hydrophobic amino acids, was also described by Garner and Behal (1975) for an alanyl aminopeptidase from human liver. Our results are also in accordance with those reported by Sharma and Ortwerth (1986), who observed a high inhibitory effect of hydrophobic amino acids and basic amino acids such as arginine on AAP activity from bovine lens, the inhibition being via competitive mode. The inhibitory effect of free amino acids on RAP was also reported by Kawata et al. (1980) as an inhibition via the competitive mode for an arginyl aminopeptidase from porcine liver. Moreover, the inhibitory effect of free amino acids on purified RAP is higher than on the RAP directly assayed in the muscle extract, probably due to the protective presence of other proteins and substrates located in the extract. The presence of substrates and inhibitors in the porcine muscle implies that both kinds of compounds fight for the same binding site in the enzyme, and the final result is a low inhibitory effect controlling its activity. AAP from porcine muscle is also inhibited via competitive mode, but its high susceptibility to hydrophobic amino acids, such as phenylalanine, tryptophan, and leucine, implies that their inhibitory effect is very strong and the substrates present in the muscle (proteins and peptides) have to compete with them.

On the other hand, the low activity recovered in dry-cured ham and loin for both aminopeptidases is due to the high concentration of free amino acids. These concentrations are usually obtained in the finished product, meaning that muscle aminopeptidases would exhibit very little activity toward the end of the process. This reduction in activity has been also reported when the effect of the curing agents (salt, nitrate, nitrite) and the long processing time of dry-cured meat products were studied (Flores et al., 1997b). Salt is the main agent that produces a remarkable effect, inhibiting AAP activity and activating RAP activity. In addition, the pH values, 6.0 and 6.2 for dry-cured loin and ham, respectively, promote the activity of AAP and RAP. The high pH of cooked ham, 6.5, also enhances their activity, although for a short time due to the high temperatures used during the processing (Toldrá et al., 1995). However, the mild temperatures during the processing of dry-cured products allow an adequate action of the aminopeptidases and a progressive generation of free amino acids. This is the case of RAP because it is less susceptible to the feedback inhibition. AAP will be responsible for the increment in free amino acids at the first steps of processing due to its broad substrate

specificity, but its activity may suffer a high regulation by hydrophobic amino acids toward the midpoint of the process.

In summary, the substrates and inhibitors present in muscle during the processing of meat products will act on the aminopeptidase activity via the competitive mode, thus regulating the generation of free amino acids.

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