CHANGES OF FREE AMINO ACID CONCENTRATION AND OF ALANYL AMINOPEPTIDASE ACTIVITY IN *BICEPS FEMORIS* DURING PROCESSING OF JINHUA HAM

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Introduction

Jinhua ham is appreciated for its characteristic flavor in most Asian countries. The development of desired aging flavor requires a long processing time. Intense proteolysis has been observed during dry-curing process (Martín, Córdoba, Antequera, Timón, & Ventanas, 1998; Molina, & Toldrá, 1992), giving rise to an important collection of free amino acids (Buscailhon, Monin, Cornet, & Bousset, 1994; Cordoba, Antequera, Garcia, Ventanas, Lopez, & Asensio, 1994; Sforza, Pigazzani, Motti, Porta, Virgili, Galaverna, Dossena, & Marchelli, 2001) that can directly contribute to flavor such as sweet, sour or bitter tastes, or indirectly contribute as precursors of volatile flavor compounds.

The free amino acids in dry-cured hams are mainly generated from muscle proteins and peptides by the actions of aminopeptidases (Toldrá, Flores, & Sanz, 1997). Muscle aminopeptidase activities have been reported in both raw and dry-cured ham with good stability even after 8 months of curing (Toldrá, Aristoy, Part, Cerveró, Rico, Motillva, & Flores, 1992). Alanyl aminopeptidase (AAP) accounts for 83% of the total porcine skeletal muscle aminopeptidase activities and demonstrates broader substrate specificity (Flores, Aristoy, & Toldrá, 1996). Therefore, it may be the aminopeptidase that takes the most important role in the generation of free amino acids during dry-cured ham processing.

The action of aminopeptides and free amino acid generation are very important for dry-cured hams because they are involved in the development of characteristic ham flavor. However, relevant information on Jinhua ham processing has not been well documented up till now.

Objectives

The main objective of the present work was tracing the changes of AAP activity and free animo acid contents in *biceps femoris* during the processing of Jinhua ham and evaluating the contributions of AAP and amino acids to the formation of Jinhua ham flavor.

Methodology

Materials

Sixty trimmed green hams weighting 6.2-6.9 kg from local cross swine (5-6 months, 90-100 kg) of Lanxi (Zhejiang province of China) were used to produce Jinhua hams. 7-amido-4-methyl-coumarin (AMC), Ala-AMC was purchased from Bachem Feinchemikalien (Bubendorf, Switzerland) and other chemical agents, purchased from Sigma (St. Louis, MO).

Jinhua ham processing

Jinhua ham processing was carried out under natural condition as prescribed by traditional processing technology in Zhejing Provincial Food Company, China. The process involves 6 stages, i.e. natural cooling, salting, soaking and washing, sun-drying, loft-aging and post-aging. After natural cooling for about 24 h, green hams were piled and salted for 30 d during which salt was added 5 times (50 mg nitrate per kg green ham was mixed in the salt used at the 2nd time) and ham piles were turned over for 7 times. After soaking and washing for 24 h and sun-drying for 20 d (including 11 days of cloudy or rainy or snowy weather), hams were transferred to aging-loft and aged for five months. Then the hams were post-aged at room temperature for two months.

Sampling

Biceps femoris was fully sampled for analysis from 5 hams randomly taken after each processing stages, i.e. prior-salting (about 24 h), end of salting (31 d), end of sun-drying (51 d), middle loft-aging (124 d), end of loft-aging (203 d) and end of post-aging (264 d). Samples were packed and stored under -40 before analyzing.

Amino acid analysis

About 5 g chopped sample was homogenized in 20 ml deionized water with a polytron (three stokes, 10 s each at 22000 r.p.m. with cooling in ice) homogenizer (IKA T18 basic, Made in IKA, German). The homogenate was deproteinized by mixing it with 20 ml of 10% sulfosalicylic acid. The mixture was maintained at 4 for 17 h and then filtered through filter paper. The filtrate was adjusted to pH 6.0 with 4N NaOH and diluted to 50ml with a buffer (100 mM sodium citrate buffer, pH 6.00). The diluted solution was ultra-filtrated through a 10 kDa ultrafiltration membrance with a Stirred Ultrafiltration Cell Model 8200 (Millipore Co., USA) before amino acid derivatization was carried out with AccQ·Fluor Reagent Kit (P/N WAT052880, Waters Co., USA) on the direction for use attached to the Kit. Amino acid derivates were detected on a Waters High Performance Liquid Chromatograph equipped with two pumps (Waters 515 HPLC pump) and a UV detector (Waters 2487 Dual λ Absorbance Detector, detecting at 254 nm). The column was an AccQ·Tag Column (Nava-Pak^{TM} C18, 3.9×150 mm, interior diameter 4 μm) and column temperature was controlled to 37 . The eluents used were: (A) AccQ·Tag^{TM} Eluent A (Borate buffer): water at 1: 10, and (B) acetonitrile: water: methanol at 45: 40: 15. The flow rate was 1 ml·min^{-1} and the flowing solvent gradient

was performed on the following procedure: initial 0% B, linear change to 1.0% B in 0.5 min, linear change to 7.0% B in 16.5 min, linear change to 10.0% B in 4 min, linear change to 33.0% B in 9 min and maintained 3min at 33.0% B, then linear change to 100% B in 1 min and maintained at 100% B for 3 min, at last linear change to 0% B in 1 min and maintained for 12 min.

Preparation of enzyme extract

The extraction was performed according to Toldrá et al. (1992) and Rosell, & Toldrá (1998) with slight modifications. About 5 g chopped sample was homogenized in 35 ml extraction buffer (50 mM sodium phosphate buffer, pH 7.5, containing 5 mM EGTA) with a polytron (three stokes, 10 s each at 23000 r.p.m. with cooling in ice) homogenizer (IKA T18 basic, Made in IKA, German). The homogenate was stirred for 60 min under 4 and then centrifuged at 22000 g for 20 min. The supernatant was filtered through fine silk cloth and finally diluted to 50 ml with extraction buffer for enzyme activity determination after shaking.

Enzyme assays

The enzyme activity was determined according to Toldrá et al. (1992) and Rosell et al. (1998), with some modifications. To 2.5 ml substrate solution (100 mM sodium phosphate buffer, pH 7.00, containing 0.33% of 30% Brij 35, 5.0 mM $CaCl_2$, 1.0 mM DTT and 0.25 mM Ala-AMC), 0.5 ml enzyme extract was added. The mixture was incubated in a water bath at 37 for 30 min and the reaction was terminated by immediate addition of 6 ml of ethanol. The generated fluorescence was determined with a Cary Eclipse Fluorescence Spectrophotometer (VARIAN, Australia) at excitation and emission wavelengths of 380 nm and 440 nm, respectively. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 nmol AMC per min at 37 , and called potential enzyme activity. Muscle AAP activity was calculated on the basis of *biceps femoris* dry matter subtracting fat and sodium chloride, i.e. (DM-Fat-Salt), and simply expressed as $U \cdot g^{-1}$.

2.6. Response surface experimental design and method

Response surface methodology (RSM) based on Box-Behnken design (BBD) was adopted to investigate the effects of processing factors on AAP activity. Four factors, i.e. temperature (Temp.), sodium chloride content (Salt), sodium nitrate content (Nitrate) and pH value (pH) (Table 1), were considered in the design and their value ranges were set according to the determining results from Jinhua ham processing (refer to Zhao, Zhou, Xu, Peng, Huan, Jing, & Chen, 2005). With Design-Expert software (version 6.0.5, Stat-Ease Inc., Minneapolis, USA), a four-factor and one-response (AAP activity) experimental design was performed and totally 29 sets of experiments were generated. A prior-salting sample was used for AAP activity determination for the convenience of control of salt and nitrate content in reaction mixture. Preparation of enzyme extracts was similar to the above mentioned procedure, but before the supernatants were diluted to 50 ml with extraction buffer, definite amount of NaCl and $NaNO_3$ were added and pH was adjusted to definite values as designed. Enzyme assay was similar to the above mentioned

procedure, except for that the buffers used in substrate solutions were different in pH value, NaCl and $NaNO_3$ content. In preparation of different substrate solutions in pH value, 200 mM sodium acetate buffer of pH 5.40, 200 mM sodium citrate buffer of pH 5.75 and 200 mM sodium phosphate buffer of pH 6.50 were used. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 nmol AMC per min at the designed reaction temperature. Muscle enzyme activity was calculated on the basis of *biceps femoris* dry matter subtracting fat and sodium chloride (DM-Fat-Salt), and expressed as $U \cdot g^{-1}$.

2.7. Statistics

Free amino acid and AAP activity data were assessed by analysis of variance using one-way ANOVA procedure of SPSS 10.0 (SPSS Inc.). The data from response surface experiments were analyzed through analysis of variance and quadratic regression, and rotatable response surface figures were drawn by 3-D surface procedures inside Design-Expert software (version 6.0.5, Stat-Ease Inc., Minneapolis, USA). The actual activity of muscle AAP (the ability of hydrolyzing proteins and peptides under actual processing condition of dry-cured ham) was estimated by the regression equation.

Results & Discussion

Changes of free amino acid contents in muscle during Jinhua ham processing

All the free amino acids detected in prior-salting hams increased significantly ($P < 0.05$) during processing except arginine and cystine (Table 2). Most free amino acids increased by 5-20 folds after processing compared with those in prior-salting hams, with the exception of arginine and cystine which concentration didn't change very much during processing ($P > 0.05$). Arginine, glutamic acid, leucine, lysine and alanine were found to be the free amino acids present in higher concentration in the final products, while cystine was the lowest. The highest increase was lysine that reached a final concentration of more than 80 times of that found in prior-salting hams, followed by aspartic acid, serine, tyrosine and isoleucine. With regard to the lengths of different processing stages, the fastest increase in the concentrations of total free amino acids took place in the second half of loft-aging and the sun-drying processes, followed by the first half of loft-aging and post-aging stages.

It has been well documented that the concentrations of all free amino acids except for arginine and histidine, increased to some extents during dry-cured ham processing (Buscailhon et al., 1994; Cordoba et al., 1994; Flores, Aristoy, Spanier, & Toldrá, 1997; Sforza et al., 2001; Ventanas, Cordoba, Antequera, Garcia, Lopez-Bote, & Asensio, 1992; Zhu, & Hu, 1993). The most abundant free amino acids were arginine, glutamic acid, leucine, lysine and alanine (Cordoba et al, 1994; Sforza et al., 2001; Zarkadas, Karatzas, Khalili, Khanizadeh, & Morin, 1988; Zhu et al., 1993). These reports generally accorded with our research on Jinhua ham. We also found that the concentration of cystine was very low and didn't change very much during processing. Cystine represents the oxidation form of cysteine that is very active and easily changes into other compounds, which may explain our result.

It is reported (Cordoba et al., 1994; Zhu et al., 1993) that the contents of most free amino acids in dry-cured ham products were 40-60 folds of those in fresh pork, which was much higher than our results. This is because our initial samples were from prior-salting hams rather than fresh hams. Prior-salting hams had been cooled under natural temperature (3-12 with daily average of 7.8) for about 24 h. Considering the relatively high temperature and strong activities of proteases in muscle with no restraining effect from salt, muscle proteins experienced considerable hydrolysis before samples were taken.

3.2. Changes of potential AAP activity during processing

As shown in Table 3, porcine muscle possessed very strong potential AAP activity, but it decreased gradually during processing from 201635.43 $U \cdot g^{-1}$ at prior-salting to 6147.11 $U \cdot g^{-1}$ at the end of post-aging. Salting, sun-drying and the second half of loft-aging processes greatly reduced AAP activity ($P < 0.05$) and about 27.11%, 35.88% and 20.27% of AAP activity losses were respectively observed during each stage. After processing, about 3.05% of prior-salting activity was remained.

Past work has revealed that the activities of all the aminopeptidases detected decreased gradually along the processing of Spain dry-cured ham and AAP showed the highest exopeptidase activity along the full process (Toldrá, Aristoy, & Flores, 2000). Our work proved the decline tendency of AAP activity during Jinhua ham processing, but the activity loss rate was much higher and the activity remains much lower than those demonstrated in Spain ham. The differences may result from different processing technologies adopted in manufacturing the two hams. Jinhua ham is salted and aged under completely natural conditions and only salt or salt and nitrate is used at salting stage, instead of salting agents mixture including glucose and vitamin C used in Spain ham salting. In addition, Jinhua ham aging temperature is relatively high (highest to 40). High temperature accelerates denaturation of enzyme proteins and intensifies chemical and biochemical reactions such as oxidation processes, resulting in fast losing of enzyme activity, while glucose activates AAP hydrolyzing activity (Toldrá, Cerveró, & Part, 1993).

Effects of main processing factors on AAP activity

Statistical results showed that temperature, pH value and salt content had significant effects on AAP activity ($P < 0.001$). Both temperature and salt content interacted with pH value on AAP activity ($P < 0.01$). However, 0-50 $mg \cdot L^{-1}$ of sodium nitrate in the reaction mixtures didn't evidently affect AAP activity ($P > 0.05$). By stepwise regression analysis, an optimal quadratic regression equation ($P < 0.001$) was generated (Eq.1). Related statistics ($R^2 = 0.99$, *Adj* $R^2 = 0.98$, *Pred* $R^2 = 0.95$, *Adeq Precision* = 49.02) indicated that the equation was well fit and could accurately predict the actual activity of AAP under practical condition.

$$\text{Ln(AAP activity)} = 32.31334 - 0.17946 \times \text{Temp} + 0.10028 \times \text{Salt} - 9.55187 \times \text{pH} - 0.00053 \times \text{Temp}^2 + 0.02052 \times \text{Salt}^2 + 0.94804 \times \text{pH}^2 - 0.04506 \times \text{Temp} \times \text{pH} - 0.09083 \times \text{Salt} \times \text{pH} \quad \text{(Eq.1)}$$

As shown in Fig.1, muscle AAP activity increased in exponential curve along with the increase of temperature from 4 to 40 and pH value from 5.00 to 6.50. The effect of temperature on AAP activity was enhanced by the increase of pH value. Increase of temperature also strengthened the effect of pH on AAP activity. Fig.2 displayed that AAP activity was activated by increase of pH value but restrained by salt, but the increase of salt content inhibited the activating effect of pH rise on AAP activity.

It is reported (Toldrá et al., 1992; Toldrá et al., 1993; Toldrá et al., 1997) that muscle AAP displayed its maximum activity at 40 and pH 7.0, salt intensely inhibited AAP activity, but 0 $mg \cdot L^{-1}$ and 50 $mg \cdot L^{-1}$ of nitrate didn't display different effects on AAP activity (Toldrá et al., 1993). These were in agreement with our result. In addition, we found that pH value interacted with temperature and salt content on AAP activity, which has not documented before.

Changes of actual activities of muscle AAP during Jinhua ham processing

AAP activity was observably affected by temperature, salt content and pH value that changed continuously during processing of Jinhua ham (Zhao et al., 2005). Therefore, it is normally very difficult to determine the actual activity of muscle AAP during processing. Using data of processing factors determined from Jinhua ham processing (refer to Zhao et al., 2005), changes of actual activity of muscle AAP during Jinhua ham processing were predicted with Eq.1 (Table 4). Table 4 revealed that muscle AAP could always keep actual activity during Jinhua ham processing and 143.05 $U \cdot g^{-1}$ actual AAP activities were retained even at the end of post-aging. Considering the extensive substrate specificity of AAP (Flores et al., 1996), it must have taken some effects in generating free amino acids during whole process of Jinhua ham processing. However, it is obvious that the spectrum of AAP activity against terminal amino acids does not accord with the observed release of free amino acids in Jinhua hams, indicating that other muscle aminopetidases may also play important roles in the generation of free amino acids during processing.

Conclusions

Muscle AAP possessed very strong potential activity that decreased gradually during Jinhua ham processing and about 3.05% of prior-salting activity was left in the products. Temperature, salt content and pH value significantly affected AAP hydrolyzing activity, which made AAP actual activity accounting for less than 5% of its corresponding potential activity at each stage of Jinhua ham processing. Even so, AAP could always show considerable actual activity. All the free amino acids but arginine and cystine increased to some extents during processing and most of the free amino acids detected in the final products were 5-20 times of those found in prior-salting hams, which might be results of the coactions of muscle AAP and other aminopeptidases.

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Tables and Figures

Table 1. Factors and levels for Box-Behnken response surface experimental design

Levels	Temperature ( )	Salt (%)	Sodium nitrate ($mg \cdot L^{-1}$)	pH value
−1	4.0	0.0	0.0	5.00
0	22.0	5.0	25.0	5.75
+1	40.0	10.0	50.0	6.50

Table 2. Results of 17 free amino acid contents in biceps femoris after each stage of Jinhua ham processing

FAA	Prior-salting	End of salting	End of sun-drying	Middle of loft-aging	End of loft-aging	End of post-aging	Prob.
Alanine	215.44±27.23^{d}	239.76±8.47^{d}	243.30±17.69^{d}	353.29±45.25^{c}	820.79±5.13^{b}	964.96±36.81^{a}	***
Arginine	1843.60±185.71	1321.84±98.92	1701.66±132.61	1646.52±198.30	1833.80±224.21	1871.83±111.06	*ns*
Aspartic acid	20.16±9.43^{c}	12.46±3.40^{c}	24.20±11.24^{c}	43.24±17.95^{c}	449.99±52.84^{b}	563.93±57.25^{a}	***
Cystine	14.99±1.72	19.26±2.14	33.33±10.97	41.01±5.86	39.89±5.20	31.65±5.81	*ns*
Glutamic acid	170.94±26.92^{d}	242.56±10.39^{d}	293.49±48.58^{d}	477.01±60.94^{c}	1033.36±51.52^{b}	1212.52±51.54^{a}	***
Glycine	80.35±7.56^{c}	105.72±3.19^{c}	138.32±17.45^{c}	281.25±31.26^{b}	407.79±39.52^{a}	426.83±32.25^{a}	***
Histidine	183.35±20.80^{c}	194.40±18.19^{c}	286.22±10.82^{b}	343.12±37.27^{ab}	387.54±41.19^{a}	365.38±37.33^{ab}	***
Isoleucine	42.72±9.88^{d}	110.78±8.41^{cd}	155.73±16.44^{c}	319.26±38.96^{b}	609.47±50.74^{a}	612.51±40.45^{a}	***
Leucine	87.97±15.50^{d}	197.05±16.53^{cd}	268.72±26.10^{c}	542.88±57.73^{b}	1096.62±88.41^{a}	1095.39±63.62^{a}	***
Lysine	12.53±3.06^{c}	32.92±7.04^{c}	79.16±28.60^{c}	43.79±9.81^{c}	469.83±60.03^{b}	1006.72±119.26^{a}	***
Methionine	44.30±6.35^{c}	85.60±5.80^{c}	117.29±12.34^{c}	241.52±30.51^{b}	420.65±40.91^{a}	394.33±29.45^{a}	***
Phenylalanine	61.56±8.88^{c}	124.26±12.50^{bc}	220.71±19.01^{b}	524.84±51.57^{a}	655.22±80.39^{a}	584.25±51.91^{a}	***
Proline	86.97±14.15^{d}	99.24±11.99^{d}	141.84±10.24^{d}	280.42±37.31^{c}	463.65±44.17^{b}	577.89±46.47^{a}	***
Serine	27.05±3.15^{c}	21.97±3.00^{c}	115.13±52.09^{c}	84.39±38.56^{c}	423.72±69.63^{b}	576.11±46.18^{a}	***
Threonine	127.54±14.76^{c}	174.09±6.43^{c}	221.83±27.61^{c}	358.06±35.56^{b}	696.01±26.32^{a}	644.98±49.78^{a}	***
Tyrosine	29.72±0.39^{b}	51.86±15.31^{b}	113.27±39.83^{b}	86.21±30.22^{b}	432.37±33.61^{a}	454.80±38.11^{a}	***
Valine	67.16±10.38^{d}	160.07±10.12^{cd}	197.79±26.47^{c}	401.52±47.31^{b}	753.69±62.45^{a}	762.28±51.89^{a}	***
Total	3116.33±316.28^{c}	3193.85±106.73^{c}	4351.99±302.56^{c}	6068.32±596.03^{b}	10994.40±612.85^{a}	12146.36±584.60^{a}	***

Note:

a. Contents of free amino acids were in $mg \cdot 100g^{-1}$ on the basis of *biceps femoris* dry matter subtracting fat and sodium chloride, i.e. (DM – Fat – Salt) and expressed as mean ± standard error.

b. Means within the same row without common superscripts differed significantly, $P < 0.05$.

c. Prob.: ANOVA results; *ns*: not significant; ***: significant, $P < 0.001$

Table 3. Results of potential AAP activity after each processing stage of Jinhua ham

	Prior-salting	End of salting	End of sun-drying	Middle of loft-aging	End of loft-aging	End of post-aging
AAP Activity ($U \cdot g^{-1}$)[a]	201635.43±8378.37[a]	146964.89±15841.88[b]	74616.12±12171.14[c]	48897.60±8522.33[c]	8030.60±2464.40[d]	6147.11±1841.59[d]
Residual (%)[b]	100.00	72.89	37.01	24.25	3.98	3.05

Note:

a. Means within the same row without common superscripts differed significantly, $P < 0.05$.

b. Residual (%) indicated the percentage of AAP activity after each stage accounting for that of prior-salting.

Table 4. Results of predicted actual AAP activity after each processing stage of Jinhua ham

	Prior-salting	End of salting	End of sun-drying	Middle of loft-aging	End of loft-aging	End of post-aging
Predicted Actual AAP Activity ($U \cdot g^{-1}$)	8333.27	2467.56	991.45	623.37	454.07	143.05
Predicted / Potential (%)*	4.13	1.68	1.33	1.27	5.65	2.33

Note:

* Predicted / potential (%) was the percentage of predicted actual AAP activity accounting for the potential AAP activity at the same processing stage.

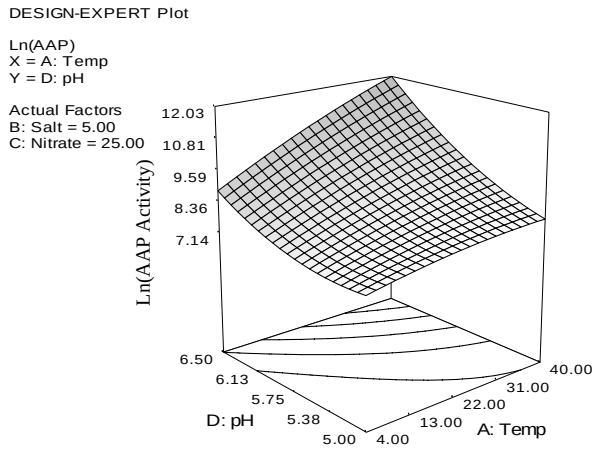

Fig.1 Effects of temperature and pH value on AAP

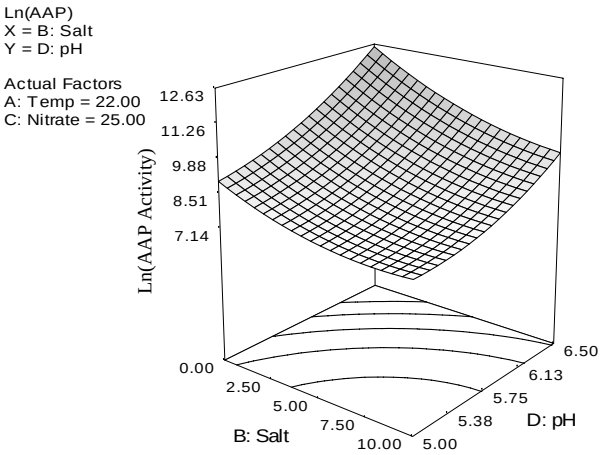

Fig.2 Effects of salt content and pH value on AAP activity