CHANGES IN CATHEPSIN B AND L ACTIVITIES DURING JINHUA HAM PROCESSING

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Introduction

Up till now, about 20 proteases have been found in skeletal muscle, of which cathepsins and calpains are the possibly crucial endopeptidases playing main roles in muscle proteolysis during dry-cured ham processing. However, calpains lost their activities after salting (Sárraga, Gil, & Garcia-Regueiro, 1993) and among cathepsins, cathepsin D lost its activity the most quickly during processing (Sárraga et al., 1993; Toldra, & Etherington, 1988; Toldrá, Rico, & Flores, 1993) and may play some role only in the first several months of processing (Toldrá et al., 1993). On the other hand, cathepsin B, L and H always kept some activities during dry-cured ham processing (Sárraga et al., 1993; Toldrá et al., 1993). It is reported that cathepsin H hardly hydrolyzes any fibrillin (Ouali, Garrel, Obled, Deval, Valin, & Penny, 1987), while cathepsin B and L possess extensive hydrolyzing activities on myofibril proteins (Parreno, Cusso, Gil, & Sarraga, 1994; Sárraga et al., 1993; Toldrá, Rico, & Flores, 1992; Toldrá et al., 1993). In addition, cathepsin B and L are rather stable during dry-cured ham processing (Parreno et al., 1994; Sárraga et al., 1993; Toldrá et al., 1992; Toldrá et al., 1993) and so they are considered to be the main endopeptidases responsible for muscle proteolysis and flavor formation in dry-cured ham.

Jinhua ham is one of the famous dry-cured hams of the world. However, little work was done on this ham. There has been no report on changes of cathepsin B and L activities during Jinhua ham processing.

Objectives

The objective of this investigation was to follow the changes of *biceps femoris* cathepsin B and L activities and their influencing factors during Jinhua ham processing using Response Surface Methodology (RSM).

Methodology

Materials

Sixty trimmed green hams weighing 6.2-6.9 kg from local crossbred swine (5-6 months, 90-100 kg) from Lanxi (Zhejiang province of P. R. China) were used to produce Jinhua ham. 7-amido-4-methyl-coumarin (AMC), Z-Arg-Arg-AMC and Z-Phe-Arg-AMC used for enzyme activity determination were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland) and other chemical agents, purchased from Sigma (St. Louis, MO).

Jinhua ham processing

Jinhua hams were processed under natural condition as prescribed by traditional processing technology in Zhejing Provincial Food Company, P. R. China. The process mainly involves 6 stages, i.e. natural cooling, dry 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 2^{nd} 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 (31d), end of sun-drying (51d), 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.

Potential enzyme activity determination

Preparation of enzyme extract

The extraction was performed refer to Koohmaraie & Kretchmar (1990), García-Garrido et al. (2000) and Rosell & Toldrá (1998), with slight modifications. Samples were thawed at 4 , trimmed off visible fat or connective tissue and finely chopped. About 5 g chopped sample was homogenized in 35 ml extraction buffer (50 mM sodium acetate buffer, pH 5.00, containing 100 mM sodium chloride, 1 mM EDTA and 2 $ml \cdot L^{-1}$ Triton X-100) 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

Enzyme activity was determined according to Parolari, Virgili, & Schivazappa, (1994), Blanchard & Mantle (1996) and García-Garrido, Quiles-Zafra, Tapiador, &

Luque-de-Castro (2000) with some modifications. To 2.5 ml substrate solution (50 mM sodium phosphate buffer, pH 6.00, containing 0.3125 mM Z-Arg-Arg-AMC, 4 mM EDTA, 2 mM DTT and 3.4 ml·L^{-1} Brij for cathepsin B assay and 50 mM sodium phosphate buffer, pH 6.00, containing 0.3125 mM Z-Phe-Arg-AMC, 4 mM EDTA, 2 mM DTT and 3.4 ml·L^{-1} Brij for cathepsin B+L assay), 0.5 ml enzyme extract was added. The mixture was incubated in a water bath at 37 for 20 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. Calibration curves were drawn with AMC under similar condition. Cathepsin L activity was calculated by cathepsin B+L activity substracting cathepsin B activity. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 nmol AMC per min at 37 . Potential muscle enzyme activity (the ability of hydrolyzing proteins under the defined condition of dry-cured ham) 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·g^{-1}.

Response surface experimental design and method

In the present study, RSM based on Box-Behnken design (BBD) was adopted. Four factors, i.e. temperature (Temp.), salt content (Salt), sodium nitrate content (Nitrate) and pH value (Table 1), were considered in the design and their value ranges were set according to the determining results from Jinhua ham processing (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 two-response (cathepsin B and L activities) experimental design was performed and 29 sets of experiments for each response were generated. For the convenience of controlling salt and nitrate content in reaction mixture, prior-salting samples were used in the experiments. 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 sodium nitrate 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, salt content and sodium nitrate 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 5.80 and 200 mM sodium phosphate buffer of pH 6.20 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 simply expressed as U·g^{-1}.

Statistics

Enzyme activities 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 stepwise 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 activities of cathepsins (the

ability of hydrolyzing proteins under actual processing condition of Jinhua ham) were estimated by gained regression equations.

Results & Discussion

Changes of potential activities of cathepsin B and L

Potential activity of cathepsin B decreased gradually during processing, from 11332.05 $U \cdot g^{-1}$ prior salting to 1055.32 $U \cdot g^{-1}$ at the end of post-aging (Table 2). The potential activity of cathepsin L changed in the similar way to that of cathepsin B with the exception that the former showed a clear rise at the sun-drying stage. At the end of post-aging, 9.31% and 13.66% of the prior-salting activities were left for cathepsin B and cathepsin L, respectively.

By far, no information on change of muscle cathepsin activity during Jinhua ham processing can be referred. Related documents revealed that cathepsin B and L lost their activities (potential activities) gradually during dry-cured ham processing and 5% to 15% of the original activities of cathepsin B and B+L remained at the end of processing (Toldrá, Rico, & Flores, 1993). These statements were generally consistent with our results on Jinhua ham. In addition, an obvious increase of cathepsin L activity during sun-drying was recorded in this study. This may be out of the specific Jinhua ham processing technology. Since Jinhua ham is dehydrated directly in sunshine after salting and soaking, chemical and biochemical reactions may take place in muscle, which may produce compounds that boost the activity of cathepsin L.

3.2. Response surface of main influencing factors affected cathepsin B activity

Response surface experiments revealed that temperature, pH value and salt content had significant effects on cathepsin B and L activities ($P < 0.01$) (Table 3 and Table 4). In addition, temperature interacted with salt content ($P < 0.001$) on cathepsin B activity and interacted with pH value as well as with salt content ($P < 0.01$) on cathepsin L activity. However, sodium nitrate content in the range of 0-50 $mg \cdot kg^{-1}$ didn't obviously affect cathepsin B or L activity ($P > 0.05$). Stepwise regression analysis produced two optimal quadratic regression equations for predicting cathepsin B and L activities individually (Eq.1 and Eq.2). Regression diagnostic analysis indicated that the regression equations were effective and very high R^2, *Adj.* R^2, *Pred.* R^2 and *Adeq. Precision* proved the effectiveness of the equations.

$$\text{Cathepsin B activity} = 106866.43 + 222.28 \times \text{Temp} - 37348.27 \times \text{pH} - 187.54 \times \text{Salt} + 3.78 \times \text{Temp}^2 + 3280.59 \times \text{pH}^2 + 18.21 \times \text{Salt}^2 - 35.77 \times \text{Temp} \times \text{pH} - 9.16 \times \text{Temp} \times \text{Salt} \quad \text{Eq.1}$$

$$\text{Cathepsin L activity} = 1300.20 + 585.72 \times \text{Temp} - 220.27 \times \text{pH} - 1440.96 \times \text{Salt} + 6.90 \times \text{Temp}^2 - 107.58 \times \text{Temp} \times \text{pH} - 14.47 \times \text{Temp} \times \text{Salt} + 265.74 \times \text{pH} \times \text{Salt} \times \text{pH} \quad \text{Eq.2}$$

Similar effects of temperature and pH value on cathepsin B and L activities were observed (Fig.1 and Fig.3). Cathepsin B and L activities increased in a quadratic curve

way along with the increase of temperature from 4 to 40 . On the other hand, the increase of pH value from 5.40 to 6.20 restrained cathepsin B and L activities to some degree. Temperature showed stronger effect at relatively lower pH value, while pH value could only influence cathepsin B and L activity at high temperature and no obvious pH effect could be observed below 10 on cathepsing B activity or below 20 on cathepsin L activity.

The effects of temperature and salt content on cathepsin B and L activities were also similar as shown in Fig.2 and Fig.4. Salt inhibited cathepsin B and L activities remarkably. The increase of salt content also weakened the effect of temperature on cathepsin B and L activities, but the increase of temperature strengthened the inhibiting effect of salt. However, when temperature was below 10 for cathepsin B or below 20 , the inhibiting effects of salt on cathepsin B and L activities were very weak.

According to our knowledge, no record of research on factors influencing cathepsin activity using RSM has been published up to now. Related studies by single-factor experiments, control experiments or simulating experiments revealed that salt notably inhibited the activities of cathepsin B and L (Rico, Toldrá, & Flores, 1991), increase of salt content reducing cathepsin B activity (Rico et al., 1991), but nitrate or nitrite showed little effect on cathepsins (Jaarsveld, Naude, & Oelofsen, 1998). The optimal pH value and temperature of cathepsin B and L were respectively 3.0 to 6.0 and 40 to 45 (Jaarsveld et al., 1998). Their activities weakened when pH value rose up and strengthened as pH dropped down (Arnau, Guerrero, & Sarraga, 1998). These results were in agreement with our studies by RSM. We also found that factors interacted with each other impacting the activities of cathepsin B and L, which should be helpful in interpreting changes of cathepsin B and L activities during dry-cured ham processing.

3.3. Changes of actual activities of cathepsin B and L during Jinhua ham processing

As a result of continuous changes of processing temperature, muscle salt content and pH value during dry-cured ham processing, muscle enzymes may seldom fully express their potential activities. Toldrá et al. (1992) simulated dry-cured ham processing conditions and studied the activities of muscle proteases, which was very useful in understanding the role of proteases in dry-cured ham processing. However, systematical investigation on changes of actual activities of cathepsin B and L along processing of dry-cured ham has not been documented. Through Eq.1 and Eq.2, changes of actual activities of *biceps femoris* cathepsin B and L during Jinhua ham processing were estimated with the factor data (refer to Zhao et al., 2005) and the results are shown in table 5. Table 5 revealed that less than 5% of the corresponding potential activity of cathepsin B and L could be displayed most of the time before half loft-aging, except for prior-salting when 8.31% of potential cathepsin B activity was exhibited. However, about 15% and 20% of cathepsin B and L potential activity, separately, showed as actual activities during the second half of loft-aging and post-aging stages. Relatively, the actual activity of cathepsin B was always higher than that of cathepsin L before half of loft-aging, but cathepsin L showed stronger actual activity than that of cathepsin B at the last two stages because a sharp increase of cathepsin L activity during the second half of loft-aging.

Conclusions

The activities of cathepsin B and L were significantly influenced by temperature, salt content, pH value and their interactions, but were not obviously affected by sodium nitrate. Cathepsin B and L could always hold high potential activities that fell gradually during Jinhua ham processing. At the end of processing, 9.31% original potential activity of cathepsin B and 13.66% original potential activity of cathepsin L were left. During most of the processing periods, only small part of cathepsin B and L potential activities were expressed as actual activities. Anyway, cathepsin B and L retained actual activities throughout processing, especially during loft-aging and post-aging periods.

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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·kg^{-1})	pH value
−1	4.0	0.0	0.0	5.40
0	22.0	5.0	25.0	5.80
+1	40.0	10.0	50.0	6.20

Table 2. Results of potential activities of cathepsin B and L after each processing stage of Jinhua ham

Time	Cathepsin B Activity		Cathepsin L Activity	
	M ± S.E. (U·g^{-1})	Remains (%)	M ± S.E. (U·g^{-1})	Remains (%)
Prior-salting	11332.05±717.67^{a}	100.00	9955.50±468.30^{ab}	100.00
End of salting	9444.73±1167.78^{ab}	83.35	8577.98±916.44^{bc}	86.16
End of sun-drying	7727.67±318.11^{bc}	68.19	11649.70±667.22^{a}	117.02
Middle of loft-aging	7314.73±822.02^{c}	64.55	6815.09±799.34^{c}	68.46
End of loft-aging	1988.61±459.57^{d}	17.55	2281.95±365.71^{d}	22.92
End of post-aging	1055.32±138.67^{d}	9.31	1359.90±212.87^{d}	13.66

Note:

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

b. M ± S.E. stands for means ± standard error.

c. Remains (%) indicate the percentage of cathepsin activity after each stage accounting for that of prior-salting.

Table 3. ANOVA results of quadratic regression model for cathepsin B response surface

Source	Sum of Squares	DF	Mean Square	*F* Value	Prob > *F*
Model	98439892.20	8	12304986.53	149.33	< 0.001
A (Temp.)	71404453.42	1	71404453.42	866.54	< 0.001
B (pH)	12376.72	1	12376.72	0.15	0.702
C (salt)	12850797.92	1	12850797.92	155.95	< 0.001
A^2	10112216.43	10	10112216.43	122.72	< 0.001
B^2	1853463.98	1	1853463.98	22.49	0.001
C^2	1394246.52	1	1394246.52	16.92	0.005
AB	265299.27	1	265299.27	3.22	0.088
AC	2718890.42	1	2718890.42	33.00	< 0.001
Residual	1648036.46	20	82401.82		
Error	58023.40	4	14505.85		
Total	100087928.67	28			

R^2=0.98 *Adj.* R^2=0.98 *Pred.* R^2=0.96 *Adeq. Precision*=44.98

Table 4. ANOVA results of quadratic regression model for cathepsin L response surface

Source	Sum of Squares	DF	Mean Square	*F* Value	Prob > *F*
Model	207758656.76	7	29679808.11	82.77	< 0.001
A (Temp.)	144972262.04	1	144972262.04	404.27	< 0.001
B (pH)	3040410.65	1	3040410.65	8.48	0.008
C (Salt)	14251914.95	1	14251914.95	39.74	< 0.001
A^2	35181814.72	1	35181814.72	98.11	< 0.001
AB	2400014.17	1	2400014.17	6.69	0.017
AC	6782345.16	1	6782345.16	18.91	0.003
BC	1129895.07	1	1129895.07	3.15	0.090
Residual	7530656.77	21	358602.70		
Error	87330.54	40	21832.63		
Total	215289313.53	28			

R^2=0.97 *Adj.* R^2=0.95 *Pred.* R^2=0.91 *Adeq. Precision*=30.57

Table 5. Predicted actual activities of cathepsin B and L after each processing stage

Cathepsin	Statistics	Prior-salting	End of salting	End of sun-drying	Middle of loft-aging	End of loft-aging	End of post-aging
Cathepsin B	Predicted activity ($U \cdot g^{-1}$)	941.71	237.31	198.18	302.37	300.58	164.70
	Activity present (%)	8.31	2.51	2.56	4.13	15.12	15.61
Cathepsin L	Predicted activity ($U \cdot g^{-1}$)	335.20	101.27	194.86	186.15	467.39	274.89
	Activity present (%)	3.37	1.18	1.67	2.73	20.48	20.21

Note: Activity present % = Predicted actual÷Potential activity×100

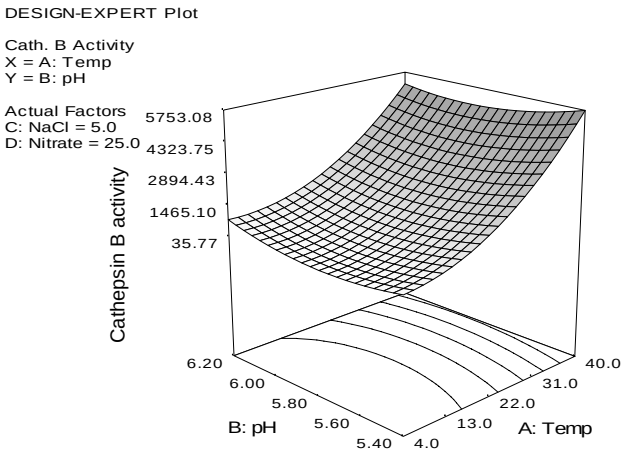

Fig.1 Effects of temperature and pH value on cathepsin B activity

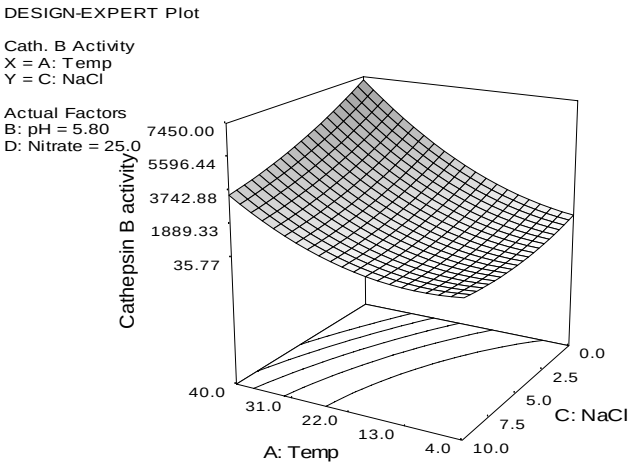

Fig.2 Effects of temperature and salt content on cathepsin B activity

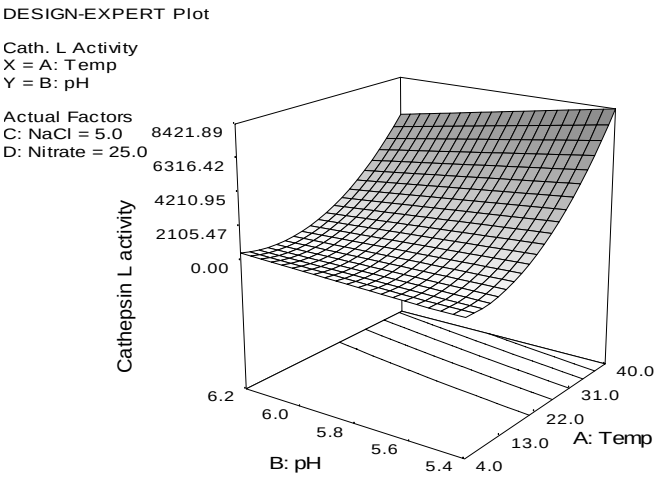

Fig.3 Effects of temperature and pH value on cathepsin L activity

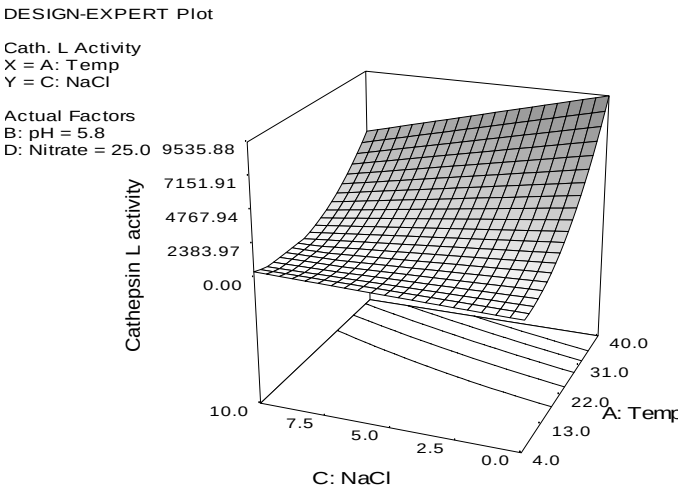

Fig.4 Effects of temperature and salt content on cathepsin L activity