Effect of Extraction Methods on the Types and Levels of Free Amino Acid of Beef *Longissimus* Muscle

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Abstract

The current study was carried out to investigate the impact of extraction conditions on the free amino acid level and type in beef *longissimus* muscle. The sample blocks were chiller aged for 1 d and 7 d at 4°C. There are three homogenization speeds (11,000, 19,000 and 24,000 rpm) for bigger and two speeds (11,000 and 13,000 rpm) for smaller homogenizer's dispersing tools were used for evaluation. Results showed that chiller ageing greatly ($p < 0.05$) increased extractable free amino acids, except cystine. Homogenization with the bigger dispersing tool at 24,000 rpm resulted in the highest free amino acid levels for both 1 and 7 d samples. Significant differences ($p < 0.05$) in the mean values of most amino acids due to the effect of speed and interactions between ageing times. However, the speed effect and interaction between ageing with homogenization speed were not significant ($p > 0.05$) for most of the amino acids except valine and isoleucine when using the smaller dispersing tools. The current data indicated that a standardized method for free amino acid types and levels of aged beef samples. In addition, the results also suggested that utilization of a big dispersing tool at high homogenization speed is a better condition for releasing free amino acid contents in beef samples.

Key words: free amino acids, beef, homogenization effect, ageing, speed, dispersing tools

Introduction

Meat is considered as the high-quality protein source because of their balances in amino acid contents, especially all the essential amino acids necessary for physical and mental well-being. On the other hand, free amino acids also contribute to meat taste and indirectly to aroma flavors by generation of volatile compounds through the Maillard reactions and Strecker degradations (Aristoy and Toldra, 2009).

The amino acid profiles can be viewed as a two-stage process. The first stage involves the sample preparation and the second stage involves chromatographic analysis. The homogenization treatment is a fundamental step of the analysis because the successful determination of free amino acids is dependent on the efficient disruption and homogenization of muscle tissues (Kivi, 2000). The efficient disruption facilitates the lysis of the starting material and release all the free amino acids contained in the meat

sample, ensuring high yield of amino acids. Incomplete disruption may results in reduced yields. Earlier studies indicated that the homogenization conditions result in different free amino acids level as a consequence of various disruption impacts. The shaft homogenizer-rotor stators is one of the most widely used tools for animal tissues (Burden, 2008), which generated the smallest fragments than CryoGrinder and Omni mixer (Hopkins *et al.*, 2004). In addition, the speed and duration of homogenization may greatly interact with homogenizer types (Hopkins *et al.*, 2004), otherwise the speed and efficiency of homogenization were greatly eroded when a smaller homogenizer was adopted (Burden, 2008).

Most of the laboratory rotor stator homogenizer's dispersers which allow, for example, keeping track of the speed range up to 27,000 rpm. In the case of dispersers, stainless steel dispersing elements are capable of processing between 0.03 and 2 L of samples with 5 to 25 mm diameter interchangeable generator probes. Sample volumes can range from 0.25 mL to 1,000 mL with the available of different size. For consistency results of free amino acid levels in beef over ageing period, it is very important to choose a right generator and dispersers for fibrous tissue samples which matches your sample volume and

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required level of dispersion. Also, the selection of homogenizer type, tools such as dispersing elements and homogenization method may have a significant impact on the outcome (Halpine, 2004). However most cases the type of homogenizer, speed and dispersing elements has not been specified in the published papers (Hopkins *et al.*, 2004). These together can result in large variations in free amino acids content between laboratories and experiments and thus consequently generating controversial data.

The objective of this study was to evaluate the effects of a sampling technique particularly homogenization on the types and levels of amino acids for differently aged beef sample. The free amino acid contents as a function of the extraction condition were assessed using a HPLC technique equipped with a diode array detector (DAD) detector. The current study emphasized the importance of standard method for consistent results and compatible data with other laboratories.

Materials and Methods

Chemicals

HPLC-grade purified water, acetonitrile and methanol were purchased from J.T Baker (USA), amino acid standards, extended amino acids, also derivatization reagents; FMOc, OPA reagent, borate buffer were purchased from Agilent technologies (Waldbronn, Germany). Triethylamine was purchased from Fluka (Belgium), sodium phosphate and hydrochloric acid was purchased from Merck (Darmstadt, Germany). Sodium tetraborate decahydrate, sodium azide and ortho phosphoric acid were purchased from Sigma (Steinheim, Germany), filter papers were from Whatman (Maidstone, England) and membrane PTFE (pore: 0.45 μm , diameter: 13 mm) was obtained from Sartorius (Goettingen, Germany).

Experimental design

Muscle sampling and extraction of free amino acids

M. longissimus (LD) muscles of Hanwoo beef was sampled from a commercial slaughter house day after slaughter and aged for 7 d at 4°C. All samples were frozen at -40°C until analyzed for both experiment 1 and 2. Sample preparation for the detection of free amino acid was performed by Aristoy and Toldra (1991) with suitable modification as described by Hwang *et al.* (2004).

Experiment 1

Briefly, approximately 1 g LD muscle tissue was homogenized in 5 mL of 0.01N HCl of 50 mL conical tube

using a rotor-stator homogenizer (Ultra turrax T 25 basic, Ika, Malaysia) with bigger S25 NK 19 G dispersing tool at low (11,000 rpm), medium (19,000 rpm) or high (24,000 rpm) for three strokes of 20 s each.

Experiment 2

For the smaller S8 N8G dispersing tools, the samples were homogenized at low speed (11,000 rpm) and high (13,000 rpm) for three strokes of 20 s each. The homogenized samples were then centrifuged at 10,000 g for 20 min at 4°C. The supernatant was filtered through 541# Whatman filter paper and stored at -80°C until use. 10 μL of ISTD stock solution (1 nmol norvaline) was added into 300 μL thawed samples and left for 30 min at room temperature followed deproteinized with 690 μL of acetonitrile then centrifuged at 10,000 g for 15 min. Two hundred micro liters of the supernatant were used for derivative step. Each analysis was done in triplicates

Instrumental analysis

The free amino acid contents in beef sample from experiment 1 and 2 were analyzed using a RP-HPLC 1200 system (Agilent Technologies Inc., USA) equipped with a diode array detector (DAD). HPLC conditions for free amino acids followed as described by Woodward and Henderson (2007). A rapid Resolution HT Eclipse XDB-C18 column (4.6 mm $\times$ 150 mm column packed with 5 μm particles Agilent Technologies Inc., USA) was used for separation of amino acids. Two hundred microlitres of the supernatant were derivatised with OPA for the primary amino acids and FMOc for the secondary amino acids; furthermore a 0.4 N borate buffer was used. Briefly, OPA-derivatised amino acids were monitored at 338 nm and FMOc-derivatised amino acids were monitored at 262 nm. The column temperature at 40°C; flow rate was 2 mL/min, using a gradient between two solvents. Solvent A was 10 mM Na₂HPO₄: 10 mM Na₂B₄O₇: 0.5 mM NaN₃ at pH 8.2 and Solvent B was Acetonitrile: Methanol: Water (45:45:10 v/v). The separation was achieved in 8.7 min; linear gradient 2% B for 1 min, 2-57% B for 6 min and 57-100% for 0.1 min then maintain for 1.3 min and re-equilibrated with 100% A for 0.2 min before next sample injections. For the calibration curves 17 primary amino acids, plus 4 extended amino acids are combined with fixed amounts of internal standards and final concentration of standard solution were 90, 225 and 900 pmol. The primary amino acids were quantified by using external standard procedures or by using norvaline as an internal standard and the secondary amino acids were

quantified using sarcosine as an internal standard. Individual free amino acid values were expressed as $\mu\text{mol/g}$. The mechanical shear effect of homogenizing conditions on beef muscle fiber was monitored using an electronic microscope at $40\times$ magnifications, coupled to a digital camera (Olympus, CKX 41, and Foculus IEEE1394, Germany).

Statistical analysis

Analysis of variance was performed using the SAS General Linear Models Procedure. Ageing and homogenizer speed and their interaction were presented by least square means interactions; ageing $\times$ speed. (SAS Institute, Cary, 2007).

Result and Discussion

Type and level of free amino acids as a function of ageing and speeds of larger dispensing tool

Total of 20 free amino acids were detected in beef samples both aged 1 and 7 d, except hydroxyproline which rarely appears in protein structures. The use of rapid resolution HT Eclipse XDB-C18 column (4.6 mm $\times$ 150 mm column with 5 μm sized particle) produces a high ana-

lytic sensitivity and shortened analytic time, the separation was achieved in 8.7 min and the total cycle time was approximately 15 min, including injector program pre-run DAD auto-balancing, and re-equilibration time.

Changes in free amino acid contents during ageing, using bigger dispensing tool (stator diameter 19 mm and rotor diameter 12.7 mm) and different speed (low-11,000; medium-19,000 and high-24,000 rpm) are shown in Table 1. The alanine (5.28 $\mu\text{mol/g}$), glutamine (3.04 $\mu\text{mol/g}$), serine (0.41 $\mu\text{mol/g}$), valine (0.32 $\mu\text{mol/g}$) and leucine (0.49 $\mu\text{mol/g}$) were the most dominant among detected amino acids, while asparagine (0.14 $\mu\text{mol/g}$) and aspartic acids (0.1 $\mu\text{mol/g}$) were found to be trace in concentrations in 1 d aged meat. The mean values of individual free amino acids significantly increased with ageing time except for cystine. An increase in amino acids in beef during ageing period has been elucidated in the previous works in that the proteolysis contributes to the breakdown of the myofibrillar protein, action of calpains and cathepsins on major myofibrillar proteins, generating protein fragments and intermediate size polypeptides, furthermore polypeptides and peptides generate free amino acids (Toldra, 2006). The final protein degradation products, such as peptides and free amino acids were thought to be

Table 1. Changes in free amino acid contents during ageing, and by using bigger homogenizer and different speed ($\mu\text{mol/g}$)

Bigger Homogenizer										
Amino acids	1 d			7 d			SEM	Ageing	Speed	Ageing ×Speed
	High	Medium	Slow	High	Medium	Slow				
ASP	0.01	nd	nd	0.20 ^{ab}	0.22 ^a	0.17 ^b	0.01	***	*	ns
GLU	0.30	0.33	0.28	2.23 ^a	1.97 ^b	1.96 ^b	0.06	***	*	*
ASN	0.14 ^a	0.07 ^b	0.09 ^b	0.77	0.69	0.73	0.02	***	*	ns
SER	0.41	0.33	0.36	2.16	1.36	1.94	0.25	***	ns	ns
GLN	3.04 ^a	1.56 ^b	2.31 ^b	8.05 ^a	6.68 ^b	6.85 ^b	0.37	***	**	ns
HIS	0.13	0.13	0.11	0.57	0.54	0.57	0.02	***	ns	ns
GLY	0.46	0.39	0.41	1.63 ^a	1.40 ^b	1.33 ^b	0.03	***	***	*
THR	0.30 ^a	0.22 ^b	0.22 ^b	1.71 ^a	1.40 ^b	1.33 ^b	0.05	***	**	*
ARG	0.26	0.20	0.20	1.36 ^a	0.97 ^b	1.09 ^b	0.03	***	***	**
ALA	5.28 ^a	5.12 ^b	4.48 ^b	9.10 ^a	7.48 ^b	6.94 ^b	1.30	**	*	*
TYR	0.20	0.18	0.18	1.45 ^a	0.99 ^b	1.04 ^b	0.07	***	*	*
CYS	0.13	0.09	0.11	0.08	0.14	0.11	0.01	ns	ns	ns
VAL	0.32 ^a	0.28 ^b	0.24 ^b	1.69 ^a	1.45 ^b	1.42 ^b	0.04	***	**	*
MET	0.17	0.14	0.13	0.77 ^a	0.71 ^b	0.67 ^b	0.01	***	**	*
TRP	0.11	0.08	0.10	0.21	0.17	0.17	0.02	**	ns	ns
PHE	0.21	0.26	0.29	1.97 ^a	1.62 ^b	1.52 ^b	0.06	***	*	**
ILE	0.28	0.27	0.23	1.23 ^a	1.05 ^b	1.04 ^b	0.03	***	***	*
LEU	0.49 ^a	0.36 ^b	0.34 ^b	1.92 ^a	1.21 ^b	1.09 ^b	0.08	***	***	**
LYS	0.35	0.32	0.26	1.96 ^a	1.72 ^b	1.64 ^b	0.04	***	**	*
PRO	0.36 ^a	0.17 ^b	0.15 ^b	0.38	0.33	0.33	0.02	***	***	*

nd, Not detected; ns, Not significant different ($p>0.05$).

* $p\leq 0.05$; ** $p\leq 0.01$; *** $p\leq 0.001$.

^{a-b}Different letters in the same row indicate significant difference at $p\leq 0.05$.

caused by the action of endogenous proteinases, oligopeptidases and aminopeptidases C, H and P (Feidt *et al.*, 1996; Moya *et al.*, 2001). This accumulation of free amino acids is known to be important in the development of meat flavors (Toldra, 2006). An increase in particular amino acids caused by ageing has been found in the previous studies, representatively Parrish *et al.* (1969) and Field *et al.* (1971) found that large increases in alanine, glutamine, serine, valine, leucine and lysine are obtained by ageing of beef for 5 d. Nishimura (1998) reported that rates increase in free amino acids of beef during the post-mortem was 3 $\mu\text{mol/g}$ meat per day, especially while large increases were in alanine, leucine, serine and valine. In addition, Migita and Nishimura (2006) suggested that amino peptidase D which newly-found distinct from amino peptidase B appears to contribute the increase in free amino acids, especially alanine and glutamine in meat during ageing.

In this study, only sulphur-amino acid like cystine was not significantly differed between ageing days. The cystine is not as essential because this can be made from methionine which is present but in a lower quantity about 0.17 $\mu\text{mol/g}$ in 1 d aged beef. Furthermore, it is probably related to which Furst *et al.* (1990) reported that cystine

reproducibility and linearity were poor when using OPA or FMOC derivatives.

The higher homogenizing speed at 24,000 rpm significantly affected the detection sensitivity of amino acids, also most amino acids tended to significantly increase with homogenizing speed however some amino acids such as serine, histidine, cysteine and proline showed a slightly increased in their mean values. Overall interactions between ageing and speed showed that significant differences for almost detected amino acids except for aspartic acid, serine, histidine, and tryptophan. This means that the mean values of amino acids increased with ageing time and big dispensing tool when high speed applied. The results suggested that the rotor turns at a high rpm; the tissues are rapidly reduced in size by a combination of extreme turbulence, cavitations and scissor like mechanical shearing occurring within the narrow gap between the rotor and the stator (Burden, 2008). Furthermore, mechanical disruption tissue generates by protein fragmenting and disrupting the sarcomere structure within myofibrils (Solomon *et al.*, 2002). Major ultra-structural changes which occur during ageing are the disappearance of the transversal alignment of the Z-disks and sarcomere fragmentation (Feidt *et al.*, 1996). This protein breakdown

Table 2. Changes in free amino acid contents during ageing, and by using smaller dispersing tool and different speed ($\mu\text{mol/g}$)

Smaller homogenizer								
Amino acids	1 d		7 d		SEM	Ageing	Speed	Ageing × Speed
	High	Slow	High	Slow				
ASP	nd	nd	0.14	0.14	0.01	***	ns	ns
GLU	0.30	0.23	1.34	1.44	0.11	***	ns	ns
ASN	0.04	0.07	0.47	0.49	0.04	***	ns	ns
SER	0.30	0.32	1.31	1.07	0.08	***	ns	ns
GLN	0.90	2.25	4.66	6.80	0.43	***	ns	ns
HIS	0.14	0.07	0.38	0.43	0.45	***	ns	ns
GLY	0.40	0.35	1.07	1.01	0.11	***	ns	ns
THR	0.22	0.16	1.04	1.06	0.09	***	ns	ns
ARG	0.20	0.16	0.87	0.84	0.06	***	ns	ns
ALA	4.40	3.61	10.31	11.14	0.97	***	ns	ns
TYR	0.17	0.15	1.22	1.02	0.08	***	ns	ns
CYS	0.12	nd	0.09	0.07	0.02	***	ns	ns
VAL	0.25 ^a	0.19 ^b	1.09 ^a	0.82 ^b	0.06	***	*	ns
MET	0.12	0.10	0.50	0.46	0.03	***	ns	ns
TRP	nd	nd	0.20	0.12	0.01	***	ns	ns
PHE	0.27	0.27	1.45	1.25	0.05	***	ns	ns
ILE	0.25	0.22	0.80 ^a	0.33 ^b	0.09	*	*	*
LEU	0.32	0.30	1.11	0.88	0.06	***	ns	ns
LYS	0.25	0.23	1.22	1.18	0.08	***	ns	ns
PRO	0.13	0.13	0.32	0.32	0.04	**	ns	ns

nd, Not detected; ns, Not significant different ($p>0.05$).

* $p\leq 0.05$; ** $p\leq 0.01$; *** $p\leq 0.001$.

^{a-b}Different letters in the same row indicate significant difference at $p\leq 0.05$.

and polypeptide production degraded to small peptides and free amino acids. Therefore, postmortem ageing and homogenizing at high rpm treatment may improve the proteolysis and that the amino acids with high concentrations were released from degraded proteins which means that efficiently enzymes may work faster on smaller particles (Nishimura, 1998).

Type and level of free amino acids as a function of ageing and speeds of smaller dispersing tool

Most of the free amino acids which released by using the smaller dispersing tool (stator diameter 8 mm, and rotor diameter 6.1 mm) at high speed were slightly higher in their mean values when compared to those by using medium and slow speed but not significantly in 1 d and 7 d ageing samples (Table 2), only valine and isoleucine were significantly differed between homogenizing speed. Mean values also were not significantly differed when the interactions between ageing with homogenizing speed. Moreover, aspartic acid and tryptophan were not detected in 1 d ageing beef samples. Cystine also not detected in homogenized at lower speed. However, when the samples were aged for 7 d, all amino acid appeared in both homogenizing speed (low and high). The absence of these two amino acids in the samples was also mentioned in the previous studies. Kivi (2000) reported that the tryptophan was lost when used ACN for deproteinization procedures. Furst *et al.* (1990) revealed that the aspartic acid and tryptophan were tough amino acids when used OPA reagent for derivatization. Moreover, aspartic acid is unstable and it is hydrolytic product of asparagines and this may be related with lower values of asparagines in lower speed. Smaller dispersing tool may also do not match to sample volume which became incomplete disruption of samples and resulted in lower amount of amino acids.

It has been established that homogenization at higher speed at 24,000 rpm is better for detecting an ageing effect on releasing amino acids than when samples were homogenized at medium and slower speeds by using a bigger dispersing tools. The several experiments revealed that the homogenization speed effected to the sample state. Hopkins *et al.* (2004) reported that the homogenization increases up to 15,000 rpm, the myofibrillar fragmentation index (MFI) values decreases compared to those obtained at slower speed such as 5000 rpm. Karumendu *et al.* (2009) revealed that the homogenization at 16,000 rpm provided the greatest ability to detect ageing differences for particle sizes between samples aged for 1 and 5 d and rate MFI values.

Furthermore, using big dispensing tool for homogenizing the both 1 d and 7 d ageing samples at high speed, result indicated the mean values of all 20 detected amino acids were higher compared to those homogenized by the small dispersing tool. This might probably related to homogenization process, the use of bigger dispersing tool effectively destroyed tissue for amino acid releases and produced a much finer particulate due to physical breakdown of protein, plasma membranes, also physical disruptions of the myofibrillar ultra structure and intermyofibrillar linkages. Furthermore, incomplete sample disruption due to smaller dispersing tool may resulted in the reduction

Fig. 1. Microstructure of the homogenized muscle sample at different speeds using bigger Rotor-stator homogenizer with S25 NK 19 G dispersing tools; A, 24,000 rpm; B, 19,000 rpm; C, 11,000 rpm. Using electronic microscope at 40x magnifications, coupled to a digital camera (Olympus, CKX 41, and Foculus IEEE1394, Germany).

Fig. 2. Microstructure of the homogenized sample at different speeds by using smaller Rotor-stator homogenizer with S8 N8G dispersing tools; Homogenized A, 13,000 rpm; B, 11,000 respectively. Using electronic microscope at 40x magnifications, coupled to a digital camera (Olympus, CKX 41, and Foculus IEEE1394, Germany).

yields significantly and application of smaller dispersing tools may reduce the sample volume (Halpine, 2004).

The microstructures of muscle fiber fragments for homogenized samples

The microstructures of muscle fiber fragments of samples formed by homogenizing using two different dispersing tools at various speeds are showed in Figs. 1 and 2. It shows that more tissue disruption formed by using the big dispersing tool compared to the small one. The muscle fiber fragment size reduced when raising the homogenizing speed. Similarly, Hopkins *et al.* (2004) reported that homogenization speed affect the size of myofibrillar fragment and examined that myofibrils for homogenized at lower speed the myofibrils were much longer and still connected. It is logical to assume that the rotor stators quickly sheared samples at the high speed, reduced the muscle mass to fragments which were further reduced in size by subsequent homogenization steps. The big dispersing tool was effective in disrupting tissue for amino acids. Probably, there are the main reasons making the mean values and detection sensitivity of amino acids

higher in the samples which were homogenized by big dispersing tool and high speeds (Tables 1 and 2).

Conclusion

Ageing of beef produced a significant increase in the mean values of free amino acids, which are important indicators relating to the eating quality such as tenderness, flavor and juiciness. Using big dispersing tool for homogenizing beef samples generated more number of amino acids and its mean value than using the small dispersing tool. Increase in homogenizing speed up to 24,000 rpm produced higher mean values of amino acids and analytic sensitivity. The current data indicated that utilization of a big dispersing tool at high homogenization speed is the better condition for releasing free amino acid contents in beef sample as comparing between ageing time samples in terms of consistent and repeatable outcomes. As a technical note, the current study emphasized that a standard method for analyzing free amino acid types and levels of aged beef samples is very important for consistent results and compatible data with other laboratories.

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