Structural changes evaluation with Raman spectroscopy in meat batters prepared by different processes

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Abstract A comprehensive study was conducted to evaluate the structural changes of meat and protein of pork batters produced by chopping or beating process through the phase-contrast micrograph, laser light scattering analyzer, scanning electronic microscopy and Raman spectrometer. The results showed that the shattered myofibrilla fragments were shorter and particle-sizes were smaller in the raw batter produced by beating process than those in the chopping process. Compared with the raw and cooked batters produced by chopping process, modifications in amide I and amide III bands revealed a significant decrease of α -helix content and an increase of β -sheet, β -turn and random coils content in the beating process. The changes in secondary structure of protein in the batter produced by beating process was thermally stable. Moreover, more tyrosine residues were buried, and more gauche–gauche-trans disulfide bonds conformations and hydrophobic interactions were formed in the batter produced by beating process.

Keywords Beating · Chopping · Myofibril fragment · Particle-sizes · Secondary structure

Introduction

Emulsified meat products are available to provide the consumer with a wide choice of textures and flavours depending on the different processing method. Historically,

the meatball products can be dated back to sixth century in Qi Min Yao Shu, China. One of such products is called kung-wan, as both the processing and eating qualities are different from other emulsified meat products. For example, products such as frankfurters or sausages show high juiciness and tenderness whilst for kung-wan, it is desirable to have good textural properties with high hardness, brittleness and elasticity (Wu and Lin 2011; Hsu and Chung 1998).

Reduction in the size of raw batter is a key procedure in making emulsified meat products, which involves the comminution of meat myofibril, endomysium and perimysium. Ultimately, the myofibrillar proteins are hydrated and swelled by adding the salt, and then the homogenous meat batter is formed (Tobin et al. 2013; Xiong 1997). The process also influences the cooking yield, texture and juiciness of emulsified meat products (Atilgan and Birol 2017; Tobin et al. 2012). The bowl cutter is the most used equipment for industrial mincing and mixing of meat, and stability of the product is maximized, which depends on a rotating bowl with a series of rotating sharp knives running (Allais et al. 2004; Brown and Toledo 1975). The beating machine has been designed to simulate the traditional way of making kung-wan in Asian communities, and to disrupt the connective tissues and myofibrillar structures with blunt blades (200 rpm) using a crushing force at variable speed (Kang et al. 2016, 2014a). However, few studies have been reported on the structural changes of protein in pork meat batters either by chopping or beating process.

Raman scattering spectroscopy can be used to provide the information of peptide backbone conformations, such as secondary and tertiary structures of meat food proteins (Li-Chan et al. 1994). It is also sensitive to the changes in covalent (disulfide linkages) and non-covalent (hydrogen

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bonding and hydrophobic interaction) bonds (Gao et al. 2015; Wang et al. 2013). Because of weak background scattering from water, it is directly applicable to aqueous systems (Shim et al. 2016; Li-Chan 1996). This method has been used to study the structural changes of meat systems during thermal gelation (Schmidt et al. 2013; Liu et al. 2011; herrero 2008a; Pedersen et al. 2003), and different processes and processing periods, such as high pressure and produced Cantonese sausage (Liu et al. 2011; Herrero 2008a). However, according to best of our knowledge, Raman spectroscopy has never been used to examine the structural changes of proteins in meat batters by different emulsified processes. Therefore, the objective of the present study was to determine the degree of disruption in pork myofibril, meat particle-size ranges and structural changes in proteins produced by either chopping or beating process.

Materials and methods

Preparation of pork batters

Pork leg meat (after 24–48 h postmortem, pH 5.78; 71.18% moisture, 20.47% protein and 7.14% fat, AOAC 2000) was procured three times in three days from local meat market (Nanjing, China). All visible connective tissue was trimmed from the meat. The lean meat was mixed and passed through a grinder (MM-12, Guangdong, China) fitted with a plate having 6 mm diameter holes. The ground meat (1.0 kg each) was packaged in double plastic (nylon/PE) bags and stored at -20°C until use within 2 weeks. Meat batters were prepared by either beating or chopping. In each replication, the ingredient only consisted of pork leg meat. Pork leg meat was thawed at 4°C overnight prior to use. For beating process, the thawed ground meat (1.0 kg each) was beaten for 15 min (about 200 rpm; final temperature less than 10°C) using a beating machine (MC-6, Shandong, China). For the chopping, the procedure was used to prepare products in a vacuum cutter bowl (Stephan UMC-5C, Germany) as reported by Lin and Lin (2004) with minor modifications. The thawed ground meat (1.0 kg each) was chopped (1500 rpm) for 30 s, followed by a 3 min rest and then chopped (1500 rpm) for 30 s, followed by a 3 min rest, prior to finishing with a high speed (3000 rpm) emulsification for 60 s (final temperature less than 10°C). Then 35 g meat batter were stuffed into 50 mL polypropylene tube, hermetically sealed and centrifuged at $500\times g$ (Avanti J-E, Beckman Coulter, CA) for 2 min to remove all remaining air bubbles. There were a total of eight tubes for each meat batter prepared by different processes. Half of the containers with each meat batter formulation were chilled at 4°C and analyzed as raw

batters. The rest of the containers in each case were cooked in a water bath at 80°C for 20 min, then the cooked batters were stored in a chiller at 4°C until further analysis.

Phase-contrast micrograph of myofibrils

Ten grams of each raw batter was blended in a glass rod for 60 s at $2-4^{\circ}\text{C}$ in 100 mL solution (0.1 M/L KCl, 2 mM/L MgCl_2 , 1 mM/L EGTA, 0.5 mM/L dithiothreitol and 10 mM/L K_2HPO_4 , pH 7.0) (Xiong et al. 2000). After blending, a drop of suspension containing the fiber fragments was immediately placed on a microscope slide and covered with a cover slip. The suspension was examined with the oil objective under a phase-contrast microscopy (BX41, Olympus, Japan).

Scanning electronic microscopy

Microstructure of raw batters were determined using scanning electron microscopy (Hitachi-S-3000 N, Hitachi High Technologies Corp., Toyoko, Japan) according to the procedure as described by Haga and Ohashi (1984). Cubic samples ($3\text{ mm} \times 3\text{ mm} \times 3\text{ mm}$) obtained from raw batters were fixed for 24 h at 4°C in 0.1 M phosphate buffer (pH 7.0) with 2.5% glutaraldehyde. The fixed samples were washed in 0.1 M phosphate buffer (pH 7.0) for 10 min, and then post-fixed for 5 h in the same buffer containing 1% osmium tetroxide. The post-fixed samples were washed three times with 0.1 M phosphate buffer (pH 7.0) for 10 min, and then dehydrated in a graded series of 50, 70, 90, 95, 100% ethanol for 10 min in each ethanol concentration, followed by dehydration twice with 100% acetone for 10 min. Each treatment was replicated in triplicated and the representative photos were used.

Particle size distribution

Ten grams of each raw batter was homogenized in a polytron homogenizer (T25 digital, IKA Ltd, Germany) at 3000 rpm for 15 s at $2-4^{\circ}\text{C}$ in 100 mL distilled water. The particle size distribution was determined using a Mastersizer laser light scattering analyzer (Mastersizer 2000, Malvern Instruments Ltd., Worcester shire, UK) during four successive readings. The particle size was expressed as $D_{3,2}$ (Surface Weighted Mean), $D_{4,3}$ (Volume Weighted Mean) and D_{10} , D_{50} , D_{90} (accumulated value to achieve volume percent of particle size of 10, 50 and 90%, respectively).

Raman spectroscopy measurement

Raman experiments of raw and cooked batters were determined using a procedure of Shao et al. (2011). All

spectra were automatically baselines corrected, smoothed and normalized against the phenylalanine band at 1003 cm^{-1} (Herrero 2008a; Moudache et al. 2017) using Labspec version 3.01c (Horiba/Jobin. Yvon, Longjumeau, France). For secondary structures, raw or cooked batters proteins were all ascertained as percentages of α -helix, β -sheet, β -turn, and random coil or unordered conformations (Alix et al. 1988). For this purpose, the water spectrum was subtracted based on the same criteria as reported previously (Alix et al. 1988; Herrero et al. 2011).

Statistical analysis

Statistical analyses were carried out using the statistical software package SPSS 18.0 (SPSS Inc., USA). For the data of meat particle-size and protein conformations, an analysis of variance (ANOVA) using the general linear model (GLM) procedure was performed, which considered as different processes (chopping or beating) a fixed effect and replicate as a random term. When significant treatment effects were found, an independent-samples *t* test was used to determine any significant difference between different processes and considered to be significant at $p < 0.05$.

Results and discussion

Microscopic examination

Phase-contrast micrograph of myofibrils between beating and chopping process (Fig. 1) showed significant effect on the morphology of raw batters. The batter produced by chopping process showed larger myofibril fragments (Fig. 1a) compared to those by the beating process (Fig. 1b). Moreover, the myofibril fragments were disrupted less than 10 sarcomeres by the beating (Fig. 1b). Some workers have been reported that products with different myofibrillar fragmentations were obtained when used different homogenizer types and speeds had product different myofibrillar fragmentation termed (Hopkins et al. 2000, 2004; Karumendu et al. 2009). The results were generated by the different mechanisms between cutter bowl and beating machine. Chopping of meat by bowl cutter depends on rotating bowl connected with a series of rotating sharp knives running ($>1500\text{ rpm}$) in a vertical plane. However, the disruption of connective tissue and myofibrils by beating machine depends on the driving force of blunt blades (about 200 rpm), and the compression, friction and disintegration of meat pieces and blunt blades (Kang et al. 2014b, 2016).

Fig. 1 Phase-contrast micrographs (a, b) of the pork myofibrils, and scanning electron micrographs (c, d) of the raw pork batters produced by either chopping or beating process

Fig. 2 Particle-size distributions of the pork meat particle produced by either chopping or beating process with homogenized at 3000 rpm for 15 s at 2–4 °C with the Omni mixer, respectively. Note C, treatment with chopping process; T, treatment with beating process

Table 1 Effects of the chopping or beating process on meat particles mean diameters of raw pork batters

Sample	D _{3,2} (μm)	D _{4,3} (μm)	D _{0,1} (μm)	D _{0,5} (μm)	D _{0,9} (μm)
C	77.60 ± 7.55	12.23 ± 0.11	4.76 ± 0.03	26.05 ± 0.48	182.02 ± 9.45
T	111.85 ± 1.50	19.78 ± 0.26	7.13 ± 0.07	65.66 ± 2.13	264.32 ± 8.00
	*	**	**	**	**

Each value represents the mean ± se. C, treatment with chopping process; T, treatment with beating process. *ns* non-significant; ** $p < 0.01$; * $p < 0.05$

Scanning electronic microscopy

Micrographs of raw batters depicted significant effect of chopping and beating process on the structures of batters (Fig. 1). In the chopped batter (Fig. 1c), the meat bundles were cut by sharpening knives, and the cut surfaces were found in good order, the diameters of meat bundles were large. However, in the beating batter, the meat bundles showed disordered myofibrils with broken endomysium and also generated some thin fibers (Fig. 1d). This was in agreement with the result of microscopic examination. In comparison to the chopping, more myofibrils, endomysium and perimysium were shattered by the beating process.

Particle-size analysis

One of the most important operations for the production of emulsified meat products is the size reduction, which especially is carried out in cutter bowl and beating machine. Figure 2 shows the particle-sizes distribution of the raw batters produced by the chopping or the beating process. A bimodal distribution for the raw batters included the major peaks of the beating and chopping treatments (located at 15 and 138 μm) and the shoulder peaks (located at 91 and 20 μm) respectively. The results indicated that the values of D_{3,2}, D_{4,3}, D_{0,1}, D_{0,5} and D_{0,9} significantly

($p < 0.01$) declined in beating process than chopping process (Table 1). That showed the particle-size of raw batters was affected by the process methods. In general, the smaller particle-size of meat batter was produced by the beating process than the chopping process.

Raman spectroscopic analysis

Raman spectra of raw or cooked meat batters produced by either chopping or beating process were found in the 400–2000 cm⁻¹ region (Fig. 3a). According to previous literature (Li-Chan et al. 1994; Alizadeh-Pasdar et al. 2002; Sánchez-González et al. 2008), the changes of intensity and frequency in the Raman bands were mainly taken as the indication for the secondary structures and local environment of raw or cooked meat batters.

Secondary structural

The Raman bands centered at 1655 and 1665 cm⁻¹ had been assigned to amide I vibrational mode made on raw and cooked batters (Krimm and Bandekar 1986; Nache et al. 2016), providing the secondary structural information about protein (Tu 1982; Song et al. 2016). The frequencies of amide I band principally involved C=O stretching, C–N stretching, Cα–C–N bending and N–H in-plane bending of

Fig. 3 Raman spectra of raw and cooked pork batters in the region 500–2000 cm^{-1} (a), 450–650 cm^{-1} (b) and 2800–3100 cm^{-1} (c). Note C, treatment with chopping process; T, treatment with beating process

peptide groups. As the amide I band is sensitive to the changes of the hydrogen bonding scheme involving the peptide linkages, it consists of overlapping band components falling in the 1650–1660, 1665–1680, 1680 and 1660–1665 cm^{-1} ranges, which are attributable to α -helix,

β -sheets, β -turn and random coil structures, respectively (Li-Chan 1996; Ngarize et al. 2004; Herrero 2008a). By knowing the frequency of peak Raman amide I band, the Alix's method was used for the quantitative determination of the secondary structure of protein (Table 2) with following equation:

$$\text{Structure \%} = a \cdot v(\text{maximum}) + b$$

where a and b are coefficients calculated for each class of structure by using the least squares method (Alix et al. 1988).

The amide I bands of raw C and T, centered at 1653 and 1656 cm^{-1} respectively (Fig. 3a), indicated a predominance of α -helix. According to Table 2, there were significant effects ($p < 0.05$) of chopping and beating processes on the values of secondary structural percentages in the raw C and T. Finally, this increased β -sheet, β -turn, random coil and decreased α -helix in the beating. The results implied that the beating process could cause more unfolding of α -helix structure than the chopping process, then ultimately transforming into β -sheet, β -turn and random coil structures (Boecker et al. 2007; Liu et al. 2011). After heating, the frequency peak positions of cooked C and T were shifted to 1660 and 1662 cm^{-1} respectively (Fig. 3a), that indicated a slightly increase in β -sheet content. The heat process showed significant effect ($p < 0.05$) on secondary structures of proteins. The α -helix content of cooked batters were found lower, and had higher β -sheet, β -turn and random coil content than the raw batters. Some workers have reported the similar results of temperature on β -sheet, β -turn, and random coil fractions with α -helix (Xu et al. 2011; Liu et al. 2011; Kumar and Karne 2017). The cooked T also had a higher β -sheet ($p < 0.01$) and random coil content ($p < 0.05$) than the cooked C. That resulted in the changes of secondary structures by beating process were stable during thermal treatment.

The amide III (1225–1350 cm^{-1}) band mainly involves C-N stretching and N-H in-plane bending vibrations of the peptide bond, and provided information about secondary structure (Herrero 2008a). Proteins with higher α -helix (1260–1300 cm^{-1}) have a weak band while β -sheet (1230–1245 cm^{-1}) generated a more intense band. The random coil structure was centered at around 1245 cm^{-1} (Herrero 2008b; Bouraoui et al. 1997). For the raw batter C (Fig. 3a), a stronger intensity peak was centered at 1297 cm^{-1} whereas the wave number (1271 cm^{-1}) was lower and had weak peak at 1232 cm^{-1} for the raw batter T. The results indicated that the use of beating process improved fraction of β -sheet at the expense of α -helix. Cooked batters of C and T had a peak at 1239 and 1242 cm^{-1} , respectively. This ultimately improved the contents of β -sheet, β -turn, and random coil fractions, and

Table 2 Percentages of protein secondary structures α -helix, β -sheet, β -turns, random coil of the raw and cooked pork batters analyzed either chopping or beating process

Sample	α -helix			β -sheet			β -turn			Random coil		
	Raw	Cooked	Sign.	Raw	Cooked	Sign.	Raw	Cooked	Sign.	Raw	Cooked	Sign.
C	73.89 $\pm$ 3.30	49.20 $\pm$ 3.28	**	5.15 $\pm$ 2.53	24.07 $\pm$ 2.51	**	12.27 $\pm$ 0.52	16.14 $\pm$ 0.29	**	9.13 $\pm$ 0.20	10.64 $\pm$ 0.20	*
T	58.70 $\pm$ 5.70	39.73 $\pm$ 3.28	**	16.79 $\pm$ 4.37	31.33 $\pm$ 2.51	**	14.65 $\pm$ 0.89	17.62 $\pm$ 0.29	*	10.06 $\pm$ 0.35	11.22 $\pm$ 0.20	*
Sign.	**	**		**	**		*	*		*	*	

Each value represents the mean $\pm$ se. C, treatment with chopping process; T, treatment with beating process. ns non-significant; ** $p < 0.01$; * $p < 0.05$

decreased the α -helix. The results implied an increase of β -sheet or random coil structures during heating. It was difficult to interpret because vibrational spectroscopy of proteins produced a complex pattern of bands in the range of amide III (Herrero 2008b; Herrero et al. 2009).

Changes of S–S stretching

The changes of S–S stretching are important for non-reversible heat gelation and correlation with the formation of disulfide bonds (Bouraoui et al. 1997). The bands generated by S–S stretching evidenced for a Raman spectrum band in the 500–650 cm^{-1} range. Proteins and peptides containing cysteine residues show a band near 510 cm^{-1} , which has been attributed to S–S stretching vibrations of disulfide bonds, that is in the ganache–ganache–ganache conformation (Li-Chan et al. 1994). As it is the lowest potential energy conformation, some natural proteins assumed the conformation of this S–S bonds conformation (Tu 1982). Bands located at 516–530 and 535–545 cm^{-1} have been assigned to S–S bonds in the conformation of ganache–ganache-trans and trans-ganache-trans respectively, or with small C–C–S–S dihedral angles (Li-Chan et al. 1994; Li-Chan 1996). Raw C with two weak bands were located at 516 and 545 cm^{-1} , and raw T with three weak bands were located at 515, 532 and 545 cm^{-1} (Fig. 3b). Compared to the chopping process, the beating could cause partial transition from ganache–ganache–ganache and/or trans-ganache-trans to ganache–ganache-trans conformation. The new bands were observed at 529 and 519 cm^{-1} for cooked C and T, respectively. These implied that heat treatment caused the change of ganache–ganache–ganache and trans-ganache-trans conformations into ganache–ganache-trans conformation. Moreover, both beating process and heat treatment had similar effect on S–S stretching, and on increased intensity. The results are in agreement with Bouraoui et al. (1997), who documented the increasing relative intensity of the band around 530 cm^{-1} in the cooked and set-cooked gels. This denoted that stretching or aliphatic chain vibrations of S–S changed, and gauche–gauche-trans disulfide bonds conformations were formed. So the cross-linking of proteins could occur largely by heating or beating process.

Changes of local environment

There are several Raman bands that can provide information about tertiary structure of proteins by local environments, such as tryptophan, tyrosil doublet, and aliphatic hydrophobic residues, the changes in Raman bands mainly provide information about hydrophobic interactions (Li-Chan et al. 1994; Herrero et al. 2009). Raman band near

Table 3 Normalized intensity values at I_{760}/I_{1003} and I_{850}/I_{830} of Raman spectra of the raw and cooked pork batters analyzed either chopping or beating process

Sample	I_{760}/I_{1003}			Sign.	I_{850}/I_{830}		
	Raw	Cooked			Raw	Cooked	Sign.
C	0.62 ± 0.02	0.65 ± 0.04	ns		1.32 ± 0.20	1.04 ± 0.06	*
T	0.56 ± 0.03	0.52 ± 0.04	ns		1.05 ± 0.02	1.18 ± 0.07	ns
Sign.	*	*			*	*	

Each value represents the mean $\pm$ se. C, treatment with chopping process; T, treatment with beating process. ns: non-significant; ** $p < 0.01$; * $p < 0.05$

760 cm^{-1} was assigned to the stretching vibration of the tryptophan residues ring (Li-Chan 1996). There was a significant ($p < 0.05$) effect on the Raman band intensity of tryptophan residues in raw and cooked C and T (Table 3). The normalized intensity of the Raman band near 760 cm^{-1} of raw C and T, and of cooked was 0.62 and 0.56, and 0.65 and 0.52, respectively. The beating process indicated improved hydrophobic micro-environment in polar aqueous solvent due to unfolding of protein compared with chopping process (Li-Chan et al. 1994; Herrero 2008a). However, the chopping or beating process non-significantly affected ($p > 0.05$) even when the normalized intensity of the Raman band near 760 cm^{-1} was taken as a function of thermal treatment.

The double Raman bands (near 830 and 850 cm^{-1}) were assigned to vibrations of the para-substituted benzene ring of tyrosine residues, and useful for monitoring the microenvironment around tyrosyl residues (Siamwiza et al. 1975; Li-Chan et al. 1994; Li-Chan 1996). On the hand, when tyrosine residues are exposed to the aqueous or polar environment, or act as simultaneous acceptor and donor of moderate to weak hydrogen bonds, the doublet bands ratio (I_{850}/I_{830}) ranges from 0.90 to 2.5. In contrary, when tyrosine residues are buried in a hydrophobic environment and act as hydrogen donors usually range from 0.7 to 1.0, but can be as low as 0.3 in the case of extremely strong hydrogen bonding to a negative acceptor (Li-Chan et al. 1994). The double Tyrosine ratio (I_{850}/I_{830}) of raw C and T were found more than 1.0 (Table 3), which indicated that both chopping and beating process caused tyrosine residues of proteins exposed to polar environment. Compared with raw C, the ratio of other treatments (raw and cooked) were significantly ($p < 0.05$) decreased. The results implied that tyrosine residues were in a more buried environment due to protein unfolding followed by aggregation during heating or beating process (Herrero et al. 2009). However, the ratio of raw T decreased non-significantly ($p > 0.05$) after heating. So the tyrosine residues were found in a more hidden environment due to protein aggregation and denaturation caused by beating process.

Stretching vibrational change of C–H

Aliphatic amino acids, peptides, and proteins showed the C–H stretching vibrations in the $2800\text{--}3050\text{ cm}^{-1}$ range of the Raman spectrum (Howell et al. 1999). The intensity of the strongest vibrations C–H band was located at around 2935 cm^{-1} (Fig. 3c). The band position of raw T shifted from 2929 cm^{-1} to 2932 cm^{-1} as compared with raw C. The polarity of the environment around hydrocarbon chains increased as the intensity of 2930 cm^{-1} band shifted to high wave number, because the unfolding of protein and aliphatic residues exposure (Leelapongwattana et al. 2008; Liu et al. 2011). The beating process unfolded α -helix significantly as compared with chopping, and exposed the hydrophobic amino acids during the processing, that lead to more proteins denaturation and thus reactivity during heating. Then all samples were shifted to 2937 cm^{-1} after thermal treatment. Xu et al. (2011) observed that the increased heating temperature of pork myofibrillar protein from 30 to $70\text{ }^{\circ}\text{C}$, which destructed the tertiary and the H-bonding secondary structures of proteins, the Raman bands had a trend of shifting to high wave number. These also resulted in protein cross-linking when the type of hydrophobic contact between aliphatic protein side.

Conclusion

The process methods were significant affected the myofibril fragments, particle-sizes of pork batters, and the secondly and tertiary structures of proteins. Compared with the chopping, the batter produced by beating had smaller myofibril fragments and particle-size. The proteins of batter produced by beating had a higher β -sheet, β -turn and random coil contents and lower α -helix content than the chopping process. Whatever beating or chopping process, α -helix content of protein decreased, while β -sheet, β -turn and random coil contents increased after heating, and the beating process also led to the higher β -sheet, β -turn and random coil structure contents than the chopping process. The use of beating process also changed the S–S stretching,

local environment and C–H stretching. The results proved that the use of beating process resulted in the greater disruption of myofibrils, and more β -sheet, β -turn and random coil formed, which led to the release of more protein into solution and therefore were available for the protein–protein interactions.

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