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Structural Evaluation of Myofibrillar Proteins during Processing of Cantonese Sausage by Raman Spectroscopy

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ABSTRACT: Structural changes of myofibrillar proteins from raw pork muscle and Cantonese sausage at different processing periods were elucidated using Raman spectroscopy. Fourier deconvolution combined with iterative curve fitting were used to analyze the amide I Raman band. Results from amide I, amide III, and C−C stretching vibrations in 890−1060 cm^{-1} showed that α-helix decreased accompanied by an increase in β-sheet structure during the first 18 h, and a rebuilding process of secondary structures was observed at the rest stage due to proteolysis. The hierarchical cluster analysis results of amide I and amide III confirmed this rebuilding process. Changes in a doublet near 850 and 830 cm^{-1} suggested that some tyrosine residues became buried in a more hydrophobic environment due to intermolecular interactions. Raman spectra in the 2855−2940 cm^{-1} region suggested that the environment of aliphatic side chains might have been changed during the final stage and further confirmed above rebuilding process.

KEYWORDS: Raman spectroscopy, myofibrillar proteins, structure, hierarchical cluster analysis, Cantonese sausage

INTRODUCTION

Myofibrillar proteins, contributing to 55−65% of total muscle proteins, are the major protein fraction responsible for many physicochemical properties of muscle foods.[1] The physicochemical state of myofibrillar proteins affects the functionality of meat systems and plays a direct role in determining the quality and value of processed meat products.[2] The myofibrillar structure is a complex system with many protein−protein interactions.[3] Meat processing consists of a series of technological steps. Information concerning meat proteins and their behavior after slaughter and during aging is extensively documented.[4,5] Salting and heat treatment are two important steps of processed meat products. Thermally induced protein−protein aggregation is considered to be the key element to transform protein to a three-dimensional matrix during meat processing.[6] Recently, the evolution of myofibrillar protein of muscle has been mapped during salting and heat treatment.[7−9] They observed that structural evaluation of myofibrillar proteins was primarily affected by salting and cooking, resulting in protein functionalities changes, such as water binding ability.

The structural changes of protein can be examined at the molecular level by several methods such as circular dichroism, fluorescence spectroscopy, nuclear magnetic resonance (NMR), infrared absorption, and Raman spectroscopy. NMR methods mainly give detailed and specific information about peptides and proteins in solution and are limited to low molecular weight proteins.[10] Infrared spectroscopy methods present problems arising from the very strong infrared absorption of water at 1650 cm^{-1}, in the middle of the amide I band.[11] Raman spectroscopy is a more suitable and direct technique that overcomes most of the above objections and can provide information on the secondary and tertiary structure of proteins in solid samples, like muscle food.[12] The Raman spectral assignments of proteins are usually based on model compounds such as amino acids or short peptides. Raman spectroscopy has been shown to be a useful tool to determine structural changes that occur during processing of meat systems.[9,11,13] It has been used to study the structure of isolated meat proteins such as myosin.[14,15]

Cantonese sausage is a semidry sausage made in China, which has gained much popularity and acceptance over the world. Cantonese sausage is oven-dried at 42−50 °C for 3 days after salting. Salting and thermal treatment during meat processing strongly influence the protein changes. However, information concerning the structural changes of myofibrillar proteins during Cantonese sausage processing is still limited. Therefore, the objective of this work was to evaluate structural changes of myofibrillar proteins and their interactions by Raman spectroscopy at the molecular level.

MATERIALS AND METHODS

Samples and Materials. Cantonese sausage samples (three replications) and raw pork muscle were collected from Jinrong Meat Products Co. Ltd. (Zhongshan, China). Raw muscle and back fat were obtained from a local commercial abattoir (Zhongshan, China), and the pigs were slaughtered at about 6 months of age following standard industrial procedures. Cantonese sausage was prepared according to the following formulation: lean pork (70 g), back fat (30 g), salt (3.5 g), sugar (12 g), wine (4 g), sodium nitrite (0.02 g), and water (20 g). These raw materials were mixed and stuffed into natural casings with a diameter of 37 mm and dried for 3 h at 50 °C with solar drying systems (Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou, China), followed by a reduction of temperature to the range of 45 °C, then dried for 21 h, and finally dried for 48 h at 42 °C. Samples (1000 g) were periodically taken at 0, 6, 18, 36, 54, and 72 h for each batch and kept at −18 °C for further use.

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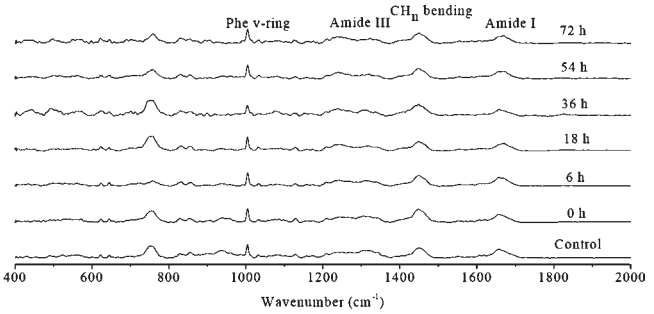

Figure 1. Raman spectra of myofibrillar proteins from raw muscle and Cantonese sausage at different processing periods in the 400−2000 cm^{-1} region.

Isolation of Myofibrillar Proteins. Myofibrillar proteins were prepared according to the method of Martinaud et al.[16] Ten grams of Cantonese sausage was homogenized in 100 mL of sodium phosphate buffer solution (20 mM) at pH 6.5 containing 50 mM NaCl, 25 mM KCl, 3 mM $MgCl_2$, and 4 mM ethylenediaminetetraacetic acid, to which a protease inhibitor (1 mM PMSF) had been added, with an Ultra-Turrax homogenizer (Beijing Jingke Huarui Instrument Co. Ltd., Beijing, China) at 8000 rpm for 1 min in an ice bath. The homogenate was sieved through the sifter (mesh 250 μm), and collagen was eliminated by filtration on sifter. The extract was centrifuged at 2000*g* for 15 min at 4 °C. The pellet was washed twice with 100 mL of distilled water. The washed myofibril pellet was freeze-dried for Raman spectroscopy use.

The purity of myofibrillar proteins was assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis according to the method of Flores et al.[17] The protein contents of myofibrillar proteins were 87−92%. The characteristic polypeptidic bands corresponding to myofibrils and to each major myofibrillar proteins were present in the gels. Results show that myofibril preparations were free of sarcoplasmic protein contamination (results not shown).

Raman Spectroscopy. Raman spectra with an excitation at 632.8 nm (laser He/Ne, less than 10mW on the sample) were obtained at room temperature, using a LABRAM-Aramis spectrometer (Horiba Jobin-Yvon, France). Spectral repeatability was smaller than 0.2 cm^{-1}, and spectral resolution was better than 1 cm^{-1}. Myofibrillar protein samples were placed on microscope slides. The laser was then focused on the samples. Raman spectra of at least three different positions were collected from 400 to 4000 cm^{-1}. The spectral data from the scans of samples in the Raman spectrophotometer were baseline corrected and normalized according to the protein phenylalanine peak at 1003 ± 1 cm^{-1}. Protein secondary structures were determined as percentages of α, β, turns, and random conformations according to Susi and Byler.[18] The relative amounts of different protein secondary structures were determined from amide I spectra by computing the areas under the bands attributed to secondary structures. The areas of the bands were calculated by integration of the corresponding fitted band. Baseline correction, normalization, derivation, curve fitting, and area calculation were carried out by means of PeakFit Version 4.12 software (SPSS Inc., Chicago, IL), OPUS 6.5, and Origin 8.0 (OriginLab Corp., Northampton, MA) software.

Statistical Analysis. Statistical calculation and hierarchical cluster analysis were investigated using the statistical package SPSS 11.5 (SPSS Inc.). Data were subjected to analysis of variance (one-way ANOVA), and mean comparisons were carried out by least-squares difference.

RESULTS AND DISCUSSION

Raman spectra of myofibrillar proteins from raw muscle and Cantonese sausage at different processing periods in the 400−2000 cm^{-1} region are shown in Figure 1. The assignments of the Raman bands have been carried out according to literature references.[11,12] The frequency and intensity changes in the Raman bands were the main indicators of changes in the secondary protein structure and of variations in the local environments of myofibrillar proteins.

Amide I. *Analysis of Amide I Spectra Profile.* The most useful Raman bands to provide secondary structural information about proteins are the amide I (1600−1700 cm^{-1}) band, which mainly involves C═O stretching and, to a lesser degree, C−N stretching, C_α−C−N bending, and N−H in-plane bending of peptide groups.[11,12] Susi and Byler[18] reported that Fourier deconvolution of the amide I Raman band combined with iterative curve fitting could yield useful quantitative conformation of protein. In this work, Fourier deconvolution and iterative curve fitting were used to analyze an amide I Raman band of myofibrillar proteins from raw muscle and Cantonese sausage at different processing periods. Deconvolved and curve-fitted amide I Raman bands of myofibrillar proteins are shown in Figure 2. For the raw myofibrillar proteins, the major feature of the amide I band was centered at 1657 cm^{-1}, indicating a predominance of the α-helical structure, and a small shoulder near 1680 cm^{-1} suggested the minor contribution of β-sheet in the myofibrillar proteins. Salting (0 h) induced a trend toward uncoiling of helices, which was confirmed by the shift of the major band to 1660 cm^{-1}. The obvious shoulder band near 1675−1680 cm^{-1} assigned to the β-sheet structure was evident at the sixth hour. As processing proceeded, the band near 1675−1680 cm^{-1} gradually became dominant except at the 36th hour. However, these samples differed in other minor features. For example, the spectra of the 54th and 72nd hours showed a minor band near 1656 cm^{-1}. It was noteworthy that, at the 36th hour, the band near 1656 cm^{-1} was higher than that near 1675 cm^{-1}, indicating that the α-helical structure increased.

The corresponding results of the curve-fitting analysis expressed as a function of percentage areas of main protein secondary structures are in Table 1. There was a decrease ($p < 0.05$) in the α-helix accompanied by an increase ($p < 0.05$) in the β-sheet structure during 0−18 and 36−72 h. However, there is a tendency for the decrease ($p < 0.05$) of the β-sheet content with simultaneous increase ($p < 0.05$) of the α-helix content during 18−36 h. These results were consistent with the amide I spectra changes (Figure 2).

Some authors have reported the changes in protein secondary structure upon addition of inorganic salts in isolated myosin solutions. Reduced α-helix observed in myosin in the presence of inorganic salts such as $CaCl_2$, $MgCl_2$, and LiBr can be explained by an increase in interchain hydrogen bonding.[15] Fourier transform infrared (FTIR) spectroscopic analysis of pork muscle, subjected to brine salting at different concentrations, has shown protein structural changes in terms of decreases in α-helix content at a salt concentration higher than 3%.[8] Studies of heat-induced structural changes in meat myofibrillar proteins, determined by FTIR spectroscopy, had indicated an increase in β-sheet structure levels.[7] Herrero et al.[9] reported that the establishment of a stable gel network upon heating is closely linked to an increasing hydrogen-bonded β-sheet structure. Studies in roasted beef indicated that the α-helical to β-sheet ratio is an important factor contributing to the shear force, tenderness, acceptability of texture, and overall acceptability of meat.[19] Moreover, Bouraoui, Nakai, and Li-Chan[20] reported that the structure change was possibly through intermolecular

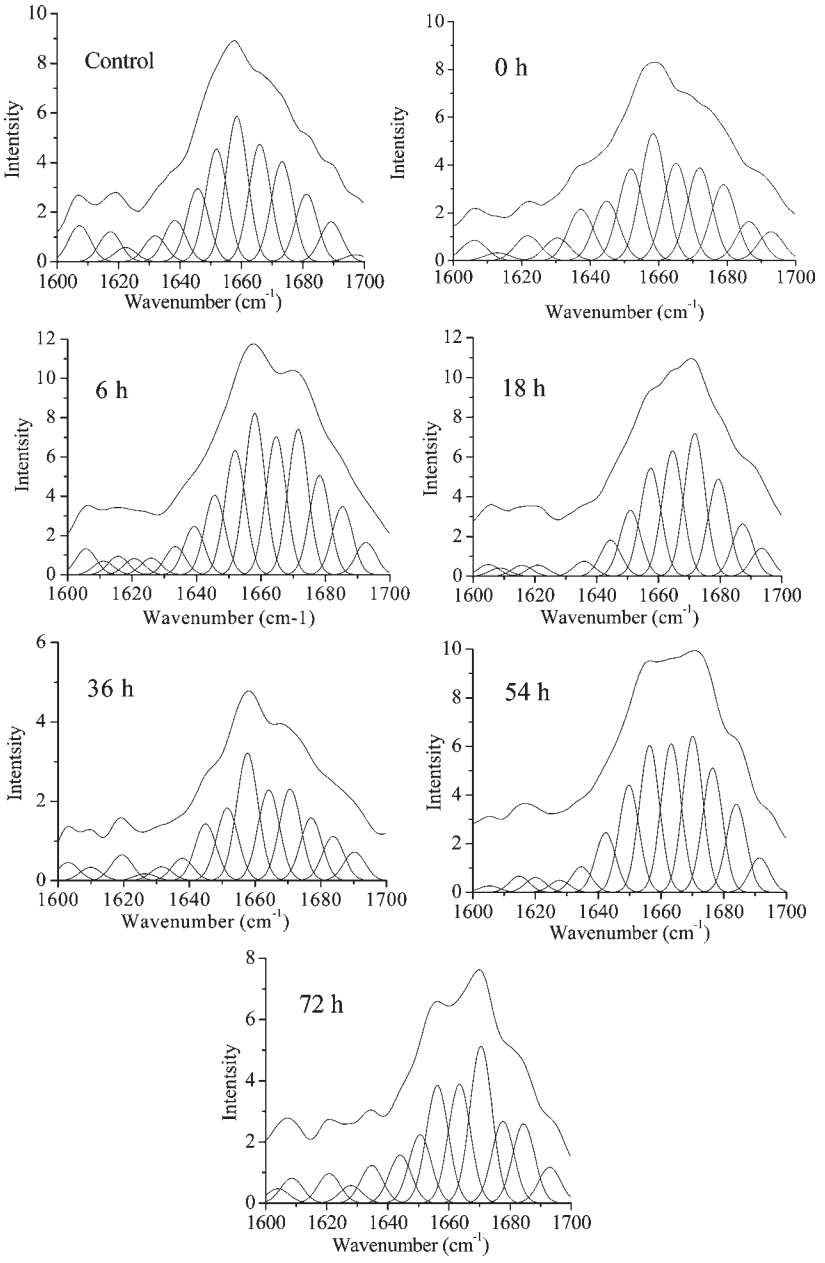

Figure 2. Deconvolved and curve-fitted Raman bands of amide I of myofibrillar proteins from raw muscle and Cantonese sausage at different processing periods.

interactions between exposed hydrophobic residues, which increased during cooking. In the present work, changes in amide I during 0−18 and 36−72 h could also be due to an unfolding of helical structures, followed by the formation of sheet structures. An increase in α-helix accompanied by a decrease in β-sheet structure during 18−36 h might be due to proteolysis of Cantonese sausage, which involves the decrease of high molecular weight proteins and the formation of polypeptides and free amino acids.[21] Enzymatic hydrolysis of proteins, especially partial hydrolysis, is often employed to improve the functionalities of food proteins.[22,23] Such functionality changes were related to alterations in protein structure, including partial unfolding, altered isoelectric point, and increased hydrophobicity. These discrepancies between the 36th hour and the other periods might be due to changes of myofibrillar proteins composition due to proteolysis. Proteolysis is an important biochemical behavior occurring during the processing of Cantonese sausage.[24] The appearance of bands with some vibration frequencies in the Ramam spectra depends on the molecular geometry and the chemical composition of substance tested.[12] Proteolysis might lead to the myofibrillar proteins undergoing a rebuilding process of secondary structures. In addition, the structure of random and other types at the 36th hour increased as compared to the others, confirming this rebuilding process.

Hierarchical Cluster Analysis of Amide I. Multivariate statistical techniques are the right tools for viewing and analyzing a matrix of complex data. Hierarchical cluster analysis consists of mathematically treating each variable as a point in the multidimensional space described by the samples.[25] When a given sample is taken as a point in the space defined by the variables,

Table 1. Determined Frequencies of Amide I Component Bands, Relative Assigned Structures, and Their Distributions for Secondary Structural Contents of Myofibrillar Proteins from Raw Muscle and Cantonese Sausage at Different Processing Periods

	frequency (cm^{-1})/structure distribution (%)						
structure[a]	control	0 h	6 h	18 h	36 h	54 h	72 h
amino acid side chains	1607 (4.46 ± 0.47 c)	1607 (2.73 ± 0.43 b)	1606 (2.56 ± 0.41 b)	1605 (1.65 ± 0.51 a)	1604 (2.69 ± 0.33 b)	1606 (0.68 ± 0.18 a)	1605 (1.50 ± 0.31 a)
	1617 (3.71 ± 0.47 c)	1613 (1.05 ± 0.19 a)	1621 (1.59 ± 0.31 a)	1609 (1.15 ± 0.17 a)	1619 (3.82 ± 0.31 c)	1615 (1.69 ± 0.32 a)	1609 (2.55 ± 0.44 b)
β-sheet	1622 (1.76 ± 0.45 a)	1622 (3.33 ± 0.32 b)	1626 (1.64 ± 0.38 a)	1616 (1.51 ± 0.48 a)	1627 (1.02 ± 0.18 a)	1620 (1.58 ± 0.25 a)	1621 (3.11 ± 0.29 b)
	1632 (3.19 ± 0.31 c)	1630 (3.03 ± 0.38 c)	1633 (3.77 ± 0.40 c)	1621 (1.57 ± 0.39 ab)	1632 (2.04 ± 0.46 b)	1628 (1.26 ± 0.33 a)	1628 (2.15 ± 0.32 b)
turn	1638 (5.11 ± 0.46 d)	1637 (6.88 ± 0.44 e)	1639 (5.41 ± 0.81 d)	1636 (2.10 ± 0.27 a)	1638 (3.30 ± 0.38 bc)	1635 (2.71 ± 0.43 ab)	1635 (4.10 ± 0.37 c)
random	1646 (9.02 ± 0.91 c)	1645 (7.97 ± 1.20 bc)	1646 (7.83 ± 0.89 bc)	1645 (5.04 ± 0.89 a)	1645 (8.44 ± 0.99 c)	1642 (6.35 ± 0.91 a)	1644 (5.90 ± 0.51 a)
α-helix	1652 (13.88 ± 0.84 d)	1652 (12.26 ± 1.81 cd)	1652 (12.24 ± 1.99 cd)	1651 (9.25 ± 0.83 ab)	1652 (10.83 ± 1.54 abc)	1650 (11.42 ± 1.52 bcd)	1651 (8.40 ± 0.58 a)
	1659 (17.91 ± 1.40 bc)	1658 (16.99 ± 0.99 bc)	1658 (15.88 ± 1.75 ab)	1657 (15.18 ± 1.53 ab)	1658 (18.99 ± 1.41 c)	1656 (15.65 ± 1.52 ab)	1656 (14.41 ± 1.73 a)
β-sheet	1666 (14.49 ± 1.84 ab)	1665 (13.01 ± 1.76 a)	1665 (13.61 ± 1.75 a)	1665 (17.61 ± 1.47 b)	1664 (14.01 ± 1.48 a)	1663 (15.81 ± 1.43 ab)	1663 (14.57 ± 0.68 a)
	1673 (12.34 ± 1.80 ab)	1672 (13.50 ± 1.03 abc)	1672 (15.81 ± 1.91 c)	1672 (20.04 ± 1.48 e)	1671 (14.13 ± 1.39 abc)	1670 (16.61 ± 2.27 cd)	1670 (19.20 ± 1.92 de)
turn	1681 (8.34 ± 1.54 a)	1679 (10.16 ± 1.13 abc)	1678 (9.78 ± 1.63 ab)	1679 (13.64 ± 1.98 d)	1677 (9.91 ± 1.00 ab)	1676 (13.23 ± 2.17 cd)	1678 (10.01 ± 1.56 ab)
	1689 (4.93 ± 0.75 a)	1686 (5.22 ± 0.84 ab)	1685 (6.70 ± 1.28 b)	1687 (7.35 ± 0.66 b)	1684 (6.57 ± 1.24 b)	1684 (9.37 ± 1.43 c)	1684 (9.70 ± 0.84 c)
	1696 (0.86 ± 0.18 a)	1693 (3.87 ± 0.84 b)	1693 (3.18 ± 1.01 b)	1693 (3.91 ± 0.59 b)	1690 (4.25 ± 0.54 b)	1691 (3.64 ± 0.78 b)	1693 (4.40 ± 0.48 b)
α-helix	31.79 ± 1.44 d	29.25 ± 0.85 cd	28.12 ± 0.40 bc	24.43 ± 0.73 a	29.82 ± 2.46 cd	27.07 ± 1.67 bc	22.81 ± 1.71 a
β-sheet	31.78 ± 1.55 a	32.87 ± 1.22 ab	34.83 ± 0.41 bc	40.73 ± 0.88 d	31.20 ± 1.45 a	35.26 ± 0.93 c	39.03 ± 0.97 d
turn	18.24 ± 1.01 a	26.13 ± 1.03 bc	25.07 ± 0.76 bc	27.00 ± 1.78 bc	24.03 ± 1.16 b	28.95 ± 1.55 c	28.21 ± 2.38 c
random	9.02 ± 0.91 d	7.97 ± 1.20 bcd	7.83 ± 0.89 bcd	5.04 ± 0.89 a	8.44 ± 0.99 cd	6.35 ± 0.91 abc	5.90 ± 0.51 ab
other	8.17 ± 0.56 d	3.78 ± 0.61 b	4.15 ± 0.12 b	2.80 ± 0.67 a	6.51 ± 0.59 c	2.37 ± 0.14 a	4.05 ± 0.64 b

[a] Susi and Byler[18] and Iconomidou et al.[32]. α-Helix, band at 1650−1652 cm^{-1}; β-sheet, bands at 1620−1627, 1628−1632, 1663−1665, and 1670−1673 cm^{-1}; turns, band at 1635−1639 cm^{-1}; random coil, band at 1642−1646 cm^{-1}; other (amino acid side chains), bands at 1604−1607 and 1609−1619 cm^{-1}; 6 h, 1621 cm^{-1} attributed to amino acid side chains; and 18 h, 1616 cm^{-1} attributed to β-sheet.

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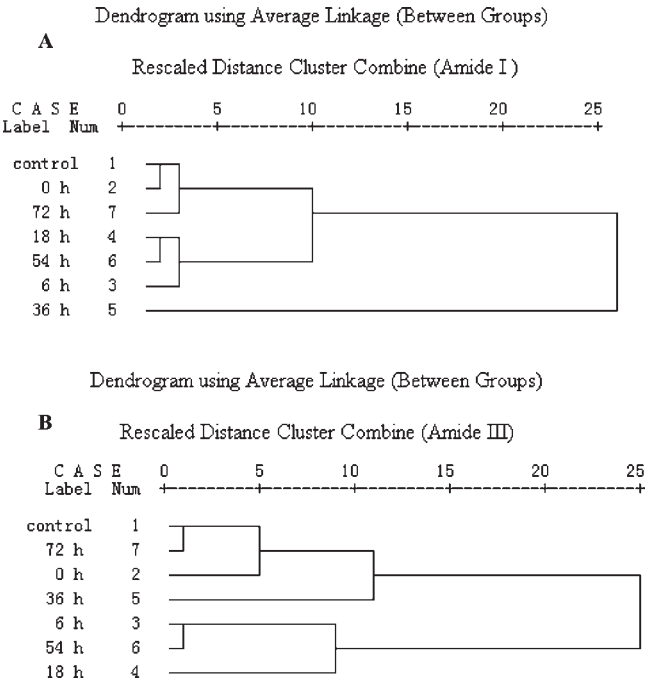

Figure 3. Dendrograms of amides I (1600−1700 cm^{-1}) and III (1220−1275 cm^{-1}) of myofibrillar proteins from raw muscle and Cantonese sausage at different processing periods (A, amide I; B, amide III).

one can calculate the distance between this point and the other points, thereby establishing a matrix that describes the proximity between all of the samples studied. Thus, hierarchical cluster analysis is a simple way of grouping the samples studied through similarity to evaluate the influence of salting and processing on the structural changes of myofibrillar proteins of Cantonese sausage.

Results of hierarchical cluster analysis for amide I are present in Figure 3A. Three clusters were observed in the dendrogram. The first cluster consisted of the three samples of control, zero hour, and 72nd hour. The second cluster consisted of the three samples of the 6th, 18th, and 54th hours. The third cluster is the sample of the 36th hour. It could be seen that control and zeroth hour were clustered together first, and then, the 18th and 54th hours were clustered. These indicated that they had a great proximity. Then, the 72nd and 6th hours were successively clustered into the group of control and 54th hour, respectively. The sample of the 36th hour was separated individually in the dendrogram from the other samples. These results further confirmed the rebuilding process of myofibrillar proteins during the processing of Cantonese sausage.

Amide III. *Analysis of Amide III Spectra Profile.* The −CO−NH− amide or peptide bond has several distinct vibrational modes, with the amide I band near 1650 cm^{-1} and the amide III band near 1250 cm^{-1}. The exact location of these bands depends on the secondary structure of the polypeptide chain. Because of possible overlap of bands from miscellaneous C−H bending and aromatic ring vibrations in the amide III region, it is recommended that both regions are analyzed to obtain a more reliable interpretation of the changes in the secondary structure of proteins.[11,12]

This spectroscopic Raman band of amide III involves C−N stretching and N−H in-plane bending vibrations of the peptide bond as well as contributions from Cα−C stretching and C═O

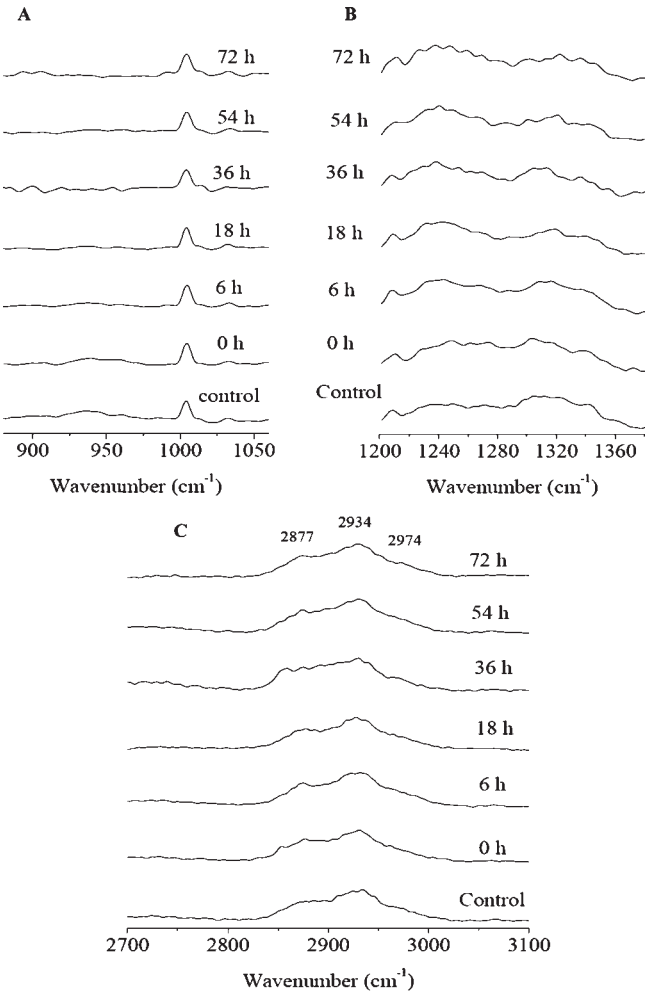

Figure 4. Raman spectra of myofibrillar proteins from raw muscle and Cantonese sausage at different processing periods. (A) The wavenumber range of 890−1100 cm^{-1}, (B) the amide III (1220−1275 cm^{-1}), and (C) the wavenumber range of 2700−3100 cm^{-1}.

in-plane bending. The amide III band is difficult to interpret by the fact that vibrational spectroscopy of proteins produces a complex pattern of bands in the 1225−1350 cm^{-1} range (Figure 4B). This is due to the fact that the characteristic frequency ranges for β-sheet (1230−1245 cm^{-1}) and random coil (1240−1255 cm^{-1}) overlap to some extent.[26] Bands near 1250 and 1308 cm^{-1} in the myofibrillar proteins from Cantonese sausage (Figure 4B) are similar to those reported at 1252 and 1304 cm^{-1} for Pacific cod myosin[20] and 1265 and 1304 cm^{-1} for myosin.[14] These two features have been attributed to the α-helical structures in the globular and fibrous conformations of the myosin head and tail regions, respectively.[14] A slight decreasing intensity except for the 36th hour was observed at the band near 1308 cm^{-1} during processing of Cantonese sausage (Figure 4B), indicating that the α-helical structure decreased. The β-sheet structures usually lead to a more intense band near 1230−1245 cm^{-1}, while random coil structures appear near 1250 cm^{-1}. There is a slight intensity increase except for the 36th hour observed near 1230−1245 cm^{-1} during the processing of Cantonese sausage (Figure 4B), suggesting that the β-sheet structure increased. Besides, the weaker shoulder band at about 1232 cm^{-1} was observed during processing except at the 36th hour and control, which was

Table 2. Normalized Intensities of the Tryptophan Bands (757 and 1340 cm^{-1}), Tyrosyl Doublet (850/830 cm^{-1}), CC Band (940 cm^{-1}), and CH Band (2934 cm^{-1}) of Myofibrillar Proteins from Cantonese Sausage[a]

	I_{850}/I_{830} (cm^{-1})	I_{940}/I_{1004} (cm^{-1})	I_{757}/I_{1004} (cm^{-1})	I_{1340}/I_{1004} (cm^{-1})	I_{2934}/I_{1004}(cm^{-1})
control	1.32 ± 0.16 a	0.50 ± 0.07 a	0.87 ± 0.06 bc	0.38 ± 0.06 a	0.19 ± 0.03 b
0 h	1.27 ± 0.20 a	0.26 ± 0.06 b	0.82 ± 0.12 bc	0.36 ± 0.06 a	0.19 ± 0.02 b
6 h	0.94 ± 0.16 b	0.19 ± 0.04 bc	0.86 ± 0.06 bc	0.30 ± 0.06 ab	0.19 ± 0.03 b
18 h	0.93 ± 0.11 b	0.17 ± 0.05 bc	0.99 ± 0.07 b	0.27 ± 0.04 ab	0.19 ± 0.03 b
36 h	0.87 ± 0.10 b	0.14 ± 0.05 bc	1.29 ± 0.12 a	0.27 ± 0.03 ab	0.25 ± 0.04 a
54 h	0.92 ± 0.11 b	0.18 ± 0.04 bc	0.67 ± 0.11 c	0.22 ± 0.02 b	0.18 ± 0.04 b
72 h	0.85 ± 0.08 b	0.10 ± 0.03 c	0.65 ± 0.12 c	0.24 ± 0.04 b	0.17 ± 0.04 b

[a] Values in a column followed by different letters are significantly different ($p < 0.05$).

considered to be related to the increase in parallel β-sheet content.[20] Raman spectra of myofibrillar proteins at the 36th hour were different to the other periods. It further confirmed the rebuilding process of Cantonese sausage protein during processing. The results from amide III were consistent with data from amide I Raman band.

Hierarchical Cluster Analysis of Amide III. A dendrogram of amide III of myofibrillar proteins from raw muscle and Cantonese sausage at different processing periods is shown in Figure 3B. It also could be divided into three clusters, the same as amide I. It was interesting to observe that the control and 72nd hour clustered together first, and then, the 6th and 54th hours clustered. Then, zeroth and 18th hours successively clustered into the group of control and 54th hours, respectively. These discrepancies to amide I might be due to distinct vibrational modes of the polypeptide chain. Amide I vibrational mode involves mainly C═O stretching vibrations and, partly, C−N stretching, Cα−C−N bending, and N−H in-plane bending of peptide groups, while amide III mode involves mainly C−N stretching and N−H in-plane bending vibrations of the peptide bond as well as contributions from Cα−C stretching and C═O in-plane bending.[11,27]

C−C Stretching Vibrations. Another way of looking at the secondary structure is the use of the C−C stretching vibrations in 890−1060 cm^{-1}. The results showed an intensity decrease trend in the Raman band near 940 cm^{-1} during processing (Table 2). Gradual loss of α-helix structure leads to broadening and weakening in the intensity of this band.[15] The changes in the spectrum profile were in agreement with this theory (Figure 4A). However, the obvious shoulder band at 900 cm^{-1} at the 36th hour was detected. The decline of the 900 cm^{-1} band intensities suggested that the α-helices are dissociated to some extent by heat treatment at 40 °C.[26] This indicated the α-helix increased during 18−36 h. These results were consistent with data from amide I Raman band.

Tryptophan Residues and Tyrosine (Tyr) Doublet Bands. Raman bands at 757 and 1340 cm^{-1} display information about the microenvironment of the tryptophan (Trp) residues. The intensity of the bands near 1340 cm^{-1} slightly decreased ($p < 0.05$) during Cantonese sausage processing (Table 2), which perhaps indicated the exposure of Trp residues. Similar findings were reported by Bouraoui, Nakai, and Li-Chan.[20] However, the I_{757}/I_{1004} showed no significant difference ($p > 0.05$) during 18 h, then increased to the maximum of 1.29 at the 36th hour, and decreased during the final phase of processing. It should be noted that the overlap of Trp band at 757 cm^{-1} with that at 755 cm^{-1} was assigned to aliphatic residues.[28]

The intensity ratio I_{850}/I_{830} was determined for the Tyr doublet at 830 and 850 cm^{-1}. It has been suggested by Yu, Jo, and O'Shea[29] that the ratio of the Tyr ring vibrations at 850 and 830 cm^{-1} (R_{Tyr}) reflects "buried" and "exposed" Tyr groups. $R_{Tyr} \geq 1$ in the myosin Raman spectrum is indicative of Tyr residues exposed on the protein surface, which interact with water molecules as a hydrogen bond donor and acceptor. If R_{Tyr} falls between 0.7 and 1.0, the Tyr residues are considered "buried". The intensity ratios I_{850}/I_{830} of control and zeroth hour were 1.32 and 1.27, respectively, suggesting that the Tyr residues of these samples were mainly exposed and able to participate in the formation of moderate or weak hydrogen bonds. During the processing of Cantonese sausage, the intensity ratios I_{850}/I_{830} decreased significantly ($p < 0.05$) to 0.85−0.94, suggesting that some Tyr residues became buried in a more hydrophobic environment. This result was in agreement with the report of Ogawa et al.,[30] who also found the tyrosyl doublet ratio had showed a decrease as an effect of heat process on lemon sole (*Microstomus kitt*) actomyosin. In the present work, the Tyr residues were less exposed to the surface during processing, probably because of intermolecular interactions during protein network formation.[31] However, Herrero et al.[9] have reported that the Tyr residues are buried in a hydrophobic environment independently of thermal treatment and NaCl content.

Aliphatic C−H Stretching Vibrations. Raman spectra in the wavenumber range of 2800−3000 cm^{-1} are assigned to C−H stretching bands. Myofibrillar proteins of control showed a distinct peak at 2885 cm^{-1} attributable to the CH_2 asymmetric stretch, along with a discernible shoulder at 2934 cm^{-1} arising from the CH_3 symmetric and/or CH_2 asymmetric stretch.[30] During processing, these two peaks shifted slightly toward lower frequencies of 2930 and 2874 cm^{-1}, and a small shoulder appeared at 2860 cm^{-1} due to CH_2 symmetric stretch (Figure 4C). Similar findings have been reported by Ogawa et al.[30] Careche and Li-Chan[12] have reported that a shift to higher wavenumbers in the C−H stretching band at 2933 cm^{-1} is observed after frozen denaturation of cod myosin, and it stems from enhanced exposure of the aliphatic residues to the aqueous environment. Ogawa et al.[30] have pointed out that the observed peak shift toward lower frequencies in the 2855−2940 cm^{-1} region suggests that the microenvironment of aliphatic side chains is changed upon heating, although it is not confirmed whether the changes can be interpreted as enhanced exposure.

The intensity of the band near 2930 cm^{-1} kept constantly until the 36th hour, then increasing to the maximum of 0.25, and thereafter decreasing at the rest stage. In addition, an increase of the β-sheet structure during 36−72 h could be observed (Table 1). It is well-known that the β-sheet structure is comprised of characteristic hydrogen bonds involving the polypeptide backbone. Therefore, Herrero et al.[9] have assumed that

thermal treatment and salting induce mainly rearrangement of hydrogen bonds resulting in β-sheet formation. Cofrades and Jiménez-Colmenero[3] have found that the molecular associations involved in gel network formation during the elaboration in frankfurter comprise β-sheet hydrogen-bonding and hydrophobic interactions, which are equally involved in protein denaturation and aggregation. Therefore, changes in the intensity of the band near 2930 cm^{-1} during processing might be due to salting and heat treatment. These results further confirmed the rebuilding behavior of myofibrillar proteins during final stage.

In conclusion, this investigation showed that Raman spectroscopy was a useful technique to evaluate the protein structural changes during Cantonese sausage processing. Decreases in the α-helix structure accompanied by increases in β-sheets were observed during 0−18 and 36−72 h. The discrepancies at the 36th hour indicated that a rebuilding process of secondary structures occurred at the rest stage. Analysis of amide III and C−C stretching vibrations in 890−1060 cm^{-1} confirmed it. The hierarchical cluster analysis results of amides I and III and the wavenumber range of 2855−2940 cm^{-1} further confirmed this rebuilding process. Changes in a doublet near 850 and 830 cm^{-1} suggested that some Tyr residues became buried in a more hydrophobic environment. Results from aliphatic C−H stretching vibrations showed that the microenvironment of aliphatic side chains might be changed during final stage.

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