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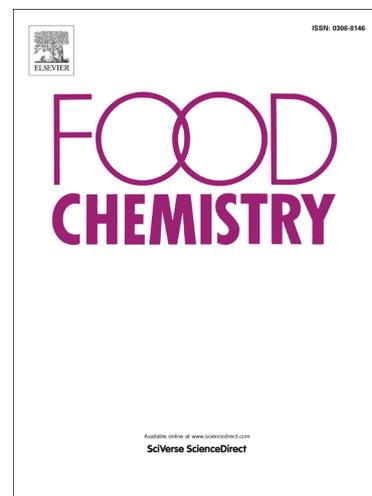
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**Changes in the myosin secondary structure and shrimp surimi gel strength  
induced by dense phase carbon dioxide**

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**ABSTRACT**

Dense phase carbon dioxide (DPCD) could induce protein conformation changes. Myosin and shrimp surimi from *Litopenaeus vannamei* were treated with DPCD at 5-25 MPa and 40-60 °C for 20 min. Myosin secondary structure was investigated by circular dichroism and shrimp surimi gel strength was determined using textural analysis to develop correlations between them. DPCD had a greater effect on secondary structure and gel strength than heating. With increasing pressure and temperature, the  $\alpha$ -helix content of DPCD-treated myosin decreased, while the  $\beta$ -sheet,  $\beta$ -turn and random coil contents increased, and the shrimp surimi gel strength increased. The  $\alpha$ -helix content was negatively correlated with gel strength, while the  $\beta$ -sheet,  $\beta$ -turn and random coil contents were positively correlated with gel strength. Therefore, when DPCD induced myosin to form a gel, the  $\alpha$ -helix of myosin was unfolded and gradually converted to a  $\beta$ -sheet. Such transformations led to protein-protein interactions and cross-linking, which formed a three-dimensional network to enhance the gel strength.

**Key words:** Dense phase carbon dioxide; Secondary structure; Gel strength; Myosin; Shrimp surimi

## 1. Introduction

Dense phase carbon dioxide (DPCD), also known as high-pressure carbon dioxide (HPCD) treatment, is a promising non-thermal food processing technology that uses pressurized CO<sub>2</sub> below 50 MPa and a treatment temperature below 60 °C. Initially, DPCD was mainly applied to inactivate microorganisms and enzymes in food (Ferrentino & Spilimbergo, 2011; Hu, Zhou, Xu, Zhang, & Liao, 2013; Liu, Guo, Liu, Liu, & Deng, 2016). However, DPCD has an impact on food qualities such as flavour, texture and colour (Zhou, Bi, Xu, Yang, & Liao, 2015). At present, there are some reports demonstrating that DPCD can induce food protein conformation changes and denaturation (Qu, Zhang, Rao, Li, & Li, 2010; Qu et al., 2012; Floren, Spilimbergo, Motta, & Migliaresi, 2012; Fernandes-Silva et al., 2013; Szerman, Rao, Li, Yang, Vaudagna, & Zhang, 2015 ; Yan, Xu, Jia, Dai, & Li, 2016), which can provide a new method for preparing protein gel products.

Gel formation is one of the most important functional properties of food protein. Protein gelation is the process of forming a network structure after protein denaturation and aggregation (Ferry, 1948; Kinsella, & Melachouris, 1976). The change in protein conformation is a prerequisite for the aggregation of protein molecules (Ferry, 1948). Although protein gelation can be induced by heat, high pressure, presence of ions, acid, urea or enzymes, the mechanism of gel network formation in every method can differ considerably (Asghar, Samejima, & Yasui, 1985; Messens, Van Camp, & Huyghebaert, 1997; Colmenero, 2002). These physical and chemical means to induce protein gelation are listed in Table 1 (Totosaus, Montejano, Salazar, & Guerrero, 2002).

In recent years, it has been reported that DPCD can induce protein into forming a gel with better qualities under suitable conditions. For example, minced mutton gel induced by DPCD

was much denser with a greater hardness and elasticity than that obtained with heat treatment (Qu, Zhang, Rao, Li, & Li, 2010). Furthermore, it was revealed that the  $\alpha$ -helix content of myosin decreased, and the  $\beta$ -sheet content increased in the gelation process, which could promote protein-protein interaction to form a dense network structure (Qu, Zhang, Rao, Li, & Li, 2010). The silk protein gel induced by DPCD had more porosity, less swelling and better mechanical properties than those obtained by citric acid (Floren, Spilimbergo, Motta, & Migliaresi, 2012). Furthermore, cross-linked three-dimensional hydrogels of marine collagen extracted from shark skin was successfully developed using DPCD at room temperature and 55 bar for 16 h while the conventional cross-linking reaction took 7 days under atmospheric conditions (Fernandes-Silva et al., 2013). DPCD treatment can also modify lamb meat proteins and lead to weak protein-protein interactions and the formation of gel-like structures (Szerman, Rao, Li, Yang, Vaudagna, & Zhang, 2015). When compared with sausage protein from sheep *longissimus dorsi* muscle treated by heating (50 or 60 °C, 15 min), DPCD treatment (10 MPa, 50 or 60 °C, 15 min) increased the hardness, elasticity and weight loss but reduced the redness of the sausages. This was a result of decreased protein solubility and a change in the myosin secondary structure (Rao et al., 2016).

In our previous study, it was also found that DPCD can induce shrimp surimi to form a gel (Qu et al., 2012). Compared with the gel induced by heating, the gel induced by DPCD had higher strength and water-holding capacity and retained more nutrient components (Qu et al., 2012). This work was aimed to explore the effects of DPCD treatment on myosin secondary structure using circular dichroism (CD) and shrimp surimi gel strength using a texture profile analyser and to discuss their correlations.

## 2. Materials and methods

### 2.1. Materials

Live *Litopenaeus vannamei* with an average size of 55–60 shrimp/kg was purchased from the Dongfeng seafood wholesale market in Zhanjiang, China. The heads, shells and intestinal glands of shrimps were removed by hand. Shrimp was washed for 10 s with cold water and minced (HR2105-90 blender, Philips (China) Investment Co., Ltd.) for 30 s.

All chemicals used in this work were of analytical grade. The Lowry Protein Assay Kit was purchased from Shanghai Labaide Biotechnology Company (Shanghai, China). 5-Triphosphate adenosine disodium (ATP), dithiothreitol (DTT) and bovine serum albumin were purchased from Guangzhou Qiyun Biotechnology Company (Guangzhou, Guangdong Province, China). The purity of CO<sub>2</sub> was 99.9%, which was purchased from Zhanjiang Oxygen Corporation (Zhanjiang, Guangdong Province, China). Other chemicals were purchased from Guangzhou Chemical Reagents Company (Guangzhou, Guangdong Province, China).

### 2.2. Myosin extraction

Myosin was extracted from shrimp meat using Liu's method (Liu et al., 2010), with some modifications. The tri-HCl buffer and  $\beta$ -mercaptoethanol in Liu's method were replaced by phosphate buffer and DTT in this experiment, respectively. All of the steps were performed at 4 °C to minimize proteolysis and protein denaturation. The shrimp mince (100 g) was extracted with 500 ml of 20 mM sodium phosphate buffer (pH 7.0) with constant agitation using a magnetic stirrer (RTC basic, IKA Works GmbH & Co.) for 10 min. The suspension was centrifuged at 5500  $\times$  g for 10 min at 4 °C using a refrigerated centrifuge (Avanti J-26 XP Centrifuge; Beckman Coulter, Fullerton, CA, USA). The procedure was performed 3 times and the supernatant was discarded. The precipitate was extracted with 300 ml of 5 mM

ATP solution (pH 6.4) containing 0.45 M KCl, 7.5 mM MgCl<sub>2</sub> and 0.15 mM DTT and the mixture was stirred slowly with a magnetic stirrer for 15 min at 4 °C. The solution was centrifuged at 10,000 × g for 10 min at 4 °C. The solution was precipitated by adding 900 ml of cold distilled water and was centrifuged at 6000 × g for 10 min at 4 °C. The precipitate was dissolved in one-fifth volume of 0.12 M Tris-maleate (pH 7.5), containing 3 M KCl and 0.6 mM DTT. The mixture was placed at 4 °C for 12 h or overnight. Next, the mixture was dissolved in one-tenth volume of 110 mM ATP (pH 7.5) containing 55 mM MgCl<sub>2</sub> and 5.5 mM ethyleneglycol bis(2-aminoethylether)tetraacetic acid (EGTA) and was placed at 4 °C for 2 h. Thereafter, 40-45% saturated ammonium sulfate was added to the mixture, which was centrifuged at 6000 × g for 10 min at 4 °C. The precipitate was dissolved in 20 mM sodium phosphate buffer (pH 7.0) containing 0.6 M KCl and was exhaustively dialysed using the same buffer solution. The solution after dialysis was centrifuged at 6000 × g and 4 °C for 10 min. The supernatant was taken as the purified shrimp myosin. The concentration of the myosin solution was 12-16 mg/ml determined by the Lowery method (Lowry, Rosebrough, & Randall, 1951), using bovine serum albumin as the standard. The myosin purity was checked using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The myosin purity in this work was greater than 95% as determined by densitometry (ChemiDoc XRS<sup>+</sup> system, Bio-Rad, USA). The myosin solution was stored at 4 °C and was used within three days. It was ensured that its electrophoretic pattern was not changed during the storage period.

### *2.3. Dense phase carbon dioxide treatment*

The DPCD system was described by Zhang (Zhang, Liu, Zhang, Ji, Gao, & Deng, 2011). The 1 litre stainless steel pressure vessel (DPCD equipment) was manufactured by Nantong Huaan Supercritical Extraction Co. Ltd. (Nantong, China).

For each experiment, a 5 ml sample of myosin solution (0.1mg/ml) was placed in a 10 ml plastic centrifuge tube. The tube was placed in the pressure vessel. After the vessel was preheated to the set temperature, the air in the vessel was evacuated by opening the CO<sub>2</sub> inlet valve for approximately 2 min. The vessel was pressurized by the plunger pump for approximately 3 min until the pressure reached the experimental setting. Next, the myosin in the vessel was held at constant pressure at equilibrium temperature during DPCD treatment. After 20 min, the vessel was slowly depressurized for 3-5 min.

For the temperature and pressure, a two-factor repeated experimental design was used. The temperatures tested were 40, 50 and 60 °C, while the pressure levels were 5, 10, 15 and 25 MPa, respectively. Three control groups were set: untreated myosin (C1), heat treatment in a water bath (C2), and 0.1-MPa CO<sub>2</sub> treatment, which used pure CO<sub>2</sub> at 0.1 MPa. The temperatures in the second and third control groups were 40, 50 and 60 °C, with a treatment time of 20 min. All of the experiments were performed in triplicate.

#### 2.4. Circular dichroism spectra analysis

Changes in the secondary structure were monitored using a Chirascan circular dichroism (CD) spectropolarimeter (plus qCD; Applied Photophysics Ltd., England). CD spectra were scanned at the far UV range from 200 to 260 nm, using a quartz cuvette of 0.1-cm light path length at 4 °C. The resolution ratio was set at 0.5 nm, while the scanning speed was 100 nm/min, and the sensitivity and response were 20 mdeg and 0.25 s, respectively. The myosin concentration for CD was 0.1 mg/ml, and the mean residue molecular weight of protein was 115 g/mol. All of the spectra measured were baseline corrected using the phosphate buffer. Measurements were carried out after treatment in triplicate.

The mean residue ellipticity [ $\theta_\lambda$ ] is the most commonly reported unit of the CD data and

the equation is given by:

$$[\theta]_{\lambda} = \frac{\theta_{\lambda} \times MRW}{10 \times d \times c}$$

where  $\theta_{\lambda}$  is the observed ellipticity (degrees) at wavelength  $\lambda$ ,  $d$  is the path length (cm),  $c$  is the protein concentration (g/ml), and  $MRW$  is the mean residue weight (115 g/mol) (Kelly, Jess & Price, 2005). The units of  $[\theta_{\lambda}]$  are  $\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ .

The secondary structure content of myosin was determined using the SELCON3 program of the CDPro package (<http://sites.bmb.colostate.edu/sreeram/CDPro/>). In SELCON3, the  $\alpha$ -helix and  $\beta$ -sheet conformations in the globular protein structures are divided into regular and distorted fractions, by considering a certain number of terminal residues in a given helical or strand segment to be distorted. The number of  $\alpha$ -helical and  $\beta$ -strand segments and their average length in a given protein are estimated from the fraction of distorted helical and strand conformations relative to the total helix and strand contents. The main advantage of this method is that it provides very good estimates of the structure of globular proteins (Greenfield, 2006; Sreerama, & Woody, 2000).

### 2.5. Preparation of shrimp surimi

Shrimp surimi was prepared according to Qu's method (Qu et al., 2012). The heads, shells and intestinal glands of fresh shrimp were removed, and the shrimp meat was washed with cold water (4 °C), ground and homogenized. The shrimp surimi was washed for 15 min with 5 volumes of cold water. The washing was repeated 3 times. Next, the shrimp surimi was wrapped with gauze and squeezed by hand to dehydrate it. The content of water in the shrimp surimi was 78~81 wt%. The shrimp surimi was blended with 3% salt, and chopped for 15 min before being stuffed into collagen casing (20 mm diameter); both ends were then sealed flatly and smoothly. The shrimp sausages were treated with DPCD and were cooled to 4 °C

immediately. The experimental design of DPCD-treated sausages is the same as that of DPCD-treated myosin. The shrimp sausages were placed at 4 °C and were stored for 12 h, and then the gel strength was determined. The content of water in the shrimp sausages was 71-73 wt%.

### 2.6. Gel strength analysis

The gel strength was analysed using the Texture Profile Analyzer (TMS-PRO, Food Technology Corporation, Virginia, USA) according to Qu's method (Qu et al., 2012). The gel was subjected to a compression test at a trigger-type button. The cylindrical probe with a 5-mm diameter was used to penetrate the sample at 20 mm. The trigger force was 0.5 N, with a test speed of 60 mm/min. The sample deformation was 60% and the gel strength (N×mm) was equal to the breaking strength (N) multiplied by the breaking distance (mm).

### 2.7. Statistical analysis

Analyses of variance (ANOVA), multiple comparisons by Tukey's HSD method and correlation analysis were carried out using JMP software (Version 10, SAS Institute Inc., Cary, North Carolina, USA). All of the data were represented as mean±S.D. All of the experiments were performed in triplicate.

## 3. Results and discussion

### 3.1. Changes in myosin CD spectra

The  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turn, and random coil are the four principal types of secondary structures of protein. CD is recognized as an excellent method to determine the secondary structure of proteins in solution. Each structure has its distinctive characteristic CD spectra. For example,  $\alpha$ -helical proteins have negative bands at 222 nm and 208 nm and a positive band at 193 nm (Greenfield, 2006);  $\beta$ -sheet proteins have negative bands at 218 nm and

positive bands at 195 nm (Greenfield, 2006); the  $\beta$ -turn structure has a positive peak at 206 nm and a strong negative peak at 180-190 nm (Chang, Wu, & Yang, 1978); disordered proteins have very low ellipticity above 210 nm and negative bands near 195 nm (Greenfield, 2006). However, the absorption peak position of secondary structure may be slightly changed due to the influence of different factors, such as protein species, protein concentration, buffer system, and instrument parameters (Kelly, Jess, & Price, 2005).

The changes in the CD spectra of DPCD-treated myosin are presented in Figure 1. The figure shows that untreated myosin had two negative bands at 222 nm and 208 nm, indicating that myosin from *Litopenaeus vannamei* was a protein rich in  $\alpha$ -helix conformation. At 40 °C (Figure 1A), the CD intensities of heat treatment (C2) and 0.1 MPa CO<sub>2</sub> treatment were similar to those of untreated myosin (C1). At 50 °C (Figure 1B), the CD intensities of C2 and 0.1 MPa were slightly higher than those of C1, but the CD intensities of C2 were similar to those of 0.1 MPa. At 60 °C (Figure 1C), the CD intensities of C2 were slightly higher than those of C1 and the CD intensities of 0.1 MPa were slightly higher than that of C2. The results indicated that heating at 0.1 MPa CO<sub>2</sub> had a significant effect on the CD intensities of myosin, and the CD intensities were slightly increased with increasing temperature, which could be due to a weak interaction between CO<sub>2</sub> and myosin at 60 °C. Compared with the three control groups, the CD intensities of DPCD-treated myosin were increased significantly, and the intensities were increased with increasing pressure and temperature. Therefore, DPCD treatment has significantly changed the myosin secondary structure; however, the greater the pressure (5-25 MPa) and temperature (40-60 °C) are, the greater the structural change will be.

### 3.2. Changes in myosin secondary structure

Secondary structural changes in myosin induced by DPCD are presented in Figure 2. It

shows that the  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turn and random coil contents of untreated myosin from *Litopenaeus vannamei* were  $58.12\pm 0.94\%$ ,  $5.85\pm 0.33\%$ ,  $13.83\pm 0.13\%$  and  $13.83\pm 0.13\%$ , respectively. Compared with untreated myosin, the  $\alpha$ -helix content of myosin treated by heating in a water bath had a declining trend with increasing temperature from 40 °C to 60 °C whereas the  $\beta$ -sheet content had an increasing trend, and the  $\beta$ -turn and random coil contents changed only slightly. Although these changes in the secondary structure were not significant ( $P>0.05$ ), the change in the trends were consistent with the results of many previous studies. For example, the  $\alpha$ -helix content of rabbit myosin was decreased significantly from 40 °C to 70 °C, the  $\beta$ -sheet content was increased significantly from 40 °C to 60 °C, and the random coil content of was increased from 40 °C to 80 °C (Han, Wu, Wang, Xu, & Zhou, 2015). Furthermore, the  $\alpha$ -helix content of porcine myofibrillar proteins was markedly decreased and the  $\beta$ -sheet,  $\beta$ -turn and random coil fraction were increased from 30 °C to 70 °C (Xu, Han, Fei, & Zhou, 2011). The  $\alpha$ -helix fraction of myosin from silver carp was gradually decreased from 5 °C to 90 °C, the  $\beta$ -sheet and  $\beta$ -turn fractions were considerably higher in the range of 35-90 °C than those in the range of 5-30 °C, and the random coil fraction tended to increase with increasing temperature (Liu et al., 2010). The  $\alpha$ -helix content of porcine myosin was decreased from 5 °C to 90 °C at pH=7.0, the  $\beta$ -sheet fraction was considerably higher in the range of 55-90 °C than in the range of 5-50 °C, the  $\beta$ -turn fraction exhibited a slight decrease above 70 °C, and the random coil fraction tended to increase with increasing temperature (Liu, Zhao, Xiong, Xie, & Qin, 2008). A decrease in the  $\alpha$ -helix content and increase in the  $\beta$ -sheet content have also been observed during the thermal gelling of Pacific whiting surimi (Bouraoui, Nakai, & Li, 1997). Although there are some differences in the changes of the  $\beta$ -turn and random coil, in general, the  $\alpha$ -helix of myosin was gradually decreased with

increasing temperature, whereas the  $\beta$ -sheet fraction was increased. This indicated that the formation of the  $\beta$ -sheet occurred simultaneously with the unfolding of the  $\alpha$ -helix during the gelation process (Liu, Zhao, Xiong, Xie, & Qin, 2008). The differences among these studied results may be because myosin was from different species, and its secondary structure was different.

Comparing untreated and heat-treated myosin, the secondary structure content of myosin treated with CO<sub>2</sub> at 0.1 MPa and 40-60 °C had minor changes, but these changes were not significant ( $P>0.05$ ). The reason may be that CO<sub>2</sub> combines with water to form carbonic acid at 0.1 MPa, which may have a buffering effect and, hence, a protective effect on myosin. However, compared with the three control groups, the  $\alpha$ -helix content of DPCD-treated myosin was decreased ( $P<0.05$ ); the  $\beta$ -sheet,  $\beta$ -turn and random coil contents were increased ( $P<0.05$ ). Therefore, DPCD treatment had a greater effect on the secondary structure of myosin than heat treatment at the same temperature. This indicates that CO<sub>2</sub> interacts with myosin under a specific pressure and temperature.

As seen in Figure 2, at a specific temperature, the  $\alpha$ -helix content of DPCD-treated myosin was decreased significantly ( $P<0.05$ ). The  $\beta$ -sheet and  $\beta$ -turn contents were increased ( $P<0.05$ ) with increasing pressure, while the pressure of DPCD had no significant effect on the random coil content of myosin ( $P>0.05$ ). This could be because the solubility of CO<sub>2</sub> in solution increases with increasing pressure (for example, the solubility of CO<sub>2</sub> in water increases from 9.71 dm<sup>3</sup>/kg to 25.63 dm<sup>3</sup>/kg when the pressure increases from 2.5 MPa to 10 MPa at 50 °C (IUPAC, 2016)). Therefore, myosin can interact with more CO<sub>2</sub> molecules. In addition, the pH of the solution is reduced with increasing pressure (Hu, Zhou, Xu, Zhang, & Liao, 2013), also leading to changes in protein conformation. Furthermore, as shown in

Figure 2, at the same pressure, the  $\alpha$ -helix content of DPCD-treated myosin was decreased significantly ( $P<0.05$ ) with increasing temperature, while the  $\beta$ -sheet,  $\beta$ -turn and random coil contents were increased ( $P<0.05$ ). At a constant pressure, the solubility of  $\text{CO}_2$  in solution decreases with increasing temperature (for example, the solubility of  $\text{CO}_2$  in water decreases from  $27.81 \text{ dm}^3/\text{kg}$  to  $23.95 \text{ dm}^3/\text{kg}$  with increasing temperature from  $40 \text{ }^\circ\text{C}$  to  $60 \text{ }^\circ\text{C}$  at  $10 \text{ MPa}$ ) (IUPAC, 2016), but higher temperature has a heating effect, which also leads to larger changes in protein conformation. Therefore, it could be concluded that the myosin conformational changes induced by DPCD have three effects: pH lowering, heating and molecular effects of  $\text{CO}_2$ , some of which can have synergy.

There have been many reports concerning the effects of DPCD on the secondary structure of proteins. Some researchers have observed that DPCD treatment causes secondary structure changes in enzymatic proteins. For instance, the  $\alpha$ -helix structure of some enzyme (e.g., lipase, alkaline protease, acid protease, and glucoamylase) was irreversibly decomposed after micro-bubbled DPCD treatment at  $35 \text{ }^\circ\text{C}$  and  $25 \text{ MPa}$  for  $30 \text{ min}$ , while there was reversible decomposition after heat treatment at  $80 \text{ }^\circ\text{C}$  for  $30 \text{ min}$  (Ishikawa, Shimoda, Yonekura, & Osajima, 1996). The  $\beta$ -structural content of DPCD-treated pectin methylesterase was decreased with increasing pressure from  $8$  to  $30 \text{ MPa}$  at  $55 \text{ }^\circ\text{C}$  for  $10 \text{ min}$  (Zhou, Wu, Hu, Zhi, & Liao, 2009). Furthermore, the  $\alpha$ -helix content of DPCD-treated myrosinase, which belongs to the family of enzymes involved in plant defence against herbivores, was decreased, and the  $\beta$ -sheet content was increased at  $22 \text{ MPa}$  and  $55 \text{ }^\circ\text{C}$  for  $60 \text{ min}$  (Yang, Liao, Hu, Zhang, Chen, & Wu, 2011). Many other researchers also found that DPCD can cause secondary structure changes in food protein. The  $\alpha$ -helix content of DPCD-treated horse muscle myoglobin was decreased from  $47\%$  to  $12\%$  with increasing pressure from  $8$  to  $30 \text{ MPa}$  at  $35 \text{ }^\circ\text{C}$  for  $30 \text{ min}$

(Ishikawa, Shimoda, Yonekura, Mishima, Matsumoto, & Osajima, 2000). The  $\alpha$ -helix content of myoglobin from lyophilized equine skeletal muscle was 57.47%, while it was 45.51% after DPCD treatment at 35 MPa and 35 °C for 30 min (Yan, Xu, Jia, Dai, & Li, 2016). Moreover, the  $\alpha$ -helix content was decreased with increasing pressure from 7 MPa to 35 MPa (Yan, Xu, Jia, Dai, & Li, 2016). Furthermore, when a whey protein isolate solution was treated with DPCD at 20 MPa and 60 °C for 1 h, the  $\alpha$ -helix content was decreased, and the  $\beta$ -sheet content was increased significantly (Xu, Yuan, Jiang, Wang, Hou, & Gao, 2011). Similarly, when a 2 wt % silk solution was treated with DPCD at 60 bar and 40 °C, the  $\alpha$ -helix content of silk protein was decreased and gradually converted to a  $\beta$ -sheet structure with increasing treatment time from 0 to 90 min (Floren, Spilimbergo, & Migliaresi, 2012). Rao et al., (2016) noticed that both heating (60 °C, 30 min) and DPCD (10 MPa, 60 °C, 30 min.) treatments can cause the conformational changes in myosin in *longissimus dorsi* muscle. These results are very much consistent with our research, especially; DPCD treatment can reduce the content of the  $\alpha$ -helix and increase the content of the  $\beta$ -sheet. Therefore, during DPCD treatment, the  $\alpha$ -helix structure of protein is decomposed easily and is converted to a  $\beta$ -sheet. These changes are very helpful for proteins to form stable gels induced by DPCD because the  $\beta$ -sheet is preferred for the protein-carbon dioxide interaction rather than the  $\alpha$ -helix (Cundari et al., 2009; Imtiaz-Ul-Islam, Hong, & Langrish, 2011). In addition, the geometrical difference between the  $\alpha$ -helix and  $\beta$ -sheet shows that more side chains of the amino acids on the  $\beta$ -sheet are exposed to the surrounding conditions, while the  $\alpha$ -helix is a more compact structure, and fewer side chains are available for hydrogen bonding to the surrounding carbon dioxide (Imtiaz-Ul-Islam, Hong, & Langrish, 2011).

However, some researchers have found that DPCD treatment had little effect on the

secondary structure of proteins because it was almost completely recovered after releasing carbon dioxide. For example, DPCD treatment caused minor disruption to the secondary structure of hen egg lysozyme, which was negligible at the working pressure of 200 bar and 40 °C (Moshashaée, Bisrat, Forbes, Nyqvist, & York, 2003). Although the secondary structure of albumin and lysozyme treated with SC-CO<sub>2</sub> had a progressive change at 2, 5, 9, 12 MPa and 40 °C, it was almost completely reverted when ambient conditions were restored (Striolo, Favaro, Elvassore, Bertucco, & Di Noto, 2003), indicating that the effect caused by DPCD treatment is reversible under the conditions investigated. These results are in contradiction with our research. It is likely that this depends on the particular protein considered and on the experimental conditions chosen (Striolo, Favaro, Elvassore, Bertucco, & Di Noto, 2003). The main reason could be due to the weak interaction between protein and CO<sub>2</sub> at a lower treatment temperature (Xu, Yuan, Jiang, Wang, Hou, & Gao, 2011).

From the previously mentioned literature, it can be concluded that DPCD treatment can cause protein secondary structure changes through the interaction between DPCD (or pressurized CO<sub>2</sub>) and protein. Moreover, it is well understood that mild pressures without CO<sub>2</sub> (up to 200 MPa) can cause proteins to undergo reversible unfolding, whereas irreversible changes can occur above 200 MPa (Balny & Masson, 1993), and a pressure without CO<sub>2</sub> below 50 MPa would not be an important factor for protein conformational changes (Yan, Xu, Jia, Dai, & Li, 2016). Therefore, DPCD should be the main factor responsible for protein conformational changes due to the effect of lowering pH and the molecular effect of CO<sub>2</sub>. In this sense, first, when CO<sub>2</sub> combines with water, it forms carbonic acid, which further dissociates to form HCO<sub>3</sub><sup>-</sup>, CO<sub>3</sub><sup>2-</sup>, and H<sup>+</sup> ions. H<sup>+</sup> reduces the solution pH. A higher pressure and temperature will enhance CO<sub>2</sub> solubility, facilitating both the acidification of the external

environment as well as contact of the proteins with CO<sub>2</sub> (Hu, Zhou, Xu, Zhang, & Liao, 2013).

Second, the molecular effect of CO<sub>2</sub> could be explained by it being a non-polar solvent that can interact with protein hydrophobic amino acid residues to expose them (Yoshimura et al., 2001) and extract water (Brown, Fryer, Norton, Bakalis, & Bridson, 2008), altering the balance of the water-protein interactions and resulting in protein conformational changes.

Many researchers have shown that DPCD can interact with basic amino acid (Lys, Arg and His) residues of proteins to form bicarbonate complexes (Yoshimura et al., 2001; Cundari et al., 2009), leading to protein conformational changes. In addition, during the quick depressurization step of DPCD treatment, extraction, aggregation and homogenization effects of CO<sub>2</sub> could also lead to protein conformational changes (Hu, Zhou, Xu, Zhang, & Liao, 2013).

### *3.3. Changes in the shrimp surimi gel strength*

Changes in the shrimp surimi gel strength induced by DPCD are presented in Figure 3. Regarding the heat and 0.1 MPa CO<sub>2</sub> treatments, the temperature had a significant effect on the shrimp surimi gel strength ( $P < 0.05$ ); however, at the same temperature, there was no significant difference between the heating treatment and 0.1 MPa CO<sub>2</sub> treatment ( $P > 0.05$ ). This indicates that the gel strength was induced by heat for both heat and 0.1 MPa CO<sub>2</sub> treatments. Although 0.1 MPa CO<sub>2</sub> treatment can change the conformation of myosin slightly (Figure 1 and Figure 2), it can not induce shrimp surimi to form a gel. This is mainly because the experimental object is myosin in solution in Figure 1 and Figure 2, while the experimental object is shrimp surimi (solid) in Figure 3. Additionally, 0.1 MPa CO<sub>2</sub> can combine with water in solution to form carbonic acid, which interacts with myosin. However, 0.1 MPa CO<sub>2</sub> is difficult to penetrate into shrimp surimi and interact with internal protein, whereas it can

possibly interact with the surface protein of shrimp surimi.

Figure 3 shows that DPCD treatment significantly increased shrimp surimi gel strength ( $P<0.05$ ) compared with thermal treatment. This is consistent with a previous study showing that DPCD can increase the textural properties (including Warner–Bratzler shear force, hardness, elasticity, cohesiveness and chewiness) of lamb sausages ( $P<0.05$ ) (Szerman, Rao, Li, Yang, Vaudagna, & Zhang, 2015). However, some studies have revealed that DPCD had little effect on the texture of meat. Choi et al. (2008) treated porcine *longissimus dorsi* muscle with CO<sub>2</sub> at 7.4 or 15.2 MPa (31.1 °C, 10 min) and observed that Warner–Bratzler shear force values were higher than those of control samples, but the increase was not significant ( $P>0.05$ ). Ferrentino, Balzan and Spilimbergo (2013) found that cooked ham treated with CO<sub>2</sub> at 12 MPa (50 °C, 5 min) had lower resistance to the compression than control samples, but there was also no significant differences between the fresh and treated samples ( $P>0.05$ ). The differences between these results may be related to factors such as meat species, water content, or treatment conditions.

In addition, shrimp surimi gel strength increased significantly with increasing pressure at the same temperature ( $P<0.05$ ). The gel strength was a maximum at 25 MPa and 50 °C, which was  $14.28\pm 0.57$  N·mm. The hardness and chewiness of minced mutton sausages treated by DPCD were increased with increasing pressure from 10 MPa to 50 MPa at 60 °C for 30 min (Qu, Zhang, Rao, Li, & Li, 2010). This is a result of two phenomena: One is that the solubility of CO<sub>2</sub> in water increases with increasing pressure. The content of water is approximately 80% in shrimp surimi, and at a higher pressure more CO<sub>2</sub> dissolves in shrimp surimi, which strengthens the interaction between CO<sub>2</sub> and protein. The other is that a higher pressure can increase the permeability to CO<sub>2</sub>, allowing CO<sub>2</sub> molecules to quickly spread into shrimp

surimi, which strengthens the interaction between CO<sub>2</sub> and proteins. At 60 °C, the gel strength was increased significantly with increasing pressure from 5 MPa to 10 MPa ( $P<0.05$ ), but the gel strength did not change with increasing pressure from 10 MPa to 25 MPa ( $P>0.05$ ). This can be related to the solubility of CO<sub>2</sub> in water. The solubility of CO<sub>2</sub> in water increases from 14.48 dm<sup>3</sup>/kg to 23.95 dm<sup>3</sup>/kg with increasing pressure from 5 MPa to 10 MPa (IUPAC, 2016). However, the solubility of CO<sub>2</sub> in water increases from 23.95 dm<sup>3</sup>/kg to only 28.35 dm<sup>3</sup>/kg with increasing pressure from 10 MPa to 25 MPa (IUPAC, 2016). These data show that the increment of solubility from 5 MPa to 10 MPa is higher than that from 10 MPa to 25 MPa at 60 °C.

At the same pressure (5-15 MPa), the shrimp surimi gel strength was increased significantly with increasing temperature ( $P<0.05$ ). This is also consistent with the previous study that textural properties (including hardness, elasticity, cohesiveness and chewiness) of lamb sausages induced by DPCD increased with increasing temperature ( $P<0.05$ ) (Rao et al., 2016). From a theoretical analysis, the solubility of CO<sub>2</sub> in water decreases with increasing temperature at a constant pressure, which should decrease the gel strength. However, the reduction in CO<sub>2</sub> solubility in water decreases significantly with increasing pressure in a stable temperature range. For example, the solubility of CO<sub>2</sub> in water decreases from 20.35 dm<sup>3</sup>/kg to 14.48 dm<sup>3</sup>/kg with increasing temperature from 40 °C to 60 °C at 5 MPa; the solubility of CO<sub>2</sub> decreases from 27.81 dm<sup>3</sup>/kg to 23.95 dm<sup>3</sup>/kg with increasing temperature from 40 °C to 60 °C at 10 MPa, the solubility of CO<sub>2</sub> decreases from 30.74 dm<sup>3</sup>/kg to 28.17 dm<sup>3</sup>/kg with increasing temperature from 40 °C to 60 °C at 20 MPa (IUPAC, 2016). Therefore, it can be inferred that the decrease in solubility of CO<sub>2</sub> caused by increasing temperature is not adequate to weaken the interaction between proteins and CO<sub>2</sub>. By contrast, the heating

effect of increasing temperature can strengthen the interaction between protein and CO<sub>2</sub> and enhance gel strength.

As shown in Figure 3, the shrimp surimi gel strength was increased significantly from 40 °C to 50 °C at 25 MPa ( $P<0.05$ ), while the gel strength was decreased from 50 °C to 60 °C ( $P<0.05$ ). The reason may be that there is an alkaline protease (the optimal temperature is 60 °C) in shrimp surimi and it can make shrimp surimi modori at 60 °C (Eakpetch, Benjakul, Visessanguan, & Kijroongrojana, 2008). Although DPCD can inactivate some enzymes, the effect of DPCD on alkaline protease needs to be further studied.

#### *3.4. Correlations between secondary structure and gel strength*

The secondary structure of myosin was determined by CD at a very low protein concentration (0.1 mg/ml), whereas the shrimp surimi gel strength was determined at a high protein concentration (approximately 4.8 g/100g). The study on their relationship may have some limitations. However, the research suggested that the CD results of globulin (at a low concentration of 0.01%) from common buckwheat were mostly consistent with the Raman data (at a high concentration of 5%) (Choi, & Ma, 2007). The correlation coefficients between the myosin secondary structure and shrimp surimi gel strength are presented in Table 2 and Table 3.

Table 2 and Table 3 show a negative correlation between the shrimp surimi gel strength and the  $\alpha$ -helix content, indicating that when the  $\alpha$ -helix content was decreased, the gel strength was increased. However, there was a positive correlation between the shrimp surimi gel strength and the  $\beta$ -sheet,  $\beta$ -turn and random coil contents, indicating that the increase in the  $\beta$ -sheet,  $\beta$ -turn and random coil contents increased the gel strength. This is consistent with gels induced by heating or high hydrostatic pressure. During heating, the  $\alpha$ -helix content was

negatively related to  $G'$  (elastic modulus) of porcine and silver carp myosin, whereas the  $\beta$ -sheet,  $\beta$ -turn and random coil contents were positively related to  $G'$  (Liu, Zhao, Xiong, Xie, & Qin, 2008; Liu et al., 2010). Furthermore, the  $\alpha$ -helix content was negatively correlated to the elasticity of rabbit myosin gel induced by high hydrostatic pressure and heating (Cao, Zhang, Wang, Zhou, & Xu, 2013). Therefore, both the formation of a  $\beta$ -sheet and unfolding of an  $\alpha$ -helix are helpful for myosin gel formation.

Moreover, Table 2 shows that, at a specific temperature, the absolute values of the correlation coefficients between the gel strength and secondary structure were  $\beta$ -sheet >  $\alpha$ -helix >  $\beta$ -turn > random coil, which indicating that pressure had a greater effect on the  $\alpha$ -helix and  $\beta$ -sheet than on the  $\beta$ -turn and random coil. This further confirms that  $\alpha$ -helix and  $\beta$ -sheet structures were more important for gel strength than  $\beta$ -turn and random coil structures. As presented in Table 3, at the same pressure, the absolute values of the correlation coefficients between the gel strength and the four secondary structures were larger, indicating that the temperature had a greater effect on secondary structure and gel strength than the pressure.

Myosin is a globular protein rich in  $\alpha$ -helices.  $\alpha$ -Helices play an important role in the gel formation of myosin since  $\alpha$ -helix unfolding is beneficial to the formation of intermolecular disulfide bonds and hydrophobic interactions (Liu, Zhao, Xiong, Xie, & Liu, 2007). Although the  $\beta$ -sheet content is lower in most native globular proteins, during thermally induced globular protein gelation, the general trend is for the  $\alpha$ -helix content to decrease, and the  $\beta$ -sheet content and number of disordered conformations to increase. Additionally, the  $\beta$ -sheet structure is more ordered and extensive than that present in the native globular molecules (Clark, Saunderson, & Suggett, 1981). In addition, the  $\beta$ -sheet is essential for protein-protein

interactions and gel network formation because it has a relatively large surface area for ordered hydrogen bonding (Choi, & Ma, 2007). The intermolecular hydrogen bonding interactions between  $\beta$ -sheets, oriented in either parallel or antiparallel  $\beta$ -sheet configurations, may act as junction zones, thus stabilizing the gel network (Wang, & Damodaran, 1991).  $\beta$ -sheets have a weaker water hydration strength than  $\alpha$ -helices, which can also promote protein-protein interactions and gel network formation (Choi, & Ma, 2007; Liu, Zhao, Xiong, Xie, & Qin, 2008). For most of the globular protein gels, the  $\beta$ -sheet conformation is an important secondary structure feature of the aggregated state (Clark, Saunderson, & Suggett, 1981). Therefore, these results and the published references suggest that  $\beta$ -sheets play a significant part in the globular protein aggregation process. Moreover, the presence of more  $\beta$ -sheets and fewer  $\alpha$ -helices can increase gel strength.

#### **4. Conclusion**

With increasing pressure and temperature upon DPCD treatment, the  $\alpha$ -helix of myosin was gradually decreased, while the  $\beta$ -sheet,  $\beta$ -turn and random coil contents, as well as the shrimp gel strength, were increased.  $\alpha$ -helix loss occurred simultaneously with the formation of  $\beta$ -sheet,  $\beta$ -turn and random coil structures. In other words, the  $\alpha$ -helices of myosin became unfolded and were gradually converted to  $\beta$ -sheet,  $\beta$ -turn and random coil structures, especially to  $\beta$ -sheets, during the gelation process. Due to  $\alpha$ -helix unfolding, active groups such as sulfhydryl and hydrophobic groups were exposed, leading to protein-protein interactions and chemical cross-linking induced by DPCD. This in turn led to the formation of a three-dimensional stable network retaining water, resulting in enhanced gel strength.

#### **Conflict of interest**

The authors have declared no conflict of interest.

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**Figure captions:**

Figure 1. CD spectra changes in myosin induced by DPCD with increasing pressure and temperature (treatment time: 20 min)

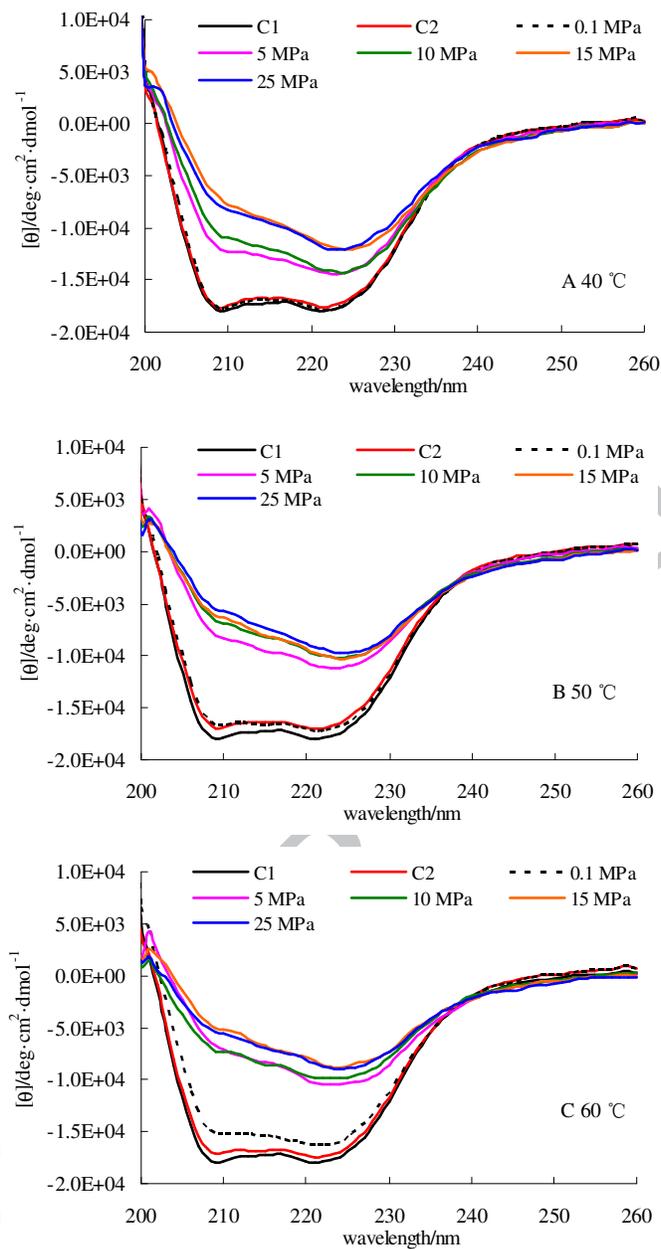
Note: C1 represents untreated myosin. C2 represents heat treatment in a water bath. Each experiment was determined in triplicate and the tested values were averaged.

Figure 2. Secondary structure changes in myosin induced by DPCD with increasing pressure and temperature (treatment time: 20 min)

Note: C1 represents untreated myosin. C2 represents heat treatment in a water bath. Each experiment was determined in triplicate, and the data were represented as mean  $\pm$  S.D.

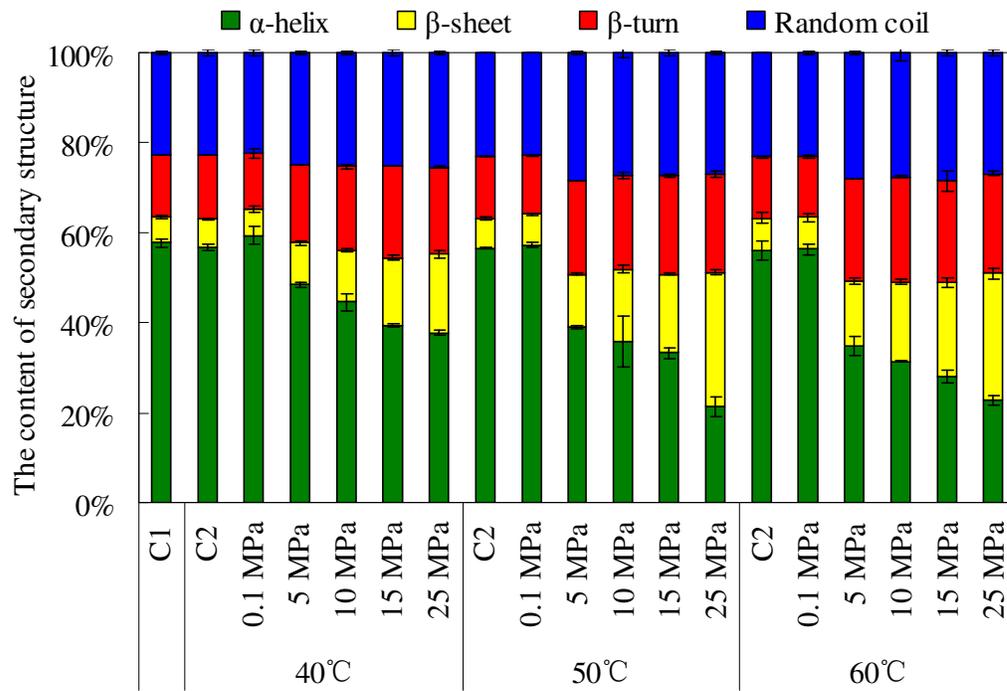
Figure 3. Changes in the shrimp surimi gel strength induced by DPCD with increasing pressure and temperature (treatment time: 20 min)

Note: The control represents heat treatment in water bath. The different lowercase letters above the bars indicate significant differences ( $P < 0.05$ ) in the gel strength at the same temperature and different pressures; the different capital letters above the bars indicate significant differences ( $P < 0.05$ ) in the gel strength at the same pressure and different temperatures. Each experiment was determined in triplicate and the data were represented as mean  $\pm$  S.D.



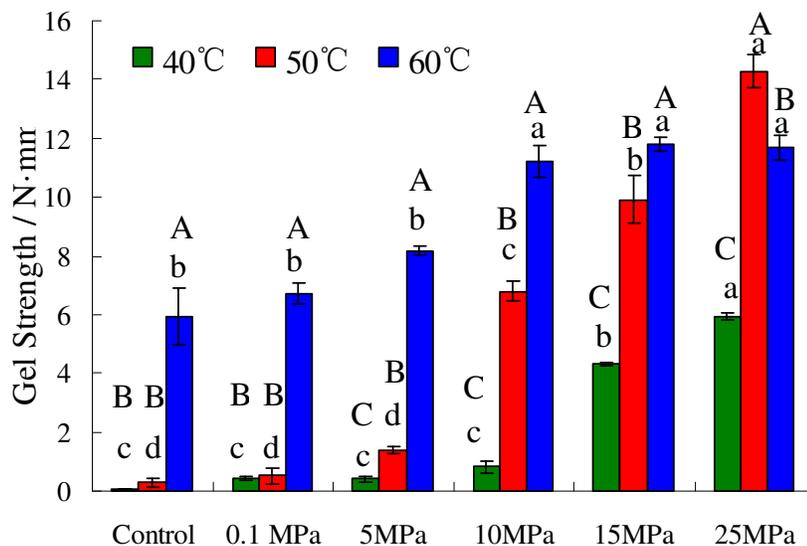
Note: C1 represents untreated myosin. C2 represents heat treatment in a water bath. Each experiment was determined in triplicate, and the tested values were averaged.

Figure 1 CD spectra changes in myosin induced by DPCD with increasing pressure and temperature (treatment time 20 min)



Note: C1 represents untreated myosin. C2 represents heat treatment in a water bath. Each experiment was determined in triplicate, and the data were represented as means±S.D.

Figure 2 Secondary structure changes in myosin induced by DPCD with increasing pressure and temperature (treatment time 20 min)



Note: The control represents heat treatment in a water bath. The different lowercase letters above the bars indicate significant differences ( $P < 0.05$ ) in the gel strength at the same temperature and different pressures; the different capital letters above the bars indicate a significant difference ( $P < 0.05$ ) in the gel strength at the same pressure and different temperatures. Each experiment was determined in triplicate, and the data were represented as means  $\pm$  S.D.

Figure 3 Changes in the shrimp surimi gel strength induced by DPCD with increasing pressure and temperature (treatment time 20 min)

Table 1 Physical and chemical means to induce protein gelation (Totosaus, Montejano, Salazar, & Guerrero, 2002)

Physical	Heat	Native protein partially unfolded by heat to form a network. Ordered matrix, by aggregation of the molecules.
	High pressure	Pressure (200–500 MPa) induces hydrophobic interactions and disulphide bonds between protein molecules, resulting in a rearrangement gel structure.
Chemical	Ion	After initial heating and salt addition, electrostatic repulsion or charges are shielded, forming a gel. Disruption of secondary structure induces a hydrophobic effect.
	Urea	Urea promotes intermolecular thiol-disulphide oxidation of thiol groups, resulting in a network formation.
	Acid	Slow pH reduction allows denaturation to form clusters or aggregates. These fractal clusters may be considered as the building blocks of the gel.
	Enzymatic	Enzyme catalyses cross-linking between protein glutamine residues to form a gel structure.

Table 2 Correlations between the secondary structure and gel strength (N x mm) at the same temperature

Temperature/ °C	$\alpha$ -helix	$\beta$ -sheet	$\beta$ -turn	Random coil
40	-0.78	0.92	0.67	0.57
50	-0.88	0.96	0.71	0.52
60	-0.90	0.87	0.77	0.74

Table 3 Correlation between the secondary structure and gel strength (N x mm) at the same

pressure

Pressure/ MPa	$\alpha$ -helix	$\beta$ -sheets	$\beta$ -turns	Random coil
0	-0.71	0.67	0.86	0.78
5	-0.81	0.92	0.84	0.55
10	-0.99	0.97	0.99	0.88
15	-0.93	0.98	0.92	0.94
25	-0.97	0.98	0.89	0.91

**Highlights**

- With increased pressure and temperature of DPCD, the  $\alpha$ -helix content of myosin was decreased.
- With increased pressure and temperature of DPCD, the  $\beta$ -sheet content of myosin was increased.
- With increased pressure and temperature of DPCD, the shrimp surimi gel strength was increased.
- The  $\alpha$ -helix content was negatively correlated with gel strength.
- The  $\beta$ -sheet content was positively correlated with gel strength.