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The effect of dense phase carbon dioxide on the conformation of hemoglobin

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ABSTRACT

Dense phase carbon dioxide (DPCD) sterilization is a non-thermal sterilization technology used to process heat-sensitive foods. Although nutritional and sensorial quality of food is preserved while unwanted microbial activity is reduced during DPCD sterilization, the effect on protein structure remains unclear. In this work, the effect of DPCD on the higher order structure and fluorescence properties of Hemoglobin (Hb) was investigated. The different conditions assessed during DPCD processing included variation in pressure, pH and heating conditions. Results from this study showed an inversely proportional correlation between α-helical content of Hb and pressure. As the pressure was lowered, the levels of α-helical content increased. The increased levels of α-helix correlated with a lower fluorescence intensity and a limited redshift in the fluorescence emission wavelength. TEM imaging showed that DPCD processing resulted in Hb with larger molecular diameters, which became smaller as the pressure increased. Interestingly, after 7-day storage at 4 °C, an increase in α-helical content was observed. Results from this work show that DPCD sterilization does impact the conformation of hemoglobin, with a notable impact on secondary and tertiary structure.

1. Introduction

Nowadays, consumers have been become more sophisticated and knowledgeable with regard to food quality. Food safety which is the main concern for people worldwide who are demanding “better” quality and longer shelf life for foods. Quality expectations include high nutritional value and sensory attributes, which are getting more stringent. According to customers' increasing demands, the food industry has been looking for advances in sterilizing technology which ensure the least deterioration of product quality, longer shelf life and the safer foods. Non-thermal technologies such as irradiation, ultra-high pressure (UHP), pulsed electric fields (PEF), pulsed magnetic fields, ultrasound, high-voltage arc discharge, and dense phase carbon dioxide (DPCD), are attracting interest and gaining acceptance as food processing methods. Recently, the combination of different non-thermal technologies has also received a great deal of attention from consumers and researchers such as a combined treatment based on high-pressure carbon dioxide and high-power ultrasound (Ferrentino, Komes, & Spilimbergo, 2015). Mostly above mentioned new technology has several limitations. DPCD technology, because of its unique and effective sterilizing effect, non-toxin, sensory and nutritional property maintenance, and mild pressure and temperature, has been thought as one of the most important non-thermal methods in food industry.

DPCD is a cold pasteurization method that affects microorganisms and enzymes through molecular effects of CO_2 under pressures below 50 MPa without exposing foods to adverse effects of heat and retaining their fresh like physical, nutritional, and sensory qualities (Damar & Balaban, 2006). The sterile medium CO_2 is a natural component of many foods, which is nontoxic, nonflammable, and an inexpensive gas. Because of its unique advantages as compared to traditional thermal technologies, DPCD has been thought as one of the most promising non-thermal methods in food industry.

Fraser (1951) initially reported the effects of DPCD on microorganism, vegetative cells, enzymes and quality attributes. A decade ago, DPCD mostly applied to liquid foods to cold-pasteurize and extend their shelf lifes as an alternative to thermal food preservation (Balaban, Ferrentino, & Spilimbergo, 2012). Gradually, with advancing technology and growing demand, researchers have put an emphasis on the applications of DPCD to solid foods including meat and meat products.

Previous studies mostly focused on the effects of DPCD on microbes in products. Wei, Balaban, Fernando and Peplow (1991) treated chicken strips inoculated with microorganisms at 14 MPa and 35 °C to obtain < 2 log reductions after 2 h. Scientists have studied the effects of DPCD on other quality attributes like color, tenderness, etc. Meurehg and Arturo (2006) treated beef trimmings with inoculated *Escherichia coli* and Salmonella. About 10 MPa for 15 min caused about 1 log reduction of *E. coli*. He also observed the effect of treatment on color, protein solubility and tenderness of the resulting product. Dagan and

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Balaban (2010) used a continuous DPCD system for pasteurization of beer and compared its physical and sensory attributes with that of fresh and heat pasteurized beer. Ferrentino, Balzan, Dorigato, Pegoretti, and Spilimbergo (2012) treated fresh-cut coconut pieces with DPCD. They evaluated the microbial, textural and microstructure effects at 8–12 MPa, 24–45 °C, for 5 to 60 min. Treatment of 15 min at 45 °C and 12 MPa resulted in 4 log CFU/g reduction of mesophilic microorganisms, lactic acid bacteria, total coliforms, and yeasts and molds. Ferrentino, Balzan, and Spilimbergo (2013) treated dry cured ham pieces at 8 and 12 MPa, 35 to 50 °C for 5–60 min. *Listeria* was completely inactivated (initial level 107 CFU/g) at 50 °C, 12 MPa, 15 min. Lower initial loads required less severe conditions for total inactivation. The process slightly affected the color and sensory attributes of the product.

Among quality factors of meat products, color plays a very key role, compared with other attributes on meat product purchasing decisions, because it tells consumers the sensorial quality information of the meat products (Tan, Zhou, Han, Li, & Qin, 2014; Xu, Zhu, Tan, Qin, & Zhou, 2016). Some studies(Chun, Min, & Hong, 2014; Neto et al., 2015) showed that the total myoglobin content and the relative proportions of oxymyoglobin, myoglobin, and metmyoglobin has a great influence on the intensity of visual color of fresh meat, which is mainly due to their ferrous oxygenated form and physical characteristics of the meat.

Our coworkers (Yan, Cui, Dai, Wang, & Li, 2010) found that the redness value of fresh meat treated by HPCD below 35 MPa had a noticeable decline compared with the control samples, which is consistent with the results of Choi et al. (2008). Another study (Yan, Xu, Jia, Dai, & Li, 2016) indicated that the relative α-helix content of Mb increase sharply after DPCD treating and the intrinsic relative fluorescence intensity of amino acid residues increased, but there is nonsignificant evidence shown that DPCD processing had a direct effect on polypeptide chains and interior structure of Mb.

The Mb plays a major part in meat color, but another hem protein Hb also has a certain influence on meat color. Hb is the iron-containing oxygen-transport metalloprotein in red blood cells of all vertebrates. It is a heterotetramer of two α-globin and two β-globin polypeptides, with a heme tightly bound to a pocket in each globin monomer (Hardison, 1996). To date, researchers have studied the effect of Hb derivatives as meat product colorant (Hhm, Ma, Jin, & He, 2017). So, it is meaningful to know the effect of DPCD on Hb.

In this paper, circular dichroism (CD) was used to analyze the change of secondary structure, fluorescence spectroscopy (FS) was used to assess changes to tertiary structure and transmission electron microscopy (TEM) was used to monitor conformational changes in hemoglobin.

2. Materials and methods

2.1. Materials

The lyophilized equine hemoglobin was purchased from Sigma Aldrich Co. (Beijing, China). One hundred grams of hemoglobin was dissolved in 100 mL of pH 7.0 phosphate buffer (NaH_2PO_4–Na_2HPO_4), stored at 0–4 °C before use. All chemicals used in the investigation were of analytical grade.

Commercially available CO_2 of 99.9% purity was purchased from Beijing Analytical Instrument Co. (Beijing, China) and used throughout the study.

2.2. Processing of Hb

DPCD processing was conducted at 50 °C for 30 min at 7, 14, 21, and 28 MPa of pressure, respectively. After setting the test temperature and time, turned on the water bath temperature system, made processing kettle temperature of DPCD device meet the test requirements and maintained. Preheat the reaction kettle to 50 °C, and then put the sample, 3 mL Hb solution in 10 mL plastic tube, into the reaction kettle. After sealing the vessel, boosted the pump, introduced carbon dioxide until the pressure reached experimental setting, then Hb in the vessel was held at constant pressure at equilibrium temperature during DPCD treatment. After 30 min opened the discharge valve, unloaded the pressure slowly for about 15 min, then took the sample out and cooled it quickly.

The heat processing was conducted at 50 °C for 30 min, and 100 °C for 15 min. For each experiment, a 3 mL Hb sample in 10 mL plastic tube was placed in a water bath until reaching the preset time.

The acid treating was conducted using phosphate buffer (NaH_2PO_4–Na_2HPO_4) at pH 4.0, 4.6, and 5.2, respectively.

All the samples were stored at 0–4 °C before use and each case repeated 3 times.

2.3. Determination of the pH value

pH meter (Hanna Instruments®, Italy) used to measure the pH value after DPCD and heat processing.

2.4. Circular dichroism spectra analysis

CD spectra were scanned at the far UV range from 250 to 195 nm with a JASCO J-810 CD spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan), using a quartz cuvette of 1.0 cm light path length at ambient temperature (20 ± 1 °C). The scanning velocity was 50 nm/min and the bandwidth was 1 nm. The Hb concentration for CD was 1 mg/mL. All spectra measured were baseline corrected using a phosphate buffer scan.

Mean residue ellipticity is the most commonly reported unit of the CD data and is measured in degrees·cm^2·$dmol^{-1}$ using the equation

$$[\theta] = \frac{0.1 \times MRW \times \theta}{P \times CONC}$$

where θ represents the measured ellipticity in degree; MRW (mean residue weight) = M / N, where M is the molecular mass of the polypeptide chain (in Da), and N is the number of amino acid residues in the chain amu for the protein in grams per mole; CONC represents the protein concentration in milligrams per liter; and P is the path length in centimeters (Kelly, Jess, & Price, 2005).

The α-helix relative fraction residual content was calculated by following formulas (Qin, Liu, & Teng, 2011).

$$\alpha\text{-helix}\% = -\frac{[\theta]_{208} + 4000}{32,980 - 4000} \times 100\%$$

2.5. Fluorescence spectroscopy analysis

The changes of the microenvironment in amino acids of Hb were investigated by fluorescence spectroscopy. Fluorescence spectra were measured using a RF-5301 spectrofluorimeter (Hitachi, Ltd. JPN) with a quartz cuvette of 1 cm optical path length at 20 ± 1 °C. The fixed excitation wavelength was 280 nm recorded from the range of 300–400 nm. The Hb concentration was 1 mg/mL. All the spectra were scanned continuously with three replicates. The relative fluorescence intensity (RFI) was calculated as follows.

$$RFI = \frac{\text{fluorescence intensity of Hb after treatments}}{\text{fluorescence intensity of Hb before treatments}} \times 100\%$$

2.6. Transmission electron microscope analysis

The microstructure changes of different processed Hb, including DPCD, heat and acid processing, was observed by H-7500 transmission electron microscope (Hitachi, Ltd. JPN). The processed sample was ultrasound dispersed evenly 1 min at 80 W of power. Take a small