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Effect of Cold Atmospheric Plasma (CAP) on Endogenous Enzyme Activity and Quality Parameters of Hairtail (*Trichiurus japonicus*)

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ABSTRACT

Cold atmospheric plasma (CAP) is a promising non-thermal technology that offers the capability of enzyme denaturation without the destruction of important components. The effect of CAP generated by dielectric barrier discharge (DBD) on endogenous enzyme activity and quality parameters of Hairtail (*Trichiurus japonicus*) was investigated. DBD voltage (30 kV, 40 kV, and 50 kV) and treatment time (2.5, 5.0, 7.5, 10, and 15 minutes) were employed. In all cases, a significant reduction in endogenous enzyme activity of Hairtail ($p<0.05$) was observed. Our results showed that the treatment time and voltage, significantly affected endogenous enzyme activity and carbonyl content. Nevertheless, the initial temperature and pH did not show significant changes after plasma treatments. From these results, we conclude that cold atmospheric plasma could be utilized as a good technology to maintain product quality and prolong the shelf life.

Keywords: Cold atmospheric plasma; dielectric barrier discharge; Hairtail; shelf life; endogenous enzyme activity

INTRODUCTION

Hairtail (*Trichiurus japonicus*) is among the most productive marine fish, can be found in the Indian Ocean, the Yellow Sea as well as the East China Sea (Hsieh *et al*., 2002; Jin *et al*., 2012). The high nutritional value and delicious taste of Hairtail resulted in increasing its consumption and production in the recent years (Luan *et al*., 2017). The Hairtail contains about 5.9 % protein and 18% fat (Hsieh *et al*., 2002). Several essential amino acids of human have been found in the Hartial's protein including, lysine, phenylalanine, threonine, tryptophan, leucine, methionine, valine, and isoleucine (Jin *et al*., 2012). It is well known that the spoilage of fish begin instantly after its death, therefore fish considered as highly perishable foods (Fellows and Hampton, 1994). Autolysis induced by endogenous enzymes and microbial activity play the main role in the quality deterioration of fish (Sequeira-Munoz *et al*., 1999;Hultmann, 2010). The degradation of myofibrillar proteins resulting from the high activity of endogenous enzymes leads to texture softening of fish muscles (Hultmann, 2010). Moreover, the enzymatic and chemical activities during postmortem handling and storage lead to the oxidation of unsaturated fatty acids, that negatively affected the nutritional value, color, flavor, as well as texture (Luan *et al*., 2017; Maqsood *et al*., 2012). Low temperatures are widely used for fish preservation, where it can control the biochemical and microbial spoilage and thus extend shelf life (Gallart-Jornet *et al*., 2007). At the same time, the low temperature promotes enzymatic hydrolysis of phospholipids and protein denaturation causing an adverse effect on fish quality (Sequeira-Munoz *et al*., 1999). Therefore, it is important to find new technologies that can be implemented to extend fish shelf life and keep the products in high quality.

Over the last years, the potential application of atmospheric cold plasma (CP) as nonthermal technology to decrease the microbial contamination and deactivate the endogenous enzymes in food products has been examined (Schlüter *et al*., 2013). Plasma can be considered as the fourth state of matter, which comes in the form of ionized gas. Dielectric barrier discharge (DBD) is commonly used to generate cold plasma (CP) through an electric field (Albertos *et al*., 2017). CP could effectively inhibit the activity of enzymes by changing the protein secondary structure (Attri and Choi, 2013; Li *et al*., 2011; Surowsky *et al*., 2013). In addition, CP has been applied to deactivate various enzymes including tomato peroxidase (Pankaj *et al*., 2013), apple polyphenol oxidase (Tappi *et al*., 2014), α-chymotrypsin (Attri and Choi, 2013), mushroom polyphenol oxidase (Surowsky *et al*., 2013), horseradish peroxidase (Surowsky *et al*., 2013), and alkaline phosphatase(Segat *et al*., 2016). However, there are very limited studies regarding the influence of Cold Atmospheric Plasma (CAP) on fish quality and endogenous enzymes activity. Therefore, this work seeks to investigate the impact of different conditions (voltages and times) of CAP on the enzymatic activity and the physicochemical characteristics of Hairtail (*Trichiurus japonicus*).

MATERIALS AND METHODS

Materials

DTNB (5,5-dithio-bis-(2-nitrobenzoic acid) was purchased from Aladdin Industrial Corporation. DNPH (2,4-Dinitrophenylhydrazine) and other reagents were purchased from Sinopharm Chemical Reagent Co. Ltd, China.

Sample Preparation

Fresh Hairtails (length 60.0 ± 5.0 cm) were purchased from a local market (Zhoushan, Zhejiang, China) and immediately transported to the laboratory in an ice box contain crushed ice. Fish samples were washed, beheaded, and gutted directly after arriving.

Plasma Treatment

Dielectric Barrier Discharge (DBD) was used to treat the fish samples as shown in Fig. 1 (Pankaj *et al*., 2013). The Alternating Dielectric (AC) Test Set, 600 series, with model number BK130/36 was supplied from Phenix Technologies, Inc., USA. The DBD plasma source was comprised of two parallel rounded aluminum plates with an outer diameter of 155 mm. Dielectric barriers in the form of polystyrene boards (2 mm thickness) in each end was used to stabilize the discharge. The distance between both the electrodes was adjusted to 75 mm. High voltage transformer was used to deliver the necessary energy that required for the generation of reactive oxidative species (ROS) from atmospheric gas. Fish samples were put in a petri dish with an 89 mm diameter and 2 mm thickness. The experiment was conducted in triplicate, and samples

were treated at voltages of 30 kV, 40 kV, and 50 kV for different times (2.5, 5.0, 7.5, 10, and 15 minutes) in a separate assay. Control was also prepared, without plasma treatment and stored at 4°C.

Fig. 1. Schematic Diagram of experimental set-up for Dielectric Barrier Discharge (DBD) of Hairtail fish (Pankaj *et al*., 2013).

Determination of Endogenous Enzyme Activity

To determine endogenous enzyme activity, a modified method from (Hernández Andrés *et al*., 2005) was used. After Cold Atmospheric Plasma (CAP) treatment, Hairtail flesh was comminuted and homogenized in cold 0.1 M sodium phosphate buffer (pH 6.0) a ratio of 1:10 (w/v) using a blender at maximum speed for 1 minute. To remove the connective tissues, 4 layers of gauze were used to filter the homogenates. After that, CAP reaction was stopped by mixing 10 mL of 10% Trichloroacetic acid (TCA) with an equal amount of homogenate. The mixture was rested for 15 minutes at room temperature, followed by centrifugation at 13,000 rpm for 3 minutes at 4°C using Hitachi Himac CF 16RX. The number of soluble oligopeptides was determined as described by (Lowry *et al*., 1951), tyrosine has been used as a standard. The absorbance was then read at 750 nm using Hitachi U-2000 UV-Vis spectrophotometer. The results were average from 3 independent determinations. The activity of the endogenous enzyme was measured by the amount of tyrosine released (µmoles) per gram of protein (µmoles Tyr/g protein) (Hernández Andrés *et al*., 2005).

Determination of Carbonyl Content

Carbonyl content of treated and untreated samples was measured according to (Xia *et al*., 2009) with slight modifications. The homogenates were subjected to dilution with 0.1 M sodium phosphate buffer pH 6.0 ratio at of 1:5 (v/v). Subsequently, 400 µl of the diluted portions were divided into two different tubes, namely sample tubes and control tubes. Both tubes were added with 80 µL 50% TCA and then centrifuged at 12,000 rpm for 3 minutes. The supernatant was discarded, then 2M HCl and 10 mM DNPH in 2M HCl were added to control and sample tubes respectively. These pellets were shaken for 15 minutes at room temperature and precipitated with TCA (10% final concentration (w/v)). Then, 1 mL of ethanol: ethyl acetate (1:1 v/v) were used to wash the pellets two times and centrifuged. After that, Washed pellets were dissolved in 1.5 mL of 6M Guanidine Hydrochloride in 20mM sodium phosphate buffer pH 6.5. The absorbance of the control was read at 280 nm, while for the sample was read at 365 nm by using Bovine Serum Albumin (BSA) dissolved in the same guanidine solution. The absorption coefficient of 22,000 $M^{-1}cm^{-1}$ was used for protein hydrazones.

Determination of Total Sulfhydryl Content

Sulfhydryl contents were determined according to (Li *et al*., 2013). One mL of homogenates was subjected to 8 mL of dissociating buffer consisted of 10.4 g Tris-HCl, 6.9 g glycine, 480 g urea, and 1.2 g EDTA per 1 L (pH 8.0) and 0.5 mL of Ellman's reagent (containing 4 mg/mL DTNB). The mixture was then incubated in the dark at room temperature for 30 minutes. Subsequently, the absorbance was read at 412 nm using Hitachi U2000 UV-Vis Spectrophotometer. To estimate the SH concentration, the absorption coefficient of 13,600 $M^{-1}cm^{-1}$ was used. The final results were expressed as µmol total SH/ mg of protein (Li *et al*., 2013).

Determination of pH and temperature.

100 mL of cold distilled water was mixed well with 10 g of Hairtail fish muscle using a blender for 1 minute at speed of 1000 rpm. The pH-meter (Sartorius PB-10) has been used to measure the pH of homogenate. While, the temperature was determined by regular thermometer (Rong *et al*., 2010).

Statistical Analysis

The results were expressed as the mean of triplicate ± standard deviation. Two-Way ANOVA of was employed for statistical analysis and Tukey significant difference procedure when applicable. The statistical analysis was carried out by IBM SPSS 21.0 and a significant level of 5% was used.

RESULTS AND DISCUSSION

Effect of Plasma on Endogenous Enzyme Activity

In fish muscles, endogenous enzymes play an essential role in the quality changes, that take place in the early stage of fresh fish storage (Hultmann, 2010). After fish harvesting, the ATP converts to hypoxanthine (Hx) as a result of endogenous enzymes activity, which leads to rigor mortis, a decline in muscle pH, as well as reducing the quality of meat (Sequeira-Munoz *et al*., 1999). The most important issue for the fish industry is muscle softening which is due to hydrolysis of different proteins by endogenous proteases (Hultmann and Rustad, 2004). Several studies indicated that cold atmospheric plasma (CAP) effectively inactivate the endogenous enzymes that cause deterioration of the product; such as polyphenol oxidase (PPO) in apple (Tappi *et al*., 2014) and peroxidase (POD) in tomato (Pankaj *et al*., 2013). Therefore, CAP was applied in this research to evaluate its capacity to deactivate the autolytic enzymes in the fresh Hairtail fish, which has a high proteolysis rate (Jiang *et al*., 2000 and Hsieh *et al*., 2002), which is similar to squid enzyme activity. Fig. 2 shows the changes in the endogenous enzymes activities of Hairtail fish subjected to CAP. The initial endogenous enzymes activities (control) were 201.95 ± 0.72 umole Tyr/ g protein, which is significantly (P>0.05) higher than all samples treated with CAP at different condition. Endogenous enzymes activities reduced significantly (P>0.05) with increasing exposure time and voltage (kV) of DBD. The lowest enzyme activities (116.94± 1.27 umole Tyr/ g protein) were observed with samples exposed to CAP at 50 kV for 15 minutes. These results are in good agreement with other studies which have shown the efficiency of CAP to control the endogenous enzymes activities (Misra *et al*., 2016). In contrast, some reports in the literature indicate an increase in enzymatic activity after plasma treatment (Chen *et al*., 2016; Lee *et al*., 2016; Li *et al*., 2011; Meiqiang *et al*., 2005; Xu *et al*., 2016). An important implication of these results is that enzyme

inactivation by CAP may be depending on enzyme type (Tappi *et al*., 2014). The most likely explanation of decline endogenous enzyme activity, that observed in the sample treated with CAP Hairtail, probably due to the fact that the cells were unable to keep up its defenses against ROS (Misra *et al*., 2016).

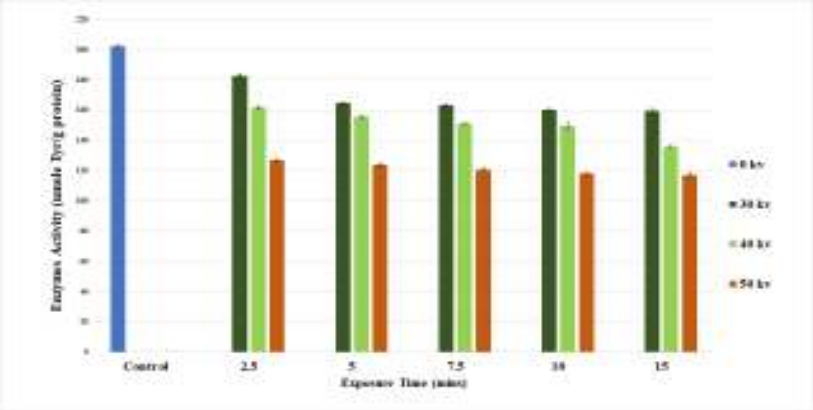

Fig. 2. Endogenous Enzyme Activity of Hairtail subjected to different DBD treatments. Value (mean ± standard deviation. n=3). Control refers to samples without CAP treatment.

Effect of CAP on Carbonyl Content

Carbonyl contents commonly used as an indication of protein oxidation, where 2,4-dinitrophenylhydrazine (DNPH) use to react with the carbonyls formed (Kolgiri and Patil, 2017). The fatty acid oxidation results in the formation of volatile carbonyl compounds (pentanal, cis-4-heptanal and trans-6-nonenal), which is responsible for changes in food quality including the color, texture, flavor, and odor characteristics (Payap, 2011;Bußler, 2017). The effect of CAP on the carbonyl content of Hairtail fish is given in Fig. 3. Our results indicated that the reduction of carbonyl content was dependent on both plasma exposure time and the voltage applied. Generally, a significant reduction was achieved with 50 kV for 15 mins, followed by 50 kV for 10 mins. These results are in line with the endogenous enzyme activity results. (Aryee *et al*., 2007;Payap, 2011) stated that the majority of lipolysis (such as phospholipase and triacyl lipase) that occurs in the stored fish, are caused by endogenous enzymes and microorganisms.

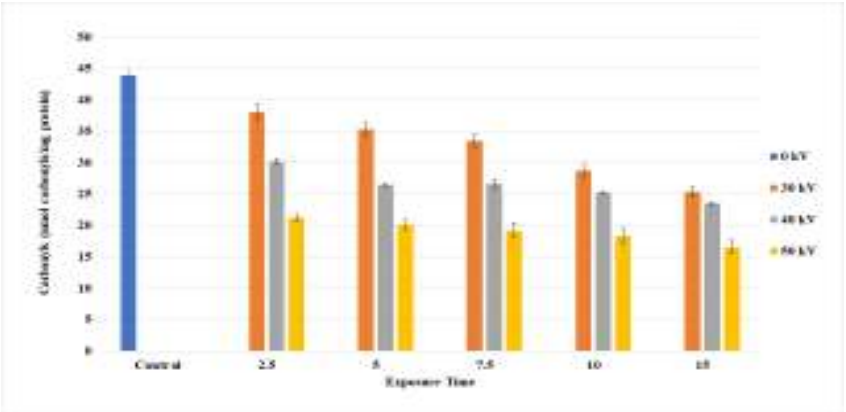

Fig. 3. Carbonyl content of Hairtail subjected to different DBD treatments. Value (mean ± standard deviation. n=3). Control refers to sample without CAP treatment.

Effect of CAP on Total Sulfhydryl (SH)

The damage caused by ROS can be measured also by determination the loss of sulfhydryl groups, which are detected by reacting Ellman reagent with free thiol groups. This reaction leads to the formation of disulfide bonds and the thiolate ion was released (which is colored) (Soladoye *et al*., 2015). The well-known amino acid that possesses sulfhydryl groups is methionine and cysteine, where both are highly susceptible to ROS (Zhou *et al*., 2016). This method has been utilized to assess another category of protein oxidation especially in meat products (Soladoye *et al*., 2015). No significant (P>0.05) difference were found in the total sulfhydryl (SH) between groups (Fig. 4). However, total SH groups at 50kV for 15 mins was significantly lower than control and other samples. It has been reported that the loss in Ca^{2+} -sensitivity of myofibrillar protein would be an indicator of the proteolytic degradation of tropomyosin and the modification of actin-myosin interaction by oxidation of sulfhydryl (SH) group of myosin (Payap, 2011).

To the best of the author's knowledge, CAP/DBD application in seafood products was still very limited, and so far the work which has been done is in microbiological aspect (Misra *et al*., 2016). As such, possible explanations of these phenomena may be due to that specific ROS combination might be needed to inactivate the enzymes, as shown in studies involving lysozyme (Kylián *et al*., 2008) and lipase (Li *et al*., 2011).

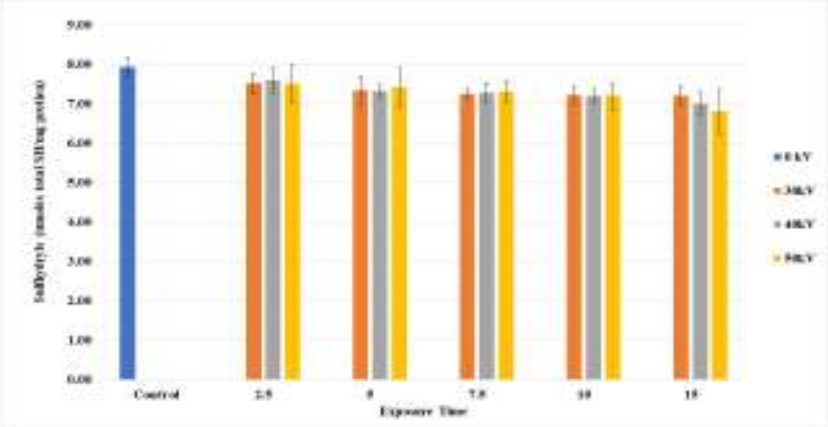

Fig. 4. Total Sulfhydryl (SH) of Hairtail subjected to different DBD treatments. Value (mean ± standard deviation. n=3). Control refers to sample without CAP treatment.

Effect of CAP on pH and temperature.

Fig. 5 presents the effect of CAP on pH of Hairtail samples. The pH behavior of Hairtail fish did not show any clear trend after plasma exposure. In general, a slight decrease in pH was observed due to the dissolution of ROS (Ercan *et al*., 2016). These results are in good agreement with (Kim *et al*., 2011) and (Ulbin-Figlewicz *et al*., 2013) who found that the cold plasma did not cause any pH changes. In addition, (Alfaro *et al*., 2013) reported that the pH is a poor indicator of fish freshness. However, the fish considered spoiled when the pH higher than 7 (Plazos *et al*., 2015).

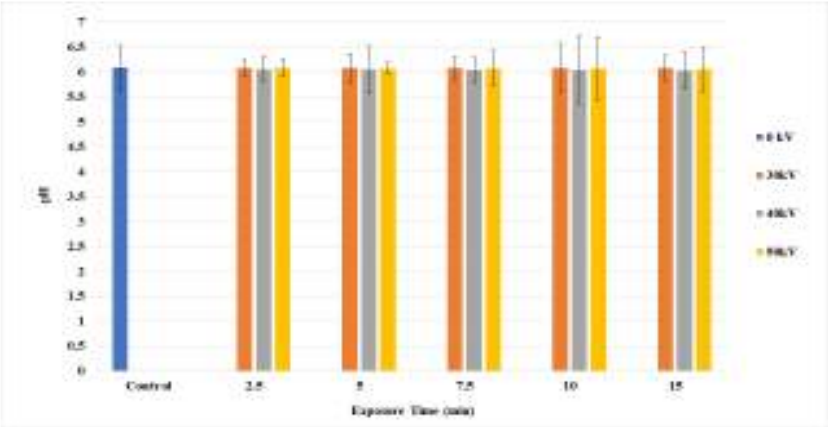

Fig. 5. pH of Hairtail submitted different DBD treatments. Value (mean ± standard deviation. n=3). Control refers to sample without CAP treatment.

In this study, all tested samples maintained values lower than the critical limit. The initial temperature of Hairtail was found to be in the range of 16-20 ˚C, where no enzyme would be denatured. pH and temperature did not change with the treatment conditions employed (Fig. 6).

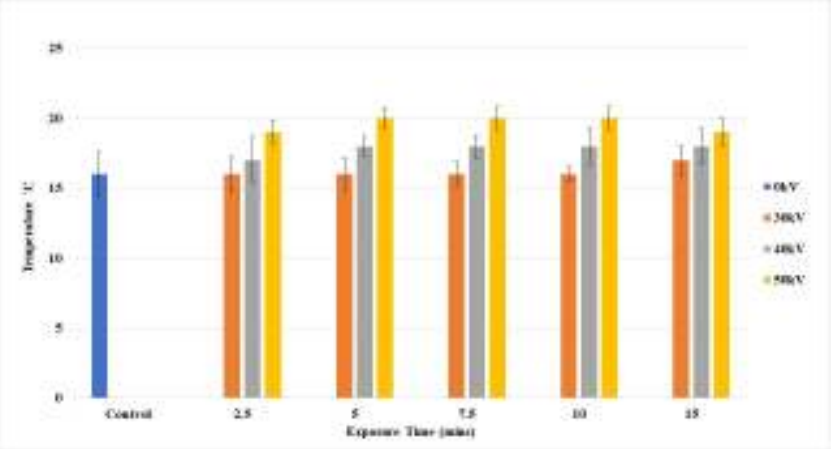

Fig. 6. Initial temperature of Hairtail submitted different DBD treatments. Value (mean ± standard deviation. n=3). Control refers to sample without CAP treatment.

CONCLUSION

DBD is shown to be a potential treatment for controlling the endogenous enzyme, that lead to extending the shelf-life and quality maintained. Our results indicate that the time and voltage used during treatment have significant effects on endogenous enzyme inactivation. Additionally, the CAP significantly decreased the carbonyl Content, while a slight reduction in the total SH was observed. However, CAP does not affect adversely physicochemical parameters such as pH and temperature. Overall, CAP could be considered as a good alternative for traditional preservation method.

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تأثير البلازما الباردة (CAP) على نشاط الانزيمات الذاتية ومعايير الجودة لاسماك الهيراتيل (trichiurus japonicus)

شيماء رضا حطب

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تعتبر تكنولوجيا البلازما الباردة (CAP) طريقة واعدة غير حرارية لوقف نشاط الانزيمات الذاتية والمسببة للفساد الاولي للمواد الغذائية. في هذ ا البحث تم دراسة تأثير البلازما الباردة والمولدة من تفريغ حاجز العازل الكهربي (DBD) علي نشاط الانزيمات الذاتية بأسماك الهيراتيل (*Trichiurus japonicus*) وكذلك تأثرها على عوامل الجودة المختلفة. قد تم تطبيق درجات جهد مختلفة (30 كيلو فولت و40 كيلو فولت و50 كيلو فولت) على فترات متباينة (2.5، 5.0، 7.5، 10، 15 دقيقة). وأظهرت النتائج انخفاض كبير (P <0.05) في نشاط الانزيمات الذاتية مع جميع المعاملات. كما اكدت تجاربنا ان نشاط الانزيمات الذاتية وكذلك محتوى الكربونيل في العينات المختبرة يتأثر بشكل كبير بالجهد والوقت المستخدمة، بينما لم تظهر درجات الحرارة الداخلية ودرجة الحموضة أي تغير بعد المعالجة بالبلازما الباردة. من هذه النتائج، نستنتج أن البلازما البارد (CAP) يمكن أن تستخدم كتكنولوجيا واعد للحفاظ علي جودة الأسماك واطالة عمرها الافتراضي.