EFFECTS OF VITAMIN C ON INHIBITION OF POLYPHENOLOXIDASE AND QUALITY CHANGES OF WHITE SHRIMP (*LITOPENAUS VANNAMEI*) DURING COLD STORAGE

ẢNH HƯỞNG CỦA VITAMIN C ĐẾN KHẢ NĂNG ỨC CHẾ ENZYME POLYPHENOLOXIDASE VÀ CHẤT LƯỢNG TÔM THẺ CHÂN TRẮNG TRONG QUÁ TRÌNH BẢO QUẢN LẠNH

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ABSTRACT

Effects of vitamin C (vit C) on inhibition of polyphenoloxidase (PPO) and quality changes of pacific white shrimp (Litopenaus vannamei) during cold storage of 7 days were investigated and compared to shrimps treated with an additive in commerce (sodium metabisulfide, SMS) and control sample (treated by water). Vit C was able to inhibit the activity of PPO enzyme that extracted from cephalothoraxes of shrimps with average molecular weight of 3.5 kDa and PPO activity of 1.25 (unit/mg enzyme). At 100 μM vit C, PPO relative activities were 21.43 %. In addition, shrimp treated with vit C exhibited lower melanosis score and TBARs value than those in the control sample ($p<0.05$). These results suggested that vit C was able to slow down the oxidation process of fat and melanosis development in white shrimp during the cold processing. Besides, the quality of shrimps was evaluated base on microbiological analysis and pH measurement. After 5 days of storage at 2^oC, the effectiveness of preventing bacterial formation in shrimp samples was higher in vit C sample, SMS sample, and control sample.

Keywords: *Inhibition of polyphenoloxydase, antioxidant activity, vitamin C, sodium metabisulfide, white shrimp, Litopenaus vannamei.*

TÓM TẮT

Ảnh hưởng của vitamin C (vit C) đến khả năng ức chế enzyme polyphenoloxydase (PPO) và chất lượng của tôm thẻ chân trắng (Litopenaus vannamei) trong 7 ngày bảo quản lạnh được nghiên cứu và so sánh với phụ gia thương mại là sodium metabisulfide (SMS) và nước (mẫu chứng). Vitamin C có khả năng ức chế hoạt tính của ezyme PPO được trích ly từ đầu tôm với khối lượng trung bình là 3,5 kDa và hoạt độ là 1,25 (unit/mg enzyme). Tại nồng độ vitamin C 100 μM, phần trăm ức chế tương đối của vitamin C là 21,43 %. Bên cạnh đó, mẫu tôm xử lý bằng vit C có điểm cảm quan biến đen và giá trị TBARs thấp hơn mẫu chứng ($p<0.05$). Cho thấy, vit C có khả năng ức chế sự hình thành biến đen và oxy hóa lipid xảy ra trong tôm. Bên cạnh đó, chất lượng của tôm trong quá trình bảo quản lạnh được đánh giá dựa trên các phân tích vi sinh và đo pH. Sau 5 này bảo quản tại 2^oC, hiệu quả ngăn chặn sự hình thành vi khuẩn trên mẫu tôm được xử lý bằng vit C cao hơn trong mẫu SMS và mẫu chứng.

Từ khóa: *Hoạt tính ức chế polyphenoloxydase, vitamin C, sodium metabisulfide, tôm thẻ chân trắng, Litopenaus vannamei.*

1. INTRODUCTION

In recent years, shrimp and shrimp products have occupied a large portion in the exported seafood products of Vietnam [1]. However, they are among the world's most perishable commodities, and their spoilage begins soon after the death. Even when they are kept in cold storage, discoloration (melanosis), oxidation, and microbial spoilage in shrimp are serious problems affecting organoleptic, nutritional and economic value

of shrimp. Melanosis is triggered by a biochemical mechanism which oxidizes phenols to quinones by polyphenoloxydase (PPO) [2]. This is followed by non-enzymatic polymerization and auto-oxidation of quinones, giving rise to dark pigments of high molecular weight. In addition, lipid oxidation is another deteriorative reaction causing the unacceptability of shrimp products. To extend the shelf-life of shrimps, melanosis inhibitors have been used. Sulfites and their derivatives are widely used as PPO inhibitor by preventing the polymerization of quinones, combining irreversibly with them, and forming colorless compounds [3]. However, sulfiting agents are known to produce allergic reactions and serious disturbances in asthmatic subjects. Therefore, safe compounds from natural origin such as ascorbic, citric acid, kojic acid, dodecyl gallate, and oxalic acid have been used as the substitutes of sulfiting agents [3-4]. In this study, we studied on effects of vitamin C on inhibition of PPO and quality changes of pacific white shrimp during 7 days of cold storage at 2^{o}C.

2. EXPERIMENTAL

2.1. Chemicals and materials

L-β-(3,4 dihydroxylphenyl) alanine (L-DOPA), Brij-35, vitamin C (L-ascorbic acid), malonaldehyde bis (dimethyl acetal), thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA) was obtained from Merck (Darmstadt, Germany).

White shrimps (Litopenaeus vannamei) with the size of 30-40 shrimps/kg were purchased from Thu Duc market, Thu Duc district, Hochiminh city. The shrimps were kept alive and transported to laboratory.

2.2 Preparation of crude PPO from the cephalothoraxes of white shrimps

The cephalothoraxes of sixty shrimp were separated, pooled and powdered by grinding with liquid nitrogen in an Electrolux blender. The powder obtained was kept in polyethylene bag and stored at -20^{o}C for not more than 2 weeks. The extraction of PPO was carried out following the method of Simpson *et al*. (1987) with a slight modification [5]. The powder (50 g) was mixed with 150 mL of extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35). The mixture was stirred continuously at 4^{o}C for 30 min, followed by centrifugation at 8000 x g at 4^{o}C for 30 min using a refrigerated centrifuge. Solid ammonium sulfate was added into the supernatant to obtain 40% saturation and allowed to stand still at 4^{o}C for 30 min. The precipitate was collected by centrifugation at 12.500xg at 4^{o}C for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2 and dialyzed against 15 volumes of the same buffer at 4^{o}C with three changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3000 x g at 4^{o}C for 30 min and the supernatant was used as "crude PPO extract".

2.2.1. Measurement of PPO activity

PPO activity was assayed using L-DOPA as a substrate according to the method of Simpson et al. (1987) with a slight modification [5]. The assay system consisted of 100 μM of crude PPO extract, 600 μM of 15 mL L-DOPA in deionized water, 400 μM of 0.05 phosphate buffer, pH 6.0 and μM of deionized water. The PPO activity was determined for 3 min at 45oC by monitoring the formation of dopachrome at 475 nm using a UV-160 spectrophotometer (Hitachi, Japan).

One unit of PPO activity was defined as an increase in the absorbance at 475 nm bay 0.001/min. Therefore, PPO activity was expressed as unit/mL. Enzyme and substrate ware prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and the deionized water was used instead.

2.2.2. Size exclusion chromatography

PPO crude extracted from cephalothoraxes of shrimps was determined molecular weight using size exclusion chromatography by HPLC 1100 (Agilent, USA) with RID detector. The samples was

eluted on a Ultrahydrogel 500 (300 mm x 7.8 mm ID) column with mobile phase of 0.5 M KNO_3, with a flow rate of 1.0 mL/min. The temperature of the column was maintained at 40 ^oC and the injection volume 20.0 μL.

2.2.3. Tyrosinase Inhibitory Activity

Vit C (1000 μM) with different volumes were mixed with crude PPO (100 μL) to obtain the final concentration of 0.01, 0.05 and 0.1% (w/v), respectively. This reaction mixture was incubated for 30 min at room temperature. Then, the assay buffer (400 μM) was added. To initiate the reaction, 600 μM of pre-incunated 15 mM L-DOPA (45^oC) were added. The reaction was conducted at 45^oC and the absorbance at 475 nm was monitored for 3 min. The control was run in the same manner, except deionized water was used instead of vit C as follows:

$$\text{Relative activity (\%)} = \frac{A-B}{A} \text{x100} \quad (1)$$

Where A: PPO activity of control; B: PPO activity in the presence of vit C.

2.3. Applying for shrimp cold storage

The shrimps were immersed in 0.05 % vitamin C, 2% SMS solution and water (control sample) at room temperature for 5 minutes, similarly. Shrimps were fished out and preserved in plastic box at 2^0C. Three shrimps from each treatment were taken every 0 days up to 7 days for evaluation of determining pH, melanosis development, bacterial count, protein analysis and lipid peroxidation inhibition.

2.3.1. pH measurement

pH measurement was performed by the method of Lopez-Caballero *et al.* (2007) with a slight modification [6]. Shrimp meat (2g) was homogenized with 10 volumes of deionized water for 1 min and the homogenate was kept at room temperature for 5 min. The pH was determined using a pH-meter.

2.3.2. Sensory evaluation

Fifteen candidates (19–22 years old) for panelists were selected from students of the Chemical and Food Technology Faculty. Candidates were carefully screened for ability to recognize and describe common aroma. Control sample (treated by water) and shrimp samples (treated by ethanol extracts) were evaluated during storage and classified according to the degree of black spot formation. The gray value in shrimp was evaluated directly using modified Montero's sensory evaluation [3]. Fifteen candidates (n=15) evaluated gray values in shrimp by levels 1 to 5 scale as follow: point 0 = no point; point 1= light (about 20% of the surface area affected shrimp); point 2 = the average (accounting for 20-40% surface area affected shrimp); point 3= significant (accounting for 40 - 60% surface area affected shrimp); point 4= very severe (60-80 % occupied surface area affected shrimp); point 5= very terrible (80-100 % occupied surface area affected shrimp).

2.3.3. Lipid peroxidation inhibition assay

MDA is considered to be the final product of the oxidation process of lipid peroxidation. TBA reacts with MDA to form a di-adduct, a red chromogen, which can be detected spectrophotometrically at 532 nm [7]. Shrimps were grinded by machine, then was mixed with 10 mL TCA 7.5% solution. The mixture was filtered about 15 min, the filtrate was mixed with TBA 0.02 M solution equal volume rate, then the mixture was heated at 100^oC for 15 min. Absorbance was measured at 532 nm by the spectrophotometer. MDA contents were calculated from standard curve built at concentrations from 0.01 to 0.05 μM and reported as mgMAD/kg shrimp. MDA content values were calculated from the mean values of data from three determinations.

2.3.4 Microbiological analysis

*Enterobacteriacea*e and *Pseudomonas aeruginosa* in shrimp samples after 5 days preserving at 2^oC were detected and determined based on bacterial count method following TCVN 5518-2:2007 (ISO 21528-2:2004) and 3347/2001/QĐ-BYT at Ho Chi Minh Institute Pasteur.

3. RESULTS AND DISCUSSION

3.1. Preparation of PPO extract

3.1.1 Determining PPO activity and average molecular weight of PPO

1.1 gram of PPO crude was obtained from 164 gram of the cephalothoraxes that separated from 800 gram of shrimps following Simpson method. Base on the results of the PPO gel chromatography, the average molecular weight of the PPO was about 3.5 kDa (Figure. 1). The results are quite low compared to the results of previous studies; PPO extracted from shrimps is 20-25 kDa [5]. This difference may be due to differences in living conditions, age of shrimp, methods of identification. In addition, it is possible that the PPO is also extracted with many impurities, not removed by the experimental method. In addition, PPO activity is 1.25 (unit/mg enzyme) with L-DOPA as a substrate according to the method of Simpson *et al*. (1987) in 2.2.1 section.

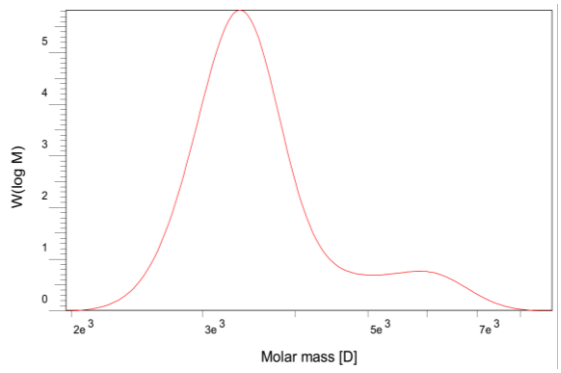

Figure 1. *Molecular weight of PPO extracted from cephalothoraxes of white shrimps*

3.1.2. Effect of vitamin C on the inhibition of PPO

The assay was carried out at different concentrations of vit C including 10, 25, 50, and 100 μM. Relative activities of samples were 5.31 %, 8.06 %, 13.19%, and 21.43 %, respectively. The increased inhibition of PPO was observed with increasing concentration ($p<0.05$). The result is suggested that vit C is able to inhibit the activity of the PPO enzyme. Vitamin C suppressed PPO by reducing the quinones formed at the end of catalytic browning by PPO. Quinone has the ability to self-polymerize to form melanin, causing the phenomenon to turn black [2].

3.2 Applying for shrimp cold storage

3.2.1 pH measurement

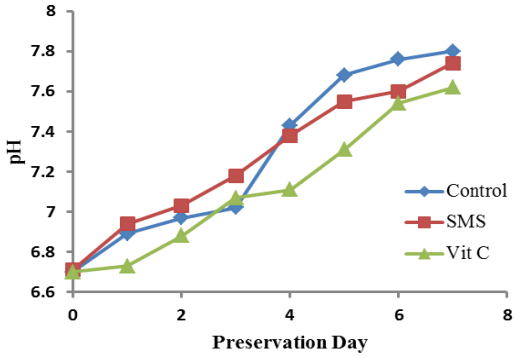

Figure 2. *The changes of pH in shrimp during cold storage*

Changes in pH of white shrimp with and without different treatments during cold storage are shown in Figure 2. As the storage time increased, pH of all shrimps increased ($p<0.05$). Within the first two days of storage, no differences in pH were noticeable among all samples ($p>0.05$). After two days of storage, those treated with vit C had the lowest pH, followed by those treated with SMS and control sample, respectively ($p<0.05$). At the end of storage, the control, shrimp treated with SMS, 0.05 % vit C had pHs of 7.80, 7.74 and 7.62, respectively. The increase in pH was associated with the accumulation of basic compounds, mainly resulted from the microbial action. The lower increase in pH of shrimp treated with vit C was in accordance with the lower microbial count.

3.2.2. Sensory evaluation

Treated shrimp samples by SMS and vit C have gray values lower than the control sample in cold storage (Figure. 3, 4). Besides, shrimp samples treated by SMS have the lowest gray values were 2.2, 2.6, and 3.7 after 3, 5, and 7 days in cold storage. Melanosis scores of the control sample after 3, 5, and 7 days were 2.9, 4.4, and 4.8, respectively. Meanwhile, gray values of treated shrimp samples by vit C were 2.3, 2.8, and 4.3, respectively. Besides, there is no significant difference between shrimps treated by SMS

and vit C after 5 days (P<0.05). In general, gray values occurred significantly after 3 days of preservation. The significant differences between control sample and vit C sample is suggested that vit C is able to prevent melanosis development in shrimp during the cold processing.

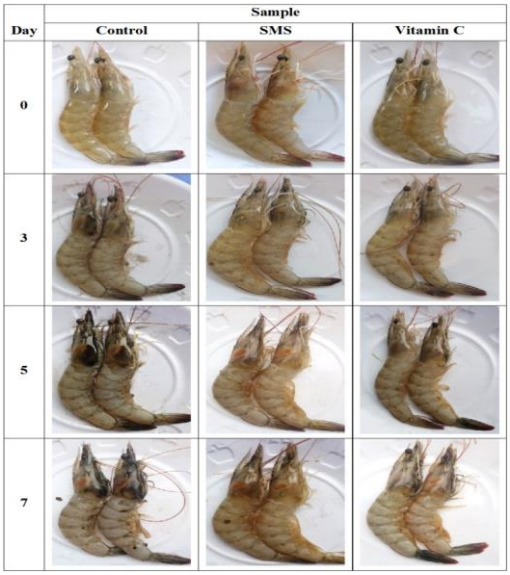

Figure 3. *Development of melanosis in shrimps during cold storage*

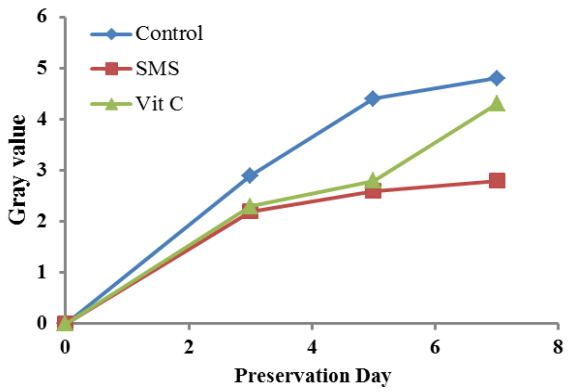

Figure 4. *Changes in the mean gray value in shrimps before and after cold storage. Results are presented in terms of mean ± confidence value (n=15, p=95%)*

3.2.3. Lipid peroxidation inhibition assay

The results in Figure.5 showed that the lowest TBARS value over the days of the SMS sample, followed by the vitamin C and the control sample, respectively. In general, the TBARs values of the extract were found to be increased from the first day to the fifth day and to decrease significantly during 5-7 days of storage at 2 ^{o}C (Figure. 5). Treated shrimp samples by vit C have TBARs values lower (p<0.05) than the control sample.

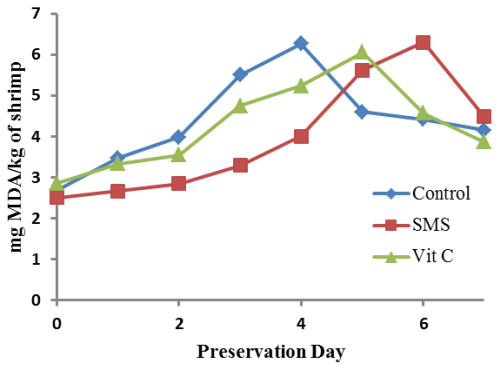

Figure 5. *The TBARs value changes of shrimp during preservation at 2^{o}C*

The increasing TBARs values (0-5 days) are as fat oxidation powerful place in the first stage, the product of the fat oxidation such as hydroperoxide that formatted and oxidized rapid into secondary products like aldehyde. The secondary oxidation products continue to be converted to all other products under the effect of enzymes and microorganisms, leading to diminished TBARs value (5-7 days) [8]. Treated shrimp samples have lower TBARs value than the control sample. These results showed that vit C able to slow the process oxidation of fat in shrimp during the cold processing.

3.2.4. Microbiological analysis

The results showed that the total aerobic macrobiotic of the vitamin C sample (2.5x10^{4} Cfu/g) was 44 times and 18 times lower than those in the control sample and the SMS sample, respectively (Table 1). This indicated that the shrimp treated with vitamin C was able to inhibit the growth of aerobic microorganisms better than that of the control and SMS samples. According to TCVN 5289: 2006 requirements of frozen seafood, total aerobic bacteria should not exceed 106 CFU / g. Thus, it can be seen that samples treated by vitamin C and SMS are suitable while control sample is not acceptable. Besides, *Pseudomonas aeruginosa* and *Enterobacteriaceae* are two pathogenic microorganisms in cryopreservation products. Results showed that the number of these two microorganisms in the vitamin C sample was

lower than in the SMS sample. From this it can be concluded that vitamin C can inhibit harmful microorganisms, aerobic bacteria such as *Pseudomonas aeruginosa* and *Enterobacteriaceae* better than additive samples.

Table 1. *The result of microbiological analysis of shrimp samples after 5 days*

Criteria	Sample		
	Control	**SMS**	**Vitamin C**
Total aerobic microorganisms (Cfu/g)	$1.1x10^6$	$4.7x10^5$	$2.5x10^4$
Pseudomonas aeruginosa (Cfu/g)	*	$7.8x10^3$	*
Enterobacteriaceae (Cfu/g)	*	$1.4x10^3$	$2.1.10^2$

** not detected.*

4. CONCLUSION

In this study, the ability of vitamin C to inhibit of PPO was studied and compared to SMS and water. Vitamin C is not only capable of reducing the formation of black spot formation and lipid oxidation in shrimp but also preventing the growth of bacteria, thereby reducing the pH value of shrimp. Firstly, vit C is strong antioxidant, can scavenge free radical. In addition, vit C is an acidulant which can affect enzyme and substrate by changing ionization state and breakdown of structural conformation, respectively [3]. Finally, ascorbic acid is highly water-soluble, which is acidic and moderately strong reducing compound. Vit C also acts an oxygen scavenger for the removal of molecular oxygen in PPO reaction. PPO inhibition by vit C had been attributed to the reduction of enzymatically formed o-quinones to their precursor diphenols [9].

REFERENCES

[1] Nguyen Xuan Duy, Nguyen Anh Tuan, *Screening of Plants with Antioxidant Activity and Application in Fishery Processing,* Can Tho University of Journal Sciences, 28, 59-68, 2013.
[2] Norman F. Haard and Benjamin K. Simpson, *Pholyphenoloxidase. In seafood enzyme utilization and influence on post-harvest seafood quality*, Marcel Dekker, 271-315, 2000.
[3] Montero P. et al, *The effect of inhibitors and high pressure treatment to prevent melanosis and microbial growth on chilled prawns (Penaeus japonicas)*, Journal of Food Science, 66, 1201-1206, 2001.
[4] Chen J.S., *et al, Inhibition mechanism of kojic acid on polyphenol oxidase*, Journal of agricultural and food chemistry, 39, 1897-1901, 1991.
[5] Simpson B.K., *et al, Phenoloxidase from shrimp (Penaues setiferus): Purification and some properties*, Journal of agricultural and food chemistry, 35, 918-921, 1987.
[6] Lopez-Caballero M.E., *et al, Quality of thawed deepwater pink shrimp (Parapenaeus longirostris) treated with melanosis-inhibiting formulations during chilled storage, International Journal of Food Science* & *Technology*, 42, 1029-1038, 2007.
[7] Singh R. and Arora S, *Attenuation of Free radicals by Acetone Extract / Fractions of Acacia nilotica L.) Willd Ex del, Journal* of *Chinese Clinical Medicine*, 2, 196-204, 2007.
[8] Angel B. E., *et al, Effects of Ergothioneine from Mushrooms (Flammulina velutipes) on Melanosis and Lipid Oxidation of Kuruma Shrimp (Marsupenaeus japonicus)*, Journal of agricultural and food chemistry, 58, 2577-2585, 2011.
[9] Walker, J.R.L., *Enzymatic browning in foods. Its chemistry and control,* Food Technological NZ, 12, 19-25.

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