

Research Article

Preservative Effect of Na₂SO₃ and Ascorbic Acid on the Inhibition of *Clanis bilineata* Meat Melanosis

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Abstract: Melanosis formation in the Meat of *Clanis Bilineata* Larvae (MCBL) treated with Na₂SO₃ in combination with Ascorbic Acid (AA) was monitored during frozen storage of 6 months. No melanosis occurred in MCBL when the meat was treated with 0.05% Na₂SO₃ in combination with 0.04% AA (p<0.05). Total viable number of Mesophilic and psychrophilic bacterial in MCBL treated with Na₂SO₃ (0.05%) in combination with AA (0.04%) decreased, while that in the control MCBL increased. Results indicated that the treatment developed in this study was a promising way to preserve MCBL during extended frozen storage.

Keywords: Ascorbic acid, *Clanis bilineata*, melanosis, Na₂SO₃

INTRODUCTION

Over the past few years, there has been renewed interest in using insects as food, mainly because of an increasing awareness that they are nutritious (Defoliart, 1992; Ladrón de Guevara *et al.*, 1995; Raksakantong *et al.*, 2010). *Clanis Bilineata* (CB), a member of the subfamily Ambulicinae (*Sphingidae*, *Lepidoptera*), is an edible insect that usually grows on soybean leaves. About 6,000 tonnes of CB Larvae (CBL) are consumed in China each year. The protein content of dry CBL is 65.5% (w/w) and the essential amino acid content is 52.84% (w/w) (Wu *et al.*, 2000). The fat content of CBL is 23.68% (w/w), and the level of unsaturated fatty acids is 64.17% (w/w) (Wu *et al.*, 2000). Linolenic acid, which is a functional fatty acid, is as high as 36.53% (w/w) of the total fatty acids (Wu *et al.*, 2000).

The meat of CBL (MCBL) is usually frozen due to seasonality. However, the main problem is melanosis formation during storage catalyzed by Polyphenoloxidase (PPO) in the presence of phenolic compounds and oxygen (Nirmal and Benjakul, 2009a). Absence of melanosis is of important economic significance for MCBL: the melanosis in MCBL decreases its sensory quality and frequently causes product rejection by the consumers. Though heating can inhibit the activity of PPO and resolve this problem, it decreases the flavour of MCBL. So far, there are not any reports focused on this problem.

Na₂SO₃ has been known to inhibit the activity of PPO, reduce oxygen content and inhibit bacterial growth (Taylor *et al.*, 1986), while Ascorbic Acid (AA) has the ability to reduce oxygen and adjust pH (Lamikanra and Watson, 2001). Therefore, the aim of this study was to investigate the inhibitory effect of Na₂SO₃ and AA on PPO and the formation of melanosis in MCBL.

MATERIALS AND METHODS

Materials: Live fourth instar larvae, which were from the same sources, and had similar properties (average weight 7.52 g; average body length 7.54 cm), were purchased from a local agricultural market (Xinqu, Jiangsu Province, China). Na₂SO₃ and AA were purchased from Fuchen Chemical Reagents Co. (Tianjin, China). L-β-(3, 4-dihydroxyphenyl) alanine (L-DOPA), Brij-35, and ammonium sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of reagent grade.

Preparation of MCBL: The larvae were washed and drowned with tap water. The heads of the dead larvae were removed by hand. The content, including meat and fluid, inside the skin was extruded from the tails with an iron bar on a slate, quickly cooled to 0-10°C, and stored until used within 2 h.

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Preparation of PPO extract from MCBL: 100 g of MCBL was grounded into powder with liquid nitrogen in a Waring blender (JJ-2, Wuxi Woshin Instruments Co., LTD., and Wuxi, China). The powder was kept in a storage bag and stored at -20°C until used within 24 h. The isolation of PPO was carried out according to the method described by Nirmal and Benjakul (2009a) with slight modifications. The powder (50 g) was mixed with 150 mL of the 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°C for 30 min, followed by centrifugation at 8,000×g for 30 min using a refrigerated centrifuge (GTR10-1, Beijing Era Beili Centrifuge Co., LTD, Beijing, China) at 4°C. Solid ammonium sulphate was added into the supernatant to obtain 40% saturation and allowed to stand at 4°C for 30 min. The precipitate was collected by centrifugation at 12,500×g for 30 min using a refrigerated centrifuge at 4°C. The pellet obtained was dissolved in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2 and dialysed against 15 volume of the same buffer at 4°C with three changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3,000×g at 4 °C for 30 min and the supernatant was used as “crude PPO extracts”.

Determination of PPO activity: The activity of PPO was determined using L-DOPA as a substrate according to the method described by Nirmal and Benjakul (2009b) with a slight modification. The assay system consisted of 100 µL of crude PPO extract, 600 µL of 15 mM L-DOPA in deionised water, 400 µL of 0.05 M phosphate buffer (pH 6.0), and 100 µL of deionised water. The PPO activity was determined for 3 min at 45°C by monitoring the formation of dopachrome at 475 nm using a UV spectrophotometer (752, Nanjing Qilin Instruments CO., LDT, Nanjing, China). One unit of PPO activity was defined as an increase in the absorbance by 0.001 at 475 nm/min/mL. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and deionised water was used instead.

Effect of Na₂SO₃ and its synergists on the activity of PPO: Na₂SO₃ was dissolved in distilled water to various concentrations (0.04, 0.06, 0.08, 0.10, 0.12, 0.14%, respectively, w/v) (Taylor *et al.*, 1986). Na₂SO₃ solution (100 µL) was mixed with crude PPO extract (100 µL) and the mixture was incubated at room temperature (25°C) for 30 min, and then, the assay buffer (400 µL, 0.05 M phosphate buffer, pH 6.0) was added. To initiate the reaction, 600 µL of preincubated 15 mM L-DOPA (45°C) were added. The reaction was conducted at 45°C and the absorbance at 475 nm was monitored for 3 min. The control was run in the same manner, except the deionized water was used instead of Na₂SO₃ solution. The sample blank was prepared by using distilled water instead of L-DOPA. One unit of

PPO activity was defined as increase in the absorbance by 0.001 at 475 nm/min/mL. Residual activity was calculated and the inhibitory activity was expressed as percent inhibition as follows:

$$\text{Inhibition (\%)} = (A-B)/A \times 100$$

where A, PPO activity of the control; B, PPO activity in the presence of inhibitor (Nirmal and Benjakul, 2012).

AA at various levels (0.02, 0.03, 0.04, 0.05, 0.06, and 0.07%, respectively) in combination with maximum inhibition level of Na₂SO₃ (0.05%, w/w) was evaluated for PPO inhibition activity on melanosis.

Treatment of MCBL: 1000 g MCBL was mixed with 0.5 g Na₂SO₃ and 0.4 g AA, frozen quickly, and stored at -8°C for time intervals (1, 2, 3, 4, 5, and 6 month). MCBL without any treatment were used as the control.

Melanosis assessment: The melanosis of MCBL was measured using a Minolta colorimeter (CS-100A, Minolta Co. Ltd, Japan) according to Lu *et al.* (2007) with slight modifications. The degree of browning was expressed by the changes in the Lightness (*L*) value.

Microbiological analysis: At different times (1, 2, 3, 4, 5, and 6 month) the samples were drawn for microbiological analysis carried out according to the method of Nirmal and Benjakul (2009b) with slight modifications. 25 g of thawed MCBL was placed in a Waring blender (JJ-2, Wuxi Woshin Instruments Co., LTD. and Wuxi, China) containing 225 mL of 0.85% saline water. After homogenization, appropriate dilutions were prepared to spread on the plate count agar medium containing 0.5% NaCl for the determination of total viable counts. Total viable counts of mesophilic and psychrophilic bacterial were determined by incubating the agar plates at 35°C for 2 days and 4°C for 7 days, respectively.

pH Measurement: 25 g of thawed MCBL was placed in a Waring blender (JJ-2, Wuxi Woshin Instruments Co., LTD., Wuxi, China) containing 225 mL of deionized water. After homogenization, the pH of the dilution was recorded using a digital pH meter (Model: PHS-3C, CD Instruments, China).

Sensory evaluation: At month 0 and month 6 of frozen storage, MCBL without and with different treatments were placed on a stainless steel tray, covered with aluminium foil and steamed for 3 min when the internal temperature reached 95°C. The cooked samples were evaluated by 18 panelists from the Department of Food Science and Technology, aged 30-45 (scientists in food science), using the 9-point hedonic scale, where 9 = like extremely; 7 = like moderately; 5 = neither like or nor dislike; 3 = dislike moderately; 1 = dislike extremely (Meilgaard *et al.*, 1990). Panelists were regular consumers of MCBL and had no allergies to MCBL. All panelists were trained for use a series of standards

of color, odor, and taste and evaluate for color, odor, taste and overall likeness.

Statistical analysis: All data are presented as mean±S.D. Statistical analysis was performed using Statgraphics Centurion XV Version 15.1.02. A multifactor ANOVA with posterior multiple range test was used to find significant differences among the effects of storage time and dipping condition on colour, firmness, and microbiological count.

RESULTS AND DISCUSSION

Effect of Na₂SO₃ without or with its synergists on PPO inhibition: The effect of Na₂SO₃ alone or with its synergist on inhibition of PPO from MCBL is shown in Fig. 1. Na₂SO₃ in combination with and without AA at various levels showed the inhibitory effect towards PPO. When Na₂SO₃ was used alone as the inhibitor toward PPO activity, maximum percent inhibition (67.3%) was obtained at 0.05% (w/v) (Fig. 1), indicating that 0.05% (w/v) of Na₂SO₃ was optimum concentration. PPO activity percent inhibition increased from 67.3 to 98.2% as AA was added at various levels to 0.05% (w/v) Na₂SO₃ from 0 to 0.04% (w/v), and did not increase further as AA level was beyond 0.04% (w/v). 0.05% (w/v) Na₂SO₃ and 0.04% (w/v) CA showed high synergy and the highest PPO inhibitory

activity. Therefore, 0.05% (w/v) Na₂SO₃ in combination with 0.04% (w/v) CA was further evaluated for PPO inhibition.

Effect of Na₂SO₃ in combination with AA on melanosis of MCBL during frozen Storage: The changes in L values of the MCBL with and without treatment of Na₂SO₃ in combination with AA during frozen storage are shown in Fig. 2. The L values for the control decreased as the storage time increased. As to the L values for the MCBL with the treatment of Na₂SO₃ in combination with AA, they did not decrease significantly (p>0.05) and were significantly lower than those of the control (p<0.05). It has been reported that sulphites inhibit browning (Taylor *et al.*, 1986). The absence of formation of melanosis in MCBL treated with Na₂SO₃ and AA was coincided with its PPO inhibitory activity as shown in Fig. 1.

Effect of Na₂SO₃ with AA on the microbiological changes of MCBL during frozen storage: The changes in psychrophilic and mesophilic bacterial count of MCBL during frozen storage due to the presence of Na₂SO₃ in combination with AA are shown in Fig. 3. In the control (sample without treatment), psychrophilic bacterial count (Fig. 3a) increased steadily in 3 months (p<0.05). However, psychrophilic bacterial count in the

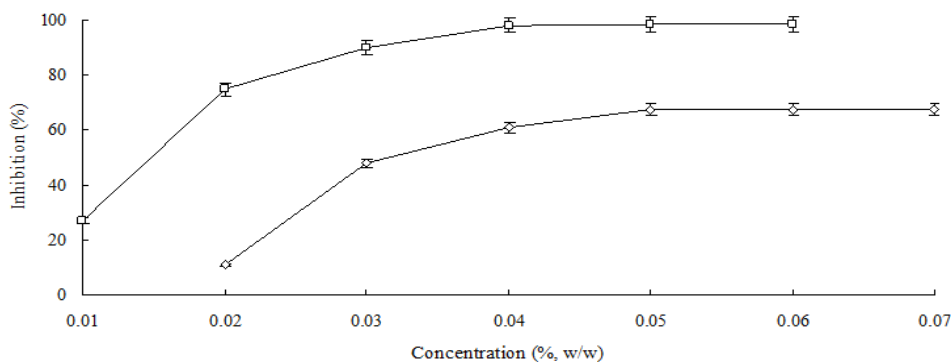


Fig. 1: Effect of Na₂SO₃ with (□) or without (◇) its synergists on inhibition of PPO activity of MCBL. Data are shown as mean ± SD (n = 3)

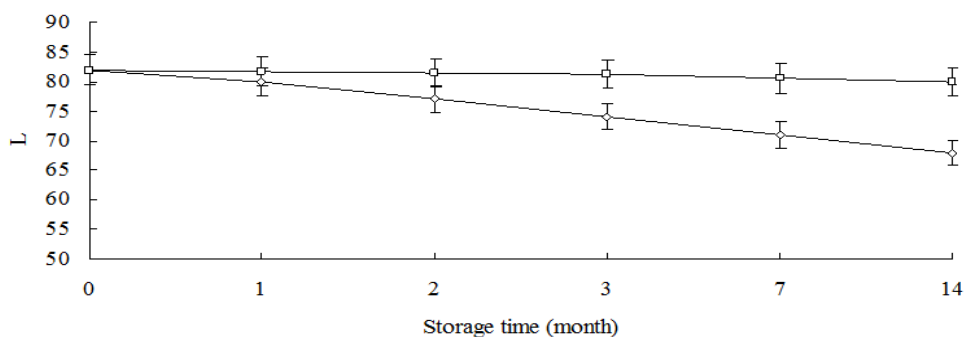


Fig. 2: Effect of Na₂SO₃ in combination with AA on melanosis formation of MCBL: control (◇); treatment (□). Data are shown as mean ± SD (n = 3)

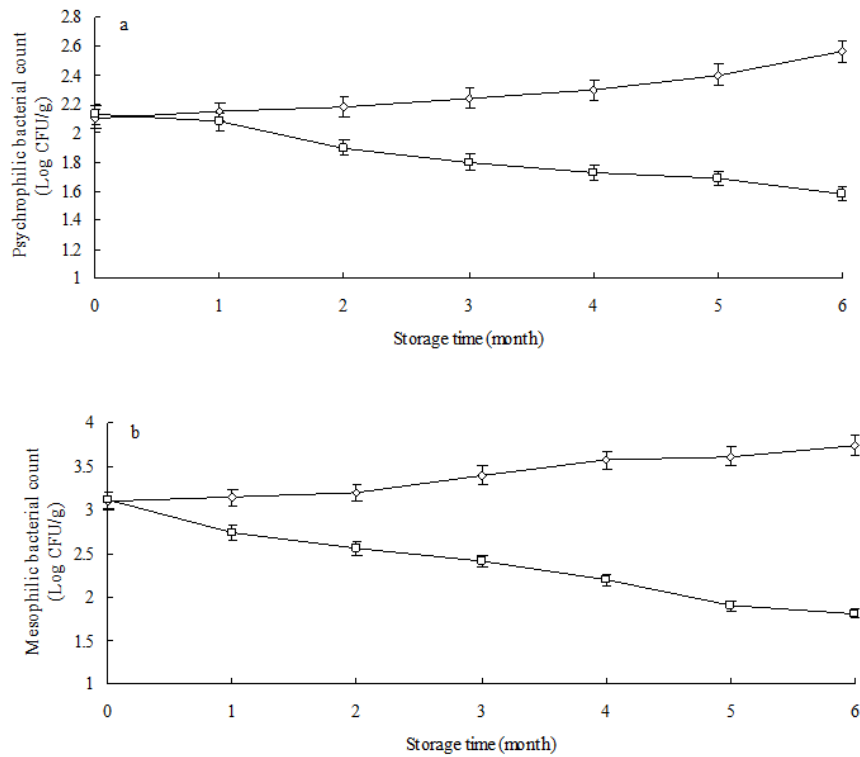


Fig. 3: Effect of Na₂SO₃ in combination with AA on the growth of microbiolism: psychrophilic bacterial, (a) mesophilic bacterial, (b) Data are shown as mean ± SD (n = 3)

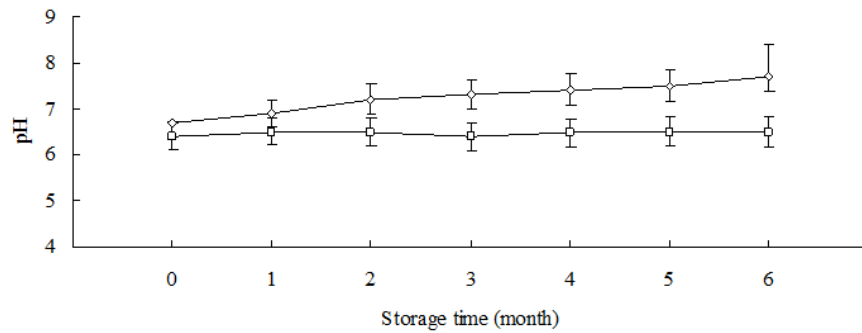


Fig. 4: Effect of Na₂SO₃ in combination with AA on pH of MCBL: control (◇); treatment (□). Data are shown as mean ± SD (n = 3)

Table 1: Effect of Na₂SO₃ in combination with AA on likeness score of MCBL during frozen storage^a

Storage time (month)	Groups	Color	Odor	Taste	Flavor	Overall
0	Control	8.7±0.43	8.1±0.51	8.3±0.45	8.0±0.48	8.8±0.42
	Treatments	8.7±0.41	7.8±0.42	8.0±0.44	7.7±0.31	8.8±0.46
1	Control	3.2±0.16	6.1±0.30	7.4±0.31	7.1±0.33	6.1±0.34
	Treatments	8.7±0.43	7.8±0.31	8.1±0.43	7.7±0.32	8.7±0.91
2	Control	1.0±0.04	1.0±0.03	3.1±0.15	2.7±0.12	3.0±0.29
	Treatments	8.7±0.46	7.8±0.31	8.1±0.43	7.7±0.32	8.7±0.41
3	Control	1.0±0.03	1.0±0.02	1.0±0.05	1.0±0.04	1.0±0.05
	Treatments	8.7±0.41	7.8±0.32	8.1±0.44	7.7±0.35	8.7±0.43
4	Control	1.0	1.0	1.0	1.0	1.0
	Treatments	8.7±0.42	7.8±0.33	8.1±0.41	7.7±0.34	8.7±0.41
5	Control	1.0	1.0	1.0	1.0	1.0
	Treatments	8.7±0.44	7.8±0.35	8.1±0.42	7.7±0.33	8.7±0.42
6	Control	1.0	1.0	1.0	1.0	1.0
	Treatments	8.7±0.43	7.8±0.36	8.1±0.40	7.7±0.37	8.8±0.40

^a Mean±S.D. from three independent experiments

MCBL treated with Na₂SO₃ and AA decreased continuously, compared with the control (p<0.05). This indicated the antimicrobial activity of Na₂SO₃ in combination with AA towards psychrophilic bacteria in MCBL even during frozen storage (Fig. 3a). In an acidic medium, Na₂SO₃ transforms into sulfite, which might damage to the microorganism by inhibiting some enzyme activities, i.e., oxidase (Taylor *et al.*, 1986). Changes in mesophilic bacterial count of MCBL with and without treatments during frozen storage are similar to those in psychrophilic bacteria (Fig. 3b).

Effect of Na₂SO₃ with AA on pH of MCBL during frozen storage: Changes in pH of MCBL with and without treatment during frozen storage are shown in Fig. 4. pH of the MCBL at month 0 was 6.7. As the storage time increased, pH of MCBL increased continuously (p<0.05). The increase in pH was attributed to the accumulation of basic compounds, mainly came from microbial action (Lopez-Caballero *et al.*, 2007). The lower pH of MCBL treated with Na₂SO₃ with AA may be the result of then acidic AA, and the steady pH may be due to the lower microbial count (Fig. 3). Results suggested that Na₂SO₃ in combination with AA might retard the microbial growth, and the spoilage or decomposition could be lowered.

Effect of Na₂SO₃ in combination with AA on sensory properties of MCBL during frozen storage: Changes in sensory properties of MCBL with and without melanosis inhibition of treatment during frozen storage are presented in Table 1. Each property of color, odor, taste, flavor, and overall likeness of cooked MCBL was evaluated at month 0, 1, 2, 3, 4, 5, and 6 of frozen storage. At month 0, no obvious differences in all attributes were observed between control and treated samples (p>0.05). After storage of 1 month, all sensory attributes in the control samples decreased noticeably (p<0.05). At month 1, the higher scores for color, flavor, and overall likeness were found in MCBL treated with Na₂SO₃ in combination with AA, compared with the control (p<0.05). After storage of 3 month, the melanosis reached highest scores, flavour as well as likeness scores of the control samples decreased to the lowest (Table 1), while those of the samples treated with Na₂SO₃ in combination with AA maintained the initial levels (Table 1). The lower melanosis score and high score of flavour as well as taste likeness scores of samples treated with Na₂SO₃ in combination with AA were also associated with the lower microbial load in those samples (Fig. 3), in comparison with the control. Therefore, it is clear that the treatment of MCBL with Na₂SO₃ in combination with AA could improve the sensory properties during extended frozen storage, which was most likely associated with low melanosis score.

CONCLUSION

This study demonstrated that Na₂SO₃ in combination with AA could be used to prevent melanosis from forming in MCBL during extended frozen storage, and the efficacy was associated with dose. At the same time, treatment of MCBL with Na₂SO₃ in combination with AA could inhibit microbial growth. Moreover, there has been no change in sensory properties of MCBL when treated with Na₂SO₃ in combination with AA throughout the whole storage time, while sensory quality of the control samples decreased significantly after 1 month of storage and continuously decreased. Therefore, treatment with Na₂SO₃ in combination with AA extended shelf-life of MCBL.

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