The Role of Monoxide Hemoglobin in Color Improvement of Chicken Sausage

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Received August 7, 2015
Revised December 10, 2015
Accepted December 13, 2015
Published online April 30, 2016

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pISSN 1226-7708
eISSN 2092-6456

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Abstract The role of monoxide hemoglobin (COHb) in improvement of chicken sausage color was investigated. COHb and NaNO₂ synergistically increased a* values. Addition of 0.1% COHb decreased the residual nitrite content in the presence of 0.001% NaNO₂. Compared with controls, the combined treatment resulted in significantly higher nitroso pigment contents while the single treatment resulted in slightly higher nitroso pigment contents. Visible spectrometry indicated that both nitrosohemochrome (NH) and hematin were the main ingredients of pigments extracted from chicken sausage treated with a combination of 0.006% NaNO₂ and 0.6% COHb. Formation of NH and hematin caused an increase in a* values and a decrease in L* values, respectively. COHb showed potential for use in meat product formulations.

Keywords: color, nitrosohemochrome (NH), hematin, monoxide hemoglobin (COHb), sodium nitrite (NaNO₂)

Introduction

Among quality factors of meat products, color more than any other affects meat product purchasing decisions because it is the first impression for consumers regarding meat products (1,2). The traditional method for improvement of meat product color involves use of nitrite due to multifunctional roles of production of a characteristic pink color, exertion of an anti-oxidative activity, contribution to development of a characteristic cured meat product flavor, and inhibition of food spoilage bacterial growth (3). However, use of nitrite in meat products faces a challenge because of potential health risks (4) and, therefore, there is considerable interest worldwide in development of food colorants from natural sources. Hemoglobin (Hb), one of the most important chromoprotein sources, exists in animal and poultry blood. Hb can be stabilized via coordination with Hb and small molecule ligands, such as carbon monoxide (CO) (1,5,6). Moreover, COHb or a combination of COHb and sodium nitrite (NaNO₂), is reported to improve the color and/or other physicochemical properties of pork sausage during storage (1,2). A similar phenomenon is also observed in mortadellas formulated with CO-treated porcine blood (7). Thus, COHb shows potential for use in meat product formulations.

However, to date, no studies are available that focus on the role of monoxide hemoglobin in improvement of meat product color. For example, there is a lack of experimental data that can demonstrate existence of (a) a reaction between COHb and NaNO₂ and/or, (b)

dissociation followed by oxidation of COHb during the process, and these changes may be correlated with the color of meat products. Therefore, the objectives of this study were (a) investigation of the reaction between COHb and NaNO₂ to form nitrosohemochrome (NH), and (b) dissociation followed by oxidation of COHb for generation of hematin. Color changes, residual nitrite contents, nitroso pigment contents, and the chemical ingredients which are related to the reactions mentioned above, were investigated. The results supply clues that are helpful for clarification of the role of COHb for improvement of chicken sausage color.

Materials and Methods

Preparation of monoxide hemoglobin (COHb) COHb was prepared following a previous procedure (1). Specifically, 8.0 g of sodium citrate (AR; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was added to 1,000 mL of fresh porcine blood. The mixture was stirred with a glass rod for 1 min. Subsequently, the mixture was filtered through gauze. The filtrate was centrifuged in a refrigerated centrifuge (CT15RT; Shanghai Tianmei Biochemical Engineering Equipment Co., Ltd., Shanghai, China) at a speed of 1,660×g for 10 min at 4°C. After centrifugation, 2 layers had formed. The supernatant was removed, and red blood cells (RBCs) were collected. Distilled water can cause swelling and rupture of RBCs. Therefore, RBCs were washed with 650 mL of a 0.9% (w/v) sodium chloride

solution twice in order to remove plasma protein residues.

CO was prepared via a reaction of concentrated sulfuric acid (AR; Sinopharm Chemical Reagent Co., Ltd.) and formic acid (AR; Sinopharm Chemical Reagent Co., Ltd.) (dry powder) at 85°C. Resulting CO was purified using a solution of 10% (w/v) NaOH (AR; Sinopharm Chemical Reagent Co., Ltd.), then added to saturate the RBCs with a vigorous stirring (JJ-1; Jiangsu province Jintan Jincheng Guosheng Experimental Instrument Factory, Jintan, China) at room temperature (25°C). Subsequently, the mixture was dehydrated via freeze-drying using an ALPHA1-4LSC freeze dryer (Martin Christ, Osterode am Harz, Germany). The resulting dry product was characterized based on visual spectral analysis with a spectrophotometer (Unico UV-2802; Unico (Shanghai) Instrument Co., Ltd., Shanghai, China) (not shown). Characteristic peaks at 411, 529, and 555 nm, which were consistent with the COHb spectrum, were observed (1), indicating that the product was COHb, the purity of which was close to 100% based on a procedure described by Liu and his cooperators (8).

Sausage preparation Frozen chicken breast meat and fresh pork back fat were purchased from a local Hypermarket of the Carrefour Group in Hefei, China. Frozen boneless chicken meat was thawed for 24 h at 2–4°C, and then the skin, visible fat, and connective tissue were removed. Trimmed chicken meat and fresh pork back fat were mixed at a ratio of 19 to 1 (w/w) then ground twice with a meat grinder (MGB-120; Yeekai, Guangdong, China) fitted with a plate containing 20 holes of diameter=6 mm. Based on an equimolar reaction between COHb and NaNO₂ (AR; Sinopharm Chemical Reagent Co., Ltd.), 6 experimental treatments were prepared as Treatment 1: control without NaNO₂ or COHb, Treatment 2: 0.1% COHb, Treatment 3: 0.2% COHb, Treatment 4: 0.001% NaNO₂, Treatment 5: 0.001% NaNO₂ and 0.1% COHb at a molar ratio of NaNO₂ to COHb of 1:1, and Treatment 6: 0.001% NaNO₂ and 0.2% COHb at a molar ratio of NaNO₂ to COHb of 1:2. In addition, other additives were also included as 5% deionized water, 2.5% sodium chloride (food grade, Anhui Salt Industry General Corporation, Hefei, China), 0.05% monosodium glutamate (Food grade, Shanghai totole Food Co., Ltd., Shanghai, China), 0.2% ginger powder (Sichuan Youjia Foodstuffs Co., Ltd., Sichuan, China), 0.15% phosphates (Food grade, Xi'an harvest Biotechnology Co., Ltd., Xi'an, China), and 0.2% white pepper powder (Sichuan Youjia Foodstuffs Co., Ltd.). Food additives were based on the meat mixture. Meat mixtures of approximately 20 g were stuffed into polyethylene casings of diameter=30 mm, then stored as raw sausages at 4°C for 24 h. Raw sausages were subsequently heated in a water bath from 70 to 80°C, then maintained at 80±1°C for 40 min for cooking. After cooling with tap water, cooked sausages were stored at 4°C overnight.

Color measurement Color measurement was carried out following a previous protocol (1). Chicken sausage color was measured using an automatic colorimeter (WB-2000 IXA; Beijing Kangguang Instrument

Co., Ltd., Beijing, China). Specifically, cooked sausages were cut to 1.0 cm lengths and color was determined immediately. Six measurements for each of 3 replicates were expressed as L*, a* and b* values. The overall lightness or darkness was determined as the L* value (0=black, 100=white). The red (positive=a+) and green (negative=a-) intensity was represented as the a* scale, and the yellow (positive=b+) and blue (negative=b-) intensity was represented as the b* scale.

Residual nitrite Residual nitrite contents were determined following the method described by the National Food Safety Standard of China for Determination of nitrite and nitrate in food (GB 5009.33-2010). Three hundred mL of 70°C distilled water was added to 5.0±0.01 g of minced chicken sausage and heated in a water bath at 100°C for 15 min for cooking. Subsequently, cooked sausage samples were cooled to room temperature in a cool water bath. The minced meat suspension was then filtered with medium filter paper and a supernatant was obtained. Then, 5.0 mL of a 10.6% (w/v) potassium ferrocyanide (AR; Sinopharm Chemical Reagent Co., Ltd.) solution and 5.0 mL of a 22% (w/v) zinc acetate (AR; Sinopharm Chemical Reagent Co., Ltd.) solution were added to the supernatant. Subsequently, the mixture was diluted with deionized water to 500 mL and allowed to stand for 30 min, then filtered with medium filter paper. After discarding 30 mL of filtrate collected at the beginning stage, the rest was used for analysis.

A diazo compound was generated via a reaction of nitrite with 0.4% (w/v) *p*-aminobenzenesulfonic acid. Subsequently, the resulting compound was coupled with 0.2% (w/v) naphthylethylenediamine hydrochloride for development of a purple compound. The absorbance of the solution at 538 nm was obtained with a UV-1600 spectrophotometer (Rayleigh Analytical Instrument Corp., Beijing, China) against a blank that contained all reagents minus meat. The nitrite content was determined following an external standard method in triplicate.

Determination of nitroso pigment contents Nitroso pigment analysis and total pigment analysis of chicken sausage were performed following a previous protocol (9). Nitroso pigments were extracted using a mixture of acetone (AR; Sinopharm Chemical Reagent Co., Ltd.) and water. Hydrochloric acid (AR; Sinopharm Chemical Reagent Co., Ltd.) was used for extraction of total pigments from chicken sausage. The nitroso pigment contents in chicken sausages were calculated from nitroso pigment and total pigment contents in triplicate.

Total pigment analysis Ten g of minced chicken sausage was placed in brown bottle and 40 mL of acetone, 2 mL of distilled water, and 1 mL of hydrochloric acid were added. The mixture was held in the dark for 1 h for extraction of all pigments. After filtration of the mixture with medium filter paper, the absorbance of the filtrate was recorded with a UV-1600 spectrophotometer (Rayleigh Analytical Instrument Corp.) at 640 nm (A_{640}) against a mixture consisting of

40 mL of acetone (AR; Sinopharm Chemical Reagent Co., Ltd.), 2 mL of distilled water, and 1 mL of HCl (AR; Sinopharm Chemical Reagent Co., Ltd.). The total pigment content was calculated as:

$$\text{Total pigments (ppm)} = 680 \times A_{640}$$

Nitroso pigment analysis To 10 g of minced chicken sausage in a brown bottle, 40 mL of acetone, and 3 mL of distilled water were added. The mixture was stirred with a glass rod for 5 min, and then filtered with medium filter paper. The absorbance of the filtrate was obtained with a UV-1600 spectrophotometer at 540 nm (A_{540}) against a mixture of 40 mL of acetone and 3 mL of distilled water.

The myoglobin content in chicken meat is low and can be neglected, unlike the myoglobin content of COHb. Theoretically, NH is the main ingredient in nitroso pigments. The nitroso pigment contents were determined as:

$$\text{Nitroso pigment (ppm)} = 290 \times A_{540}$$

Determination of pigment amounts extracted from sausages of different treatments

Pigment extraction from chicken sausage was carried out following a previous method (10) with modification. Specifically, 20.0 g of minced chicken sausage treated with 0.6% COHb or a combination of 0.6% COHb and 0.006% NaNO₂ in a brown flask was defatted via extraction using 100 mL of petroleum ether (AR; Sinopharm Chemical Reagent Co., Ltd.) in the dark at 4°C for 24 h. Subsequently, sausage samples were subjected to extraction using 150 mL of acetone for 10 min. Extracts were subsequently filtered through medium filter paper and acetone was removed from filtrates prior to subsequent extraction using 30 mL of ethyl acetate (AR; Sinopharm Chemical Reagent Co., Ltd.) twice. Then, separating the organic layer with a separatory funnel and removing ethyl acetate with a rotary evaporator (RE-52A; Shanghai Yarong Biochemical Instrument Factory, Shanghai, China), the molten pigment was obtained for subsequent analysis.

Visible absorption spectra of the pigment samples were measured using a spectrophotometer (1). Spectra of pigments diluted using ethyl acetate (AR; Sinopharm Chemical Reagent Co., Ltd.) were recorded with a spectrophotometer (Unico UV-2802; Unico (Shanghai) Instrument Co., Ltd.) from 300 to 700 nm at a scanning rate of 100 nm/min with a spectrophotometer resolution of ± 0.5 nm. Ethyl acetate was used as a blank. The measurement was repeated twice.

Statistics analysis Statistical analysis was performed using Microsoft Office Excel 2003. Data were expressed as mean $\pm$ standard deviation (SD). An analysis of variance (ANOVA) was used to determine significance differences between sausage samples at $p < 0.05$. When a significant effect was obtained, Duncan's multiple range test was used for comparison of sample means.

Results and Discussion

Color The objective of this study is to clarify the role of COHb in improvement of meat product color in the presence of NaNO₂. In order to avoid interference of endogenous pigments, such as myoglobin, chicken breast was used as a raw material for preparation of sausage due to low pigment content (11).

Sausages treated with COHb were visually darker and sausages containing more added COHb were darker (Fig. 1). Similarly, the color of mortadellas reportedly became darker when COHb was added (7), possibly related to changes in the Hb content. Mielnik and Slinde (12) observed that blood imparted a dark color for sausages to which it was added. Slinde (13) speculated that the color of black salami sausage could be attributed to oxidation of Hb. In this study, COHb, or Hb existing in added COHb, dissociated, which led to a darker sausage. However, for sausages treated with NaNO₂ alone, darkening resulted in a change in the final color to brown. Sausages treated with a combination of COHb and NaNO₂ were darker than controls, but lighter than sausages treated with COHb alone.

Addition of COHb resulted in a significant ($p < 0.05$) reduction in L* values in both the presence and absence of NaNO₂ (Fig. 2), compared with controls. Reduction in L* values suggested that sausages were visually darker, as mentioned above. Sausages with more added COHb exhibited significantly ($p < 0.05$) lower L* values, indicating that more COHb may have dissociated, consistent with previous reports (1,2,7). Addition of NaNO₂ had no significant ($p > 0.05$) effect on L* values in both the absence and presence of COHb.

Sausages treated with COHb alone were visually redder than controls (Fig. 1). COHb does not easily oxidize to hemichrome owing to a strong affinity between CO and Hb (14), and formation of hemichrome decreased sausage redness. Thus, a redder color may have been due to the stability of COHb. Sausages treated with a

Fig. 1. Visual color of sausages with different treatments

Fig. 2. CIE color values of sausages with different treatments ($n=3$)

combination of COHb and NaNO₂ were redder than sausages treated with COHb alone, indicating that COHb and NaNO₂ exerted a synergistic influence on the visual color of chicken sausage, in agreement with previous reports (2,7). The synergistic effects of COHb and NaNO₂ on visual redness may have been due to the yield of NH by a reaction between added COHb and NO. Under conditions in this study, reductants in ginger powder and chicken meat may have been enough to reduce nitrite to NO. The yield of NH was also related to lower residual nitrite contents in sausages treated with a combination of COHb and NaNO₂ than the total of residual nitrite contents in both sausages treated with the same level of COHb and in sausages treated with NaNO₂ (see below) and (b) a combined treatment with COHb and NaNO₂ increased sausage nitroso pigment contents (see below).

Addition of COHb alone led to a significant ($p<0.05$) increase in a^* values, compared with controls (Fig. 2). Effects of COHb on a^* values depended on COHb levels. Sausages with more COHb exhibited significantly ($p<0.05$) higher a^* values, compared with controls, regardless of the absence or presence of NaNO₂, in agreement with visual colors. Addition of NaNO₂ alone did not significantly ($p>0.05$) increase a^* values, compared with controls perhaps because chromoprotein levels in chicken breast are low (11). Therefore, addition of NaNO₂ alone did not form enough pinkish-red NH to increase a^* values. NH is a product of a reaction between chromoproteins, mainly hemoglobin and myoglobin, and NO, followed by thermal denaturation (15). Usually, NH exhibits a pinkish-red color that can be characteristic of cured meat products (16). However, addition of NaNO₂ resulted in a significant ($p<0.05$) increase in a^* values in the presence of COHb, indicating that COHb and NaNO₂ had a synergistic influence on a^* values. Similarly, COHb and NaNO₂ reportedly synergistically increased a^* values of chicken sausage (2) and mortadella (7).

Treatment with COHb or a combination of COHb and NaNO₂, resulted in significantly ($p<0.05$) lower b^* values than for controls

Fig. 3. Residual nitrite contents of sausages with different treatments ($n=3$)

(Fig. 2). Sausages with more COHb added exhibited significantly ($p<0.05$) lower b^* values in the presence and absence of NaNO₂. Significantly ($p<0.05$) lower b^* values were observed in COHb-treated pork sausage (2), compared with controls, while only slight variations in b^* values were reported in 100 g meat loaves with up to 7 g of blood addition (12). Compared with sausages treated with the same levels of COHb alone, sausages treated with a combination of COHb and NaNO₂ exhibited significantly ($p<0.05$) lower b^* values.

A combined treatment of COHb and NaNO₂ decreased L^* values and increased a^* values, indicating that (a) formation of NH probably occurred via a reaction between COHb and NaNO₂, and (b) dissociation followed by oxidation of COHb also occurred during processing of chicken sausage. Therefore, the residual nitrite content, the pigment content, and pigment spectra were further investigated.

Residual nitrite content Addition of NaNO₂ caused significant ($p<0.05$) increases in residual nitrite contents of all sausages, compared with controls (Fig. 3). Addition of COHb also increased residual nitrite contents, but the increment was not significant ($p>0.05$), compared with controls. Sample 5 exhibited significantly ($p<0.05$) lower residual nitrite contents than Sample 4. Also, residual nitrite contents in Sample 6 were significantly ($p<0.05$) lower than the total nitrite content in Samples 3 and 4. Residual nitrites in meat products and other foods are a potential health risk (17) and partial replacement of nitrite with COHb contributed to enhancement of chicken sausage safety. Binding of CO and Hb is reversible (18), and the affinity of NO to heme iron is stronger than the affinity of CO (19). Thus, formation of NF may have been responsible because COHb and NaNO₂ synergistically increased a^* values (see above) and nitroso pigment contents (see below).

On the other hand, residual nitrite contents in Sample 5 reached 2.31 ppm, which was higher than for Sample 2 or 3. Moreover, further increases in COHb contents did not decrease residual nitrite contents in Sample 6. Dissociation of COHb (20), oxidation of Hb (21), and binding of NO with ferrous iron rather than with ferric iron in the

Fig. 4. Pigment contents of sausages with different treatments ($n=3$)

porphyrin ring located in Hb (22), may have caused COHb to partially reaction with NaNO_2 . This can also be confirmed by the fact that nitroso pigment contents in Samples 5 and 6 were less than 0.01 g per 1 kg of total weight, a theoretical value if NaNO_2 reacts fully with COHb (see below).

Pigment contents Addition of COHb alone significantly ($p<0.05$) increased total pigment contents, compared with controls. Moreover, sausages with more added COHb exhibited significantly ($p<0.05$) higher total pigment contents (Fig. 4). Addition of NaNO_2 alone did not significantly ($p>0.05$) affect total pigment contents in both the presence and absence of COHb.

Addition of COHb significantly ($p<0.05$) increased nitroso pigment contents in the presence of NaNO_2 , perhaps due to formation of nitrosomyochromes via a reaction of COHb and NO. Formation of nitrosomyochromes was observed in meat products processed using *Lactobacillus fermentum* fermentation, such as *L. fermentum* AS1.1880 (23) and *Lactobacillus* spp. (24). However, COHb had no significant ($p>0.05$) effect on nitroso pigment contents in the absence of NaNO_2 . Therefore, NaNO_2 was necessary for formation of nitroso pigments.

Addition of NaNO_2 reportedly significantly increased nitroso pigment contents in beef frankfurters (9). Addition of NaNO_2 alone had no significant ($p>0.05$) influence on nitroso pigment contents, compared with controls, in this study. Different effects of NaNO_2 on nitroso pigment contents may be due to different meat materials. Chicken breast is usually considered to be white muscle (11), while beef is considered to be red muscle (25). Usually, pigment contents in white muscle are low and may have been insufficient for formation of NH in this study. Therefore, NaNO_2 treatment had no obvious influence on nitroso pigment contents.

COHb was also necessary for an increase in nitroso pigment contents in chicken sausage. Binding of CO and Hb is reversible (18), and NO has a stronger affinity to heme iron than CO (19). Thus, replacement of CO in COHb by NO may have occurred in this study and

Fig. 5. Visible absorption spectra of pigments ($n=2$)

contributed to elevated nitroso pigment contents. Replacement of CO by NO of COHb in sausage may have been related to a decrease in residual nitrite contents and an increase in a^* values in sausages treated with a combination of COHb and NaNO_2 (see above).

In theory, nitroso pigment contents in Sample 5 were not lower than 1 mg/g if added COHb reacted completely with NaNO_2 . However, nitroso pigment contents were 33.83 ppm and 38.57 ppm in Sample 5 and 6 sausages, respectively, indicating that COHb did not react fully with NaNO_2 . Dissociation followed by oxidation of COHb may have occurred in this study. An incomplete reaction between COHb and NaNO_2 can also account for: (a) the samples treated with a combination of COHb and NaNO_2 had lower L^* values than the one treated with NaNO_2 alone (see above), and (b) the samples treated with a combination of COHb and NaNO_2 had lower residual nitrite contents than the one treated with NaNO_2 alone (as mentioned above) for the combined treatment.

Pigment spectra Each derivative myosin (Mb) has a characteristic absorbance spectrum (26). Similarly, each derivative Hb also has a characteristic absorbance spectrum (27). Characteristic absorbance spectra can be used to determine the main pigment ingredients in specific meats. Therefore, pigment extracts from the two sausage samples treated with 0.6% COHb or a combination of 0.6% COHb and 0.006% NaNO_2 , respectively, were analyzed using visible spectrometry in order to reveal changes in COHb added to chicken sausage during processing.

For visible spectral analysis of pigment extract of sausage treated with 0.6% COHb (Pigment 1), no obvious characteristic peak was found in the wavelength range of 450 nm to 650 nm (Fig. 5). The main ingredient of Pigment 1 was not a Mb derivative, based on comparison of characteristic peaks (26), perhaps due to a low Mb content in chicken meat. The Mb content in 100 g of chicken meat is less than 31 mg (28). Therefore, an Hb derivative became the dominant pigment ingredient when 0.6% COHb was added. In

addition, 3 characteristic peaks at 411, 529, and 555 nm were reported in the visible spectrum of COHb (1). Therefore, the main ingredient of Pigment 1 was not ascribed to COHb. The Hb derivative denatures under heating (29), and binding of Hb and CO is reversible (20). Thus, the main ingredient of Pigment 1 may have been a disassociation product of COHb due to thermal denaturation.

Three characteristic peaks located at 475, 537, and 634 nm were observed in the visible spectrum of pigment extract of sausages treated with a combination of 0.6% COHb and 0.006% NaNO₂ (Pigment 2), different from Pigment 1. Addition of NaNO₂ affected the chemical structures of pigment extracts. Peaks at 475 and 537 nm in the visible spectrum of Pigment 2 were reportedly similar to two characteristic peaks of NH, while another peak located at 634 nm was similar to a peak characteristic of acid hematin (30). Thus, NH and acid hematin may be the ingredients of Pigment 2. Therefore, CO of the COHb in chicken sausage was partially replaced by NO, and then thermally denatured to generate NH. Meanwhile, other added COHb was involved in dissociation, followed by oxidation, to generate hematin. The former caused an increase in a* values, and the latter led to a decrease in L* values. Also, added COHb partially reacting with NaNO₂ led to a reduction in residual nitrite contents and an increase in nitroso pigment contents.

In conclusion, addition of both COHb and NaNO₂ synergistically increased a* values and nitroso pigment contents and synergistically decreased residual nitrite contents. Both NH and hematin were the main ingredients of pigments extracted from chicken sausage treated with a combination of 0.6% COHb and 0.006% NaNO₂, which may have contributed to an increase in a* values and a decrease in L* values, respectively. COHb has potential for use in meat product formulations.

Acknowledgments This work was supported by the National Natural Science Foundation of China (21272051, 21542008) and the Anhui Science and Technology Department (1208085MC43).

Disclosure The authors declare no conflict of interest.

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