Nitrosation of Hemoglobin from Animal Blood as a Colorant of Meat Products

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(Received June 17, 1992)

Abstract The purpose was examination of the following: 1) reaction conditions for the preparation of stable nitrosohemoglobin (NOHb) obtained by the nitrosation of hemoglobin (Hb) from animal blood and 2) the possibility of augmenting meat product color by the addition of NOHb which can be prepared easily in the native state and followed by cooking. More than 80% of total Hb in a mixture of 100 mM NaNO₂-100 mM sodium ascorbate (NaAsA) was nitrosated, and NO₂⁻ was found present at 3,600 ppm on the 10th day of storage at 2°C. The nitrosation of Hb in the mixture (25 mM NaNO₂-25 mM NaAsA, pH 4.5) proceeded rapidly, and the color forming ratio was 85% even at 14 days of storage. Added nitrite could not be detected after 3 days of storage. 0.5% and 1% of the NOHb reaction mixture were added to porcine muscle with 2% NaCl, 200 ppm NaNO₂ and 0.1% NaAsA. The sausage was prepared for assessment of color formation. The following results were obtained: Nitroso heme pigments were formed to a greater extent than those of the control (without addition of NOHb) meat product; NOHb in the Hb reaction mixture had a qualitative effect on meat color formation.

Anim. Sci. Technol. (Jpn.) 63 (12) : 1247-1252, 1992

Key words : animal blood, hemoglobin, color formation, nitrosohemoglobin.

Most animal blood from slaughterhouses is used for fertilizer and animal feed even though it is a valuable source of protein. There are many problems involved in the use of blood as food material. The red cell fraction, erythrocytes, though high in nutritional value, is difficult to use as a food component compared to that of the plasma fraction, according to reports on the utilization of iron by protease treatment¹⁾, fermented seasoning^{5,9)} and decolorized globin¹⁶⁾. The use of erythrocytes in food is hindered most due to the dark brownish color it imparts following heat treatment even when present in a minute amount. The reason for this is that Hb (hemoglobin), the major part of the erythrocytes, is converted to heat-denatured methemoglobin. Attempts have been made to stabilize Hb so that it will retain

its red color as a coloring agent for processed meat products. MARUYAMA⁷⁾ carried out the nitrosation of Hb with nitrite and ascorbate, cooked the pigment and solubilized the heme group by the decomposition of globin portion with protease treatment. SHAHIDI and PEGG¹²⁻¹⁴⁾ conducted the nitrosation of Hb from animal blood and obtained nitrosohemochrome by heating NOHb (nitroso hemoglobin). Though it was the purpose in these studies to use this pigment as a colorant for meat products, heat-denatured NOHb (nitrosohemochrome) was noted to be unstable and oxidized to a brown color with the passage of time¹⁵⁾. ITO²⁾ carried out the electrolytic reduction of heme proteins to obtain meat colorants and augment the red color of meat products through their addition during curing.

To utilize the red cell fraction from slaughtered animals, this investigation was made of the following using commercial Hb powder : 1) reaction conditions for preparing stable NOHb and 2) the possibility of augmenting meat product color by the addition of NOHb readily obtainable in the native state and then by cooking.

Materials and Methods

Preparation of Hb solution : Hb powder from cattle blood (Harimex Co. Ltd.) was dissolved in H_2O , and glucose at concentrations of 18% and 40% respectively. The solution was stored overnight at $2^\circ C$. The mixture was subsequently centrifuged at $9,000 \times G$ for 10 min at $4^\circ C$ and the supernatant was used as Hb solution.

Preparation of Hb reaction mixture : Hb reaction mixtures were prepared by mixing the Hb solution (50 g) with NaCl (2%), $NaNO_2$ (3.1–100 mM) and sodium ascorbate (NaAsA, the same level as $NaNO_2$). The final weight of each mixture was 53 g, and each was analyzed for NOHb formation during storage at 2 or $20^\circ C$.

Preparation of pork sausage after adding the Hb reaction mixture : 0.5 and 1% Hb reaction mixtures after several days of storage were added to porcine muscle (*M. longissimus thoracis*, relatively lighter color) with 2% NaCl, 0.02% $NaNO_2$ and 0.1% NaAsA. Sausage was prepared for color assessment and comparison was made with a control sample containing all the curing agents but NOHb.

Color forming ratio determination : The color forming ratio (CFR) of each mixture was determined by the method of the authors^{10,11}, and expressed as % NOHb to total Hb. In the case of sausage to which NOHb had been added, total and nitroso heme pigments were extracted with 75% acetone–0.7% HCl and 75% acetone respectively, in the same manner as for the Hb reaction mixture. Hunter values were also measured by a color difference meter (Nip-

pon Denshoku Kogyo Co. Ltd., Model ND-1001 DP).

Determination of residual nitrite : The NOHb extract obtained with 75% acetone was used to determine nitrite content according to the method of MIRNA and SCHÜTZ⁸.

Results and Discussion

Effects of $NaNO_2$ and NaAsA concentrations on Hb nitrosation and residual nitrite : The concentrations of $NaNO_2$ and NaAsA for this study were decided on based on the reports of LEE and CASSENS⁶) and SHAHIDI and PEGG^{13–15}). More than 80% of the total Hb of a mixture (pH 7.3–7.6) of 100 mM $NaNO_2$ –100 mM NaAsA was nitrosated on the 1st day of storage (Fig. 1). CFR of the reaction mixture showed no change even after 1 month of storage at $2^\circ C$ or 10 days at $20^\circ C$ in the dark. Though NOHb in the mixture remained stable, NO_2^- content was 3,600 ppm at 10 days of storage at $2^\circ C$, this being 76% of the original amount. NOHb content in the reaction mixture with 25 mM

Fig. 1. CFR of the Hb reaction mixture.

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NaNO_2 -25 mM NaAsA was 80% in CFR at 8 days of storage, and its formation occurred in essentially the same manner at 100 mM.

This value is in agreement with general type CFR of cooked cured meat products^{10,11}. Residual nitrite decreased in the 25 mM NaNO_2 -NaAsA reaction mixture with storage, and NO_2^- was 480 ppm after 8th day of storage (data not shown). No residual nitrite was detected in the reaction mixture of 12.5 mM and was below the level of NaNO_2 -NaAsA after reaction for 3 days. However, CFR of these mixtures did not exceed 80%, indicating the nitrosation of Hb not to easily proceed with decrease in nitrite and ascorbate content. The

Fig. 2. Effects of pH on CFR and residual nitrite content of the Hb reaction mixture (25 mM NaNO_2 -25 mM NaAsA).

* Adjusted with lactic acid.

Fig. 3. Effects of temperature on CFR and residual nitrite content of the Hb reaction mixture (25 mM NaNO_2 -25 mM NaAsA, pH 4.5).

Fig. 4. Absorption spectra of the Hb reaction mixture.

following experiments were thus conducted to determine the conditions most suitable for using a 25 mM NaNO₂-25 mM NaAsA reaction mixture.

Effects of pH on CFR and residual nitrite content of the reaction mixture: Fig. 2 shows CFR and NO₂⁻ of the reaction mixture with 25 mM NaNO₂-25 mM NaAsA at varied pH adjusted with lactic acid. Compared with the control sample with unadjusted pH, CFR of the pH 4.5 mixture exceeded 90% by the 3rd day of storage at 2°C. More than 85% in CFR was maintained even for 14 days. Added nitrite could not be detected after 3 days of storage. CFR of the reaction mixture at pH 4.5, 20°C had decreased on the 10th day of storage, and no residual nitrite could be detected during storage (Fig. 3).

The presence of an intermediate (nitrosoascorbic acid) as reported by IZUMI *et al.*^{3,4)} may possibly be the reason for the absence of free nitrite. They consider that such an intermediate may be formed by the interaction of

nitrite and ascorbic acid with concomitant increase in the rate constant of this reaction with decrease in pH in model meat-curing brine. This appeared to be the case in the present study. Nitrite would thus appear to easily react with ascorbate with decrease in pH, with consequent acceleration of the nitrosation of Hb.

The absorption spectra of NOHb formed with the 25 mM NaNO₂-25 mM NaAsA reaction mixture are shown in Fig. 4. Before the reaction, spectral scanning indicated a pattern for the formation of oxy- and met-hemoglobin due to the presence of both pigments. At 5 days, 2°C and pH 4.5, the typical spectral pattern of NOHb was observed. Most Hb was confirmed to be nitrosated under the experimental conditions used.

Effects of Hb reaction mixture on experimental sausages: The Hb reaction mixture (CFR: 86.8%) was added to minced porcine muscle, and sausage was prepared (Table 1). Following 0.5 and 1% additions of the reaction

Table 1. Effect of addition of Hb reaction mixture to experimental sausages (I)

Sample ¹	pH	Hunter value			CFR (%)	Residual NO ₂ ⁻ (ppm)	
		L	a	b			
① Control { NaCl 2(%) NaNO ₂ 0.02 NaAsA 0.1	5.87	60.2	14.5	8.2	86.8	15.3	
② ①+Hb reaction mixture ²	0.5%	5.88	56.5	15.2	8.4	89.0	16.0
③ ①+Hb reaction mixture ²	1.0%	5.84	54.0	15.6	8.5	86.2	17.1

¹ Added to porcine muscle (*M. longissimus thoracis*) → Stuffed into polyvinylidene chloride casing (50 mm) → Cooked at 75°C for 60 min.

² Contained 10.96% Hb, 2% NaCl, 25 mM NaNO₂, 25 mM NaAsA and 40% glucose (pH 4.8), and kept for 10 days at 2°C after preparation (CFR=86.8%).

Table 2. Effect of addition of Hb reaction mixture to experimental sausages (II)

Sample	Addition level of Hb (%)	Nitroso heme pigment (NOHP) ¹		Increase in NOHb		Increase rate of NOHP (B-A)/A × 100 (%)
		-Hb (A) (%)	+Hb (B) (%)	Observed (B-A) (%)	Calculated ² (%)	
Pork	0.5	0.129	0.185	0.056	0.049	43.4
sausage	1.0	0.129	0.225	0.096	0.097	74.4

¹ Expressed as the level of NOHb. ² Level of NOHb from Hb reaction mixture added.

mixture, Hunter a-values as well as CFR increased. Redness was also greater than that of the control. In Table 2, the formation of nitroso heme pigments can be seen to be greater than that of the control meat product (without NOHb). The nitroso heme pigments increased to 43.4 and 74.4% respectively after adding Hb at 0.5 and 1%. NOHb in the reaction mixture thus augments the color of cooked cured meat products. Although similar results were obtained by SHAHIDI and PEGG¹²⁻¹⁴⁾, their preparation was denatured NOHb by preheating, and it has been pointed out that such a preparation is unstable due to oxidation with time¹⁵⁾. Residual nitrite in the sausage did not increase significantly by the addition of the reaction mixture, which is in good agreement with IZUMI and CASSENS³⁾. They injected brine containing NaNO₂ and ascorbic acid at pH 4.0 or 4.5 and as a result could obtain bacon with an extremely small amount of residual nitrite.

The direct preparation of NOHb from Hb of animal blood should be conducted so that NOHb can be used as a colorant in meat processing and reduce nitrite content in processed meat products.

Acknowledgement

The present study was supported in part by a research grant from the Ito Foundation, Tokyo. The authors thank Mitsubishi Kasei Co. Ltd. for providing the cattle hemoglobin powder.

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屠畜血液ヘモグロビンのニトロソ化と肉製品への利用

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屠畜血液のヘモグロビン (Hb) のニトロソ化反応条件について，まず検討を行なった．牛血液粉末より調製した Hb 溶液に NaNO_2 とアスコルビン酸ナトリウム (NaAsA) をそれぞれ 100 mM 添加した場合，反応 1 日目において全 Hb の 80% 以上がニトロソ化した．この反応液中のニトロソヘモグロビン (NOHb) はかなり長時間安定に保たれたが， NO_2^- は 2°C において 10 日目に約 3,600 ppm が残存した．反応条件について検討し得られた 25 mM NaNO_2 -25 mM NaAsA ，pH 4.5 の Hb 反応液では，Hb のニトロソ化が速やかに進行し，発色率は 2°C で貯蔵 14 日目においても 85% 以上を保持し，一方， 20°C では 10 日目において明らかな減少が観察された．貯蔵中の Hb 反応液に NaNO_2 は検出されなかった．次に，この条件で調製した Hb 反応液を，原料肉に対し 0.02% NaNO_2 -0.1% NaAsA とともに 0.5 および 1% 添加し試作したソーセージの発色状態を調べた．その結果，Hb 反応液の添加によって発色は増強され，定量したソーセージ中のニトロソヘム色素の増強割合から，添加した Hb 反応液中の NOHb は，ほぼ定量的にソーセージの発色に寄与していることが確認された．

日畜会報，63 (12) : 1247-1252, 1992