Extractability of native nitroso heme pigments from cured meat

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Introduction

It is well known that the characteristic pinkish color in meat products is due to the nitrosation of endogenous heme pigments to which nitrite has been added. The colored pigments are generally extracted and measured spectrophotometrically. A widely used method for this is Hornsey's acetone procedure (1956). Okayama and Nagata (1978) showed that cooked cured meat pigments, i.e., denatured nitroso heme pigments, could be quantitatively extracted with 75% acetone by this procedure with slight modification. The absorbance of the extract was measured with much greater sensitivity at 395 nm and expressed as color forming ability (CFA). However, very little information is available for the quantitative extraction of native (i.e., undenatured) nitroso heme pigments (NOHP) from muscle which shows cured meat color when not subjected to heat.

In this research, an examination was made of the following: 1) extractability with 75% acetone of nitrosomyoglobin (NOMb) prepared using muscle pigment myoglobin (Mb), 2) extractability of native NOHP from commercial raw ham and cured meat with water, using 75% acetone, and 3) the effects of endogenous factors on extractability.

Materials and Methods

Preparation of NOMb. NOMb was prepared in a brown test tube from a reaction medium containing 0.4mM equine Mb (Sigma Chemical Co., type 1), 50mM $NaNO_2$ and 50mM sodium ascorbate (NaASC) in M/35 Veronal-acetate buffer (pH 5.5) according to the procedure of Lee and Cassens (1976) with slight modification. The reaction medium constituents were mixed and incubated at room temperature (22-23°C) in a dark room. The time course of the absorption spectrum of NOMb formed was recorded immediately following a 1:10 dilution of the solution with the buffer. The maximum formation of NOMb was determined by monitoring the optical density at 547 nm.

Extractability of NOMb with 75% acetone. One ml of the reaction medium containing NOMb was mixed with 1.5 ml of distilled water and 7.5 ml of acetone to make the final concentration of acetone 75%, and the NOMb was extracted at 0°C for 10 min with occasional stirring. The extract was filtered through Toyo No.6 filter paper and extractability was evaluated from the absorption spectrum. Metmyoglobin (MetMb) was also prepared from a mixture containing 0.4mM Mb and 50mM $NaNO_2$; a portion of it was heated at 75°C for 60 min to form denatured MetMb (DMetMb). Both of their extractabilities with 75% acetone were investigated.

Recovery of added NOMb from cured meat product. The NOMb solution was added to pork sausage and its recovery with 75% acetone was investigated. Pork sausage was prepared from minced porcine skeletal muscle (M. longissimus thoracis, 24 hr postmortem). The minced muscle was adjusted to pH 5.5 with lactic acid and mixed with brine containing sodium chloride and sodium nitrite to give levels of 2% NaCl and 100 ppm $NaNO_2$. After being stuffed into a polyvinylidene chloride

coefficient of NOMb at 547 nm [13.3 (Fox and Thomson, 1963)].

Figure 2 shows the absorption spectra of 75% acetone extracts from the NOMb reaction medium incubated for 60 min. DNOMb was also prepared by heating the reaction medium at 75°C for 60 min and extracted with 75% acetone. No significant difference in the absorption spectra of the 75% acetone extracts could be observed between NOMb and DNOMb and they had the same optical density at 395 nm, one of the absorption maxima. Hornsey (1956) reported that the extractability of nitrosohemoglobin (NOHb) with 80% acetone (measured at 540 nm) was only half that of denatured NOHb. However, all NOMb was solubilized and extracted with 75% acetone under the present conditions. Anderton and Locke (1955) and Hornsey (1956) confirmed that DNOMb exists in acetone solution as a nitroso heme-acetone complex. Judging from the results in Fig. 2, NOMb as does DNOMb changes into a nitroso heme-acetone complex in 75% acetone. MetMb and DMetMb could not be extracted with 75% acetone (data not shown), thus indicating that the oxidized heme pigments exerted no influence or interference toward the absorbance measurement of NOMb or DNOMb.

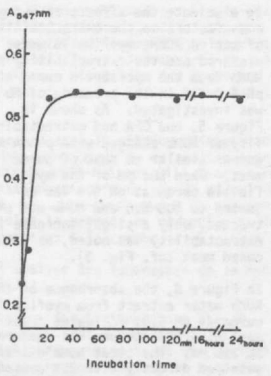

Fig. 1. Formation of NOMb from a reaction medium containing Mb, nitrite and ascorbate at pH 5.5. At specified intervals, a 1 ml sample was diluted to 10 ml with the buffer solution and the absorbance was quickly monitored at 547 nm.

Table 1 shows the recovery of the added NOMb solution (60 min incubation) from pork sausage. Recovery from the sample to which 1 and 2 ml of NOMb had been added was 98.3 and 99.4%, respectively (Average 98.8%). These results show that NOMb can be quantitatively extracted with 75% acetone from pork sausage.

Table 2 shows the extractability of native NOHP from commercial raw hams. Since raw hams in practice are processed at lower temperature (≲ 20°C), most of their heme pigments were considered to be in the native state. However, the extractability of native NOHP was generally low and varied, ranging from 8 to 75%. No increase in extractability was observed even when the homogenate was kept overnight at 0°C. The

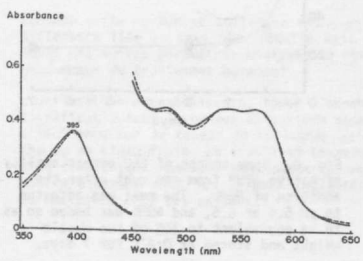

Fig. 2. Absorption spectra of 75% acetone extracts from the reaction medium. The reaction medium contained NOMb (---) or DNOMb (——). To record the spectra from 350 to 450 nm, each extract was diluted 1:10 with 75% acetone.

casing, the mixture was cooked at 75°C for 60 min. The NOMb solution following incubation for 60 min was diluted 1:4 with M/35 Veronal-acetate buffer (pH 5.5) and quickly mixed with 5 g of the ground sausage preparation in a 50 ml volumetric flask. 37.5 ml of cold acetone were then added to the mixture which was made to a final volume of 50 ml using distilled water. The extract obtained at 0°C over a period of 30 min was measured for absorbance at 395 nm and the recovery of added NOMb was determined from the absorbance value.

Commercial raw hams purchased from 7 meat packers and trimmed free of external fat and connective tissues were minced twice through a plate with holes 3.2 mm in diameter. The NOHP was extracted with water and the degree of extraction was determined using the 75% acetone.

Preparation of cured meats. Normal porcine skeletal muscle (M. longissimus thoracis, 24 hr postmortem) ranging in pH from 5.5 to 5.7 was minced twice and mixed with brine so as to contain 100 ppm $NaNO_2$, 0.1% NaASC and 2% NaCl. The amount of added brine was 10% by weight of the minced muscle and the mixture was stored at 0-2°C for 7 days in a dark room and measured for extractability of native NOHP. Meat samples cured at pH 5.0-6.5 were also prepared with lactic acid and NaOH.

Preparation of myofibrils. Minced porcine muscle was homogenized with three times the volume of 0.85% NaCl solution and maintained at 0°C for 60 min. The homogenate was then centrifuged at 10,000 x g for 60 min. The resulting residue was washed twice with cold 0.85% NaCl solution and made up to the original weight of the muscle sample. The sample was adjusted to pH 5.0-6.5 and cured in the presence of 0.1% Mb by the same procedure described in the preparation of cured meat and analyzed for extractability of native NOHP (NOMb).

Extractability of native NOHP with water. Five grams each of the commercial raw ham, cured meat and cured myofibrils with Mb were homogenized with 12.5 ml of water in a brown stoppered centrifugal tube and held at 0°C for 60 min with occasional shaking. The tube was then centrifuged at 4,500 rpm for 15 min. The supernatant was mixed with 37.5 ml of cold acetone in a 50 ml volumetric flask and made up to 50 ml with distilled water. After being kept at 0°C for 30 min, the suspension was filtered through Toyo No.6 filter paper and the filtrate was measured for absorbance at 395 nm. This extraction procedure was repeated twice and the native NOHP content was estimated from the sum of the absorbances of the 75% acetone extracts at 395 nm. Acetone was added directly to 5 g of the sample to extract the total NOHP and the 75% acetone extract was measured for absorbance at 395 nm (CFA (Ando, 1973; Sakata et al., 1980)]. The percentage of water extracted NOHP to the total NOHP was difined as the extractability of native NOHP. The raw porcine muscle and myofibrils were each adjusted to pH 5.5 or 6.5 and then mixed with the NOMb solution prepared by the above method in an amount equivalent to 10% of the sample weight. The resulting mixtures were kept at 0-2°C for 7 days in the presence of 2% NaCl. During this period, the extractability of NOHP was determined.

Results and Discussion

The NOMb spectrum with maximal absorbance at 547 and 578 nm was observed during incubation of the reaction medium containing Mb, $NaNO_2$ and NaASC. The absorbance at 547 nm (β peak) rapidly attained a maximum at which it remained as long as 24 hr (Fig. 1). Mb in the reaction medium after 20 min incubation was judged perfectly nitrosated on the basis of a calculation using the mMolar extinction

Table 1. Recovery of added NOMb from cured meat product

Sample	Absorbance at 395 nm				Recovery (%)
	-NOMb(A)	+NOMb(B)	Calculated	Recovered (B-A)	
Pork sausage	0.432	0.604[a)]	0.175	0.172	98.3
	0.432	0.780[b)]	0.350	0.348	99.4
					Av. 98.9

a) One ml of NOMb soln. was added to the sample; b) two ml of NOMb soln. were added; values shown as the mean of three experiments.

reason for the low extractability was considered to be that the denaturation of the heme pigments occurred during meat processing and/or that native heme pigments could not be sufficiently extracted by the present procedure.

For confirmation of these possibilities, water extraction carried out on the cured meat. As evident from Table 3, the extractability of native NOHP was low (about 25%) as also noted in the case of the raw hams (cf. Table 2), and no significant difference was observed in extractability between cured meat sample with or without NaCl (data not shown). Most of the heme pigments could be easily extracted with water from uncured raw meat. However, extracting native NOHP from cured meat was found to be difficult in spite of its high solubility in water. These findings are evidence that the extractability of NOHP is low in raw hams.

Figure 3 shows the CFA and extractability of native NOHP from meat cured for 7 days at pH 5.0-6.5. The CFA decreased but the extractability increased in proportion to pH at the time of curing. This indicates that instances of higher extractability from raw ham samples (Table 2) may possibly be due to the high pH at the time of curing. Even when the pH of meat cured at pH 5.5 rose to 6.5 at the time of water extraction, there was only a slight increase in extractability. The extractability of NOMb added to the raw meat at pH 5.5 also remarkably decreased with time, whereas all of the NOMb added at pH 6.5 could be extracted without adsorption to the meat (Fig. 4).

Table 2. Extractability of native NOHP from raw hams

Sample	Absorbance at 395 nm		Extractability (A/B, %)
	Extracted NOHP (A)	Total NOHP (B)	
A	0.094	0.646	14.6
B	0.221	0.596	37.1
C	0.068	0.457	14.9
D	0.072	0.606	11.9
E	0.068	0.661	10.3
F	0.514	0.689	74.6
G	0.064	0.781	8.2

Table 3. Extractability of native NOHP from cured meats

Sample	Absorbance at 395 nm		Extractability (A/B, %)
	Extracted NOHP (A)	Total NOHP (B)	
A	0.071	0.306	23.2
B	0.071	0.302	23.5
C	0.065	0.430	15.1
D	0.180	0.495	36.4
E	0.047	0.184	25.5
F	0.098	0.442	22.2
			Mean ± SD 24.4 ± 6.9

To elucidate the effects of endogenous factors on the extractability of native NOHP, myofibrils were prepared and the extractability of NOMb from the myofibrils cured at pH 5.0-6.5 in the presence of Mb was investigated. As shown in Figure 5, the CFA and extractability of NOMb changed with pH in a manner similar to that of cured meat. When the pH of the myofibrils cured at pH 5.5 was adjusted to 5.0-6.5 and NOMb was extracted, only a slight increase in extractability was noted, as with cured meat (cf. Fig. 3).

In Figure 6, the absorbance of the NOMb water extract from myofibrils cured at pH 5.5, following treatment with 75% acetone was measured at 395 nm. The total NOMb extract obtained directly with 75% acetone was also measured at 395 nm. The total NOMb gradually increased with curing time but the absorbance of extracted NOMb with water was considerably low though there was a slight increase in the first two days of curing.

In the case of NOMb added to the myofibrils (Fig. 7), a remarkable variation in the extractability of NOMb with pH (5.5 and 6.5) similar to that observed for raw meat was noted (cf. Fig. 4). These results indicate that a reaction between myofibrils and native NOHP occurs during meat curing.

Bendall and Wismer-Pedersen (1962) reported that myofibrillar proteins in pale, soft and exudative (PSE) porcine muscle are tightly surrounded by denatured sarcoplasmic proteins. Scopes (1964) noted that denatured sarcoplasmic proteins in PSE muscle lowered the extractability of myofibrillar proteins by binding with them. In our previous paper (Sakata et al., 1981; 1983), the decline in color formation of PSE muscle may possibly have resulted from an interaction between heme pigments and myofibrils in muscle postmortem under the conditions of low pH and relatively high temperature. These physicochemical characteristics of muscle proteins under PSE conditions are not considered to have any direct relation to the phenomenon observed in cured meat in this paper, since no denaturation occurred. However, it may be assumed that myofibrils react with heme pigments, one class of sarcoplasmic proteins, under certain conditions. Such an interaction may result in no virtual loss of NOHP from cured meat during water soaking in the course of meat processing.

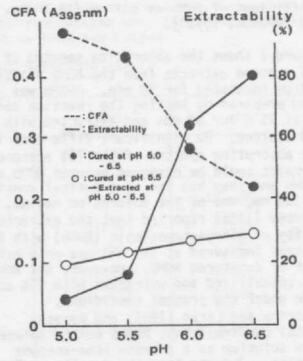

Fig. 3. Effect of pH on the extractability of native NOHP from cured meat and its CFA. The meat was cured with 100 ppm $NaNO_2$, 0.1% NaASC and 2% NaCl at 0-2°C for 7 days in a dark room.

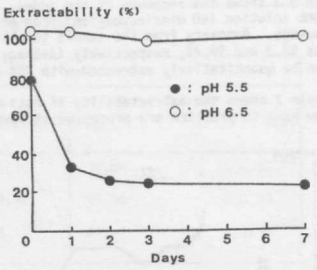

Fig. 4. Time course of the extractability of native NOHP from raw meat after the addition of NOMb. The meat was adjusted to pH 5.5 or 6.5, and NOMb was added so as to be equivalent to 10% of the muscle weight and stored at 0-2°C for 7 days.

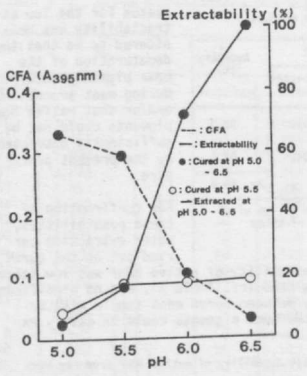

Fig. 5. Effect of pH on the extractability of NOMb from cured myofibrils and its CFA. The myofibrils were cured with 100 ppm $NaNO_2$, 0.1% NaASC and 2% NaCl in the presence of 0.1% Mb at 0-2°C for 7 days in a dark room.

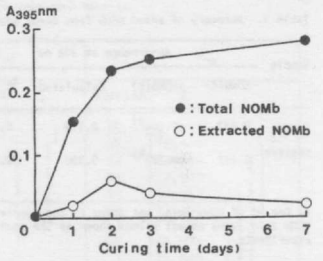

Fig. 6. Total and extracted NOMb from myofibrils cured in the presence of Mb at pH 5.5, and 0-2°C in a dark room.

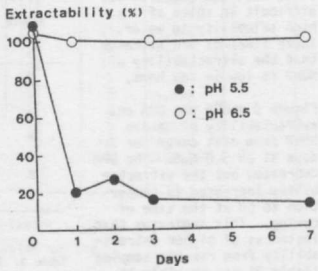

Fig. 7. Time course of extractability of NOMb from myofibrils after addition of NOMb. The preparation and manner of addition of NOMb were the same as in Fig. 4.

The myofibrillar proteins affecting the extractability of native NOHP and a method for separating native NOHP from denatured NOHP quantitatively are being investigated.

Conclusions

It was confirmed that native NOHP could be quantitatively extracted with 75% acetone from processed meat products in the same manner as denatured NOHP. The decline in extractability of native NOHP from cured meat with water accompanying a decrease in pH was found to result from an interaction between native NOHP and myofibrils in cured meat. The present study has also shown that native NOHP cannot be completely extracted under our experimental conditions. A different method must be devised for the separate and simultaneous determination of both native and denatured NOHP.

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