MARKETING AND PRODUCTS

Effects of Sodium Chloride, Phosphate, and Dextrose on the Heat Stability of Purified Myoglobin, Hemoglobin, and Cytochrome c

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ABSTRACT Eight combinations of sodium chloride (0, 2.5%), sodium tripolyphosphate (0, .5%) and dextrose (0, 1%) were dissolved in phosphate buffer containing .5 mg myoglobin (Mb), hemoglobin (Hb), or cytochrome c per milliliter. These solutions were heated to 68 C, 74 C, 80 C, or 85 C to determine ingredient effects on the heat stability of purified Mb, Hb, and cytochrome c.

Salt significantly decreased the heat stability of Mb at all temperatures studied, and Hb at 68 C and 74 C. However, salt significantly increased the heat stability of cytochrome c. Phosphate increased the heat stability of Mb but decreased that of cytochrome c due to a pH increase. Dextrose increased the heat stability of Hb at 68 C, and that of cytochrome c at 85 C.

(Key words: salt, phosphate, myoglobin, cytochrome c, heat stability)

INTRODUCTION

Meat color is a result of the concentration of heme pigments, reactions of the pigments with gaseous elements or compounds, and the structural properties of muscle proteins (Potthast, 1987). The most important meat pigment is myoglobin (Mb). The amount of Mb in meat can vary greatly by kind of animal, age, sex, and muscle types within a carcass (Ginger et al., 1954; Rickansrud and Henrickson, 1967; Froning et al., 1968; Nocito et al., 1973). Fleming et al. (1960) reported that in a well-bled beef rib eye muscle as much as 95% or more of the pigment is accounted for by Mb.

Niewiarowicz et al. (1986) reported that the combined hemoglobin (Hb) and Mb in turkey breast and leg meat were .75 mg/g and 2.65 mg/g meat, respectively. The amount of Hb can vary depending on bleed-out, slaughter method, and preslaughter conditions.

The concentration of cytochrome c is very small compared to that of Mb, but it can play a very important role in the color of the breast meat in birds. The amount of cytochrome c varies greatly among kinds of organs. Of all organs, the heart has a higher cytochrome c concentration than that in other organs (Potter

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and Dubois, 1942; Drabkin, 1950; Lamberg and Barrett, 1973). Schollemeyer and Klingenberg (1962) reported that the amount of cytochrome c in the breast muscle of the pigeon was as much as 720 µg/g meat, whereas the white and smooth muscle of rabbit contain 24 mg/g meat fraction. Gonzalez-Cadavid and Campbell (1967) reported that rat liver contains 123 µg of cytochrome c/g meat and rat muscle contains 110 µg/g meat (Williams and Thorp, 1969).

In fresh meat, color is determined by the state of the heme group in the pigment. Enzymatic reduction of metmyoglobin (met-Mb) was studied by many researchers. Stewart et al. (1965) reported that Mb-reducing activity was increased with the pH increase from 5.1 to 7.1 and with a temperature increase from 3 C to 35 C. The metMb reductase was purified from the muscle of tuna, dolphins, and bovine heart, and was also characterized. Shimizu and Matsuura (1971) reported that metMb reductase could reduce metMb, ferricytochrome c and methemoglobin (metHb) in the presence of β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), β-nicotinamide adenine dinucleotide phosphate (NADH), or methylene blue. With metHb or metMb reductase, the activity of NADPH as an electron donor is higher than that of NADH. The structure of globin also has a great effect on the state of the heme (Perutz et al., 1976). Low temperature, low pH, and a mitochondrial respiration inhibitor could maintain the bright

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red color in prerigor muscle (Cornforth and Egbert, 1985; Cornforth et al., 1985; Egbert and Cornforth, 1986). Claus et al. (1984) reported that electrical stimulation minimized the darkening effect of hotboned meat. Shikama and Sugawara (1978) observed that the rate of autoxidation of oxymyoglobin (MbO₂) increases rapidly with increasing hydrogen ion concentration.

Upon heating, heme proteins show a different behavior from that of fresh meat. It was suggested that the heme protein present in cooked meat could complex with some of the denatured proteins or a carboxylate ion of the denatured globin molecules or NADH (Tappel, 1957; Tarladgis, 1962; Ledward, 1971, 1974). Dymicky et al. (1975) and Akyounoglou et al. (1963) also showed that amino acids, amino acid esters, pyridine derivatives, and other related compounds also could react with heme, and could form ferric or ferrohemochromes. Ledward (1971) described that in aqueous Mb solution, increasing temperature leads to conformational changes in the heme environments, and this change may allow Mb to be attacked by the denatured proteins on the heme.

Jank and Froning (1973) reported that the addition of Kena increased, but lowering the pH decreased, the heat stability of a crude Mb extract. The metMb appeared to be more stable to heat denaturation than ferrous Mb or MbO₂. Cornish and Froning (1974) reported that 50% heat denaturation of turkey Mb was accomplished at 78 °C, and cytochrome c could resist denaturation at 105 °C. Draudt (1969) reported that in a ground beef system metMb was the least stable and 50% of the metMb was denatured after 30 min of heating at 44 °C. But in purified form, metMb was one of the most stable forms of Mb, and 50% of the purified Mb was precipitated at 62 °C to 65 °C after 30 min of heating.

When ferrous cytochrome c solution was heated to 100 °C at pH 7.4 to 10.2 with no reducing substances it was immediately oxidized, but in solution with sodium dithionite, the ferrous cytochrome c remained reduced. On cooling, the oxidized cytochrome absorption band returned to its original position and intensity (Keilin, 1966). Keilin (1966) also described that native cytochrome c does not combine with carbon monoxide but heat-denatured cytochrome c does. Cytochrome c undergoes marked changes in its properties when heated but these changes are to a large extent reversible. Moreover, oxidized and reduced cytochrome c are affected differently by heat. The heat stability of ferrous-cytochrome c is higher than that of ferricytochrome c and about 80% of heat-denatured ferrous-cytochrome c reverted to its original native state. Because of these large differences in the behavior of native and denatured heme pigments, the study of heat stability of Mb and cytochrome c becomes a very important consideration in processed meat. The objectives of this study were to investigate the effects of added salt, phosphate, and dextrose on the heat stability of purified Mb, Hb, and cytochrome c in a model system.

**MATERIALS AND METHODS**

**Purification of Myoglobin, Hemoglobin, and Cytochrome c**

*Sample Preparation for Purification.* Samples were prepared using the method described by Brautigan et al. (1978) with some modification. Frozen turkey hearts (~29 °C) were partially thawed in a 3 °C cold room and ground through a 9.5-mm plate, and the ground turkey heart meat was homogenized in a Waring blender (Fisher Scientific, Fairlawn, NJ) for 5 s with 1 vol of 3% (wt/vol) Al₂(SO₄)₃·17H₂O solution. The homogenate was centrifuged at 1,140 x g for 20 min and the supernatant was collected. This solution was dialysed against 20 to 30 vol of distilled water for 16 to 20 h in a 3 °C cold room with four to five changes of water, and the precipitants were removed by centrifugation at 6,000 x g for 30 min.

*Cytochrome c Purification.* The prepared clear red solution was passed through a bed of Amberlite IRC-50 (Mallinckrodt Chemical Works, St. Louis, MO) equilibrated with 50 mM phosphate buffer, pH 7.0, to trap cytochrome c. Cytochrome c was displaced with 0.5 M NaCl in the 50 mM phosphate buffer, pH 7.0, and then dialysed against distilled water for 40 h in a 3 °C cold room with five changes of water. After dialysis the precipitants were spun down at 6,000 x g for 30 min at 4 °C and the supernatant was concentrated with polyethylene glycol and passed through a G-50 gel filtration column to remove impurities. The main fraction of cytochrome c was collected after the G-50 gel filtration column and dialysed, and finally passed through a mixed bed ion-exchange resin and then freeze-dried.
Myoglobin and Hemoglobin Purification. The resulting solution after passing through an Amberlite IRC-50 cation exchange column was concentrated with polyethylene glycol and was passed through a Sephadex G-50 gel filtration column (Pharmacia Fine Chemicals, Piscataway, NJ) to remove Hb and other proteins. The crude Mb solution was purified either by using a DEAE-Sephadex anion exchange column or a CM-Sephadex cation exchange column. The main fraction of each ion-exchange column was collected and dialysed against distilled water for 2 days with five to six changes of water. After passing through a mixed bed ion exchange resin, the solution was freeze dried. The Hb was purified from turkey blood using the method described by Riggs (1981).

Gel Electrophoresis. The SDS-PAGE was carried out according to the method described by Greaser et al. (1983) to check purity of Mb, Hb, and cytochrome c. Gels of 15 or 20% acrylamide were used, and the purity of pigments was confirmed by a single band for each pigment in the SDS-polyacrylamide gel.

Heat Denaturation Study of Myoglobin, Hemoglobin, and Cytochrome c

Sample Preparation. The Mb, Hb, or cytochrome c was dissolved in .1 M phosphate buffer, pH 6.3 at concentrations of .5 mg/mL. The same concentration of pigment solutions was used to exclude protein concentration effects on heat denaturation rate. Four milliliters of the Mb, Hb, or cytochrome c solution were transferred to test tubes and eight combinations of samples were made with two levels of NaCl (0, 2.5%), two levels of sodium tripolyphosphate (0, .5%), and two levels of dextrose (0, 1%). The 2.5% NaCl and .5% sodium tripolyphosphate levels were chosen because they are common levels of these ingredients in meat products. After preparation of four sets of Mb, Hb, and cytochrome c solutions with additives, they were heated in a 95°C water bath. Samples were taken from the water bath when the thermometer inserted in a sample tube reached 68 C, 74 C, 80 C, or 85 C, respectively. These temperatures were chosen because they included the usual end-point temperatures for products like turkey rolls and roasted turkeys. After cooling to room temperature (ca 22 C), samples were centrifuged at 1,140 x g for 30 min to precipitate the denatured pigments.

Absorption Spectrophotometry. After centrifugation, about 2 mL of each supernatant were transferred to a cuvette and a few granules of sodium hydrosulfite were added to reduce pigments. The absorption maxima of each solution from 600 to 380 nm were recorded.

Statistics. The heat denaturation study was replicated four times. Analysis of variance using the statistical analysis system (SAS, 1986) was used to determine the differences due to the main effects of salt, phosphate, dextrose, and their interactions on heat stability of Mb and cytochrome c. For Hb, only main effects were measured. Values in the top line of each table indicate the average of 32 samples. The values (+ or −) for main and interaction effects in the tables were expressed as the increase or decrease in each parameter caused by the added ingredient (e.g., for a salt main effect, an A556 of −0.043 (average of .265 in Table 1, 74 C temperature) means the actual A556 with added salt was .243 (average of 16 samples). 287 (average of 16 samples) without salt. For the interaction effect, the actual A556 of (0% salt x 0% phosphate) + (2% salt x .5% phosphate)/2 was .280, and that of (0% salt x .5% phosphate) + (2% salt x 0% phosphate)/2 was .250. Duncan's multiple range test was used to determine significant differences in mean values (Steel and Torrie, 1960). The interaction term was used as the test statistic to measure significance.

RESULTS AND DISCUSSION

Temperature. Tables 1, 2, and 3 show the average absorbance values of samples after heat treatments. As expected, the heat stability of Hb was much lower than that of Mb and cytochrome c. About 50% of the Hb was denatured at 68 C. At 74 C, about 90% of the Hb was denatured. Large portions of Mb (65%) and cytochrome c (75%) were denatured at 85 C, but almost all of the Hb (90%) was denatured at 74 C or higher. With a 74 C heat treatment, only 15% of the Mb and 7.5% of the cytochrome c were denatured. The heat denaturation rate of Mb and cytochrome c have shown similar patterns. Up to 80 C, Mb denatured faster than cytochrome c, but at 85 C a larger portion of cytochrome c was denatured. Satterlee and Zachariah (1972) and Cornish and Froning (1974) reported that 50% heat denaturation of turkey Mb was accomplished at around 78 C. Draudt (1969) reported that 50% of the purified Mb was precipitated at 62 C to 65 C after 30 min of heating.
HEAT STABILITY OF MEAT PIGMENTS

TABLE 1. Effects of sodium chloride, phosphate, dextrose, and their interactions on the 556-nm absorbance of x-ray-heated myoglobin (Mb)\(^1\)^\(^2\)

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>n</th>
<th>68 C</th>
<th>74 C</th>
<th>80 C</th>
<th>85 C</th>
<th>(A_{556})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>32</td>
<td>16</td>
<td>.297(^a)</td>
<td>.265(^b)</td>
<td>.199(^c)</td>
<td>.114(^d)</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride (S)</td>
<td>1</td>
<td>16</td>
<td>-.004*</td>
<td>-.043**</td>
<td>-.084**</td>
<td>-.035**</td>
<td></td>
</tr>
<tr>
<td>Phosphate (P)</td>
<td>1</td>
<td>16</td>
<td>+.002</td>
<td>+.028*</td>
<td>+.068**</td>
<td>+.068**</td>
<td></td>
</tr>
<tr>
<td>Dextrose (D)</td>
<td>1</td>
<td>16</td>
<td>+.002</td>
<td>-.002</td>
<td>+.004</td>
<td>+.004</td>
<td></td>
</tr>
<tr>
<td>(S \times P)</td>
<td>1</td>
<td>16</td>
<td>+.003</td>
<td>+.030**</td>
<td>+.032**</td>
<td>-.008**</td>
<td></td>
</tr>
<tr>
<td>(S \times D)</td>
<td>1</td>
<td>16</td>
<td>-.001</td>
<td>+.003</td>
<td>+.003</td>
<td>-.003</td>
<td></td>
</tr>
<tr>
<td>(P \times D)</td>
<td>1</td>
<td>16</td>
<td>-.004</td>
<td>-.003</td>
<td>-.005</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(S \times P \times D)</td>
<td>1</td>
<td>16</td>
<td>+.001</td>
<td>-.004</td>
<td>+.004</td>
<td>-.003</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a-d}\)Average values with no common superscripts are significantly different \((P<.05)\).

\(^1\)A_{556} \text{ of Mb before heating was .31.}

\(^2\)Changes in \(A_{556}\)-values (+ or -) caused by adding the respective ingredient compared with all samples without the ingredient.

*Significant at \(P<.05\).

**Significant at \(P<.01\).

Although each of these results shows some differences due to their conditions, most of the purified Mb would stay denatured until the temperature reached approximately 74 C in a model system.

The heat stability of cytochrome c in this study was quite different from that reported by Cornish and Froning (1974), who indicated that in 10 mM phosphate buffer, pH 7.0, the purified cytochrome c could resist denaturation at 105 C. This high stability of cytochrome c in their study could be caused partly by the buffer system. A lower buffer concentration might play an important role by decreasing the heat stability of cytochrome c. Myer (1968) observed that partial unfolding of cytochrome c could occur at 53 C and become predominant at 82 C, but Keilin (1966) explained that heat alteration of ferrous cytochrome c is largely reversible even after a 100 C treatment. The heat stability of ferricytochrome c is much lower than that of the ferrous form, and about 80% of heat-denatured ferrocyahtochrome c can be reverted to its original native state after cooling (Keilin, 1966). This regeneration of cytochrome c may play a very important role in the color of uncured cooked meat such as oven-roasted turkey breast meat.

Although Hb comprises only 10 to 20% of the total pigment content of well-bled meat (Fleming et al., 1960; Rickansrud and Hendrickson, 1967), it was shown to be a very important factor in the color of cooked meat (Table 2). The heat denaturation rate of Hb changed greatly between 68 C to 74 C, which is the normal end-point temperature range of cooked meat.

The concentration of pigments and other proteins may also affect the heat denaturation rate of Mb and cytochrome c. Draudt (1969) reported that the heat stability of Mb in a meat system was very low but Cornish and Froning (1974) indicated that the interaction between pigments could increase their heat stability.

Salt. Salt significantly decreased the heat stability of Mb under all temperature conditions (Table 1). Salt also significantly increased the heat denaturation of Hb at 68 C and 74 C (Table 2). With added salt, about 22 and 50% of the Mb was denatured at 74 C and 80 C, respectively. The decrease in heat stability of Mb and Hb with added salt could be caused by the chloride ion. As described by Ledward (1971), the Mb in aqueous solution would undergo some conformational change caused by heat, and the unfolding of polypeptide chains could expose the heme group to aqueous solution. It is well established that the heme group of Hb or Mb in acetone-HCl solution can bind to Cl\(^-\) and make chlorohematin (Falk, 1964). The exposure of the heme group by heating may have provided a good chance for a chloride ion to react with heme in the salt-added samples. Subsequent
bond cleavage between proximal histidine (F8) and iron could result in apomyoglobin and chlorohemmin. Chlorohemmin is not soluble in aqueous condition in the pH 6.3 range, and apomyoglobin would be destabilized more easily than intact Mb. Although the most favorable condition for the heme iron and chloride ion reaction is far different from the conditions in this study, the possibility of hemin formation is still high, as no other materials are available for the reaction with heme.

Unlike in Mb, salt significantly (P<0.01) increased the heat stability of cytochrome c when the temperature was 80°C or higher (Table 3). With salt, only 6% of the cytochrome c was denatured at 74°C, and about 15% of cytochrome c was denatured at 80°C. The difference between reactions of Mb and cytochrome c with salt could be caused partly by their differences in heme environment (Takano et al., 1973) but cannot be explained well at this point. The material that can react with cytochrome c should have a very high affinity to the heme iron ligand. Only -CN, -N₃, -NO, and -F can make a complex with cytochrome c by attacking the iron-methionine bond under certain conditions (Potter, 1940; George et al., 1967). Fung and Vinogradov (1968) reported that Cl⁻ stabilized

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**TABLE 2. Effects of sodium chloride, phosphate, and dextrose on the 556-nm absorbance of heat-treated hemoglobin (Hb).**

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>n</th>
<th>68°C</th>
<th>74°C</th>
<th>80°C</th>
<th>85°C</th>
<th>(A556)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>.195a</td>
<td>.042b</td>
<td>.019c</td>
<td>.010d</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1</td>
<td>8</td>
<td>-.046**</td>
<td>-.006**</td>
<td>-.001</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>1</td>
<td>8</td>
<td>+.003</td>
<td>0</td>
<td>0</td>
<td>+.001</td>
<td></td>
</tr>
<tr>
<td>Dextrose</td>
<td>1</td>
<td>8</td>
<td>+.019**</td>
<td>+.002</td>
<td>-.002</td>
<td>-.001</td>
<td></td>
</tr>
</tbody>
</table>

a-d Average values with no common superscripts are significantly different (P<0.05).

1A556 of Hb before heating was 36.

2Changes in A556 values (+ or -) caused by adding the respective ingredient compared with all samples without the ingredient.

**Significant at P<0.01.**

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**TABLE 3. Effects of sodium chloride, phosphate, dextrose, and their interactions on the 550-nm absorbance of heat-treated cytochrome c.**

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>n</th>
<th>68°C</th>
<th>74°C</th>
<th>80°C</th>
<th>85°C</th>
<th>(A550)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>32</td>
<td>16</td>
<td>1.120a</td>
<td>1.055b</td>
<td>.881c</td>
<td>.291d</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride (S)</td>
<td>1</td>
<td>16</td>
<td>+.002</td>
<td>+.034</td>
<td>+.150**</td>
<td>+.105**</td>
<td></td>
</tr>
<tr>
<td>Phosphate (P)</td>
<td>1</td>
<td>16</td>
<td>-.007</td>
<td>-.064</td>
<td>-.240**</td>
<td>-.167**</td>
<td></td>
</tr>
<tr>
<td>Dextrose (D)</td>
<td>1</td>
<td>16</td>
<td>-.012</td>
<td>+.048</td>
<td>+.032</td>
<td>+.060**</td>
<td></td>
</tr>
<tr>
<td>S × P</td>
<td>1</td>
<td>16</td>
<td>+.004</td>
<td>+.074</td>
<td>+.240**</td>
<td>+.071**</td>
<td></td>
</tr>
<tr>
<td>S × D</td>
<td>1</td>
<td>16</td>
<td>+.004</td>
<td>-.004</td>
<td>-.004</td>
<td>+.004</td>
<td></td>
</tr>
<tr>
<td>P × D</td>
<td>1</td>
<td>16</td>
<td>-.003</td>
<td>+.038</td>
<td>+.039</td>
<td>-.018</td>
<td></td>
</tr>
<tr>
<td>S × P × D</td>
<td>1</td>
<td>16</td>
<td>+.002</td>
<td>-.033</td>
<td>-.017</td>
<td>+.018</td>
<td></td>
</tr>
</tbody>
</table>

a-d Average values with no common superscripts are significantly different (P<0.05).

1A550 of cytochrome c before heating was 1.14.

2Changes in A550 values (+ or -) caused by adding the respective ingredient compared with all samples without the ingredient.

**Significant at P<0.01.**
two forms of ferricytochrome c and prevented a disruption of the central coordinates (heme- ligand) of ferricytochrome c at low pH.

**Phosphate.** Phosphate significantly increased the heat stability of Mb at 74 C or higher (Table 1). This increase in heat stability by phosphate could be somewhat related to the pH increase by added phosphate. Satterlee and Zachariah (1972) observed that a pH increase could increase the heat stability of Mb. Janku and Froning (1973) also reported that the addition of Kena increased the heat stability of turkey Mb, and the heat denaturation of pigments could be increased by reducing the pH of the solution.

The heat stability of cytochrome c was significantly (P<.01) decreased by added phosphate in the 80 C and 85 C heat treatments (Table 3). Butt and Keilin (1962) noted that the degree of irreversible heat denaturation of oxidized cytochrome c (Fe3+) increased as the pH was raised from 7.2 to 10.2. By adding phosphate (.5%) to the .1M phosphate buffer, pH 6.3, the pH was increased by .4. Therefore, the increase in pH by added phosphate affected the heat stability of Mb and cytochrome c differently.

Added salt and phosphate have contradictory effects on the heat stability of Mb and cytochrome c. The reason for this cannot be explained at this point. When both salt and phosphate were added together the heat stability of Mb and cytochrome c became intermediate (Figures 1 and 2).

**Dextrase.** Dextrase significantly (P<.01) increased the heat stability of Hb at 68 C (Table 2). Dextrase also significantly (P<.01) increased the heat stability of cytochrome c in the 85 C heat treatment (Table 3).

**Interactions.** The only interaction effect found in this study was the salt × phosphate interaction in both Mb and cytochrome c. The salt × phosphate interaction significantly (P<.01) increased the heat stability of Mb at 74 C and 80 C but decreased it at 85 C (Table 1). The salt × phosphate interaction significantly increased the heat stability of cytochrome c at 80 C and 85 C (Table 3).

Overall, added salt stabilized cytochrome c and destabilized Mb, but added phosphate greatly decreased the heat stability of cyto-
chome c and increased that of Mb. When both salt and phosphate were added, the stabilizing effects of salt on cytochrome c and phosphate on Mb were stronger than the destabilizing effects of salt on Mb and phosphate on cytochrome c, respectively (Figures 1 and 2). Denaturation of pigment exposes its heme to the outer environment, and oxidized heme will be predominant. Because the color strength of undenatured pigments is higher than that of the denatured form, the heat stability can have a strong effect on the color of meat.

REFERENCES


HEAT STABILITY OF MEAT PIGMENTS


