Studies on "Green" Tuna—IV. Effect of Cysteine on Greening of Myoglobin in the Presence of Trimethylamine Oxide

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As reported previously¹⁾, the ordinary tuna meat reveals on cooking a desirable pink color, but a tannish-green color due to greening of Mb if TMAO is incorporated into it before cooking.

The precipitate obtained by heating a solution containing crystalline tuna MMb and TMAO, however, was not green, and some additional factor was postulated to be required for the greening of Mb. The factor might be the key to elucidation of the green pigment and mechanism of "greening".

To locate the factor, the mixed solution of tuna MMb and TMAO was heated with a number of reducing substance and color of the resulting precipitate was examined. The results in these experiments clearly indicated that cysteine is one of the necessary factors for greening of Mb.

Experimental

Effect of egg albumin on greening of Mb in the presence of TMAO. Crystalline MMb was prepared from the red muscle of tuna in the usual manner²⁾. It was dissolved in 0.1 M phosphate buffer (pH 6.5) to a final concentration of 140 mg%. To a 5 ml portion of this solution were added 1 g of egg albumin and 20 mg of TMAO·2H₂O. The mixture was readjusted to pH 6.5 by addition of sodium hydroxide. A test tube (18×180 mm) containing the mixture was covered with a piece of paraffine paper and kept in a boiling water-bath for one hour. The mixture lacking TMAO was treated under the same condition and served as control. After being cooled in tap water, the coagulum formed was examined with the naked eye on its color and submitted to the measurement of absorption spectrum with a Beckman DU spectrophotometer equipped with an opal glass apparatus after being reduced with sodium hydrosulfite¹⁾.

Effects of reducing substances on greening of Mb. A 20 ml portion of 0.1 M phosphate buffer (pH 6.5) containing 30 mg of MMb and 80 mg of TMAO·2H₂O was

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Abbreviations used: TMAO...Trimethylamine oxide, MMb...Metmyoglobin, Mb...Myoglobin, Hb...Hemoglobin.

placed in a 50 ml flask. The reducing substances, listed in Table 1 and each equivalent to TMAO, were dissolved respectively in a minimum quantity of water with the exception of hydrogen sulfide, adjusted to pH 6.5, and poured into the flask. Immediately after the transference, air in the flask was evacuated off for 3 minutes by an aspirator and then replaced by nitrogen gas. This procedure was repeated once for a satisfactory expulsion of oxygen. The flask was then stoppered and heated in a boiling water-bath for one hour. Hydrogen sulfide was bubbled from the top of a fine glass tube into the mixture throughout the heat treatment. After being cooled in tap water, the contents of the flask were filtered quickly by suction, and color of the precipitate and filtrate was observed.

Results and Discussion

Although tuna meat develops a tannish-green color on cooking in the presence of TMAO as mentioned above, the purified Mb did not turn green when heated with TMAO. This fact strongly suggested that some additional factor must be involved in the "greening" phenomenon and that the factor must be present sufficiently in meat but little in Mb itself, if any. It was also a problem whether the factor is involved in the part of protein or in the other constituents of the muscle.

In the first experiment, tuna meat was replaced on trial by a colorless protein, egg albumin, and a mixture of crystalline MMb and egg albumin was heated with and without TMAO. The coagulum was faintly pink in the absence of TMAO, whereas faintly yellowish green in its presence, indicating the apparent discoloration of Mb. Absorption spectra of these coagula were determined after reduction with sodium hydrosulfite and are shown in Fig. 1. The spectrum of the coagulum showing a faintly pink color reveals two absorption maxima at $555\sim 560$ and $525\sim 530\text{ m}\mu$ like hemochrome. On the contrary, that of the discolored one showing a faintly yellowish green color has no absorption maximum in the visible region and bears a considerable resemblance to that observed previously on the "green" tuna meat¹. These results indicate that the greening of Mb occurs in egg albumin as in tuna meat.

There arises another question as to why crystalline MMb does not show greening when heated with TMAO alone, although it belongs to a category of protein like egg albumin. In this connection, the amino acid composition of Mb may be a subject of discussion. It is generally recognized that mammalian Mb's³ are characterized by the lack of sulfur-containing amino acids, but tuna Mb⁴ has been reported to contain one mole of cysteine in its molecule. The amount of cysteine, however, is obviously far less than in meat proteins, egg albumin⁵, and other proteins. In the above-mentioned systems, those involving either meat protein or egg albumin may be far richer in SH-group than that involving only Mb as protein. Thus, the amount of cysteine residue should be considered.

It has been also known, on the other hand, that the coexistence of some reducing

substance is indispensable for formation of green pigments from Hb or Mb in oxidation with hydrogen peroxide or molecular oxygen⁶). If it is also true for our case, a reducing power of egg albumin attributable to cysteine residues is supposed to play a part in greening of Mb due to TMAO.

In the second experiment, egg albumin was then replaced by cysteine or other reducing substances, and the heat treatment was carried out under anaerobic condition, since the muscle should be kept practically in anaerobic state on cooking. In this connection, SIEDLER *et al.*⁷) reported that Mb gives a green pigment like sulfmyoglobin when heated with cysteine in the presence of air at 60°C for one hour. This greening reaction, however, is thought to be different from that induced by TMAO, and might be avoided under the anaerobic condition adopted in the present study.

As shown in Table 1, Mb produces the reddish brown precipitate when heated

Fig. 1. Absorption spectra of the coagula reduced by sodium hydrosulfite.
a...Coagulum from a solution containing MMb and egg albumin
b...Coagulum from a solution containing MMb, egg albumin, and TMAO.

Table 1. Effects of reducing substances on the greening of Mb.

Reaction mixture	Color	
	Precipitate	Filtrate
MMb	Reddish brown	Colorless
MMb + TMAO	Brown	Colorless
MMb + Cysteine	Brown	Faintly yellow
MMb + Cysteine + TMAO	<i>Greenish</i> brown	Yellowish brown
MMb + Glutathione	Brown	Faintly yellow
MMb + Glutathione + TMAO	Faintly <i>greenish</i> brown	Yellowish brown
MMb + Thioglycolic acid	Brown	Faintly pink
MMb + Thioglycolic acid + TMAO	Faintly <i>greenish</i> brown	Brown
MMb + Hydrogen sulfide	Brown	Colorless
MMb + Hydrogen sulfide + TMAO	Faintly <i>greenish</i> brown	Colorless
MMb + Ascorbic acid	Brown	Faintly yellow
MMb + Ascorbic acid + TMAO	Brown	Reddish brown
MMb + Sodium hydrosulfite	Reddish brown	Colorless
MMb + Sodium hydrosulfite + TMAO	Brown	Red

alone or with sodium hydrosulfite, but the brown precipitate with TMAO or most of the reducing substances. Since Mb did not give any green pigments in the presence of reducing substance, the greening induced by atmospheric oxygen as mentioned above must have been excluded in this experiment.

Among reducing substances tested in the presence of TMAO, cysteine was effective in yielding the greenish brown precipitate from Mb. Sulfur compounds, such as glutathione, thioglycolic acid and hydrogen sulfide, gave the faintly greenish brown precipitate, but the discoloration was not so marked as in the case of cysteine. These results show that the natural "green" tuna can be represented by a model system consisting of Mb, TMAO, and cysteine. The cysteine residues which will be exposed on the surface of heat-denatured protein molecule may participate into the "greening" phenomenon of tuna meat, as supported by the fact that the "greening" of tuna proceeds only after the fish was cooked to some extent⁸⁾.

The insoluble nature of the green precipitates obtained here prevented the detailed examinations on their spectra. However, it has been recently found that a detergent, sodium dodecyl sulfate, is a good solvent for the precipitate and a further study on characterization of the green precipitate is now under progress.

Summary

To find out an additional factor participating in greening of Mb with TMAO, effects of egg albumin, cysteine and some other reducing substances were examined. The mixed solution was heated under anaerobic condition and color of the precipitate and filtrate was observed on the green tinge. The results obtained are as follows:

1. Egg albumin caused greening to some extent and cysteine the most intense greening. Glutathione, thioglycolic acid and hydrogen sulfide also induced a slight greening but were not so effective as cysteine. Ascorbic acid and sodium hydrosulfite were ineffective.
2. The natural "green" tuna could be represented by a model system consisting of Mb, TMAO and cysteine.

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References

- 1) C. KOIZUMI and Y. HASHIMOTO: This Bull., **31**, 439~447 (1965).
- 2) K. SCHMID: *Helv. Chim. Acta.*, **32**, 105~114 (1949).
- 3) M. INOUE, S. ONOKI, Y. IMAI, and A. TSUGITA: *Tanpakushitsu Kakusan Kōso*, **9**, 315~384 (1964).
- 4) S. KŌNOSU, K. HASHIMOTO, and F. MATSUURA: This Bull., **24**, 563~566 (1958).
- 5) G.R. TRISTRAM: In "The Protein" (H. NEURATH and K. BAILEY, ed.), Vol. 1, 217 (1953).
- 6) R. LEMBERG and J.W. LEGGE: *Hematin Compounds and Bile Pigments*, Interscience Publishers, Inc., New York (1949).
- 7) A.J. SIEDLER and B.S. SCHWEIGERT: *J. Agr. Food Chem.*, **7**, 271~274 (1959).
- 8) Y. SASANO, H. ONO, T. TAWARA, and K. HIGASHI: This Bull., **27**, 586~592 (1961).