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In Vitro Formation of Methemoglobin by Lipophilic Fractions in Fishes and the Causative Substance

JUNKO SAJIKI and KATSUHIRO TAKAHASHI

The Public Health Laboratory of Chiba Prefecture, 666-2 Nitonacho,
Chiba City 280, Japan

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In examining substances causing hemoglobin (Hb) denaturation *in vitro* obtained from lipophilic fractions of fishes, the transformation of oxyhemoglobin (O₂Hb) to methemoglobin (MetHb) was observed in the ether extracts of *Engraulis japonica* (anchovy) and the viscera of *Cololabis saira* in six fish species. Removal of acidic substances from ether extracts of anchovy viscera by pretreatment with Na₂CO₃ resulted in suppression of Hb denaturation activity, suggesting that the substance causing Hb denaturation was acidic. The transformation of hemoglobin was observed in three fractions separated by thin layer chromatography: Fr. 1, found at the origin contained primarily phospholipids; Fr. 2 consisted of two bands between fractions of phospholipids and free fatty acids; and Fr. 3 was composed of free fatty acids. Addition of α -tocopherol to the sample completely suppressed the transformation of O₂Hb to MetHb, and the usual brownish precipitation caused by protein denaturation did not appear. The concentrations of free eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contained in ether extracts were directly proportional to magnitude of the MetHb formation index. These results indicated that the main acidic substances causing blood Hb denaturation *in vitro* were polyunsaturated fatty acids such as EPA and DHA which were present in abundance in anchovy viscera.

Keywords — methemoglobin formation; lipophilic fraction; fishes; α -tocopherol; polyunsaturated fatty acid; eicosapentaenoic acid; docosahexaenoic acid

Introduction

We previously showed that an acidic substance changing oxyhemoglobin (O₂Hb) to methemoglobin (MetHb) existed in lipophilic fractions of bivalves, and was toxic to mice by intraperitoneal injection.¹⁾ It was thought that the change could be caused by any acidic substance other than okadaic acid,¹⁾ because okadaic acid, which is also a lipophilic substance identified as a causative agent of human diarrhea and mouse lethal toxicity, was not able to induce Hb denaturation at concentrations up to the mouse lethal dose. On the other hand, transformation of Hb was induced by the addition of polyunsaturat-

ed fatty acids (PUFA) such as linoleic acid (LA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to red blood cells *in vitro*.²⁾ These results suggested that Hb denaturation observed in bivalves could be attributable to PUFA. Hb denaturation *in vitro* was also observed in the lipophilic fraction of some fishes. The present study deals with the detection and determination of hemoglobin denaturing substances contained in the extracts of several fish species.

Materials

Fishes used in the experiment were *Trachurus japonicus* (Japanese mackerel), *Sebastes macrochir* (Kichiji rockfish), *Loligo loligo bleck-*

eri (squid), *Katsuwonus pelamis* (Arctic bonito), *Cololabis saira* (mackerel pike) and *Engraulis japonica* (Japanese anchovy).

Sheep red blood cells (Kohjin Co., Ltd.) were used as the substrate for hemoglobin tests.

Reagents and Instruments

Special grades of methanol and acetonitrile (Wako Pure Chemical Ind., Ltd.) were used for high performance liquid chromatography (HPLC). Other chemicals used were also special grade. EPA (C_{20:5}) and DHA (C_{22:6}) used as standard materials (Sigma Chemical Co., Ltd.) for HPLC had purities of 99% or higher. Standard phospholipids, free fatty acids and triglycerides used for thin layer chromatography (TLC) were also purchased from Sigma. DL- α -Tocopherol (Wako Pure Chemical Ind., Ltd.) was used as an antioxidant.

Silica gel plates (60F₂₅₄, 200 mm×200 mm and 50 mm×200 mm, Merck) were heated for 2 h at 110°C for preparation and preserved in a desiccator prior to use.

Spectrophotometry was performed with a Hitachi 557 dual wavelength spectrophotometer (Hitachi Co., Ltd.). The HPLC system and the UV detector for the determination of EPA and DHA were Shimadzu Co., Ltd. models LC-6A and SPD-6A, respectively, and were connected to a Shimadzu C-R6A recorder.

Injection samples for HPLC were filtered through a Millipore filter (FH-type, 0.5 μ m) before use.

Experimental Methods

1. Lipid Extract Procedure from Fishes and Preparation of Samples for Hb Denaturation Test — Tissue samples of 100 g (80 g for viscera) were taken from each of the fishes and were homogenized in 300 ml acetone. The extraction was repeated twice with 200 ml acetone each time. After evaporation of acetone, the lipid was re-extracted from the residue with 200 ml of diethylether. Aliquots of ether corresponding to 1 g wet weight of fish were placed in a crucible to measure the amount of crude lipid by heating at

120°C for 1 h and cooling for 30 min in a desiccator. To prepare the sample for determination of MetHb formation, an ether solution corresponding to 4 g wet weight of fish was put into a test tube. After evaporating the ether under N₂ gas, the residue was suspended in 1 ml phosphate buffered saline (PBS, pH 7.4) containing 1% Tween 60.

Extraction of lipid from fish material added with 0.5% wet weight α -tocopherol was carried out in the same manner as the non-treated sample above.

In order to remove acidic substances from the ether extract, 100 ml of 0.5 M Na₂CO₃ was added to 100 ml ether extract, and the alkaline layer was discarded after shaking for separation. After repeating the same procedure once more, the ether was washed with water repeatedly to adjust the pH between 6 and 7.

2. Fractionation of Lipids in the Ether Extract by TLC and the Preparation of Samples for Hb Test

— Ether extracted lipid from anchovy viscera (corresponding to 0.2 g of total lipid) was applied on two silica gel plates (200 mm×200 mm) and developed with a solvent consisting of petroleum ether–ethyl ether–acetic acid (74:15:1, v/v/v) according to the method of Ozawa *et al.*³⁾ On another silica gel plate (50 mm×200 mm), the same sample was developed together with lipid standards for analyzing phospholipids, free fatty acids and triglycerides if contained. A third plate was left to stand in a box saturated with iodine vapor for visualization. Various lipid fractions were scraped into Erlenmeyer flasks from the portions of gel on the two untreated plates corresponding to the *R_f* values of the standard lipids (Fig. 2). Lipids were extracted from individual portions of gel. Lipids in the fraction between phospholipid and free fatty acid were extracted three times with 30 ml methanol, and other non-polar lipids such as triglyceride and cholesterol ester were extracted three times with ethyl ether. After evaporating the solvents under N₂ gas, lipids were weighed. Test samples were then prepared by adding 1 ml of PBS to each lipid fraction already dissolved in

0.5 ml methanol.

3. Determination of Methemoglobin Formation — After thrice rinsing sheep red blood cells with PBS (pH 7.4), a 2% suspension in PBS was prepared. Samples of lipid fractions were added to the blood cell suspension at a definite concentration. After standing for 1 h at room temperature, 0.5 ml of reacted solution was taken out and hemolyzed by the addition of distilled water. The spectrum change of the sample was immediately scanned by dual wavelength spectrophotometry. MetHb formation was measured by the method previously reported.⁴⁾ Change of Hb was represented as MetHb formation index (MFI): *i.e.*, the ratio of the amount of MetHb produced by the test sample to the total amount of transformed MetHb contained in the blood suspension. This was measured by the ratio of absorbance difference (ΔA_s) between 630 nm and 700 nm in the sample to the difference (ΔA_c) of the

same sample after addition of a small amount of potassium ferricyanide.

4. Determination of Free EPA and DHA — Concentrations of EPA and DHA in ether extracts of anchovy viscera were assayed according to HPLC methods previously reported.⁵⁾ The residue extracted with ether was dissolved in methanol to make equivalents of 9.4 mg wet weight of viscera per ml. Ten μ l of filtrate from Millipore filtering was applied to HPLC. Assay conditions of HPLC were as follows: column size: ODS (4.6 mm i.d. $\times$ 250 mm), mobile phase: acetonitrile/methanol/water/phosphoric acid (53:20:17:0.1, v/v/v/v), flow rate: 1.2 ml/min, assay wavelength: 195 nm.

Results

1. Biochemical Properties of Ether Extracts from Fish

MFI, total crude lipid content and pH of

TABLE I. Characteristics of Ether Extracts from Various Kinds of Fish

Kind of fish	Lipid (%)	pH	MetHb formation index (MFI)
<i>Trachurus japonicus</i> (fresh, whole)	5.6	3.5	0
<i>Sebastolobus macrochir</i> (fresh, whole)	3.6	3.7	0
<i>Loligo loligo bleckeri</i> (fresh, whole)	1.4	4.1	0
<i>Katsuwonus pelamis</i> (fresh, muscle)	1.1	3.5	0
(fresh, viscera)	1.5	3.6	0
<i>Cololabis saira</i> (fresh, viscera)	33.3		0.12
<i>Engraulis japonica</i> (fresh, whole)	1.5	3.6	0.08
(fresh, whole)	1.1	3.6	0
(fresh, whole)	5.6	3.1	0
(fresh, whole)	3.6	4.5	0
(fresh, whole)	1.1	3.6	0
(fresh, whole)	1.2	4.0	0.14
(fresh, viscera)	2.0	4.1	0.09
(fresh, viscera)	2.2	4.4	0.13
(fresh, viscera)	1.6	3.7	0.12
(fresh, viscera)	2.1	3.7	0.13
(fresh, viscera)	1.9		0.22
(fresh, viscera)	2.0		0.19
(dry, whole)	3.3	3.0	0.17
(dry, viscera)	7.5	3.1	0.33

Fig. 1. The Change of Hemoglobin Spectrum before (Control) and 1 Hour after Addition of Ether Extract from Viscera of *Engraulis Japonica* Treated or Untreated with Na_2CO_3

— Na_2CO_3 ,
 - - - + Na_2CO_3 ,
 — Control.

Lipid (%), pH and MFI of ether extract without Na_2CO_3 treatment were 7.5, 3.1 and 0.33, whereas those with the treatment were 6.6, 7.4 and 0.14, respectively.

fishes are shown in Table I. Hb denaturation was observed in viscera of *Cololabis saira* and in both viscera and whole body tissue of *Engraulis japonica* (anchovy). No hemoglobin denaturation was observed in other fishes. MFI of *Cololabis saira* was 0.12. As shown in Table I, Hb denaturation by anchovy was observed in two out of six whole body samples. Though various MFI values were obtained, all viscera of anchovy showed activities of MetHb formation. MFI of dry anchovy was 0.17 for whole body and 0.33 for viscera. Fish species varied in their content of crude lipid, with the highest levels in viscera of *Cololabis saira* and the lowest in fresh anchovy, *Katsuwonus pelamis* and *Loligo loligo bleckeri*. All suspensions had low pH values, ranging from 3.0 to 4.5.

When suspension of ether extract from dry anchovy viscera was added to blood, MetHb peaks occurred at 500 and 630 nm replacing O_2Hb peaks at 540 and 578 nm, as

Fig. 2. Thin Layer Chromatogram of Ether Extracted Lipids from Viscera of *Engraulis Japonica*

Solvents:

petroleum ether/diethyl ether/acetate (74:15:1, v/v/v).

A: lipid standards; (a) phospholipid, (b) free fatty acid, (c) triblyceride.

B: ether extracts from viscera of *Engraulis Japonica* showing MetHb formation index of 0.22.

shown in Fig. 1, resulting in a MFI of 0.33. However, this Hb change became as small as MFI 0.14 when acidic substances were removed by Na_2CO_3 treatment.

2. Biochemical Properties of Various Lipids in Ether Extract of Anchovy Viscera Fractionated by TLC

TLC of lipids in anchovy viscera with 0.22 MFI is shown in Fig. 2. These lipids were classified into five fractions: Fr.1 at the origin was composed mainly of phospholipids; Fr.2 consisted of unknown substances possessing polarity between phospholipid and free fatty acid with R_f values between 0.08 and 0.16; Fr.3 was mainly free fatty acids; Fr.4 was mainly triglycerides; and Fr.5 included a substance corresponding to cholesterol ester, judging from previous

TABLE II. Lipid Content and MetHb Formation Index in Lipid Fractions of Viscera of *Engraulis Japonica* Separated by TLC

No. of fraction	Lipid (mg)	MFI	MFI/mg lipid
1	43.5	0.16	$3.68(\times 10^{-3})$
2	49.6	0.29	5.85
3	53.2	0.29	5.45
4	85.1	0	0
5	4.8	0	0

The crude ether extract showed an MFI of 0.22.

reports.³⁾ Lipid content and MFI of each fraction were examined (Table II). Recovery of lipids extracted from TLC to the original solution was 118.1%. When 100 μ l of each lipid sample was added to 1 ml of 2% blood suspension, MetHb formation was observed in Fr. 1, 2 and 3, and was especially prominent in Fr. 2 and 3 which contained free fatty acids (Table II). As shown in Table II, relative MFIs to the lipid content of Fr. 1, 2 and 3 calculated were 3.68×10^{-3} , 5.85×10^{-3} and 5.45×10^{-3} , respectively. From these data, it is clear that the activity of MetHb formation in Fr. 2 is almost the same as that in Fr. 3. No Hb change was observed in Fr. 4 or 5.

Addition of α -tocopherol at 0.5% to two extracts of anchovy viscera whose MFI were 0.22 and 0.19, respectively, resulted in complete suppression of Hb transformation and brownish precipitate formation, which is a product of hemoglobin denaturation.

3. Concentrations of EPA and DHA in Ether Extracts from Anchovy Viscera

Since the main PUFAs in ether extracts from anchovy viscera were EPA and DHA,⁵⁾ concentrations of these fatty acids were determined by HPLC in ether extracts from various anchovy viscera. As shown in Table III, concentrations of EPA and DHA in viscera at 0.33 MFI (sample 1) were respectively 5.7 and 3.4 times higher than those in viscera showing no activity of Hb change (sample 4).

Discussion

We previously reported that Hb denatur-

TABLE III. Eicosapentaenoic Acid and Docosahexaenoic Acid Concentrations in Ether Extracts of 4 Samples of Viscera of *Engraulis Japonica* at Different MetHb Formation Index

Sample No.	MFI	EPA (mg/g ^{a)})	DHA (mg/g ^{a)})
1	0.33	7.25	5.7
2	0.13	3.39	3.8
3	0.12	3.36	2.5
4	0	1.27	1.7

a) mg/g wet weight of viscera of *Engraulis Japonica*.

ation was induced by addition of unsaturated fatty acids such as EPA but not by stearic acid, a saturated fatty acid.²⁾ The more double bonds contained in a fatty acid, the more intensive was the action on hemoglobin. In view of such experimental results, unsaturated fatty acids contained in bivalves were considered to be the causative agents in the acidic substance showing Hb denaturation activity *in vitro*.

It was clear that Hb denaturation *in vitro* took place not only in shellfish but also certain fishes. Considering that free fatty acids (Fr. 3) separated by TLC caused potent Hb transformation, which was suppressed by addition of α -tocopherol, it is most probable that Hb denaturation is due to oxidative reactions catalyzed by heme iron on polyunsaturated fatty acids, such as EPA and DHA, which are abundantly contained in fish.

It is known that radical oxygens such as H_2O_2 and O_2^- participate in the oxidation of O_2Hb to MetHb.^{6,7)} The mechanism of α -tocopherol suppression of Hb denaturation was considered to be the same as the suppression of protein denaturation due to inhibition of radical oxygen and lipoperoxide formation by a catalytic reaction *via* heme iron.

It was interesting that Hb denaturation was observed not only in Fr. 3 but also in Frs. 1 and 2, which contain more hydrophilic substances than free fatty acids. Although substances contained in these fractions of anchovy are not yet known, except phos-

pholipid, it has been generally accepted that oxides of unsaturated fatty acids such as linoleic acid (LA), EPA and DHA must be present due to their smaller *R_f* values than the acids themselves.^{8,9)} In a further experiment applying TLC in the same solvent system used in this report, an autooxidized EPA was located at the origin and this denatured larger amounts of Hb than did EPA itself (data not presented). In the Hb denaturation observed in Fr. 1, phospholipids cannot be ignored because those in sardine consist chiefly of polyunsaturated fatty acids like EPA and DHA.¹⁰⁾

It is generally known that hydrolysis by lipase occurs in fish meat very soon after death, followed by the formation of a large amount of free fatty acids.¹¹⁾ Thus, the extensive Hb denaturation observed in dry anchovy compared to fresh tissue is thought to be due to the release of free fatty acids and synthesis of oxides from unsaturated fatty acids while the anchovy was exposed to the air for a few days to dry.

Recently, Fujii *et al*¹²⁾ reported that iron-catalyzed lipid oxidation was initiated by the presence of small amounts of lipid peroxides. Since oxidation of free PUFA released from

esterified lipids such as phospholipid or triglyceride was accelerated by the presence of peroxides already formed in dry fish, MetHb formation was enhanced. In this study, MFIs varied across samples, as shown in Table I, and MFI in sample No. 4 in Table III was zero, even though the amounts of DHA and EPA were more than 50% of sample No. 3. These discrepant data suggested that the formation of MetHb was not only dependent on PUFA but also on those oxides.

Examination of the relationship between MFI level and PUFA concentration in terms of the results of our previous report²⁾ (*e.g.*, in sample No. 1 in Table III) indicates that 0.33 MFI is too low. The low value of actual measured MFI in fish lipid can be interpreted as indicating that antioxidants such as α -tocopherol, formed and contained in fish tissue,¹⁰⁾ may play a role in suppressing MetHb formation.

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References

- 1) J. Sajiki and K. Takahashi, *Eisei Kagaku*, **35**, 414 (1989).
- 2) J. Sajiki and K. Takahashi, *Eisei Kagaku*, **36**, 51 (1990) (in Japanese with English abstract).
- 3) A. Ozawa, E. Nakamura, H. Jimbo, T. Fujita, A. Hirai, T. Terano, T. Hamazaki, Y. Tamura and A. Kumagai, *Bunseki Kagaku*, **32**, 174 (1983) (in Japanese with English abstract).
- 4) J. Sajiki and K. Takahashi, *Bulletin Public Health Laboratory of Chiba Prefecture*, **13**, 9 (1989).
- 5) J. Sajiki and K. Takahashi, *Lipids*, "submitted."
- 6) B. Goldberg and A. Stern, *Arch. Biochem. Biophys.*, **178**, 218 (1977).
- 7) R.E. Lynch, G.R. Lee and G.E. Cartwright, *J. Biol. Chem.*, **251**, 1015 (1976).
- 8) P.T. Gamage, T. Mori and S. Matsushita, *Agric. Biol. Chem.*, **35**, 33 (1971).
- 9) S.-Y. Cho, K. Miyashita, T. Miyazawa, K. Fujimoto and T. Kaneda, *Nippon Suisan Gakkaishi*, **53**, 813 (1987).
- 10) N. Tsukuda, *Nippon Shokuhin Kogyo Gakkaishi*, **27**, 210 (1980) (in Japanese).
- 11) N. Tsukuda, *Bull. Tokai Reg. Fish Res. Lab.*, **94**, 51 (1987) (in Japanese with English abstract).
- 12) T. Fujii, Y. Hiramoto, J. Terao and K. Fukuzawa, *Arch. Biochem. Biophys.*, **284**, 120 (1991).