Studies on a Blue-Green Serum Pigment of Eel—III. Amino Acid Composition and Constituent Sugars*

Katsumi YAMAGUCHI, Kanehisa HASHIMOTO,
and Fumio MATSUURA**

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In the previous papers^{1,2)}, the authors reported the isolation and characterization of a blue-green pigment from eel serum, as well as the identification of its chromophore as biliverdin. Experiments on the nature of protein and sugar moieties are presented in this paper, as a part of a general investigation on the molecular structure of this complicated chromoprotein.

The results show that the chromoprotein resembles some mammalian serum protein in amino acid composition, and that it contains small amounts of both N-acetylneuraminic acid and glucosamine, too. The details will be given below.

Experimental

Material: The blue-green chromoprotein was highly purified from the serum of eel, *Anguilla japonica*, by the method reported previously¹⁾.

Preparation of protein moiety: Since the chromoprotein contains appreciable amounts of lipid as described in the previous paper²⁾, it was first delipidated in the following manner. A solution of the purified chromoprotein was treated with approximately ten volumes of a HCl-acetone mixture³⁾. Most of the lipid as well as biliverdin were readily extracted, while the residual protein precipitated in white flock. The precipitates were filtered, and washed several times with acetone. The precipitates were then extracted with ether, and chloroform/methanol (1:3)⁴⁾ for 24 hrs. each with mechanical stirring to remove residual lipid. The protein moiety obtained was grey. All the operations were carried out in a cold room at 4°C.

Isolation of sugars: A sialic acid was isolated and crystallized from the protein moiety according to the methods reported by SVENNERHOLM⁵⁾ and ENOMOTO *et al.*⁶⁾ with minor modifications. The isolation of hexosamine was also attempted essentially by the method of IZUMI⁷⁾.

Methods of analysis:

(1) Amino acid analysis: The protein moiety was hydrolyzed with 6N HCl at 110°C for 24 hrs. in a sealed evacuated tube and analyzed. For assay of tryptophan,

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** Lab. of Marine Biochem., Fac. of Agr., The Univ. of Tokyo, Bunkyo-ku, Tokyo (山口勝巳・橋本周久・松浦文雄: 東大農学部).

hydrolysis was conducted in a saturated $\text{Ba}(\text{OH})_2$ solution at 121°C for 7 hrs. in a sealed evacuated tube. A HITACHI model KLA-2 automatic amino acid analyzer was used. Nitrogen content was determined by the micro-Kjeldahl method. Moisture and ash contents were measured as usual.

(2) Sugar analysis:

Color reactions: After being hydrolyzed with 2N HCl at 100°C for 2 hrs., the protein moiety was examined for the Molisch, anthrone, Fehling, Benedict, orcin-HCl (Bial's), and Seliwanoff reactions⁶⁾. The Elson-Morgan reaction was also tested for hexosamine⁷⁾. The direct Ehrlich, Bial, diphenylamine, resorcinol, and thiobarbituric acid reactions for sialic acid⁷⁻⁹⁾ were tested on a specimen hydrolyzed with 0.05N H_2SO_4 at 80°C for 1 hr.

Paper chromatography: The protein moiety was hydrolyzed as described above and passed through a Doulite A-4 column to remove hydrogen ion. The eluate was concentrated under reduced pressure and used for chromatography. Ascending paper chromatography was carried out using Toyo filter paper No. 51 of 40×2 cm, at room temperature unless specified. Solvent systems employed were; (A) ethylacetate/pyridine/water (2:1:2), (B) ethylacetate/acetic acid/water (3:1:3), and (C) n-butanol/acetic acid/water (4:1:5). In addition, (D) n-butanol/pyridine/water (6:4:3) and (E) n-butanol/n-propanol/0.1N HCl (1:2:1) were used for sialic acid. Another system, phenol/ammonia^{7,10)}, was also used for hexosamine in chromatography at 37°C . After chromatographic runs, spots were developed with an ammoniacal silver nitrate solution, and/or with a ninhydrin solution⁶⁾.

Thin-layer chromatography: According to STAHL *et al.*¹¹⁾, thin-layer chromatography was conducted using a Kieselguhl G plate in a solvent system of 65% isopropanol/ethylacetate (35:65). The anisaldehyde-sulfuric acid reagent as well as an ammoniacal silver nitrate solution were sprayed for color development.

Melting point: Melting point of the sialic acid crystals obtained as above described was determined with a micro melting-point apparatus (YANAGIMOTO Co., Ltd., Tokyo).

Infrared spectrum: Infrared spectrum of the eel sialic acid was measured in compressed KBr with a KOKEN model DS-301 infrared spectrophotometer.

Determination of sialic acid: Sialic acid content of the protein moiety was determined according to the thiobarbituric acid method¹²⁾.

Determination of hexosamine: Hexosamine content of the protein moiety was determined using the Elson-Morgan reaction⁷⁾.

Results and Discussion

Amino acid composition: As shown in Table 1, the amino acid composition of the eel pigment is featured by relatively high contents of aspartic acid, glutamic acid, lysine, leucine, and histidine, and a very low content of half cystine. It would be

interesting to compare the amino acid pattern of the pigment to those of the other serum proteins of the same animal. However, the data on the latter are not available, which is true not only for eel, but also for the other fishes. The data available seem to be confined to those of mammalian, especially human serum proteins. Accordingly, the amino acid composition of the eel pigment was necessarily compared with those of some human serum proteins, and was found to resemble that of β_1 -lipoprotein among them^{13,14}), as shown in Table 1. Since this eel chromoprotein is regarded as a kind of lipoprotein, it may be reasonable that the pigment and human β_1 -lipoprotein show the resemblance in amino acid composition.

The total nitrogen, 12.40%, was very close to that calculated from the

amino acid composition, 12.37%. However, the total of amino acid contents was as low as 82.3%, or 74.2% after correction for the added water in hydrolysis, indicating that the rest, 25.8%, is to be attributed to substances other than amino acid. The most probable substances might be sugars and the following experiment was conducted.

Constituent sugars: All the sugar reactions for the hydrolyzate of the protein except Seliwanoff's were positive, indicating the presence of sugar. So, with the hydrolyzates obtained under various conditions, paper chromatography was carried out using solvent systems (A), (B), and (C), resulting in the appearance of two spots of sugar. The same number of spots appeared also in thin-layer chromatography. The spot with smaller R_f value was suspected to be sialic acid, because of rather frequent tailing in chromatography. This supposition was indirectly supported by the formation of large quantities of humin during acid hydrolysis of the protein preparation⁹). Thus, the glycoprotein was examined for the sialic acid-specific color

Table 1. Amino acid composition of glycoprotein moiety of eel pigment, in comparison to that of a human serum lipoprotein. (g amino acid / 100 g of protein).

Amino acid	Eel glycoprotein		Human β_1 -lipoprotein ¹³⁾
Lysine	7.74	9.40*	7.58
Histidine	6.10	7.40	3.00
Arginine	2.52	3.06	4.29
Aspartic acid	9.56	11.62	10.93
Threonine	4.01	4.88	5.84
Serine	3.97	4.83	6.60
Glutamic acid	10.58	12.85	14.05
Proline	2.66	3.23	3.34
Glycine	2.82	3.45	2.79
Alanine	3.54	4.30	4.20
Cystine/2	0.58	0.70	0.61
Valine	4.56	5.65	5.54
Methionine	1.92	2.34	2.08
Isoleucine	3.40	4.13	6.18
Leucine	7.21	8.77	11.93
Tyrosine	4.40	5.35	4.15
Phenylalanine	4.09	4.97	6.71
Tryptophan	1.05	1.27	0.88
Amide NH ₃	1.52	1.85	1.82
Total	82.32	100.0	102.5

* In this column are given amino acid contents in the left column, as multiplied by 100/82.32 for the convenience of comparison.

reactions, and all these were positive. The spot with larger Rf value, on the other hand, seemed to be a hexosamine because it was positive to both ammoniacal silver nitrate reagent and ninhydrin solution. In addition, the HCl-hydrolyzate of the glycoprotein was positive in the Elson-Morgan reaction, indicating the presence of hexosamine⁷⁾. The hexosamine was thought to be either glucosamine or galactosamine based on its Rf value. For further confirmation of the above result, the following experiments were carried out to isolate and examine these sugars.

Fig. 1. Sialic acid crystals from the blue-green serum pigment of eel.

i) Identification and determination of sialic acid: From 3 g of the glycoprotein, 5.0 mg of rod-shaped crystals were obtained (Fig. 1). The sialic acid crystals were melted and decomposed at 175–180°C, turning black. The melting point determined here was rather close to that of N-acetylneuraminic, N-glycolylneuraminic, or 4-O-acetyl-N-acetylneuraminic acid⁸⁾. However, the Egriwe's reaction was found to be negative for the crystals, excluding the possibility of N-glycolylneuraminic acid⁸⁾. Based on these results, together with the paper chromatographic behavior (Table 2) and infrared spectrum (Fig. 2), the sialic acid was identified as N-acetylneuraminic acid^{6, 8, 9, 15, 16)}. The content in the glycoprotein moiety was 1.4%.

Table 2. Paper chromatography of the crystalline sialic acid from the eel pigment.

Solvent	Rf			
	Sialic acid of eel pigment	NANA*	NGNA*	ONDANA*
Ethylacetate/pyridine/water (2:1:2)	0.15	0.16	0.14	0.25
Ethylacetate/acetic acid/water (3:1:3)	0.13	0.15	0.07	0.33
n-Butanol/acetic acid/water (4:1:5)	0.09	0.09	0.19	0.45
n-Butanol/pyridine/water (6:4:3)	0.18	0.20	0.14	0.32
n-Butanol/n-propanol/0.1 NHCl (1:2:1)	0.48	0.45	0.34	—

* NANA: N-acetylneuraminic acid

NGNA: N-glycolylneuraminic acid

ONDANA: 4-O-acetyl-N-acetylneuraminic acid

Rf values of these three acids were cited from literature^{8, 15)}.

ii) Identification and determination of hexosamine: Crystallization of the hexosamine was tried, but in vain. From the paper chromatographic data using the amorphous preparation, the hexosamine was identified as glucosamine^{7, 10)} (Table 3). In this connection, since IZUMI⁷⁾ reports a complete conversion of N-acetylglucosamine into glucosamine on heating in more concentrated than 2N HCl at 100°C for 2 hrs., the

Fig. 2. Infrared spectrum of the crystalline sialic acid of eel in compressed KBr.

Table 3. Paper chromatography of the purified hexosamine from the eel pigment.

Solvent	R_{glucose}		
	Hexosamine of eel pigment	Glucosamine*	Galactosamine*
n-Butanol/pyridine/water (6:4:3)	0.81	0.78	0.68
Phenol-ammonia	1.47	1.52	(Tailing)

* Authentic specimens.

following experiment was carried out to check the glucosamine under consideration in this respect; The glycoprotein preparation was moderately hydrolyzed with 0.01N HCl at 100°C for 10 mins.¹⁷⁾, neutralized and passed through a Dowex 50W-X8 (H^+ form, 50–100 mesh) column. The eluate was examined for N-acetylhexosamine by the modified Reissig method of Morgan-Elson reaction⁷⁾, giving a negative result. Thus, the glucosamine was evidenced to be non-acetylated in the glycoprotein. The glucosamine content in the moiety was determined to be 0.3%.

Based on the results obtained here, it may be pointed out that not only the amino acid composition but also the sugar moiety resemble those of human β_1 -lipoprotein¹³⁾.

Now, 78.5% of the glycoprotein moiety can be accounted for, on the basis of the total of amino acid (74.2%), N-acetylneuraminic acid (1.4%), glucosamine (0.3%), and ash (2.6%). A difference, 21.5%, still remains to be explained. As mentioned above, the chromoprotein was delipidated by successive treatments of acetone, ether, and chloroform/methanol (1:3) at low temperature to prevent possible modification. Therefore, some amounts of cohesive conjugated lipid might have been left remaining in the glycoprotein moiety, even after those treatments. In this connection, HILLYARD *et al.*¹⁸⁾ report that even mild treatments such as salting-out often make the delipidation of lipoprotein difficult. Actually, the glycoprotein moiety liberated the ether insoluble conjugated lipid, though slowly, when was repeatedly extracted with a hot chloroform/methanol (2:1) according to the method of FOLCH¹⁹⁾. Therefore, the greater

part of the unexplainable portion could be accounted for by such cohesive lipid.

Summary

The blue-green pigment of eel was analyzed for amino acid composition and constituent sugars. It contained relatively large amounts of aspartic acid, glutamic acid, lysine, leucine, and histidine. Small amounts of both N-acetylneuraminic acid and glucosamine were detected as constituent sugars. The composition, as a whole, resembled those of β_1 -lipoprotein of human blood serum.

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