Studies on the Haptoglobin of Eel—II Physico-chemical Properties

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The haptoglobin (Hp) isolated from eel serum was analyzed for several physico-chemical properties. The molecular weight was determined to be 97,000 and 93,500 by the sedimentation equilibrium and Sephadex TLC method, respectively. Eel Hp exhibited only a single absorption maximum at 280 nm with a specific extinction coefficient of 10.4, over the u.v. to visible region. The isoelectric point was measured to be 3.7 by the isoelectric focussing method. In amino acid composition, the contents of acidic amino acids were particularly high, while those of several amino acids such as Met, His, Trp were low. The amino acid residues totalled 72.18 g per 100 g of Hp. The remainder was found to be mostly accounted for by constituent sugars such as galactose, sialic acid, glucosamine, *etc.*

The above properties of eel Hp, as a whole, are fairly comparable to those of mammalian Hp.

As reported in the preceding paper,¹⁾ eel haptoglobin (Hp) was isolated in a highly pure state by a method mainly consisting of Sephadex gel filtration, and batch adsorption and column chromatography on DEAE-cellulose.

This time we examined some physico-chemical properties of this piscine Hp. Results obtained showed that eel Hp resemble the mammalian Hp in all the properties analyzed: molecular weight, isoelectric point, amino acid composition, constituent sugars, *etc.*

Experimental

Eel Hp Hp was isolated and purified from the serum of eel *Anguilla japonica* by the method described previously.¹⁾ The Hp specimens thus prepared were checked for homogeneity by starch gel electrophoresis. After desalting by dialysis, they were kept frozen around -20°C until analyzed.

Methods of analysis *Molecular weight* Molecular weight determinations were made by both sedimentation equilibrium method and Sephadex-thin layer chromatography (TLC) method. In the former, the Hp specimen was previously dialyzed against 0.1 M phosphate buffer pH 7.0, and a Hitachi model UCA-1A ultracentrifuge was employed at about 10,000 rpm. In calculation of molecular weight, a value of 0.75 was assumed for the partial specific volume of eel Hp. The Sephadex-TLC was performed on Sephadex

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G-100 according to MORRIS,²⁾ using the molecular weight markers (Mann Res. Lab.) and human hemoglobin (Hb) as a control.

Absorption spectrum The absorption spectrum was measured in distilled water over the u.v. to visible region with a Beckman model DU spectrophotometer. An aliquot of the solution was dried at 105°C until a constant weight was attained and the dry matter content obtained was used in calculation of the specific extinction coefficient of eel Hp.

Isoelectric point The isoelectric point was determined by the isoelectric focussing method,³⁾ employing an LKB Produkter's apparatus with a 110 ml electrolysis column. The carrier ampholyte for pH 3–5 was used. The starting condition of electric current was 400 V and 2 mA. After the current reached an equilibrium, 2 g fractions were collected and assayed for $E_{280\text{ nm}}$ and pH.

Amino acid composition Eel Hp was hydrolyzed with 6 N HCl at 110°C for 24 hr in a sealed evacuated tube and analyzed with a Hitachi model KLA-2 amino acid analyzer. Tryptophan was assayed by the spectrophotometric method of GOODWIN *et al.*⁴⁾

Constituent sugars The neutral sugar and amino sugar were analyzed as follows, referring to the method of IMANARI *et al.*⁵⁾ Eel Hp (usually 3–4 mg) was hydrolyzed with 2.5 N trifluoroacetic acid at 100°C for 7 hr. The lyzate was charged onto Amberlite CG-4B (AcO⁻ form) and CG 120 (H⁺ form) columns for separation into the neutral sugar and the amino sugar fractions.

The former fraction was treated with NaBH₄ to convert the sugars into corresponding sugar alcohols, which were further converted into trifluoroacetyl derivatives by treatment with trifluoroacetic acid anhydride, and subjected to gas liquid chromatography (GLC). On the other hand, the amino sugar fraction was similarly treated with NaBH₄ and trifluoroacetic acid anhydride to convert them into trifluoroacetyl derivatives of corresponding amino sugar alcohols, and analyzed by GLC.

GLC was performed by a Shibata 4B gas chromatograph equipped with a hydrogen flame ionization detector, using a 4 mm × 1.8 m column packed with 2% XF-1105 Gas-Chrom P. The flow rate of nitrogen carrier gas was 30 ml/min and the temperature was 100–150°C with an increment rate of 5°C/min. Peaks were identified as usual by cochromatography with the authentic sugar specimens and quantitated by use of their calibration curves established previously.

The sialic acid content of eel Hp was determined by the thiobarbituric acid method.⁶⁾

Results and Discussion

Molecular weight The molecular weight of eel Hp was determined to be about 97,000 by the sedimentation equilibrium method. On the other hand, a value of 93,500 was obtained for the molecular weight by the Sephadex-TLC method. These values for

eel Hp are in the range hitherto reported for various mammalian Hp's, 60,000–100,000.⁷⁻¹⁰⁾

Absorption spectrum As shown in Fig. 1, eel Hp exhibited an absorption maximum at 280 nm with a shoulder at 290 nm in the u.v. region, but not in the visible region at all. The specific extinction coefficient ($E_{1\%}^{1\text{cm}}$) at 280 nm was calculated to be 10.4. This value

Fig. 1. Absorption spectrum of eel Hp.
Solvent: water

Fig. 2. Isoelectric focussing of eel Hp.

Table 1. Amino acid composition of eel Hp in comparison of those of three mammalian Hp's

	Grams of residue/100 g Hp			
	Eel	Human ⁽¹⁾ (Hp 1-1)	Dog ⁽⁹⁾	Rabbit ⁽¹²⁾
Asp	9.28	9.27	8.94	8.33
Thr	4.65	4.36	3.97	4.45
Ser	5.11	3.36	3.14	4.63
Glu	9.07	9.90	10.37	10.11
Pro	3.66	4.02	4.17	3.26
Gly	3.00	2.65	3.08	2.37
Ala	3.31	3.43	3.38	3.68
Cys (1/2)		2.13	1.52	1.64
Val	5.69	6.20	7.19	5.09
Met	1.25	1.05	1.93	1.14
Ile	2.96	3.32	3.07	3.42
Leu	6.99	6.30	5.95	6.41
Tyr	2.44	4.95	5.38	5.94
Phe	3.73	2.64	3.12	3.74
His	1.05	3.00	2.97	2.99
Lys	3.82	8.16	7.96	6.37
Arg	4.84	2.26	1.45	3.72
Trp	1.33	2.96	2.74	2.20
Total	72.18	74.84	80.33	75.65

is somewhat smaller than 11.6–11.8 for several mammalian Hp's.^{8,11)}

Isoelectric point The electric current reached an equilibrium at 750 V and 1.5 mA, 48 hr after the isoelectric focussing was started. As seen in Fig. 2, a single peak appeared in respect of $E_{280\text{ nm}}$, demonstrating again high homogeneity of the preparation of eel Hp analyzed. The isoelectric point, 3.7, of this Hp, as obtained from this curve is slightly lower than those for mammalian Hp's, 3.9–4.2.^{8,11)}

Amino acid composition As seen in Table 1, eel Hp is rich in acidic amino acids, and poor in several amino acids such as Met, His, Trp, *etc.* Comparing to the mammalian Hp's^{9,11,12)} cited for reference, eel Hp is somewhat higher in the contents of Ser, Leu, Arg, *etc.*, and lower in those of Tyr, His, Lys, *etc.*, though there is a general resemblance in the amino acid composition among the four Hp's. The total of amino acids in this piscine Hp was 72.18 g/100 g, suggesting the possibility that it is also a glycoprotein as is the mammalian Hp.

Constituent sugars The chromatogram of the neutral sugar fraction treated as mentioned above is presented in Fig. 3. Three peaks appeared and were identified

Fig. 3. Gas chromatographic identification of the neutral sugars (above) and amino sugar (below) of eel Hp. Both sugar fractions were treated with NABH_4 and trifluoroacetic acid anhydride, and the trifluoroacetyl derivatives formed were chromatographed. See the text for further details.

Table 2. Constituent sugars of eel Hp, as compared to those of several mammalian Hp's

	Grams of residue/100 g Hp			
	Eel	Human ⁽¹¹⁾ (Hp 1-1)	Dog ⁽⁹⁾	Rabbit ⁽¹²⁾
Fucose	0.6	0.2	0.17	0.3
Mannose	1.6	} 7.79	1.77	} 6.6
Galactose	4.6		3.62	
Glucosamine	4.1	5.3	5.47	5.6
Sialic acid	4.3	5.3	3.93	5.4
Total	15.2	18.59	14.96	17.9

to be fucose, mannose, and galactose in the order to elongated retention time. On the other hand, a single peak appeared in the case of amino sugar fraction, as seen also in Fig. 3. It was identified as glucosamine.

The contents of those four sugars in eel Hp is given in Table 2, together with the data of some mammalian Hp's.^{9,11,12)} The content of sialic acid is also included. It is clear from the table that the constituent sugars of eel Hp are rather similar, qualitatively as well as quantitatively, to those of the mammalian Hp.

Thus, it may be concluded that eel Hp is fairly comparable to the mammalian Hp in physico-chemical properties.

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