Studies on the Haptoglobin of Eel—I Purification of Haptoglobin

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Haptoglobin (Hp), a hemoglobin (Hb)-binding protein, was isolated and purified from eel serum by a method which consists of Sephadex G-25 gel filtration, DEAE-cellulose batch adsorption, DEAE-cellulose column chromatography, and Sephadex G-100 gel filtration. The Hp specimen thus purified was homogeneous in starch gel electrophoresis at pH 8.4. The yield averaged about 20 mg per 100 ml of eel serum. It was noticed during the purification that, unlike rabbit Hp, prolonged standing in NaCl solution and adsorption to DEAE-cellulose results in a decrease of Hb-binding capacity.

As will be published elsewhere, haptoglobin (Hp), a hemoglobin (Hb)-binding serum protein, is widely distributed in fishes as in mammals. In contrast to the mammalian Hp which binds Hb in 1:1 molar ratio, the fish Hp seems to bind more than one mole of Hb per mole, as far as hitherto reported: KOEHN¹⁾ suggested, on the basis of some electrophoretic studies with catostomid serum, that its Hp molecule possesses more than one Hb binding sites. AIZAWA²⁾ observed, by use of a partially purified eel Hp, that it binds roughly two moles of Hb per mole. On the other hand, few information is available at present of physico-chemical properties of fish Hp.

The situation being mentioned above, it seemed interesting from the viewpoint of comparative biochemistry to isolate and characterize fish Hp, and this series of study was undertaken, taking for convenience the eel as the source of Hp. The present paper deals with its purification.

Experimental

Serum and hemoglobin By heart puncture or by cutting the head, blood was collected from a number of live specimens of the eel *Anguilla japonica* with or without heparin as the anticoagulant. After centrifugal separation from the red cells, the serum (or plasma) was used immediately for purification of Hp as described below. The serum was also provided from the rabbit which was previously injected with turpentine (1.5 ml/kg of body weight) to raise the Hp level,³⁾ and used for purification of Hp for comparison.

Component F of eel Hb was isolated from the above sedimented red cells by starch

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block electrophoresis,⁴⁾ converted into the cyanmet form, and used for detection and purity check of eel Hp as given below. Rabbit Hb was also prepared and used for the same purpose. Throughout the present study, was used the Hb solution of several percent, as determined spectrophotometrically using a molar extinction coefficient of cyanmet Hb at 540 nm, 11,500, by DRABKIN.⁵⁾

As occasion demanded, the serum and the red cells, washed with saline, were mixed with about an equal volume of a glycerol-5% citrate mixture (2:3, v/v),⁶⁾ and kept frozen around -20°C . Just before use, the glycerol-containing cakes were thawed and dialyzed against several changes of large amounts of cold distilled water.

Detection and purity check of Hp In the course of purification, Hp was detected by comparing the electrophoretic patterns of the test solution with and without Hb added. The procedure adopted is briefly as follows.

To begin with, protein concentration of the test solution was estimated either by u.v. spectrophotometry or by biuret method and, to its aliquot was added the Hb solution so that the protein to Hb ratio was approximately 1:1 or more. The mixture thus prepared was electrophoresed for 3 hr in a starch gel at 13 v/cm in a low temperature room. The test solution without Hb added and the Hb solution were also run. As the gel and bridge solutions, 0.018 M Tris-0.015 M borate buffer and 0.18 M Tris-0.15 M borate buffer, both containing 0.01% KCN and at pH 8.4, were used respectively. After completion of the electrophoresis, the gel was sliced horizontally. One of the sliced gels was stained with amido black 10B for protein and the other with benzidine for hemo-protein. By comparing those patterns, the protein band which was made benzidine-positive and whose mobility was changed upon addition of Hb, was judged as Hp.

The purity of Hp preparation at each purification step was estimated by comparing the intensity of the Hp band thus detected and that of other protein band(s) in the amido black stained pattern.

Purification of Hp After the preliminary examination, Hp was isolated and purified from the eel serum by the following method which is an improvement of the method of CONNELL *et al.*⁷⁾ and consists of Sephadex G-25 gel filtration, DEAE-cellulose batch adsorption, DEAE-cellulose column chromatography, and Sephadex G-100 gel filtration. All the experimental operations were performed in a low temperature room at $2-4^{\circ}\text{C}$.

Pretreatment The freshly prepared or deep-frozen serum (usually 50 ml) was adjusted to pH 4.6 with 1 M CH_3COOH , and the precipitates formed were eliminated by centrifugation.

Sephadex G-25 gel filtration The pretreated serum was subjected to gel filtration through a Sephadex G-25 column (5×60 cm) which was previously treated with 0.1 M NaCl and thoroughly washed with distilled water. The flow rate was fixed at 12 ml/min. The elution curve obtained is presented in Fig. 1. Since Hp was detected exclusively

Fig. 1. Sephadex G-25 gel filtration of eel serum.

in the first peak, the brownish-colored fractions as indicated by both arrows were combined and purified further.

DEAE-cellulose batch adsorption The DEAE-cellulose was treated as usual with 0.1 N NaOH and 0.1 N HCl, and finally suspended in distilled water at about 25 mg/ml, adjusting to pH 4.6. About 40 ml of the suspension, equivalent to *ca.* 1 g of dry DEAE-cellulose, was added to the above crude Hp solution, stirred for 15 min, and filtered to collect the Hp-adsorbing DEAE-cellulose. After washing with small amounts of distilled water, the DEAE-cellulose cake was suspended in distilled water and poured into a glass filter (2×10 cm). The Hp fraction as

checked by the above method was eluted with 0.2 M NaCl. The adsorption-elution procedure was repeated, usually three to four more times, until practically no Hp was detected in the eluate. During these operations, special care was taken so as to minimize the standing time of Hp in 0.2 M NaCl for the reason described in *Discussion*. Finally, all these Hp-containing brown eluates were combined and dialyzed against 0.03 M acetate buffer at pH 4.6.

DEAE-cellulose column chromatography The dialyzed solution was loaded onto a DEAE-cellulose column (2.5×40 cm) which was previously treated with 0.1 N NaOH and bufferized with 0.03 M acetate buffer at pH 4.6. Then the column was successively washed with about 500 ml each of 0.149 M acetate buffer pH 5.4, 0.188 M acetate buffer pH 5.6, and finally 0.2 M NaCl. Care was taken to initiate the washing immediately after

Fig. 2. DEAE-cellulose column chromatography of crude eel Hp.

the crude Hp was adsorbed on the column for the reason given below. As seen in Fig. 2, three peaks appeared. The Hp was detected in the fractions on and after the shoulder of the last peak. Those fractions were combined, desalted by dialysis, and concentrated by ultrafiltration.

Sephadex G-100 gel filtration The concentrated solution was subjected to gel filtration on a Sephadex G-100 column (2.5×100 cm) previously washed thoroughly with distilled water. As shown in Fig. 3, two peaks appeared. The fractions of the fast peak were brown and non-Hp. The slow peak, consisting of colorless fractions, was found to contain Hp, and hence the fractions as indicated by both arrows were combined and concentrated by means of ultrafiltration.

Starch gel electropherograms of the Hp solutions at respective purification steps are schematically presented in Fig. 4. As seen there, the eel Hp specimen finally obtained was homogeneous. Its yield was about 20 mg per 100 ml of blood serum. A homogeneous rabbit Hp was also isolated by the same procedure in the yield of about 90 mg from 100 ml of serum.

Fig. 3. Sephadex G-100 gel filtration of crude eel Hp.

Fig. 4. Purification of eel Hp, as seen from starch gel electropherograms. Amido black stain.

Discussion

The first two steps in the above procedure, Sephadex G-25 gel filtration and DEAE-cellulose batch adsorption, were adopted after CONNELL *et al.*⁷⁾ with some modifications. They showed that human Hp is satisfactorily purified by these two steps and further purified by fractionation with ammonium sulfate. This was almost true for rabbit Hp. In the case of eel Hp, however, large amounts of contaminating proteins were still present in the Hp preparation even after the batch adsorption step was finished (Fig. 4), as has already been pointed out by AIZAWA.²⁾ In addition, eel Hp, unlike human Hp, was found to remain soluble in a fairly high concentration after fractionation with 50% ammonium sulfate. Therefore, the fractionation step was replaced by DEAE-cellulose

column chromatography and Sephadex G-100 gel filtration, as mentioned above, resulting in attainment of a highly purified eel Hp. AIZAWA also adopted DEAE-cellulose column chromatography for further purification of eel Hp, using simultaneously continuous gradients of pH and acetate buffer concentration. However, we found that a stepwise elution as described above is more effective in the purification of eel Hp. In addition, Sephadex G-100 gel filtration was found to be very useful in further purification of this Hp.

During the examination of the purification procedure of eel Hp, it was noticed that its Hb-binding capacity is liable to decrease as the time of its contact with NaCl and the time of its adsorption onto DEAE-cellulose are prolonged: The binding capacity of eel Hp specimen purified under sufficient care in these respects was more than two moles per mole, in contrast to about one mole per mole in that prepared otherwise. As far as rabbit Hp is concerned, however, such difference was not recognized. The Hb-binding behaviors of eel Hp will be reported later in detail.

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