キハダマグロ眼球のヒアルロン酸の特性

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Characterization of Hyaluronic Acid of Yellowfin Tuna Eyeball

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Hyaluronic acid was isolated from yellowfin tuna eyeball. Electrophoretic behavior of the hyaluronic acid confirmed to that of authentic hyaluronic acid. The hyaluronic acid obtained here almost digested by hyaluronidase.

The intrinsic viscosity was 10.85 dl/g. The viscosity average molecular weight calculated from the intrinsic viscosity was 5.6×10^5. The sedimentation constant in 0.5 M NaCl solution was 7.9 S. The value of the intrinsic viscosity and the molecular weight were similar to that of hyaluronic acid (fraction V) fractionated from bovine viterous body by Laurent *et al.* The sedimentation constant of our fish hyaluronic acid was larger than that of their bovine hyaluronic acid.

Hyaluronic acid, one of the mucopolysaccharides, has been found in many different connective tissues, such as human umbilical cord, bovine vitreous body, and cock comb. The physicochemical properties of hyaluronic acid from these connective tissues have been studied by many investigators.[1] However, no hyaluronic acid isolated from fish has so far been studied. This paper dealt with the isolated hyaluronic acid from yellowfin tuna eyeball and with hydrodynamical characterization of the hyaluronic acid, its viscosity, and sedimentation behavior.

Materials and Methods

Preparation of Hyaluronic Acid

Hyaluronic acid of the fish eyeball was prepared by essentially the same method as usual for the preparation of glucosaminoglycans from urinary excretion reported by Ohkawa *et al.*[2] The outline is as follows: Eyeballs were taken from frozen-stored yellowfin tuna *Thunnus albacares* which had been frozen immediately after capture. After thawing, the eyeballs were cleaned up these surrounding tissues and washed with distilled water. Corneas were cut out. The vitreous body, vitreous humor, and crystalline lens were collected and homogenized without buffer at 5°C. The homogenate was filtered with a gauze. About 700 ml of the filtrate was obtained from 20 eyeballs. An equal volume of cold acetone was added to the filtrate (700 ml) and the mixture was stirred overnight at 4°C. After centrifugation at 6,000 × g for 30 min, the precipitate was collected. This process was repeated twice. The precipitate was dried with a rotary evaporator to remove the acetone, and then, digested with 1% Actinase E, a commercial preparation of proteinase(s) from *Streptomyces griseus* (Kaken Pharmaceutical Co.). The digestion was carried out in 0.1 M Tris-HCl buffer solution containing 50 mM NaCl (pH 7.8) at 37°C for about 24 h. The reactant was heated at 80°C for 15 min. After the centrifugation at 6,000 × g for 15 min, the supernatant was dialyzed against distilld water.

Cetylpyridinium chloride (CPC) of 3% solution was added drop by drop to the dialyzed supernatant with stirring slowly until the complex of hyaluronic acid and CPC was formed. When white precipitate formed and unbreakable bubbles were formed on the surface of the liquid, the addition of CPC was stopped. The precipitate was dissolved in 0.5 M NaCl solution with stirring overnight at 4°C.

To the dializate was added 10 ml of 1 M potassium thiocyanate (KSCN) and about 5 g of celite. After the mixture was stirred vigorously and insoluble matter was removed by centrifugation at 10,000 × g for 15 min, the supernatant was filtered with a glass filter of 3G3. The supernatant was dialyzed against distilled water, then the hyaluronic acid was precipitated by the ad-

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dition of 4 volumes of 95% ethanol containing 10% CH_3COOK. The precipitate was dried over P_2O_5 *in vacuo*.

Electrophoresis of Hyaluronic Acid

Two dimensional electrophoresis was employed to the sample using a cellulose acetate membrane, 10 cm × 10 cm. In the first electrophoresis, 0.1 M pyridine-0.4 M formic acid buffer solution (pH 3.0) was used. In the second one, 0.1 M barium acetate (pH 8.0) was used as a supporting medium. As staining and decoloring reagents, 0.1% alcian blue 8GX in 0.1% acetic acid and 0.1% acetic acid were used. The following mucopolysaccharides were used as authentic markers; chondroitin 4-sulfate, dermatan sulfate, and hyaluronic acid originated from testes of bovine which were purchased from Seikagaku Kogyo Co., Tokyo.

Digestion Test by Enzyme

To confirm that the preparation obtained was hyaluronic acid, the digestion test was made. Hyaluronic acid at the concentration was 1.25 mg/m*l* was digested by 1 unit of hyaluronidase (EC 3.2.1.35) in 0.05 M sodium acetate solution at 50°C for 48 h. Aliquot of the reaction mixture was subjected to electrophoresis on a cellulose membrane with the authentic hyaluronic acid, using the above 0.1 M pyridine-0.4 M formic acid as a supporting electrolyte.

Viscosity Measurement

Viscosity measurement was made for hyaluronic acid in 0.5 M NaCl solution at 20°C using an Ubbelohde type viscometer with a flow time of *ca.* 120*s* for water.

Ultracentrifugation

To determine the sedimentation constant of the hyaluronic acid, the sedimentation velocity measurements were performed on a Hitachi 282 ultracentrifuge. As a solvent, 0.5 M NaCl solution was used. Double sectorial cells were used for all measurements. The rotor speed was set at 55,000 rpm, and the temperature was maintained at 20°C. The sedimentation constant was calculated by the usual method.[3]

Results and Discussion

Figure 1 shows the results of the digestion test of the samples and the hyaluronic acid. As is seen in the figure, the samples obtained in this study was almost digested by a hyaluronidase. However, the hyaluronidase (EC 3.2.1.35) is generally known as the enzyme to digest both hyaluronic acid and chondroitin sulfate. The two dimensional electrophoresis pattern of hyaluronic acid is shown in Fig. 2. It is clear that

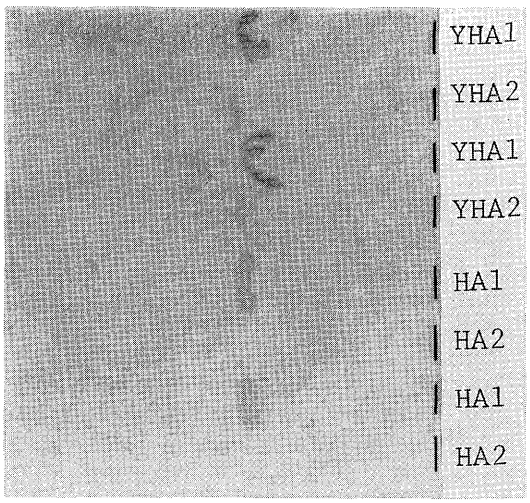

Fig. 1. Electrophoresis patterns of hyaluronic acid. YHA1, hyaluronic acid from yellowfin tuna eyeball without treatment of hyaluronidase; YHA2, hyaluronic acid from tuna eyeball after digestion with hyaluronidase. HA1, standard hyaluronic acid without treatment of hyaluronic acid after digestion with hyaluronidase.

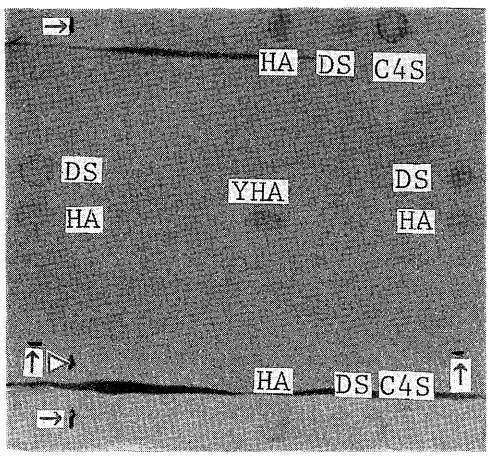

Fig. 2. Two-dimensional electrophoresis pattern of hyaluronic acid of yellowfin tuna eyeball. C4S, chondroitin 4-sulfate; DS, dermatan sulfate; HA, standard sample of hyaluronic acid; YHA, hyaluronic acid from yellowfine tuna eyeball. Arrows indicate the points of the origin of the standard mucopolysccharide and the triangle indicates the point of the origin of hyaluronic acid from tuna eyeball.

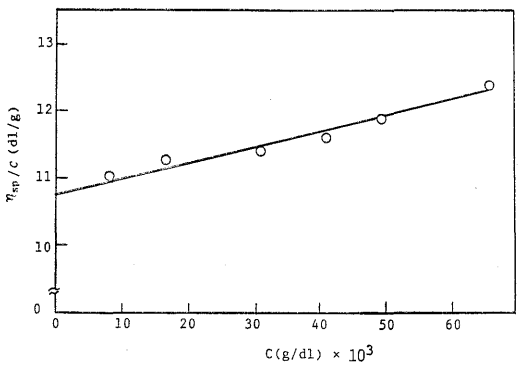

Fig. 3. Plot of the reduced viscosity of hyaluronic acid in 0.5 M NaCl solution versus concentration.

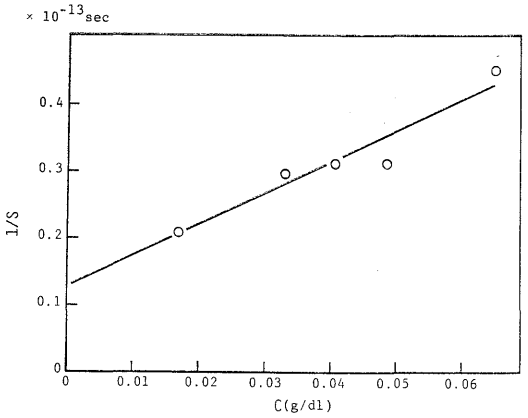

Fig. 4. Concentration dependence of the reciprocal of the sedimentation coefficients of hyaluronic acid in 0.5 M NaCl solution.

the migration position of samples on the two dimensional electrophoresis corresponded to that of authentic hyaluronic acid alone. Thus, the sample purified from yellowfin tuna eyeball was confirmed to be hyaluronic acid.

Figure 3 shows the concentration dependence of reduced viscosity of hyaluronic acid from yellowfin tuna eyeball in 0.5 M NaCl solution. The intrinsic viscosity was found to be 10.85 dl/g. The viscosity average molecular weight was calculated to be 5.6×10^5 from the equation, $[\eta]=0.036\times M^{0.78}$, proposed for the relation between intrinsic viscosity $[\eta]$ and molecular weight (M) of hyaluronic acid by Laurent *et al.*[4] The sedimentation constant of the hyaluronic acid from yellowfin tuna eyeball was calculated to be 7.9S from the plot of concentration dependence of the sedimentation coefficient of hyaluronica cid as shown in Fig. 4. This value is somewhat larger than that of 6.2S measured for the hyaluronic acid from cock comb.

Hyaluronic acid has been prepared from many different tissues by a variety of purification methods and is usually obtained as polydisperse molecular substances. Moreover, various values of the molecular parameters such as molecular weight, intrinsic viscosity, and sedimentation constant, have been reported by many investigators for hyaluronic acid.[5-9] It has been considered that the reason for the difference in the molecular parameters of hyaluronic acid might be attributed to be difference in factors such as the sources, the age and breed, and the method of purification employed.

Laurent *et al.*[4] fractionated bovine vitreous body hyaluronic acid and obtained two major molecular species of hyaluronic acid whose molecular weights were 1.5×10^6 (fraction III) and 4.1×10^5 (fraction V). The sedimentation constant was 7.2S for the former and 3.8S for the latter and the intrinsic viscosity was 24.5 dl/g for the former and 8.8 dl/g for the latter. Our values for the molecular parameters of hyaluronic acid, the intrinsic viscosity and the molecular weight, are similar to that of the latter, except for the sedimentation constant. As it may be considered that the hyaluronic acid obtained in this study exists as polydisperse in solution, the fractionation of hyaluronic acid of fish eyeball remains to be carried out.

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