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Isolation and characterization of hyaluronic acid from the liver of marine stingray *Aetobatus narinari*

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ABSTRACT

Although hyaluronic acid research pursuits ahead in exploring its biomedical perspective, very limited investigations were carried out in their isolation shape view point, furthermore, most of the investigations were targeted towards the terrestrial source. To swerve from that, the present study was projected through the marine superstore, where in high molecular weight hyaluronic acid of 13, 65,863 Da was isolated from the liver of stingray *Aetobatus narinari*. The purified HA was confirmed at the preliminary level by their stains all dye binding nature. Their analytical composition including carbon, hydrogen, nitrogen, N-acetyl glucosamine, glucuronic acid contents was analysed. The HA was characterized by agarose-gel electrophoresis, FTIR, HPTLC, and ^{1}H NMR. The DPPH radical scavenging activity of HA and its reducing power was evident to all the tested concentrations, but lower than that of ascorbic acid. HA showed significant inhibition against the proliferation of cells, substantiating its influence in regulation of cell functions.

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1. Introduction

Hyaluronic acid (HA), a biopolymer of global interest is a very attractive component for biomaterial research from several points of view. Hyaluronan, an anionic linear polysaccharide was formerly known as acid mucopolysaccharide and is now called as glycosaminoglycans [1] is a non sulphated glycosaminoglycan distributed widely throughout connective, epithelial and neural tissues, as well as the synovial fluid, skin, and cartilage. Unlike a lot of other ingredients typically found in nutritional supplements, hyaluronic acid is a substance that occurs naturally in the human body. It is a high molecular weight (10^5 and 10^7 kDa) polysaccharide [2] with an unbranched backbone composed of alternating sequences of (1-4) glucuronic acid and (1-3)-N-acetyl glucosamine moieties.

In animals and man, the half-life of hyaluronan in tissue, ranges from less than one to several days. HA breaks down to short molecules during the metabolic pathway through the tissues, lymphatic's, lymph nodes, blood, liver and kidney in series [3,4]. Enzymatic degradation cleaves the hyaluronic acid macromolecule into small polymers, each comprising of variable lengths of dimer chains, many of which appear to modulate wound healing, although studies have indicated that most of the effects attributed to the molecule are applicable to only a few products.

Because of its high biocompatibility and its common presence hyaluronan is gaining popularity in anti-ageing treatments, nutritional supplements, and medical treatments. Hyaluronic acid being a viscous slippery substance is multifunctional glue which occurs mainly in the extracellular matrix and is thought to take part in many biological functions like cell proliferation, differentiation and tissue repair. In synovial fluid, the elevated concentration of high molecular weight hyaluronic acid provides necessary lubrication for the joint and serves as a shock observer, reducing friction of the moving bones and diminishing wear and tear of the joints [5]. Hyaluronic acid is now recognized to play important roles in embryogenesis, signal transduction and cell motility, and is associated with cancer invasiveness and metastasis [6]. Large matrix polymers of hyaluronic acid are space filling, anti-angiogenesis and immunosuppressive, whereas the intermediate sized polymer comprising 25–50 disaccharides are inflammatory, immunostimulatory and highly angiogenic [7]. As pointed above, the biological properties of smaller hyaluronic acid fragments may be quite distinct and even opposite of those of the larger precursor molecules. Focus has moved recently to HA polymers as drug delivery devices with studies suggesting a number of molecules might be used as gel preparations for drug transport. Since the application of the polysaccharide in the medicinal field has been increased, the interest in isolation of HA has been augmented to greater extents.

The importance of this polysaccharide has increased significantly as the problem of the search for substitutes of the vitreous body was formulated at the end of the 1950 and the expediency of the utilization of HA solutions for this purpose was substantiated

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[8,9]. The expanding application of HA derived therapeutics emphasizes the impetus to change the pace for the world's rummage of source target for HA isolation. Since HA has received much attention lately as a natural source of health and beauty, this stab of isolation of HA from marine source was carried out to endow with an alternative source for hyaluronic acid (HA) isolation.

2. Materials and methods

2.1. Collection of animal

The liver of stingray *Aetobatus narinari* was collected from Parangipettai (latitude 11°29′N; longitude 79°46′E) fish processing company during February and March. The animal was dissected, and the liver was freshly collected and transferred to the laboratory in an ice box. The liver was stored at −20 °C until further use.

2.2. Isolation of glycosaminoglycans

Isolation of GAGs was done according to the procedure reported elsewhere [10]. The 250 g of tissue was taken and defatted with acetone and dried at 60 °C for 24 h. The 5 g of pellet was solubilized (1 g/20 ml) in the 100 mM sodium acetate buffer pH 5.5 containing 5 mM EDTA and 5 mM cysteine. 100 mg of papain was added per gram of tissue, and the solution was incubated for 24 h at 60 °C in a stirrer. After boiling for 10 min, the mixture was centrifuged at 5000 × *g* for 15 min and three volumes of ethanol saturated with sodium acetate were added to the supernatant and stored at 4 °C for 24 h. The precipitate was recovered by centrifugation at 5000 × *g* for 15 min and dried at 60 °C for 6 h.

2.3. Purification of GAGs

The crude sample (about 715 mg) was dissolved in 10 ml of 0.05 M NaCl. After centrifugation at 10,000 × *g* for 10 min, the supernatant was applied to a column (1 cm × 20 cm) packed with DEAE Cellulose® A-25 anion-exchange resin equilibrated with the same NaCl solution. The GAGs were eluted with a linear gradient of NaCl from 0.05 to 1.2 M at a flow of 1 ml/min. Fractions of 2 ml were collected.

2.4. Hyaluronic acid determination

The 96 well assay for quantification of uronic acid was performed by following [11]. Serial dilution of standard and sample of 50 μl was pipetted into a 96 well plate. 200 μl of a solution of 25 mM sodium tetraborate in sulphuric acid was added. The plate was heated for 10 min at 100 °C in an oven and cooled at room temperature for 15 min. 50 μl of 0.125% carbazole in absolute ethanol was carefully added and heated at 100 °C in an oven. After cooling at room temperature for 15 min, the plate was read in the micro plate reader (Softmax, USA) at a wavelength of 550 nm.

Two volumes of ethanol were added to the collected fractions corresponding to single species of polysaccharides evaluated by hyaluronic acid assay and precipitated at 4 °C. The dried material with the purified GAGs then dissolved in 10 ml water, transferred to a dialysis bag and dialysed against running tap water for 24 h, lyophilized and solubilized in distilled water for further characterization.

2.5. Agarose-gel electrophoresis

Discontinuous agarose-gel electrophoresis using barium acetate/0.05 HCl was performed according to the procedure reported elsewhere [12]. Agarose-gel was prepared at a concentration of 0.5% in 0.04 M barium acetate buffer pH 5.8 with a thickness of about 4–5 mm. Samples of 2–20 μl dissolved in distilled water were loaded into the wells. The run was in 0.05 M HCl for 180 min at 200 mA and in 0.04 M barium acetate for 60 min at 100 mA. After migration, the plate was soaked in a solution of 0.2% cetylpyridinium chloride. After drying, the plate was stained with freshly prepared toluidine blue (0.2% in ethanol–water–acetic acid, 50:49:1) for 30 min, and destained with ethanol–water–acetic acid (50:49:1). Further the plates were stained with stains all (25 mg in 500 ml ethanol–water (50:50) overnight in the dark and destained with water) to reveal HA.

2.6. Spectroscopic investigation of the hyaluronic acid

Spectroscopic investigation of the hyaluronic acid in solution was carried out [13]. Briefly distinct concentrations of HA were prepared in deionized water with concentrations ranging from 5 to 25 μg/ml. The dye was prepared by dissolving 5 mg stain all in 43 ml of water and 7 ml of methanol. The absorbance spectrum of several solutions of both the dye and sample hyaluronic acid with different concentrations was scanned between 320 and 950 nm by using Shimadzu spectrophotometer.

2.7. Compositional analysis

Carbon, hydrogen and nitrogen content of the samples was determined by Perklin Elmer CHNS/O analyser (USA). The uronic acid and N-acetyl glucosamine contents of the sample were determined [14,15].

2.8. GPC

The molecular weight of the isolated HA from *A. narinari* liver was determined with GPC–HPLC system by using the RI detector with column TSK G2000 SW& TSK G 3000 SW joined in series.

2.9. HPTLC

The isolated HA from *A. narinari* liver was dissolved in the phosphate buffer pH 7.0 and analysed in HPTLC with some modifications [16]. Standard and samples were loaded on a pre coated silica gel-60 TLC plate. The mobile phase consisting of n-butanol/formic acid/water, 4:8:1 (v/v) was used. After derivatization, the plate was stained by dipping in diphenylamine–aniline–phosphoric acid regent (1 ml of 37.5% HCl, 2 ml of aniline, 10 ml of 85% phosphoric acid, 100 ml of ethyl acetate and 2 g diphenylamine) for 3 s and heated at 150 °C for 10 s. TLC plate was scanned and analysed by CAMAG Scanner 3. The spectral scanning was carried out with D2 and W light. The scanning was started from 200 nm to 400 nm in absorption measurement mode.

2.10. FTIR

The solid sample of HA was subjected to IR spectroscopy (instrument) which helped to find the presence of different amino, carboxyl and hydroxyl groups of the sample. One part of the sample was mixed with ninety nine parts of dried KBr and then compressed to prepare a salt disc (3 mm diameter). These discs were analysed under IR-spectrophotometer. The absorption was read between 400 and 4000 cm^{-1}.

2.11. ^1H NMR spectroscopy

^1H NMR spectra were obtained using a Bruker Avance 400 nuclear magnetic spectrometer (Bruker DRX 500, Rheinstetten, Germany) operated at 400 MHz. The samples were pre-lyophilized three times with D_2O and finally prepared by dissolving 5 mg in

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