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Extraction, purification and characterization of hyaluronic acid from Rooster comb

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Abstract: Hyaluronic acid, is extract by different procedures from various sources like pig, rabbit, oxes and human are available, but these processes have certain imitations like low yield, and also it requires the killing of these animals which is against the experimental ethics. In the present study, we have carried out the extraction of hyaluronic acid from cock's comb which was further analyzed with qualitative test, viscosity, UV absorption, endotoxin detection assay. Also, the protein contamination of extracted hyaluronic acid was determined by using SDS-PAGE of hyaluronic acid was studied for checking the protein contaminants and it was noted that there were no bands observed in the well loaded with extracted hyaluronic acid sample indicating that the final extract of hyaluronic acid is not contaminated with the protein. The extraction and purification of hyaluronic acid by using the method reported here give pure hyaluronic acid. The viscosity of extracted hyaluronic acid was found to be 2.55 poise which is economical and can be used for industrial production of hyaluronic acid having clinical applications.

Keywords: Hyaluronic acid, Osteoarthritis, protein contamination Rooster combs SDS-PAGE

INTRODUCTION

Hyaluronic acid is a long chain polysaccharide found in all mammals. It is present in loose connective tissue, skin, the eye and synovial fluid where it secreted continuously by the synovial membrane into the joint space and comprises the major macro-molecular part of the synovial fluid. It is highly concentrated at the surface of the articular cartilage and the superficial layers of the synovial membrane. In the synovial fluid, HA acts as both a lubricant and a shock absorber (Balazs , Watson , Duff IF, and Saul 1967) Due to the meshwork it forms with aqueous solutions, it acts as a semipermeable barrier regulating metabolic exchanges between cartilage and the synovial fluid, and a viscoelastic shield around synoviocytes and adjacent nerve endings (Moreland, 2003) Through its molecular size HA hinders the free movement of lytic enzymes and inflammatory mediators, and enhances chondrocyte metabolism (Goldberg, Buckwalter 2005) Osteoarthritis is associated with a decrease in concentration and average molecular weight of native HA in synovial fluid(Pelletier and Martel-Pelletier, 1993). .

Hyaluronic acid has been shown to decrease dryness in the skin and offering great potential for the revitalization of the skin. It keeps skin smooth and lubricates the joints. It also plays a critical role in improving skin condition and wounds. It plays a key role both in tissue hydration and lubrication. The compound takes care of the life of skin, so the skin becomes vibrant, supple, moist and looks healthy (Mason *et al.,* 1982). Deficiency of hyaluronic acid in human body creates problems related to joints (osteoarthritis), heart valves and eyes. Hyaluronic acid as an essential structural element in the matrix plays an important role for tissue architecture by immobilizing specific proteins (aggrecan, versican, neurocan, brevican, CD44 etc.) in desired locations within the body. It is implicated in many biological processes including fertilization, embryonic development, cell migration and differentiation, wound healing, inflammation, growth and metastasis of tumor cells and whenever rapid tissue turnover and repair are occurring (Csoka *et al*., 1997). The function of Hyaluronic acid may be partly regulated and dependent on its chain length, e.g. angiogenesis is presumably induced by small HA oligosaccharides, whereas high molecular weight Hyaluronic acid exerts inhibitory effects (West et, al., 2000). At physiological concentrations, Hyaluronic acid molecules form a random network of chains. Such a network may act as a sieve and regulate the distribution and transportation of plasma proteins. Hyaluronic acid abnormalities are common threats in connective tissue disorders (Weiss *et al*., 1980).

Hyaluronic acid is produced by type B lining cells of the membrane joints. It is synthesized by a class of integral membrane proteins called hyaluronan synthe-

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tase of which vertebrates have three types namely HAS1, HAS2, HAS3 (Prehm *et al.,* 1983). These enzymes lengthen hyaluronan by repeatedly adding glucuronic acid and N-acetyl glucosamine to the nascent polysaccharide as it is extruded through the cell membrane into the extracellular space. Hyaluronic acid is degraded by a family of enzymes called hyaluronidase. Because of its varied applications, through the present study, we are suggesting the economic way of isolating the hyaluronic acid from one of the waste material that is roosters comb of slaughter house in the form of sodium salt.

MATERIALS AND METHODS

Rooster combs and maintenance: Rooster combs are obtained from the local market immediately after the slotting of chickens. in clean plastic polypropylene bags in cold condition.

The collected rooster combs were washed under tap water to remove all dirt and blood stains followed by distilled water wash. The rooster combs were weighed, and 50 grams of rooster combs were taken in Hyaluronic acid extraction. To start with the experiment the Rooster combs were cut into small pieces with the help of clean stainless steel chopper. The collected pieces of combs were ground in an electric grinder were suspended in 100 ml acetone and were stored at 8 ^{0}C (Swann, 1968).

After 24 hours the acetone was squeezed from the cock's comb, and additional acetone was added. This was repeated ten times at 24 hours interval. After the last extraction, the remaining acetone was evaporated in a steam of air. The weight of dried and deflated combs was found to be 8.35 gm. The dried form of combs was then extracted ten times successively with 100 ml of 5% of Sodium acetate. Each time the viscous fluid was squeezed through several layers of cheesecloth. The final dried combs material was pure, white and fibrous in appearance. This dried material was precipitated with sodium saline citrate. This mixture was squeezed with cheesecloth. At last the obtained precipitate was purified by using the procedure suggested by (Meyer and Palmer, 1979).

Removal of pyrogen from the hyaluronic acid: From the purified extract Pyrogen was removed by centrifuging it at 150000 rpm for 20 minutes and the sediment was removed. The sodium hyaluronate in the form of the gel after taking out the supernatant is washed by adding 70% (v/v) water-soluble organic solvent. By this operation, the sodium hyaluronate turns from gel to powder. Further, the extract was subjected to vacuum drying whereby sodium hyaluronate is obtained. It was reported that extraction by this method removes the pyrogens at a level of 0.0015 to 0.003 EU/mg or less (Prescott *et al*., 2003). The obtained powdery sodium hyaluronate was allowed to dry under aseptic conditions and was confirmed by using the confirmative test.

Confirmative test: The obtained sample of hyaluronic acid was mixed with toludine blue (Ramalingam *et al.*, 1970). From the observation (green color formation) it was confirmed that the obtained sample consists of hyaluronic acid.

Identification of hyaluronic acid: One of the methods used for identification of Hyaluronic acid is viscosity (Eiji Shimada *et al*., 1975) which was done by using Oswald's viscometer. Hyaluronic acid is identified by observing the viscosity of extracted liquid (Chester, 1964). Water is used as a standard whose viscosity is known.

UV spectral analysis of extracted hyaluronic acid: Hyaluronic acid absorbs maximally at 190-200 nm wavelength (Lapcik, 1992). To confirm the obtained sample is hyaluronic acid the pattern of absorption of hyaluronic acid was studied using Spectrophotometer.

Endotoxin detection assay of extracted hyaluronic acid: Before starting with the endotoxin detection assay, all glassware was soaked overnight in cleaning agent, E-Toxa-Clean (Sigma), rinsed in pyrogen-free water and oven dried at 300 °C. Finally, the purified extract of hyaluronic acid from Rooster comb hyaluronan was analyzed for endotoxin, using the Limulus amoebocyte lysate assay (E-Toxate, Sigma).

As hyaluronic acid is a polysaccharide, while following the purification protocols of hyalronic acid for its clinical applicaons, proteins act as one of the unwanted and major contaminants. Thus, it is important to have a hyaluronic acid at the end of purification without protein contamination. In the present study, we have checked the protein contamination in the final extract of hyaluronic acid by using SDS PAGE (He, 2011).

RESULTS AND DISCUSSION

Hyaluronic acid is present in appreciable quantities in the cock's comb and can be isolated in relatively pure form in present study The obtained hyaluronic acid was confirmed by using qualitative confirmative test / Toluidine blue test which was positive that is in blue

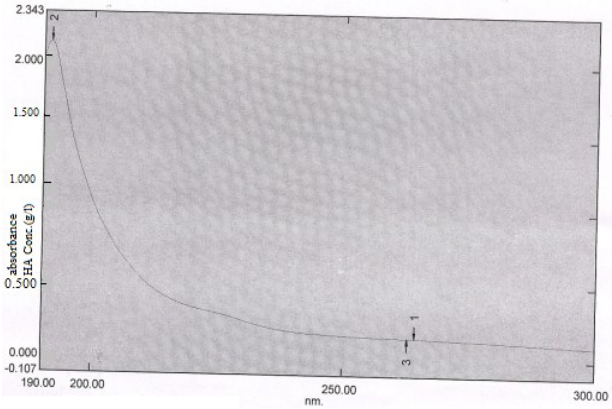

Fig. 1. *UV spectral analysis of extracted hyaluronic acid showing absorption maxima at 191.4 nm.*

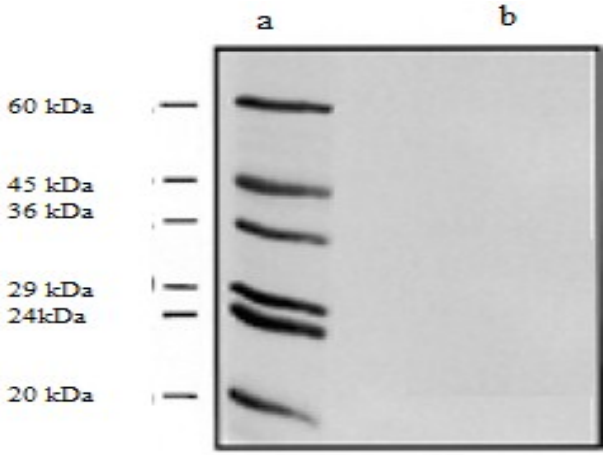

Fig. 2. *SDS PAGE of hyaluronic acid extract (a): Molecular weight markers,-bands show contamination with protein(b) No bands showing hyaluronic acid extract in pure form.*

colour formation is same as (Ramalingam *et al*., 1970). The viscosity was used for the base of identification of Hyaluronic acid. The viscosity of extracted hyaluronic acid was found to be 2.55 poise by using water as a standard (Eiji Shimada *et al*., 1975) so now it is partially purified. Thus, it is expected to check the purity of extracted hyaluronic acid for which UV absorption where the extract Hyaluronic acid sample showed maximum absorption at 191.4 nm (Fig.1). Similar results of absorption of hyaluronic acid were reported by Lapcik (1992) where they got maximum absorption at 200 nm of Hyaluronic acid copper (II) complexes spectroscopic characterization (UV spectra of pure hyaluronic acid solution before and after addition of copper ion) .In the endotoxin detection assay we check the protein contamination in the final extract of hyaluronic acid by using SDS PAGE (Fig. 2) bands are observed it is contaminated by protein(molecular weight markers) no bands are observed hyaluronic acid extract is in pure form same as a result as of (Laemmli, 2011).

Conclusion

The results obtained shows that the applied methodology is effective for extracting and purifying hyaluronic acid. The quantitative and qualitative analysis shows that viscosity of extracted hyaluronic acid was found to be 2.55 poise and SDS PAGE (Laemmli, 2011). of hyaluronic acid extracted from showed that no bands observed in well loaded with extracted hyaluronic acid sample indicating that the final extract of hyaluronic acid is not contaminated with the protein gives pure hyaluronic acid. Thus the hyaluronic acid obtained from chicken combs may be used as a co-product from the poultry industry for research and clinical applications

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