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Purification and Structure Identification of Hyaluronic Acid

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Abstract: Polysaccharide produced by mutated strain of Streptococcus zooepidemicus was purified by the procedures including Savage method, quaternary ammonium compound precipitation, DEAE-cellulose(DE52) chromatography and Sephadex G-75 gel filtration. The structure of the purified polysaccharide has been characterized by means of chemical composition analysis, ^{13}C NMR spectrum, infrared spectrum and circular dichroism (CD). All the results showed that the purified polysaccharide was hyaluronic acid (HA). The single helix conformation of the purified HA was determined by Congo red experiment. The molecular weight of the HA was about 1.16×10^6D, which was measured by viscosity method.

Keywords: Streptococcus zooepidemicus, hyaluronic acid, structure identification.

Chemically, hyaluronic acid is a member of glycosaminoglycans. It is alternating and repeating unit of D-glucuronic acid and N-acetyl-D-glucosamine to form a linear chain with the molecular weight up to 106D[1]. In addition to application in cosmetic and surgery fields, HA is also used for drug delivery, coatings and implantation of organs, and therapeutics owing its ability of modified cellular behavior[2]. Through chemical mutation, we obtained a mutated strain of *Streptococcus zooepidemicus* (J18), which is capable of producing the polysaccharide in high yield[3].

Streptococcus zooepidemicus (J18) mutated by N-methyl-N′-nitro-N-nitrosoguanidine (NTG), was fermented under the optional condition for 24 h[3]. The cells and the other insoluble components in the fermented mixture were first removed by centrifugal separation and the polysaccharide was precipitated by alcohol and collected by centrifugation. Savage method was used to remove the proteins, and with quaternary ammonium precipitation to remove low molecular weight contaminants. Then the polysaccharide was purified by DEAE-cellulose (DE52) anion-exchange column chromatography and Sephadex G-75 gel filtration. The content of the polysaccharide was measured according to the carbazole method and the content of the protein was measured by means of comassic brilliant blue binding method. The total recovery of polysaccharide was 59.65 % with less than 0.1% protein.

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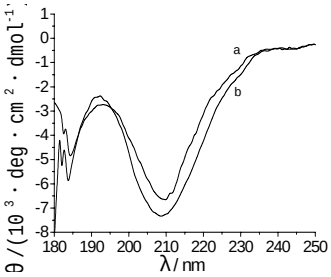
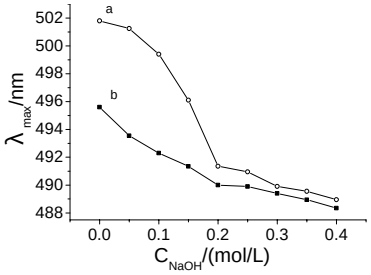
Figure 1 Circular dichroic spectrum of purified HA and standard HA



a. Purified Polysaccharide b. Standard HA

Figure 2 Change in the absorption maxium of the congo red-HA



a. Congo red-HA complex b. Congo red

Carbazole method[4] was used to assay the content of D-glucuronic acid and the content of N-acetyl-D-glucosamine was assayed with Elson-Morgan method[5]. It was found that the hydrolyzed products of HA existed in ratio of 1:1.

The IR (KBr) spectrum of the purified HA was measured and it showed absorption bands at 3400(OH,NH), 2900(CH), 1560 ～ 1620(C=O,CN), and 1400 ～ 1320 cm^{-1}(C-O,COO-), which were precisely in accordance with the IR spectrum of standard HA. The circular dichroism (CD) of the purified polysaccharide showed two negative Cotton effects at 209.57 nm and 184.32 nm, respectively, indicating that the purified polysaccharide was a polyglucosamine with *O*-3 glucosaminoside aminosugar transfer structure (see **Figure 1**). The ^{13}C NMR spectrum (δppm，dissolved in H_2O) of the polysaccharide showed the signals at 173.70(-COO-), 102.71～100.14($\overset{-O}{-O}$CH-), 68.00～82.09 (>CHOH), 53.85 (>CHNH), 60.25 (-CH_2OH) and 22.03 (-CH_3). From the above results, we can conclude that the polysaccharide produced from J18 was HA.

The complex of HA with Congo red was scanned by UV/Vis spectrum. The λ_{max} could red shift in the alkali solution with different concentrations. The result showed the red shift of λ_{max} of HA complex with Congo red, but there is not a metastable state in the curve(see **Figure 2**). This was extremely similar to the reported result of the other single helix polysaccharide[6].

The average molecular weight of HA was assayed by means of measuring the viscosity at 25°C[7], and was calculated according to the empirical formula: $[\eta]= 3.6\times10^{-4}Mr^{0.78}$. It was about 1.16×10^6 D.

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