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A METHOD FOR THE PURIFICATION OF BOVINE VITREOUS BODY HYALURONIC ACID

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Summary

Bovine vitreous body hyaluronic acid was treated with activated CH-Sepharose 4B after purification with cold 5% trichloroacetic acid. By this method a highly purified hyaluronic acid can be prepared. Contaminating serum proteins were completely removed. The remaining small amounts (0.3%) of the protein contaminants were found, by immunological methods, to be non-plasma proteins.

Introduction

Hyaluronic acid is a glycosaminoglycan of the type (-GlcNAc-GlcUA-)$_n$ where GlcNAc is *N*-acetyl-D-glucosamine and GlcUA is D-glucuronic acid. This polymer is a constituent of various connective tissues and is always found associated with protein. In the vitreous body, hyaluronic acid is found together with collagen, serum proteins, and non-plasma proteins [1]. The main problem in the isolation of pure hyaluronic acid is, therefore, the separation from these proteins; until now various purification methods have been applied. For the isolation of purified hyaluronic acid from vitreous body, precipitation with cetylpyridinium chloride [2], Dowex 50 cation-exchange resin [3], and DEAE-Sephadex [4] have been used. Proteolytic enzymes such as papain are now commonly used for the isolation of pure hyaluronic acid [5,6]; however, even after repeated proteolysis, hyaluronic acid is always associated with a small amount of protein.

In this study, we have attempted to isolate pure hyaluronic acid from bovine vitreous body without the use of proteolytic enzymes. The bulk of the contaminant proteins was removed by treatment with cold trichloroacetic acid [7].

We have used activated CH-Sepharose 4B to eliminate more proteins from this treated hyaluronic acid.

Materials and Methods

Purification with trichloroacetic acid. Hyaluronic acid from bovine vitreous body was purified with trichloroacetic acid as described earlier [7].

Treatment with activated CH-Sepharose 4B. 25 mg trichloroacetic acid purified hyaluronic acid were dissolved in 10 ml 0.1 M $NaHCO_3$, containing 0.5 M NaCl, and mixed with 1 g activated CH-Sepharose 4B (Pharmacia AB, Uppsala). The mixture was rotated for 1 h at room temperature, filtered on a sintered glass filter, dialyzed against H_2O, and precipitated with acetone, after addition of NaCl to a final concentration of 1%.

Column chromatography. Column chromatography fractionation of hyaluronic acid was done using Dowex 1-X2 (200—400 mesh, Cl^-, Serva No. 41031 and a column of 2.0 × 45 cm). Hyaluronic acid was solubilized in 0.1 M NaCl, at a concentration of 1 mg/ml. After application of the sample, elution was effected using a 0.1—2.0 M gradient of NaCl. Hyaluronic acid was identified by determination of uronic acid by the method of Bitter and Muir [8]. The fractions which eluted at 0.5—0.65 M NaCl were collected, dialyzed against H_2O, concentrated under reduced pressure, precipitated with acetone, and dried in air.

Protein determination. The protein concentration was determined with Folin-Ciocalteu's phenol reagent (Merck, Darmstadt, No. 9001) by the method of Lowry et al. [9] using bovine serum albumin as standard.

Labeling experiments. Labeling experiments with NaB^3H_4 were performed as described earlier [7].

Viscosity measurements. Viscosity measurements were performed in a KPG-Ubbelohde viscometer (Schott u. Gen./Mainz) at 20.0°C and the mean values of kinematical viscosity were evaluated in cSt. Solutions of 2 mg/ml hyaluronic acid in 0.1 M NaCl were prepared using the method of Bitter and Muir [8] for uronic acid determinations.

Immunization. Red New Zealand rabbits were immunized intradermally with emulsified mixtures of equal parts of 5 mg/ml hyaluronic acid in H_2O and complete Freund's adjuvant (Behring Institute, Marburg/Lahn). Booster injections were given 3 weeks after the initial inoculation. Blood was collected from the ear 7 days after the last injection.

Immunological techniques. Double immunodiffusion was performed according to the technique of Ouchterlony [10]. Proteins were quantified by immunoelectrophoresis in agarose gel containing antibodies (the so-called 'rocket' technique) according to Laurell [11]. For better results, hyaluronic acid solutions were treated with hyaluronidase (Merck, Darmstadt, No. 4508) prior to electrophoresis. The precipitates were stained with Coomassie Brillant Blue R-250.

Results

By treatment with cold 5% trichloroacetic acid, bovine vitreous body hyaluronic acid was purified to a protein content of about 1.2%, as determined

by the method of Lowry et al. [9]. Because of the high protein content of about 20% of the starting material, the recovery of purified hyaluronic acid was 55% of the dry weight of the starting material.

By the technique of Ouchterlony [10] and the 'rocket' technique [11] using antisera against whole bovine serum or bovine serum albumin, one can prove that bovine serum proteins are still present in this hyaluronic acid preparation.

By treatment with activated CH-Sepharose 4B, as described in this paper, we were able to remove more proteins from the trichloroacetic acid purified hyaluronic acid. A first incubation with activated CH-Sepharose 4B provides a hyaluronic acid with a protein content of 0.6% protein, a second treatment yields hyaluronic acid with about 0.3% protein. The recovery of this preparation was 80%.

Using Ouchterlony's method or the 'rocket' technique with antisera against whole bovine serum or bovine albumin, even at different concentrations of antigen or antiserum, we were not able to identify serum proteins contaminating these hyaluronic acid preparations. Therefore, we assumed that we had eliminated serum proteins from this hyaluronic acid preparation and we conclude that the remaining protein must be of non-plasma protein origin.

This can be proved by raising rabbit antisera against this protein which accompanies bovine vitreous body hyaluronic acid. After two injections of an emulsion of hyaluronic acid treated with activated CH-Sepharose 4B with complete Freund's adjuvant, it was possible to obtain precipitating antibodies against the residual protein of hyaluronic acid preparations. This antiserum pre-

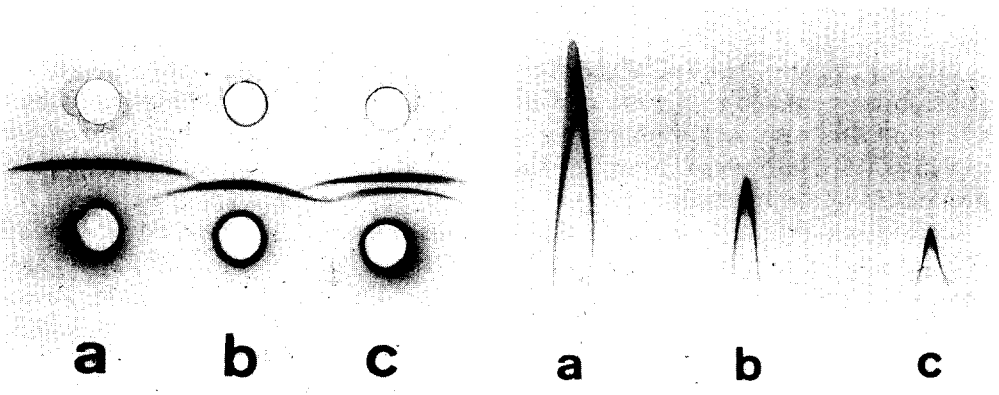

Fig. 1. Double immunodiffusion technique according to Ouchterlony. The upper wells contained the antigens, the lower wells the antisera. (a) Bovine serum albumin/bovine serum albumin antiserum. (b) Hyaluronic acid treated with trichloroacetic acid and activated CH-Sepharose 4B/antiserum against this hyaluronic acid preparation. (c) Mixture of bovine serum albumin and hyaluronic acid treated with trichloroacetic acid and activated CH-Sepharose 4B/mixture of bovine serum albumin antiserum and antiserum against hyaluronic acid treated with trichloroacetic acid and activated CH-Sepharose 4B.

Fig. 2. Quantitative immunoelectrophoresis in agarose gel containing antibodies (rocket technique) against hyaluronic acid treated with trichloroacetic acid and activated CH-Sepharose 4B. The wells contained equal amounts of: (a) trichloroacetic acid-purified hyaluronic acid without treatment with activated CH-Sepharose 4B; (b) trichloroacetic acid-treated hyaluronic acid once incubated with activated CH-Sepharose 4B; (c) trichloroacetic acid-treated hyaluronic acid twice incubated with activated CH-Sepharose 4B. The decreasing length of the precipitates corresponds with decreasing amounts of the protein that contaminates hyaluronic acid.

cipitates with bovine vitreous body homogenate, trichloroacetic acid treated hyaluronic acid, and activated CH-Sepharose 4B purified hyaluronic acid, but not with whole bovine serum or bovine serum albumin. Fig. 1 shows that our antiserum forms only one precipitin line with hyaluronic acid and that the precipitate is not identical with the bovine serum albumin precipitin line.

This antiserum against the non-plasma protein enabled us to monitor the elimination of this protein from our hyaluronic acid preparations (Fig. 2). Since the length of the precipitates of the 'rocket' technique is reduced according to the removal of the protein that contaminates the hyaluronic acid, quantitative immunoelectrophoresis in agarose gel containing antibodies shows a decreasing length of the precipitates with increasing purification of the hyaluronic acid preparations.

This quantitative immunological method shows good agreement with the method of Lowry et al. when observing the different steps of this purification procedure.

The best purification of the hyaluronic acid, however, was achieved by a combination of Dowex 1-X2 column chromatography and treatment with activated CH-Sepharose 4B. The trichloroacetic acid-treated hyaluronic acid was at first purified by Dowex 1-X2 column chromatography, where the 0.5—0.65 M NaCl fraction yielded a protein-poor hyaluronic acid which was free of other glycosaminoglycans. By 2-fold treatment of this hyaluronic acid preparation with activated CH-Sepharose 4B, it is possible to obtain a bovine vitreous body hyaluronic acid with a protein content of about 0.1% as determined by the method of Lowry et al.

To assess whether depolymerization occurs during the purification steps we performed labeling experiments with NaB^3H_4. After reduction with NaB^3H_4 we found only a small increase in radioactivity with trichloroacetic acid treated hyaluronic acid (2470 c.p.m. per 20 μg uronic acid) and trichloroacetic acid and activated CH-Sepharose 4B treated hyaluronic acid (2649 c.p.m. per 20 μg uronic acid) compared with untreated hyaluronic acid (2251 c.p.m.) per 20 μg uronic acid).

Also the viscosity of hyaluronic acid solutions, containing 2 mg/ml hyaluronic acid in 0.1 M NaCl, is not greatly reduced by treatment with trichloroacetic acid. The kinematical viscosity of the starting material amounted to 2.36 cSt, the viscosity of the trichloroacetic acid treated hyaluronic acid was 2.17 cSt, and the trichloroacetic acid and activated CH-Sepharose 4B treated hyaluronic acid showed a kinematical viscosity of 2.14 cSt.

Discussion

By our mild procedure without proteolytic enzymes, purified hyaluronic acid without contaminating serum proteins can be obtained within 8 h. Reduction with NaB^3H_4 was performed to investigate whether trichloroacetic acid degrades hyaluronic acid with the generation of end groups reducible by NaB^3H_4. We found only a small difference in radioactivity between the starting material and trichloroacetic acid and activated CH-Sepharose 4B treated hyaluronic acid. Also viscosity measurements show that cold 5% trichloroacetic acid produces only small changes in the chain length of hyaluronic acid. There-

fore, we conclude that by this method hyaluronic acid can be isolated with little degradation.

The remaining traces of protein represent what appears to be a single non-plasma protein and immunological studies on this protein can be performed with this hyaluronic acid preparation. Non-plasma proteins have been described by Laurent et al. [12], who found some of these proteins in bovine vitreous humour by immunological methods. We suggest that the residual protein in our hyaluronic acid preparation is one of these non-plasma proteins. Our method removed serum proteins and facilitates the preparation of a specific antiserum against one non-plasma protein. The role of this protein remains unclear, and the question arises whether this protein has a function in the formation of the vitreous body gel.

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