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Polyacrylamide Gel Electrophoresis에 있어서 酸性 Mucopolysaccharide의 移動度의 變化

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Variation of the Electrophoretic Mobility of Acid Mucopolysaccharides in Polyacrylamide Gel Electrophoresis

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要　約. 酸性 mucopolysaccharide는 鯨胎兒의 nucleus pulposus에서 分離하였다.

Gel electrophoresis에 있어서 酸性 mucopolysaccharide의 分離는 0.03%의 hexamine cobaltic chloride를 含有하는 0.05 *M* 醋酸소—다 緩衝溶液(pH 4.8)中에서 가장 좋았다.

Spacer gel 上의 mucopolysaccharide 溶液을 40% sucrose 溶液으로 덮었을 때 mucopolysaccharide의 移動度에 變化가 나타나는 것을 觀察하였으며 이 效果는 醋酸소—다 緩衝溶液에 hexamine cobaltic chloride을 加하면 消失하는 것을 觀察하였다.

Abstract. Acid mucopolysaccharides were isolated from nucleus pulposus of whale embryo.

The separation of acid mucopolysaccharides was most excellent in 0.03% hexamine cobaltic chloride in 0.05 *M*-sodium acetate buffer solution at pH 4.8.

Changes in electrophoretic mobility of acid mucopolysaccharides were observed when the sample solution on top of spacer gel were covered with 40% sucrose solution.

This effect was not observed by the addition of hexamine cobaltic chloride to the buffer solution.

Introduction

For the separation of mucopolysaccharides [1,2] and estimation of their molecular weight[3,3a] polyacrylamide gel electrophoresis was used.

In the study of the molecular weight destribution of acid mucopolysaccharides in polyacry lamide gel column, author noticed that the separation of acid mucopolysaccharides was most excellent at pH 4.8(*Fig.* 1).

Also their mobility varies by different volume and density of the sample solution. Usually 2.5 to 10 μl of 1% mucopolysaccharide solutions which were made routinely in water were used.

In all samples under the same electrophoretic conditions, the degree of change in mobility was different and dependent on the type of

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polysaccharide used (*Fig.* 1).

Changes in electrophoretic mobility of acid mucopolysaccharides were observed when the sample solution at the top of spacer gel were covered with 100 μl of 40 % sucrose in 0.05 *M*-sodium acetate buffer solution (*Fig.* 2).

This effect was not observed by addition of hexamine cobaltic chloride($Co(NH_3)_6Cl_3$) to the buffer solution (*Fig.* 3).

Experimental

Methods and Materials. A Bucheler disc electrophoresis apparatus(Fort Lee, New Jersey) with a constant voltage power supply was used. The following stock solutions were prepared according to Clark and Veis[11,12].

Solution *A*: 1.0 *M*-Potassium hydroxide, 48 m*l*; glacial acetic acid, 17.2 m*l*; N, N, N′, N′-tetramethylenediamine(TEMED), 4.0 m*l*; water to make 100 m*l*.

Solution *B*: 1.0 *M*-Potassium hydroxide, 50 m*l*; glacial acetic acid, 4.0 m*l*; water to make 100 m*l*.

Solution *C*: Acrylamide, 20 g; N, N′-methylenebisacrylamide, 0.8 g; water to make 100 m*l*.

Solution *D*: Acrylamide, 10 g; N, N′-methylenebisacrylamide, 2.5 g; water to make 100 m*l*.

Solution *E*: Riboflavin, 0.004 g; water to make 100 m*l*.

Separator Gel Ssolution: Mix one part solution *A*, two parts solution *C*, four parts solution *E*, one part water.

Spacer Gel Solution: Mix one part solution *B*, two parts solution *D*, one part solution *E*, four parts water.

Preparation of the Gel: Gel polymerization was accomplished as described by Ornstein and Davis[13]. Glass columns(7.5×0.5 cm I.D.) were filled with separator gel solution to the heigth of 4.5 cm and photopolymerized for 45 min. On top of the above described separator gel, the spacer gel solution was added to a depth of 1 cm and it was photopolymerized for 90 min. Before polymerization, water was carefully layered on top of each gel solution through very fine bore tubing attached to a syringe. After polymerization of the gel, the water was poured off from the tubes.

Analytical Methods. The modified Elson-Morgan method[4] was used for the determination of hexosamine.

Amino acid analysis was performed by the method described by Moore *et al.*[5].

Protein was determined by the method of Lowry *et al*[6]., uronic acid by the carbazole method[7], and hexose by anthrone method[8].

Types of acid mucopolysaccharides were determined by infrared spectra.

Isolation of Mucopolysaccharides. Nucleopulposus of whales were obtained from a whale embryo and it was packed in ice.

They were sliced and homogenized with 95 % ethanol in Waring Blendor in a cold room and washed with several changes of acetone and then dried and stored.

Protein polysaccharides thus isolated from the nucleopulposus of the whales were found to contain about 30 % proteins(Folin Method).

Mucopolysaccharides with a protein content of the order of 2~3% were obtained by proteolysis of the protein polysaccharides.

The following procedure was used for isolation of acid mucopolysaccharide.

a) Digestion with Papain[14]

A dispersion of protein polysaccharide was made in 0.1 *M* potassium acetate buffer(pH 5.0) to 5 %. It was digested with papain(10 mg/g) (african papaya, Colbiochem.) for 12 hrs at 60 °C.

A small amount of L-cysteine hydrochloride

(44 mg/g) was added to prevent oxidative degradation of the enzyme papain, a few drops of toluene were added as a bacteriostatic.

After the enzymic digestion, the enzyme was denatured by immersing the container in boiling water bath for 3∼4 min.

b) Incubation with Alkali

After papain digestion, the solution was rendered 0.45 *M* in potassium hydroxide.

The mixture was kept at room temperature (25 °C) for 24 hrs in nitrogen atmosphere.

After the incubation, the solution was neutralized to pH 7.0 by means of glacial acetic acid.

c) Pronase Digestion[15]

After alkali treatment, the solution was diluted with 0.1 *M* calcium acetate solution (pH 7.2) (200 m*l*/g). Pronase (10∼12 mg/g) which was filtered through 0.45 μ millipore was added.

The mixture was kept at 37 °C for 24 hrs in the presence of toluene. The solution was dialyzed for 24 hrs with several changes of distilled water and then concentrated in vacuo and lyophilized.

The mucopolysaccharides thus obtained have, in general, the following composition:

Subunits of KS—
- Protein : 2∼3 %
- Galactose : 15∼20 %
- Glucosamine : 15∼20 %

Subunits of CS—
- Protein : 2∼3 %
- Uronic acid : 15∼20 %
- Galactosamine : 15∼20 %

KS : Keratan sulfate

CS : Chondroitin sulfate

d) Alcohol Fractionation of the Mucopolysaccharides

Mucopolysaccharides obtained after papain, alkali and pronase digestion were fractionated into two fractions by the method of Schubert[9].

A 4∼5 g/100 m*l* aquous solution of the mucopolysaccharide was made. Barium chloride was added on gm/gm basis.

Ethanol (anhydrous) was added to make 15% solution. If there was any turbidity, it was removed by centrifugation.

The solution was further diluted with water to make about 1% solution of mucopolysaccharide.

Then, ethanol was added to obtain 25 % alcoholic solution. It was left at 4 °C for 16 hrs. The salted out fraction was removed by centrifugation (Servall, at 37000 g).

The residue was dissolved in water, and passed through Dowex 50-H^+ form, 50∼100 mesh.

The effluent was neutralized to pH 7, and then dialyzed against double distilled water and lyophilized.

The fraction obtained after the above procedure was predominant in chondioitin sulfate.

To the supernatent, ethanol was added to make a 75% solution.

It was left overnight at 4 °C and the fraction thus precipitated was seperated by centrifugation and isolated as discribed above.

The result was predominant in keratan sulfate. It was purified through Dowex 1-Cl^- form, 50∼100 mesh.

Elution (10) was carried out successively with solutions containing NaCl at 0.5 *M*, 1.0 *M*, 1.7 *M*, 2.0 *M*, 3.0 *M* and 5.0 *M*.

After dialysis to remove salts and concentrating to small volumes, the fractions were lyophilized.

Acid mucopolysaccharides were obtained as a sodium salt.

Chondroitin sulfate was generally completely eluted in fractions of 0.5 *M* to 1.7 *M* NaCl.

Keratosulfate was contained in the 2.0 *M* to 5.0 *M* NaCl fractions.

Gel Electrophoresis

Procedure I. 5 to 10 μl of 1 % mucopolysaccharide solutions were layered on the spacer gels. The mucopolysaccharide solutions layered on the spacer gels were carefully covered with water to fill the columns completely.

Analytical data of acid mucopolysaccharides

Analysis	CS-A	CS-C	KS
Hexuronic acid %	24.5	24.6	—
Glucosamine %	—	—	25
Galactosamine %	25.3	25.2	
Hexose %	5	4.8	26.2
Protein %	<1.0	<1.0	<1.0

CS-*A* : Chondroitin sulfate *A*,
CS-*C* : Chondroitin sulfate *C*,
KS : Keratan sulfate

The columns were then inserted into the upper electrode vessel. The upper and lower electrode vessels were filled with 0.03 % hexamine cobaltic chloride in 0.05 *M* sodium acetate buffer (pH 4.8). A current of 4 mA per gel column was applied for 2 hr. The gel columns were stained in 0.1 % Azure A in 6 % acetic acid for 1 hr. Destaining was carried out electrophoretically in a 6 % acetic acid solution using a current of 6 mA per column (*Fig.* 1).

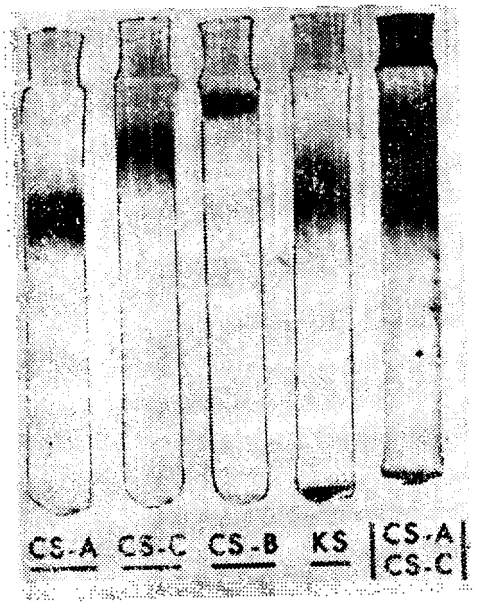

Fig. 1. Electrophoresis of mucopolysaccharides using procedure I. A current of 4 mA per gel column was applied in 0.03 % hexamine cobaltic chloride in 0.05 *M* sodium acetate buffer (pH 4.8) for 2 hrs.
CS-*A* : Chondroitin sulfate *A*
CS-*B* : Chondroitin sulfate *B*
CS-*C* : Chondroitin sulfate *C*

Procedure II. A slightly modified procedure I was applied in procedure II. Mucopolysaccharide solutions layered on the spacer gels were carefully covered with 100 μl of 40 % sucrose in 0.05 *M* sodium acetate buffer solution and with water completely. The columns were then inserted into the upper electrode vessel.

The electrophoretic runs, staining and destaining of the gels were carried out as described above (*Fig.* 2).

Fig. 2. Electrophoresis of mucopolysaccharides using procedure II. In the cases of CS-A2 and KS 2, each sample was covered with 100 μl of 40 % sucrose solution, repectively.
CS-*A* 1 and KS 1 were controls.

Procedure III. The mucopolysaccharide layers on the spacer gels were carefully covered with 100 μl of 40 % sucrose in 0.05 M sodium acetate buffer solution and with water completely. The columns were then inserted into the upper electrode vessel. The upper and lower electrode vessels were filled with 0.03 % hexamine cobaltic chloride, $Co(NH_3)_6Cl_3$, in 0.05 M sodium acetate buffer solution (final pH 4.8). A current of 1 mA per gel column was applied for 15 hr. The gel columns were then stainned and destainned as in procedure I (*Fig.* 3).

Results and Discussion

Chondroitin sulfate *A*, *C*, and keratan sulfate were isolated from the nucleus pulposus of whale embryo.

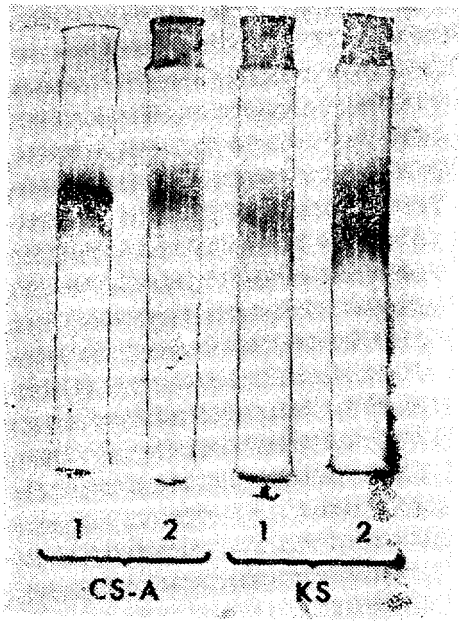

Fig. 3. Electrophoresis of mucopolysaccharides using procedure III.
A current of 1 mA per gel column was applied in 0.03 % hexamine cobaltic chloride in 0.05M sodium acetate buffer solution (final pH 4.8) for 15 hrs.
CS-*A*1 and KS 1 were controls.

An interesting fact is that keratan sulfate from whale embryo contains about 5 % sialic acid and its molecular weight is 4,000 (viscosity measurments).

In procedure I, electrophoresis was carried out in 0.03 % hexamine cobaltic chloride in 0.05 M sodium acetate buffer solution (final pH 4.8). Mucopolysaccharide solutions layered on the spacer gel were covered with water.

The typical electrophoretic patterns of acid mucopolysaccharides are shown in *Fig.* I. Chondroitin sulfate *A*, *B*, *C* and keratan sulfate migrate without much spreading the moving boundaries.

In procedure II, electrophoresis was carried out in 0.05 M sodium acetate buffer solution at pH 4.8. Mucopolysaccharide solutions layered on the spacer gel were covered with 100 μl of 40 % sucrose in 0.05 M sodium acetate buffer solution, and with water (CS-*A* 2, KS 2).

In controls, sample solutions layered on the spacer gel ware covered with water only (CS-*A* 1, KS 1).

The electrophoretic patterns of CS-*A* 2 and KS 2 migrate slower than controls as shown *Fig.* 2.

In procedure III, however, when the electrophoresis was carried out in 0.03 % hexamine cobaltic chloride in 0.05 M sodium acetate buffer solution, this effect was not observed (*Fig.* 3).

These observed phenomena can be interpreted as due to the ionic strength.

That is, in procedure II, the stream of Na^+ ion was hindered by thick neutral sugar, sucrose wall, therefore the migration of acid polysaccharides was slowered.

On the other hand, in procedure III, the ionic strength was strengthened with Co^{++} ion and inhibition effect of concentrated sucrose solution was disappeared.

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