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Isolation of Seromucoids from Human Serum

JOHN GOA

Department of Medical Biochemistry, University of Gothenburg, Sweden *

A mixture of seromucoids has been prepared by means of fractional precipitation with perchloric acid and phosphotungstic acid. The seromucoids were separated into three fractions by means of chromatography on DEAE-cellulose. Each fraction was separated into subfractions by preparative paper electrophoresis, a total of seven fractions being obtained. One fraction was shown to be identical with MP-1 of Winzler or α_1-acid glycoprotein of Schmid. With the unfractionated seromucoid mixture six to eight precipitation lines were obtained immunologically by gel precipitation technique. Some chemical data of the proteins are given, — they are all rich in carbohydrate.

According to Winzler [1] seromucoids means a heterogeneous group of serum proteins left in solution by perchloric acid and precipitated by phosphotungstic acid. These proteins are rich in carbohydrate, which can be split from the protein only by very drastic treatment, and differ from other glycoproteins by their higher solubility.

Zanetti [2], Bywaters [3] and Rimington [4] prepared proteins of this type from the filtrate after heat coagulation of the bulk of the serum proteins. Winzler *et al.*[5] prepared them by ammonium sulphate precipitation of the perchloric acid filtrate of serum, and by means of electrophoresis found three components. The largest component moved like α_1-globulin at pH 8.4, the two other and smaller components moved slower [6]. The first component was subsequently prepared in pure state by ammonium sulphate precipitation of serum [7], and the chemical composition was studied. It was called M-1 or MP-1.

From Cohn-fraction VI, Schmid [8,9] isolated an α_1-acid glycoprotein which was shown to be identical with Winzlers MP-1. Later on Schmid isolated another carbohydrate-rich protein [10,11] which moved like α_2-globulin at pH 8.6. This protein, however, was heterogeneous in acetate buffer at pH 3.5 where it showed three components.

* Present address: Central Laboratory, Rikshospitalet, Oslo, Norway.

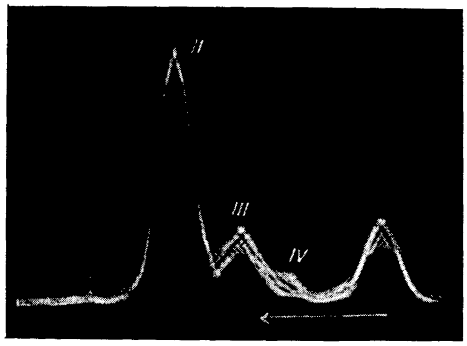

Fig. 1. Moving boundary electrophoresis of seromucoids. Barbital buffer pH 8.6, $\mu = 0.1$. Temp. + 1.5°C. Potential: 7.60 V/cm. Time:115 min. descending.
Mobility: I -8.2×10^{-5} cm²/V sec.
II -5.7×10^{-5} cm²/V sec.
III -3.5×10^{-5} cm²/V sec.
IV -1.9×10^{-5} cm²/V sec.

Of the seromucoids mentioned here, only Winzlers MP-1 or Schmids α_1-acid glycoprotein has been characterized. This protein has also been called orosomucoid [1].

During recent years chromatography on different ion exchangers has been used for isolation and purification of many proteins. Peterson and Sober [12] introduced several derivatives of cellulose, of which diethylaminoethyl-(DEAE)-cellulose has been especially useful. In the present study a combination of fractional precipitation, chromatograhy on DEAE-cellulose and preparative paper electrophoresis has been used for isolation of some carbohydrate-rich serum proteins which are not precipitated by perchloric acid.

METHODS OF PREPARATION

Fractional precipitation

One volume of serum or plasma was diluted with one volume of 0.9 % sodium chloride and precipitated by addition of two volumes of 1 M perchloric acid (final concentration 0.5 M). The precipitate was immediately centrifuged off or filtered. To the filtrate was added 1/20 volume of 2 % phosphotungstic acid in 2 N hydrochloric acid, the precipitated

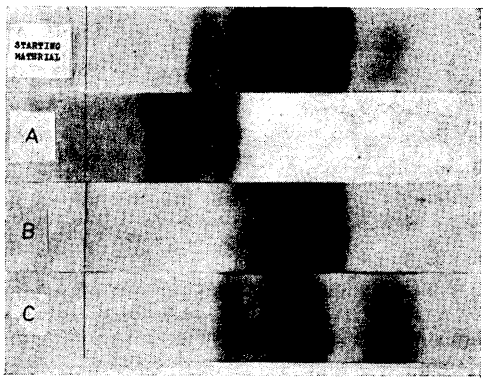

Fig. 2. Paper electrophoresis of seromucoids in barbital buffer pH 8.6. Starting material is the serum proteins left in solution by perchloric acid. A, B, and C are the fractions obtained by chromatography on DEAE-cellulose. The fractions are concentrated before electrophoresis and there is no quantitative relation between the amount of protein on the papers and the proportions in which they are present in the starting material.

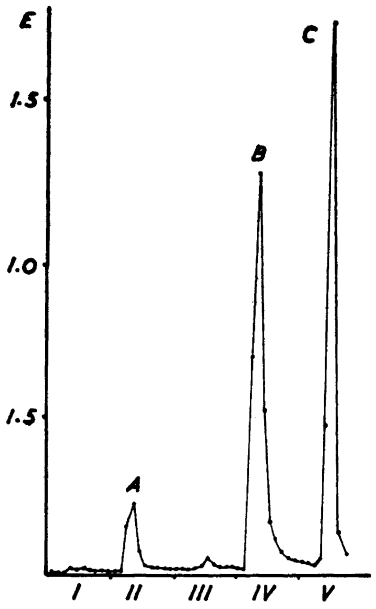

Fig. 3. Elution curve of 110 mg seromucoids. The Roman figures indicate the eluting buffers. I = 0.005 M phosphate, II = 0.005 M phosphate + 0.08 N sodium chloride, III = 0.05 M phosphate, IV = 0.05 M phosphate + 0.1 N sodium chloride and V = 1 N sodium chloride + 0.1 N sodium hydroxide. In all phosphate buffers pH was 6.8–6.9.

seromucoids were centrifuged off and dissolved by dropwise addition of 0.1 N sodium hydroxide to pH 6–7. To the solution obtained was added three times its volume of 95 % ethanol, and centrifuged. The precipitate was dissolved in distilled water and dialyzed. At pH 8.6 it consists electrophoretically of four fractions as shown in Figs. 1 and 2.

Chromatography

DEAE-cellulose was prepared exactly according to Sober *et al.*[13] The nitrogen content of the dried powder was 1.3 %. The cellulose ion exchanger was equilibrated with 0.005 M phosphate buffer pH 6.9 and packed in chromatographic columns (25 × 200 mm, fitted with a G2 sintered glass disk). The length of the cellulose columns were 6–7 cm. The proteins were eluted stepwise by increasing the ionic strength of the eluting buffer.

Step I. About 200 mg seromucoids were applied on each column at room temperature. All the protein was adsorbed to the cellulose, and an addition of 100 ml 0.005 M phosphate buffer resulted in no elution of protein.

Step II. The composition of the eluting buffer was changed to 0.005 M phosphate + 0.08 N sodium chloride, pH 6.9. The protein eluted with this buffer was called fraction A, and a paper electrophoresis strip of this fraction is shown in Fig. 2.

Step III. When no more protein was eluted in step II, some 100 ml 0.05 M phosphate buffer, pH 6.8 was allowed to pass the column, only traces of protein being eluted.

Step IV. The eluting buffer was now changed to 0.05 M phosphate + 0.1 N sodium chloride. The protein eluted was called fraction B and showed electrophoretically one large fraction (about 90 %), followed by a small one (Fig. 2).

Step V. After elution of fraction B about 40 % of the applied protein was left on the column. This protein was eluted with 1 N sodium chloride + 0.1 N sodium hydroxide and called fraction C. Electrophoretically it consists of three components (Fig. 2).

The effluent was collected in 10 ml samples and read in a Beckman DU spectrophotometer at 278 mμ. An elution curve is shown in Fig. 3.

Table 1. The table shows the approximate amounts of the different seromucoids isolated from 1 litre human serum. It also shows the percentage of hexose and hexosamine and the glucosamine/galactosamine ratio in the preparations.

Fraction	mg per litre serum	Hexose in per cent of protein	Hexosamine in per cent of protein	Glucosamine/ galactosamine ratio
A-1	20	6.6	7.1	1 : 0.12
A-2	20	5.1	5.6	1 : 0
B-1	250	16.2	11.3	1 : 0
B-2	25	10.6	10.6	1 : 0.28
C-1	25	7.2	3.5	1 : 1.10
C-2	100	9.3	10.4	1 : 0.35
C-3	75	7.8	8.3	1 : 0.50

Preparative paper electrophoresis

The fractions eluted from the column were dialyzed and concentrated by pervaporation to a concentration of 4—5 % protein. About 0.5 ml of the concentrated solution was placed evenly along a line transversely on 10 × 40 cm Munktell No. 150/20 filter paper strips, and run for 6—7 h with a voltage of 300 V as described previously [14]. The buffer used was barbital pH 8.6, ionic strength 0.05. When the electrophoresis was completed the paper was dried in the air and a strip cut out longitudinally and stained with bromophenol blue. With this strip as reference the protein fractions were cut out, eluted from the paper in 0.9 % sodium chloride, dialyzed and lyophilyzed.

RESULTS

Each of the fractions from the chromatographic column could be separated into subfractions by means of preparative paper electrophoresis. Fraction A was separated into two subfractions, the fastest called A-1, the slowest A-2. Fraction B was also separated into two subfractions, the fastest called B-1, the slowest B-2. Fraction C was separated into three subfractions, which were called C-1, C-2, and C-3, according to their mobilities.

All the isolated subfractions were homogeneous in paper electrophoresis at pH 8.6. The amounts prepared from one litre human serum are given in Table 1.

Table 2. Comparison between analytical data of fraction B-1 and "acid glycoprotein" of Schmid [9].

	Nitrogen per cent of protein	*Hexose* per cent of protein	*Hexosamine* per cent of protein	*Glucosamine/ galactosamine* ratio
Fraction B-1	10.6	16.2	11.3	1 : 0
Acid glycoprotein (own preparation)	11.3	16.4	11.3	1 : 0
Acid glycoprotein (values given by Schmid)	10.7	17.2	11.5	

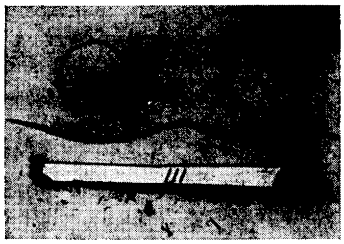

Fig. 4. Immunological identification test of fraction B-1 and "acid glycoprotein". In I 15 µl 5 % fraction B-1, in II 15 µl 5 % "acid glycoprotein" and in III 20 µl antihuman serum from rabbit (Behringwerke). The precipitation lines are stained with Amidoblack.

One of these fractions is well known beforehand, *viz.* fraction B-1, which is also present in largest amount in serum. It is identical with α_1-acid glycoprotein of Schmid or MP-1 of Winzler. To show the identity, α_1-acid glycoprotein was prepared according to the description of Schmid [9]. The protein thus prepared and fraction B-1 were analyzed for nitrogen (micro Kjeldahl), hexose [14], hexosamine [15] and the glucosamine/galactosamine ratio [16]. The values are given in Table 2 together with values given by Schmid [9].

In paper electrophoresis fraction B-1 had the same mobility as α_1-acid glycoprotein, and by moving boundary electrophoresis it moved like α_1-globulin with the mobility -5.7×10^{-5} cm^2/V sec. in barbital buffer pH 8.6 and ionic strength 0.1. In acetate buffer pH 4.5 and ionic strength 0.1 it moved with the mobility -3.2×10^{-5} cm^2/V sec.

Immunological experiments with gel precipitation technique according to the principle of double diffusion as worked out by Ouchterlony [17,18], were carried out with fraction B-1 and α_1-acid glycoprotein. With anti-human serum from rabbit and 2 % agar as diffusion medium, the two preparations gave a common precipitation line indicating identity (Fig. 4).

Fig. 5. The seromucoid preparation (15 µl, 5 %) precipitated in agar against antihuman serum from rabbit (Behringwerke). Four precipitation lines are seen (stained with Amidoblack).

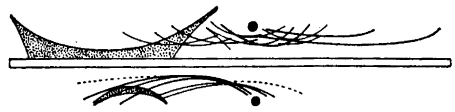

Fig. 6. Immune electrophoresis of normal serum (upper part) and a 10 % solution of the unfractionated seromucoid mixture (lower part).

Of other seromucoids described earlier, *e.g.* MP-2 and MP-3 of Winzler *et al.*[5] or α_2-glycoprotein of Schmid [11] there are no chemical data for comparison.

The content of hexose, hexosamine and the glycosamine/galactosamine ratio for all the fractions are given in Table 1. The values are not corrected for ash and water content except for fraction B-1. It will be seen that they are all rich in carbohydrate.

The possibility exists that some of the fractions described are artefacts or in some way modified proteins. Thus de Vaux St-Cyr *et al.*[19] found a lower mobility of orosomucoid prepared by perchloric acid than with trichloroacetic acid precipitation. However, with the immunological double diffusion gel technique the unfractionated seromucoid mixture showed four precipitation lines (Fig. 5), and by immunoelectrophoresis according to Grabar [20] it showed five distinct lines, one diffuse and two week lines (Fig. 6). When run simultaneously with normal serum the precipitation lines were found in the region of α-globulins (Fig. 6). In a similar preparation de Vaux St-Cyr *et al.*[19] found six precipitation lines.

In a previous paper [21] it has been reported that fraction C-2 and C-3 have antitryptic effect. Work is in progress on preparation and purification of the fractions in a preparative scale.

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