THE ISOLATION AND PARTIAL CHARACTERISATION OF A NEW α-MACROGLOBULIN FROM HUMAN PREGNANCY SERUM

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1. Introduction

In the course of an investigation into serum protein changes in human pregnancy [1] it was observed that when immunoelectrophoresis was carried out with anti-pregnancy serum an additional precipitin line, in the fast α_2- globulin region, consistently appeared; this was not present when commercial polyvalent antiserum was used.

The possible occurrence of 'pregnancy-specific components' in serum has been noted previously by immunoelectrophoresis [2, 3] but the actual identification of any of these has not been achieved.

The present paper reports the isolation and partial characterisation of a new α-macroglobulin from human pregnancy serum. This is the additional α_2-globulin that can be shown by immunoelectrophoresis.

2. Materials and methods

2.1. *Materials*

Serum was prepared from women in or after their 35th week of pregnancy, as previously described [1], and antiserum to it was raised in rabbits using Freund's complete adjuvent (Difco). Commercial polyvalent antiserum was supplied by Dakopatts.

The DEAE-cellulose (DE52) and CM-cellulose (CM52) were products of Whatman, while Dextran sulphate was obtained from Pharmacia. The isoelectric focusing column (vol 110 ml) and pH 4–6 ampholine carrier ampholytes were supplied by LKB Produkter AB. Analar grade reagents were used wherever possible.

2.2. *Methods*

Gel filtration of the macroglobulin was carried out on a column (1.6 cm × 96 cm) of 8% agarose (Bio-Gel A-1.5 m) equilibrated with 0.02 M phosphate buffer, pH 7.4, containing 0.1 M NaCl. The flow rate was 9.5 ml/hr and the effluent was continuously monitored for protein content at 280 nm. The void volume was determined with blue Dextran 2000 (Pharmacia) and the gel column calibrated against proteins of known Stokes radius and molecular weight [4]. These included bovine thyroglobulin (Sigma), horse spleen apoferritin (Serva), glutamate dehydrogenase (Sigma), catalase (Boehringer) and human gamma globulin (Serva) [5–8].

Tracing the α-macroglobulin throughout the extraction procedure was achieved, in the first place, by using immunoelectrophoresis with absorbed anti-pregnancy serum. This absorbed anti-serum was prepared by the addition of a 25% solution of pooled serum, from 15 non-pregnant women, to the rabbit anti-pregnancy serum in a dilution series. When the ratio of the above reactants was greater than 9:1, respectively, only one precipitin line was apparent, corresponding to the later characterised macroglobulin.

The sedimentation equilibrium experiment was performed using the long-column meniscus depletion technique of Chervenka [9] on a Spinco model E ultracentrifuge at 20°. The sample was dialysed against 1% KCl.

Hydrolyses for amino acid analyses were carried out under N_2 in sealed tubes, with constant boiling HCl, at 110° for 24 hr. For subsequent analysis, a Locarte amino acid analyser was used. Cysteine was determined as cysteic acid after performic acid oxidation at 0° [10].

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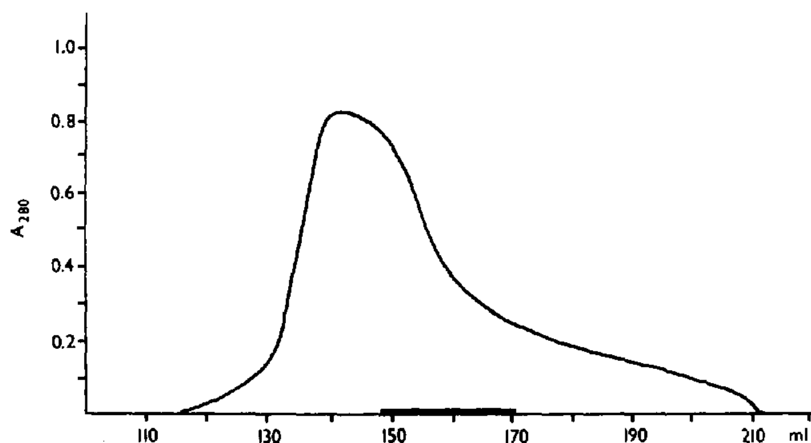

Fig.1. Gel filtration of protein fraction from Step 4 on a column of 8% agarose. The fraction indicated by the black bar was collected.

Tryptophan was determined colorimetrically [11]. Neutral sugars were released by hydrolysis in 1 N HCl for 4 hr at 110° and quantitated by gas-liquid chromatography [12], α-methyl-D-glucoside being used as an internal standard. Sialic acid was estimated as *N*-acetylneuraminic acid by the method of Warren [13] after hydrolysis in 0.05 N H_2SO_4 at 90° for 1 hr. Hexosamines were determined by gas-liquid chromatography, as previously described [14]. Immunoelectrophoresis was performed in 1% agar (Difco) with 0.06 M barbitone buffer pH 8.6 for 2 hr at 15 V per cm.

3. Results and discussion

The purification procedure for the α-macroglobulin is as follows (unless otherwise stated, all steps were performed at 4°):

Step 1. Removal of β-lipoproteins: 2 ml of a 10% dextran sulphate solution were added per 100 ml serum and, after the addition of 10 ml 1 M $CaCl_2$, the precipitate that formed was removed by centrifugation [15].

Step 2. $(NH_4)_2SO_4$ fractionation: The supernatant was brought to 1.5 M with saturated $(NH_4)_2SO_4$ solution, pH 6.5. After stirring for 2 hr at 0°, the precipitate was removed by centrifugation at 2,500 *g* for 15 min. Further $(NH_4)_2SO_4$ solution was then slowly added to this supernatant to give a final conc. of 2.0 M and this was allowed to stir for 12 hr at 0°. The precipitate was recovered by centrifugation, at 5,000 *g* for 30 min, redissolved in 0.0175 M phosphate buffer, pH 6.3 and dialysed exhaustively against the same.

Step 3. DEAE-cellulose chromatography: The dialysed fraction was applied to a column (2.5 cm × 30 cm) of DEAE-cellulose, equilibrated with 0.0175 M phosphate buffer, pH 6.3. The column was then eluted, at a flow rate of 100 ml/hr, with 200 ml volumes of this buffer, 0.04 M phosphate, pH 5.9 and 0.1 M phosphate, pH 5.8 [16]. The material that was removed with the latter buffer was collected and dialysed exhaustively against 0.015 M phosphate, pH 6.2.

Step 4. CM-cellulose chromatography: The dialysed solution was applied to a column (2.5 cm × 20 cm) of CM-cellulose equilibrated with 0.015 M phosphate buffer, pH 6.2. The elution was performed with the equilibrating buffer at a flow rate of 100 ml/hr and the α-macroglobulin recovered in the unadsorbed fraction. Saturated $(NH_4)_2SO_4$ solution was added to this fraction to a concentration of 2.0 M and the precipitate collected by centrifugation. The precipitate was then dissolved in 0.02 M phosphate buffer, pH 7.4, containing 0.1 M NaCl.

Step 5. 8% Agarose gel filtration: 5 ml samples of solution were subjected to gel filtration on a column (2.6 cm × 55 cm) of Bio-Gel A-1.5 m equilibrated with the above buffer and having a flow rate of 20 ml/hr. The fraction indicated in fig. 1 was collected and treated with $(NH_4)_2SO_4$ solution as in step 4. The

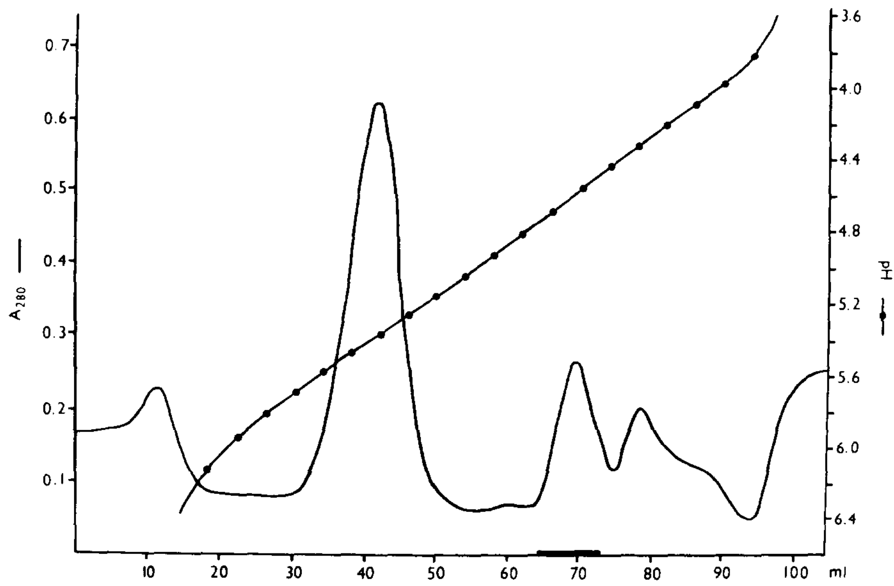

Fig.2. Isoelectric focusing of the partially purified α-macroglobulin from Step 5, on pH 4–6 carrier ampholyte. The fraction indicated by the black bar contained the pure protein.

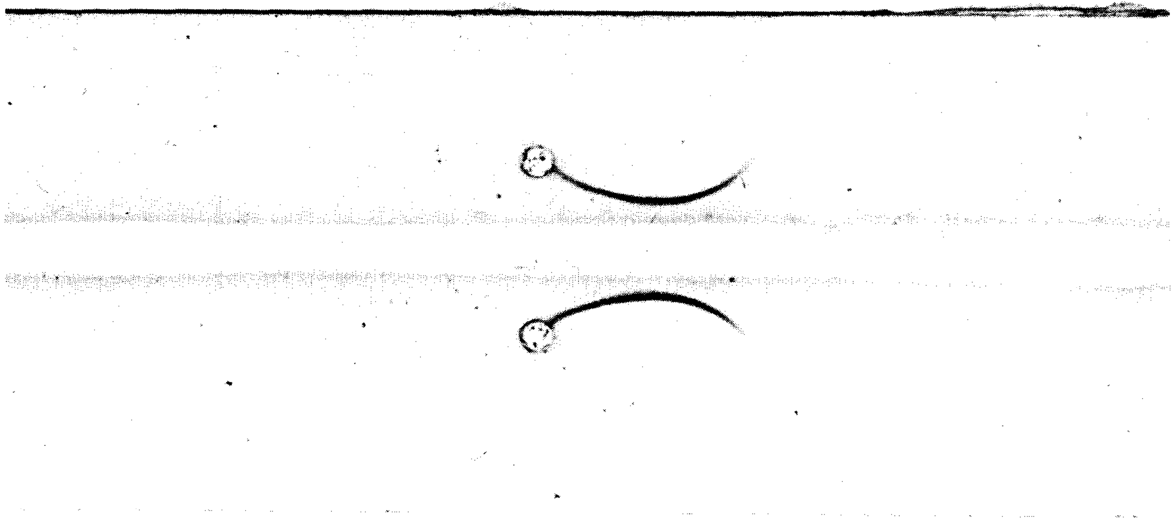

Fig.3. Immunoelectrophoresis of purified α-macroglobulin against rabbit anti-pregnancy serum.

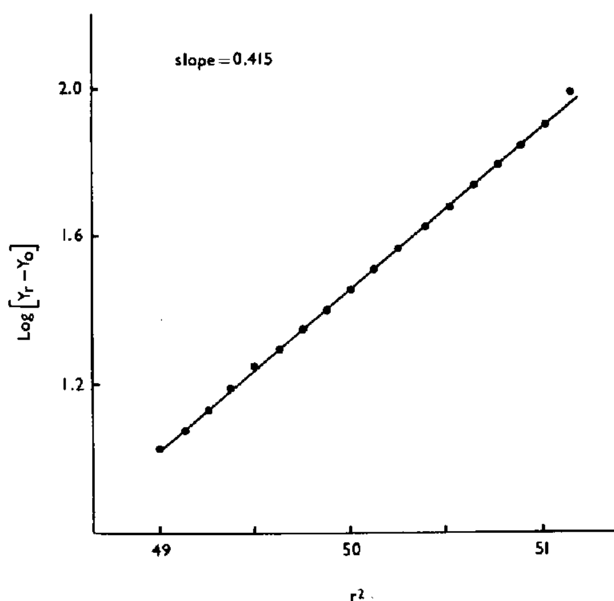

Fig. 4. Plot of the natural logarithms of the differences between fringe displacements (Y_r) and the zero concentration level (Y_0) against the square of the distances from the centre of rotation (r) from the sedimentation equilibrium experiment.

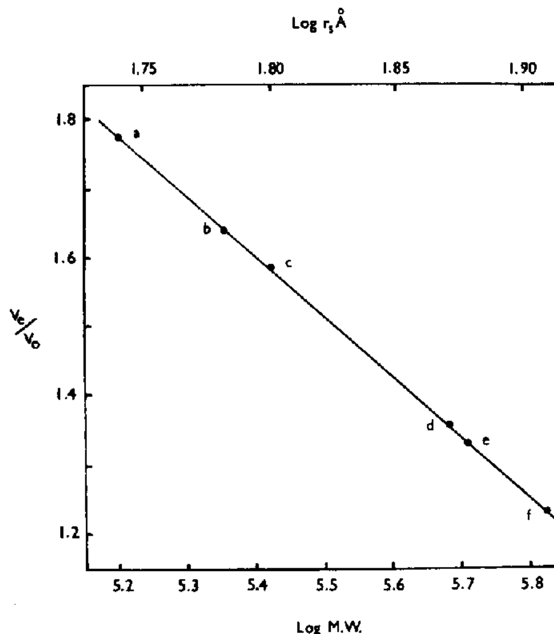

Fig. 5. Plot correlating the elution volume to the Stokes radius and molecular weight, of 5 proteins and the α-macroglobulin on an 8% Agarose column. Experimental details are described in the text. a) Gamma globulin; b) catalase; c) glutamate dehydrogenase; d) apoferritin; e) α-macroglobulin; f) thyroglobulin. V_e = Solute elution volume. V_0 = Void volume.

precipitate was dissolved in and dialysed against 1% glycine solution.

Step 6. Isoelectric focusing: The partially purified α-macroglobulin was subjected to isoelectric focusing [17] and the elution profile obtained is shown in fig. 2. The sucrose gradient contained 1% (w/v) carrier ampholytes and 0.5% (w/v) Brij 35 nonionic detergent. A potential of 500 V, rising to 700 V, was applied to the column for 48 hr. The fraction indicated in fig. 2 (isoelectric point pH 4.6) was collected, dialysed against 0.0175 M phosphate buffer, pH 6.3 and applied to a small column (1.5 cm × 3 cm) of DEAE-cellulose. The column was eluted with the same buffer until all the detergent (detectable by measuring its absorbance at 250 nm) had been eliminated and then the pure glycoprotein was removed with 0.4 M phosphate buffer, pH 5.2.

The α-macroglobulin, purified as above, showed only one precipitin line in immunoelectrophoresis (fig. 3) when reacted against anti-pregnancy serum and gave no reaction at all with commercial antiserum. Homogeneity is also indicated by the linear sedimentation equilibrium plot shown in fig. 4. The average

Table 1
Amino acid composition of the α-macroglobulin.

Aspartic acid	98.94
Threonine	63.77
Serine	95.82
Glutamic acid	111.63
Proline	48.50
Glycine	86.84
Alanine	78.45
Valine	65.28
Half-cystine	16.94
Methionine	Trace
Isoleucine	46.57
Leucine	77.54
Tyrosine	31.56
Phenylalanine	33.28
Lysine	57.27
Histidine	23.44
Arginine	41.83
Tryptophan	22.32

Values are expressed as residues of amino acid per 1000 residues.

Table 2
Carbodydrate composition of the α-macroglobulin.

Glucosamine (as free base)		2.49
Total hexose		6.00
Glucose	2.24	
Galactose	2.25	
Mannose	1.51	
Fucose	Trace	
Sialic acid		2.55

Values are expressed as grams per 100 grams protein.

value for the slope was calculated by least squares treatment of significant experimental points. It was not possible to determine the partial specific volume, due to the limited amount of sample available; if, however, a value of 0.725 (often taken as an average for proteins) is assumed, then a $\overline{M}w$ of 506,000 can be calculated for the α-globulin. Support for this figure was given by the gel chromatography on 8% agarose. From the calibration graph shown in fig. 5 a molecular weight of 507,000 and a Stokes radius of 72 Å can be estimated.

Amino acid analysis of the α-macroglobulin (table 1) shows that it contains relatively large amounts of aspartic acid, serine, glutamic acid, glycine, alanine and leucine but only small amounts of histidine, tryptophan and half-cystine, with a trace of methionine. The carbohydrate composition is shown in table 2. Glucose, galactose and mannose were the main neutral sugar components and were in a molar ratio of 3:3:2, respectively. This α-globulin is unusual when compared with other serum proteins in having glucose in its complement of neutral sugars. Glucosamine was the only hexosamine present and glucuronic acid was detectable at a very low concentration.

To date, this α-macroglobulin appears to be peculiar to the maternal circulation although an antigen reacting with its specific antiserum has been shown in the sera of several tumour-bearing patients [18]. The biological function of this serum protein is unknown but further investigations are being carried out to ascertain its possible implication in the foetal–maternal relationship.

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References

[1] W.H. Stimson, Clin. Biochem., in press.
[2] J. Hirschfeld and U. Söderberg, Nature 187 (1960) 332.
[3] J.W.W. Studd, J.D. Blainey and D.E. Bailey, J. Obstet. Gynaec. Brit. Comm. 77 (1970) 42.
[4] H. Determann and W. Michel, J. Chromatog. 25 (1966) 303.
[5] T.C. Laurent and J. Killander, J. Chromatog. 14 (1964) 317.
[6] A.P. Kulkarni and K.N. Mehrotra, Anal. Biochem. 38 (1970) 285.
[7] J. Marrink and M. Gruber, FEBS Letters 2 (1969) 242.
[8] H. Edelhoch, J. Biol. Chem. 235 (1960) 1326.
[9] C.H. Chervenka, Anal. Biochem. 34 (1970) 24.
[10] S. Moore, J. Biol. Chem. 238 (1963) 235.
[11] M.K. Gaitonde and T. Dovey, Biochem. J. 117 (1970) 907.
[12] C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, J. Am. Chem. Soc. 85 (1963) 2497.
[13] L. Warren, J. Biol. Chem. 234 (1959) 1971.
[14] W.H. Stimson, FEBS Letters 13 (1971) 17.
[15] A. Van Dalen, H.G. Seijen and M. Gruber, Biochim. Biophys. Acta 147 (1967) 421.
[16] H.A. Sober and E.A. Peterson, Federation Proc. 17 (1958) 1116.
[17] H. Haglund, in: Methods of Biochemical Analysis, Vol. 19, ed. D. Glick (Interscience, 1971) p.1.
[18] W.H. Stimson, Lancet 1 (1972) 684.