IMMUNOCHEMICAL MEASUREMENT OF PROSTATIC BINDING PROTEIN

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1. Introduction

As shown previously [1,2] rat ventral prostate contains a large amount of prostatic binding protein which is secreted by this organ and plays a major role in the non-specific steroid binding in prostatic cytosol. Up until now we have studied this protein by its binding activity but the quantitative use of this parameter was hindered by the marked effect of various factors (e.g. delipidation) on the binding activity. Here we describe the production of a monospecific antiserum against prostatic binding protein and its use in quantitative measurements by radial immunodiffusion [3].

2. Materials and methods

Adult male Wistar rats were used in this study. Cytosol of ventral prostate was prepared in Tris–HCl buffer (50 mM Tris, pH 7.3 at 4°C) as described in a previous paper [2], to which we refer for most materials and methods used in this paper. [7-^{3}H]Pregnenolone (24 Ci/mmol) was obtained from New England Nuclear (Boston, USA).

2.1. *Measurement of pregnenolone binding*

This steroid was chosen because of its preferential binding to prostatic binding protein [2]. The sample (0.05 ml) was added to 0.25 ml of a solution of [^{3}H]pregnenolone (0.1 μCi/ml) in buffer containing 1% human serum albumin (Behringwerke, Marburg/Lahn, Germany). After 3 h of incubation at 0°C, 0.2 ml of a suspension of dextran-coated charcoal (4 g charcoal, 0.2 g dextran/100 ml water) was added. The samples were incubated for 20 min at 0°C, centrifuged for 10 min at 0°C and the radioactivity in the supernatant (bound steroid) measured.

2.2. *Small scale purification of prostatic binding protein*

Two ml of delipidated prostatic cytosol (12.8 mg protein/ml) were applied to a column (10 × 0.7 cm) of DEAE cellulose (Whatman DE 52) equilibrated with Tris buffer. The column was eluted with a linear 0–0.3 M KCl gradient in buffer. The absorbance at 280 nm and pregnenolone binding activity were measured in all fractions (50 × 2 ml) of this column. The same experiment was repeated on another aliquot (2 ml) of dilipidated cytosol. The fractions of both experiments containing most of the binding activity were combined, concentrated on an Amicon PM-10 membrane and applied to a Sephadex G-100 column (84 × 1.5 cm) which was eluted with buffer in 2.1 ml fractions. The fractions (37–40) containing most of the binding activity were combined and used for immunisation.

2.3. *Immunisation*

Three ml of the prostatic binding protein ($A_{280\,nm}$ 0.707) preparation was emulsified in 6 ml complete Freund's adjuvans (Difco Laboratories). Two rabbits were immunised by intradermal injection of 3 ml of this emulsion at multiple sites [4]. Booster injections were given 4 weeks later. The rabbits were bled from an ear vein 8 weeks after the first injection and bled out 8 weeks later.

2.4. *Immunochemical quantitation of prostatic binding protein by radial immunodiffusion [3]*

A series of circular wells (3 mm diameter) were punched out from an agarose gel (10 × 10 × 0.1 cm) containing 1% agarose (Indubiose A 37, Industrie Biologique Française, Gennevilliers, France) and 2% antiserum in Tris buffer. These wells were filled with exactly 5 μl sample or standard solution. To each plate a set of control samples was applied, corresponding to dilutions of the standard preparation (see below) containing 0.25, 0.5, 1, 1.5 and 2 arbitrary units/ml. The test samples were also diluted within this range. The diluent was 2% rat plasma in Tris buffer. After 2 days of diffusion in a moist chamber at room temperature, the plates were washed and stained as described by Mancini et al. [3]. Then the diameter of the circular immunoprecipitates was measured in two perpendicular directions under tenfold optical magnification. The concentration of prostatic binding protein was calculated using slope and intercept, determined by linear regression of a standard curve of concentration versus square diameter.

2.5. *Preparation of arbitrary standard*

A preparation of cytosol (14.7 mg protein/ml) from ventral prostates of adult rats was considered to contain 100 arbitrary units/ml. It was diluted tenfold by addition of 1 vol. rat plasma and 8 vol. buffer containing 1‰ sodium azide. The rat plasma, which does not contain prostatic binding protein itself was added to avoid possible losses by adsorption or proteolysis. Aliquots (0.2 ml) of this diluted cytosol were stored at −20°C and diluted further before use.

3. Results

3.1. *Small scale purification of prostatic binding protein*

The high concentration of prostatic binding protein in rat prostatic cytosol made it possible to purify this protein by a relatively simple technique. For this purpose ion exchange chromatography, on DEAE-cellulose was combined with gel filtration on Sephadex G-100 (fig.1). Delipidated cytosol [2] was used as starting material. In the eluate of the DEAE-cellulose column there was already a striking correlation of binding activity and protein ($A_{280\,nm}$). This correlation was even more marked in the fractions from the

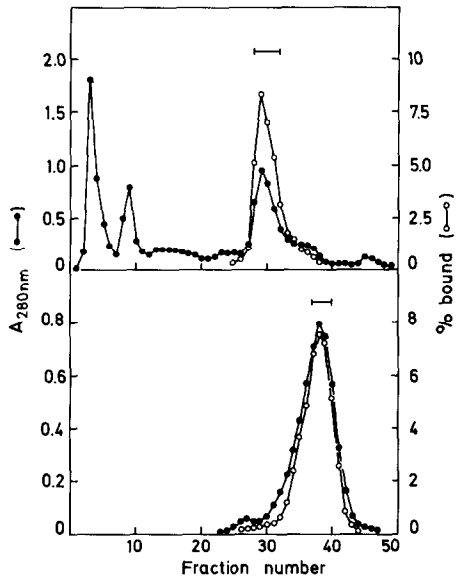

Fig.1. Purification of prostatic binding protein. The upper part of the figure shows the DEAE-cellulose chromatography of delipidated cytosol (see Materials and methods). The fractions containing most of the binding activity (horizontal bar) were analysed further by gel filtration on Sephadex G-100 (lower part). The top fractions of the peak of binding activity were combined (horizontal bar) and used for immunization.

Sephadex G-100 column. The fractions corresponding to the peak of binding activity contained only prostatic binding protein when analysed by polyacrylamide gel electrophoresis (5% acrylamide) in Tris-glycine buffer [2] and two subunits of smaller molecular weight (estimated M_r 14 000 and 17 000; fig.2) in SDS-containing gels, according to Weber and Osborn [5]. The top fractions (37–40) of this peak were combined and used for immunisation.

3.2. *Production of antisera against prostatic binding protein*

Both rabbits responded to immunisation by the formation of antibodies. The specificity of these antisera was checked by the double immunodiffusion technique of Ouchterlony [6] and by crossed immunoelectrophoresis [7]. Purified prostatic binding protein or total prostatic cytosol formed a single immunoprecipitation line with these antisera by the Ouchterlony technique (fig.3(A)). These precipitation

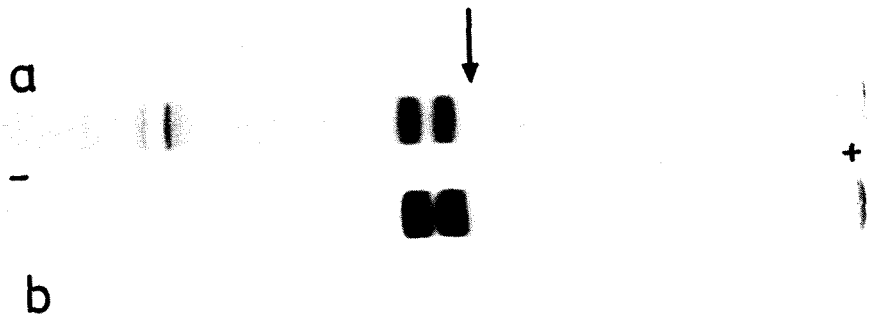

Fig.2. Polyacrylamide gel electrophoresis in SDS-containing gels. The method of Weber and Osborn [5] was followed using 10% acrylamide and 0.1% SDS in phosphate buffer. The samples consisted of 25 μl total prostatic cytosol (a) or purified prostatic binding protein (b) diluted, respectively, twenty-fold and four-fold with sample buffer, containing no mercaptoethanol. The arrow shows the position of cytochrome *c*.

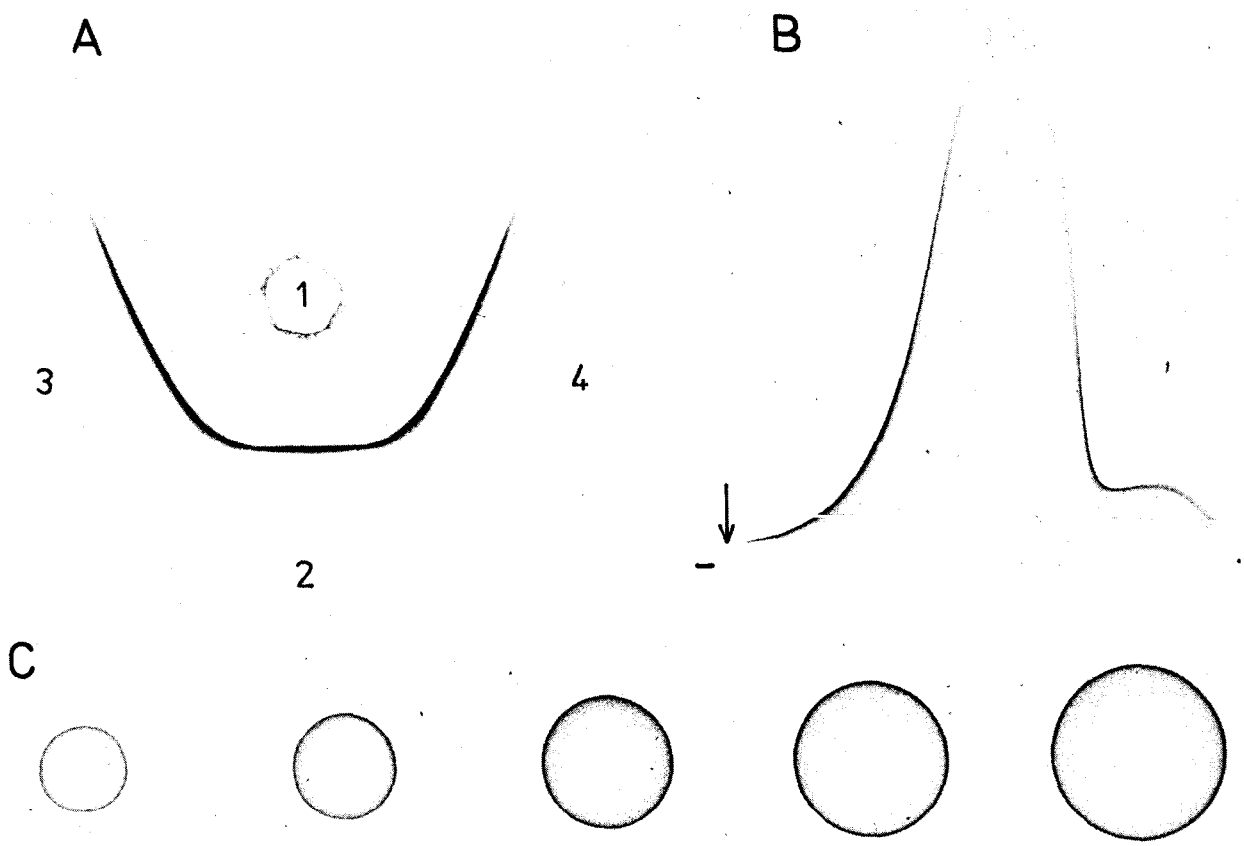

Fig.3. (A) Double immunodiffusion pattern according to Ouchterlony [6] well 1 contains 10 μl antiserum; well 3, 5 μl of total prostatic cytosol (dilution 1:20), wells 2 and 4, 5 μl of a solution of purified prostatic binding protein. (B) Crossed immunoelectrophoresis [7]. The sample (5 μl of twenty-fold diluted prostatic cytosol) was run first for 3 h at 100 V in a 2% agarose gel containing no antiserum and thereafter for 20 h at 40 V in a perpendicular direction in a gel containing 1.5% antiserum. The buffer in this experiment consisted of sodiumdiethylbarbiturate (43 mM), sodium acetate (43 mM) and HCl to pH 8.2. (C) Single radial immunodiffusion, corresponding to the points of the standard curve.

lines showed complete fusion as expected for immunological identity. Both subunits prepared by polyacrylamide gel electrophoresis in SDS-containing gels also produced similar immunoprecipitation lines.

In crossed immunoelectrophoresis [7] prostatic cytosol contained a large peak of immunologically reacting material, preceded by a much smaller one with higher electrophoretic mobility (fig.3(B)). The complete fusion of the precipitation lines of both peaks indicates that they correspond to immunologically closely related forms. Additional evidence of the specificity of the antiserum forms the precipitation of 60.9% of [^{3}H]pregnenolone bound in diluted delipidated prostatic cytosol (protein: 1.1 mg/ml) by incubation for 30 min at 0°C with 2 vol. antiserum. Finally, the antiserum gave no reaction with rat serum or cytosol prepared from rat kidney, lung, liver, thymus, testes, epididymes and uterus.

3.3. *Quantitative estimation of prostatic binding protein by radial immunodiffusion*

After incubation the antigen in test or control samples formed clearcut circular immunoprecipitates in the antibody-containing agarose gels (fig.3(C)). A very good linear correlation existed between the amount of antigen and the square diameter of these circles with correlation coefficients for the standard curve better than 0.999. The coefficient of variation for 20 samples measured in duplicate was 4.8%. Finally, the sensitivity of the assay posed no problem, since the samples usually had to be diluted 10–200 fold. The results of this assay are expressed in arbitrary units, since there was not enough purified prostatic binding protein for calibration on the basis of weight. For purified prostatic binding protein, prepared as described above, a concentration of 1 unit/ml corresponds to an $A_{280\,nm}$ of 0.036 and a protein concentration of 0.044 mg/ml in the Lowry method [8] using human serum albumin as reference.

In prostatic cytosol of 6 months old male rats the concentration of prostatic binding protein was 11.50 ± 2.04 (n = 4) units/mg protein. When we use the conversion factor of 0.044, this corresponds to 50.6% of the total protein in prostatic cytosol, measured by the Lowry method. This estimate is higher than the estimate of 15–20% obtained on the basis of steroid binding [2]. This may indicate that a part of prostatic binding protein shows no binding activity even in delipidated cytosol. The estimate of 50%, on the other hand, is compatible with the relative intensity of the corresponding protein bands in SDS–polyacrylamide gel electrophoresis.

3.4. *Effects of various factors on the concentration of prostatic binding protein*

As shown previously [2], the binding of steroids of prostatic binding protein increases several times by delipidation, whereas incubation with dithiothreitol at 37°C results in an almost complete loss of binding activity. These factors had only a small effect on the immunochemically measured concentration of prostatic binding protein. Indeed, after delipidation or treatment with dithiothreitol, these values were, respectively, 108.9% and 92.3% of the control value.

4. Discussion

In this paper we describe a small scale purification of prostatic binding protein and the production of a monospecific antiserum against it. This antiserum was used for the quantitative measurement of prostatic binding protein by the method of radial immunodiffusion [3]. This technique is more specific and more reliable than the measurement of binding. Indeed, various factors which enhance (delipidation) or destroy (reducing reagents, low pH) the binding activity have little or no effect on the immunochemically determined concentration. Furthermore, the immunochemical measurement is easier to perform and requires only a very small sample. By use of this method, we could confirm that prostatic binding protein is a major constituent of prostatic cytosol and specific for this organ. We hope to use this technique for a more detailed study of prostatic binding protein as a function of age and hormonal treatment and for the further characterization of this protein. Finally, the antiserum will allow us to perform some other experiments on the localization and production of prostatic binding protein.

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