Partial Purification of Avian Vitellogenin by Chromatography on Hydroxyapatite

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Vitellogenin is the yolk precursor protein found in the blood of laying oviparous vertebrates.[1] Its synthesis in the liver can be induced also in immature and male animals by estrogens.[1] In the blood, vitellogenin is a lipoglycophosphoprotein with a molecular weight of about 500 000, of which 12 % is lipid. Native avian vitellogenin contains two monomer polypeptide chains with a molecular weight of about 240 000.[2] In the ovary, vitellogenin is taken up by the growing oocyte within which it is proteolytically cleaved into lipovitellin (molecular weight 170 000) and two slightly different phosvitins (molecular weights 28 000 and 34 000).[2] Vitellogenin is the only phosphoprotein detectable in the blood of laying hen and estrogen-stimulated rooster. It contains approximately 3 % phosphorus by weight.[2] Most of the phosphorus is in the phosvitin moiety, which is composed of about 50 % clustered serine residues, over 90 % of which appear to be phosphorylated.[2] The present study was undertaken to examine the chromatographic behavior of vitellogenin on hydroxyapatite, which has a high affinity for phosphoproteins.[3] We found that vitellogenin is strongly bound to hydroxyapatite and can thus be partially purified from plasma with this procedure.

Experimental. White Leghorn roosters weighing about 2 kg were obtained from a local hatchery and fed *ad libitum*. When roosters were treated with estradiol-17β benzoate (Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.), they were injected i.m. in leg muscles with 40 mg in 4 ml of sesame oil. Blood was collected by cardiac puncture into syringes containing heparin or EDTA at 4 days from the estrogen injection. Plasma was separated from blood by centrifugation at 3000 *g* for 15 min and used immediately.

Hydroxyapatite (Hypatite C) was purchased from Clarksen Chemical Co., Williamsport, Pa., U.S.A. Sephadex G-100 was from Pharmacia Fine Chemicals Inc., Uppsala, Sweden. Human γ-globulin was obtained from Kabi, Stockholm, Sweden. Phosvitin and bovine serum albumin were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Adenosine 5'-[γ-^{32}P]triphosphate (37 MBq/ml) was purchased from the Radiochemical Centre, Amersham, England. Reagents for polyacrylamide gel electrophoresis were purchased from Eastman Kodak Co., Rochester, N.Y., U.S.A. All other analytical grade chemicals were from E. Merck AG, Darmstadt, West Germany.

The column (0.9 cm × 19 cm) of hydroxyapatite was equilibrated at 4 °C with a buffer containing 0.015 M potassium phosphate, pH 6.4. A 3 ml plasma sample was directly applied onto the column and washed with 150 ml of the equilibration buffer. The column was then eluted at 4 °C with a 400 ml linear gradient from 0.015 to 1.5 M potassium phosphate, pH 6.4. The flow rate was about 24 ml/h. Fractions of 4 ml were collected and the absorbance of the effluent at 280 nm was continuously monitored with an Uvicord III absorbance monitor. Protein concentration in each fraction was determined by the method of Lowry *et al.*[4] with bovine serum albumin as a standard. Selected fractions were concentrated by dialysis and lyophilization for enzymatic phosphorylation.

The purification of phosvitin kinase from non-estrogenized rooster liver and the phosphorylation of vitellogenin and phosvitin with [γ-^{32}P]ATP were performed as previously described.[5] Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis in 5 % (w/v) gels was performed according to the method of Weber and Osborn.[6] The gels were stained for protein with Coomassie brillant blue. For radioactivity determinations, the gels were cut into 2 mm slices by use of a gel slicer, each slice incubated overnight with 50 μl of water and 0.5 ml of NCS solubilizer and counted in a toluene-based scintillant.[5] Gel filtration on Sephadex G-100 was performed in 2.4 cm × 100 cm columns in a buffer containing 0.5 M NaCl, 0.01 M $MgCl_2$ and 5 mM sodium acetate, pH 5.

Results and discussion. Vitellogenin is present in the plasma of estrogen-stimulated roosters but absent from the plasma of unstimulated animals.[2] Fig. 1 shows a typical hydroxyapatite purification of vitellogenin from estrogen-stimulated rooster plasma. A strongly bound protein peak is eluted from the column at about 1.1 M potassium phosphate concentration. Phosvitin from egg yolk is also eluted in this region. This agrees with a previous report of elution of phosvitin at about 1.2 M potassium phosphate concentration.[3] However, plasma from unstimulated roosters yields no material, which elutes in this position and the standard proteins, bovine serum albumin and human γ-globulin, are eluted at the beginning of the gradient.

The last component eluting from the column was characterized by molecular weight estimates and by its ability to act as acceptor for phosphate with phosvitin kinase.[2] The incorporation of ^{32}P by phosvitin kinase into trichloroacetic acid precipitable material in selected fractions indicates that specific substrate sites are only present in the last

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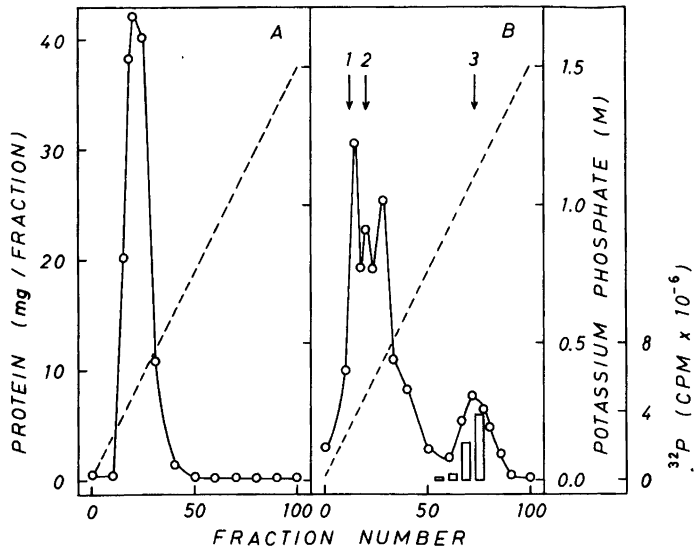

Fig. 1. Hydroxyapatite chromatography of plasma from (A) unstimulated rooster and (B) rooster injected with estradiol-17β benzoate 4 days previously. A 3 ml plasma sample was applied directly onto the column and eluted as described in the text. The elution positions of human γ-globulin (1), bovine serum albumin (2) and egg yolk phosvitin (3) are indicated by arrows. Duplicate 0.01 ml aliquots from every fifth fraction were subjected to *in vitro* phosphorylation by phosvitin kinase. The acid precipitable ^{32}P-radioactivity is indicated by open columns.

eluting peak (Fig. 1B). The material in this position was pooled, dialyzed and lyophilized, and subjected to *in vitro* phosphorylation by phosvitin kinase. Gel filtration of the labeled protein indicates a molecular weight of about 500 000, which agrees with the molecular weight of avian plasma vitellogenin.[7] However, several radioactive bands were observed when the labeled protein was electrophoresed on SDS-polyacrylamide gels, which indicates that the isolated vitellogenin was partially degraded. The rapid degradation of plasma vitellogenin during purification has been previously observed even in the presence of the serine protease inhibitor phenylmethanesulfonyl fluoride.[2,7] Nevertheless, the molecular weight of the largest radioactive band in SDS-polyacrylamide gels was found to be about 240 000, which approximates with the molecular weight of vitellogenin monomer.[2,7]

The evaluation of the purity of the isolated vitellogenin is somewhat difficult, since SDS-polyacrylamide gel electrophoresis is not suitable due to the generation of proteolytic cleavage fragments from vitellogenin during purification. A faintly stained albumin band was present in the SDS-polyacrylamide gels from the leading edge of the peak, but comparable gels from the trailing edge of the peak revealed no staining material with a molecular weight less than about 90 000 (phosvitin does not stain with Coomassie brillant blue[2]). These results and the fact that the only new protein species induced by estrogens is vitellogenin,[1] demonstrate that hydroxyapatite chromatography can be used in the isolation and purification of avian plasma vitellogenin.

Other methods used in the isolation and purification of plasma vitellogenin include affinity chromatography,[7] chromatography on TEAE- and DEAE-cellulose and various precipitation methods (e.g. by Mg^{2+} in the presence of EDTA).[8] TEAE-cellulose chromatography is no longer recommended, because the ion exchange material has been found to bind vitellogenin irreversibly.[8] Albumin and vitellogenin are eluted close to each other in DEAE-cellulose chromatography and this may cause cross-contamination depending on the protein load.[8] The strong binding of vitellogenin to hydroxyapatite demonstrated in this study may, in this respect, offer some advantages over DEAE-cellulose.

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