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ISOLATION OF TURBOT (*SCOPHTHALMUS MAXIMUS*) VITELLOGENIN BY HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY

CHRISTER SILVERSAND* and CARL HAUX
Department of Zoophysiology, University of Göteborg, P.O. Box 25059, S-400 31 Göteborg (Sweden)
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SUMMARY

The use of high-performance anion-exchange chromatography on a Mono Q column for isolation of a glycolipophosphoprotein, vitellogenin, from turbot plasma has been evaluated. The method is an effective, rapid one-step procedure, which gives a pure preparation of vitellogenin as assessed by electrophoresis, [^{32}P]orthophosphate incorporation and amino acid composition.

INTRODUCTION

In egg laying vertebrates, a large hepatically derived glycolipophosphoprotein serves as the macromolecular precursor to the egg yolk proteins[1–8]. This glycolipophosphoprotein, vitellogenin (VTG), is synthesized by the liver in response to circulating estrogens. Normally, this synthesis occurs in female oviparous animals during the oocyte development, but it can also be induced in female, male and juvenile animals by administration of estradiol-17β[7,8]. VTG is secreted by the liver into the blood and transported to the ovaries, where it is incorporated into the growing oocytes[2–4,7,8]. Inside the oocyte, VTG is cleaved and processed to form the two major yolk proteins: lipovitellin (lipoprotein) and phosvitin (phosphoprotein)[3,4,6,7]. These yolk proteins form the principal nutritive reserve of the egg, and constitute the food supply for the developing embryo before it is capable of feeding.

The VTG molecule is a complex, high-molecular-weight plasma protein, containing variable amounts of lipids, carbohydrates, phosphates and metal ions[1,6,10]. In teleost fish, the total lipid content of VTG is about 20%[6,11,12]. VTG is of wide physiological and biochemical interest because the vitellogenic process is an unique developmental system, which allows the study of many of the facets of cell biology. VTG provides a useful tool for detailed analysis of protein induction and regulation of gene expression[13], and the uptake of VTG into maturing oocytes is an excellent system for studies of the mechanism of receptor mediated endocytosis[8,9]. Further, VTG has a vital role as a nutritional source for the embryo during embryogenesis.

Research centered on vitellogenesis and related processes often requires purified, intact VTG. The molecule has therefore been isolated from several vertebrates and insects, and a multitude of different separation techniques have been used, in-

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cluding DEAE-cellulose chromatography[14], selective precipitation[14], ultracentrifugal separation[2], high-pressure liquid chromatography[15,16] and electrophoresis[14]. These methods are usually tedious and yield adequate isolation results only if two or more methods are combined. Furthermore, the purification of VTG from certain teleosts has demonstrated that VTG is sensitive to degradation during isolation procedures[7,11]. Disintegration may occur despite the use of proteolytic inhibitors, such as phenylmethylsulphonyl fluoride or aprotinin.

In the present study, the isolation of VTG from estradiol-17β-treated turbot (*Scophthalmus maximus*) plasma using a Mono Q anion-exchange column connected to a fast protein liquid chromatography (FPLC) system has been investigated. The purity of the turbot VTG isolated was evaluated by native acrylamide gel electrophoresis. To compare the results obtained in this study with those from earlier purification methods, VTG was also isolated from rainbow trout (*Salmo gairdneri*) plasma, a species from which VTG has been purified previously[5,11,12,16]. In addition, a partial characterization of turbot VTG was performed.

MATERIALS AND METHODS

Apparatus and chemicals

As anion exchanger, Mono Q (Pharmacia, Sweden), based on monodisperse 10-μm spheres was employed. The gel was supplied packed in HR 5/5 columns (50 mm × 5 mm I.D.). Ion-exchange chromatography was performed with an FPLC system consisting of a GP 250 gradient programmer, P-500 pump, V-7 injection valve, solvent mixer, prefilter, sample loop of 1000 μl, UV-1 UV monitor with an HR low-dead-volume flowcell and a Rec-482 recorder, all from Pharmacia (Sweden). Estradiol-17β and bovine serum albumin were obtained from Sigma Chemicals (U.S.A.), [^{32}P]orthophosphate from Amersham (U.K.), acrylamide, Coomassie Brilliant Blue (R-250) and tris(hydroxymethyl)aminomethane (Tris) from Merck (F.R.G.), Trasylol (aprotinin with 10^4 trypsin inhibiting units/ml) from Bayer Leverkusen (F.R.G.), glass microfibre filters (GF/B; diameter 24 mm) from Whatman (U.K.), filters (22 μm) from Millipore (France) and Instagel scintillation cocktail from Packard (The Netherlands). The electrophoresis calibration kits were obtained from Pharmacia (Sweden). All other chemicals were of analytical grade.

Fish, hormone treatment and sampling

A total of 20 juvenile turbot (*Scophthalmus maximus*), with an average weight of 80 g, were obtained from a marine fish hatchery (Øye Havbruk, Øyestranda) in southern Norway. The fish were transported to the laboratory and acclimatized for 2 weeks in 50-l aquaria with aerated sea-water at a temperature of 18°C. The water was renewed every second day, and the photoperiod was 12 h light/12 h dark. No food was given during the experiments. In order to induce VTG synthesis, ten turbot were injected intraperitoneally (i.p.) twice a week with estradiol-17β (E-17β) dispersed in peanut oil. The dose level was 20 mg E-17β kg^{-1} week^{-1} and the volume injected each time was 0.2 ml. Control fish were not injected.

To monitor the presence of VTG in each turbot, both hormone treated and control fish were injected i.p. with 250 μCi of carrier-free [^{32}P]orthophosphate 24 h prior to blood sampling.

Two weeks after the first hormone injection, blood was taken from the caudal vessels, using a cold and heparinized syringe. The blood was centrifuged and the resulting plasma collected. To avoid protease activity, a trypsin inhibitor, aprotinin (0.2 ml Trasylol), was injected i.p. 30 min before blood was taken. All preparative procedures were carried out at 4°C.

In parallel to the turbot, a total of ten juvenile rainbow trout (*Salmo gairdneri*), obtained from a local hatchery (Antens laxodling AB), were kept and treated in the same way as the turbot, except that the rainbow trout were kept in fresh water at 8°C.

Isolation procedure

The plasma preparation obtained was either loaded directly onto the Mono Q column or first processed by selective precipitation of VTG before chromatography. The ion-exchange chromatography was carried out as follows.

All solutions pumped onto the Mono Q column contained Trasylol (1%, v/v) and were degassed and filtered through 0.22-μm filters. Before a sample was loaded, the column was equilibrated with three volumes of 20 m*M* Tris–HCl, pH 8.0 (buffer A). Immediately after the blood was centrifuged, 1.0 ml of plasma was diluted in 10 ml buffer A, and 0.50 ml of this dilution was injected onto the equilibrated column. Unbound substances were eluted with 5.0 ml of buffer A. The bound plasma proteins were separated by a 15-ml linear gradient from 0.00 to 0.50 *M* NaCl or by a 17-ml stepwise gradient of 0.00, 0.32, 0.30, 0.50 *M* NaCl. To ensure that no other proteins remained on the column, it was eluted with three volumes of 1.0 *M* NaCl. The absorbance at 280 nm was measured. The flow-rate through the column was 1.0 ml min^{-1} and fractions of 0.50 ml were collected. The chromatographic procedure was carried out at 4 and 20°C. To optimize the chromatographic conditions, buffers with three different hydrogen ion concentrations (pH 7.5, 8.0 and 8.5) were tested.

To concentrate VTG from plasma prior to chromatography, it was precipitated by mixing 1.0 ml of plasma with 4.0 ml of 20 m*M* EDTA, pH 7.7, and by subsequently adding 0.30 ml of 0.50 *M* $MgCl_2$[14]. After centrifugation at 5000 *g* for 15 min, the precipitate was dissolved in 1.0 ml of 1.0 *M* NaCl. A sample of 0.50 ml of the dissolved precipitate was diluted in 5.0 ml of buffer A and 1.0 ml was then applied on the Mono Q column.

Phosphate and plasma protein determination

Protein labelled with [^{32}P]orthophosphate was measured by a slight modification of the method described by Mans and Novelli[17]. Samples of 200 μl of each of the chromatographic fractions were applied to individual glass micro filters placed at the bottom of scintillation vials. After the discs had been dried, protein was precipitated with 1.0 ml of a cold mixture of 10% (v/w) trichloroacetic acid (TCA) and 1% (v/w) phosphotungstic acid, $H_3[P(W_3O_{10})_4]$. The discs were left for 1 h at 4°C and then washed three times with 4 ml chloroform–methanol (2:1, v/v) and dried overnight. To each vial, 10 ml of scintillation cocktail were added and the samples counted for radioactivity in a β-scintillation counter (LKB 215 Rackbeta). Total plasma protein was determined by the biuret method[18] using bovine serum albumin as a reference.

Gel electrophoresis

To assess the purity of the VTG preparation obtained by FPLC on a Mono Q

ion exchanger, native discontinuous polyacrylamide gel electrophoresis (PAGE) was carried out. Samples of the main fractions chromatographed and plasma from both E-17β-treated and control fish were electrophoresed on the same kind of gel. Native PAGE was performed according to the procedure of Chrambach *et al.*[19], system 2860.O.X, on a gradient slab gel (80 mm × 0.75 mm, LKB 2050 midget electrophoresis unit) with 5–10% (w/v) and 2% bis-acrylamide. To determine the molecular weight (MW) of turbot VTG, PAGE in the presence of sodium dodecyl sulphate (SDS) and mercaptoethanol was performed according to Laemmli[20], on a gradient slab gel (80 mm × 0.75 mm) with 5–15% (w/v) total acrylamide concentration and 2% bisacrylamide. The gels were stained with Coomassie Brilliant Blue R-250 in 40% methanol–10% acetic acid in water and with silver staining[21]. The MW was estimated by comparing the mobility of the unknown proteins with those of marker proteins. The logarithm of MW was plotted against electrophoretic mobility, and linear regression was used to estimate the MW of the samples. [^{32}P]Orthophosphate-labelled proteins in the native polyacrylamide gels were detected autoradiographically using an X-ray film (Agfa Osray). The film was exposed for 2 weeks at 8°C.

Amino acid analysis

The purified VTG was hydrolysed at 110°C in 6 *M* HCl in vacuum-sealed tubes for 24 or 72 h. Samples for cysteine, methionine and proline determination were treated by oxidation. Quantitative amino acid analysis was performed by ion-exchange chromatography at the Central Amino Acid Analysis Laboratory, Institute of Biochemistry, Uppsala, Sweden.

RESULTS

Isolation procedure

After administration of E-17β to juvenile turbot, there was a rapid increase in total plasma protein concentration. The protein levels increased from 3.5 ± 0.2 g per 100 ml (n = 5) in control fish to 10.0 ± 0.2 g per 100 ml (n = 8) in estradiol-treated fish.

Plasma from E-17β-treated turbot chromatographed on a Mono Q column, at 4°C, gave a large and apparently homogeneous absorbance peak, appearing as the last component (Fig. 1B). This peak, which was eluted at a chloride ion concentration of 0.34 *M*, was absent when plasma from control fish was subjected to chromatography (Fig. 1A). When measuring the protein-bound [^{32}P]orthophosphate activity in the different fractions chromatographed, it appeared that all radioactivity was associated with this major absorbance peak. Since VTG is known to be a phosphorus-rich protein and will incorporate [^{32}P]orthophosphate during synthesis[1,7,11,14], we tentatively identified the absorbance peak at 0.34 *M* NaCl as VTG.

The chromatographic procedure was performed at two different temperatures, 4 and 20°C. At room temperature (20°C) the major absorbance peak, present when plasma from estrogenized turbot was chromatographed, became irregular and dissociated (chromatogram not shown). When the FPLC system was moved to a cold-storage room at 4°C, the elution profile changed and the main fraction appeared homogeneous as in Fig. 1B. Thus, during the rest of the study all chromatographic work was carried out at 4°C. To select the best buffer conditions, 20 m*M* Tris–HCl

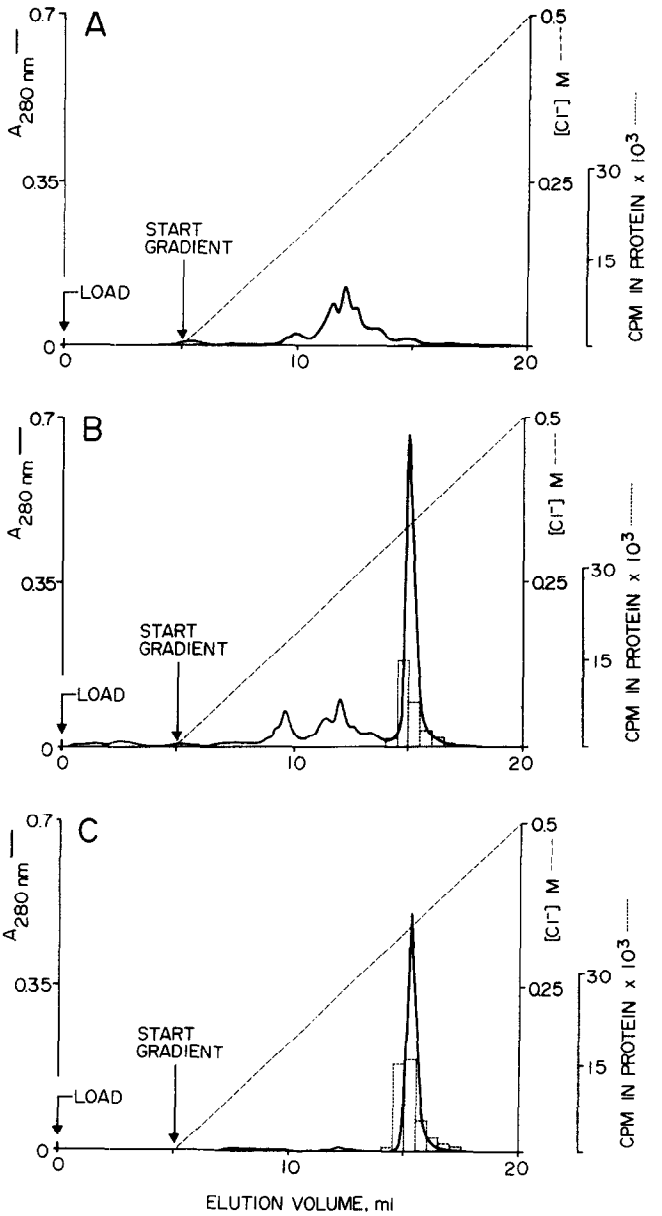

Fig. 1. Elution profiles after Mono Q chromatography of (A) plasma from control turbot, (B) plasma from estradiol-17β-treated turbot and (C) protein precipitated with Mg^{2+}–EDTA from plasma of estradiol-17β-treated turbot. The fish were injected intraperitoneally with 250 μCi of [^{32}P]orthophosphate 24 h prior to blood sampling. Elution buffer: 20 m*M* Tris–HCl, pH 8.0; gradient, 0.00–0.50 *M* NaCl. Absorbance at 280 nm (——) land cpm in protein (- - - - -) were measured.

buffer at three different pH values, 7.5, 8.0 and 8.5, was examined. No significant change in resolution was observed, and only small alterations of the elution positions of the various plasma proteins. We selected pH 8.0 in subsequent studies, in order to stay inside the buffering pH range not too far from the pK_a value for the Tris buffer.

In order to increase the resolution and reach baseline separation between VTG and other plasma components, a complex stepwise gradient was used (Fig. 2). The gradient was stopped at a chloride ion concentration of 0.32 *M*, and a negative gradient step of 2.0 ml from 0.32 to 0.30 *M* was introduced before the gradient continued to 0.50 M. Alternatively, baseline separation was achieved by using a selective precipitation step with $MgCl_2$ in the presence of EDTA prior to chromatography. Other proteins in the plasma were reduced relative to VTG (Fig. 1C).

The elution positions of VTG and the other plasma proteins were dependent upon the sample concentration loaded onto the Mono Q. At lower protein concentrations, between 0.10 and 1.00 mg, VTG were eluted reproducibly at 0.34 *M* Cl^-. With progressively higher loads, the VTG peak were eluted earlier. Sample loads of up to 10 mg total plasma protein were used, and the elution ion strength of VTG showed a displacement of 0.02 *M* Cl^- to 0.32 *M*, compared to the chromatogram shown in Fig. 1B.

Electrophoresis and autoradiography

Fig. 3 shows the native electrophoretic patterns of plasma from control and estrogenized turbot and of the various VTG fractions prepared chromatographically. When plasma was electrophoresed and stained with Coomassie Brilliant Blue, two new bands, I and II, appeared during estradiol treatment (Fig. 3, lanes a and b). These two bands were absent in control fish plasma (Fig. 3, lanes c and d). The apparent MW of bands I and II was estimated as 530 000 and 275 000 respectively. When the chromatographed VTG fractions were electrophoresed on native gel two protein bands were visible at the same position and with the same relative mobility, R_m, as those of bands I and II in plasma from E-17β-treated fish. All plasma protein bands, except I and II and a thin band situated just beneath band I, disappeared after the isolation procedures (Fig. 3, lane e). When plasma was selectively precipitated with $MgCl_2$ prior to chromatography and when the stepwise gradient was used, the thin

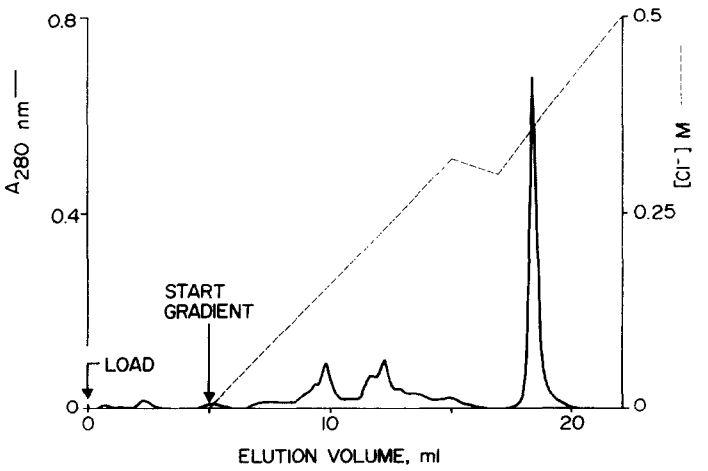

Fig. 2. Elution profiles after Mono Q chromatography of plasma from estradiol-17β-treated turbot using a stepwise gradient. Elution buffer: 20 m*M* Tris–HCl, pH 8.0; gradient 0.00, 0.32, 0.30, 0.50 *M* NaCl. Absorbance at 280 nm (——) was measured.

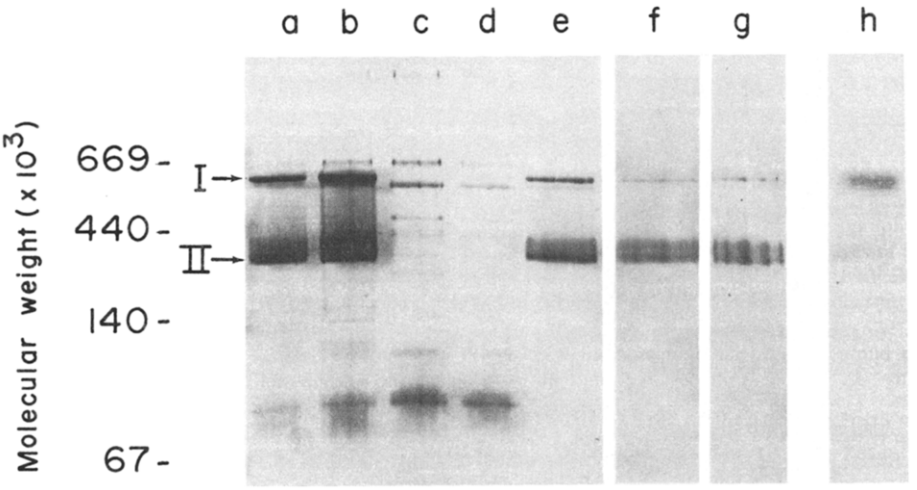

Fig. 3. Native polyacrylamide gel electrophoresis patterns of plasma proteins from turbot on a 5–10% (w/v) total acrylamide concentration gradient slab gel with 2% bis-acrylamide. Lanes: a, plasma (0.05 μl) from estradiol-17β-treated fish; b, plasma (0.10 μl) from estradiol-17β-treated fish; c, plasma (0.10 μl) from control fish; d, plasma (0.05 μl) from control fish; e, vitellogenin (VTG) obtained by Mono Q chromatography using a linear salt gradient; f, VTG obtained by Mono Q chromatography using a non-linear gradient; g, VTG precipitated with Mg^{2+}–EDTA and chromatographed on Mono Q column; h, autoradiography of the gel for samples a, b, e–g. VTG dimer and monomer are designated I and II, respectively. Numbers to the left refer to the molecular weights of the standard proteins.

band close to band I disappeared (Fig. 3, lanes f and g). The native gels were also stained with silver to see if other proteins were present in the purified fractions that were not stained by Coomassie Brilliant Blue; none was discernible. To ensure that bands I and II on the native gel were VTG, and that no other [^{32}P]orthophosphate-binding proteins were present that had not been stained by the methods used in this study, the gel was autoradiographed on an X-ray film (Fig. 3, lane h). After incubation, two labelled bands were visible on the autoradiogram. These two bands were equivalent to bands I and II on the gel. Both bands consisted of VTG. The MW of turbot VTG was estimated by SDS-PAGE by comparing the mobility of the unknown plasma proteins with those of marker proteins (Fig. 4). VTG from turbot has a MW close to 185 000.

Amino acid analysis

The results of the amino acid analyses of purified turbot VTG and the amino acid composition of VTG from two other teleost species are shown in Table I. The amino acid composition of turbot VTG is similar to that of VTG from goldfish[7] and rainbow trout[22].

To evaluate whether the isolation procedure developed in the present study can be used as a general method for purifying VTG from other fish species, plasma from vitellogenic rainbow trout was applied to a Mono Q column (Fig. 5). Rainbow trout plasma provided elution profiles very similar to those achieved with turbot plasma, the peak eluting at a chloride ion concentration of 0.35 *M*.

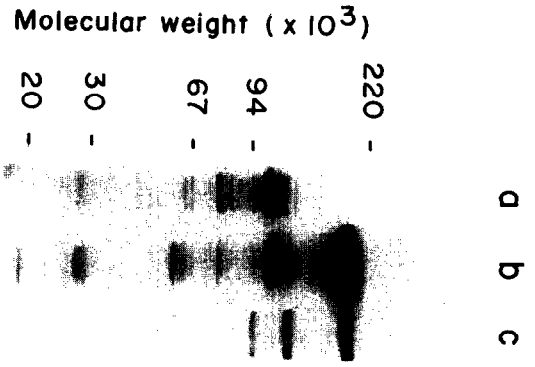

Fig. 4. SDS–PAGE patterns of turbot plasma proteins on a 5–15% (w/v) total acrylamide concentration gradient slab gel with 2% bisacrylamide. Lanes: a, plasma from control fish (0.20 μl); b, plasma from estradiol-17β-treated fish (0.20 μl) and c, vitellogenin obtained by Mono Q chromatography. Numbers to the left refer to the molecular weights of the standard proteins.

DISCUSSION

The present study demonstrates the advantage of using a Mono Q anion-exchange column connected to an FPLC system for isolation of turbot VTG. The

TABLE I

AMINO ACID COMPOSITION OF VITELLOGENIN FROM TURBOT, RAINBOW TROUT AND GOLDFISH

ND = Not determined.

Amino acid	*Mol % of total amino acid*		
	Turbot	*Rainbow trout*[a]	*Gold fish*[b]
Asp	7.8	8.5	6.5
Thr	5.2	5.0	5.5
Ser	7.5	7.6	6.9
Glu	10.1	11.6	11.9
Pro	4.5	5.3	5.5
Gly	4.6	4.3	4.6
Ala	12.0	11.8	12.8
Cys/2	1.7	ND	ND
Val	7.6	7.2	6.9
Met	2.8	2.6	2.0
Ile	6.3	5.5	6.6
Leu	10.2	9.6	10.8
Tyr	2.9	3.0	2.6
Phe	3.0	4.1	2.9
His	2.2	2.1	2.3
Lys	7.1	7.2	7.0
Arg	4.4	4.6	4.9

[a] Data from ref. 22.
[b] Data from ref. 7.

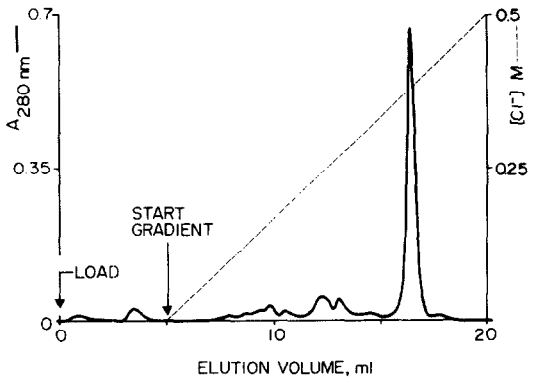

Fig. 5. Elution profile obtained after plasma from estradiol-17β-treated rainbow trout was subjected to Mono Q column chromatography. Elution buffer: 20 m*M* Tris–HCl, pH 8.0; gradient 0.00–0.50 *M* NaCl. Absorbance at 280 nm (——) was measured.

method is an effective one-step procedure, which gives a pure preparation of VTG in a short processing time. This is also exemplified by the isolation of VTG from rainbow trout, and presumably VTG from other species would be similarly purified.

The phosphorylation of VTG in combination with the very low level of other phosphorylated proteins in plasma makes the determination of [^{32}P]orthophosphate a widely used method for detecting and quantifying VTG during purification[1,3,7,14]. Since all [^{32}P]orthophosphate was associated with one absorbance peak in the chromatogram, present only in E-17β-treated fish, this peak was identified as VTG. By measuring [^{32}P]orthophosphate in all chromatographed fractions, it was possible to verify that there were no other proteins rich in phosphorus that eluted in other fractions. This verification is critical, as earlier investigations have shown that phosphorylated fragments, or the highly phosphorylated subunit of VTG, phosvitin, can dissociate from the VTG molecule during the isolation procedure[7,11]. Furthermore, phosvitin is difficult to detect spectrophotometrically because of its low absorbance at 280 nm, due to a general lack of aromatic amino acids[6,7,11].

When 10 μl plasma from E-17β-treated turbot were chromatographed, VTG eluted from the column as the last protein at a chloride concentration of 0.34 *M*. Chromatography of identical plasma samples verified the excellent reproducibility of the method. However, the elution position of VTG, as for the other plasma proteins, was dependent upon the sample load, and the proteins were eluted earlier when the sample load was increased. In this study, sample loads of up to 10 mg total plasma protein, which is 40% of the total recommended capacity for the column, were used without any reduction of the separation capacity.

The elution patterns, and thus the separation results, can be changed by using different gradient slopes. The linear salt gradient used initially provided reliable and reproducible chromatographic profiles and was used to test the effect of pH, salt and temperature. When the chemistry of the chromatography was fully optimized, a more complex gradient was introduced to improve the separation. With this stepwise gradient, the absorbance curve reached zero before VTG was eluted. Thus, the FPLC system gives the opportunity to program complex gradients in order to isolate certain peaks.

Temperature had a significant influence on the elution profile. When chromatography was carried out at room temperature (20°C), the absorbance peak for turbot VTG became irregular. These results were consistent, even when the trypsin inhibitor aprotinin was present. When chromatography was instead performed at a lower temperature, 4°C, VTG did not disintegrate and the peak was homogeneous. According to earlier investigations, VTG from some teleosts proved to be sensitive to proteolysis during isolation[7,11]; thus, precautions to prevent proteolytic activity are essential. In the present study, all preparative and chromatographic procedures were therefore carried out at low temperature and in the presence of the trypsin inhibitor aprotinin. In addition, each fish was treated with an injection of aprotinin half an hour before sampling.

When plasma from E-17β-treated turbot was electrophoresed on native gel, two new protein bands, I and II, became visible. Both bands contained [^{32}P]phosphorylated proteins, indicating that they consisted of VTG. The MW of band I was about twice that of band II, suggesting that bands I and II consist of VTG in dimeric and monomeric forms. Further evidence in support of this suggestion is that bands I and II exhibited the same patterns upon electrophoresis in the presence of SDS. The finding that turbot VTG exists in dimeric form in plasma and appears as both dimeric and monomeric forms on native electrophoresis is in accord with earlier work on other fish species[7,23].

To assess the purity of the VTG preparations obtained by chromatography, the different VTG peak fractions were applied to native PAGE. When the VTG preparation obtained by chromatography with a linear salt gradient (Fig. 1A) was applied to native electrophoresis, two bands appeared with the same R_m as those of bands I and II from plasma of estrogenized turbot. All other plasma components disappeared except for the thin band adjacent to band I. This band was present in both E-17β-treated and control turbot, indicating that this protein is not induced by E-17β. Use of the more complex stepwise gradient to elute the proteins from the Mono Q column, or inclusion of the selective precipitation step prior to chromatography, resulted in the disappearance of this faint protein band.

The MW of turbot VTG was estimated by SDS-PAGE to be 185 000. This value is very similar to that of VTG from other fish species, *e.g.*, MW 170 000[12,24] for rainbow trout. The difference observed between the MW on SDS and on native PAGE may be due partly to the relatively high lipid content of VTG. The lipid fraction is non-covalently bound to VTG and is therefore removed during treatment with SDS. This is in line with the finding made when purified turbot VTG was analyzed for its amino acid composition. Thus, when VTG was analyzed, only 68% (w/w) of the preparation consisted of amino acids; the rest was probably predominantly lipids. Even if the MW, provided by native electrophoresis, may be subject to considerable error, the calculation of the MW of SDS-treated turbot VTG by subtracting 32% (non-peptide content) from the value obtained by native PAGE results in a value of 187 000. This is very close to the MW estimated by SDS-PAGE.

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