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Note

Determination of vitellogenin in serum of rainbow trout (*Salmo gairdneri*) by high-performance gel permeation chromatography

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Vitellogenin is a female-specific lipophosphoprotein occurring in serum of oviparous vertebrates during maturation. It is synthesized in the liver and transported to the ovaries, where it is deposited in the oocytes. Vitellogenin is the main precursor of the egg yolk proteins and lipids. The initiation and maintenance of protein synthesis are mainly controlled by female sex steroid hormones (oestrogens). Vitellogenesis has been demonstrated in many vertebrates, including the teleost fishes [1–3]. Immature fish of both sexes show very small amounts of phosphoprotein in the serum.

The vitellogenin molecule has several chemical characteristics that serve as a basis for its identification and quantification. The serine residues in the peptide chains are highly phosphorylated, and vitellogenin contains specific lipid and carbohydrate components. Moreover, vitellogenin has been shown to bind calcium and magnesium [4]. Determination of alkali-labile protein phosphorus is a method widely used to detect and quantify vitellogenin in serum [5]. Analysis of protein-bound calcium has also been applied to determine vitellogenin [4,6]. The protein complex has highly antigenic properties, and this feature is used in the radioimmunoassay methods [3,7,8].

Methods for measuring serum vitellogenin in fish were reviewed by Ng and Idler [9]. For studies concerning vitellogenesis where both quantitative and qualitative aspects are of interest, a rapid and non-destructive separation technique is warranted. DEAE-cellulose electrophoresis and Sephadex gel permeation chromatography have been used [3,7,10], but these methods are often cumbersome and time-consuming.

This paper reports on the separation and isolation of vitellogenin in rainbow trout (*Salmo gairdneri*) serum using high-performance gel permeation chroma-

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tography. The method was evaluated by artificially induced vitellogenesis (administration of 17β-estradiol) and applied to naturally maturing fish. The type of gel exclusion column used has also proved suitable for human lipoprotein separation [11].

EXPERIMENTAL

Gel permeation

An LKB (Bromma, Sweden) Ultrapac TSK-G 4000 SW (600 mm$\times$7.5 mm I.D.) gel permeation column with a TSK-GSWP (75 mm$\times$7.5 mm I.D.) guard column were used to separate the serum proteins. The other components were: LKB 2150 HPLC pump, LKB 2151 variable-wavelength detector, Rheodyne 7125 injection valve with a 200-μl sample loop and Spectra-Physics SP4270 integrator. When the column is used for analytical purposes the injected sample should not contain more than 5 mg of protein, according to the manufacturer. The sample size can be increased ca. ten-fold for semipreparative use.

Degassed Tris buffer (Sigma, St. Louis, MO, U.S.A.) pH 7.2, containing 0.1 *M* potassium chloride and 0.05% sodium azide was used as eluent in an isocratic system at a flow-rate of 1 ml/min. The detection wavelength was 280 nm. The column was calibrated with proteins of known relative molecular masses from 160 000 to 670 000 (Pharmacia Fine Chemicals Gel filtration calibration kit).

Induction of vitellogenesis

Individually marked rainbow trout ($n=31$) of both sexes with an initial weight ($\pm$S.D.) of 2.6$\pm$0.9 kg were held in a circular basin (6 m^3) with a salinity of 29 ppt at 6°C. Fifteen fish (hormone group) were injected intraperitoneally with peanut oil containing 2 mg/ml 17β-estradiol (1.0 mg/kg fish) and sixteen fish (control group) with pure peanut oil (0.5 ml/kg). The fish were anaesthetized with benzocaine [12] and weighed, and blood samples taken before hormonal injection. Blood was collected at days 0, 2, 5, 14 and 21 from *ductus cuvieri*, according to a method described by Lied et al. [13]. About 5 ml of blood were withdrawn from each fish and allowed to clot for 3 h prior to centrifugation at 3000 *g* for 10 min. Serum samples were stored at −20°C until analysed. Serum was diluted 1:2 (v/v) with the eluent buffer and filtered (Millipore AAWP, 0.8 μm) prior to injection.

Naturally maturing fish

In 54 naturally maturing rainbow trout blood was collected at irregular intervals and treated according to the same procedure.

Phosphoprotein phosphorus and protein assay

Phosphoprotein phosphorus was analysed following a procedure described by Wallace and Jared [14]. Determination of phosphorus in the hydrolysates was carried out by means of a Technicon RA-1000 random access analyser using a standard colorimetric phosphomolybdate method. Total protein in serum was analysed according to a Technicon RA-1000 adapted Biuret method. The content

of protein in the fractions sampled after chromatography was determined with the Coomassie Brilliant Blue method using bovine serum albumin as standard [15].

RESULTS

Gel permeation chromatography

Fig. 1 shows the serum protein profiles of one rainbow trout at days 0, 2, 5, 14 and 21 after injection of a single hormone dose. A new peak at day 14 at an elution volume of ca. 18 ml indicated the presence of vitellogenin in serum. The area of this peak (percentage of total) was 19.3% at day 14 and 33.1% at day 21. The content of total protein in these samples were 32 and 25 g/l, respectively. The amount of vitellogenin is thus 6.2 g/l at day 14 and 8.3 g/l at day 21. The vitellogenin was not baseline-separated from a small peak, present in immature fish, eluting slightly earlier with an area of less than 5%.

The other fish in the hormone group showed similar results with vitellogenic

Fig. 1. Serum protein profiles from one rainbow trout at days 0, 2, 5, 14 and 21 after intraperiteonal injection with 17β-estradiol (chromatogram 1, 2, 3, 4 and 5, respectively). Arrow indicates the vitellogenin peak.

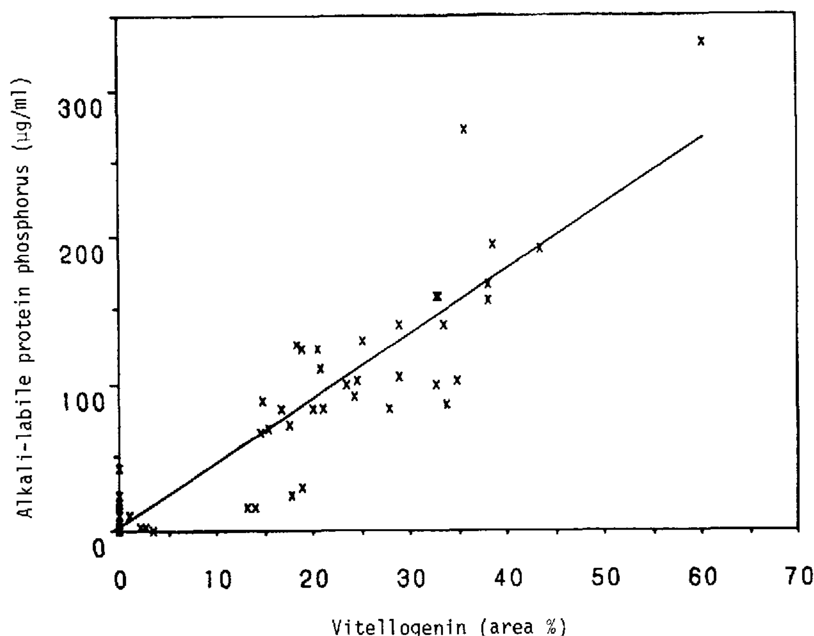

Fig. 2. Determination of vitellogenin in sera from naturally maturing rainbow trout by high-performance gel permeation chromatography compared with the alkali-labile protein phosphorus method. Regression analysis showed a significant relation ($p < 0.05$) expressed by the equation $y = 2.35 + 4.39x$ ($r = 0.91$, $n = 54$). The S.D. of the coefficient was 0.28.

activity after two weeks, while no vitellogenic response were detected in fish from the control group during the experiment.

Reproducibility and protein recovery

One sample of pooled sera from maturing rainbow trout was analysed eight times to test the reproducibility of the analysis. The mean and S.D. of the vitellogenin peak were 23.9 ± 0.4 area per cent. The area variation was less than 2%, and the elution volume was less than 1.3% for all peaks with an area greater than 1% of the total.

To evaluate the protein recovery, a serum sample was injected and fractionated into twenty fractions each of 2 ml, sampled from the front of the void volume peak. The serum sample contained 56.9 g/l protein, and on analyses of the fractions 53.8 g/l was recovered (95%).

Vitellogenin correlation to phosphoprotein phosphorus

The relation between the area percentage of vitellogenin calculated from the gel permeation chromatogram and the analyses of alkali-labile phosphorus in naturally maturing rainbow trout is presented in Fig. 2. Linear regression analysis revealed a significant relationship ($p < 0.05$), expressed by the equation $y = 2.35 + 4.39x$ ($r = 0.1$, $n = 54$). The correlation coefficient was 0.96 when vitellogenin was expressed as the percentage of the total protein determined in serum by the standard Biuret method.

DISCUSSION

Gel permeation chromatography of serum from oestrogen-treated and control fish confirmed the appearance of protein with high relative molecular mass in fish injected with 17β-estradiol (Fig. 1). This protein was not detected in fish injected with pure peanut oil. The experimental concept with hormone injection applied in the present study has frequently been applied to demonstrate vitellogenin synthesis in fish [9]. The new peak appearing in the chromatogram of serum proteins from oestrogen-treated fish eluted at ca. 18 ml, just behind ferritin (M_r=440 000) from the standard calibration kit. Various relative molecular masses for vitellogenin between 390 000 and 600 000 have been reported in the salmonids [9]. Norberg and Haux [10] reported 440 000 in the rainbow trout by use of traditional gel chromatography of purified vitellogenin. It was thus concluded that the new peak appearing in the high-performance gel permeation chromatogram of serum from hormone-injected fish in the present investigation was vitellogenin.

As the time from hormonal injection to serum vitellogenin detection varied considerably among individuals in the present study, it has been found most appropriate to present data from one single fish with a typical vitellogenic response (Fig. 1). Vitellogenin was not detected in serum of any fish five days after injection. In the sample presented here, vitellogenin was present in significant amounts after two weeks and increased thereafter until day 21. No samples were collected any later.

Vitellogenin determined by the method outlined was significantly correlated ($p<0.05$) with the alkali-labile protein phosphorus assay in naturally maturing rainbow trout (Fig. 2). The correlation coefficient increased from 0.91 to 0.96 when vitellogenin was calculated relative to the amount of total protein in the sample and then correlated to alkali-labile protein phosphorus. This indicates that the best quantification of vitellogenin is obtained by taking into consideration both the peak area percentage and total protein. The lipid content of vitellogenin (ca. 20%) is not incorporated in the values given. Values reported for vitellogenin in serum of salmonid fish vary somewhat according to the methods used. The levels reported here agree with earlier findings in salmonids [9].

Indirect quantification of vitellogenin by analysis of serum alkali-labile protein phosphorus has proved significant in several experiments with both naturally maturing and oestrogen-treated fish [5,6,16]. However, by application of the protein phosphorus method, it is not possible to isolate vitellogenin in its native form. This is necessary where the object of the study also implies chemical characterization of the compound.

According to Ng and Idler [9], there are significant correlations between the stage of gonadal development, the serum estrogen level and the serum vitellogenin concentration in fish. Thus the present method is a valuable tool in research concerning studies on sexual maturation and vitellogenesis, as it is easy to carry out and non-destructive to vitellogenin. Vitellogenin from rainbow trout has been shown to be homogenous with no proteolysis after deep-freezing and storage in plasma or serum samples [3,10]. With automatic injection and fraction

collection, a large number of samples can be run consecutively. The chromatogram also gives information on the other serum proteins, of which albumin has been associated with vitellogenesis [5,17,18].

REFERENCES

1 P.A. Plack, D.J. Pritchard and N.W. Fraser, Biochem. J., 121 (1971) 847.
2 R.A. Wallace, in R.E. Jones (Editor), The Vertebrate Ovary, Plenum Press, New York, 1978.
3 C.M. Campbell and D.R. Idler, Biol. Reprod., 22 (1980) 605.
4 B.T. Bjørnsson and C. Haux, J. Comp. Physiol., 155B (1985) 347.
5 C. Haux, Dissertation, University of Gøteborg, Gøteborg, 1985.
6 R.E. Bailey, J. Exp. Zool., 136 (1957) 455.
7 Y.P. So, D.R. Idler and S.J. Hwang, Comp. Biochem. Physiol., 81B (1985) 63.
8 P.A. Copeland, J.P. Sumpter, T.K. Walker and M. Croft, Comp. Biochem. Physiol., 83B (1986) 487.
9 T.B. Ng and D.R. Idler, in W.S. Hoar, D.J. Randall and E.M. Donaldson (Editors), Fish Physiology, IXA, Reproduction, Associated Press, New York, 1983.
10 B. Norberg and C. Haux, Comp. Biochem. Physiol., 81B (1985) 869.
11 R. Vercaemst, M. Rosseneu and J.P. Van Biervliet, J. Chromatogr., 276 (1983) 174.
12 G. Wedemeyer, J. Fish. Res. Board Can., 27 (1970) 909.
13 E. Lied, J. Gjerde and O.R. Brækkan, J. Fish. Res. Board Can., 32 (1975) 699.
14 R.A. Wallace and D.W. Jared, Can. J. Biochem., 46 (1968) 953.
15 M. Bradford, Anal. Biochem., 72 (1976) 248.
16 J.C.A. Craik and S.M. Harvey, J. Fish. Biol., 25 (1984) 293.
17 G.E. Shul'man (Editor), Life Cycles of Fish: Physiology and Biochemistry, Wiley, New York, Toronto, 1974.
18 J.R. Tata and D.F. Smith, Prog. Horm. Res., 35 (1979) 47.