Purification of vitellogenin from the plasma of Indian freshwater murrel, *Channa punctatus* (Bloch) by different methods: A comparative study

Neeta Sehgal and SV Goswami*

Department of Zoology, University of Delhi, Delhi 110 007, India

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Plasma from estrogenized, [125I] NaH2PO4-injected murrel, *Channa punctatus* was collected in the presence of proteolytic inhibitors and subjected to different separation procedures singly or in combination, viz., gel filtration chromatography on Ultrogel AcA 34, ion-exchange chromatography on DEAE sephacel, or selective precipitation with dimethylformamide or with Mg2+: EDTA in order to isolate vitellogenin from other plasma proteins. The results show that chromatography on Ultrogel or DEAE sephacel yields intact vitellogenin whereas prior precipitation with DMF or with Mg2+: EDTA results in either co-precipitation of other plasma proteins or in the cleavage of phosphitin-like material from the native vitellogenin molecule.

The egg yolk of fishes is derived from a bloodborne precursor, vitellogenin, which is synthesised only in the liver of females under estrogenic influence during periods of reproductive activity. This protein is absent in males, thereby suggesting femalenesspecificity. Hepatic synthesis of vitellogenin can be induced in both sexes at any time of the year by administration of estrogen.5,6

Vitellogenin is a high molecular weight protein that contains, in addition to the polypeptide backbone, several peripheral moieties such as carbohydrates, lipids and phosphate. Vitellogenins of several fishes can selectively bind calcium and iron.7,12 These characteristics have been employed by many investigators to devise procedures for selective isolation of vitellogenin from the blood of several fish species. These procedures include ultracentrifugation, selective precipitation with dimethylformamide (DMF)8 or with Mg2+ in the presence of a chelating agent14, gel filtration chromatography, ion-exchange chromatography, affinity chromatography, or a combination of these procedures.5,27,31

Considerable heterogeneity has been reported in the physico-chemical characteristics of piscine vitellogenins.5,24,32,33 This heterogeneity may be attributed to species-dependent variations in the vitellogenin molecule as well as to differences in the isolation procedures.5 The present investigation evaluates a variety of procedures for isolation of vitellogenin from the plasma of the Indian freshwater murrel, *Channa punctatus*.

Materials and Methods

Adult specimens of *C. punctatus* (body wt: 100-160g) were collected from the backwaters of the river Yamuna around Delhi (Lat. 28°35' N, Long. 77°12' E) during the preparatory period (February) and acclimated to laboratory conditions (25°C±1°C; L:D:: 12:12) for at least seven days prior to initiation of experiments.5,6 Fishes were fed *ad libitum* on minced beef throughout the experimental duration. Water in the aquaria was renewed daily with dechlorinated tap water. Unless otherwise specified, all chemicals used were of analytical grade and were obtained from Sigma Chemical Co., St. Louis, U.S.A. and British Drug House Laboratory, Bombay, India.

Our preliminary experiments have shown that plasma vitellogenin level increases significantly in both sexes of murrel injected with 5 μg and 10 μg of estradiol-17β (E2); no further increase was observed with higher dosages (20-40 μg). In addition, vitellogenin titre increased progressively after 4th and 5th injection, after which no statistically significant increase was evoked. Therefore, murrels of either sex were injected daily with 10 μg of E2 intramuscularly for five days. Each fish was injected with 0.25 mCi of
Anion exchange chromatography

Counted in a Packard column and equilibrated with the starting buffer alone. The disks were air-dried and radioactivity was counted in aliquots of column fractions.

Two step method

Gel filtration followed by anion exchange chromatography

Plasma from E2-treated murrel was fractionated on Ultrogel AcA 34. Fractions from the vitellogenin peak (V/Vo = 1.42) were pooled and dialysed for 18 hr against the starting buffer at 4°C and applied to DEAE sephacel column. Adsorbed material was eluted with a linear gradient of 0 to 0.4 M NaCl. Absorbance at 280 nm was monitored in fractions.

Anion exchange followed by gel filtration chromatography

Estrogenized plasma was applied to DEAE sephacel columns and eluted as described above. Fractions eluting at 0.22 M NaCl were pooled, concentrated by dialysing against sucrose and chromatographed on Ultrogel AcA 34. Fractions of 5 ml were collected and the absorbance read at 280 nm.

Precipitation followed by gel filtration chromatography

(i) To 5 ml plasma at 0°C, 1.85 ml of chilled DMF was added and pH adjusted to 6.0 with 0.2 M acetic acid. The mixture was allowed to stand for 45 min at 0°C. The precipitate was dissolved in 12.5 ml of DMF (3.5 ml DMF in 0.15 M NaCl) and reprecipitated by adjusting the pH to 6.0. The precipitate was washed thoroughly with distilled water, dissolved in 1 M NaCl, 50 mM Tris-Cl buffer at pH 7.5 (buffer A) and chromatographed on Ultrogel AcA 34. Aliquots of fractions were processed for absorbance and radioactivity as described above.

(ii) In yet another method, vitellogenin was precipitated from the murrel plasma by mixing 7.5 ml of plasma with 30 ml of 20 mM EDTA, pH 7.7 at 0°C followed by addition of 2.25 ml of 0.5 M MgCl2. After centrifugation, the pellet was dissolved in 3 ml of buffer A and reprecipitated by addition of 30 ml distilled water. The precipitate was dissolved in buffer A. After dialysis against the same buffer, the sample was chromatographed on Ultrogel AcA 34.
column. Aliquots of fractions were evaluated for absorbance and radioactivity as described above.

Results

One step method

Gel filtration chromatography

Plasma from E2-treated murrel subjected to gel filtration chromatography on Ultrogel AcA 34 resolved into five major absorbance peaks in the fractionation range (Fig. 1). A small peak eluting at the bed volume possibly represents Tris. The first peak (peak I) eluted within the void volume appeared milky. It contained lipoproteins with high absorbance values but low protein content. Peak III (Ve 235-245 ml) consisted of fractions with high protein content and accounted for the entire 32P activity. Almost the entire alkali labile phosphorus was present in this peak only. The characteristic elution position, high protein content, alkali labile phosphorus and 32P activity indicate that this peak represents vitellogenin. In addition, absence of any protein at this elution position in the gel profile of plasma from untreated male murrel confirms it to be vitellogenin6.

Absorbance peak IV had virtually no alkali labile phosphorus and 32P activity. A few fractions following peak IV contained very little 32P activity but no alkali labile phosphorus. Fractions of peaks II and V were totally devoid of radioactivity as well as alkali labile phosphorus (Fig. 1). The profile indicates that presence of 32P in protein is restricted to only those fractions that contain alkali labile phosphorus.

Measurement of 32P has been taken as an indirect index of vitellogenin.

Anion exchange chromatography

An aliquot of pooled plasma was subjected to anion exchange chromatography on DEAE sephacel employing a two-chambered linear gradient. Vitellogenin was the last protein to elute from the column at 0.22 M NaCl as indicated by 32P activity (Fig. 2). That intact vitellogenin was indeed isolated is evident as no phosvitin-like material with 32P activity was obtained in any subsequent fraction.

Two step method

Gel filtration followed by anion exchange chromatography

Pooled vitellogenin fractions from Ultrogel AcA 34 columns were subjected to anion exchange chromatography on DEAE sephacel. Vitellogenin eluted from the column as a single absorbance peak at 0.22 M NaCl (Fig. 3).

Anion exchange followed by gel filtration chromatography

Fractions eluting at 0.22 M NaCl after DEAE sephacel chromatography when rechromatographed on Ultrogel yielded a single absorbance peak (Fig. 4).

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Fig. 1—Gel filtration chromatography on Ultrogel AcA 34 of plasma proteins from murrel treated with E2. [Fishes were given a single i.p. injection of 0.25 mCi of [32P] NaH2PO4 24 hr before blood sampling. Proteins were eluted at 4°C, with 0.1 M Tris buffer containing 0.1 M KCl and 0.2% sodium azide, pH 7.5.]

Fig. 2—Anion exchange chromatography on DEAE sephacel of plasma from murrel treated with E2. [Fishes were given a single i.p. injection of 0.25 mCi of [32P] NaH2PO4 24 hr before blood sampling. Proteins were eluted with a linear NaCl gradient (0-0.4 M) in 50 mM Tris-Cl, pH 7.5.]
Precipitation followed by gel filtration chromatography

(i) An aliquot of radiolabelled E₂-treated plasma was treated with DMF to precipitate vitellogenin. When DMF precipitate was chromatographed on Ultrogel AcA 34, several of the fractions were milky and eluted within the void volume. In addition a phosvitin-like component eluted after the major vitellogenin peak (Fig.5A).

(ii) Mg²⁺: EDTA precipitated protein when subjected to gel filtration eluted as a single ³²P-containing peak followed by a few fractions with ³²P counts but little absorbance at 280 nm (Fig.5B).

Discussion

Vitellogenin has been isolated and partially characterised from several fish species.²⁻⁵ Interestingly fish vitellogenins display much greater variability in molecular weights, degree of phosphorylation and lipidation and subunit composition than vitellogenins of amphibian or avian species.⁶ While some of these
variations may reflect habitat- or species-related differences in the vitellogenin molecule, others may well be the result of different methodologies employed for isolation and purification of vitellogenin from blood. Fish vitellogenins are reported to be highly susceptible to proteolytic degradation. Failure to observe stringent precautions against proteolysis can also result in cleavage or degradation of the native vitellogenin molecule. In the present study, Aprotinin and PMSF were used to prevent proteolysis. The results show that murrel vitellogenin can be isolated from other plasma proteins by gel filtration chromatography on Ultrogel AcA 34. Vitellogenin isolated by this procedure was found to be intact when subjected to electrophoresis (Sehgal and Goswami, unpublished). Gel filtration chromatography on a variety of media has been used to isolate vitellogenin from other plasma proteins in several fishes such as Gadus morhua, Salmo gairdneri, Platichthys flesus, Anguilla japonica, Heteropneustes fossilis and S. trutta. Ion exchange chromatography on DEAE sephacel using Tris-Cl buffer (pH 7.5) with a linear salt gradient can also be employed as a single-step procedure for obtaining intact vitellogenin from murrel plasma. Several workers have employed ion exchange chromatography to isolate vitellogenin in different types of fish, such as Carassius auratus, H. fossilis, Fundulus heteroclitus, Oncorhynchus mykiss and S. trutta, Cyprinus carpio, Morone saxatilis, G. morhua, O. mykiss, Scophthalmus maximus and Anarchichas lupus.

Selective precipitation has occasionally been used as a preliminary step to enrich vitellogenin in the plasma. DMF precipitates protein by creating a hydrogen bond between the amide and protein moiety thereby increasing the apolarity of the protein and decreasing its solubility in aqueous medium. Alternatively, amides may serve to exchange hydrogen with water of the medium. DMF has been successfully employed to isolate vitellogenin from the blood of Xenopus, Chrysemys, C. auratus and Pleurodeles. DMF has the advantage of precipitating vitellogenin along with its associated Ca (ref. 49). However, DMF is toxic and calcium ions may activate proteolytic enzymes, de Vlaming et al. have reported that DMF-precipitated vitellogenin from goldfish was contaminated with other serum proteins. According to Ansari et al., precipitation by DMF yields denatured vitellogenin which is unsuitable for in vivo uptake studies or for in vitro cell culture experiments. Data presented in the present study indicates that precipitation with DMF yields vitellogenin, which has to be purified further, especially to remove the lipoproteins. Radioactivity is mainly associated with vitellogenin but a few fractions eluting immediately after vitellogenin have 3P counts but no absorbance at 280 nm and possibly represent phosvitin, which has been cleaved from the vitellogenin molecule.

According to Wallace, serum proteins form soluble complexes with vitellogenin in the presence of calcium - a mechanism that keeps vitellogenin in solution in the blood. Divalent cations can complex and precipitate vitellogenin only when other serum proteins are absent. Bifunctional chelating agents like EDTA or EGTA displace such proteins and precipitate the divalent salt of vitellogenin from a complex mixture of proteins. This characteristic of vitellogenin has been employed by Wiley et al. to precipitate undegraded vitellogenin from Xenopus plasma by Mg2+ in the presence of EDTA. Recently, vitellogenin from several fish species such as rainbow trout, sate, Atlantic cod, striped wolfish and Atlantic halibut has been precipitated with slight modifications of the molar ratio of EDTA: MgCI2. de Vlaming et al. did not succeed in precipitating vitellogenin from the goldfish serum by Mg2+: EDTA method and attributed this failure to the low phosphorus content (0.70%) of goldfish vitellogenin. This assumption appears untenable as the technique has been successfully employed on sea trout and rainbow trout whose phosphorus content of vitellogenins is similar to that of the goldfish vitellogenin. Furthermore, in our experiments, Mg2+ EDTA effectively precipitated murrel vitellogenin though its phosphorus content (0.4%) is lower than that of goldfish vitellogenin (Sehgal and Goswami, unpublished). However, in the murrel, this procedure results in the cleavage of phosvitin-like material from the vitellogenin molecule. In addition, reprecipitation of vitellogenin with distilled water, which is essential for removing non-specific trapped proteins including lipoproteins, occasionally fails either due to lipoproteins forming soluble complexes with vitellogenin or low initial concentration of vitellogenin in the sample. The importance of initial concentration of vitellogenin in the successful isolation of intact vitellogenin has been reported in rainbow trout.
Some investigators have used two separation procedures at tandem, which fractionate proteins on the basis of two different physico-chemical characteristics of the molecule to yield homogenous preparation of vitellogenin\textsuperscript{14,27-29,31}. Carnevali and Belvedere\textsuperscript{16} used a three-step method for isolating carp vitellogenin – selective precipitation followed by DEAE cellulose chromatography followed by fast protein liquid chromatography. Results of the present study on murrel show that second purification step is not necessary and that undegraded vitellogenin can be isolated by gel filtration as well as by anion exchange chromatography. As fish vitellogenins are highly susceptible to proteolysis, additional steps of purification may degrade the protein.

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