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One-step, non-denaturing purification method of carp (*Cyprinus carpio*) vitellogenin

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Abstract

A new single-step purification procedure was developed to purify carp (*Cyprinus carpio*) vitellogenin (VTG), from estradiol-treated carp plasma. This method was performed by high performance liquid weak anion-exchange chromatography, using a discontinuous elution gradient of NaCl (0–0.5 M, steps of 12.5 mM/4 min). SDS and native-PAGE analysis, of treated-fish plasma and purified solution, showed the appearance of a 370 kDa phospholipoprotein, composed of two 130 kDa monomers, with all VTG characteristics. The sequencing of a 130 kDa monomer confirmed that it was carp VTG. Consequently, this procedure is a rapid method, permitting high quantities of non-denatured carp VTG to be obtained.

Keywords: Purification; *Cyprinus carpio*; Vitellogenin

1. Introduction

Vitellogenin (VTG) is a dimeric phospholipoglycoprotein, specific to oviparous female vertebrates such as female fish. This protein is a precursor of the egg yolk proteins and, usually, estrogens induce its synthesis in the liver. This protein is then transported to the ovary through the blood stream and incorporated into the oocytes [1,2]. Abnormal production of VTG by male and immature fishes has been used as a bioindication of xenoestrogenic impact [3,4]. For example, total or partial feminisation, or demasculinisation have been described in different studies [5–12]. Male and immature fishes possess the VTG gene, but normally do not synthesise enough estrogen to induce its expression [4]. Xenoestrogens have the capacity to bind to the estrogen receptor in males [13–16], activating VTG synthesis [17–20].

Several methods based on the high surface charge of VTG have been developed in order to purify oviparous vertebrate VTG: ultracentrifugation [21]; dimethylformamide precipitation [22], selective precipitation using Mg^{2+} or Ca^{2+}–EDTA and/or ion-exchange chromatography [23]. None of these methods are applicable to fish: generally fish-VTG(s) are less phosphorylated than those of other vertebrates. Moreover, these methods do not allow for complete separation of VTG from other plasma components. Nowadays, most of the methods used for fish VTG purification are based on two-step chromatographic methods, in which proteins are separated according to charge and/or size of the molecule. Most of these procedures consist of an anion-exchange or a hydroxylapatite chromatography techniques followed by gel filtration or vice versa. The protein elution mode used in the anion-exchange chromatography step, is usually a continuous (or linear) gradient of NaCl from 0 to 0.5 M. These methods have allowed pure and non-degraded VTG from different fish species as *Cyprinus carpio* [24–26], *Morone saxatilis* [27], *Dicentrarchus labrax* [28], *Pleuronectes vetulus* [29], *Gobio gobio* [30], *Leuciscus cephalus* [30], and *Oncorhynchus mykiss* [30] to be obtained. However, these methods are time-consuming and the numerous purification steps induce a decrease in purification efficiency. Some lately authors used an one-step chromatographic methods, but it was not applicable to carp VTG [31] or VTG obtained was not completely pure [32].

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The aim of this study was the develop a new rapid method for extraction and purification of carp VTG, in a single-step, in order to produce high quantities of non-denatured and pure protein.

2. Materials and methods

2.1. Fish

Ten mirror carp *C. carpio*, with an average mass of 1 kg, were purchased from a commercial fish farm (Saulnoy GAEC pisciculture, France). They were reared in two 500 l-aquaria provided with running fresh tap-water at about 20° C. The fish were held on an 8 h light/14 h dark photoperiod, with oxygenation and fed on commercial food.

2.2. Vitellogenin induction

VTG synthesis was induced in five carp by intraperitoneal injections of 10 μg/g body weight of 17β-estradiol (Sigma), dissolved in 100 μl methanol:chloroform (v:v). Two injections were given at 10-day intervals. The other five carp received two intraperitoneal injections of only methanol:chloroform (untreated carp). A week after the last injection, blood samples (about 7 ml) were collected from the caudal sinus and immediately placed in heparinised tubes containing a 5% antiprotease mixture (General Use, Sigma) in order to prevent proteolytical breakdown of the VTG. Blood samples were centrifuged at 1500 × *g*, 4° C, for 15 min to separate blood cells and plasma was drawn off, and stored at −20 °C before purification. After blood collection, fish were killed and dissected in order to determine the sex by histological observations.

2.3. Vitellogenin purification

2.3.1. Purification of vitellogenin by weak anion-exchange chromatography

The VTG purification method was based on a high performance liquid chromatography (HPLC) technique, using a 600S Waters Controller Chromatography System (Waters, France). The system consists of several units, including a 626 Pump (Waters), a Photodiode Array Detector 996 (Waters), at two wavelengths: 254 and 280 nm. The system was piloted by a computer running with Millenium^{32} software. The system was connected to a Model 2128 Fraction Collector (Bio-Rad).

VTG was purified in a single-step by weak anion-exchange chromatography, using a 0.75 cm × 5 cm DEAE column (301, VHP 575P, VYDAC). The column was equilibrated with 25 mM Tris–HCl buffer, pH 8.5. One hundred microlitres of plasma samples were injected into the system with a calibrated loop of 200 μl. Proteins were eluted by a discontinuous gradient of NaCl with steps of 12.5 mM per 4 min from 0 to 0.5 M NaCl. The flow rate of elution was 1 ml/min and the running time was 56 min. Fractions were collected every 2 ml and stored at −20 °C before the protein analysis.

In order to minimise proteolysis, chromatography was carried out bellow 9 °C and buffers were supplemented with a 2.5% antiprotease mixture (General Use, Sigma (v:v)). Contamination of the column was prevented by using buffers supplemented with 0.05 % sodium azide (Sigma (w:v)), filtered through a 0.22 μm filter (Millipore©; France) and degassed.

Purification of proteins was controlled by measuring absorbence at 254 and 280 nm (AU), temperature (°C), pressure (psi) and flow rate (ml/min) continuously.

2.3.2. Ultrafiltration of fractions containing vitellogenin

After the chromatographic step, purified fractions containing vitellogenin were pooled and concentrated to a final volume of 1 ml, by ultrafiltration at 3000 × *g* and 4 °C, for 20–30 min, using Vivaspin 6 Falcons with a 50 kDa molecular mass cut-off membrane (Vivascience, France).

2.4. Electrophoresis analysis

2.4.1. SDS-PAGE and native-PAGE electrophoresis

Discontinuous polyacrylamide gel electrophoresis was carried out in slabs of 0.75 mm thickness, using a Mini Protean II (Bio-Rad). The resolving gel contained 7.5% acrylamide for SDS-PAGE or 6% for native-PAGE. The stacking gel contained 4% of the same solution, according to Laemmli (1970) [33].

For SDS-PAGE analysis, samples were mixed with a Laemmli buffer (Bio-Rad, 0.5 M Tris–HCl, pH 6.8, 10% glycerol, 0.02% bromophenol blue) containing 0.1% SDS (sodium dodecyl-sulphate (w:v)) and supplemented with 5% β-mercaptoethanol (v:v). Before loading, SDS samples were heated at 100 °C for 1.5 min. For native-PAGE, samples and proteins markers were diluted in 0.5 M Tris–HCl, pH 6.8, 40% glycerol (v:v) and 0.02% bromophenol blue (w:v) (without SDS and β-mercaptoethanol), and without heating.

Protein markers (Sigma) loaded in electrophoretic gels were: dimeric urease 545 kDa, monomeric urease 272 kDa, myosine 205 kDa, β-galactosidase 116 kDa, phosphorylase 97.5 kDa, albumin 66 kDa, ovalbumin 45 kDa and carbonic amylase 29 kDa. The logarithm of molecular mass markers was plotted against electrophoretic mobility and linear regression was used to calculate the relative molecular mass of the samples.

Migration was performed under 200 V, for 45 min. Gels were specifically stained and finally air-dried (Bio-Rad Gel Air Dryer).

2.4.2. Gels stained

2.4.2.1. Coomassie blue. Coomassie blue reacts with protein amine groups [33]. Gels were fixed in an acetic

acid:ethanol:water mixture (1:4:4 (v:v:v)), for 20 min with shaking. Then, gels were transferred to a 0.15% Coomassie blue solution (v:v) overnight. After two water washings, gels were destained in an acetic acid:ethanol:water mixture (1:4:4 (v:v:v)) and dried.

2.4.2.2. Sudan black. Sudan black is a lipoprotein specific colorant [34]. Gels were stained in a solution, composed of sudan black (500 mg), acetone (20% (v:v)), acetic acid (15% (v:v)) in ultrapure water, with shaking. Gels were then destained in acetic acid:acetone:ultrapure water (15:20:70 (v:v:v)) mixture and air-dried.

2.4.2.3. Methyl green. Methyl green specifically stains phosphoproteins [35]. Briefly, gels were placed into a 10% SSA (sulfosalycilic acid (w:v)) fixative solution for 12 h. Gels were then transferred to a 10% SSA solution containing 0.5 M $CaCl_2$ for 1 h. After a rapid rinse in demineralised water, they were placed into a 0.5N NaOH solution, at 60 °C for 30 min. After two washes for 10 min in a 1% aqueous ammonium molybdate solution (w:v), gels were transferred into a 1% aqueous ammonium molybdate solution containing 1 N HNO_3 for 30 min. Finally, gels were stained with 0.5% methyl green (w:v) diluted in a 7% acetic acid solution (v:v). After 30 min, gels were destained with a 10% SSA solution at 60 °C and then stored in a 7% acetic acid solution until dried.

2.5. Amino acid sequencing

The amino acid sequencing of the carp VTG monomer was evaluated on a 476A Applied Biosystems (Applera Corp, Foster city, CA, USA). Forty micrograms of plasma proteins were separated by SDS-PAGE (7.5%). After electrophoresis, proteins were transferred on a polyvinylidene difluoride (PVDF) membrane, for 1 h at 100 V at 4 °C, with 25 mM tris, 192 mM glycine, 20% methanol (v:v), pH 8.3, as transfer buffer, using a mini transblot cell apparatus (Bio-Rad) [36]. The PVDF membrane was stained with a 0.15% Coomassie blue solution (v:v). After 5 min, it was destained in an acetic acid:methanol:water mixture (1:4:4 (v:v:v)) and air-dried. 130 kDa bands were then excised and transferred to 476A Applied Biosystems. The N-terminal amino acid sequence was determined with an Edman N-terminal sequencer [37].

3. Results

3.1. Induction of vitellogenin synthesis

Treatment of males with estradiol (E2) induces an increase in plasma protein concentration from 23.06 ± 4.26 mg/ml in untreated male to 60.25 ± 2.59 mg/ml in E2-treated male. In females, an increase was also observed, from 28.60 ± 2.44 to 52.37 ± 9.75 mg/ml (Fig. 1).

Examination of plasma from both groups (E2-treated and untreated) by native-PAGE (6%) showed an overexpressed protein band of about 370 kDa in E2-treated group. SDS-PAGE results showed that this protein was composed of two monomers of about 130 kDa each (Fig. 2A). With sudan black and methyl green stains, gels demonstrated that the two forms of the protein (monomeric and dimeric) were present in plasma and both consisted of a phospholipoprotein (Fig. 2B). These results indicate that this protein possesses electrophoretic characteristics consistent with VTG [24–30].

3.2. N-terminal amino acid sequencing

To confirm these results, the N-terminal amino acid sequence of the protein band was studied. Two sequences were obtained: X-Gln-Ile-Asn-Leu-Val-Pro-Glu-Phe-Thr-Pro-Gly (Seq 1); X-Asp-Arg-Ala-Leu-His-Pro-Glu-Val-Arg-Met-Val (Seq 2). According to BLAST protein database search program [38], these two sequences correspond to two parts of total the carp *C. carpio* VTG sequence (Fig. 3).

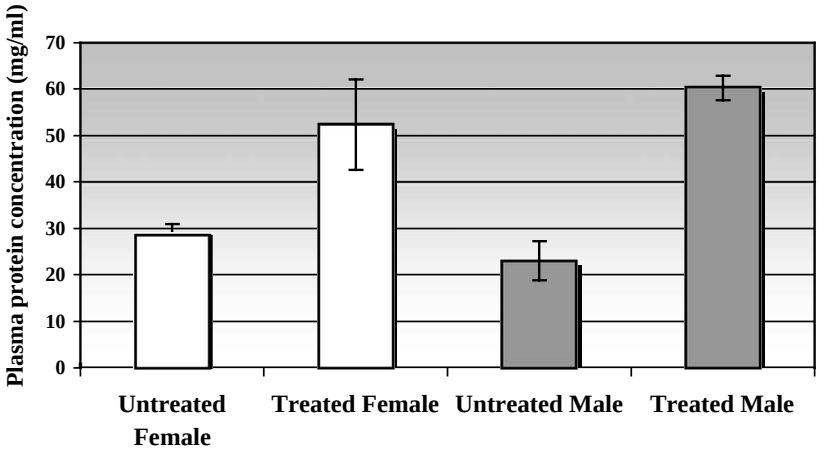

Fig. 1. Effects of 17β-estradiol treatment on total plasma protein concentration in male and female carp *Cyprinus carpio*.

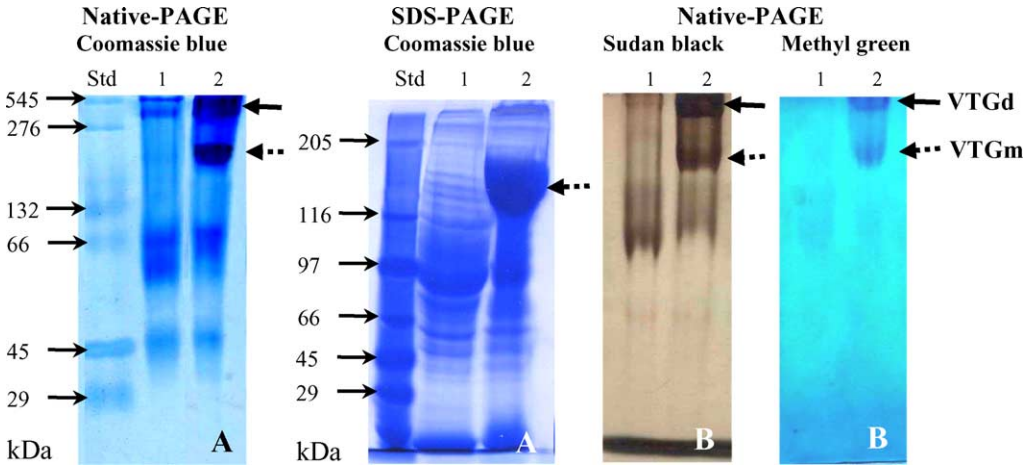

Fig. 2. (A) Analysis of plasma protein composition, by native-PAGE 6%, and SDS-PAGE 7.5%, stained with Coomassie blue, of untreated (1) and E2-treated (2) carp. (B) Analysis of plasma phospholipoprotein composition by native-PAGE (6%) stained with sudan black and methyl green, of untreated (1) and E2-treated (2) carp. Solid arrows: dimeric VTG; dotted arrows: monomeric VTG.

3.3. Vitellogenin purification

Carp VTG from E2-treated fish plasma, was purified by a single-step weak anion-exchange chromatography. The specificity of this new method is due to the elution mode which is discontinuous with steps of 12.5 mM per 4 min from 0 to 0.5 M of NaCl.

Elution profiles on weak anion-exchange chromatography from untreated and E2-treated fish plasma (Fig. 4A and B) showed a dominant peak, eluted at 1 min. The red coloration suggests this fraction contains haemoglobin among other molecules such as albumin [24]. Several small peaks were eluted between the 6th and 16th min, corresponding to other proteins contained in a normal plasma. In contrast, the three final peaks, which were eluted at 19–20, 23–24 and 27–28 min, respectively, were present only in E2-treated male or female fish.

3.4. Electrophoresis

Protein fractions, eluted between 19 and 20, 23 and 24, and 27 and 28 min were analysed by SDS-PAGE and Native-PAGE electrophoresis in order to estimate the protein composition and the presence of VTG in each fraction (Fig. 5).

SDS-PAGE electrophoresis revealed the presence of two protein bands in E2-treated fish which were absent in the untreated fish fractions (Fig. 5A). The 130 kDa major protein band was suspected to be monomeric VTG. The 94 kDa protein could correspond to a breakdown product of VTG, which is always very unstable. In spite of protease inhibitors and low temperature used during all chromatography and analysis steps, the presence of a second band may be due to SDS action [24,27,29].

In fact, native-PAGE followed by Coomassie blue stain, revealed a pure and non-denatured protein of about 370 kDa, from E2-treated fish fractions (Fig. 5B). Other results obtained with sudan black and methyl green stains, confirmed that this 370 kDa protein was a phospholipoprotein (Fig. 5B). Consequently, these results suggest that this purified protein from plasma of E2-treated fish was *C. carpio* VTG. Moreover, these results confirmed that the VTG denaturation observed by SDS-PAGE, occurred during SDS-PAGE electrophoresis process and not during VTG purification.

4. Discussion

The aim of this study was to develop a rapid and non-denaturing purification procedure for *C. carpio* VTG, to permit pure protein to be obtained with high efficiency.

The procedure need stimulation of VTG production by a 17β-estradiol (E2) treatment, according to Tyler and Sumpter [24]. This hormone is known to be the most potent estrogen for the induction of VTG synthesis in fish [17–20]. Results obtained after E2 administration were similar in males and females, increasing the total amount of proteins. VTG genes seem to be regulated in the same way by estrogens in males and females [39].

The first results showed that E2-treatment induced the synthesis of a protein, with a molecular mass of about 370 kDa (by native-PAGE), which appeared to be composed by two 130 kDa monomers (SDS-PAGE). Moreover, this protein presented all characteristics of a phospholipoprotein, by sudan black and methyl green. These results were in accordance to those found in carp [24–26] and others fish [24,27–30]. According to the amino acid sequence, the protein purified by our method, corresponded to a monomer of *C. carpio* VTG.

Various chromatographic methods have been used for isolation and purification of fish VTG, and involve procedures ensuring purity of proteins and prevention of breakdown. The first procedures developed, used a two-step chromatographic procedure, with anion-exchange or hydroxylapatite chromatography followed by gel filtration

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   1 mravvlaltv alvasq[qinl vpeftpg]kty vynyealllg glpheglara gikvnskvhl
  61 savtentflm klmdpliyey agiwpkdpfv patkltsala aqlqipikfe yangvvgkvf
 121 apagvsptvl nlhrgilnil qlnlkktqni yelqeagaqg vcrthyvise dpkanhitvt
 181 kskdlshcqe rivkdvrlay tercaecter ikslietaty nyimkpasag vliteatvee
 241 vhqfspfnei hgaaqmeakq tlafveiekt lvvpikadyl argslqyefa teilqtpihl
 301 mkisdapaqi ievlkhlvan nvamvhedap lkfvqliqll rvstleniea iwaqfkdkpa
 361 yrrwlldalp svgtpviikf ikekflagel tlpefiqalv valqmvtadl dtiqltasla
 421 mhekiakmpa lrevvmlgyg smiarhcvav ptcsaellrp iheiaaeats kndireitla
 481 lkvlgnaghp aslkpimkll pglrtaassl plrvqvdail alrniakkep klvqpvalql
 541 [vldralhpev rmv]acivlfe skpsvalvss lagalktetn mhvvsfaysh iksltritap
 601 dmaavagaan vaiklmsrkl drlsfrfsra lqldyyhtpl migaagsaym indaatilpr
 661 avvakarayl agaaadvlei gvrtegiqea llkspaades vdritkikrt lralanwkdl
 721 ptdqplasvy ikflgqevaf vkidktiiee aipivtgpkp rellkralka lqegiawqya
 781 kpllaaearr ilptavgvpm elslytaava aasvnvkati tpplpeeiet mtleqlkktd
 841 vqlqaearps ialqtfavmg vntaliqaav margkirtia pvkvaaradi lkgnykveal
 901 pvevpehiat lsfetlavvr nieeptaert vplvpelavq nsqthsdyls senqdevpvr
 961 apapfdktlc lavpyieikg cvelhshnaa firndplyyi igqhsaratv araegpaver
1021 lelevqvgpr aaerllkqis lideetpegk afllklkeil etedknrpvs sesrsssssr
1081 snrsssssss ssssssssss msssrvskta timepfrkfh kdrylaphga skkvssgssa
1141 ssferiqkqa kflgnavppv faviaravrv dhkllgyqla ayfdkptarv qivvssiaen
1201 dnlkicvdga llskhkvtak lawgpecqqy avtakaeag vvgtfpaarle lewerlpitv
1261 ttyakkmskh iymaafqagf rlervmnsek eieltlalpn qrslnvifri pemtlsrmgi
1321 hlpyaipinp dgslsiqide dilswiqrhi kee
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Fig. 3. Amino acid sequence of carp *Cyprinus carpio* vitellogenin (AF414432_1) obtained with BLAST, protein database search program. Two bold amino acid sequences were obtained with Edman N-terminal sequencer.

or vice versa [24,27–30]. But they presented a disadvantage of being time-consuming. Recently, two authors developed an one-step chromatographic method to purify fish VTG. Nevertheless, Nishi et al. obtained pure VTG from Japanese medaka (*Oryzias latipes*) using only one-step anion-exchange chromatography [31], but Tyler and Sumpter showed that this technique is not applicable to purify VTG carp [24]. In the case of Shi et al. [32], this author obtained carp VTG by a membrane chromatographic method but it was not absolutely pure.

In our case, the purification method selected was an weak anion-exchange chromatography (HPLC) using a discontinuous step gradient. In fact in linear gradient, the differences in retention of sample compounds often lead to irregular spacing of peaks in chromatogram. Spacing of peaks can be improved and time analysis decreased by optimising subsequently several parts of chromatogram. This optimisation results in a segmented gradients with a few subsequent linear gradient steps of different duration and steepness [40]. By using our purification method, involving a discontinuous step gradient, three protein peaks were observed only in treated fish plasma samples. According to native-PAGE and SDS-PAGE, these three peaks corresponded to the same protein (VTG). The difference in time-elution of the three peaks could be due to a difference in protein charges, resulting in little post-translational processing (phosphorylation or glycosylation) of carp VTG monomers. These differences were not observable in electrophoresis, in contrast to the results found by Roubal et al. with English sole (*P. vetulus*) [29], and Tao et al. with striped bass (*M. saxatilis*) [27], which

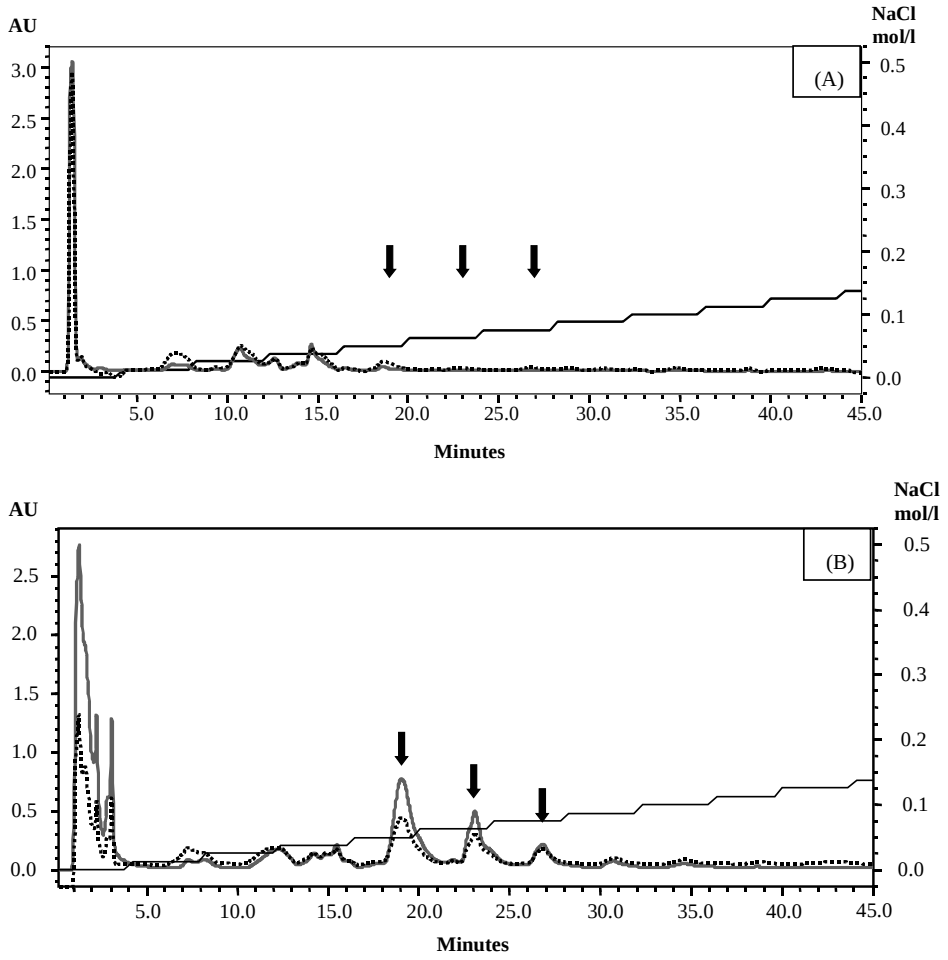

Fig. 4. Elution profiles of plasma proteins from (A) untreated carp and (B) treated carp, at 254 nm (dotted line) and 280 nm (solid line), which were purified by weak anion-exchange chromatography, with a discontinuous gradient of NaCl from 0 to 0.5 M with steps of 12.5 mM per 4 min (thin line). The arrows represent the VTG peaks at 19–20, 23–24 and 27–28 min.

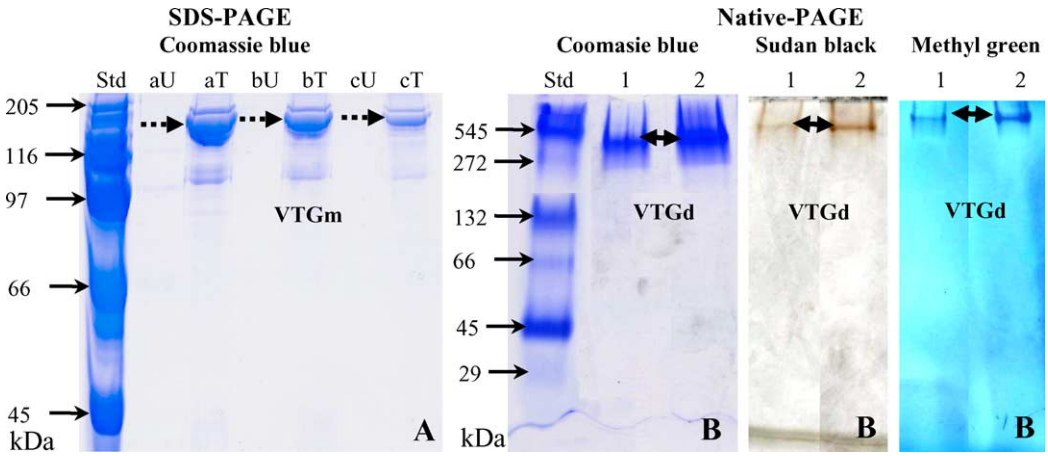

Fig. 5. (A) Protein composition analysis by SDS-PAGE 7.5% of purified fractions 19–20 (a), 23–24 (b) and 27–28 (c) from untreated (U) and E2-treated (T) carp. (B) Protein composition analysis by native-PAGE 6% of VTG purified solution before ultrafiltration (1) or after ultrafiltration (2) stained with Coomassie blue, sudan black and methyl green. Solid arrows: dimeric VTG; dotted arrows: monomeric VTG.

demonstrated two different dimeric bands. In our case, only one protein band was observed after chromatography purification, from the native-PAGE.

According to Tyler et al. [41], the maximal plasma concentration of VTG, produced by vitellogenic female carp, was about 1 mg/ml. Consequently, the quantity obtained after E2-treatement was almost 17-fold higher than the level found in vitellogenic females. This over-production was also observed in *D. labrax* [28], and *P. vetulus* [42]. This phenomenon could be explained by the fact that the high concentration of 17β-estradiol used, induced more VTG gene transcription than physiological levels.

As a conclusion, the discontinuous elution mode used in the anion-exchange chromatography method developed in this study, permitted carp VTG purification in a single-step procedure. The integrity of purified VTG was maintained and the high quantities of VTG obtained were sufficient to produce monoclonal antibodies [43] which could be used in immunological tests.

Acknowledgements

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