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Membrane chromatographic method for the rapid purification of vitellogenin from fish plasma

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Abstract

A membrane chromatographic method was developed for the rapid purification of vitellogenin (Vtg) from the plasma of 17β-estradiol induced loach (*Misgurnus angaillicaud atus*) and carp (*Cyprinus carpio*). The time required for the proposed procedure is less then 10 min at a flow-rate of 5 ml/min of the mobile phase, and 0.5 ml of fish plasma could be separated in one cycle. Multistep gradient elution was more suitable for the separation than linear gradient elution. Under optimized conditions, a single Vtg peak can be obtained and its identity was confirmed by SDS–PAGE and gel-permeation chromatography assessment. This method is rapid and easy to operate compared to conventional HPLC and FPLC columns for Vtg separation.

Keywords: Vitellogenin

1. Introduction

Recently, concerns have been raised about natural or synthetic chemicals that can interfere with vertebrate endocrine systems [1–3]. Most of those studies focused on the chemicals called xenoestrogens that can trigger agonist or antagonistic responses by binding to estrogen receptors. The measurement of vitellogenin (Vtg) in oviparous vertebrates is generally agreed to be a good indicator for agonist or antagonistic effects and it is proposed as one of several endpoints in the fish assay for endocrine disruptor screening [4–7].

Vtg is a serum lipophosphoglycoprotein that serves as the major precursor to the egg-yolk proteins of oviparous vertebrates [8]. The production of Vtg takes place in the liver and is under the receptor-mediated regulation of 17β-estradiol; both males and females have such a mechanism [9]. Normally, Vtg can only be detected in sexually active females; male or juvenile oviparous vertebrates could not synthesize Vtg since they have lower or negligible levels of estrogen to reach the threshold required to induce Vtg production. But in vivo and in vitro studies have proved that Vtg could be induced in male or juvenile fish on exposure to estrogen or estrogen mimic

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chemicals [7,10,11]. Recent researches have found blood Vtg in male fish collected form natural waters, for example, the river Mississippi [12], the Hudson River at Lake Luzerne, NY, and the Mohawk River at Frankfort, NY [13], the Escambia River in Florida [14], the River Lea in North London and the River Aire in Yorkshire [15], and the Tyne and Mersey estuaries in the UK [16].

The main techniques for Vtg determination currently are radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA) and Western blots [17]. All of those techniques need a significant amount of purified Vtg for antibody production and for standards in sample determination. Meanwhile, due to the complexity of the Vtg molecule and variation for different species, the purified Vtg for each species is a top priority.

Various approaches such as anion-exchange chromatography based on DEAE–cellulose or DEAE–agarose [18,19], ultracentrifugal separation [20], and selective precipitation [18] has been developed for the purification of Vtg. Although generally used during recent decades, those methods were time-consuming and tedious. The rapid method for the purification of Vtg was mainly based on high-performance anion-exchange chromatography [5,21]. In some approaches, gel-permeation chromatography was applied to further separate the degradation products from the native form of Vtg [22].

Membrane chromatography is a newly developed separation method. Most recently, Ghosh [23] has reviewed its characteristics, separation chemistries and applications, and suggests that it is suitable for the purification of proteins that are not the major components of their respective naturally occurring matrices. It has been used for the purification of monoclonal antibody [24], serum antibody [25], serum albumin [26], enzymes [27], etc. In this study, we have developed a rapid and simple method for the purification of Vtg from fish plasma using membrane chromatography based on commercially available strong ion-exchange membrane adsorbers (MAs) integrated in cartridges. The separation parameters of this method were compared with that of anion-exchange chromatographic column methods, and the feasibility was tested by two species of fish: loach (*Misgurnus angaillicaud atus*) and carp (*Cyprinus carpio*).

2. Experimental

2.1. Chemicals and instruments

17β-Estradiol and heparin was obtained from Sigma (St. Louis, MO, USA), aprotinin was from Boehringer (Mannheim, Germany), a high-molecular-mass calibration kit for SDS electrophoresis was from Pharmacia (Amersham Pharmacia Biotech, UK), other chemicals used for SDS electrophoresis were from Sigma, quinaldine sulfate was from the Beijing Chemical Company. All the chemicals were of analytical-reagent grade. Buffers and sample solutions were prepared with deionized water and filtered with a 0.22-μm filter.

The ready-to-use unit Sartobind™ MA Q15 (Sartorius, Goettingen, Germany) is a strongly basic anion exchanger with a quaternary ammonium group. The membrane material of this unit is supported crosslinked regenerated cellulose with an effective adsorption area of 15 cm^2. Weak anion-exchange adsorber (DEAE–Sepharose CL 6B) was bought from Pharmacia, and packed into a 24.5×2.5 cm column in the laboratory. The pump used in this work was Agilent 1100LC G1312A Binary pump (Agilent, Germany), which was controlled by an Agilent 1100LC workstation. A UV monitor 8823A with a 280-nm filter (Binda, Beijing) and a WDL-95 chromatographic workstation (a kindly gift from Professor Zou Hanfa) were used for online monitoring and data recording. A Shimadzu UV-120-02 spectrophotometer (Shimadzu, Japan) was used for recovery studies and a gel-permeation column Zorbax GF-250 (Agilent) was used for protein analysis.

2.2. Fish

Adult male ($n=60$) and female ($n=20$) loaches, with an average mass of 20 g, and five 1-year old carp, with an average mass of 200 g, were bought from the market. They were maintained in the laboratory for 1 week prior to use.

2.3. Vtg induction

The fish were anesthetized in a bath of quinaldine sulfate (40 mg/l) and injected i.p. (5 μg/g) on day 0

and 7 with 17β-estradiol dissolved in a solution of 0.9% NaCl and ethanol (1:1, v/v). Noninduced fish were injected i.p. with the solution of 0.9% NaCl and ethanol only. After 14 days exposure, the fish was anesthetized with quinaldine sulfate (40 mg/l), blood was collected from the caudal vein with heparinized syringes, and transferred to 1.5-ml centrifuge tubes containing 1 μl aprotinin (2.5 TIU (trypsin inhibiting units)) and 6 μl heparin (30 USP units). Then, the blood was centrifuged at 3000 *g*, 4 °C for 20 min plasma was collected and stored at −80 °C until purification.

2.4. Purification of Vtg by DEAE–Sepharose column

The purification of Vtg by anion-exchange chromatography on a DEAE–Sepharose column was carried out as previously described [19], except for some minor modification according to the manufacturer's information. Briefly, 0.5 ml of plasma was mixed with 1.5 ml of buffer A (25 m*M* Tris–HCl buffer containing 0.07 *M* NaCl and 50 TIU/l aprotinin, pH 8.3) and applied to the DEAE–Sepharose column equilibrated with two bed volumes of buffer A in advance. Separation was performed at 4 °C with a flow-rate of 0.8 ml/min. After washing the unbound proteins with 1.5 bed volumes of buffer A, the gradient elution was started with buffer B (25 m*M* Tris–HCl buffer containing 1 *M* NaCl and 50 TIU/l aprotinin, pH 8.3). Aliquots of the peak fraction of Vtg (identified based on the previous isolations [19,28]) was collected and stored at −80 °C.

2.5. Purification of Vtg by membrane chromatography

The fish plasma was diluted with several volumes of buffer C (25 m*M* Tris–HCl buffer containing 0.07 *M* NaCl, pH 8.3) and filtered with a 0.22-μm filter. Then, it was loaded on the anion-exchange membrane (Sartobind MA15 Q) that was equilibrated with 10 ml buffer C in advance. After washing the membrane with 5 ml of buffer C, the proteins were eluted with a multiple-step gradient to 1 *M* NaCl using buffer D (25 m*M* Tris–HCl buffer containing 1 *M* NaCl, pH 8.3). Aliquots of the peak fraction of Vtg was collected and stored at −80 °C.

2.6. Regeneration

The DEAE–Sepharose column was regenerated with two bed volumes of buffer B at a flow-rate of 0.8 ml/min and the anion-exchange membrane was regenerated with 10 ml of 1 *M* NaOH by reverse washing at a flow-rate of 5 ml/min.

2.7. Protein assays

Protein concentration was determined following the method of Bradford at 595 nm [29]. Plasma samples from membrane chromatographic fractions were analysed by gel permeation chromatography and SDS–PAGE (5% gel). For gel-permeation chromatography analysis, the column was calibrated for molecular mass determination with thyroglobulin (relative molecular mass (M_r) 640 000), IgG (M_r 158 000), and BSA (M_r 66 000). For SDS–PAGE analysis, samples were diluted in sample buffer (20 m*M* phosphate buffer, pH 7.2, containing 2% SDS, 2% mercaptoethanol, 20% glycerol, and 0.04% Bromophenol Blue) at a ratio of 1:1 and heated at 100 °C for 3 min. After electrophoresis, separated proteins were stained with Coomassie Brilliant Blue R-250, and the bands were compared to high-molecular-mass electrophoresis standards (53 000–220 000) and corresponding proteins absent or present in plasma from normal male and female fish.

3. Results and discussion

In membrane chromatography, the transport of solutes to their binding sites take place predominantly by convection, so the mass transfer efficiency on membrane adsorbers is higher than that of the traditional LC columns. Therefore, it is possible to reduce separation time by increasing flow-rate. In order to investigate the effect of flow-rate on the sample separation, we separated the loach plasma at different loading and elution flow-rates in the range of 1–5 ml/min. The results indicated that basically identical elution chromatograms were obtained at different loading flow-rates, which means the protein binding efficiency of MA was not influenced by the feed flow-rate in this range. The time required for the sample separation was decreased with the increasing

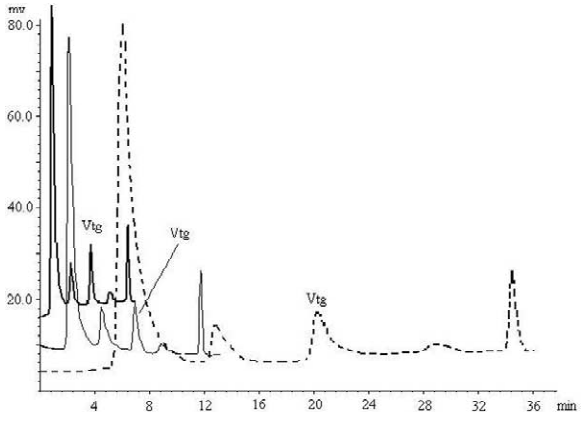

Fig. 1. Effect of elution flow-rate on the sample separation; the loading flow-rate is 5 ml/min. The elution flow-rate of chromatograms with the broken, thin and thick lines are 1, 3 and 5 ml/min, respectively.

of the elution flow-rate, but as shown in Fig. 1, no apparent change of peak shape and the resolution was observed. The reason for this is that the high mass transfer efficiency of MA, which makes the proteins binding on or dissociating from the MA very fast and independent of the flow-rate over a wide range [23]. For routine separations in this experiment, a flow-rate of 5 ml/min was adapted and the total process time was less than 10 min.

To select the best buffer solution, three solutions of 25 m*M* Tris–HCl buffer containing 0.07 *M* NaCl at pH 7.8, 8.3 and 8.9 were examined. Results indicated that no significant changes in the peak shape, peak area and resolution were observed. In order to compare with the anion-exchange chromatography method [19], we selected pH 8.3 in subsequent studies.

Generally, the protein binding capacity of membrane chromatography is lower than that of column chromatography (see Table 1). In order to get a larger capacity, two Sartobind MA Q15 units were connected in series. Experiments showed that about two-fold of capacity was achieved while little effect on the resolution was observed. The number of membrane modules which can be connected in series was limited, because the nonideal flow distribution in the inlets and outlets of each module would increase the overall dispersion of the separation system. For example, when three membrane modules were connected in series, the chromatogram peak broadened greatly and the resolution of each peak was decreased as shown in Fig. 2. Although we found that the membrane chromatography with three modules in series could also give good protein recovery and peak resolution by using multistep gradient elution, and increasing the elution volume of each step, we did not connect three membrane modules in the experiment because the process time was increasing also.

The amount of sample plasma that could be applied onto the membrane absorbers during loading process was studied and the result indicated that the protein breakthrough was negligible when less than 0.5 ml plasma was applied on two membrane modules that were connected in series. Therefore, 0.5 ml was selected as the sample volume in this experiment.

The minimum dilution ratio of the sample was selected as 4. A higher dilution ratio did not affect

Table 1
Technical specifications

Parameter	Weak anion-exchange chromatography column	Sartobind MA Q15 reusable units
Material	DEAE–Sepharose CL 6B adsorber	Supported crosslinked regenerated cellulose
Bed volume/effective adsorption area	100 ml[a]	15 cm^2/units
Protein binding capacity[b]	Thyroglobulin: 2 mg/ml HAS: 170 mg/ml α-Lactalbumin: 150 mg/ml	BSA: 0.8 mg/cm^2 HAS: 0.9 mg/cm^2 [26]
Operating temperature	4 °C	Rome temperature

[a] For the 24.5×2.5 cm column packed in our laboratory.
[b] Data from manufacturer's information unless specially noted.

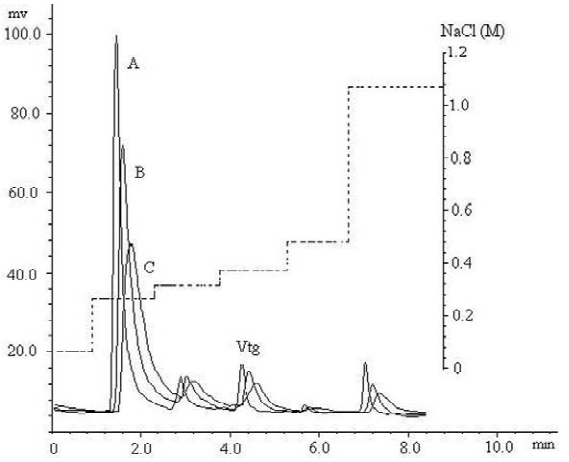

Fig. 2. Separation of loach plasma with different number of membrane modules connected in series. (A) One membrane module; (B) two membrane modules; (C) three membrane modules.

the protein binding but increased the process time of purification; a lower dilution ratio was not studied because we believe that the high salt concentration of the sample solution will decrease the binding capacity.

Although linear gradient elution usually resulted in a sharpening effect on the signal for column chromatography or for separation of several standard proteins by membrane chromatography [34], poor peak shape and low resolutions were generated in this experiment. Good resolution and peak shape were obtained only by multiple-step gradient elution (see Fig. 3b), similar to what was reported by other researchers [26,32].

Fig. 3a shows the purification of Vtg by anion-exchange chromatography on a DEAE–Sepharose column, Vtg was the second major elution peak fractions after a linear gradient of NaCl was applied, as observed previously for other teleosts [19,30]. Fig. 3b is the chromatogram using membrane chromatography, the fraction containing Vtg was assumed to be the third major peak, because there is no peak for untreated male fish. The purified Vtg obtained by means of these two methods mentioned above was confirmed by SDS–PAGE (see Fig. 4), the major band in lanes 1 and 4 was also present in the lane of female loach but absent in the lane of control male fish. The M_r of loach Vtg by SDS–PAGE is about

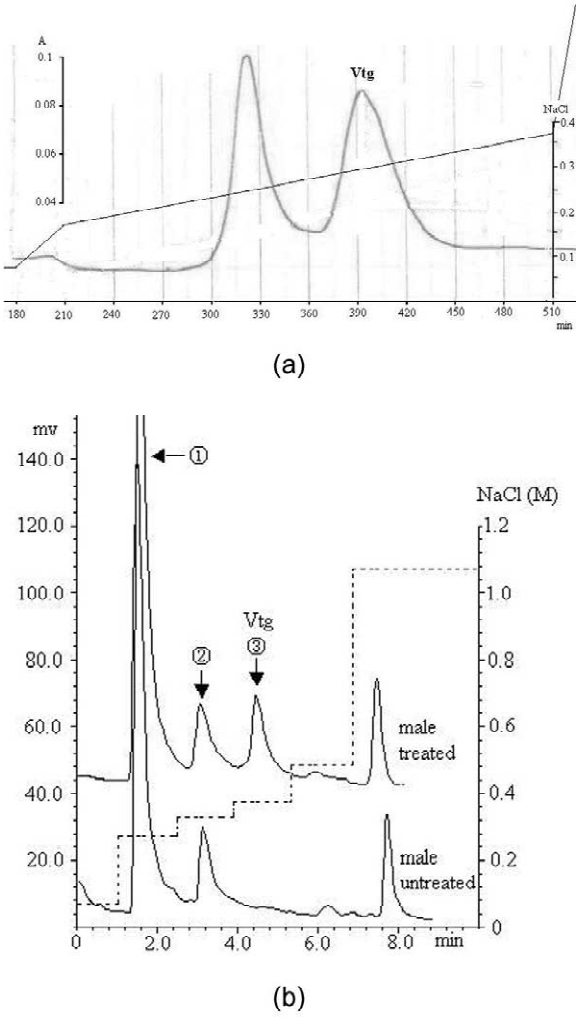

Fig. 3. Separation of plasma proteins from male loach induced with 17β-estradiol. (a) Separation with DEAE–Sepharose CL 6B anion-exchange column chromatography, The linear gradient elution by NaCl in Tris–HCl buffer was first from 0.07 to 0.17 *M*, than from 0.17 to 0.37 M; (b) separation with Sartobind MA15 Q strong anion-exchange membrane chromatography, the multistep gradient elution by NaCl in Tris–HCl buffer were 0.07, 0.27, 0.33, 0.37, 0.47 and 1.07 *M*, respectively.

160 000, which is similar to that of other teleosts, for example, 150 000 for *Cyprinus carpio* [31], 156 000 for *Pimephales promelas* [19], 170 000 and 160 000 for *Fundulus heteroclitus* and *Pagrus major*, respectively [21]. According to Fig. 4, we can also conclude that the purity of the Vtg fractions collected from membrane chromatography is identical to that

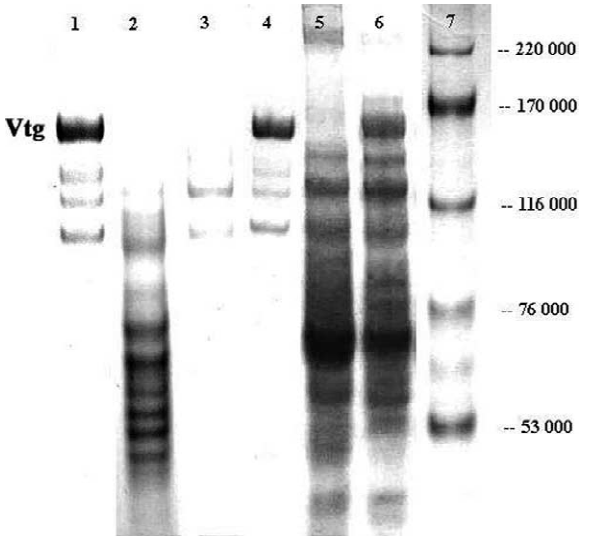

Fig. 4. SDS–PAGE analysis of plasma fractions. Lanes: 1=Vtg purified by DEAE–Sepharose CL 6B anion-exchange column chromatography (Fig. 3a); 2=proteins from peak 1 in Fig. 3 (b); 3=proteins from peak 2 in Fig. 3 (b); 4=proteins from peak 3 in Fig. 3 (b); 5=plasma from control male loach; 6=plasma from sexual mature female loach; 7=molecular mass markers.

of column chromatography. The identity of Vtg was further confirmed by gel-permeation chromatography. The relative molecular mass of the purified Vtg was calculated to be 418 000 with thyroglobulin, IgG, and BSA as molecular mass standard. The results are in accordance with those obtained by gel permeation chromatography from other fish species, for example, 442 000, 435 000 and 424 000 for *Oncorhynchus mykiss*, *Gobio gobio*, and *Leuciscus cephalus*, respectively [22].

Because standard loach Vtg is not available, we use the purified Vtg to evaluate the recovery of membrane chromatography. In detail, the Vtg fraction separated by membrane chromatography (about 5 ml) was collected and dialyzed in the bath of deionized water for 12 h. Then, after measuring the Vtg solutions concentration by the method of Warburg and Christon [33], 3 and 1 ml of this solution were transferred to tubes, respectively, and after adding 0.5 ml control male loach plasma, the matrix were diluted to 10 ml with buffer C. After loading and separation by membrane chromatography, the Vtg fraction was collected and the volume and concentration were measured. The result of recovery is shown in Table 2. The recovery is lower than that of other proteins in a previous report [32]. We think the reason for this is that some of the Vtg may have aggregated with serum albumin [34] and some Vtg may have degraded during dialyzing.

Table 2
Vtg recovery by membrane chromatography

Test	Protein added (mg)	Protein collected (mg)	Recovery (%)	RSD (%)
1	1.425	1.067	74.9	6.6
2	0.475	0.335	70.5	9.1

$n=3$.

The feasibility of membrane chromatography for the purification of Vtg from other species was tested in carp, a widely accepted fish for laboratory and field studies. The separation conditions were similar to that of loach, the chromatogram is shown in Fig. 5a and each major peak was identified by SDS–PAGE (see Fig. 5b). The electrophoresis pattern of purified carp Vtg (lane 1) was in good agreement with what has been reported previously [17].

4. Conclusion

In this study, the Vtg from 17β-estradiol treated male loach and sex immature carp were purified by commercially available strong ion-exchange MAs integrated in cartridges and the identity was confirmed by SDS–PAGE and gel-permeation chromatography. The result shows that it is a cheap and efficient substitute for conventional HPLC and FPLC columns for Vtg separation. Because of the more efficient mass transfer characteristics of MAs over the particle adsorbers that were used in conventional LC columns, membrane chromatography can be operated under a higher flow-rate without impeding the binding capacity and separation efficiency [17,26]. At a flow-rate of 5 ml/min, the whole process time was <10 min. According to the manufacturer's information, the dynamic binding capacity and recovery are not affected by flow-rate, so, if fast flow-rate pump was used, a shorter process time can be achieved.

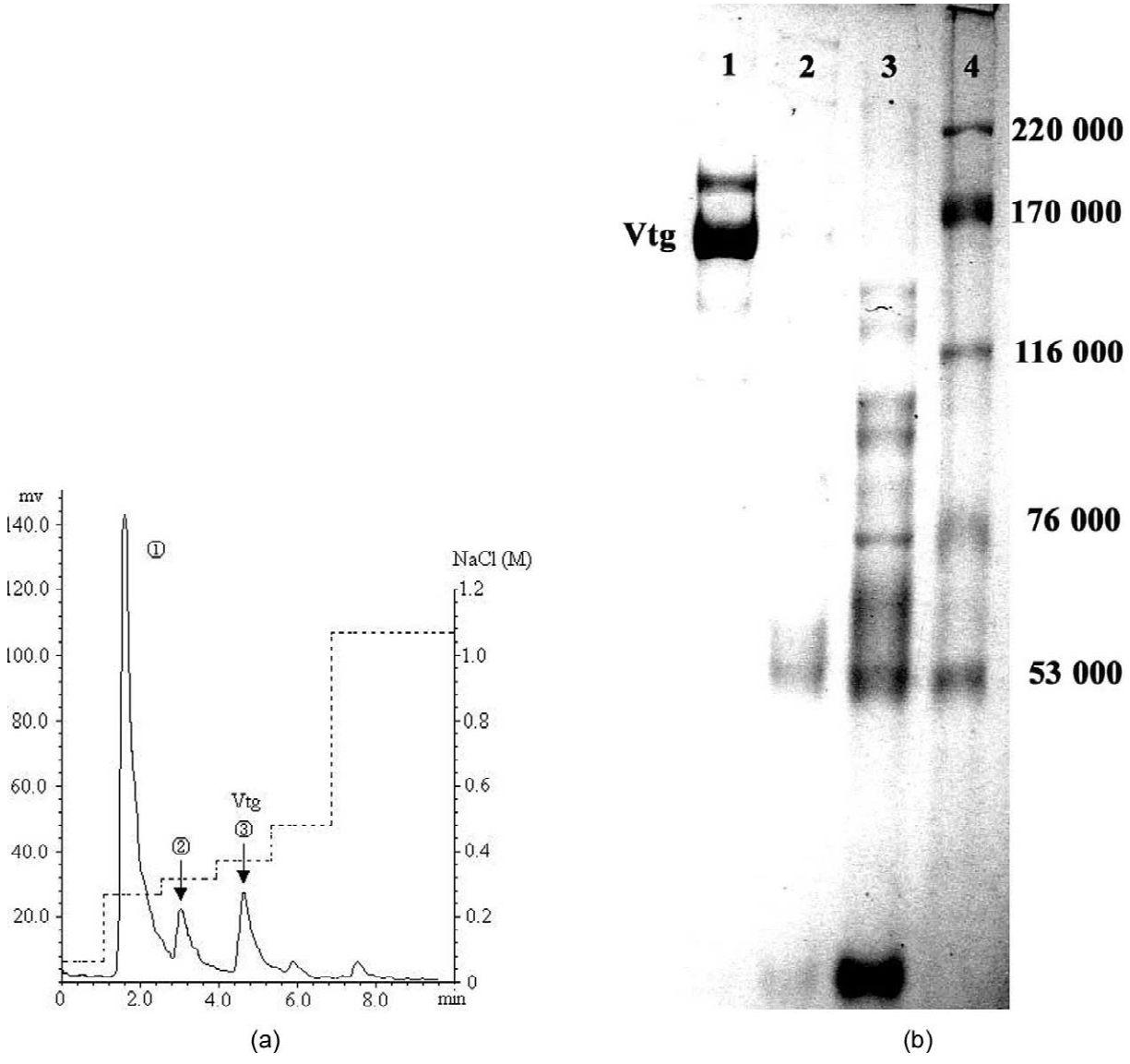

Fig. 5. Membrane chromatography separation of plasma proteins from male carp induced with 17β-estradiol (a), and analysis of separated proteins by SDS–PAGE with Coomassie Staining (b). Each major peak in (a) was identified by SDS–PAGE in (b). Lanes: 1 = peak 3; 2 = peak 2; 3 = peak 1; 4 = molecular mass markers. The multistep elution by NaCl in Tris–HCl buffer was as in Fig. 3b.

Since Vtg is very susceptible to proteolytic degradation, even in the presence of antiproteolytic agents [5], the speed at which the protein is purified from the fractions is of great importance. This can easily be achieved by membrane chromatography, because of the very short equilibration and separation time. Moreover, the binding capacity of membrane chromatography can be easily improved simply by adding membranes into the cartridge or by connecting several commercial cartridge units in series, thus the sample obtained can be purified in one step.

In conclusion, this study provides a rapid and

convenient method for the purification of Vtg from fish plasma.

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