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Separation and detection of vitellogenin in fish plasma by capillary zone electrophoresis

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Abstract

A method for coupling capillary zone electrophoresis (CZE) with rapid membrane chromatography purification (RMCP) was established for the analysis of vitellogenin (VTG) in male fish plasma induced with 17ß-estrodiol. CZE analyses of purified VTG were performed in a buffer containing 25 mM sodium borate (pH 8.4). A 50 μm i.d. fused-silica capillary was used for separation and the detection was carried out by UV-diode array at 214 nm. Inter- and intra-assay variabilities of the proposed method were less than 10.06 and 1.95%, respectively. The method has good linear relationship over the scope of 15–2250 μg/ml with a correlation coefficient of $R^2 = 0.9965$ and a detection limit of 7.0 μg/ml. The established CZE method was also applied to directly separate and identify VTG from fish plasma. The results indicated this method could minimize interferences from plasma proteins, allowing the detection of at least 62.5 μg/ml of VTG proteins in total proteins. This is a rapid and easy method to determine the quantity and purity of VTG compared to Bradford method and SDS-PAGE.

Keywords: Capillary zone electrophoresis; Vitellogenin; Fish plasma

1. Introduction

Recently, more and more attention has been focused on many of environmental toxicants, also known as endocrine disrupters, which constitute a wide group of chemical compounds that are able to antagonize the effects of endogenous hormones such as estrogens and xeno-estrogens [1]. The estrogenic and xino-estrogenic activity of many chemical is due to their capability of interacting with the estrogen receptor (ER), and the ER plays a key role as a ligand-inducible transcription factor that regulate some genes to produce proteins such as vitellogenin (VTG) [2].

VTG is a complex protein that serves as the precursor protein to the production of yolk in oviparous vertebrates. The production of VTG takes place in the liver and is under the receptor-mediated regulation of 17ß-estrodiol; both males and females have such a mechanism [3]. It is normally absent or present at very low levels in male fish because of its low level of estrogen, but due to the constitutive presence of the gene, VTG can be induced upon estrogenic exposure [4,5]. In vivo and in vitro studies have proved that VTG could be synthesized in male and juvenile fish on exposure to estrogen and estrogen mimics [6]. It was demonstrated that VTG could be a good biomarker for xeno-estrogenic exposure.

The main techniques for VTG determination currently are enzyme linked immunosorbent assay (ELISA) [7–9], radioimmunoassay (RIA) [10,11], Western blots [12], HPLC [13] and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [14]. In many related articles, immunoassays were applied in VTG analysis as the commonly used approach. It appears that immunological techniques applied to VTG proteins have a great potential for developing into sensitive, specific, rapid and reasonable

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quantitative methods. But selection of suitable antibody still remained the major problems of specificity and limitation that only a single specific protein can be assayed. SDS-PAGE is the most common methods used to study VTG protein but the results are difficult to quantify and the technique is rather time consuming. HPLC is a good choice if not considering the price of column. Thus, else method such as CE and mass spectrometry (MS) would be desirable to be developed as a reference method for VTG in external detection and quality assessments.

There are various fish specifies being used in different labs to study the VTG induction by estrogens or xino-estrogens. It is necessary for many labs to purified VTG by themselves and select detection methods because of different test fishes [15,16]. In this paper, we developed a CZE method for the analysis of VTG proteins in fish plasma followed rapid membrane chromatographic purification (RMCP). Capillary zone electrophoresis (CZE) is an established technique that has been utilized to the separation of proteins interest in complex biological fluids including plasma in the past decade [17–19]. The exceptional power of separation and resolution, rapid analysis time, economy of reagents and miniscule sample requirements has made CZE an attractive method. Compare to the ELISA method, it provides a rapid quantitative method to determine the concentration of VTG in fish plasma but no limit of various antibodies of VTG in ELISA analysis. This CZE method can also be applied to determine the purity of the VTG standard and identify the existence of VTG in fish plasma. This CZE method can be routinely employed as a complimentary tool to HPLC and ELISA in the risk assessment of environment endocrine disruptors.

2. Materials and methods

2.1. Chemicals

17ß-estrodiol (E2), bovine plasma albumin (BSA, 66 kDa) and heparin were obtained from Sigma (USA), and aprotinin was from Boehringer (Germany). Coomassie blue G-250, Protein molecular weight markers (containing Thyroglobulin, 669 kDa; Ferritin, 440 kDa; Catalase, 232 kDa; Lactate dehydrogenase, 140 kDa; Albumin, 66 kDa) was obtained from Amresco (USA). All other chemicals were reagent-grade compounds obtained from commercial sources.

2.2. Animals and treatment

Adult loaches with an average weight of 15–20 g for experiments were purchased from Beijing Dazhongsi market and cultured in the laboratory. Before the experiments, the fish were domesticated for 2 weeks. Then, males were injected 10 μg/g body weight with 17ß-estradiol previously dissolved in ethanol and re-suspended in 0.9% (w/v) saline solution every 3 days. Exposures were with E2 for 30 consecutive days in a semi-static system (water changes twice a week).

2.3. Preparation of fish plasma

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 aprotinin (2.5 TIU) and 6 heparin (30 USP units). The blood was subsequently centrifuged at 3000 rpm, 4 °C for 30 min to separate plasma, the plasma thus obtained was centrifuged at 11,000 rpm, 0 °C for 20 min to separate fat. The plasma was collected and stored at −20 °C until analysis.

2.4. Isolation and purification of VTG

VTG was purified from plasma of E2-exposed male loaches by membrane chromatography. This procedure is a modified version of the method described by Guoqing Shi [20]. The fish plasma was diluted with several volumes of buffer A (20 mM phosphate buffer containing 70 mM NaCl, pH 6.5) and filtered with a 0.2-μm cellulose acetate filter. Then, it was loaded on the anion-exchange membrane (Sartobind SQ15, Sartorius, Goettingen, Germany) equilibrated with 10 ml buffer A in advance. After washing the membrane with 5 ml of buffer A, the proteins were eluted with a multiple-step gradient to 1 M NaCl using buffer B (20 mM phosphate buffer containing 1 M NaCl, pH 6.5), which were 0.07, 0.21, 0.30, 0.49 and 1.0 M, respectively. Aliquots of the peak fraction of VTG was collected and stored at −20 °C. Fractions containing VTG were desalted by selective diffusion through a semipermeable membrane with a MWCO of 10,000. Proteins were made into powder using freeze dryer and stored at −20 °C.

The protein concentration was determined by Coomassie protein assay and detected at 595 nm [21].

2.5. SDS-PAGE

Plasma samples from chromatographic fractions were analyzed by SDS-PAGE (7.5% gel). Samples were diluted in sample buffer (125 mM tris-HCl, pH 7.2, containing 2% SDS, 2% mercaptoethanol, 20% glycerol and 0.04% Bromophenol blue) at ratio 1:1 and heated to 95 °C for 10 min. After electrophoresis, separated proteins were stained with Coomassie Blue G-250, and the bands were compared to high-molecular-mass electrophoresis standards.

2.6. Capillary electrophoresis

The CZE experiments were performed on an HP^{3D} CE system with a diode array UV detection system (Hewlett-Packard, Wilmington, DE, USA). HP^{3D} CE ChemStation software was used for instrumental control, data acquisition and data analysis. In all experiments, a constant voltage of 20 kV was applied, the temperature was set at 20 °C, and the detection was made at 214 nm. The sample was injected into the capillary by the vacuum system at 50 mbar for10 s.

Fused silica capillary (40 cm × 50 μm i.d.) used in this report was obtained from Yongnian optic fiber plant (HeBei, China). Running buffer was prepared from 50 mM stock solutions of boric acid and 50 mM sodium borate, which were prepared with deionized water. These solutions were mixed in the ratio of 1:1 and adjusted from pH 7.0 to 10.0 with a concentrated sodium hydroxide. In order to reduce protein absorption to the capillary wall that interferes with the reproducibility of the CZE measurement, the capillary was systematically flushed for 1 min with 0.1 M NaOH, 3 min with deionized water and then with running buffer for 5 min before each analysis. CZE analysis of each sample was repeated three times.

3. Results and discussion

3.1. Purification of VTG

There are several ways to purify VTG that rely on the physical-chemical properties of this unusual protein [22]. A direct approach is to bind VTG to anion exchanger, such as DEAE agarose [23,24]. The method we used was binding VTG to anion-exchange membrane described previously [20]. In order to reduce the loss of VTG in purification process, there was a little change to the method described by Guoqing Shi. In this experiment, we used SQ15 weak anion-exchange membrane substituted for MA15 Q strong anion-exchange membrane. Phosphate buffer with pH 6.5 was used instead of pH 8.3 Tris-HCl and the multistep gradient elutions by NaCl in phosphate buffer were 0.07, 0.21, 0.30, 0.49 and 1.0 M, respectively.

Some plasma proteins (Fig. 1, peak a) did not bind on the membrane under conditions of pH 6.5 and 0.21 M salinity with the sample loaded on it and hence were eluted in dead value. However, VTG (Fig. 1, peak c) and other proteins (Fig. 1, peaks b and d) were separated and eluted with a multiple-step gradient to 1 M NaCl using buffer B. The eluted fractions were analyzed by SDS-PAGE followed by a Coomassie blue-staining (Fig. 2). The results indicated the most VTG was eluted in peak c. Comparing the results of Guoqing Shi, the recovery could be enhanced when the MA15 Q strong anion-exchange membrane (the recovery is about 80%) was replaced by SQ15 weak anion-exchange membrane (the recovery is about 103%). It could keep the most amount of VTG of plasma in fraction c. In this experiment, the VTG standard was further purified using SQ 15 membrane time after time until it was single band in SDS-PAGE (Figs. 2 and 5).

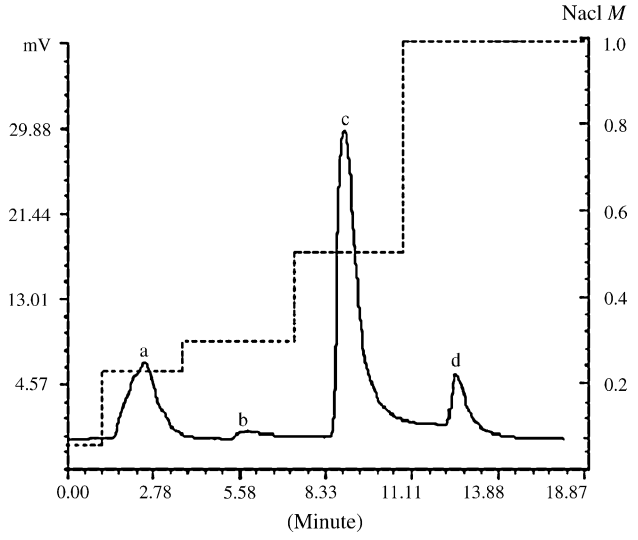

Fig. 1. Separation of plasma proteins from E2-induced male loach with SQ 15 weak anion-exchange membrane chromatography. The multi-step gradient elution by NaCl in phosphate buffer was 0.07, 0.21, 0.30, 0.49 and 1.0 M, respectively.

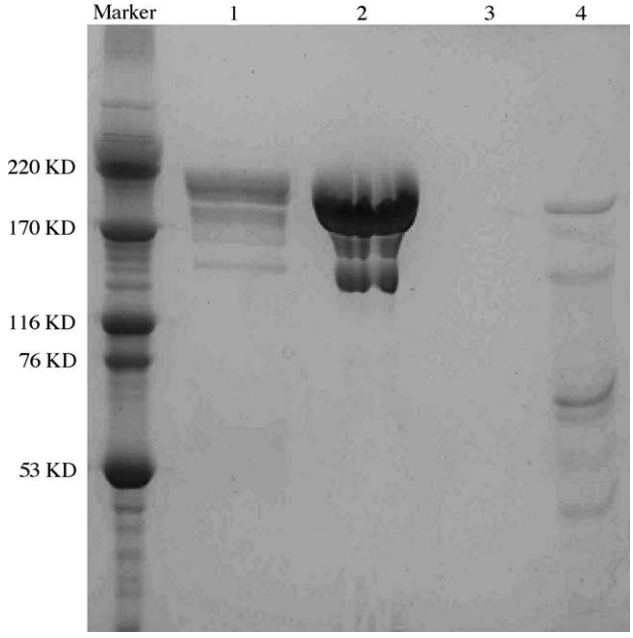

Fig. 2. SDS-PAGE analysis of protein fractions eluted with RCMP. Lanes: 1 = protein from peak d (Fig. 1, d); 2 = protein from peak c (Fig. 1, c); 3 = protein from peak b (Fig. 1, b); 4 = protein from peak a (Fig. 1, a); Marker: molecular mass marker (the molecular weights were 220, 170, 116, 76, 53 kDa from top to bottom).

3.2. CZE analysis of VTG

In general, the prerequisite for CE analysis of proteins is that the bindings of the proteins to capillary surface is reduced or eliminated. Therefore, Borate buffers with different pH were tested. When the pH of running buffer was higher than 7.4, minor peak and major peak were absolutely separated from purified VTG samples although which exited in SDS-page gel were single bands. The minor peak was not confirmed whether it was impurity protein or the production of VTG degradation since VTG is very susceptible to protein degradation [25]. For best results, it was suggested to detect the purity of VTG at the beginning of each experiment. The percents of minor peaks were 15.3, 19.9 and 16.6% ($n = 3$)

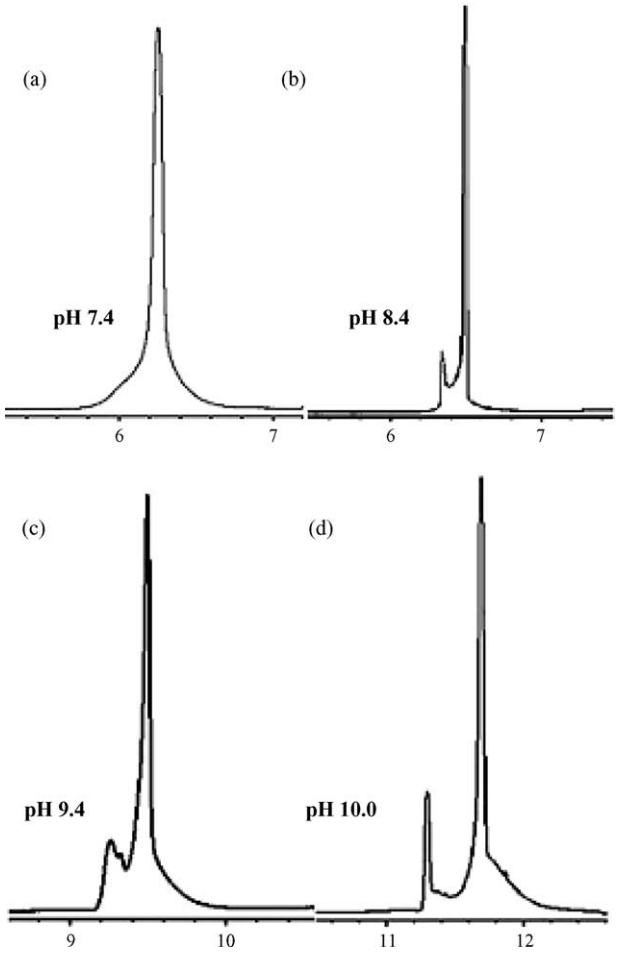

Fig. 3. Electropherograms of VTG protein in running buffer with different pH. (a) pH 7.4; (b) pH 8.4; (c) pH 9.4 and (d) pH 10.0.

in pH 8.4, 9.4 and 10.0 running buffer. The separation time was obviously prolonged when the running buffer was higher than pH 9. The electropherograms of purified VTG were different in different pH running buffers (Fig. 3). Considering rapid analysis time, well separation and perfect peak-shape, the pH of the borate buffer in the subsequent experiments was chosen to be 8.4, after comparing the relationship between migration time and peak area (Fig. 4). VTG in the plasma

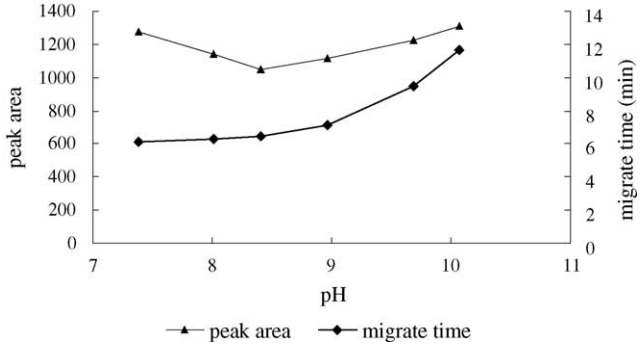

Fig. 4. Effects of different running buffer pH on the migration time and peak area of the VTG standard.

could also be successfully separated in such a suitable analytical condition. The pH of the VTG sample solutions did not cause significant change in the migration time and peak area.

An advantage of this combined method (CZE and RMCP) is that, depending on the required purity of the VTG, the influence of interfused proteins can be avoided.

3.3. Identification of VTG in the loach plasma

Fig. 5 showed the comparison of VTG between the patterns of the control plasmas and E2-induced plasmas indicated that CZE methods could be applied to the identification of VTG protein in fish plasma. Except the peak of

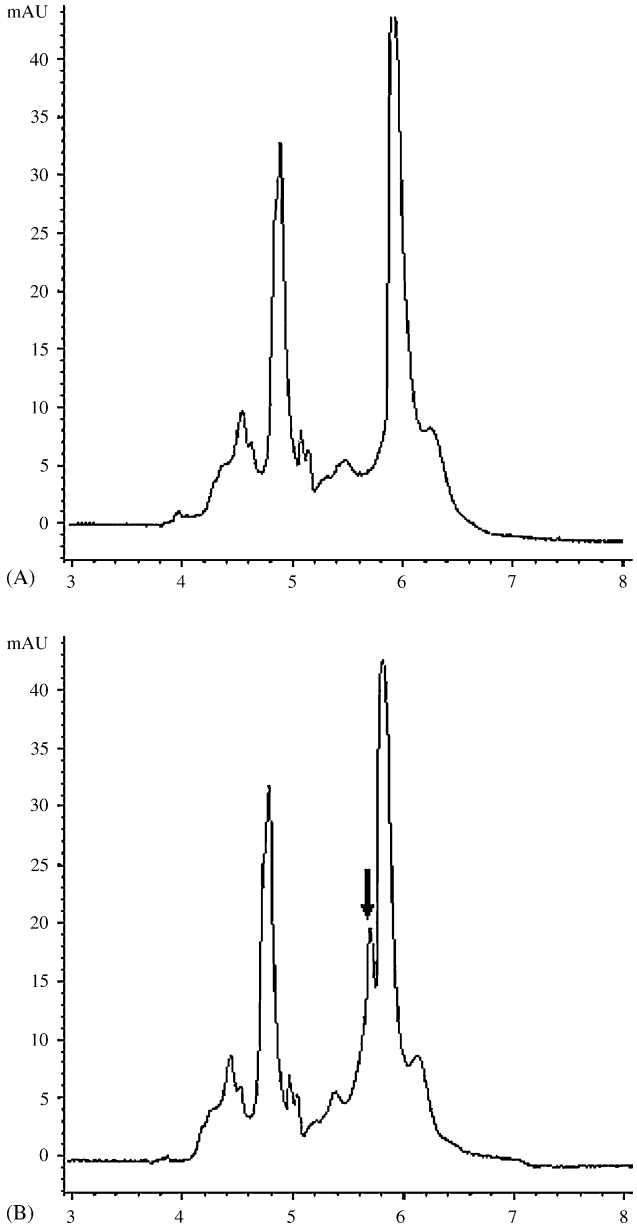

Fig. 5. CZE analyses of loach plasma proteins obtained from untreated male loach and loach treated with 17ß-estrodiol. (A) Control male loach plasma, (B) male loach plasma induced with E2 for 10 days.

VTG, the other peaks of loach plasma could not be identified due to bare of enough information. Since we were attempting to determine VTG in treated male loach plasma of a poorly-characterized biofluid, it was not possible to unambiguously identify components based solely on their mobility relative to standards under one set of separation conditions [26]. In order to gain stronger indication of new peaks, VTG at several different concentrations were added into control male plasma and separated in the pH8.4 system (Fig. 6). Control plasma containing low proportions of VTG isolate was found difficult to avoid completely the interferences from other proteins and the quantitation of VTG at low level is unreliable. Initially, experiments were carried out with control male plasma containing 0, 62.5, 125, 187.5 and 250 μg/ml VTG protein in total protein. However, the appearance of peak was hardly visible in plasma containing less than 62.5 μg/ml VTG protein. No proteins peaks were found at the nearly time in the electropherograms of control plasma (0 μg/ml) devoid of VTG proteins. The results still indicted that it was the peak of VTG. So the new peak appearance might be an indictor of the presence of VTG in male loach plasma. The sensitivity of this direct determination of VTG is very similar to the result of HPLC [13], but much lower than the ELISA method [8].

3.4. Quantitation of VTG by CZE

A concentrated stock solution of purified VTG (2.25 mg/ml) was prepared in pH8.4 borate buffer, and calibration standards were made by diluting appropriate amounts of the stock solution with buffer. With this standard prepared, linearity and detection limit were determined. The calibration curve of VTG in the range from 15 to 2250 μg/ml was demonstrated in Fig. 7. Each point of the curve represents mean peak area of VTG standards measured in three measurements. Peak area (y) could be expressed by the equation $y = 1.09564x + 44.35354$ ($R^2 = 0.9965$). The limit of detection, which was three-times the baseline noise level, was estimated to be 7.0 μg/ml.

Triplicate measurements were carried out for each VTG sample to determine the method precision. Precision results were shown in Table 1. In the intra-day assay, the coefficients of variation of the relative peak area were from 0.97 to 1.95% in three different concentrations, respectively. In the inter-day assay, the reproducibility was equal or less than 10.06% for different concentrations.

The CZE method for the identification of VTG of E2 induced male loach and its quantitation presented in this work was compared to conventional Bradford method. VTG were purified from 50 μl plasma of two male loaches that were induced by E2. Protein concentration was determined

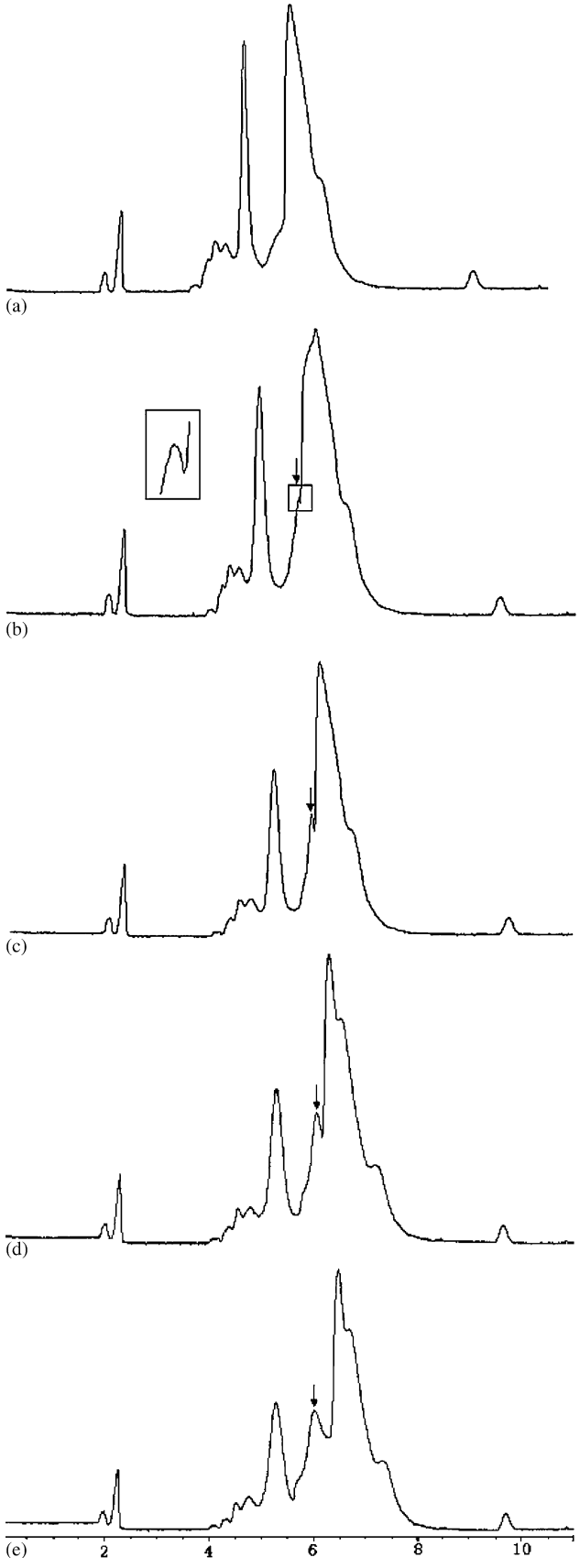

Fig. 6. CZE electropherograms of samples of fish plasma containing (a) 0, (b) 62.5, (c)125, (d) 187.5 and (e) 250 μg/ml of VTG protein in total protein. Arrows point out the indicator of the presence of VTG protein in fish plasma. 5: Further purified VTG standard with Q15 membrane.

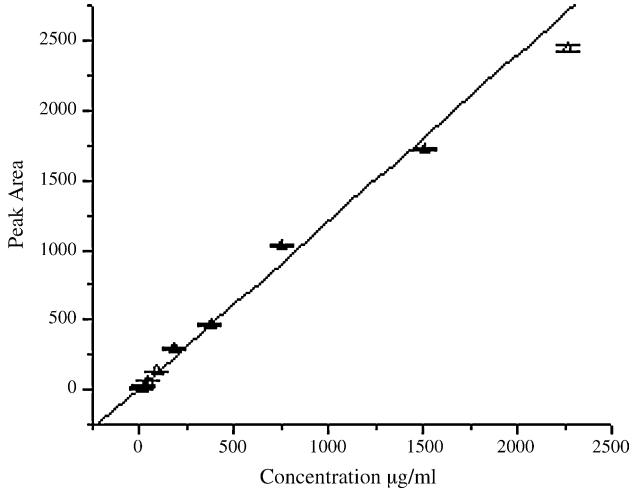

Fig. 7. Calibration graphs for peak areas of VTG protein vs. μg/ml VTG in solution. $y = 1.09564x + 44.35354$, $R^2 = 0.99651$.

Table 1
Precision of CZE method for the detection of purified VTG standard

Nominal concentration (μg/ml)	R.S.D. of peak area ($n = 3$)	
	Intra-assay[a] (%)	Intra-assay[a] (%)
40	1.95	1.95
170	1.41	1.41
500	0.97	0.97
1300	1.00	1.00

[a] Values represent mean from three samples.

by the methods we mentioned. The results of two methods were showed in Table 2. An excellent correlation of the CZE method with results from total protein detection in Bradford method was achieved in quantitative analysis. The results indicated that separation, quantification and detection with this CZE system were reproducible and reliable for the measurement of VTG. In the conventional Bradford method, quantification of the protein is the total proteins based on the dye bind, whereas in CZE method the separated protein fractions are quantified respectively based on the absorbance measurements at the wavelength of 214 nm [27]. The advantage of CZE method is more selective and accurate than Bradford method that other interferential proteins could be eliminated.

Table 2
Comparison between CZE and Bradford methods for the quantification analysis of VTG purified with RMCP

Sample[a]	VTG concentration (μg/ml)	
	CZE[b] (±S.D.)	Bradford[b] (±S.D.)
1	277.2 (±7.6)	271.2 (±11.3)
2	457.6 (±12.4)	478.3 (±15.2)

[a] Sample was VTG purified with RMCP from two E2-induced male loach.
[b] Values represent mean from three times.

4. Conclusions

The exceptional power of separation and resolution of complex mixtures of analytes form biological samples, rapid analysis time, economy of reagents, minimal sample preparation and miniscule sample requirements have made CZE an attractive method for both forensic and clinical laboratories. As a result, CZE has been routinely employed as a complimentary tool to HPLC [28].

In this study, a method coupling CZE with RMCP has been successfully used in the analysis of VTG protein in the plasma of E2-induced male loach. It has been proved to be simple and convenient, as well as sensitive and selective, as an alternative approach for the determination of VTG in fish plasma to other methods, e.g. ELISA.

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