A Simple Method for Purification of Low Levels of Beluga (*Huso huso*) Vitellogenin

Mahdi Paktinat[1], Mahvash Khodabandeh[2],
Bagher Mojazi Amiri[1], Hamid Farahmand[1]

1 Department of Fisheries, Faculty of Natural Resources, University of Tehran, Karaj, Iran
2 Industrial and Environmental Biotechnology Department, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

Abstract

The beluga sturgeon (*Huso huso*) is one of the most valuable commercial fish species in the Caspian Sea and has become highly endangered due to overfishing, loss of natural habitat and water pollution [2]. Vitellogein as a major beluga sturgeon yolk precursor, can be used as a suitable marker to monitor the contamination of the aquatic environments. In this study, we tried to compare some methods for purification of low levels of beluga vitellogenin. The efficiency of selective precipitation (EDTA-Mg+2), anion exchange chromatography and preparative electrophoresis in purification of low vitellogenin levels were compared. SDS electrophoresis analysis of the obtained fractions showed that DEAE-based chromatography and selective precipitation were unable to purify low levels of beluga vitellogenin. Preparative electrophoresis showed an appropriate result and low level of vitellogenin was purified efficiently by this method. These data suggest that preparative electrophoresis is a simple and efficient procedure for the purification of low levels of beluga Vtg.

Keywords: Beluga, vitellogenin, purification, endocrine disruptor

1. Introduction

Endocrine disrupting chemicals (EDCs) are considered to be a major source of *pollutants* in the aquatic environments and may interfere with the endocrine system and produce adverse developmental, reproductive, neurological, and immune effects. Fish, because of their constant and direct exposure to EDCs present in the aquatic environment are the most affected by EDCs [4]. This long-life exposure can influence reproduction functions including reduced fertility in both male and female, reduced fecundity, change in mating behaviors, partial sex reversal and skewed population sex ratios [10]. Certainly in this case, beluga sturgeon because of long reproductive cycle is a susceptible specie in contaminated environments. So regarding to the above mentioned issues, study on the effects of the endocrine disruptors on the reproductive functions in such endangered species is necessary. Vitellogenin (Vtg) is a dimeric phosphoglycolipoprotein with high molecular weight and the major yolk precursor protein synthesized by the liver in response to circulating estrogen, released into the bloodstream and taken up by growing oocytes via receptor-mediated endocytosis, where it undergoes proteolytic cleavage to form yolk proteins [8].

Vtg synthesis in normal conditions is confined to reproductive females, but it can be induced in either sex or every age by exposure to estrogens. Due to this trait, Vtg is a suitable marker to monitor the contamination of the aquatic environments with estrogenic compounds and often used as a biomarker of endocrine disruption in fish [1]. With regard to the significant importance of Vtg in screening and testing for endocrine disruption in fish, many studies have focused on the purification of this protein and then use of it as antigen in the production of antibody. So, setting up the appropriate methods for Vtg purification is important. Several purification methods such as ultracentrifugation, precipitation by dimethylformamide, selective precipitation using EDTA-Mg+2, ion exchange chromatography and gel filtration based on the physicochemical properties of Vtg have been developed [3]. Despite the efficiency of these methods in isolation of Vtg from most of the oviparous animals, some methods may be less efficient for purification of fish Vtg, due to high lipid content or less phosphorylation of fish Vtg [6]. Moreover, it's necessary to consider that Vtg obtained from various species respond to these methods differently. Therefore, optimization of Vtg purification methods is considered as an essential part of research about this protein. Accordingly, this study was designed to optimize the beluga Vtg purification methods and compare the performance of these methods for the purification of beluga Vtg.

2. Materials and methods

2.1. Plasma preparation

Blood plasma was obtained from naturally vitellogenic females and males reared at the Rajaee Sturgeon Hatchery Center (Sari, Mazandaran, Iran). Blood samples were collected from the caudal vein of the anesthetized fish using heparinized tubes. Plasma was then separated by centrifugation at 4000 rpm (4 °C) for 10 minutes. Aprotinin (Sigma #A6279) was added to the plasma samples (20 μL, 0.2 TIU aprotinin/1000 μL plasma), and all samples were stored at -70 °C until assay.

2.2. Purification of Vtg by DEAE-Sepharose, anion-exchanger resin

Anion exchanger resin (DEAE Sepharose CL-6B, Pharmacia) packed into 10 × 1.2 cm column and purification carried out according to the procedure described by Shi et al. [11]. Briefly, 0.5 ml of plasma sample were diluted with equal volume of buffer A (Tris-Cl 20 mM, pH: 9.0, NaCl 0 M) and centrifuged at 13000 rpm (4 °C) for 15 min. The obtained supernatant was loaded on the column equilibrated with the buffer A. Purification was performed at the room temperature with a flow rate of 36 ml h^{-1}. Unbound proteins were removed by an additional washing step with 2 column volumes of the buffer A. Bound proteins were eluted using a gradient of 0-0.8 M NaCl in 20 mM Tris-Cl (pH: 9.0), within 15 column volumes. Eluted fractions were collected at a volume of 4 ml and the elution profile was monitored at 280 nm. Fractions containing significant amounts of protein were evaluated for the presence of Vtg by SDS-PAGE.

2.3. Purification of Vtg by selective precipitation (EDTA-Mg^{+2})

Vtg precipitated from the plasma samples by method of Wiley et al. with some modifications [13]. Briefly, 0.5 ml of plasma was mixed with 2 ml of 20 mM EDTA. Then, precipita-

tion was performed with adding 0.1 ml of 0.5 M $MgCl_2$ in this mixture. The precipitate was collected by centrifugation at 5000 rpm for 15 minutes at 4 °C and the supernatant was discarded. The obtained precipitate was re-dissolved in 1 ml of the buffer containing 1 M NaCl, 50 mM Tris-Cl and centrifuged at 13000 rpm for 30 minutes at 4 °C to remove any insoluble materials. The precipitate was discarded and supernatant fraction containing purified Vtg used for following assay. In order to determine the purity of Vtg, the obtained supernatant was subjected to the SDS-polyacrylamide gel electrophoresis.

2.4. Purification of Vtg using preparative electrophoresis

Selective precipitated samples were dissolved in the 125 µl sample buffer containing SDS and β-mercaptoethanol and loaded on a preparative gel (5 % stacking gel over 7.5 % resolving gel) with high molecular weight marker and electrophoresed at 120 V. After electrophoresis, the section related to the marker was cut and bands were visualized with staining. After comparing the stained section with the main section, the Vtg and contiguous slices were excised from the gel and liquefied in a glass-glass tissue grinder in 3 ml of Tris-Cl 50 mM (pH: 7.5) and shook overnight to extract proteins from the gel slices. Obtained fractions were centrifuged at 5000 rpm (4 °C) for 20 minutes and the supernatant were evaluated for the presence of Vtg by SDS-PAGE.

2.5. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE performed with a 5% stacking gel and 7.5% separating gel according to Lämmli [5]. Gels were stained with 0.1% Coomassie Brilliant Blue G-250 for the visualization of proteins and the molecular mass of the individual protein bands was estimated using high molecular weight marker (Pharmacia Biotech, Piscataway, NJ).

2.6. Determination of Vtg concentration in purified samples

The absorbance of the each purified sample was measured at 280 and 260 nm and the following equation was used for calculating the Vtg concentration in the purified samples.

Vtg concentration = (1.55 × O.D. 280 nm) – (0.76 × O.D. 260 nm)

3. Results

3.1. Purification of Vtg by anion-exchange chromatography

Anion-exchange chromatography on DEAE-Sepharose was carried out for the purification of beluga Vtg. Result presented in fig. 1.A show that this column *was not able* to separate Vtg from plasma proteins. Accordingly, we tried to optimize chromatography conditions to purify Vtg (e.g. binding and elution buffers concentration, pH and volume). Fig. 1.B *shows the best result* of anion-exchange chromatography. Eluted Fractions containing significant amounts of protein were evaluated for the presence of Vtg by denaturing gel electrophoresis (fig. 1.B). As shown in fig. 1.B this anion-exchanger resin was not able to purify low plasma Vtg levels.

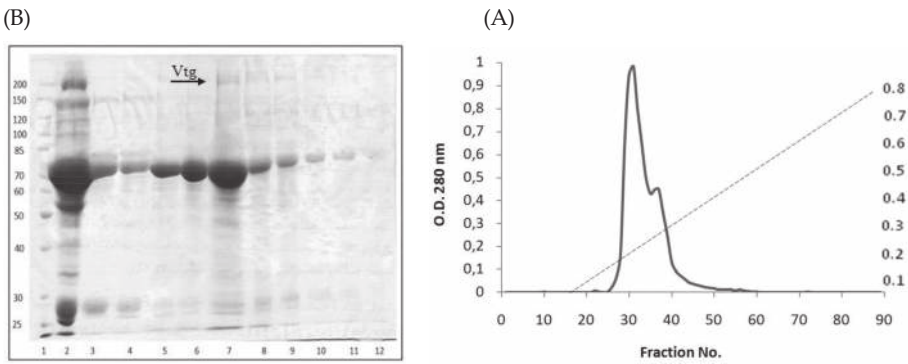

Fig 1. A: Elution profiles of plasma proteins from vitellogenic beluga on a DEAE-Sepharose chromatography column. Proteins bound to the column were eluted with a gradient of 0-0.8 M of NaCl in 20 mM Tris–HCl buffer. B: SDS-PAGE analysis of elution peak fractions in 7.5% reducing gel stained with coomassie brilliant blue. Lanes 1- molecular weight markers; 2- vitellogenic beluga plasma sample; lanes 3-12 are different elution fractions [24-42] from the column.

3.2. Purification of Vtg by selective precipitation (EDTA-Mg^{+2})

We observed that the addition of bivalent cations to plasma samples containing Vtg in the presence of EDTA was caused in the precipitation of the plasma Vtg (Fig. 2). The *SDS-PAGE results show* that Vtg has a molecular weight of about 200 kDa. A visible impurity of albumin with a molecular weight of approximately 75 kDa was present. Therefore, presence of impurities in the purified samples represents the inefficiency of this method for the purification of beluga Vtg. Nevertheless, this method could be a suitable way to identify Vtg among the plasma proteins.

3.3. Purification of Vtg by preparative electrophoresis

After termination of the preparative gel electrophoresis, slices related Vtg were excised from the gel. Proteins were extracted from the gel slices and electrophoresed for evaluating of the Vtg presence. As shown in fig. 3, purified Vtg was appeared as single major band of 200 kDa, along with some minor bands with lower apparent mass (fig. 3). It seems to be the degraded products of Vtg. With respect to protein patterns obtained from SDS-PAGE, this method has considerable ability in the purification of the beluga Vtg. To validate this method, we determined the concentration of protein in the purified sample that contained the appropriate levels of Vtg (232 μg/ml).

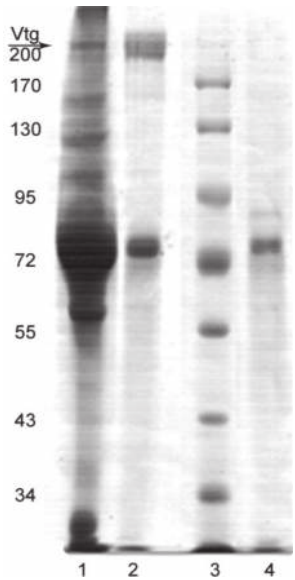

Fig 2. SDS-PAGE (7.5%) of male and female beluga Plasma. 1- beluga female plasma; 2- selective precipitated (EDTA-Mg^{+2}) beluga female plasma; 3- High molecular weight markers; 4- selective precipitated (EDTA-Mg^{+2}) beluga male plasma.

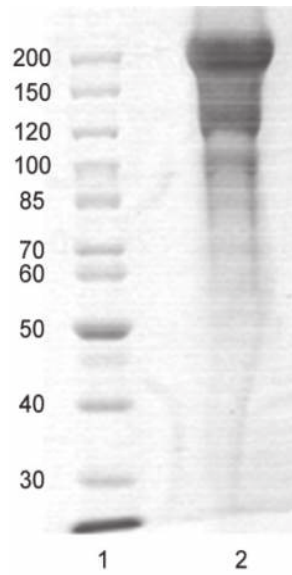

Fig 3. SDS PAGE (7.5%) of purified Vtg. 1- high molecular weight markers; 2- purified beluga Vtg.

4. Discussion

In the current study we investigated the efficiency of the anion-exchange chromatography, selective precipitation and preparative electrophoresis for the purification of the low levels of beluga Vtg.

The anion-exchange chromatography was the first method which used for the purification of beluga Vtg. *As represented in the results (fig. 1), DEAE based column was completel*y unable to purify low Vtg levels and modification of some operational features couldn't to change the results. Various chromatographic methods have been used for the isolation of the fish Vtg [7] and some have used DEAE-Sepharose for purification of fish Vtg [9]. Low levels of Vtg in loading samples and low performance of DEAE-Sepharose for isolation of this levels of Vtg may be the *main reasons* why this column was *not* very *effective* in purification of beluga Vtg. As presented in fig. 2, selective precipitation was caused to the significant precipitation of the plasma Vtg. But structural similarity between Vtg and albumin in calcium binding properties was caused to the precipitation of the plasma albumin. Wiley H. S. [13] applied the selective precipitation procedure for purification of Xenopus laevis Vtg. Low plasma Vtg levels and less phosphorylation of fish Vtg might be the main reseems of the current results.

This method is specific for calcium binding proteins such as Vtg that carries the large amounts of the calcium ions. So, applying this method to the male and female plasma can help with the identification of Vtg.

Purification of beluga Vtg using preparative electrophoresis was the last procedure which was used in this study. According to our results (fig. 3), preparative electrophoresis was an appropriate procedure for purification of beluga Vtg. In this procedure, selective precipitation (EDTA-Mg^{+2}) showed good efficiency with regard to the precipitation of Vtg from beluga plasma samples, helping to acquire highly pure Vtg from the preparative electrophoresis. Inoculation of Vtg prepared from preparative electrophoresis has some advantages and disadvantages. Disadvantages of this procedure are partial degradation and the loss of secondary and tertiary structures of Vtg [12]. On the other hand, the Vtg purification method that has been developed in this study has clear advantages with regard to speed, low cost and simplicity when compared to current purification methods.

In conclusion, the efficiency of chromatographic methods are depend on the using of an appropriate resin that can be costly. In addition, they presented a disadvantage of being time consuming and generally are inefficient for the purification of low levels of plasma Vtg. So, such procedures need to stimulate of the Vtg production by an estrogen treatment. Selective precipitation of Vtg needs to the very high concentrations of plasma Vtg and essentially is efficient in purification of Vtg with high contents of the phosphate groups. Finally, preparative electrophoresis is a suitable method for the purification of the low levels of fish Vtg, especially when combined with selective precipitation (EDTA-Mg^{+2}). However, the loss of native structure of Vtg is the main disadvantage of this method.

5. References

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