CHARACTERIZATION OF GLYCOPROTEIN FRACTION FROM CARP PITUITARIES ISOLATED USING CONCANAVALIN A AS THE AFFINITY LIGAND

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Glycoproteins that have affinity to Concanavalin A were isolated from the acetone-dried pituitaries of common carp (*Cyprinus carpio* L.). Two fractions of glycoproteins were separated using gel chromatography on Superdex 75HR. The fraction with lower molecular weight (30 000) corresponding to the carp gonadotropin cGtH II was composed of two subunits as determined using SDS-PAGE. This protein fraction was further divided into four components using reversed-phase HPLC. Two fractions were pure α and β subunits of cGtH II as follows from immunodetection and from determination of *N*-terminal amino acid sequences. The other two were a mixture of α and β subunits as was also revealed by *N*-terminal analysis. Capillary electrophoresis was also used for characterization of isolated glycoproteins.

Key words: Hormones; Glycoproteins; Gonadotropins; Carp pituitary; Capillary electrophoresis.

The extracts from carp pituitaries are widely used in fish farming for induction of artificial stripping in various fish species[1]. Nevertheles, the hypophyzation method is not always effective[2]. Gonadotropins are supposed to be the active substances of the pituitary extract evoking the biological response. Gonadotropins (GtH) are pituitary glycoprotein hormones, composed of two subunits α and β, controlling gametogenesis and gonadal steroidogenesis[3]. Recently, two different gonadotropins (GtH I and GtH II) have been found in certain fish species[4–6].

With the aim to study the carp pituitary glycoprotein hormones we used the ConA-coupled Sepharose to prepare a glycoprotein fraction from carp pituitaries. We determined the physico-chemical properties of the prepared glycoproteins.

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EXPERIMENTAL

Material

Acetone-dried carp pituitaries were purchased from Fisheries Nemanice. Protein molecular weight standards were from Serva (Heidelberg), Nitroblue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate was from Sigma (St. Louis). ConA Sepharose 4B and Superdex 75HR columns were from Pharmacia (Uppsala), μBondapack C18 column was purchased from Millipore (Milford). Anti-β-carp-GtH II antibody was kindly provided by Breton[7] (INRA Rennes), RB IgG(AFF)-Alk Phosp was from Dako (Glostrup).

Methods

Isolation. The glycoprotein fraction was purified from acetone-dried carp pituitaries. Total of 100 acetone-dried pituitaries (400–500 mg) were homogenized with distilled water. The homogenate was centrifuged and the supernatant was lyophilized. The water extract was applied to a ConA Sepharose 4B column equilibrated with 0.05 M Tris-HCl buffer (pH 8) containing 0.5 M NaCl, 0.1 mM $MnCl_2$ and 0.1 mM $CaCl_2$. The glycoproteins adsorbed on the column were eluted with 0.5 M methyl α-D-glucoside in the same buffer. The concentration of proteins was monitored by measuring absorbance at 280 nm and using the method of Bradford[8].

The glycoprotein fraction from affinity chromatography was applied onto a Superdex 75HR column equilibrated with 0.05 M phosphate buffer containing 0.15 M NaCl. The column was also used for molecular weight determination. Bovine serum albumin (BSA) (m.w. 66 000), ovalbumin (43 000) and cytochrome *c* (12 500) were used as the molecular weight standards.

The 30 000 protein fraction from gel chromatography was further purified (after incubation in 0.1% aqueous trifluoroacetic acid (TFA) at 40 °C for 1 h) using rpHPLC on a μBondapack C18 column equilibrated with 0.1% TFA; the protein fractions were eluted with acetonitrile solution of variable concentration (6 min 0–24%, next 20 min 24–32% of acetonitrile in 0.1% aqueous TFA).

SDS-PAGE. The isolation steps were monitored using SDS-PAGE in 12.5% polyacrylamide gel[9] under reducing conditions. Lysozyme (m.w. 14 400) and its polymers were used as molecular weight markers. After SDS-PAGE the proteins were either stained by Coomasie Brilliant Blue or electroblotted to a poly(vinylidene fluoride) (PVDF) membrane. The membranes were immunostained with anti-β-carp-GtH II antibody (dilution 1/10 000), followed by alkaline phosphatase-conjugated antirabbit IgG antibody (RB IgG(AFF)-Alk Phosp)(dilution 1/400) and Nitroblue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate as a substrate system.

Capillary zone electrophoresis (CZE). The 30 000 protein fraction was analyzed using CZE. The analyses were performed on a home-made apparatus[10] equipped with an untreated fused silica capillary (i.d. 0.050 mm, o.d. 0.200 mm, effective length 198 mm, total length 300 mm, polyimide outer coating) and a UV detector (205 nm). As background electrolyte 0.04 M Tris-tricine buffer (pH 8.1) was used, concentration of samples was 1 mg/ml. The sample solutions were introduced into the capillary by siphoning effect (500 Pa, 5–15 s). The applied voltage was 10 kV and the current was 13 μA, temperature 23 °C.

The N-terminal amino acid sequence was determined on a Protein Sequencer LF 3600 (Beckman Instruments).

RESULTS

The glycoproteins of carp pituitaries were obtained by affinity chromatography of the water extract on ConA Sepharose column. The glycoprotein fraction made about 10% of the water-extracted proteins and was further separated by gel chromatography on Superdex column into two protein fractions (G1, G2). The ratio of protein concentration in fractions G1 and G2 was approximately 1 : 2. The G2 fraction was composed of two subfractions (G21, G22) (Fig. 1a).

Molecular weight of the G21 and G22 fractions, as determined by gel chromatography, was 33 000 and 18 500, respectively (Fig. 1b). Both fractions G21 and G22 were resolved by SDS-PAGE into two major bands corresponding to molecular weights of 25 000 and 16 000 (Fig. 1c). The concentration of the 25 000 component was higher in the fraction G22.

The G21 and G22 fractions were subjected to reversed phase chromatography on a μBondapack C18 column. By this procedure, the glycoprotein fractions G21 and G22

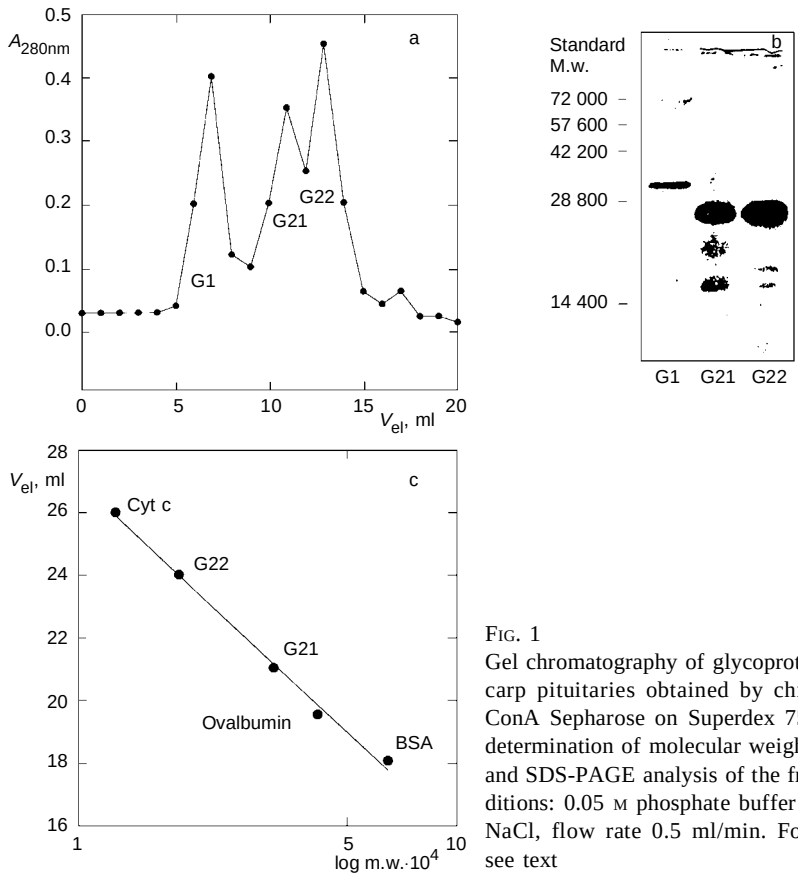

FIG. 1
Gel chromatography of glycoprotein fraction from carp pituitaries obtained by chromatography on ConA Sepharose on Superdex 75HR column (a), determination of molecular weight of fractions (c) and SDS-PAGE analysis of the fractions (b). Conditions: 0.05 M phosphate buffer containing 0.5 M NaCl, flow rate 0.5 ml/min. For further details, see text

were further separated into four and three components, respectively. Three out of four components of fraction G22 had the same elution times as the three components of fraction G21 (1 18, 2 19, 3 22 and 4 24 min in Fig. 2).

The glycoproteins separated by the methods mentioned above were analyzed by SDS-PAGE. The electrophoreograms were either stained by Coomasie Blue or electroblotted to a PVDF membrane that was immunostained with anti-β-cGtH II antibody. Positive interaction with antibody of β-subunit of cGtH II was observed in the water extract of carp pituitary, in fraction G2 from the gel chromatography and in fractions 3 and 4 from rpHPLC (Fig. 3).

The *N*-terminal amino acid sequences of the separated fractions were determined. Ten complete sequenation cycles were carried out. The sequence of Ser-Tyr-Leu-Pro-Pro-X^1-Glu-Pro-Val-X^2- was found in the fraction 4, *N*-terminal sequence in fraction 1

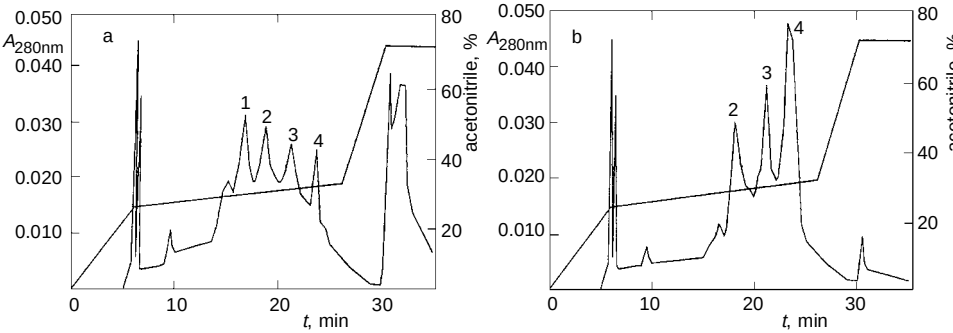

FIG. 2

Reversed-phase HPLC of glycoprotein fractions (G21 (a), G22 (b) – for details, see text) from carp pituitaries on a μBondapack C18 column. Conditions: proteins were eluted by discontinuous gradient 0 to 24 to 32% acetonitrile in 0.1% TFA, flow rate 1 ml/min

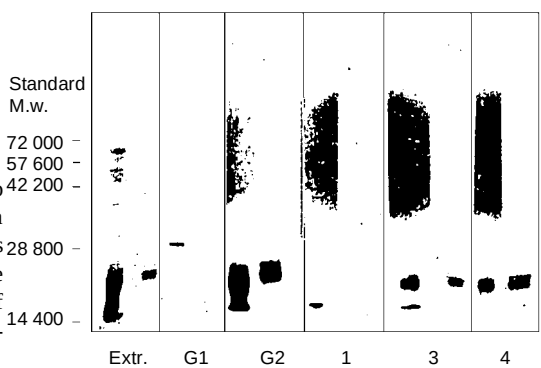

FIG. 3

SDS-PAGE of protein fractions of carp pituitaries. Water extract, fractions from gel chromatography (G1, G2), fractions from rpHPLC (1, 3, 4). – Coomasie Blue staining of gel, + immunoblot of gel using anti-β-cGtH II. Standard molecular weight markers are shown

was Tyr-Pro-Arg-Asn-Asp-Met-Asn-Asn-Phe-Gly-. Both fractions 2 and 3 proved to have two *N*-terminal residues Tyr and Ser.

The glycoprotein fractions G21 and G22 from gel chromatography were characterized by capillary electrophoresis, too (Fig. 4). This method confirmed the results obtained by SDS-PAGE and rpHPLC analysis. Fraction G22 represented a purified β-subunit of carp GtH II (fraction 4 of rpHPLC analysis). Fraction G21 was separated into two fractions, the second one being identical with that in fraction G22.

DISCUSSION

Gonadotropins were usually isolated from pituitaries of different fish species using ion exchange chromatography[4,5]. A partially purified carp gonadotropin was isolated by

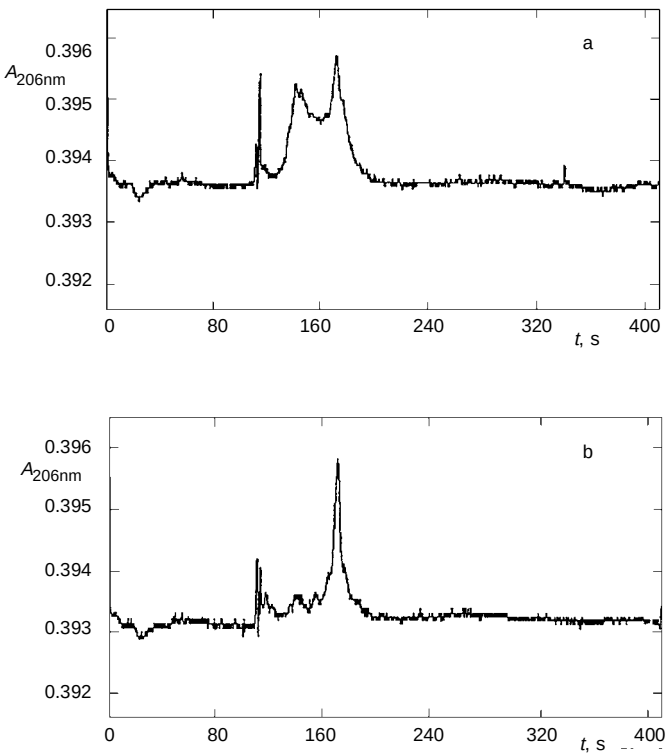

FIG. 4

Capilary electrophoresis of glycoprotein fractions of carp pituitary obtained by affinity chromatography on ConA Sepharose and gel chromatography on Superdex. a Fraction G21, b fraction G22, see Fig. 1. Conditions: capillary i.d. 0.05 mm, o.d. 0.2 mm, effective length 198 mm, total length 300 mm, sample concentration 1 mg/ml, buffer 0.04 M Tris-tricine pH 8.1, voltage 10 kV, current 13 μA

Burzava-Gerard[11] in 1971. Chang *et al.*[12] determined the amino acid sequence of the carp gonadotropin using cDNA sequenation in 1988. Van der Kraak isolated two gonadotropins from carp pituitaries[13]. The form corresponding to the previously isolated gonadotropin was named cGtH II or maturational gonadotropin, the novel form was named GtH I. However, the carp GtH I was not studied in detail.

We chose a different aproach to the isolation of carp pituitary glycoprotein hormones using affinity chromatography on ConA Sepharose as the main isolation step. The glycoprotein fraction was further divided into three fractions (G1, G21 and G22) by means of gel chromatography.

The G2 fraction obtained by gel chromatography consisted of two subunits of molecular weights 25 000 and 16 000. The 25 000 component was β subunit of so-called carp maturational gonadotropin isolated previously[11,13]. We have proved this by immunoblotting with specific anti-β-cGtH II antibody. The 16 000 component was α subunit. A sum of molecular weights of the subunits determined by means of SDS-PAGE would be 41 000, which does not coincide with the molecular weight of the G2 fraction, determined as about 30 000 by gel chromatography. A similar feature was observed in other gonadotropins[4,6]. This is assumed to be due to the glycoprotein character of the gonadotropins.

The fraction G2 was composed of two subfractions named G21 and G22. The subfraction G22 was enriched with β subunit. We suppose that partial disociation of the gonadotropin subunits occurred during the isolation procedure.

The gonadotropin subunits were finally isolated by rpHPLC which afforded highly purified α and β subunits. The other two fractions obtained in rpHPLC were identified as intact gonadotropin. We presume that the gonadotropin exists in conformational isoforms with different stability of the tertiary structure.

The only *N*-terminal amino acid sequences detected in the fractions obtained by rpHPLC of G2 fraction were the *N*-terminal amino acids of the carp gonadotropin cGtH II. The sequences we have found correspond completely to those deduced by Chang *et al.*[12] from cDNA sequenation. The residue of cysteine (X^1) cannot be identified by the method used[14]. The same is true for the asparagine residue in the position 10 (X^2), as this is a glycosylation site in the gonadotropin molecule[12].

We have not proved the presence of GtH I or other glycoprotein of carp pituitary in the G2 fraction.The homogeneity of the separated fractions was further proved by capillary electrophoresis.

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