CHICKEN PITUITARY GLYCOPROTEINS: NEW ISOLATION METHOD

Helena RYŠLAVÁ[a1], Jana KREŠLOVÁ[a2], Jana BARTHOVÁ[a3,*] and Tomislav BARTH[b]

[a] *Department of Biochemistry, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic; e-mail:* [1] *ryslava@prfdec.natur.cuni.cz,* [2] *kreslova@natur.cuni.cz,* [3] *barthova@prfdec.natur.cuni.cz*

[b] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic; e-mail: barth@uochb.cas.cz*

Received April 20, 1999
Accepted July 1, 1999

A new method for isolation of glycoproteins from chicken pituitaries was applied. The procedure consist of chromatography on ConA-Sepharose and by HPLC on S Hyper D and Vydac C4 columns. The hormonal activity of the glycoproteins was tested by determining their stimulatory effect on cAMP or testosterone production. Molecular weights of the products of tryptic cleavage of the hormone were determined using mass spectrometry (MALDI TOF). A comparison of the values obtained with theory shows that the protein is the β-unit of chicken luteinizing hormone.

Key words: Chicken pituitary; Glycoproteins; Hormones; Gonadotropins; Proteins; Mass spectrometry; Ion-exchange chromatography; HPLC.

Gonadotropins (GtH) (the follicle stimulating hormone, FSH and luteinizing hormone, LH) are hormones that control gametogenesis and gonadal steroidogenesis[1,2]. GtH together with the thyreotropic hormone belongs to the family of glycoprotein hormones. Molecular structures of GtH from different animal species are similar[3]. All the hormones are hetero dimers composed of a common α-subunit and a specific functional β-subunit[4].

We proposed new very efficient procedure for isolation of the glycoprotein hormone from chicken pituitaries consisting of affinity, reverse phase and ion-exchange chromatography. The hormone was identified by SDS electrophoresis, and by molecular weight determination (MALDI) of its tryptic cleavage products. Its hormonal activity was tested.

EXPERIMENTAL

Material

Chicken heads were obtained from RESOS, Prague, acetonitrile, trypsin were purchased from Merck, Darmstadt and lysozyme, Vydac C4, phenylmethonesulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and Medium 199 from Sigma, U.S.A. Coomasie Brilliant Blue R-250 and tris(hydroxymethyl)aminomethane (Tris), analytical grade, trifluoroacetic acid (TFA) were products of Serva, Heidelberg and Con A-Sepharose 4B, S-Hyper D 10 of Beckman Inst., U.S.A. Minicolumn C 18 was from Tessek, Prague. RIA tests cAMP and testosterone were acquired from Immunotech, France. Lysozyme and its polymers were used as molecular weight standards. Polymers of lysozyme were prepared by the method of Payne[5].

Methods

Isolation. The pituraries were excised from chicken heads and immediately placed in cold acetone. The glycoprotein fraction was extracted from acetone–dried chicken pituitaries. One gram of pituitaries was homogenized with 40 ml of homogenization medium (6% ammonium acetate, pH 5.1, 40% ethanol, 1 mM EDTA, 0.005% PMSF). The homogenate was centrifuged and the supernatant was diluted with two volumes of ethanol, the precipitate was dissolved in water and lyophilized. The extract was applied on a ConA-Sepharose column 4B (1 × 14 cm) equilibrated with the starting buffer (0.05 M Tris-HCl, pH 7.8, 0.15 M NaCl, 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$). Glycoproteins were eluted from the column with a solution of methyl-α-D-glucopyranoside in the starting buffer with linearly increasing concentration of ligand (0–500 mmol/l).

Reverse phase HPLC (RP-HPLC). Proteins were dissolved in aqueous 0.1% TFA (1–2 mg/ml), incubated 1 h at 37 °C and applied on a Vydac C4 column (0.46 × 25 cm) equilibrated with 0.1% TFA. Proteins were eluted with a solution of acetonitrile in 0.1% TFA (linearly increasing concentration 0–100%, 60 min).

Ion-exchange chromatography (IE-HPLC) was performed on a S-Hyper D10 column (4.6 × 100 mm) equilibrated with 4 mM ammonium acetate, pH 5.5. Proteins were eluted with a solution of ammonium acetate of increasing concentration (0–1 mol/l, 40 min).

The protein concentration was monitored by measuring absorbance at 280 nm and by using Lowry method[6].

SDS-PAGE. Polyacrylamide gel (15%) under reducing conditions was used[7]. Lysozyme (14 500) and its polymers were used as molecular weight markers. The proteins were stained by Coomasie Brilliant Blue R-250.

Positive ion MALDI (Matrix-assisted Laser Desorption/Ionisation) *mass spectra* were measured on a BIFLEX time of flight mass spectrometer (Bruker–Franzen, Bremen, Germany) in linear mode. The method[8] was used for the analysis of proteins purified by ConA-Sepharose and RP-HPLC chromatography. Protein solutions were mixed (1 : 1) with a matrix solution (saturated aqueous solution of sinapinic acid in 30% acetonitrile and 0.1% TFA), 1 µl of the mixture was applied to the target and dried at laboratory temperature. The method enables determination of peptide molecular weight with accuracy of one unit or even better.

Hydrolysis of proteins with trypsin. Protein zones separated by SDS-PAGE were cut out from electrophoreogram and the strips were washed with 800 µl of 100 mM Tris-HCl buffer, pH 8.1, in 50% aqueous acetonitrile for 20 min at 37 °C. The strip was then homogenized and

the homogenate was concentrated in vacuum to 20% of the starting volume. Five μg of trypsin in 100 μl of buffer (100 mM Tris-HCl, pH 8.1, 1 mM $CaCl_2$ and 10% acetonitrile) were added and the mixture was incubated overnight at 37 °C. The enzyme reaction was stopped by adding 360 μl of 2% TFA and incubated for 1 h at 60 °C. The peptides were separated using adsorption on 50 μl of silica gel C18, from which the peptides were eluted with 250 μl of 60% acetonitrile containing 0.1% of TFA. The solution was dried in vacuum and analyzed by MS-MALDI. The spectra were calibrated using monoisotopic $[M + H]^+$ ion from the peptide standard (bombesin, Aldrich).

Determination of Biological Activity

cAMP formation: 100 mg of carp or chicken gonads and different amounts of a protein fraction were incubated in 1 ml of Medium 199 for 2 h at 25 °C in an oxygen-enriched atmosphere. The protein content in tested fractions was as follows: pituitary extract 0.5–50 mg, glycoprotein fraction (from affinity chromatography) 5–20 mg and proteins adsorbed or non-adsorbed on ion-exchanger 2–20 mg. The concentration of cAMP in the solution was then determined by RIA.

Testosterone formation: 100 mg of carp gonads and different amounts of a protein fraction (see above) were incubated in 1 ml of Medium 199 for 6 h at 25 °C. The concentration of testosterone was then determined by RIA.

RESULTS AND DISCUSSION

Chicken pituitary gonadotropins have been studied by several authors[9–11]. Most of the procedures for preparation of gonadotropins consisted of fractionation of the extract by ion-exchange chromatography, thus achieving the separation of FSH from LH (refs[10–12]).

We propose a new efficient procedure for isolation of gonadotropin. According to our experience the best procedure proved to be the extraction with the solution of following composition: 6% ammonium acetate, pH 5.1, 40% ethanol, 1 mM EDTA, 0.005% PMSF. We then fractionated the extract using an S Hyper D column assuming that FSH is not adsorbed on the column. The proteins adsorbed on the ion-exchange resin were eluted with solutions of increasing ionic strength; six protein fractions were obtained (Fig. 1a). The proteins were characterized by SDS PAGE (Fig. 1b).

As it has been established that chicken GtH is a glycoprotein, we used affinity chromatography on a ConA-Sepharose column for its isolation. This method has been successfully used for purification of gonadotropins from other animal species[13,14]. Some glycoproteins were eluted from the column at low methyl glucoside concentrations (Fig. 2). A considerable portion of glycoproteins was eluted at much higher concentrations of the ligand. Glycoproteins interact with concanavalin A by means of the mannose core of their oligosaccharide chain, but the presence of another saccharide moi-

ety in the chain (*e.g.* sialic acid) is also important for the interaction[13]. The saccharide component of the glycoprotein we have prepared could be rather heterogeneous.

After affinity chromatography, the glycoproteins were separated using RP-HPLC on a Vydac C4 column; two main protein fractions were obtained (Fig. 3), molecular weight of the second fraction was 12 947 (as determined by MALDI mass spectrometry).

GtH activity was detected using an *in vitro* method. The hormone binds to receptors in the target tissues, thus activating adenylate cyclase which catalyzes the synthesis of cAMP; the process finally results in the production of steroid hormones. Individual protein fractions were incubated with target cells from carp or chicken gonads and concentrations of cAMP and testosterone were determined. Data presented in Table I were calculated from results obtained for three different amounts of tested proteins, each of them was measured three times. Table I presents one typical experiment. The highest hormone activity, as far as the stimulation of cAMP formation was concerned, was found in the pituitary extract containing a complex mixture of proteins and low-molecular-weight substances. Approximately the same activity was detected in the protein fraction prepared by ionex chromatography (Fig. 1). The effect of gonadotropins on target cells, *i.e.*, Leyding cells and seminiferous tubule cells of rat testes, was followed by Rao and Ramachandran[15] and by Dufau *et al.*[16]. Our results proved that

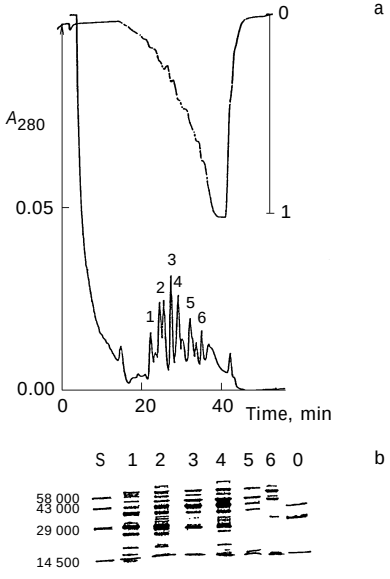

FIG. 1
Ion-exchange chromatography of chicken pituitary proteins on S-Hyper D. a: Elution profile of proteins from the column. Elution solution: ammonium acetate, pH 5.5, of increasing concentration (0–1 mol/l). Flow rate: 1 ml/min; 7 mg of proteins were applied. b: SDS-PAGE of protein fractions. S Molecular weight standard, 0 proteins not adsorbed on column, 1–6 protein fractions (Fig. 1a)

chicken gonadotropins are able to stimulate cAMP formation in chicken as well as carp gonads.

Yu *et al.*[17] investigated the biological activity and found that the active sites of avian and fish gonadotropins has a high degree of homology. The same is true for the receptor molecules in gonads. The avian luteinizing hormone stimulated testosterone or 17β-estradiol production in fish gonads. Stimulation was also observed in our experiments, albeit lower by one

TABLE I
The effect of protein fractions from chicken pituitaries on cAMP and testosterone formation in carp and chicken gonads[a]

Protein fraction	cAMP pmol/μg of protein		Testosterone ng/μg of protein
	Carp gonads	Chicken gonads	Carp gonads
Extract	31.6	23.6	0.2
From affinity chromatography	3.7	12.2	0.2
Unadsorbed proteins from IE chromatography	3.5	13.5	–
Adsorbed proteins from IE chromatography	9.2	29.6	0.03

[a] In all experiments, 100 mg of gonad tissue were used.

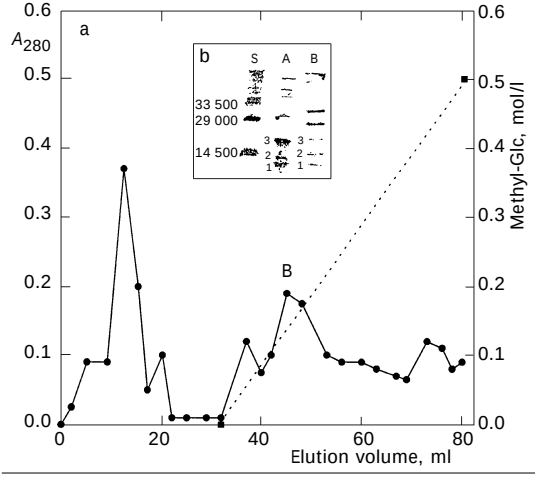

FIG. 2
Chromatography of chicken pituitary proteins on Con A-Sepharose 4B column. a: Elution profile of proteins from the column. Buffer system: 0.05 M Tris-HCl, pH 7.8, 0.15 M NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$. Flow rate: 0.25 ml/min; 10 mg of proteins were applied. b: SDS-PAGE of chicken pituitary glycoproteins. S Molecular weight standard, A proteins extracted from pituitaries with ammonium acetate, B glycoproteins isolated by affinity chromatography

order of magnitude. Testosterone may have been further metabolised to estrogen in our experimental conditions.

The glycoproteins prepared by chromatography on a ConA-Sepharose column were then separated by SDS-PAGE (Fig. 2-inset). Three fractions, the molecular weight of which corresponded to that of chicken GtH (proteins marked 1–3 in Fig. 2b), were hydrolyzed with trypsin. Molecular weights of the peptides formed were determined by mass spectrometry (MALDI TOF). All three protein fractions analysed contained peptides of m.w. 1 937.0; 2 163.1 and 2 510.3. Molecular weights of the three peptides in the hydrolyzates correspond well with the theoretical molecular weights of peptides generated by the β-unit of chicken LH.

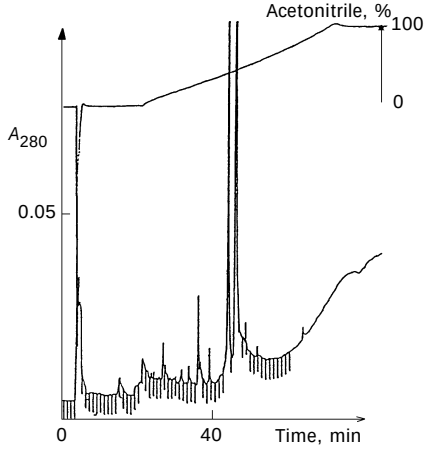

FIG. 3
RP-HPLC of chicken pituitary glycoproteins on a Vydac C4 column. Elution: 0.1% aqueous solution TFA containing increasing concentration of acetonitrile. Flow rate: 0.7 ml/min; 100 μg of proteins were applied

Suported partly by Ministry of Education of the Czech Republic (grant No. VS 96141).

REFERENCES

1. Bonsfield G. R., Butnev V. Y., Gotschalll R. R., Baker V. L., More W. T.: *Mol. Cell Endocrinol.* **1996**, *125*, 3.
2. Goudeon A.: *Endocr. Rev.* **1996**, *17*, 121.
3. Koide Y., Papkoff H., Kawauchi H.: *Eur. J. Biochem.* **1996**, *240*, 262.
4. Pierce J. G., Parsons F. T.: *Annu. Rev. Biochem.* **1981**, *50*, 465.
5. Payne J. V.: *Biochem. J.* **1973**, *135*, 867.
6. Lowry H. O., Rosebrough N. J., Farr A. L., Randall R. J.: *J. Biol. Chem.* **1951**, *193*, 256.
7. Laemli U. K.: *Nature* **1970**, *227*, 680.

8. Otto A., Thiede B., Müller E. C., Scheller C., Wittman-Liebold B., Jungblut P.: *Electrophoresis* **1996**, *17*, 1643.
9. Stockell Hartree A., Cunningham F. J.: *J. Endocrinol.* **1969**, *43*, 609.
10. Godden P. M. M., Scanes C. G.: *Gen. Comp. Endocrinol.* **1975**, *27*, 538.
11. Sakai H., Ishii S.: *Gen. Com. Endocrinol.* **1980**, *42*, 1.
12. Talbot R. T., Sharp P. J., Harvey S., Williams J. B., Dunn I. C., Sterling R. J., Bahr J. M.: *Br. Poult. Sci.* **1988**, *26*, 81.
13. Dufau M. L., Tsuruhara K. J., Catt K. J.: *Biochim. Biophys. Acta* **1972**, *278*, 281.
14. Hulová I., Barthová J., Ryšlavá H., Kašička V.: *Collect. Czech. Chem. Commun.* **1998**, *63*, 434.
15. Rao A. J., Ramachandran J.: *Life Sci.* **1975**, *17*, 411.
16. Dufau M. L., Tsuruhara T., Horner K. A., Podesta E., Catt K. J.: *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 3419.
17. Yu J. Y. L., Shen S. T., Liu C. T., Weng F. C., Peng H. K, Liu F. G.: *Aquaculture* **1995**, *135*, 59.