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SEPARATION OF SUBUNITS OF PIKE EEL GONADOTROPIN BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

Subunits of pike eel gonadotropin were dissociated in propionic acid and separated by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B with a high yield (79%). Isolated subunits were homogeneous, as tested by sodium dodecyl sulphate disc electrophoresis and by N-terminal analysis. The biological activities of two subunits, S-I and S-II, were 0% and 2.8% of native molecule, respectively, as assayed by stimulation of androgen production on carp testes. Reassociated molecules restored about 75% activity.

INTRODUCTION

It is well known that mammalian gonadotropins exist in the form of two dissimilar subunits. The dissociation of subunits could be achieved by incubation in 8–10 *M* urea or guanidine hydrochloride, 8 *M* urea in 0.05 *N* hydrochloric acid or in 1 *M* propionic acid. The dissociated subunits may be separated by counter-current distribution, fractional precipitation by salt or ethylene glycol, gel filtration, ion-exchange chromatography in the presence of a dissociation agent, affinity chromatography using specific antisera to the subunit or by hydrophobic interaction chromatography.

Recently, pike eel gonadotropins (pGTH) have been isolated and purified in our laboratory and it has been found that pGTH exists in the form of two subunits, as observed by sodium dodecyl sulphate (SDS) disc electrophoresis and N-terminal amino acid analysis[1]. At present the knowledge of piscine gonadotropins is still fragmentary. In addition to pGTH, carp gonadotropin (cGTH) has been isolated and its subunits have been dissociated either by 1 *M* propionic acid or 8 *M* urea and separated by ion-exchange chromatography on DEAE-cellulose[2]. When pGTH was subjected to dissociation and separation as described for cGHT, it gave very low recoveries of two subunits (12–15%).

This paper describes a procedure for isolation of two subunits of pGTH in high yield and purity by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B.

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MATERIALS AND METHODS

Materials

The purified preparation of pGTH used was fraction DE-80 as described in a previous paper[1]. Phenyl-Sepharose CL-4B and octyl-Sepharose CL-4B were purchased from Pharmacia (Uppsala, Sweden) and propionic acid was a product of Junsei Chemical Co. (Tokyo, Japan). Phenyl isothiocyanate, dimethylaminoazobenzene isothiocyanate and other reagents for N-terminal analysis were obtained from Pierce (Rockford, IL, U.S.A.).

Dissociation of pGTH

Prior to separation of subunits, pGTH was dissociated in 1 *M* propionic acid at a concentration of 4 mg/ml for 24 h at 35°C. After incubation, propionic acid was removed by lyophilization.

Separation of subunits

Equilibration and regeneration of phenyl-Sepharose CL-4B were performed as suggested by the manufacturer. Briefly, the gel was washed with one bed volume of distilled water, followed by one bed volume of ethanol, and then washed with twice the bed volume of *n*-butanol. After rewashing successively with one bed volume each of ethanol and distilled water, the gel was equilibrated with 0.1 *N* acetic acid (starting buffer). The lyophilized dissociated pGTH (9.4 mg) was dissolved in 0.1 *N* acetic acid and applied to an equilibrated phenyl-Sepharose CL-4B column (13 × 1.8 cm I.D.). The elution was started with 0.1 *N* acetic acid and followed by 0.1 *M* ammonium acetate at a flow-rate of 15 ml/h. The protein content was monitored by the absorbance at 280 nm. Each fraction was pooled and lyophilized.

Reassociation of subunits

Equal amounts of subunits (at a final concentration of 2 mg/ml) were incubated in 0.05 *M* sodium phosphate buffer (pH 7.4) at 25°C for 24 h. At the end of incubation, the mixture was quickly frozen with dry-ice and stored in a freezer for further characterization.

Characterization of subunits

The homogeneities of various preparations were tested by SDS polyacrylamide disc electrophoresis in the presence of 1% β-mercaptoethanol[3]. Amino acid analysis was carried out essentially according to the accelerated procedure of Spackman *et al.*[4] with a Yanako LC-5A or a Jeol 6AH amino acid analyser after hydrolysis with 6 *N* hydrochloric acid containing 0.2% phenol at 110°C for 24 h in an evacuated sealed Pyrex tube.

N-Terminal amino acid analysis was performed by the Edman method[5] with high-performance liquid chromatographic identification[6] and by the dimethylaminoazobenzene thiohydantoin (DABTH) method with polyamide thin-layer chromatographic identification[7].

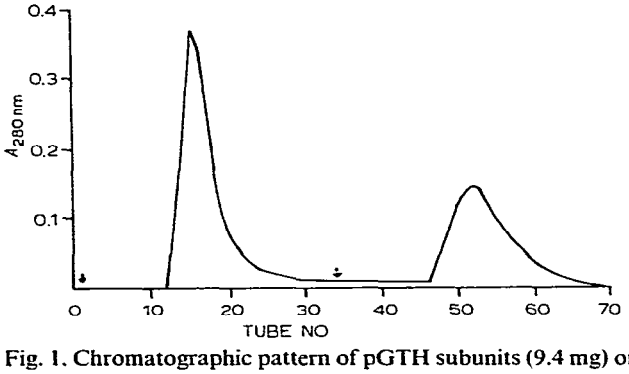
Bioassay of pGTH activity was carried out by stimulation of androgen production on carp testes *in vitro*[8].

RESULTS AND DISCUSSION

The separation of dissociated subunits of pGTH by fractional precipitation or gel filtration has not been successful. Although ion-exchange chromatography on a DEAE-cellulose column can separate two subunits with 0.05 and 0.12 *M* ammonium hydrogen carbonate containing 4 *M* urea, the yield is not more than 15%. Recently, α- and β-subunits of human pituitary thyrotropin have been successfully separated by hydrophobic interaction chromatography on pentyl-Sepharose-4B[9].

The separation of pGTH subunits by hydrophobic interaction chromatography on a phenyl-Sepharose CL-4B column was very successful, as shown in Fig. 1. The starting buffer, 0.1 *N* acetic acid, desorbed one subunit, S-I, and another subunit, S-II, was eluted by 0.1 *M* ammonium acetate. The recoveries of S-I and S-II were 3.9 and 3.5 mg, respectively, from 9.4 mg of pGTH (about 79%).

The SDS polyacrylamide disc electrophoretic patterns of S-I, S-II and native pGTH are shown in Fig. 2. Based on this electropherogram, complete separation of subunits was achieved by one-step hydrophobic interaction chromatography on phenyl-Sepharose CL-4B. It is known that the subunits are usually held together by non-covalent forces such as hydrophobic, ionic and hydrogen bonds. In the present work, the ready dissociation of pGTH in propionic acid suggests that hydrophobic



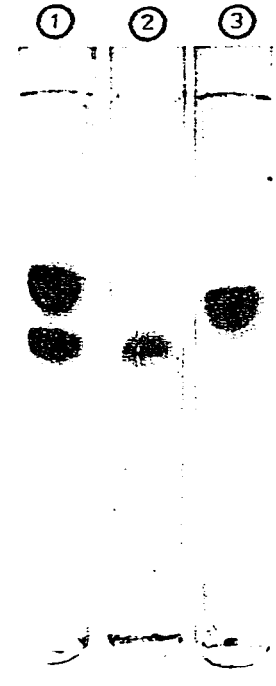


Fig. 1. Chromatographic pattern of pGTH subunits (9.4 mg) on a phenyl-Sepharose CL-4B column (13 × 1.8 cm I.D.). S-I (3.9 mg) was eluted with 0.1 *N* acetic acid (left arrow) and S-II (3.5 mg) was desorbed with 0.1 *M* ammonium acetate (pH 6.8) (right arrow). The flow-rate was 15 ml/h and 2.3 ml per tube were collected.

Fig. 2. SDS gel electrophoresis of pGTH and subunits. 1 = Native pGTH; 2 = S-I; 3 = S-II.

bonds may be of great importance in stabilizing the native pGTH molecule, which may be the reason why the separation of subunits was so successful on phenyl-Sepharose CL-4B.

When octyl-Sepharose CL-4B was used, no protein component was eluted under the same conditions. It is obvious that the octyl group is more hydrophobic than the phenyl group and selective desorption of two subunits of pGTH cannot be achieved in octyl-Sepharose CL-4B under the condition employed.

As the hydrophobic bonds are concluded to be major non-covalent forces stabilizing native pGTH from the above experiments, the direct separation of subunits on phenyl-Sepharose CL-4B was tried without prior incubation in propionic acid. The elution pattern was similar to Fig. 1, but only partial separation of two subunits was achieved. This indicated that phenyl-Sepharose CL-4B could dissociate subunits of pGTH by hydrophobic interaction.

Further chemical and biological characterization of subunits S-I and S-II and the reassociated molecule will be published elsewhere[10], but preliminary results are given in Table I. S-I showed no biological activity whereas S-II showed about 2.8% of native pGTH activity as assayed by stimulation of androgen production on carp testes *in vitro*. It is difficult to say whether it is caused by contamination or by intrinsic activity of S-II at present. The reassociated molecule restored about 75% of native pGTH activity.

TABLE I

PROPERTIES OF pGTH AND SUBUNITS

Amino acid	*Residues per 100 residues**		
	pGTH	*S-I*	*S-II*
Lys	5.4	6.3	3.8
His	2.6	3.0	1.8
Arg	6.3	4.7	5.8
Asx	9.4	9.6	9.6
Thr	7.8	6.9	7.3
Ser	9.0	6.5	9.4
Glx	8.4	10.5	9.1
Pro	10.7	7.9	11.1
Gly	3.1	3.9	2.6
Ala	3.0	4.7	2.7
½ Cys	9.7	11.2	9.1
Val	5.5	5.0	8.6
Met	1.7	1.7	1.6
Ile	3.4	3.8	4.1
Leu	6.1	5.8	6.7
Tyr	3.9	3.6	4.2
Phe	4.0	5.1	2.8
Biological activity**	100%	0%	2.8%
N-Terminus	Tyr Ser	Tyr	Ser

* Tryptophan not determined.

** Assayed by androgen production of carp testes *in vitro*.

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