Purification of a pituitary gondotropic hormone from a teleost fish, carp (Cyprinus carpio L.)

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PURIFICATION OF A PITUITARY GONADOTROPIC HORMONE FROM A TELEOST FISH, CARP (Cyprinus carpio L.)

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Summary. -- A method of purification for a pituitary gonadotropin from a teleost fish (Cyprinus carpio L.) is described. The specific activity is 1.5 mg LH-NIH-S-1/mg measured by the frog spermiation test. The study concerning the homogeneity of this preparation leads us to suggest a quaternary structure.

The purification of pituitary stimulants which control the reproductive function in Vertebrates has been the object of extensive studies, especially with relation to Mammals (beef, sheep, pig, rat, man).

Different authors [1, 2] have been able to obtain the luteinizing hormone to a high degree of purity in various species. The same obtains for the folliculo-stimulant hormone (FSH) [3, 4], although in the latter, the values of the different chemical characteristics present much greater variability than for the LH [5], allowing one to suppose that
the state of purity of FSH might be less satisfactory than for LH.

Comparable work has been carried out by HARTREE on a Bird, the chicken.

Among the lower Vertebrates, purifications of gonadotropic factors have been undertaken on a small number of species. OTSUKA was the first with an attempt to fraction the pituitary bodies of Salmon. BRETON purified a gonadotropic fraction starting with pituitary bodies of Roach. YAMAZAKE and DONALDSON studied the action of a hypophyseal gonadotropin purified from Salmon on hypophysectomied Carp.

We previously described the purification of a gonadotropic fraction present in the pituitary body of Carp whose specific activity determined by the frog spermiation test was elevated to $0.3 \text{ mg LH-NIH-S-1/mg}$ (*). The first results

(*) The dosages were realized at a time of year (October to February) when the sensitivity of the Frog to LH is greatest

$$D_{50} = 1.8 (0.9-3.8) \mu g$$

Here we describe a purification technique which led us to the preparation of a highly purified fraction of specific activity equal to $1.5 \text{ mg LH-NIH-S-1/mg}$ (*). The first results
concerning the study of the homogeneity of this fraction are also described.

MATERIAL AND METHODS

The separation material used for this study is an acetone powder of Carp pituitary bodies furnished by the Stoller fisheries (Spirit Lake, Iowa, U.S.A.).

The frog spermiation test \( I_{27} \) was used to follow the activity during preparation as was previously described \( I_{107} \).

We have used the following techniques:

- alcoholic percolation method of BATES et al. \( I_{137} \).
- columnar chromatography according to the method described by SOBER et al. \( I_{147} \).
- filtration through Sephadex G100 and G50 according to FLODIN and KILLANDER \( I_{157} \).
- analytical electrophoresis on polyacrylamide gel according to ORNSTEIN \( I_{167} \) (Canalco apparatus).
- preparatory electrophoresis on polyacrylamide gel according to ORNSTEIN in which the preparatory system had been developed by JOVIN et al. \( I_{177} \) (Buchler apparatus).
- ultracentrifugation at sucrose gradient according to MARTIN and AMES \( I_{187} \) and applied under the conditions described by FONTAINE and CONDLIFTE \( I_{197} \). In the present
case we have used the Spinco Mod. L/L2 ultracentrifuge.

Centrifugation is continued for 15 hours at 65,000 rpm.

Fractions of 0.133 ml (10 drops) are recovered and the protein dose is realized / for each fraction by the method of

TABLE I

Purification Procedure

Alcohol percolation
  ↓
Soluble fraction for 57 per cent ethanol
  ↓
2 per cent NaCl
  ↓
Concentration
Filtration through Sephadex G50  passage from buffer
  ↓
tri-HCl  pH 7.8
0.05 M in tri
temp. = 4°C
  ↓
Fraction not delayed
Chromatography on DEAE-C  equilibrium in buffer
  ↓
tri-HCl  pH 7.8
0.05 M in tri
temp. = 4°C
  ↓
Active fraction elected by a gradient of ionic force
(buffer/NaCl 0.4 M) for a conductivity comprised between
3.1 and 3.9 mMHO.
Concentration

**Filtration through Sephadex G100** in buffer trisphosphate

- pH 7.2
- 0.05 M in tr
- temp. = 4°C

Active fraction elected for a coefficient of exclusion $K_D$ comprised between 0.26 - 0.30.

**Preparative electrophoresis on polyacrylamide gel**

- Migration toward the anode in buffer triglycine pH 10.3
- intensity $I = 40mA \ V=200\ volts=4°C$

Active fraction for $Rf = 0.54$

**Concentration**

**Filtration through Sephadex G100** in buffer bicarbonate of ammonium $NH_4HCO_3$

- 0.01 M temp. = 4°C

Active coefficient peak of exclusion $K_D = 0.28$

Lycophilization

LAWRY et al. [20] by means of the Technicon automatic analyser [21]. The experimental procedure followed for purification is summarized in Table I.
TABLE II

Schedule of Recoveries in Proteins and Activity during the Principal Stages of Purification starting from One Gram of Raw Pituitary Powder.

<table>
<thead>
<tr>
<th>Stages</th>
<th>Designation of Fractions</th>
<th>Proteins (mg)</th>
<th>Specific Activity mg LH-NIH-S-1/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>separated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pituitary powder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>percolation</td>
<td>57% ethanol</td>
<td>198</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>2% NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>elected for a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-C</td>
<td>resistivity</td>
<td>29.2</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>between 3.1-3.9 mMHO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>elected for</td>
<td></td>
<td>24.8</td>
<td>0.50</td>
</tr>
<tr>
<td>Gl00</td>
<td></td>
<td>K_D = 0.32</td>
<td></td>
</tr>
<tr>
<td>Fraction A</td>
<td></td>
<td>3</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Electro-</td>
<td>B</td>
<td>8.6</td>
<td>0.87</td>
</tr>
<tr>
<td>phoresis</td>
<td>C</td>
<td>5.2</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>7.3</td>
<td>0.65</td>
</tr>
</tbody>
</table>
RESULTS

I. -- Preparation

We have summarized in Table II the schedule of recoveries in proteins and activity throughout the principal stages of purification.

1 -- By the alcohol percolation method we were able to extract the gonadotropic fraction by solutions saturated in NaCl and containing 76 to 57 per cent ethanol. This method permits in one single stage the obtaining of a fraction representing only 20 per cent of the separation material and whose specific activity is 0.20 mg LH-NIH-S-1/mg with a yield comprising between 70 and 90 per cent.

2 -- Chromatography on the exchange of cellulose ions DEAE-C (fig. 1) constitutes the second stage of purification. The yield in proteins is, according to the experiments, comprised between 50 and 60 per cent and we obtain a yield of activity of the same order. The TSH for these conditions of buffer and pH can only be partially separated from the gonadotropic fraction \[ \frac{22}{22} \]. In the case of the Eel, by chromatography on CM-C one can separate with a satisfactory yield the thyrotropic hormone from the gonadotropic hormone \[ \frac{23}{23} \].

We have applied analogous conditions to Carp: the chromatography
on CM-C is realized in citrate-phosphate buffer pH 5.8 (0.04 M in \( \text{PO}_4^3 \)). The adsorbed proteins are elected by NaCl M. The research into activities of the adsorbed and unadsorbed

![Chromatography on DEAE-C](image)

**Fig. 1.** -- Chromatography on DEAE-C of the active fraction obtained from the fraction 57 per cent ethanol - 2 per cent NaCl of alcohol percolation after G50.

Column 22 x 2 cm.

Buffer Tri-HCl 0.05 M in tri-pH 7.8.

Linear gradient of election.

++---+ Proteins (optic density 276 nm).

\( \cdots \cdots \) Gonadotropic activity in terms of LH-NIH-S-1 (frog spermiation test).

\( \dagger \) Activity is equal to or less than this value.

\( \land \cdots \land \) Thyrotropic activity (radioactive iodine fixation \( \text{I}^{131} \text{in Trout } \sqrt{227} \)).

----- Conductivity mMHO.
fractions show that 70 per cent of gonadotropic activity remains unadsorbed but that the thyrotropic activity is equally present in the two fractions. Chromatography on CM-C consequently has not been retained.

During the different preparations effected, we have conserved, after the chromatography on DEAE-C, the proteins elected in the two first tiers of the peak of activity, thus sacrificing the yield in activity for a less great contamination from TSH.

\[ \text{Separation} \quad \text{Bromophenol front} \]

FIG. 2 — Analytical electrophoresis on polyacrylamide gel of the fraction obtained before preparative electrophoresis.

Buffer Triglycine pH 8.3 \( 0.005 \text{ M in tri.} \)

Duration \( 1\frac{1}{2} \text{ hours.} \)

Intensity \( I = 2.5 \text{ mA.} \)

The protein bands are revealed by Amido-Schwartz.
The active fraction recovered represents only about 40 per cent of the separation activity. The specific activity is then 0.34 mg LH-NIH-S-1/mg.

3 -- Purification is followed by filtration through Sephadex G100 after concentration of the preceding fraction. The election diagram shows the presence of two peaks whose coefficients of exclusion are respectively $K_1 = 0.28$ and $K_2 = 0.40$. The biological activity is associated with the proteins first elected.

Analytical electrophoresis through polyacrylamide gel of the fraction obtained after G100 proves the heterogeneity of this fraction. One can see three bands, each defined by its separation coefficient ($R_f$: 0.80; 0.54 and 0.50) (fig. 2).

4 -- In order to continue purification, we have applied a preparative electrophoresis on polyacrylamide gel (fig. 3). This procedure permits good separation of the more electro-negative fraction, but that of the two very close $R_f$ bands is much more difficult to obtain. We observe, during the different electrophoreses carried out, the existence on the elective diagram of a shoulder at the end of the protein peak. The maximum of the biological activity is found again in the first part of the peak corresponding to the elected proteins for a mean separation coefficient of 0.54.
FIG. 3. — Preparative electrophoresis on polyacrylamide gel of the active fraction after G100. The progression is toward the anode, the front is shown by bromophenol in buffer tri-glycine 0.36 M in tri-pH 10.3.

\[ V = 200 \text{ volts at the beginning of the experiment} \quad I = 40 \text{ mA, kept constant.} \]

--- Concentration gel column \( h = 2 \text{ cm.} \) (1.25% monomeric acrylamide).

--- Resolution gel column \( h = 3.8 \text{ cm.} \) (7.5% monomeric acrylamide).

--- At the foot of the column the proteins are carried in tri-HCl buffer (0.1 M in tri) pH 8.1 to one output of 26 ml/h.

\[ \text{Optic density of proteins 276 mp.} \]

\[ \text{Biological activity in terms of LH-NIH-S-11.} \]

\[ \uparrow \text{ All frogs respond to the injected dose.} \]

\[ \uparrow \uparrow \text{ No frogs respond to the injected dose.} \]
(*) Optic density after election of a protein peak does not return to 0.00 because of the inclusion of certain particles of gel adsorbed at 276 μM.

The latter will be eliminated in the gel peak during the filtration through Sephadex which follows.

The most active fraction (C) is, after concentration, filtered through Sephadex G100 in buffer NH₄HCO₃ 0.1 M (fig. 4). The protein peak corresponds to a mean coefficient of exclusion $K_D = 0.28$. The specific activity of the lycophilized powder is 1.5 mg LH-NIH-S-1/mg. Fractions (A), (B) and (D) are equally preserved lycophilized after filtration through Sephadex G100 in buffer NH₄HCO₃. Fraction (A), alone, does not possess any biological activity and is elected for an exclusion coefficient of $K_D = 0.40$.

II. -- Study of the homogeneity of the active fraction.

The state of homogeneity of fraction (C) possessing the strongest activity has been studied by ultracentrifugation in a sucrose gradient and by analytical electrophoresis on polyacrylamide gel.

-- The ultracentrifugation of fraction (C) is represented graphically (fig. 5). Shoulder E appeared, suggesting the
FIG. 4 -- Filtration through Sephadex G100 of fraction (C) after preparative electrophoresis on polyacrylamide gel in buffer $NH_4HCO_3$ 0.01 M.

Column $h = 48$ cm.

d = 2 cm.

+--++ Protein optic density at 276 mμ.

o----o Conductivity mΩH0.
presence in the preparation of contaminating proteins of very slight molecular weight. Relative to the pepsinogen, chosen as the standard in the ultracentrifugation experiment, the rate of sedimentation respectively of the principal protein peak and shoulder E are 2.2 and 1.3 corresponding to values around 27,000 and 15,000 for the molecular weights. We have sought to which fraction of the preparative electrophoresis this shoulder E corresponded. The recoveries of the different fractions by filtration through Sephadex G100 have shown us that only fraction (A) presented, on the one hand, the same coefficient of exclusion and on the other hand, the same sedimentation rate (fig. 5) as shoulder E.

--- We have effected analytical electrophoresis of fractions (A) and (C) (fig. 6). We observe that fraction (A) leads effectively to the most electronegative Rf band = 0.80. As for fraction (C), we find again a coloured band for a mean Rf of 0.54, but in addition, the Rf band = 0.80 is always present when the separation of fractions (A) and (C) is obtained at the time of preparative electrophoresis.

All of these results suggest that a certain proportion of the molecules of fraction (C) would be transformed in (A)
FIG. 5. -- Ultracentrifugation on sucrose gradient (15 hours at 65,000 rpm), Spinco Model L/L2 Rotor SW 65 centrifuge.

-- Proteins (D.O. at 750 μμ after Lowry-Folin reaction on a Technicon autoanalyser).
FIG. 6. -- Analytical electrophoresis on polyacrylamide gel of fraction (A) and (C) lyophilized after G100.

Buffer Triglycine pH 8.3 0.005 M in tri

Duration 1½ hours

Intensity I = 2.5 mA

The bands of proteins are revealed by Amido-Schwartz.
FIG. 7. -- Ultracentrifugation on sucrose gradient (15 hours at 65,000 rpm), Spino Mod. L/L2 Rotor SW 65 centrifuge).

Buffer pH 2.2 (HCl 0.01 M - KCl 0.01 M).

Buffer pH 7.4 (phosphate NaCl 0.002 M - NaCl 0.1 M).

Buffer pH 10.6 (NaOH 0.01 M - Glycine 0.01 M).

-- Proteins (D.O. 750 mp after Lowry-Folin reaction by means of the Technicon analyser).
with loss of biological activity. In order to verify this hypothesis we realized ultracentrifugations at differing pH in order to determine if one complete transformation from (C) to (A) could be obtained. The results are shown in Figure 7.

We observe that each degradation is not provoked when the protein is incubated at pH 7.4 and 10.6, but at pH 2.2 a degradation of the protein into elements of different molecular encumbrance appears. We find again a rate of sedimentation of 1.5 very close to that of fraction (A).

**DISCUSSION**

The purification method which we have perfected for separating a raw pituitary extract from Carp has led us to the separation of a fraction of elevated specific activity. The latter is close to those obtained for the LH of Mammals [24].

The chromatographies on DEAE-C and CM-C point out the acid character of this fraction and show the existence of an important difference as opposed to the same hypophyseal extracts of Mammals [25]: passage through CM-C does not lead to the dissociation of the gonadotropic activity in two hormones of distinct biochemical characters. Moreover, a
more selective separation method like preparative electrophoresis no longer separates it into two different active fractions. These facts tend to confirm the hypothesis of the protein uniqueness of the gonadotropic fraction of the pituitary body of Carp 10, 11.

Comparable work has been attempted in other species of teleost fish. BRETON 8 described the purification of a gonadotropic fraction of Roach using the method applied by HERMIER 3 for the preparation of an FSH from Sheep. The biological activity of this fraction is studied by different tests of the gonadotropic activity of lower Vertebrates: the latter, no matter what test is used, is found again in the fraction corresponding to the FSH of Sheep with a very small yield in activity; moreover, filtration through Sephadex G100 of the raw hypophyseal powder from Roach leads to the election of a unique peak of gonadotropic activity.

YAMAZAKI and DONALDSON 8 have studied the gonadotropic activity of a fraction purified from Salmon pituitary bodies on hypophysectomied Carp. The gonadotropic fraction was extracted by precipitation in alcohol at 40°, then precipitated with 85%; alcohol. Purification is completed by two successive passes through Sephadex G100. These authors put forth equally
the hypothesis for the existence of a single pituitary
gonadotropic hormone in the Pacific salmon Oncorhyncus
tschrwytscha.

The comparison of these gonadotropic preparations
derived from hypophyseal extracts from different species
of teleost fish is made difficult on the one hand because
of the different biological doses which are used and the
absence of a standard gonadotropic hormone active in all
fish, and on the other hand because the biochemical charac-
teristics of these fractions are not known.

The results of the study of the state of purity of our
gonadotropic preparation suggest that the observed hetero-
geneity does not seem to convey the existence of two independent
protein entities but corresponds rather to a transformation
of the active molecule (C) into an inactive molecule (A).
Various hypotheses might account for this transformation.

Electrophoresis can cause a denaturing of the active
molecule. The FSH of Sheep in particular loses 70 per cent
of its activity after electrophoresis on starch or Pevikon.
This inactivation is mainly attributed to a denaturing of the
molecule \( \sqrt{3} \). Also, lyophylization in a volatile buffer such
as bicarbonate of ammonium causes a strong diminution of the
specific activity of this latter hormone. Preparative electrophoresis that we have realized does not cause loss of notable activity; we find again 80 to 90 per cent of the activity of separation.

ORNSTEIN evokes the possibility that a dissociation of certain enzymatic or hormonal proteins might be produced under the influence of the difference of increased potential which exists at the moment of the passage into the gel of concentration. A like possibility appears to account better for the facts observed, the molecular weight of the inactive fraction (A) is two times weaker than that of fraction (C); the incubation of active fraction (C) at pH 2.2 causes the appearance of a protein of different molecular encumbrance whose rate of sedimentation is now equal to that of inactive fraction (A). Similar studies for ovine LH have led to the demonstration of a quaternary structure for LH. Recent results have shown that folliculo-stimulant and thyro-stimulant hormones possess equally the same quaternary structure, be it the association of two sub-units in one dimere possessing the biological activity.

In the particular case of the gonadotropic hormone of a Fish, the Carp, the existence of a quaternary structure...
analagous to that of pituitary glycoprotein hormones of Mammals can be envisaged. The heterogeneity observed on the different tests used would not reflect the presence of an impurity but rather the existence of a dissociation of one part of the native molecules possessing biological activity.

Acknowledgements

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We wish also to thank Mr. Y. A. FONTAINE for the support he gave us toward the realization of this work.

SUMMARY

A purification method leading to the preparation of a gonadotropic hormone from the pituitary body of a teleost fish, Carp (Cyprinus carpio L.) is described. The specific activity of the final powder, measured by the frog spermiation test, is 1.5 mg LH-MIH-S-l/mg. The study of the homogeneity of the purified fraction suggests the existence of a quarternary structure for this hormone.
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