THE ISOLATION OF BOVINE THYROTROPHINS BY ISOELECTRIC FOCUSSING

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1. Introduction

No adequately characterized homogeneous pituitary thyrotrophin (TSH) preparations have yet been described, although several highly purified materials have been reported [1–3]. All current preparations are polydisperse when examined by gel electrophoresis at pH 8–9. The individual components appear to be very similar in their properties, and several appear to be biologically active, although the ratio of activities has never been determined precisely. No method is available for the preparation of the components in amounts sufficient for their characterization. The present paper reports the isolation of four biologically active thyrotrophins from an extract of bovine anterior pituitary glands by the method of isoelectric focussing [4] in the pH region 8.2–8.8.

2. Methods

TSH concentrates were prepared from acetone-dehydrated bovine anterior pituitary glands by the method of Dedman, Fawcett and Morris [3] which involves extraction at pH 8 and chromatography on Amberlite IRC-50 and CM-cellulose. The specific activities were in the range 15–20 units/mg. TSH activity was determined by the *in vitro* ^{131}I discharge method of Brown and Munro [5], adapted for rat thyroids.

Isoelectric focussing was carried out in the LKB 8100 apparatus at 4° at 1–2 watts for 24–36 hr. The pH gradient was produced with LKB Ampholine, pH range 7–10, and zone stability was ensured with a 0–50% sucrose density gradient. On completion of zone focussing, 1 ml fractions were collected from the apparatus and absorption at 280 nm and pH measured on each fraction. Suitable fractions were then recombined for re-focussing, which was carried out under identical conditions. Appropriate zones were freed from sucrose and Ampholine by adsorption on Amberlite IRC-50, elution with 1 M NaCl, desalting on a granulated 11 X 3 polyacrylamide gel column and freeze dried.

Analytical zone electrophoresis was carried out on 2 mm thick slabs of 8 X 3 polyacrylamide gel in a new discontinuous cathodic migration buffer system (Morris, unpublished). This system uses a potassium phosphate buffered gel with a running pH of 6.0 and a histidine phosphate electrode buffer pH 6.7. It gives better resolution of the TSH components than either anodic migration at pH 9 or cathodic migration at pH 4.3.

Mannose, fucose, glucosamine and galactosamine were determined after appropriate acid hydrolysis by reduction of alkaline triphenyltetrazolium chloride. The individual sugars were separated by thin-layer chromatography on Eastman K 511 V polycarbonate-coated sheets by the method of Moczar, Moczar, Schillinger and Robert [6].

3. Results

A typical isoelectric fractionation of a TSH concentrate prepared by CM-cellulose chromatography [3] is shown in fig. 1a. Four major biologically active components, α, β, γ and δ thyrotrophins, as well as

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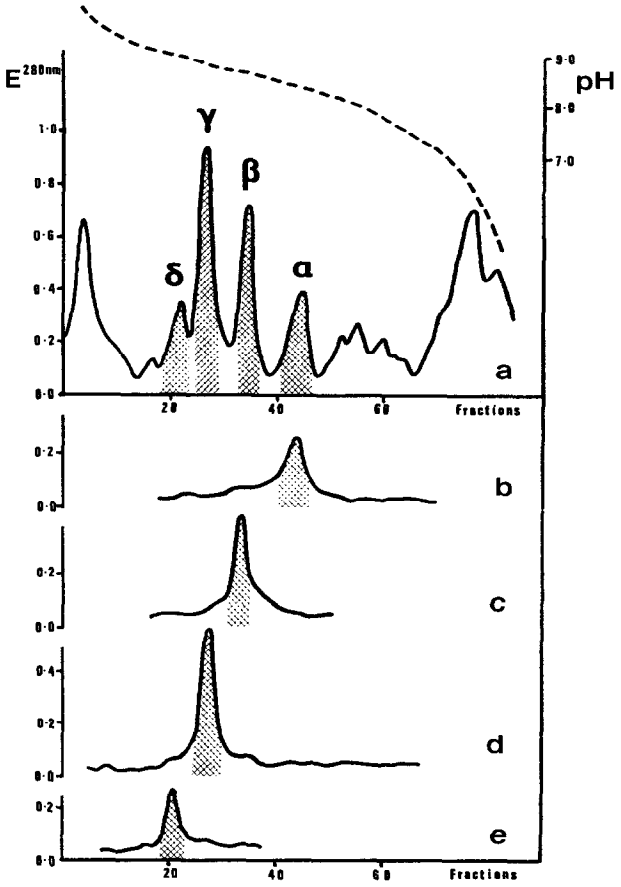

Fig. 1a. Isoelectric focussing of a TSH concentrate at pH 7–10. b. Re-focussing of α thyrotrophin. c. Re-focussing of β thyrotrophin. d. Re-focussing of γ thyrotrophin. e. Re-focussing of δ thyrotrophin. Hatched areas indicate fractions combined for re-focussing or recovery.

several inert or very weakly active fractions are present. The mean values of the isoelectric points of the various components (measured at 25°) were: α pH 8.25–8.30, β pH 8.55–8.60, γ pH 8.65–8.70, δ pH 8.8. The further purification of the active components by repeated isoelectric focussing is shown in fig. 1b–e. Some properties of the isolated components are collected in table 1. The biological activities are expressed in terms of the International Thyrotrophin preparation with the fiducial limits at $P = 0.95$. Exophthalmogenic activity (Dedman, Fawcett and Morris [7]) was demonstrated in α thyrotrophin, the only component examined. The δ component was obtained in insufficient amount to permit characterization, although its TSH activity was confirmed.

Table 1
Properties of bovine thyrotrophins.

Substance	α	β	γ
TSH potency	30.5 (24–39)	21 (11–39)	63 (43–90)
Mannose %	6.2	4.2	4.5
Fucose %	0.70	0.93	1.03
Glucosamine %	6.75	5.1	7.35
Galactosamine %	3.8	3.8	3.1

A comparison of the electrophoretic mobilities of α, β, γ and δ thyrotrophins in polyacrylamide gel at pH 6 is shown in fig. 2. The figure also illustrates the complex pattern given by the TSH concentrate used as starting material. The components are grouped into two closely similar pairs, which can also be identified, partially resolved, in the concentrate. The mobilities are however in agreement with the order of their isoelectric points, the α component (pI 8.3) with the smallest relative net charge at pH 6 being the slowest, while the δ component (pI 8.8) with the largest net charge at pH 6 being the fastest.

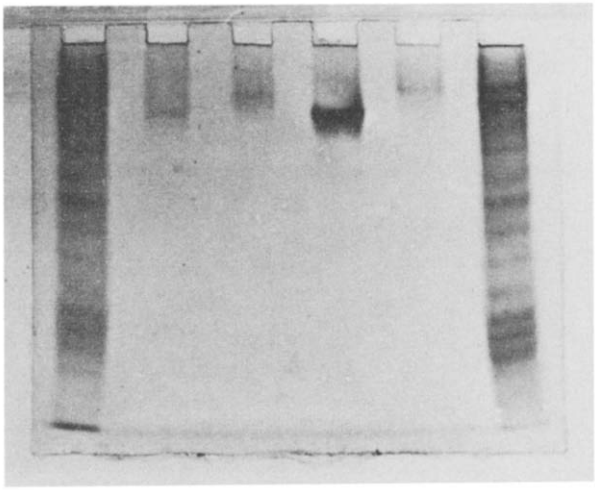

Fig. 2. Polyacrylamide gel electrophoresis of thyrotrophins at pH 6. 2½ hr at 150 v, 0.02 a. Cathode at bottom Left to right, TSH concentrate, δ thyrotrophin, β thyrotrophin, γ thyrotrophin, α thyrotrophin, TSH concentrate. Stained with 1% Naphthalene Black in 10% aqueous acetic acid.

4. Discussion

Figs. 1 and 2 show that the individual α, β, γ and δ thyrotrophins can be isolated in substantially homogeneous form by repeated isoelectric focussing. A more detailed study of their chemical and biological properties must await the isolation of larger quantities, but some speculation as to the causes of the differences between them appears to be warranted now.

The most striking difference is the high biological potency of the γ form, at least twice that of the α and β forms. The γ form is stable, retaining its TSH activity unchanged for at least six months. In view of the known instability of highly purified TSH [1,2,3] the lower potencies of the α and β forms may only reflect intrinsic differences in stability. If however the differences are real, they suggest that the γ form may be the initial product of hormone synthesis in the pituitary gland, the other forms being conversion or degradation products. Since separation by isoelectric focussing depends solely on differences in net charge and is virtually independent of differences in molecular size or shape, the primary variable in the thyrotrophins must also be net charge. This mechanism is also confirmed by their very similar migration rates in thin-layer chromatography on Sephadex G-100 and also by preliminary sedimentation velocity experiments. Both these methods however indicate some aggregation.

A possible mechanism for the differences in net charge would be hydrolysis of amide groups in γ thyrotrophin, although this would not account for the existence of the more basic δ component.

If any of the forms are artefacts, conversion must occur in the isolated gland, since isoelectric focussing experiments with crude extracts reveal the presence of multiple TSH activity zones, at pI values identical with those of the α, β, γ and δ components. The formation of multiple components is not prevented by addition of inhibitors of proteolytic activity to the extraction medium. A genetic origin for the components is precluded by their presence in an extract from a single pituitary gland.

The data of table 1 suggest that there may be differences in the monosaccharide compositions of the components, but confirmation of the reality of these differences must await the collection of more extensive analytical data. α and γ thyrotrophins appear to have the highest carbohydrate contents of any TSH preparations hitherto reported.

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