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Ovine luteinizing hormone

VI. Analysis of the misclassification errors in the separation of intrapituitary isohormones by chromatofocusing

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ABSTRACT

Luteinizing hormone (LH) in extracts of the ovine (o) anterior pituitary gland elutes as eight or more distinct peaks when analyzed by chromatofocusing on pH 10.5–7 gradients [Keel *et al.*, *Biol. Reprod.*, 36 (1987) 1102]. In order to examine the efficacy of this approach to identify the distinct charge isomers of oLH, a pool of pituitary extracts was de-salted by flow dialysis and chromatofocused on a pH 10.5–7 gradient. The immunoreactive oLH eluted in nine distinct peaks which were coded with letters beginning with the most basic form. The fractions corresponding to each peak were pooled, dialyzed and lyophilized. Each peak was then re-chromatofocused on a pH 10.5–7 gradient except for the immunoreactive oLH eluting in peak A′ because of the small amount present in this peak. Each peak, except for F and H, also consisted of a small percentage of immunoreactive oLH associated with adjacent peaks. This was plausible because chromatofocusing does not generally yield baseline resolution of peaks. Peak H eluted in a broad manner and was contaminated with significant amounts of isohormones F, G and Z. In contrast, peaks B, E, F, G and Z almost completely eluted in the anticipated regions. Thus, it appears that analysis of oLH charge isomers by chromatofocusing yields minimal misclassification errors and that the misclassification errors observed are associated with molecular forms which comprise a relatively small percentage of the oLH in pituitary extracts.

INTRODUCTION

Many, if not all, of the glycoprotein hormones exhibit charge heterogeneity [1–4]. One method frequently used to analyze the charge isomers of a particular glycoprotein hormone is chromatofocusing [1–4]. Although this method has been utilized by a variety of researchers, only a limited number have examined the efficacy of this approach by re-chromatofocusing each peak (for example, see Blum *et al.* [4]). In this study, we examined the ability of pH 10.5–7 chromatofocusing gradients to resolve the intrapituitary isohormones of ovine luteinizing hormone (oLH) as judged by re-chromatofocusing each peak identified in the original elution profile.

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MATERIALS AND METHODS

Extraction of anterior pituitary tissues

Anterior pituitary from ewes were extracted in a Tris-buffered saline solution supplemented with 0.5% (v/v) Triton X-100 and protease inhibitors [3]. Extracts were centrifuged at 100 000 *g*, aliquoted and frozen at −70°C. To prepare the original profile, four 0.5-ml aliquots obtained from different animals were pooled and supplemented with 3 mg cytochrome *c* and 4 mg myoglobin.

Chromatofocusing and radioimmunoassays

The pool of pituitary extract was de-salted by flow against water, diluted to 2% Pharmalyte 8-10.5–HCl, pH 7.0 (Pharmacia/LKB, Piscataway, NJ, USA), and chromatofocused on a pH 10.5–7 gradient using 20 ml of resin as previously described [3]. The immunoreactive oLH in each fraction was determined by radioimmunoassay [3]. This profile was divided into distinct peaks which were coded with letters beginning with the most basic form [1,3] (Fig. 1). The fractions corresponding to each peak were pooled, supplemented with 4 mg cytochrome *c* plus 3 mg myoglobin and dialyzed in SpectraPor 1 dialysis tubing (6000–8000 mol.wt. cut off; Spectrum Medical Industries, Los Angeles, CA, USA) against several changes of distilled water. After lyophilization, the resultant proteins from each peak were redissolved in 1:45 Pharmalyte

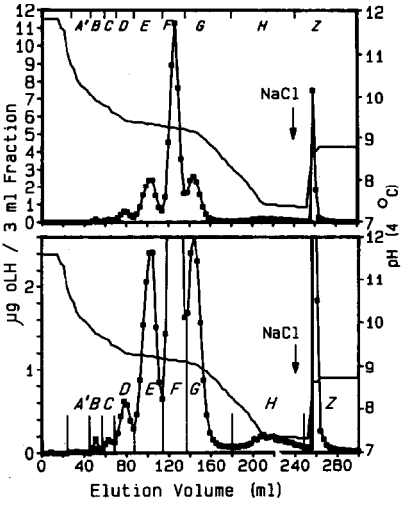


Fig. 1. Elution profile of the immunoreactive oLH in a pool of pituitary extracts chromatofocused on a pH 10.5–7 gradient. A pool of pituitary extracts (2 ml equivalent to 200 mg tissue) was supplemented with 3 mg cytochrome *c* plus 4 mg myoglobin, de-salted by flow dialysis against water and diluted to 2% Pharmalyte 8-10.5–HCl, pH 7.0. The column was eluted with diluted Pharmalyte at 10 ml/h and 3-ml fractions were collected. The oLH concentration in each fraction (■) was quantitated by radioimmunoassay and the pH gradient (solid line) was monitored with a flow-through pH monitor. Each peak of immunoreactive oLH was coded with a letter beginning with the most basic form [1,3]. Materials bound at the lower-limiting pH of 7.0 were eluted with 1 *M* NaCl and coded as peak Z. The same data is plotted in both panels except that the ordinate has been expanded to illustrate peaks with lower amounts of oLH. The distribution of oLH among the peaks is given in Table I. Remaining experimental details are presented in the text.

8-10.5–HCl, pH 7.0, and re-chromatofocused on pH 10.5–7 gradients. Columns containing 20 ml resin were utilized for peaks D–Z while columns with volumes of 10 ml resin were utilized for peaks B and C. Peak A' was not re-chromatofocused because of the extremely small amount of immunoreactive oLH in this peak. Columns (10 ml), 26 × 0.7 cm I.D., were eluted at 4 ml/h and fractions of 1.5 ml were collected. Each fraction was neutralized with a concentrated Tris–HCl solution as described in the previous paper [3]. The oLH concentration of each fraction was again determined by radioimmunoassay [3]. In all gradients, the pH was monitored with a Pharmacia/LKB pH monitor. All dialysis and chromatographic procedures were performed at 4°C.

RESULTS AND DISCUSSION

When a pool of ovine pituitary extracts was chromatofocused on a pH 10.5–7 gradient, the immunoreactive oLH eluted in nine distinct peaks (Fig. 1). These peaks were coded with letters beginning with the most basic form [1,3]. In this experiment, 127.2 μg oLH were loaded on the column and 81.9 μg were quantitated in the various fractions for a recovery of 64%. The distribution of oLH among these peaks is given in Table I. In general terms, the distribution of immunoreactive oLH was comparable to that previously observed [1,2] when materials eluting in the flow-through region were excluded from the calculations [3].

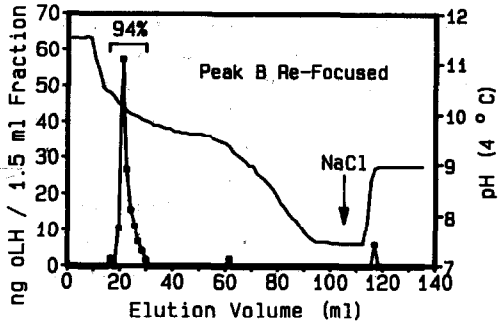
In order to examine the efficacy of chromatofocusing to separate and identify the distinct charge isomers of oLH, each peak from the original elution profile was re-chromatofocused on a pH 10.5–7 gradient (Figs. 2–9). Because of the relatively small amounts of oLH present in peaks B and C, they were re-chromatofocused on 10- rather than 20-ml columns. More than 90% of peak B re-focused as isohormone B (Fig. 2; Table I). Approximately 75% of peaks C (Fig. 3), D (Fig. 4) and E (Fig. 5) returned in the expected regions with the remainders primarily eluting as more acidic

TABLE I

ELUTION pH VALUES AND DISTRIBUTION OF IMMUNOREACTIVE oLH IN THE ORIGINAL CHROMATOFOCUSING PROFILE AND AFTER RE-CHROMATOFOCUSING

Peak	Original profile		Upon re-chromatofocusing		
	Elution pH (4°C)	Amount eluted in this peak (%)	Elution pH (4°C)	Re-focused in this peak (%)	Immunoreactive oLH recovered (%)
A'	10.38	0.1	N.D.[a]	N.D.	N.D
B	9.87	0.4	10.17	94	87
C	9.72	0.7	9.99	74	59
D	9.42	3.1	9.36	77	101
E	9.33	15.6	9.57	68	80
F	9.24	47.8	9.33	98	97
G	9.15	15.7	9.21	89	60
H	≈7.4	3.9	≈7.9	24	72
Z	<7.40	12.5	<7.40	81	61

[a] N.D. = Not determined.



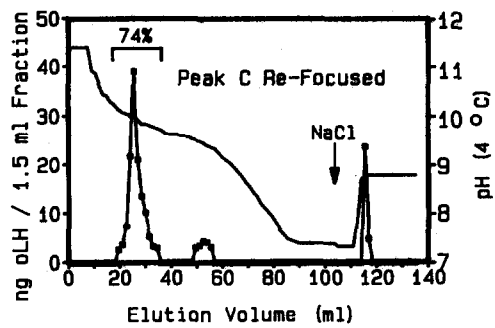
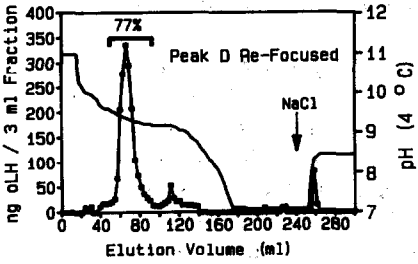


Fig. 2. Re-chromatofocusing of peak B from the original profile on a pH 10.5–7 gradient using a 10-ml column. The column was eluted at 4 ml/h and 1.5-ml fractions were collected. See the text and legend of Fig. 1 for experimental details.

Fig. 3. Re-chromatofocusing of peak C from the original profile on a pH 10.5–7 gradient using a 10-ml column. See the text and legends of Figs. 1 and 2 for experimental details.



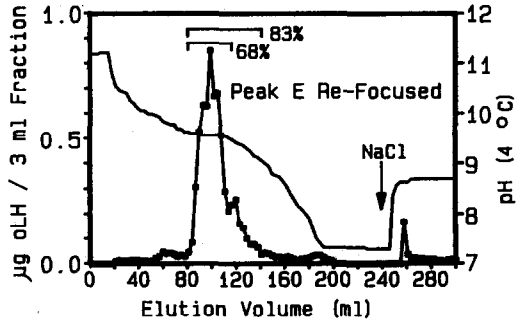
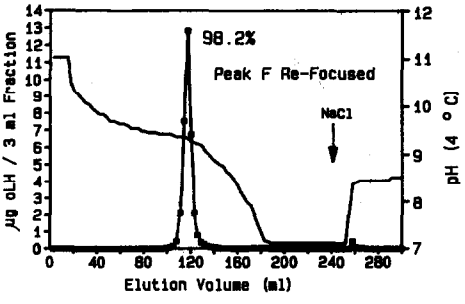


Fig. 4. Re-chromatofocusing of peak D from the original profile on a pH 10.5–7 gradient. See the text and legend of Fig. 1 for experimental details.

Fig. 5. Re-chromatofocusing of peak E from the original profile on a pH 10.5–7 gradient. See the text and legend of Fig. 1 for experimental details.



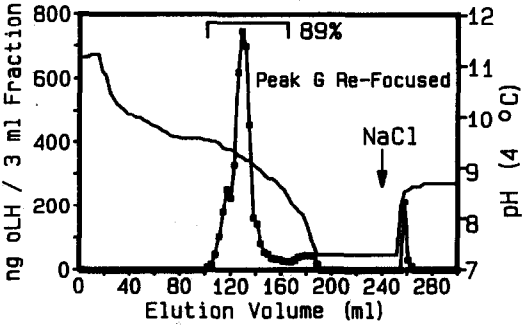


Fig. 6. Re-chromatofocusing of peak F from the original profile on a pH 10.5–7 gradient. See the text and legend of Fig. 1 for experimental details.

Fig. 7. Re-chromatofocusing of peak G from the original profile on a pH 10.5–7 gradient. See text and legend of Fig. 1 for experimental details.

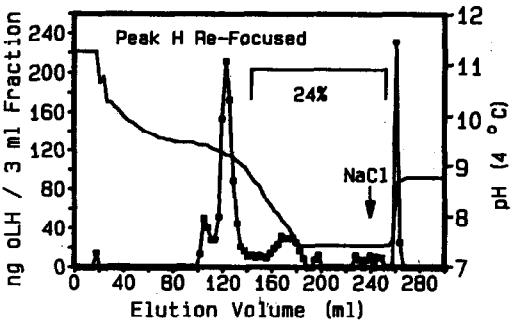


Fig. 8. Re-chromatofocusing of peak H from the original profile on a pH 10.5–7 gradient. Note that slightly less than 25% re-focused in the expected region. See the text and legend of Fig. 1 for experimental details.

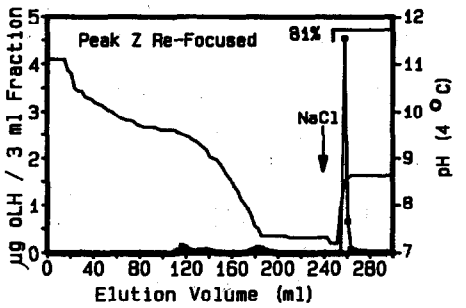


Fig. 9. Re-chromatofocusing of peak Z from the original profile on a pH 10.5–7 gradient. See the text and legend of Fig. 1 for experimental details.

forms. The pH of the tube containing the highest oLH concentration in peak E was slightly higher when re-chromatofocused than in the original profile (Table I). This was attributed to slight variations in the pH gradients between chromatographic runs, particularly as monitored with a flow-through electrode at 4°C, rather than as a shift in elution position. Some of the oLH in peaks C, D and E were bound to the column when re-chromatofocused, but eluted with 1 *M* NaCl. The absolute amounts of oLH eluting in this fashion were relatively small and may represent oLH non-specifically bound to the resin at low ionic strength.

Peak F was the predominant form and comprised almost 50% of the immunoreactive oLH in the pituitary (Fig. 6, Table I). When this peak was re-chromatofocused, essentially all of the immunoreactive oLH was again recovered in a peak corresponding to the elution position in the original profile (Fig. 6). The second-most abundant form of immunoreactive oLH was peak G. When peak G was re-chromatofocused, approximately 90% eluted in the anticipated region (Fig. 7) with the remainder eluting in the more acidic peaks H and Z.

When we revised our method slightly and began loading the pituitary extracts in Pharmalyte rather than triethylamine [3], a noticeable amount of oLH eluted as the pH gradient dropped off sharply. This peak was coded as H and is the fifth most abundant form of oLH observed in the original profile (Fig. 1, Table I). Typically, peak H elutes in a broad band as the pH gradient reaches the lower limit and, frequently, some oLH continues to elute even though the pH gradient has plateaued. When the immunoreactive oLH eluting in this manner (peak H) was re-chromatofocused only about 25% again eluted in the anticipated region (Fig. 8). Peak H appears to be primarily composed of a mixture of isohormones G and Z as well as small amounts of F. As noted previously [2,5], a large majority of the materials bound to the column at the lower-limiting pH of 7.0 (peak Z) again bound when re-chromatofocused (Fig. 9).

In any chromatographic system, the possibility of selective losses exists. The recovery of the oLH in each peak of the original profile (Table I) was similar to those

typically observed (60–85%) when intrapituiraty oLH is chromatofocused [1–3,5] suggesting there were no losses of specific isoforms.

Based on the data presented above, the charge isomers of oLH are adequately resolved by chromatofocusing. Some peaks were contaminated with forms eluting in adjacent regions but this is because chromatofocusing does not generally yield baseline resolution of the peaks of immunoreactive oLH. It would appear that there are at least eight distinct charge isomers of intrapituitary oLH which elute in the peaks coded A'–G and Z in the figures. The primary peak which appears to be subject to misclassification is peak H. Only 25% of peak H re-chromatofocused in the anticipated region. Peak H is somewhat unusual because it elutes rather broadly as the pH gradient drops sharply and continues to elute even after the pH gradient has plateaued. Peak H may represent molecular forms of oLH which are weakly bound at the lower-limiting pH of 7.0 rather than a unique molecular form.

If one considers the percentage of hormone originally classified and re-chromatofocused in each respective peak, approximately 86% of the oLH was correctly classified in the original profile. Furthermore, the misclassified portion was generally associated with adjacent peaks. Thus, it appears that analysis of the charge isomers of oLH by chromatofocusing yields minimal misclassification errors and those which are observed are associated with molecular forms which comprise a relatively small percentage of the oLH in pituitary extracts. Thus, chromatofocusing is an effective method to separate the charge isomers of the glycoprotein hormones.

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