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## Note

# Comparison of chromatofocusing and isoelectric focusing in recovery of specific activity from biological fluids

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Purification and fractionation of proteins from biological fluids is facilitated by their different isoelectric points. Isoelectric focusing (IEF) by electrophoresis in carrier ampholytes through a Sephadex column provides a successful method of protein separation for a number of biologically active compounds[1]. This method utilizes protein net charge in a pH gradient developed in the column by the passage of a current. The possibility of producing a pH gradient using an ion-exchange column has recently provided techniques for separation with resolution and recovery comparable to those of electrophoretic systems[2–4]. With ampholyte displacement column chromatography, a pH gradient is produced by mixing buffers of different pH or by employing the buffering action of an ion exchanger and a running buffer initially adjusted to one pH through a column previously adjusted to another pH[2]. Here, the comparative results of these two procedures in the fractionation of the same biologic fluids are presented.

## EXPERIMENTAL

### *Sample preparation*

Slowly thawed pooled human (hFF) and porcine (pFF) follicular fluid (30 ml each) were fractionated by dropwise addition of an equal volume of saturated ammonium sulfate and continuous agitation at 4°C. After 2 h, the precipitate was pelleted, the supernatant discarded, and the pellet resuspended (2:1) with 10% ammonium sulfate. After 12 h of additional agitation and centrifugation (3000 *g*, 30 min), the supernatant was dialyzed (Visking dialysis tubing) against 0.025 *M* Tris–HCl, pH 7.5 (buffer A) for 16 h. Insoluble material was removed by centrifugation.

An agarose-immobilized textile dye matrex gel, Orange A (Dyematrix, Amicon, Lexington, MA, U.S.A.), was prepared according to the manufacturer's instructions[5]. Columns (23 × 9 mm I.D., 2 ml bed volume) containing matrex gel Orange A were charged with 500 mg of the dialyzed retentate in 0.5 ml of buffer A, and allowed to equilibrate for 30 min at 4°C. Unbound material was eluted with 5 column-bed volumes (10 ml) of 20 m*M* Tris–HCl, pH 7.5. Bound material was eluted with 10 ml of 1.5 *M* potassium chloride in buffer A. Eluent fractions were dialyzed overnight against water in preparation for isoelectric focusing and against 0.025 *M* imidazole, pH 7.4, for ampholyte displacement chromatography.

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*Isoelectric focusing*

Eluents bound matrex to gel Orange A (13% of the total protein charge) were further purified by isoelectric focusing using a Sephadex G-15 support matrix as previously described[6]. The apparatus consisted of a 30 × 4 cm I.D. water-jacketed glass column containing a 20 × 2.5 cm Sephadex G-15 bed supported by a 8 × 2.5 cm PTFE elution plug under a 25-mm Millipore filter. The column was previously equilibrated with carrier ampholytes containing 2% of pH 3–10, 2% of pH 2–4, and 12.5% glycerine. Cytochrome *c* was used as an internal marker protein (p*I* 10.5). A second Millipore filter was placed on the top of the Sephadex bed. A 10-ml polyacrylamide solution of 14% acrylamide, 0.3% Bis, and 50 μl N,N,N′,N′-tetramethylethylenediamine (polymerized by the addition of 100 μl of 10% ammonium persulfate) was then poured over the filter. Upon completion of polymerization (*ca.* 20 min), the column was inverted, the PTFE plug removed, and a second acrylamide plug layered over the bottom filter. After polymerization (*ca.* 20 min) of the bottom plug, the column was returned to its upright position and lowered into anode buffer containing 1% sulfuric acid. The remaining upper portion of the column was filled with 1% ethanolamine. The column was cooled by recirculating water at 1–4°C throughout the procedure. Isoelectric focusing was initiated at 800 constant volts (16 mA) and allowed to proceed to equilibrium as monitored by an eventual decline in the milliamperage to 2.5 mA (*ca.* 6–8 h). Thereafter, the acrylamide plugs were replaced with PTFE plugs for column elution. Column fractions (1 ml) were collected and monitored for absorbance at 280 nm (ISCO, Lincoln, NE, U.S.A.). Fractions for bioassay were dialyzed against buffer A to remove ampholytes prior to testing in the bioassay. Protein concentrations were determined by the method of Lowry *et al.*[7].

*Ampholyte displacement chromatography*

Material bound to matrex gel Orange A in imidazole buffer (10 ml) was applied by gravity to Polybuffer Exchanger 94 (16 ml, equilibrated to pH 7.4 with 0.025 *M* imidazole in buffer A) column (20 × 1 cm I.D.). The column was washed with 40–50 ml of 0.025 *M* imidazole buffer, pH 7.4, and then the sample was added in 40 ml of imidazole buffer. The column was then eluted with degassed Pharmalyte Polybuffer 74–HCl (adjusted to pH 4.0 with hydrochloric acid) at 20 ml/h, 4°C and monitored as described for IEF. Finally, the column was washed with 20 ml of 1 *M* sodium chloride to elute proteins that were not displaced by the low ionic strength Polybuffer.

Control determination of chromatography fractions containing inhibitory activity was performed by heating (56°C for 30 min) or trypsin digestion (10 mg%) of representative samples for 3 h.

*Bioassay*

Sprague-Dawley rats (23 days old, 45–55 g) were received from Hormone Assay Labs., Chicago, IL, U.S.A.) two days after hypophysectomy, and were kept at 25°C with intervals of 14 h light and 10 h darkness[8–9]. Animals were caged in groups of three and given rat chow and water *ad libidum*. Diethylstilbesterol (DES)-containing silastic implants (1 × 5 mm: *ca.* 10 μg DES), prepared as described previously[8], were inserted subcutaneously in the hypophysectomy incision immediately after hypophysectomy. Assay design was 3 rats at each dose of unknown. An interval of 96 h after hypophy-

sectomy, animals were given gonadotropins (Serono, 1 IU: 1 IU, LH:FSH) dissolved in 0.15 *M* sodium chloride with 1% bovine serum albumin and/or equal volumes of test fractions in two divided daily doses. A further 24 h after the initial injection, animals were sacrificed by decapitation, and the ovaries were removed, trimmed and weighed (Roller-Smith balance). Rat trunk serum estradiol-17$\beta$ determinations were performed by methods previously described[10].

Control determination (no injected test fractions) for unstimulated ovarian weight were 34.7 ± 3.2 mg per rat and 129.0 ± 30.5 mg per rat for LH/FSH-stimulated. Control levels of trunk serum estradiol were 12.5 ± 0.7 pg/ml for unstimulated, 118.5 ± 21 pg/ml for LH/FSH stimulated. Where indicated, 100% inhibition equals ovarian weight and/or serum estradiol concentration of mean unstimulated control values. Zero percent inhibition equals ovarian weight or serum estradiol concentration of LH/FSH stimulated control rats.

Data analysis was performed using Students *t* test and Duncan's multiple range analysis.

## RESULTS AND DISCUSSION

A chromatogram of hFF after ammonium sulfate precipitation, dialysis, matrex gel Orange A pseudo-ligand, chromatography and isoelectric focusing is shown in Fig. 1. A single peak of absorbance is apparent between pH 4 and 6.8. When the same material was fractionated using chromatofocusing (Fig. 2), absorbance peaks were noted at pH 7, 6.5, 5.2, 4.5 and 4.2, as well as a large amount of material which eluted in the salt wash (1 *M* sodium chloride). Table I compares the biological activity of the fractions from both preparative techniques. As can be seen with isoelectric focusing, the majority of biological activity was present between pH 4 and 5. In contrast, biological activity recovered with ampholyte displacement chromatography is present between 6.5 and 6.0 as well as 4.5 and 5.0. When similar preparations of pooled pFF

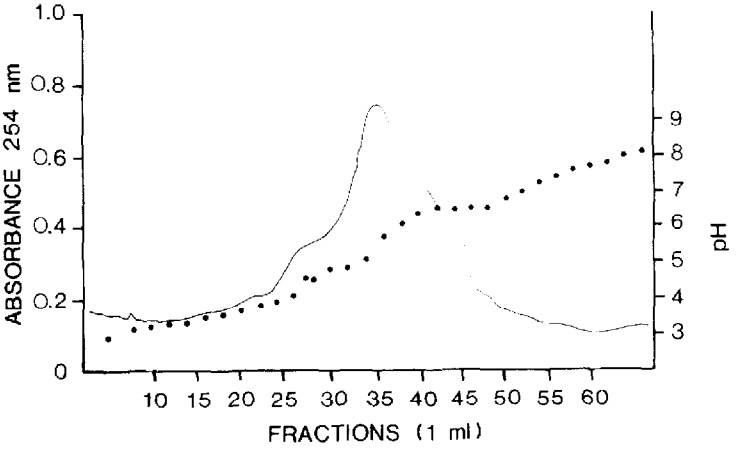


Fig. 1. Isoelectric focusing chromatogram of extracted human follicular fluid. Biological activity as determined by inhibition of rat ovarian weight augmentation with LH/FSH stimulation was found at pH 3.5–4.5 (see Table I).

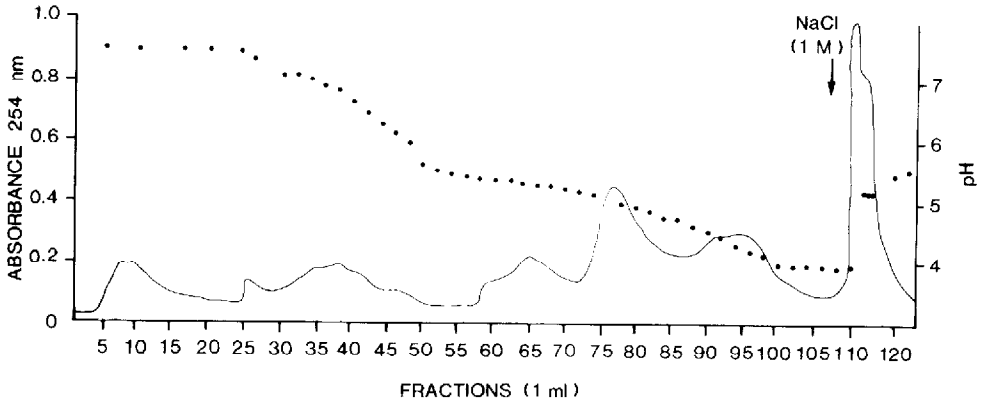


Fig. 2. Ampholyte displacement chromatogram of extracted human follicular fluid. Biological activity as determined by inhibition of rat ovarian weight augmentation with LH/FSH stimulation was found at pH 3.5–4.0 and 6.0–6.5 (see Table I).

utilizing the same bioassay were fractionated by isoelectric focusing, a single peak of biological activity was found in the pH 4–4.5 range from material bound to matrex gel Orange A (Fig. 3, 82 ± 7% inhibition of ovarian weight gain). Further, when similarly extracted pFF was fractionated by ampholyte displacement chromatography, no such activity could be eluted from the chromatofocusing column using the standard Polybuffer exchange system, but was recoverable with high salt (1 *M*) column elution (Fig. 4). Chromatography fractions heated or incubated with trypsin contained no activity in the bioassay.

Although preparative fractionation and purification can be readily and easily performed utilizing both amphoteric separation techniques, the two procedures are not directly interchangable. These results are in agreement with those of Verbalis, who noted higher p*I* determinations of neurophysins in a mixed neurophysin preparation with IEF as compared to CF[11]. A major disadvantage of isoelectric focusing has been precipitation of proteins near the isoelectric point, thus limiting the amount of protein

TABLE I

BIOLOGICAL ACTIVITY (PERCENTAGE INHIBITION OF OVARIAN WEIGHT) RECOVERED FROM ISOELECTRIC FOCUSING (IEF) AND CHROMATOFOCUSING (CF) OF EXTRACTED HUMAN FOLLICULAR FLUID

| *pH* | *IEF* | *CF* |
|---|---|---|
| 3 | 0–2 | — |
| 3.5 | 50±12 | — |
| 4 | — | 78±12 |
| 4.5 | 90±8 | 91±8 |
| 5 | 15±12 | 0 |
| 5.5 | — | 10±4 |
| 6 | 0–2 | 60±5 |
| 6.5 | 18±6 | 55±8 |
| 7 | 22±5 | — |
| 7.5 | 0–2 | 30±5 |

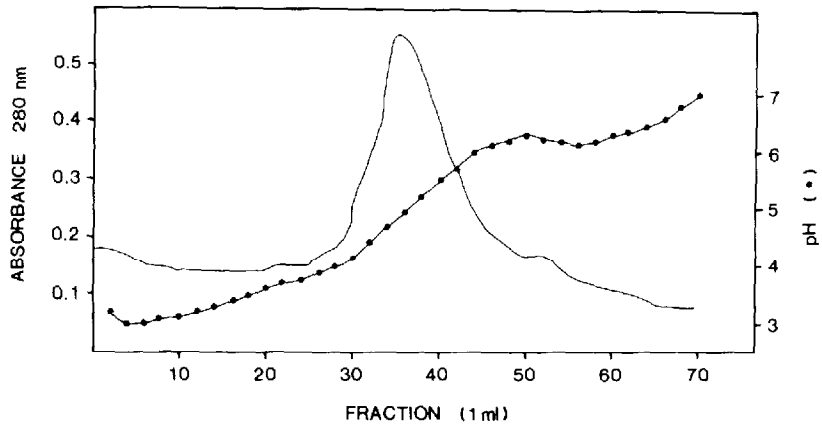


Fig. 3. Isoelectric focusing chromatogram of extracted porcine follicular fluid. Biological activity as determined by inhibition of rat ovarian weight augmentation with LH/FSH stimulation was found at pH 3.5–4.5 (50 ± 12%, $X$ ± S.E.M.).

processed in each application. The capacity for ampholyte displacement columns is potentially much larger at the p$I$ since the amount of column space at the p$I$ is a function of all the amphoteric compounds in solution. The total capacity for the commercially available gel is 300 m$M$/ml of gel[2,4].

In conclusion, isoelectric focusing and ampholyte displacement chromatography are both useful techniques for preparative-scale purification of proteins from biological fluids; however, as shown by the bioassay and purification schemes reported here, the results are not directly interchangable. Ampholyte displacement chromatography appears to be particularly well suited to seperation of follicular fluid inhibitory proteins. Because of the acidic p$I$ values of these factors, most of the proteins are eluted with the starting buffer leaving the active material bound to the ion-exchange resin. Fur-

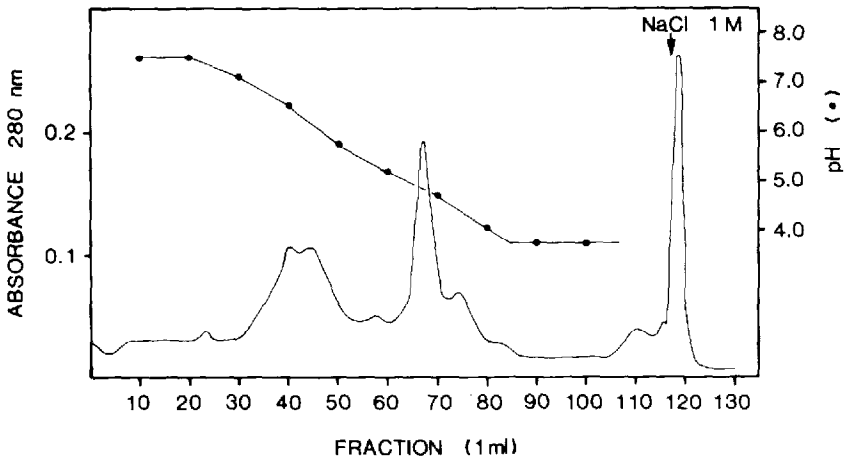


Fig. 4. Ampholyte displacement chromatogram of extracted porcine follicular fluid. Biological activity as determined by inhibition of rat ovarian weight augmentation with LH/FSH stimulation was only found in the eluent following 1 $M$ sodium chloride elution (78 ± 3%, $X$ ± S.E.M.).

thermore, the pH gradient can be altered easily to increase resolution over a given pH range allowing separations which were not resolved by IEF (see also ref. 11). The reproducibility, yield and ease of altering the pH elution gradients make this method ideal for the preparative isolation of biologically active factors from complex biological preparations. However, it is clear that the elution pH by ampholyte displacement chromatography is not necessarily equivalent to the p*I* of the proteins as determined by isoelectric focusing.

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