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Note

Separation of neurohypophyseal proteins by reversed-phase high-pressure liquid chromatography

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The class of neurohypophyseal proteins collectively called the "neurophysins" has recently occupied the attention of many investigators [1]. Separation of these molecules from various pituitary preparations using open column liquid chromatography has been investigated and standard gel electrophoretic methods used as analytical techniques [2].

The physical properties of these molecules are, briefly: they form aggregating systems in aqueous solutions, are all of monomer molecular weight ∿ 10,000 D and contain an unusually high proportion of disulfide bonds. However, the property which makes them of special interest to high-pressure liquid chromatography (HPLC) is their rather high solubility in aqueous methanol [3].

The theory of HPLC as applied to columns consisting of microparticulate packing coated with non-polar stationary phases has received much attention. The form of liquid—liquid chromatography using these columns and sometimes called "reversed-phase" is commonly thought to be most appropriate for separation of nonionogenic compounds [4]. On the other hand, it has also been shown recently that separation of polar compounds can be achieved using a non-polar stationary phase and elution with aqueous solutions containing no organic component [5]. To a practical biochemist involved in chromatographic separation of *proteins* the theoretical prediction of which columns and eluents to use in a particular case is therefore by no means clear. Indeed, it is commonly thought that protein separations require ion-exchange/sieving type packings. Be that as it may, it seems intuitively clear that proteins such as the neurophysins might exhibit a competition between solubility in a non-polar stationary phase and a partially organic mobile phase and thus be separable on that basis. The present work shows that this is true, and leads to a convenient analytical separation (which can presumably be scaled up to preparative level using a

larger column) which takes only a fraction of the time needed for a polyacrylamide gel electrophoresis (PAGE) determination, is quantitative without staining or labelling, and is at least several times more sensitive in detection. The column packing used is one commonly employed for separation of low-molecular-weight peptides.

EXPERIMENTAL

The neurophysins used in these experiments were prepared from fresh frozen bovine posterior pituitary glands in a manner previously described [2] and which is in accord with the results of other workers insofar as their purity as determined by analytical PAGE [1]. The HPLC experiments reported here were performed using a Micromeritics Model 7000B chromatograph equipped with a Model 785 variable wavelength UV detector. Solvent methanol was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and water was glass distilled from the deionized laboratory water supply. J.T. Baker (Phillipsburgh, N.J., U.S.A.) reagent grade sodium acetate was used for the buffer preparation. The HPLC column was packed in this laboratory using a Micromeritics Model 705B slurry packer. The column dimensions were 25 × 4.6 mm I.D. and the packing was E.M. Labs. (Elmsford, N.Y., U.S.A.) designation "RP-18". Using standard tests the column was found to be about 90% as efficient (expressed as number of theoretical plates and height equivalent of theoretical plates) as similar commercial columns available from several manufacturers. The standards were low-molecular-weight compounds.

Chromatographic conditions were: sample injection as 2 mg/ml solutions in phosphate buffer, pH 5 (our standard conditions for neurophysin solutions), detection in a 10-mm path length cell (volume = 10 μl) at 215 nm, elution with a mixture of 0.01 *M* acetate buffer (pH 5.7) and methanol. It was found that the ratio of buffer to methanol was very critical (see data below), and with detection at this wavelength the most practical way of obtaining stability was to mix a large proportion of the buffer—methanol mixture of approximately correct ratio with small increments of pure methanol in the chromatograph's solvent blending system to bring about the final composition.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatograms which result from injection of neurophysins I and II along with scans of analytical PAGE gels of the same components. To be noted in the chromatograms is the strong solvent front due to the phosphate buffer (taken to be the void volume, V_0). In the neurophysin I chromatogram the peak immediately following this front is real and accounts for 26% of the total UV-absorbing area. Injection amounts for neurophysins I and II differ in order to make the figure clearer for publication since peak widths vary. The PAGE scans show what would ordinarily be considered homogeneous proteins of each kind. Using gel isoelectric focussing (experiments not shown) it was shown that neurophysin I presents itself as two bands while neurophysin II remains homogeneous even in that method of

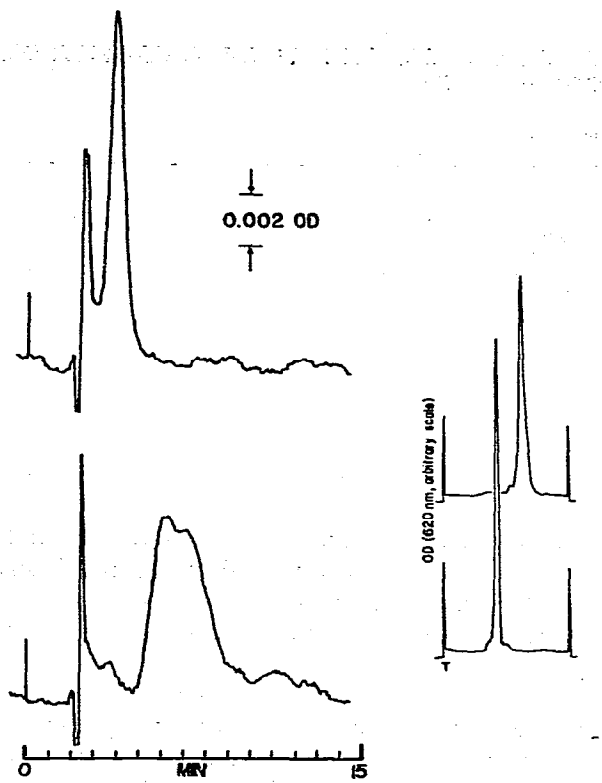

Fig. 1. Main figure: HPLC chromatograms, flow-rate 1 ml/min, detection at 215 nm, eluent composition; 48.4% 0.01 *M* acetate buffer (pH 5.7), 51.6% methanol. Upper: neurophysin I, 2 μg injected as phosphate buffer (pH 5) solution. Lower: neurophysin II, 6 μg injected as phosphate buffer (pH 5) solution. Inset figure: PAGE scans, 7.5% acrylamide gels, Tris—glycine buffer (pH 7.9), stained with amido black. "T" indicates top of gel. Upper: neurophysin I, 50-μg load. Lower: neurophysin II, 50-μg load.

separation. Table I gives the HPLC relative elution volume values (elution volume, V_e, for each component/V_o as defined above) for the major peaks obtained as a function of eluent composition.

It is clear from the above that the neurophysins can be easily separated from each other. It should be noted that the intrinsic detector sensitivity (0.005 a.u.f.s.) is about four times greater than that used in these experiments. This makes the minimum detectable amount of neurophysin about 0.3—1.0 μg. Aside from the tremendous decrease in time per determination over PAGE one of the advantages of this method is that detected signal is much more easily related to amounts of protein than by staining. The wavelength of 215 nm was chosen because it is the isosbestic point at which random and helical peptide bond absorptions, from the $\pi \rightarrow \pi^*$ transition, are equal [6]. Thus, at this wavelength UV absorption is approximately independent of protein composition and the residue molar adsorptivity (neglecting contributions from tails of aromatic residue near UV-bands) is 10^3 l·mole^{-1} ·cm^{-1} ·residue^{-1} [6]. Using this value and the number of residues in the neurophysins (approximately 100 [7]) the observed integrated absorbances account very closely for the

TABLE I

V_e/V_0 VALUES FOR ELUTION OF THE MAJOR COMPONENTS OF NEUROPHYSINS I AND II (NPI AND NPII) VS. ELUENT COMPOSITION

A: 0.01 *M* acetate buffer (pH = 5.7); B: methanol

Composition	V_e/V_0			
	NPI_1	NPI_2	$NPII_1$	$NPII_2$
48.4% A 51.6% B	1.11	1.69	2.70	3.12
49.5% A 50.5% B	1.18	2.33	4.17	5.00
50.5% A 49.5% B	1.61	4.76	8.33	10.0

total protein injected. Finally, the chromatograms (not shown) near the limit where the proteins are almost totally retained by the column show dramatically that PAGE derived, "chromatographically pure", protein is by no means pure as far as HPLC is concerned, without recourse to two-dimensional electrophoretic experiments.

The advantages of this HPLC system over currently used methods are obvious, and it seems hard to believe that this is the only protein system to which this reversed-phase method can be applied. It must be emphasized that success with proteins using UV detection at high sensitivity can only be hoped for with a detector tuned very closely to the steep shoulder of the peptide bond absorption band which has its maximum at 190 nm, but which is obscured at that wavelength by solvent absorption.

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