Journal of Chromatography, 254 (1983) 309–314
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,370

Note

Heterogeneity of human pituitary neurophysins by ampholyte displacement chromatography

JOSEPH G. VERBALIS
Department of Medicine, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15261 (U.S.A.)
(Received September 15th, 1982)

The neurophysins are cysteine-rich polypeptide proteins of molecular weight $\approx$ 10,000 dalton found in mammalian posterior pituitary glands. Much evidence suggests that nearly all species have separate neurophysins associated with each of the two major neurohypophyseal hormones, vasopressin and oxytocin[1]. However, electrophoretic separations of pituitary neurophysins have resulted in the identification of more than two neurophysins in several species, including the cow[2], pig[3], rat[4] and human[5]. While these additional neurophysins were initially felt to be artifacts of degradation during the process of extraction, recent evidence demonstrating that the neurophysins and their respective associated hormones are initially synthesized as larger 20,000-dalton precursor molecules which are then enzymatically cleaved within neurosecretory granules[6] has provided a more likely explanation for some of these additional neurophysin forms as intermediates in the processing of the precursor molecules to their final products.

Much of the neurophysin literature is confusing because of the variety of nomenclature used to describe the neurophysin forms isolated by electrophoresis from different species[1]. This paper describes a simple method for reproducible preparative separation, identification and isolation of multiple neurophysin forms found in human posterior pituitary tissue using ampholyte displacement chromatography.

EXPERIMENTAL

Tissue extraction

Desiccated human neurohypophyseal tissue was extracted in 0.1 *M* hydrochloric acid at 4°C as described previously[5]. The supernatant was chromatographed on a 100 × 2.5 cm Sephadex G-75 column eluted with 0.1 *M* formic acid at 4°C, and the protein peak coeluting with iodinated nicotine-stimulated neurophysin [^{125}I]NSN, as marker, was pooled and lyophilized[7]. A 300-mg amount of the lyophilate was dissolved in 20.0 ml of 0.025 *M* piperazine (Sigma, St. Louis, MO, U.S.A.) adjusted to pH 5.2 with hydrochloric acid. By radioimmunoassy (see below) this contained 13.3 mg/ml of human neurophysin (6.9 mg/ml of NSN and 6.4 mg/ml of estrogen-stimulated neurophysin, ESN). All further separations were done with this crude neurophysin peak.

0021-9673/83/0000–0000/$03.00

Ampholyte displacement chromatography

A 100 × 0.9 cm chromatofocusing column was packed with PBE 94 (Pharmacia, Uppsala, Sweden) and the column was equilibrated with 0.025 *M* piperazine hydrochloride, pH 5.2, at 4°C. Following application of 10.0 ml of the crude neurophysin peak, elution was begun with Polybuffer 74 (Pharmacia) diluted 1:10 in distilled and adjusted to pH 3.9 with hydrochloric acid. The elution rate was controlled by a peristaltic pump adjusted to a flow-rate of 30 ml/h, and 2.5-ml fractions were collected until the final pH was reached, after which the column was washed with 200 ml of 0.1 *M* hydrochloric acid. A finer separation of the middle range neurophysins was accomplished using the same column but by adjusting the pH of the starting buffer to 5.5, eluting with a 1:8 dilution of Polybuffer 74 at pH 4.0, and collecting 1.0-ml fractions at a flow-rate of 15 ml/h. Previously isolated preparations of human neurophysin II, III and IV[5] were dissolved in 2.0 ml of 0.025 *M* piperazine at a concentration of 10 μg/ml, and the samples run on the same chromatofocusing column as described above for the crude neurophysin peak. All fractions were analyzed for pH (Beckman Model 3500 pH meter), protein content by ultraviolet absorption at 280 nm (Beckman DB-G spectrophotometer) and neurophysin content by radioimmunoassay (see below).

Neurophysin isolation

The fractions containing the various neurophysin peaks were pooled and the protein precipitated by the addition of ammonium sulfate (Fisher Scientific, Pittsburgh, PA, U.S.A.) to 100% saturation. The precipitates were separated by centrifugation (2000 *g*, 20 min), washed with 20.0 ml of saturated ammonium sulfate, and recentrifuged. The final precipitates were then dissolved in 15.0 ml of 0.1 *M* hydrochloric acid and dialyzed extensively against 0.1 *M* formic acid (Thomas Spectrapor No. 1 dialysis membrane) prior to lyophilization.

Isoelectric focusing

Samples for isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) were dissolved in 0.1 *M* hydrochloric acid at a concentration of 0.5 mg/ml. 50 μl of 75% sucrose was added to 100-μl aliquots of the samples to yield a final sucrose concentration of 25%. Polyacrylamide gels were cast in 125 × 5.5 mm borosilicate glass tubes using 7.5% T–2.5% C, 5% glycerol, an ampholyte mixture consisting of a 2:1 ratio of Pharmalyte 4–6.5 to Pharmalyte 2.5–5 (Pharmacia) at a final ampholyte concentration of 5.0%, and polymerized under ultraviolet light following the addition of 0.1 mg riboflavin per 30.0 ml. The samples were applied as 50-μl aliquots and overlayered with 100 μl of 20% sucrose followed by 100 μl of 10% sucrose. The gels were focused at 22°C for 20 h at 250 V (with a maximum initial current of 0.5 mA per gel) using a buffer system consisting of 0.06 *M* sulfuric acid at the cathode (upper reservoir) and 0.02 *M* calcium hydroxide plus 0.04 *M* sodium hydroxide at the anode (bottom reservoir). Following focusing the gels were manually sliced at 2-mm intervals and the slices individually eluted in 1.0 ml of 0.1 *M* sodium chloride solution. The eluates were then radioimmunoassayed for neurophysin content as described below. The pH gradient was determined by corunning identical gels using known p*I* standards (Pharmacia Low p*I* Calibration Kit, p*I* 2.5–6.5) prepared in the same way as the samples.

Radioimmunoassay

Neurophysin content of the chromatographed fractions and eluted gel slices was determined by direct radioimmunoassay of the fractions by previously described specific radioimmunoassays for the human neurophysins: NSN, which has been shown to be the human vasopressin-associated neurophysin, hAVP-Np, and ESN, which has been shown to be the human oxytocin-associated neurophysin, hOT-Np[5,8].

RESULTS AND DISCUSSION

Fig. 1 shows the elution pattern of the crude neurophysin peak following ampholyte displacement chromatography. Analysis of the O.D. 280 protein peaks reveals that the majority of the extracted proteins elute at a pH > 5.2, but this protein peak contains no significant neurophysin immunoreactivity. All of the neurophysins appear to be bound by the chromatofocusing ion-exchange resin until subsequent displacement at lower p*I* values by the ampholyte elution. As shown in Fig. 1, several neurophysin-immunoreactive peaks eluted in the area from pH 4.5 to 3.9. To resolve the peaks better in the 4.2 to 4.0 region a second run was made on a smaller starting sample using a narrower pH gradient as shown in Fig. 2. Table I summarizes the elution pH and percentage of total neurophysin immunoreactivity for the major neurophysin peaks. The final overall yield from the chromatographic separation, pooling, precipitation and dialysis was 22% for NSN proteins and 35% for ESN proteins as determined by radioimmunoassay.

To determine the relationship of these ampholyte displacement peaks to previous separations using preparative disc gel electrophoresis, stored aliquots of electrophoretically isolated human neurophysins II, III and IV[5] were also analyzed on the chromatofocusing column. Human Np II eluted at the pH of the 3.95 NSN peak, hNp III at the pH of the 4.11 ESN peak and hNp IV at the pH of the 4.42 NSN peak, suggesting an inverse correlation between electrophoretic mobility and elution pH. Previous amino acid analysis of hNp II and IV has shown them to have equivalent peptide maps with the exception of an additional Arg, Ala C-terminal

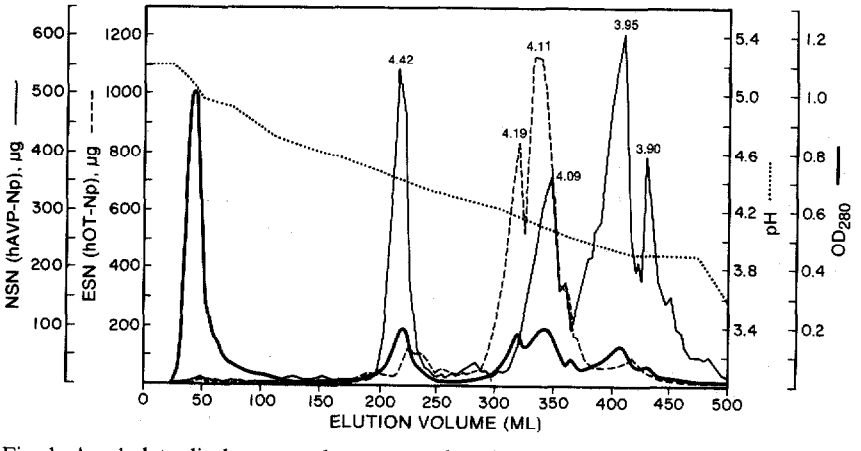

Fig. 1. Ampholyte displacement chromatography of human pituitary neurophysins showing NSN and ESN immunoreactivities, optical density at 280 nm (——) and pH (·····) of the elution fractions.

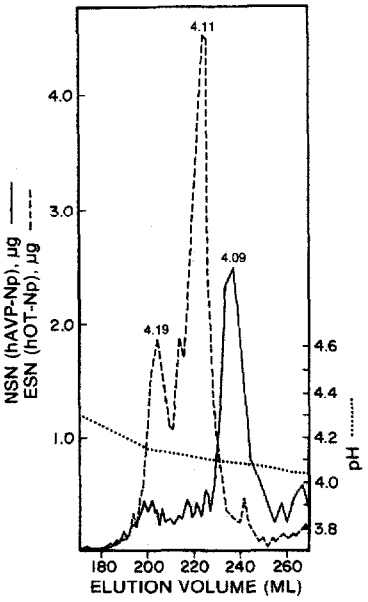

Fig. 2. Chromatogram utilizing a narrower pH gradient to separate the middle range neurophysins.

TABLE I

HUMAN PITUITARY NEUROPHYSINS ISOLATED BY AMPHOLYTE DISPLACEMENT CHROMATOGRAPHY

Chromatofocusing elution pH	*Neurophysin type*	*% of eluted neurophysin*	*pI by IEF-PAGE*
4.42	AVP-Np	9.6	5.04
4.19	OT-Np	17.7	4.60
4.11	OT-Np	30.6	4.56
4.09	AVP-Np	9.4	4.56
3.95	AVP-Np	15.1	4.45
3.90	AVP-Np	8.5	4.35

extension on hNp IV[9]. The recently reported amino acid sequence of the bovine vasopressin–neurophysin precursor molecule also contains an Arg-Ala sequence in positions 108–109 which represents a cleavage site between bovine neurophysin II and the glycosylated carboxy terminal peptide of the common precursor molecule[10]. It seems likely then that human Np IV similarly represents an incompletely processed human neurophysin precursor possessing an extra C-terminal dipeptide. Whether this structure is the result of an incorrect cleavage, or rather more likely just represents incomplete enzymatic processing of the precursor molecule remains to be determined. The two ESN proteins found here have not been individually resolved in previous reports of human neurophysins, probably because of their very similar p*I* values. The significance of this double ESN peak is not known, but may represent a similar phenomenon with the small peak of ESN eluted at the slightly higher pH representing

a basic C-terminal extension of the final product. Likewise, the NSN peak eluted at pH 4.09 has not been previously described. Presumably this is because its p*I* is sufficiently similar to ESN that electrophoretic separation was difficult in the past and any NSN activity in this area was interpreted as immunological crossreactivity with the larger nearby ESN peak. However, the ability to separate this peak from ESN immunoreactivity with a narrower pH gradient shows unequivocally that this NSN peak represents a unique protein. Characterization of this neurophysin as a potential incompletely processed precursor form *versus* a neurophysin degradation product must await amino acid analysis.

Ampholyte displacement chromatography appears to be particularly well suited to separation of neurophysin proteins. Because of the acidic p*I* values of these peptides the majority of non-neurophysin proteins are eluted with the starting buffer leaving the neurophysins bound to the ion-exchange resin. Furthermore, the pH gradient can be altered easily to increase resolution over a given pH range allowing separation of neurophysins which were not resolved by previous methods. The reproducibility, yield and ease of altering the pH elution gradients make this method ideal for the preparative isolation of multiple neurophysin forms from neurohypophyseal tissue. 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. Fig. 3 shows the profile of the same crude neurophysin peak run on a polyacrylamide isoelectric focusing gel. The relative position of the neurophysin peaks is similar to that from the chromatofocusing column, but all of the p*I* values are higher than the ampholyte displacement elution pH. IEF-PAGE of the major neurophysin peaks which were isolated by chromatofocusing resulted in the p*I* values shown in Table I. Using these data a classification system can be devised whereby all neurophysins can be identified by the species, the type of neurophysin (OT-Np or AVP-Np, where hormone specific radioimmunoassays are available), and the p*I* to allow subclassification of the different neurophysins. By this classification the two major human posterior pituitary neurophysins are hAVP-Np, p*I* 4.45, and hOT-Np, p*I* 4.56, with an incompletely processed hAVP-Np, p*I* 5.04, and several other peaks possibly also representing incompletely processed forms: hAVP-Np, p*I* 4.56; hAVP-

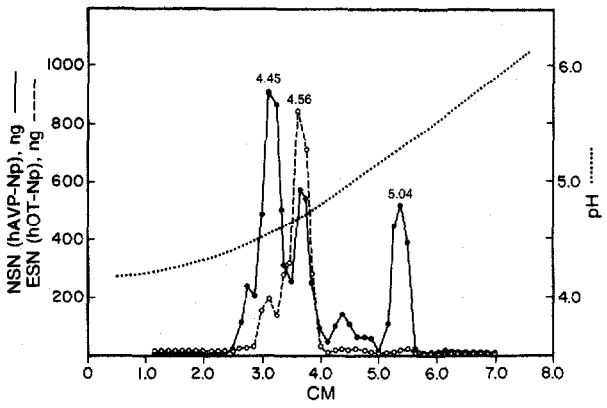

Fig. 3. Polyacrylamide isoelectric focusing gel of human pituitary neurophysins with radioimmunoassay of eluted gel slices for NSN and ESN activity.

Np, p*I* 4.35, and hOT-Np, p*I* 4.60. This method of classification has the advantage of combining criteria for the structure, function and chromatographic behavior of these proteins while avoiding the confusion created by different sequential numbering schemes for various types of separation methods. Hopefully, similar determinations in other species will establish more uniformity in the classification and future analysis of the neurophysins.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Alan G. Robinson for his continued support and encouragement, Darina Sipula for technical assistance and Jane Pennebaker for manuscript preparation. This work was supported by NIH grant RO1-AM16166; the author is a Research Associate in the Veterans Administration Career Development Program.

REFERENCES

1 A. G. Robinson, J. G. Verbalis, J. A. Amico and S. M. Seif, in S. M. McGann (Editor), *Endocrine Physiology III, Internal Review of Physiology*, Vol. 24, University Park Press, Baltimore, MD, 1981, p. 1.
2 J. DeMay and F. Vandesande, *Eur. J. Biochem.*, 69 (1976) 153.
3 K. W. Cheng and H. G. Friesen, *J. Biol. Chem.*, 246 (1971) 7656.
4 S. M. Seif, A. B. Huellmantel, M. P. Platia, C. Haluszczak and A. G. Robinson, *Endocrinology*, 100 (1977) 1317.
5 A. G. Robinson, *J. Clin. Invest.*, 55 (1975) 360.
6 M. J. Brownstein, J. T. Russell and H. Gainer, *Science*, 207 (1980) 373.
7 J. G. Verbalis and A. G. Robinson, *Brain Res.*, 237 (1982) 504.
8 A. G. Robinson, C. Haluszczak, J. Wilkins, A. B. Huellmantel and C. G. Watson, *J. Clin. Endocrinol. Metab.*, 44 (1977) 330.
9 M. T. Chauvet, J. Chauvet, R. Acher and A. G. Robinson, *FEBS Lett.*, 101 (1979) 391.
10 H. Land, G. Schütz, H. Schmale and D. Richter, *Nature (London)*, 295 (1982) 299.