ISOLATION IN HIGH YIELD OF SEPARATED RAT NEUROPHYSIN-I AND NEUROPHYSIN-II BY COMBINED CATION AND ANION EXCHANGE CHROMATOGRAPHY

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1. Introduction

Rat posterior pituitary contains three major neurophysin proteins [1–3]. To separate rat neurophysin-I and -II on an analytical [1,2] or preparative scale [3,4] it was necessary until now to add high concentrations of the dye bromophenol blue.

In this communication the isolation and separation on a preparative scale of rat neurophysin-I and -II in the absence of any added dye are reported. The method is the same as that recently introduced for the isolation of bovine neurophysin in a very high degree of purity [5] and canine neurophysins [6]. The approach has the advantage to drastically reduce at an early step of isolation the protein content of the crude preparation by removing at once unrelated neutral and basic proteins from the acidic neurophysin-like proteins.

2. Materials and methods

Acrylamide and *N,N'*-methylene-bisacrylamide (Bis) were purchased from Bio-Rad Laboratories, Richmond, Calif. and were recrystallized before use [7]. Ammonium persulfate, *N,N,N',N'*-tetramethylenediamine (TEMED), Cellex-CM, and riboflavin were also obtained from Bio-Rad Laboratories and were used without further purification. Tris(hydroxymethyl)aminomethane (Tris), Coomassie Brilliant Blue R, bromophenol blue were bought from Sigma Chemical Company, St Louis, Mo. and Sephadex G-75, G-25 and DEAE–Sephadex-A50 from Pharmacia Fine chemicals, Piscataway, N J. All other chemicals used were reagent grade products from Fisher Scientific Company.

2.1. *Protein determination and amino composition*

Protein concentration was determined according to the method of Lowry et al. [8] and amino acid composition using hydrolysis times of 24 h, 48 h and 72 h as described by Moore [9].

2.2. *Analytical polyacrylamide gel electrophoresis (PAGE) of rat neurophysin proteins*

Protein solution (50 μg/25 μl) was applied on a 7.5% gel (5.5 × 70 mm) with a running pH of 8.8, 5.9 and 4.0 at a current of 2.5 mA/gel for 1 h [10]. Protein was detected by staining with 0.05% (w/v) Coomassie Brilliant Blue in 12.5% trichloroacetic acid (TCA) [11]. After 4 h staining the gel was destained for 24 h in 10% TCA.

2.3. *Biological activities of purified neurophysins*

Hormone content at various steps in the neurophysin preparation was determined by the vasodepressor assay on conscious White leg-horn roosters [12] according to the procedure of Coon [13]. The antidiuretic activity was monitored on mature male Sprague-Dawley rats weighing 200–300 g [14,15]. A match design was used for the bioassays using USP Posterior Pituitary Reference Standard as reference.

2.4. *Immunological analysis of purified neurophysin proteins*

Ouchterlony double diffusion was carried out in 1% agarose according to Audhya and Walter [5], using an antiserum raised against porcine neurophysin in rabbit [16].

2.5. *Extraction of acid-soluble proteins from rat posterior pituitary tissue*

Lyophilized rat posterior pituitary glands were obtained as a gift from Dr A. F. Parlow of the NIAMDD Rat Pituitary Hormone Program. The glands were extracted twice in 0.1 N HCl at 4°C [17]. The crude hormone protein complex was precipitated along with several other proteins by slow saturation with 10% NaCl. The precipitate was dissolved in 0.05% acetic acid and dialysed against distilled water for three days using a spectrapor-3 (mol wt cut-off 3500) membrane tubing. The retentate was then lyophilized (1.9 g).

2.6. *Isolation of crude neurophysin proteins using Diaflo filter membrane*

The lyophilized proteins were dissolved in 300 ml 0.1 M formic acid. The solution was filtered in an Amicon Standard Stirred Cells apparatus (model 402) at 50 psi using a Diaflo PM-30 filter membrane (Amicon Corporation) at 4°C. The volume was reduced to about 80 ml and then replenished to 300 ml with 0.1 M formic acid. Three such repeated operations were performed. The filtrates were pooled and lyophilized (480 mg). The protein was assayed for vasodepressor activity [12,13].

2.7. *One-step separation of the acidic neurophysins from neutral and basic proteins by column chromatography*

Gel filtrations on bead-type Sephadex G-75 (particle size 40–120 μm) and Sephadex G-25 (particle size 50–150 μm) were carried out at room temperature in an all-glass column (2.2 × 165 cm) in 0.1 N formic acid. The flow rate was 25 ml/h (Sephadex G-75) and 35 ml/h (Sephadex G-25), respectively. Ion-exchange chromatography was performed on a CM-cellulose (Cellex-CM, hydrogen form) column (1.5 × 40 cm) equilibrated with ammonium acetate buffer, pH 4.9, 10 mM. After application of the protein in a small amount of 40 mM ammonium acetate buffer, at pH 7.4, it was eluted with the same buffer at a flow rate of 30 ml/h. Approximately 4 ml fractions were collected. Fractions containing the neurophysin proteins were pooled and lyophilized, taken up with distilled water and again lyophilized.

2.8. *Separation of rat neurophysin-I and -II by anion exchange chromatography*

DEAE–Sephadex-A50 was equilibrated with 0.06 M Tris–HCl buffer, pH 7.9, and was packed in an all-glass column (2.6 × 40 cm). After the column was charged with the protein, which has been previously dialysed against Tris–HCl buffer (0.06 M, pH 7.9), it was washed with the same buffer for a period of 6 h. The neurophysins were then eluted with a continuous NaCl ionic-strength gradient (0.0–0.4 M) in Tris

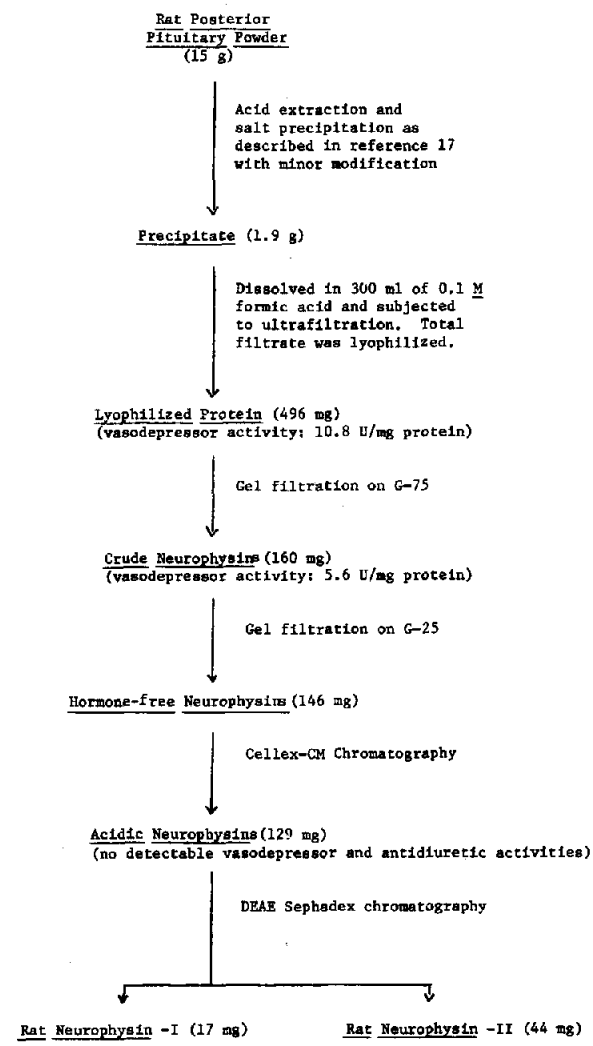

Fig.1. Flow sheet of the isolation procedure for rat neurophysin proteins. Yields and bioassay results are given in parentheses.

buffer (0.06 M, pH 7.9) at a flow rate of 8 ml/h for a theoretical volume of 800 ml. Fractions (4 ml) were collected and protein concentration determined.

3. Results and discussion

Fifteen grams of lyophilized rat posterior pituitary powder gave upon extraction with 0.1 N HCl, a yield of 4.1 g of acid soluble protein as determined by Lowry's method. After precipitation with 10% NaCl, at pH 4.0, and lyophilization, 1.9 g material was recovered (fig.1). Because of its relatively high lipid content this material was excluded from the polymer and instead adhered to the inner surface of the column upon attempted gel filtration on Sephadex G-75. Therefore, the ultrafiltration technique with a PM-30 filter was used to exclude any protein higher than 30 000 dalton. The protein (496 mg) recovered from the filtrate exhibited an avian vasodepressor activity of 10.8 U/mg protein and had a total lipid content of 76 μg/mg protein. This material was consecutively subjected to gel filtrations on Sephadex G-75 and G-25 (fig.1).

The neurophysin protein (146 mg) was then separated from other basic and neutral proteins by cation exchange chromatography using Cellex-CM. The neurophysins (129 mg) emerged in the first ultraviolet-absorbing, major peak, which was followed by two additional ultraviolet-active peaks. The three neurophysins were then separated by anion-exchange chromatography on DEAE-Sephadex A-50 using a continuous NaCl gradient as shown in fig.2. Two of these neurophysins, rat neurophysin-I (17 mg) and -II (44 mg), were characterized. Neither protein exhibited any detectable avian vasodepressor activity (tested at a dose of 1 mg) and anti-diuretic activity (tested at a dose of 0.1 mg). Both neurophysins were homogeneous proteins by the following criteria: PAGE at pH 8.8 (see insert in fig.2), 5.9 and 4.0; immunodiffusion using non-specific antibodies prepared in rabbits against porcine neurophysin [16]. The amino acid composition was in general agreement with those reported [3,4].

The partial amino acid sequences of the first 33 residues of both rat neurophysin-I and -II have been determined using these preparations [18,19].

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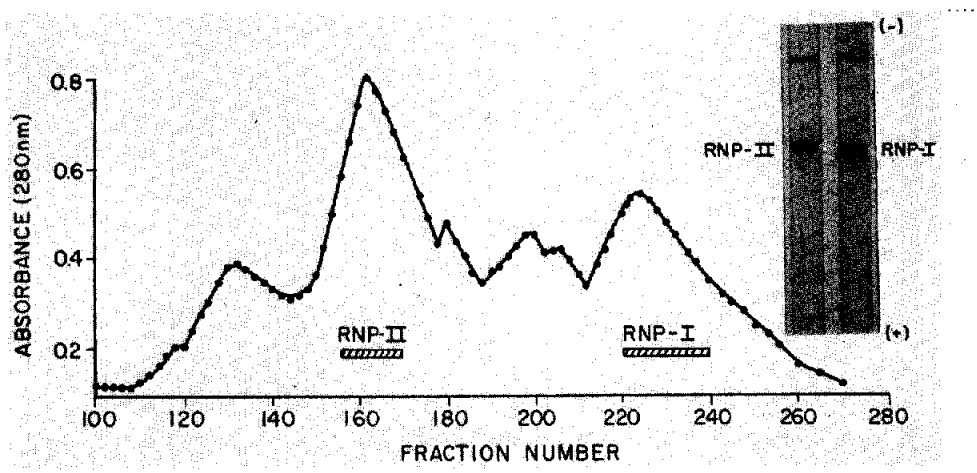

Fig.2. Dissociation of rat neurophysin-I from -II by ion-exchange chromatography. A column of DEAE Sephadex A-50 (2.6 × 40 cm) was charged with 125 mg of neurophysin protein mixture dissolved in 2.2 ml Tris–HCl buffer. The protein was eluted with Tris–HCl buffer containing increasing concentrations of sodium chloride (0.0–0.4 M over 800 ml). Fractions indicated by the shaded bars were pooled for isolation of rat neurophysins-I and -II. Insert: Analytical PAGE profiles of rat neurophysin-I and -II at a running pH 8.8.

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