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The purification of synthetic oxytocin and analogues by gel filtration on Sephadex G-15

The procedures most commonly employed for the purification of laboratory synthesized oxytocin and analogues have been counter current distribution[1] and partition chromatography on Sephadex G-25[2]—methods which entail the careful selection of a suitable solvent system for the purification of each individual analogue. The present communication describes a purification procedure by gel filtration[3] on Sephadex G-15 which requires the use of just two standard solvents. It is an adaptation of the gel filtration procedure on Sephadex G-25, described by PORATH AND SCHALLY in the purification of oxytocin isolated from natural sources[4]. The procedure is both rapid and effective, having already been used in this laboratory for the purification of several synthetic analogues of the neurohypophysial hormones.

Experimental

The synthetic oxytocin used in the purification was prepared by the Merrifield method of solid phase peptide synthesis[5] as described previously[6]. The crude lyophilized material obtained after reductive deprotection and oxidation of the protected nonapeptide intermediate served as the starting material for the purification. At this stage the sample contained, in addition to the active peptide, inorganic salts and some dimeric peptide. The first step in the purification entailed desalting of the peptide with 50% acetic acid. In a typical run the procedure used was as follows: The crude lyophilized powder (300 mg, obtained by reduction of 75 mg of protected intermediate) was dissolved in 2.0 ml of 50% acetic acid and applied to the top of a column of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, particle size 40–120 μ, column size 110 × 1.2 cm) which had been pre-equilibrated with 500 ml of 50% acetic acid. The sample was then washed into the column with an additional one ml of 50% acetic acid and eluted with 50% acetic acid at a rate of 8–10 ml per hour. Two ml aliquots were collected using an automatic fraction collector. The eluate was assayed for peptide by the method of LOWRY *et al.*[7] and the salts were located by flame photometry for potassium and sodium, and by precipitation with silver nitrate for chloride. The peptide material was eluted in two partially resolved peaks (Fig. 1), clearly separated from the salt (tubes 60–80). The second peak (tubes 31–37), containing most of the active peptide as detected by oxytocic assay[8], was pooled, diluted with two volumes of distilled water and lyophilized. Meanwhile the column was being re-equilibrated with 500 ml of 0.2 *N* acetic acid over a period of 20 h. The lyophilized powder (35 mg) from peak 2 was dissolved in 2.0 ml of 0.2 *N* acetic acid, applied to the column, washed with a further one ml of 0.2 *N* acetic acid and then eluted and collected as before, this time at a rate of 12–15 ml per hour. The active peptide emerged as a single nearly symmetrical peak preceded by a small amount of dimer (Fig. 2). The contents of this main peak (tubes 44–51) were pooled, diluted with two volumes of distilled water and lyophilized to give the desired product as a white fluffy powder (30 mg). This material was found to have an oxytocic activity of approx. 480 U/mg on the isolated rat uterus[8], and was adjudged pure by the criteria of electrophoresis, thin

layer and paper chromatography, optical rotation and microchemical and amino acid analyses[6].

In addition to oxytocin, the method has also been used to purify glumitocin (4-Ser 8-Gln oxytocin)[9], 8-Phe and 8-Gln oxytocins[10] as well as several other analogues of oxytocin. The total time required to carry out one such purification, starting with a column which had been pre-equilibrated with 50% acetic acid, was approx. 64 h, *i.e.* 12 h for each of the two elution steps and two 20 h re-equilibration periods.

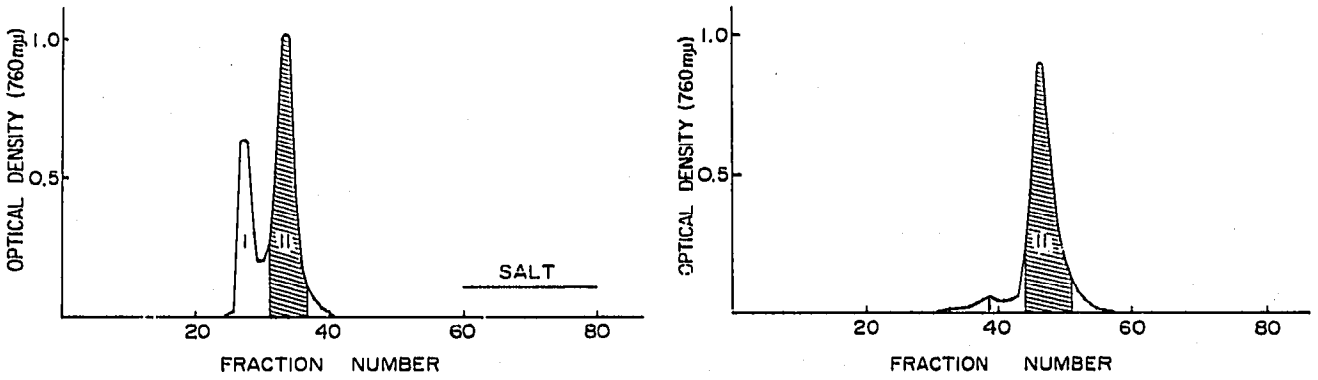

Fig. 1. Elution profile for first step in purification of synthetic oxytocin. 300 mg crude lyophilized material (dimeric peptide, active peptide and salt) dissolved in 2 ml of 50% acetic acid and applied to a column of Sephadex G-15. Eluting solvent: 50% acetic acid, flow rate 8–10 ml per hour; fraction size 2 ml. Peptide measured by LOWRY method; (I, dimer; II, oxytocin). Salt located by standard determinations as mentioned in text. Material from shaded area under peak II lyophilized.

Fig. 2. Elution profile for second step in purification of synthetic oxytocin. 35 mg of active peptide plus small amount of dimer dissolved in 2 ml of 0.2 *N* acetic acid and applied to a column of Sephadex G-15. Eluting solvent: 0.2 *N* acetic acid; flow rate 12–15 ml per hour; fraction size 2 ml. Peptide measured as in Fig. 1; (I, dimer; II, oxytocin). Material from shaded area under peak II lyophilized.

A number of further points are worth noting with regard to the development of this procedure. (1) In preliminary experiments, when only 0.2 *N* acetic acid was used for elution, the salt present in the crude lyophilized sample retarded the peptide material on the column, resulting in the active peptide being eluted with the salt. This led to the incorporation of a second step in which 50% acetic acid was used to desalt the peptide. (2) Elution with 50% acetic acid resulted in contamination of the final product with trace amounts of Sephadex dissolved out by this solvent. By reversing the two steps *i.e.* by eluting first with the 50% acetic acid solution and following with the 0.2 *N* acetic acid elution, these impurities were removed and thus the problem was eliminated. (3) In our experience, as much as 300 mg of crude lyophilized material can be applied to a column of the above size to give a satisfactory separation by this procedure.

Conclusion

Gel filtration on Sephadex G-15 can be used for the rapid and effective purification of those neurohypophysial hormones and their analogues which, because of their individual solubility characteristics, cannot readily be purified by either counter current distribution or partition chromatography until a suitable solvent system is first found. Furthermore, since the solvents which are used to effect purification of the

desired peptide by this procedure, *i.e.* acetic acid and water, are both readily removed by lyophilization, the final product is obtained in the highest possible state of purity free from contamination by eluting solvents. Thus the problem of contamination of the final product by less volatile solvents such as pyridine and benzene, which are commonly used in partition systems, is obviated. We feel that these distinct advantages, coupled with the inherent speed and simplicity of gel filtration, make this procedure a useful complement to the aforementioned methods for the purification of those synthetic analogues of the neurohypophysial hormones in which the only contaminants present are inorganic salts and dimer.

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1 E. SCHRÖDER AND K. LÜBKE, *The Peptides*, Vol. II, Academic Press, New York and London, 1966, pp. 320–335; references cited therein.
2 D. YAMASHIRO, *Nature*, 201 (1964) 76.
3 J. PORATH AND P. FLODIN, *Nature*, 183 (1959) 1657.
4 J. PORATH AND A. V. SCHALLY, *Endocrinology*, 70 (1962) 738.
5 R. B. MERRIFIELD, *J. Am. Chem. Soc.*, 85 (1963) 2149.
6 M. MANNING, *J. Am. Chem. Soc.*, 90 (1968) 1348.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 R. D. MUNSICK, *Endocrinology*, 66 (1960) 451.
9 M. MANNING, T. C. WUU, J. W. M. BAXTER AND W. H. SAWYER, *Experientia*, 24 (1968) 659.
10 M. MANNING, T. C. WUU, J. W. M. BAXTER AND W. H. SAWYER, *FEBS 1968 Meeting, Prague*, Abstr. 824, p. 206.

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