Isolation of the epidermal growth factor from the shrew submaxillary gland

T.T. Yip, Y.Y. Tam, Y.C. Kong, M.C. Belew* and J. Porath*

Department of Biochemistry, University of Hong Kong, Shatin, NT, Hong Kong and **Institute of Biochemistry, Biomedical Center, University of Uppsala, Uppsala, Sweden*

Received 4 June 1985

The epidermal growth factor (EGF) from the submaxillary glands of the Chinese Shrew (*Suncus murinus*) is purified to apparent homogeneity by using a sequence of four chromatographic steps, viz. gel filtration on Sephacryl S-200, affinity chromatography on immobilized Ni, hydrophobic interaction on phenyl-Sepharose CL-4B and reverse-phase HPLC. An 800-fold increase in specific activity and an overal recovery of 46% were achieved. The most effective step in its purification is the successful use of immobilized metal ion affinity chromatography (IMAC). This method was very selective, reproducible and requires a minimum of sample pre-treatment prior to chromatography.

Shrew *EGF* *Immobilized metal ion affinity chromatography* *Radioreceptor assay*

1. INTRODUCTION

The original method used in the purification of mouse epidermal growth factor (EGF) involved multiple and lengthy steps [1]. The method was later modified and the use of Biogel and DEAE-cellulose chromatography [2], reverse-phase HPLC [3] and high-resolution ion-exchange chromatography [4] gave much better results. However, a more efficient method for the purification of EGF is needed, especially when the highest possible purity is required for detailed studies of the biological and chemical properties of the isolated EGF.

The shrew (*Suncus murinus*) submaxillary gland is a rich source of EGF according to our investigations (Yip et al., to be published). Although EGF from several other species have been reported, the EGF from the shrew has not been studied until now. Here, its isolation from a crude extract of the submaxillary gland by using a sequence of 4 chromatographic procedures is described.

Abbreviations: Ni-TED, Ni^{2+} immobilized on tricarboxymethylethylenediamine (TED) covalently linked to Sepharose 6B; IMAC, immobilized metal ion affinity chromatography

2. MATERIALS AND METHODS

Shrews were captured from the wild. Their submaxillary glands were excised, rinsed with distilled water, blotted dry, and immediately frozen. 1 g of the glands (wet wt) was added to 9 ml cold distilled water containing 5 mM phenylmethylsulfonyl fluoride and homogenized in a Polytron PT 45-50 at maximum speed for 30 s. The homogenate was centrifuged at 40000 × *g* for 50 min. This procedure was repeated twice on the pellet. The supernatants were combined and lyophilized.

Standard mouse EGF preparations were obtained from Collaborative Research. Iodination was carried out according to the standardized chloramine T method. The radioreceptor assay (RRA) for EGF was performed according to the method of Imai et al. [5]. The standard binding curve was usable in the range 0.1–20 ng EGF. The shrew EGF preparations gave binding curves that were identical to the standard throughout the entire range.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

Sephacryl S-200, phenyl-Sepharose CL-4B, and Sepharose 6B were products of Pharmacia, Uppsala. The metal chelating gel (tricarboxymethylethylenediamine, TED, coupled to Sepharose 6B) was synthesized according to the method of Porath and Olin [6]. HPLC was carried out on an LDC Constametic IIG system. The column (4 × 250 mm) was packed with Lichrosorb RP-18 (5 μm beads) obtained from Merck AG, FRG.

3. RESULTS AND DISCUSSION

Fig.1 shows the fractionation obtained after chromatography of the crude extract from the shrew submaxillary gland on Sephacryl S-200. The major components, which are inactive, are eluted near the void volume of the column. The activity peak is associated with the minor components that elute between fractions 40 and 50 with an apparent M_r between those of hemoglobin and cytochrome *c*. The recovery of applied material was about 90%, while the recovery in activity was about 65% in the pooled fractions.

The active material was pooled and K_2SO_4 was dissolved in it to a final concentration of 0.5 M. This was then applied to an Ni-TED column and the fractionation obtained is shown in fig.2. About

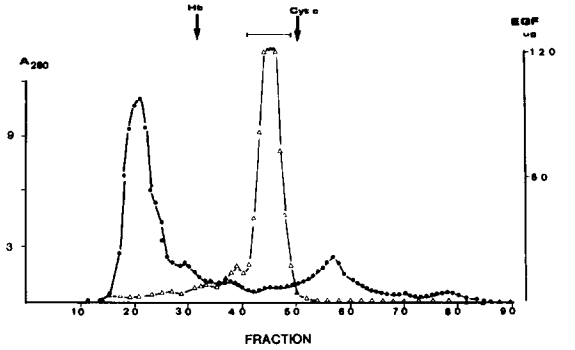

Fig.1. Gel filtration of the crude extract from the shrew submaxillary glands on a column (3.2 × 130 cm) of Sephacryl S-200. The column was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 7.4. The flow rate was 10.7 ml·cm^{-2}·h^{-1} and fractions of 20 ml were collected. (●——●) Absorbance at 280 nm, (Δ——Δ) concentration of EGF (μg/fraction) determined by RRA on a 30-fold dilution of each fraction. The horizontal bar at the top shows the fractions that were pooled. Hb and Cyt *c*, elution positions of hemoglobin and cytochrome *c*, respectively.

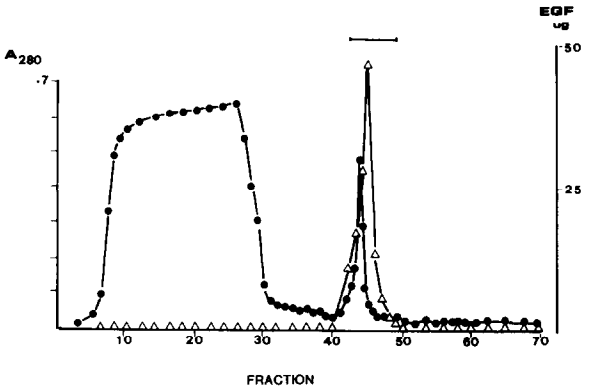

Fig.2. IMAC of the activity pool from fig.1 on Ni-TED. The column (2 × 15 cm) was equilibrated with 50 mM Tris-HCl buffer, 0.5 M in K_2SO_4, pH 7.4, and maintained at a flow rate of 23 ml·cm^{-2}·h^{-1}. Solid K_2SO_4 was dissolved in the pooled sample to give a final concentration of 0.5 M prior to application to the column. Unadsorbed material was washed out with equilibrating buffer and the adsorbed, active material was eluted by 0.1 M Na acetate buffer, 0.5 M in K_2SO_4, pH 6.0. (●——●) Absorbance at 280 nm, (Δ——Δ) concentration of EGF (μg/fraction). Pooled fractions are indicated by the horizontal bar.

70% of inactive material was eluted unadsorbed from the column. The active material, adsorbed to the column, was recovered almost quantitatively (table 1) by elution with 0.1 M sodium acetate buffer, 0.5 M in K_2SO_4, pH 6.0. However, the activity peak did not correspond well with the protein peak, indicating heterogeneity and the necessity for further purification. The total recovery of applied material or activity was better than 90%. By this step alone, the active material was purified by a factor of about 18 as estimated from its specific activity (table 1).

The active pool from the previous step was purified further by a factor of 3 after chromatography on phenyl-Sepharose CL-4B (see fig.3 and table 1). The activity was adsorbed and an attempt was made to elute it by decreasing the salt concentration in a linear gradient from 0.5 to 0 M K_2SO_4 in 0.1 M Na acetate, pH 6.0. The total gradient volume was about 1.5-times the total column volume. However, most of the activity remained adsorbed to the column and was eluted by washing of the column with the 0.1 M Na acetate buffer, pH 6.0. Further material or activity was not eluted by washing the column with 60%

Table 1

Summary of the purification scheme for shrew EGF

Sample	Total volume (ml)	Total A_{280}	Protein (mg)	EGF (μg)	Specific activity	Yield (%)
Crude extract	70	1231	882	1080	1.22	100
Sephacryl S-200 pool	185	121	78.6	695	8.84	64.4
Ni-TED pool	100	7.6	4.0	650	163	60.2
Phenyl-Sepharose pool	119	2.3	1.2	600	500	55.6
RP-HPLC pool	1	0.4	0.5	500	1000	46.3

ethylene glycol in the above acetate buffer. The recovery of applied material and activity was almost quantitative (table 1).

The final purification of the EGF to apparent homogeneity was achieved by chromatography of the active pool from the previous step on a column of reverse-phase HPLC (fig.4). The active pool from the phenyl-Sepharose CL-4B column was dialyzed against distilled water, lyophilized, and redissolved in 200 μl distilled water. Aliquots of 50 μl were applied to the HPLC column, followed by elution with a linear gradient from 20 to 32% acetonitrile in 50 mM triethylammonium acetate buffer, pH 5.6. The flow rate was 0.5 ml/min and the total gradient time was 30 min. The activity

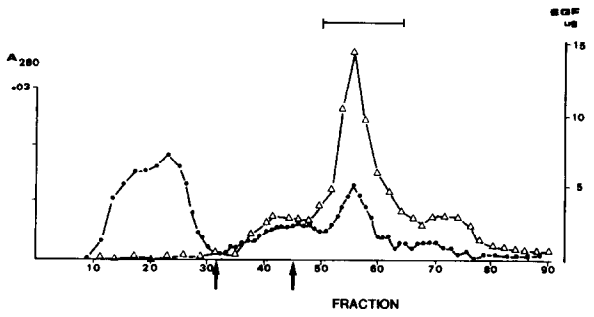

Fig.3. Chromatography of the active pool from fig.2 on phenyl-Sepharose CL-4B. The column (2.5 × 15 cm) was equilibrated with the buffer used in fig.2 and the flow rate was maintained at 23 ml·cm^{-2}·h^{-1}. Fractions of 7 ml were collected. The arrows at tubes 30 and 45 indicate the start and end of the linear gradient. (●—●) Absorbance at 280 nm, (Δ—Δ) concentration of EGF (μg/fraction). Horizontal bar indicates fractions that were pooled.

was mainly confined to the major protein peak that emerged near the end of the gradient (as indicated by arrows in fig.4). A very minor activity peak was also eluted just after this main peak. When the major activity peak was pooled and rerun on a regenerated column under the same conditions as above (see inset in fig.4) only one symmetrical peak was eluted at a position corresponding to that of the major peak A in fig.4 showing the chromatographic homogeneity of this highly purified preparation. This material was very potent in biological assays such as enhancing eyelid opening and in promoting mitogenesis of human fetal lung fibroblasts grown in vitro. Compared with the starting crude extract, this preparation was at least 800-times more potent (table 1). The high overall yield of the active, purified material indicates the suitability and effectiveness of the procedure we adopted for purifying the shrew EGF. It could well be adopted for isolating EGFs from other sources, especially since the procedures we employed were very reproducible and the whole purification step can be performed in 2 or 3 days.

Of the purification procedures we have used, that based on IMAC [6,7] was the most effective and the recovery in activity was almost quantitative. The principles and basic mechanisms of this versatile method for protein fractionation have recently been published [6,8] and its usefulness is further extended by the results of Sulkowski et al. [9] which indicate the effectiveness of IMAC methods in assessing the topography of a protein molecule with respect to the accessibility of histidine and, possibly, tryptophan residues.

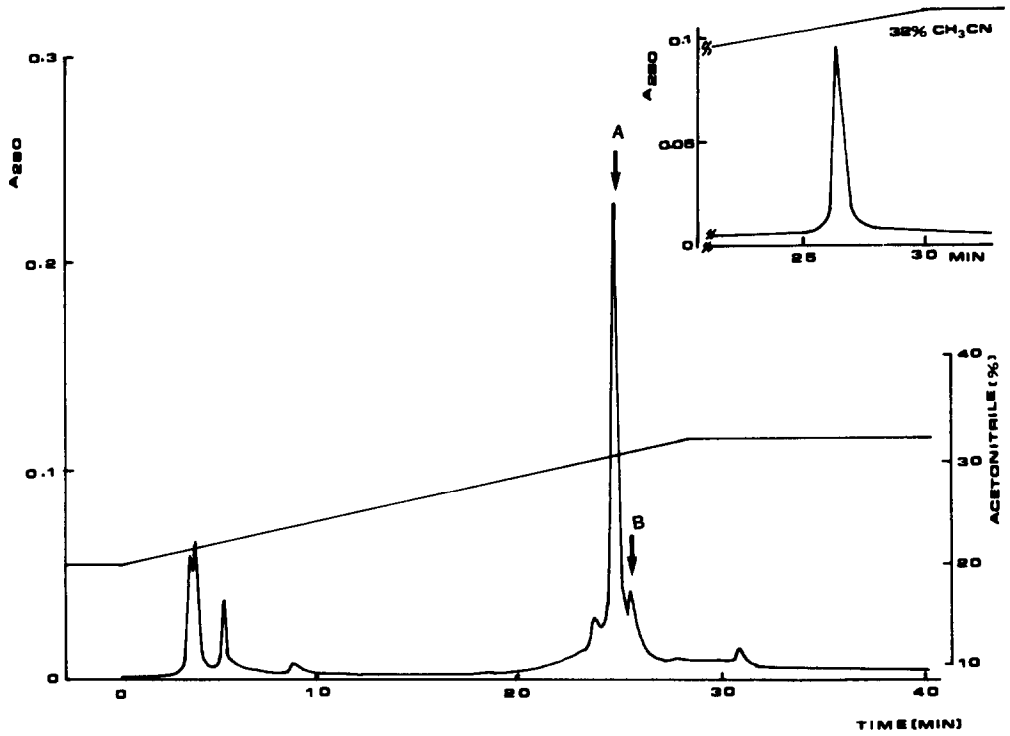

Fig.4. Final purification of the pooled active material from fig.3 on a reverse-phase HPLC column. One major peak (A), accounting for about 85% of the EGF activity and a minor peak (B) were separated. The inset shows the chromatographic homogeneity of the major peak after rerunning the pooled material in peak A on the same HPLC column, using a shallow gradient.

The fact that the shrew EGF binds to the hydrophobic adsorbent, phenyl-Sepharose CL-4B, might indicate structural similarities with EGFs from other species. The results of Holladay et al. [10] indicate that mouse and human EGF have a high content of tyrosine residues, a large proportion of which are exposed indicating the hydrophobic nature of these proteins. Further studies on the purified shrew EGF are expected to elucidate in more detail its relationships to other known and well characterized EGFs.

ACKNOWLEDGEMENTS

T.T.Y. was supported by grants from the Carl Trygger Foundation and the Kevin Hsu Research Fund (Y.C.K.) during his stay at the Institute of Biochemistry, Biomedical Center, University of Uppsala, Sweden. This work was also supported in part by grants from the Swedish Natural Science Research Council.

REFERENCES

[1] Cohen, S. (1962) J. Biol. Chem. 237, 1555–1562.
[2] Savage, C.R. jr and Cohen, S. (1972) J. Biol. Chem. 247, 7609–7611.
[3] Matrisian, L.M., Larsen, B.R., Finch, J.S. and Magnum, E. (1982) Anal. Biochem. 125, 339–351.
[4] Burgess, A.W., Lloyd, C.J. and Nice, E.C. (1983) EMBO J. 21, 2065–2069.
[5] Imai, Y., Tsushima, T., Sasaki, N. and Matsuzaki, F. (1979) in: Growth and Growth Factors (Shizume, K. and Takano, K. eds) pp.275–288, University of Tokyo Press, Tokyo.
[6] Porath, J. and Olin, B. (1983) Biochemistry 22, 1621–1630.
[7] Porath, J., Carlsson, J., Olsson, I. and Belfrage, G. (1975) Nature 258, 598–599.
[8] Sulkowski, E., Vastola, K., Oleszek, D. and Von Muenchhausen, W. (1982) Anal. Chem. Symp. Ser. 9, 313–322.
[9] Sulkowski, E. (1985) Trends Biotechnol. 3, 1–7.
[10] Holladay, L.A., Savage, C.R. jr, Cohen, S. and Puett, D. (1976) Biochemistry 15, 2624–2633.