Purification of the enhancing factor from mouse intestines

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A unique polypeptide, called enhancing factor (EF), which enhances the binding of labeled epidermal growth factor (EGF) to cells, has been isolated. It has been purified to homogeneity from the acid-soluble proteins of mouse intestines. Earlier, EF was partially purified by two cycles of gel-permeation chromatography on Bio-Gel columns. We now report the final purification of EF on high-performance liquid chromatography (HPLC), using a reverse-phase column (μBondapak C18). The purity of the protein was confirmed when a single peak was obtained in HPLC. Also, a single protein band was obtained in SDS-PAGE. Purified EF has the same properties in vitro as those reported earlier for partially purified EF.

Growth factor *HPLC* *Receptor modulation*

1. INTRODUCTION

A polypeptide, which enhances the binding of labeled epidermal growth factor (EGF) to the cell membrane in a radioreceptor assay, has been isolated from normal mouse intestines. This polypeptide is termed 'enhancing factor' (EF) [1]. EF binds to the cell membrane via its own receptor and in turn provides a binding site for EGF [2]. In vitro, EF makes EGF available to quiescent cells, even in the absence of functional EGF receptors, thereby stimulating DNA synthesis [2]. In the earlier studies, EF was partially purified, after acid/ethanol extraction of mouse intestines, and gel filtration of the acid-soluble proteins on Bio-Gel columns [1]. Here, the total purification to homogeneity of the EF using HPLC is described.

2. MATERIALS AND METHODS

EF was extracted from normal mouse small intestines following the acid/ethanol extraction procedure of Roberts et al. [3]. It was partially purified on Bio-Gel P-100 and P-60 columns and tested in the ^{125}I-EGF-receptor binding assay on fixed A431 cells as described in [1,2]. Fractions eluted from the Bio-Gel P-60 column showing enhancing activity greater than 50% were pooled (EF-P-60) and subjected to purification by HPLC (Waters Associates). 2–3 mg EF-P-60 was lyophilised, reconstituted in 100 μl of 0.1% trifluoroacetic acid (TFA) (Fluka) in water and injected into a semipreparative reverse-phase (RP) column (μBondapak C18; Waters, 7.8 × 300 mm) equilibrated with 0.1% TFA. A gradient of acetonitrile (Spectrochem, India, HPLC grade) with 0.1% TFA was applied at a flow rate of 1 ml/min for 40 min (fig.1). Elution was monitored at 280 nm. Each peak was collected separately and tested in the ^{125}I-EGF-binding radioreceptor assay as in [1]. The peak showing activity (EF-HPLC-I) was rerun on the same column, under identical conditions. The peak with activity was again collected (EF-HPLC-II) and subjected to electrophoresis and amino acid analysis.

Protein concentration was determined as described by Hartree [4], using bovine serum albumin (BSA) as standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 17.5% slab gel in the buffer system of Laemmli [5]. Standard markers including BSA (M_r 68000), carbonic anhydrase (M_r 29000), RNase (M_r 13800)

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and insulin (M_r 6000) were used. Proteins were stained with 0.25% Coomassie brilliant blue R250. 2.5–5 nM EF was hydrolysed in 500 μl of 6 N HCl under vacuum at 110°C for 22 h. Amino acid analysis was performed on an LKB amino acid analyser.

3. RESULTS AND DISCUSSION

The partially purified EF from two cycles on the Bio-Gel column was further purified on HPLC on an RP column. Fig.1 shows the protein profile recorded at 280 nm and the gradient of acetonitrile with 0.1% TFA. Each peak was collected separately and tested in the A431 radioreceptor assay as described [1]. Only one peak, which eluted with a retention time of 30.08 min (47% acetonitrile), showed enhancing activity. This peak (EF-HPLC-I) was collected and recycled through the same RP column, under identical conditions. The EF peak eluted as a single peak with a similar retention time of 29.86 min (46.6% acetonitrile) (fig.2) and showed a 90% increase in the binding of ^{125}I-EGF in the radioreceptor assay.

EF, eluted at different steps in the purification procedure, has been analysed on SDS-PAGE (fig.3). EF eluted from Bio-Gel P-100 (EP-P100)

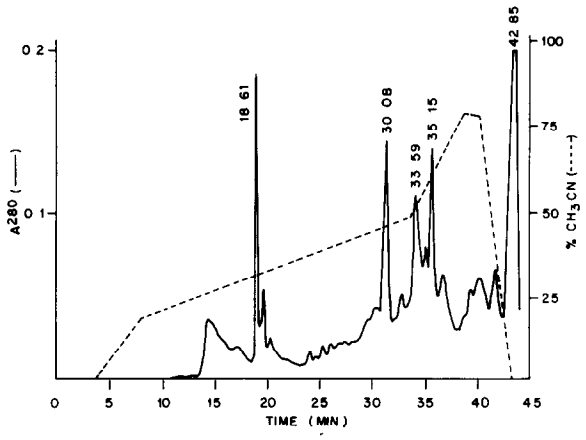

Fig.1. HPLC profile of EF-P-60 on a μBondapak C18 column. 2–3 mg lyophilised protein was reconstituted in 100 μl of 0.1% TFA and injected. Elution was performed with a 40 min, 0–80% acetonitrile gradient in 0.1% TFA (– – –), at a flow rate of 1 ml/min. Absorbance was measured at 280 nm (——). Peak fractions were collected, lyophilised and tested in the ^{125}I-EGF-binding assay as described in [1].

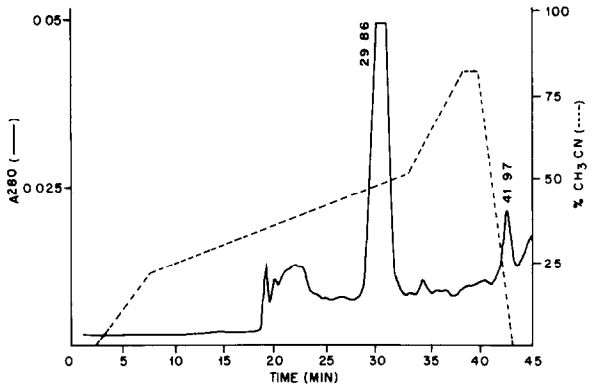

Fig.2. Rechromatography of EF-HPLC-I on a μBondapak C18 column under conditions identical to those described in fig.1. The 30.08 min peak obtained from the 1st cycle HPLC was collected, lyophilised, reconstituted in 0.1% TFA and injected.

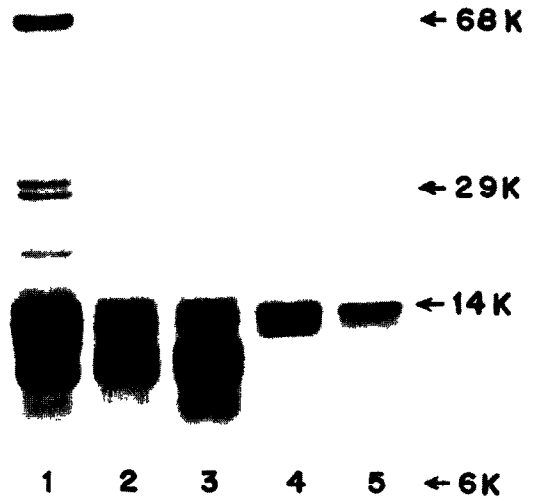

Fig.3. SDS-PAGE of EF at different steps of purification. 50 μg protein was dissolved in 60 μl sample buffer with 2-mercaptoethanol and heated for 3 min at 100°C. The samples were then applied to a 1.5 mm, 17.5% acrylamide gel with a 3% acrylamide stacking gel. The gel was fixed in methanol:acetic acid:water (5:1:5) and stained with Coomassie brilliant blue. Lanes: 1, acid-soluble proteins from mouse intestines; 2, EF-P-100; 3, EF-P-60; 4, EF-HPLC-I; 5, EF-HPLC-II.

and Bio-Gel P-60 (EF-P-60) shows 3–4 bands whereas EF-HPLC-II purified after 2 cycles of HPLC shows a single major protein band. EF has an M_r of 14000 as calculated on 17.5% SDS-PAGE, using standard M_r markers. However, when calculated from the Bio-Gel P-100 elution profile, EF elutes immediately after cytochrome *c* (M_r 12500) and has an apparent M_r of 12000. The purified EF gives a single peak when injected on the RP column in HPLC. As seen from table 1, EF has been purified 2000-fold from the crude intestinal acid-soluble proteins. Approx. 500 ng purified EF is obtained from 1 mg acid-soluble proteins. There is a substantial loss in activity which could be due to specific loss of the EF protein or its denaturation or to separation of its activator during purification. The exact reason, which is not known, is being investigated.

The amino acid composition of the purified polypeptide is shown in table 2. The number of residues per molecule is assigned tentatively, assuming an apparent M_r of 14000. The amino acid composition is distinct from other growth factors like TGF-β [6,7] isolated under identical conditions. Purified EF, like EF-P-60 at high concentrations [2], stimulates DNA synthesis in quiescent cells and acts synergistically with EGF when sub-mitogenic concentrations of both EF and EGF are added (not shown). Preliminary studies show that 18 h after hepatectomy, the acid-soluble extract of liver passed through RP-HPLC, has a small peak of enhancing activity. This activity elutes at a similar concentration of acetonitrile to intestinal EF. EF appears to play a role in cell proliferation by modulating the action of growth factors. To our knowledge, this is the first report of purification of a local modulator of growth factors from the intestine of normal mouse.

Table 1

Stepwise purification of EF from intestines of mice

Purification step	Total protein (mg)	Total units[a]	Specific activity (U/mg)
Acid-soluble extract	69.6	–	–
EF-P-100	14.8	1850	125
EF-P-60	6.0	1200	200
EF-HPLC-I	0.079	26	329
EF-HPLC-II	0.035	70	2000

[a] 1 U activity is defined as the amount of protein (mg) required for 50% enhancing activity in ^{125}I-EGF-receptor binding assay, using fixed A431 cells, as described in [1]

Table 2

Amino acid analysis of EF

Amino acid	No.of residues/mol (mean ± SD)	Amino acid	No.of residues/mol (mean ± SD)
Aspartic acid	9 ± 0.5	methionine	3 ± 0
Threonine	5 ± 0.5	isoleucine	3 ± 0
Serine	9 ± 0.5	leucine	7 ± 0.8
Glutamic acid	14 ± 0.8	tyrosine	4 ± 1.0
Proline	4 ± 0.8	phenylalanine	5 ± 0.4
Glycine	14 ± 1.9	histidine	3 ± 0
Alanine	9 ± 1.6	lysine	15 ± 1.0
Cysteine	3 ± 0	arginine	6 ± 0.5
Valine	3 ± 0.8		

EF was hydrolysed in 6 N HCl at 110°C for 24 h in sealed, evacuated ampoules. Values are based on 4 separate determinations of 4 different preparations. The number of residues per mole is based on an apparent M_r of 14000

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