Journal of Chromatography, 305 (1984) 295—308
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1924

THE USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ISOLATION AND CHARACTERISATION OF MOUSE AND RAT EPIDERMAL GROWTH FACTORS AND EXAMINATION OF APPARENT HETEROGENEITY

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(First received June 20th, 1983; revised manuscript received August 31st, 1983)

SUMMARY

Various epidermal growth factor preparations obtained from the mouse submaxillary gland (mEGF), have been separated into a number of components by reversed-phase high-performance liquid chromatography (HPLC). It is shown here, however, that when the mEGF is isolated rapidly, using only reversed-phase HPLC for trace enrichment and high-resolution fractionation, it is a single molecular species as determined with several ion-pairing solvent systems, provided that proteolysis is inhibited in the original extracts. This indicates that the minor components of mEGF that have been reported are artefacts formed during the isolation procedure, and are of no biological significance. The products of deliberate mild degradation of mEGF are shown to produce similar chromatographic profiles to those observed in samples of mEGF prepared in the absence of proteolytic inhibitors. Rat EGF has been isolated in a similar manner, and is shown to share many of the properties of the major tryptic digestion product of mEGF.

INTRODUCTION

Since first being isolated [1] epidermal growth factor (EGF) has been shown to have effects on a wide variety of cells in culture [2]. In spite of its importance in the study of control of cell proliferation, it is only readily available from the mouse [3], although lengthy schemes for purification have been published for the rat [4] and human [5] factors. Mouse EGF (mEGF)

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has previously been shown to chromatograph on a reversed-phase high-performance liquid chromatographic (HPLC) system, which is capable of a high degree of resolution [6] and has been separated into a number of components [7—9]. However, since HPLC is sufficiently efficient to separate oxidation products of peptides [10], it is necessary to distinguish between naturally occurring variants of a protein, which might be of biological interest, and artefactually generated heterogeneity, which appears to be the cause of relaxin variants [11].

We have examined the heterogeneity of mEGF preparations, and show that mouse and rat EGFs each comprise a single molecular species when prepared rapidly.

EXPERIMENTAL

Materials

Epidermal growth factor was receptor-grade material from Collaborative Research (Waltham, MA, U.S.A.). Pepstatin A was obtained from Sigma (St. Louis, MO, U.S.A.), bovine serum albumin (BSA) was Fr V reagent grade from Miles Labs. (Stoke Poges, U.K.). Acetonitrile was HPLC S-grade from Rathburn Chemicals (Walkerburn, U.K.).

Animals

Submaxillary glands were obtained from adult male C57 black mice, or from adult male Sprague—Dawley rats weighing between 150 and 250 g which were stimulated with testosterone as described by Moore [4].

Submaxillary glands were extracted in 0.05 *M* acetic acid [3] or with 0.155 *M* sodium chloride made to pH 2.1 with hydrochloric acid, supplemented with 10 μg/ml Pepstatin A. The extract was frozen and thawed, and centrifuged at 60,000 *g* for 1 h at 4°C. The resulting supernatant was collected by decantation through glass wool to remove floating fat.

Radioimmunoassay of mEGF

For the preparation of ^{125}I-labelled EGF 10 μg of mEGF (Collaborative Research) was labelled with 0.5 mCi of Na^{125}I to a specific radioactivity of between 30 and 40 μCi/μg by the method of Hunter and Greenwood [12] using 50 μg of chloramine T as oxidant and a reaction time of 1 min. The active, labelled hormone was eluted from a column (12 ml) of Sephadex G-25 equilibrated in 0.05 *M* phosphate buffer, pH 7.5, containing 0.5% (w/v) BSA.

The method of radioimmunoassay used was as previously described [13], except that the 0.2 ml of 25% (v/v) lamb serum in phosphate-buffered saline (PBS) was added post-incubation. The incubation period was 60—70 h at 4°C. The antiserum used was obtained commercially (Collaborative Research) and used at a final dilution of 1:15,000.

Receptor-binding assay for mEGF

The procedure used was as previously described [13]. The target cells were a squamous carcinoma head and neck cell line LICR-LON-HN-1 [14] which has been shown to possess membrane EGF receptors, having $7.5 \cdot 10^5$ sites per cell [15].

Bioassay

Rama 27, a clonal line of cells from the stroma of normal rat mammary gland [16] was used to assay the ability of EGF to increase DNA synthesis. Rama 27 cells were plated at a density of $2 \cdot 10^5$ cells per well in Sterilin 6-well plates in Dulbecco's Modified Eagles Medium (MEM) supplemented with 5% Fetal Calf Serum. After 24 h they were washed twice and refed with Dulbecco's MEM supplemented with 250 μg/ml BSA, 48 h later they were again refed with Dulbecco's MEM supplemented with 250 μg/ml BSA, and growth factors were added. After a further 18 h, [^{3}H] thymidine was added at a concentration of 0.4 μCi/ml (1.5 μM) and incubated for 1 h. The cells were then washed twice with PBS (Ca^{2+}- and Mg^{2+}-free), twice with ice cold 5% trichloroacetic acid and twice with cold ethanol. They were allowed to dry, incubated with 0.1 *M* sodium hydroxide at 37°C for 30 min, aliquots were taken, and counted in Instagel (Packard) in a Packard liquid scintillation counter.

HPLC methods

Initial fractionation using a bulk trace-enrichment technique was performed on a column 7.5 cm × 4.6 mm I.D. of Partisil 10 ODS (Whatman, Maidstone, U.K.) as described by Nice et al. [17].

Gradient reversed-phase HPLC was carried out on ODS Hypersil (C_{18} bonded phase, 5 μm particle size, 9 nm pore size, Shandon Southern) or Ultrapore RPSC (C_3 bonded phase, 5 μm particle size, 30 nm pore size, Altex Scientific) as indicated, on a column 15 cm × 4.6 mm I.D. using 0.155 *M* sodium chloride adjusted to pH 2.1 with hydrochloric acid as primary solvent, and acetonitrile as secondary solvent, using an Altex 324-40 system.

Analytical reversed-phase HPLC was also performed, where indicated, using 0.1% (w/v) trifluoroacetic acid in water as primary solvent, and 0.1% trifluoroacetic acid in acetonitrile as secondary solvent. Retention times on the gradient reversed-phase HPLC system can vary with the age of the column and with protein load. The relative positions of standard proteins do not alter, however, so each column was regularly standardised with a number of peptides and proteins.

Unless otherwise stated, gradient reversed-phase HPLC was performed using the following programme: primary solvent alone, 5 min; gradient of 2% per min secondary solvent, 5 min; then a gradient of 0.75% per min secondary solvent. Isocratic reversed-phase HPLC by the method of Matrisian et al. [9] used a solvent system of 0.05 *M* acetic acid brought to pH 5.6 with triethylamine, with 26% acetonitrile (v/v). Flow-rates of 1 ml/min at 45°C were used for all reversed-phase HPLC separations.

Size exclusion chromatography was performed on an Altex Spherogel TSK 3000 SW column, 30 cm × 7.5 mm I.D., at a flow-rate of 0.5 ml/min in 0.1 *M* phosphate buffer, pH 6.5, containing 0.3 *M* sodium chloride and 20% (v/v) acetonitrile. The acetonitrile was used to avoid hydrophobic interactions with the stationary phase [18]. Ion-exchange HPLC was performed on an Altex Spherogel-TSK IEX-545 DEAE column, 15 cm × 6.0 mm I.D., using 0.05 *M* Tris · HCl, pH 8, in 20% acetonitrile, as primary solvent and a gradient of 10 m*M* per min of sodium chloride. Size exclusion and ion-exchange chromatography were carried out at ambient temperature. Eluted materials were

detected by UV absorbance (LDC Spectromonitor III) or endogenous tryptophan fluorescence (254/340 nm, Schoeffel FS-970).

Modification of growth factors

Oxidation was carried out in halide-free solution with a 1:1000 dilution of fresh 30 vol hydrogen peroxide for 30 min to 2 h at room temperature. Under these conditions, methionine residues are oxidised to the sulphoxide form [19]. Deamidation was attempted by incubation of EGF in 0.01 *M* hydrochloric acid at 37°C for 1—2 days, conditions which result in deamidation of the N-terminal asparagine in the insulin A chain [20]. Digestion of EGF with trypsin and with chymotrypsin was performed as described by Savage et al. [21]. Amino acid analyses were performed on a Biotronik LC 2000 analyser.

RESULTS

Rapid isolation of EGF

The crude extract of mouse submaxillary gland was first fractionated using a previously developed trace enrichment technique described above. The EGF activity partitioned cleanly into the cut between 20% and 60% acetonitrile (Fig. 1). The acetonitrile was evaporated in a stream of nitrogen, and the active fraction applied to the 5-μm C_{18} column. On eluting with a gradient of acetonitrile, well defined UV absorbing peaks, with a single major peak of EGF

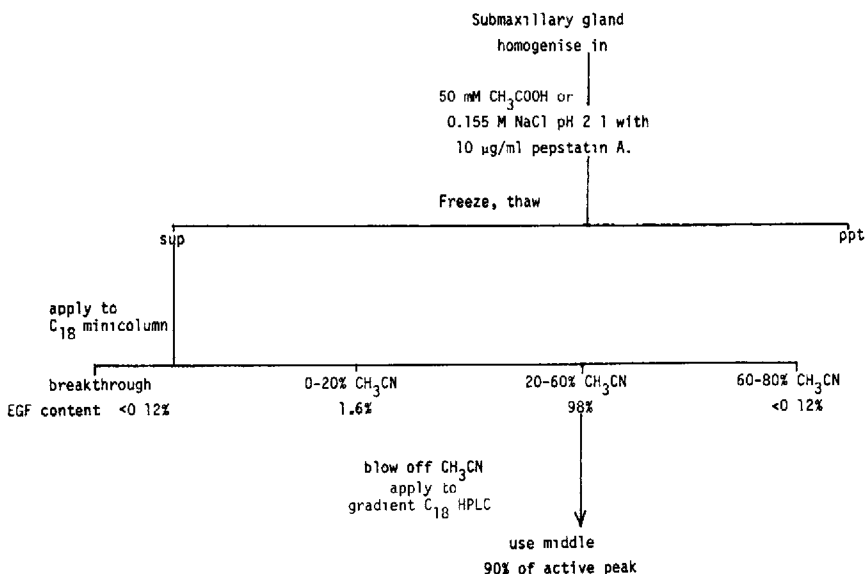

Fig. 1. Procedure for preparation of EGF. The initial homogenate was frozen, and thawed, and centrifuged at 60,000 *g* for 1 h at 4°C. The supernatant was decanted through glass wool to remove floating fat, before application to the minicolumn. Of the EGF present in the original extract 98% was recovered in the fraction retained in the presence of 0.155 *M* sodium chloride, pH 2.1, 20% acetonitrile, but eluted by 0.155 *M* sodium chloride, pH 2 1, containing 60% acetonitrile. This fraction, after removal of the acetonitrile, was subjected to reversed-phase HPLC, as shown in Figs. 2 and 3, to yield pure EGF

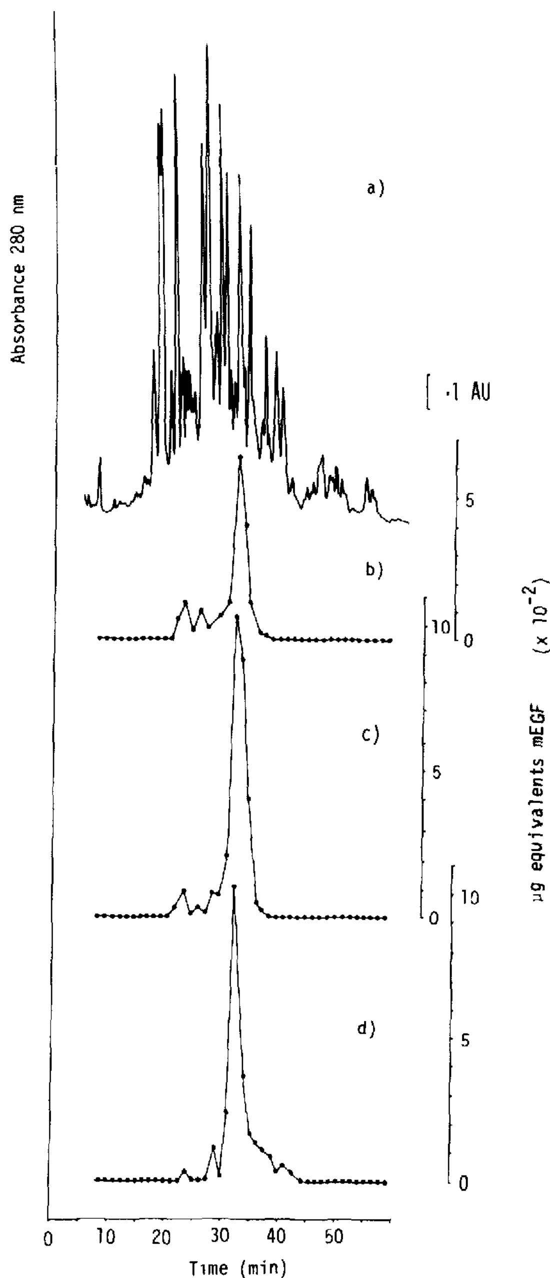

Fig. 2. Elution of mouse EGF (20 to 60% acetonitrile minicolumn cut) from C_{18} reversed-phase column. A sample containing 20 mg of protein was obtained by elution of an acetic acid mouse submaxillary gland extract from a C_{18} minicolumn as described in Fig. 1. The acetonitrile content of the sample was reduced by passing a stream of nitrogen over it prior to loading on a C_{18} reversed-phase gradient HPLC column. Elution was carried out using an acetonitrile gradient of 0.75% per min, and a flow-rate of 1 ml/min (gradient shape is the same as that shown in Fig 4a). Fractions (1 ml) were collected. Aliquots of 10 µl were taken from each fraction and diluted as required for assay, (a) absorbance at 280 nm; (b) radioimmunoassay; (c) radioreceptor assay; (d) bioassay. The combined results are shown for a single chromatogram.

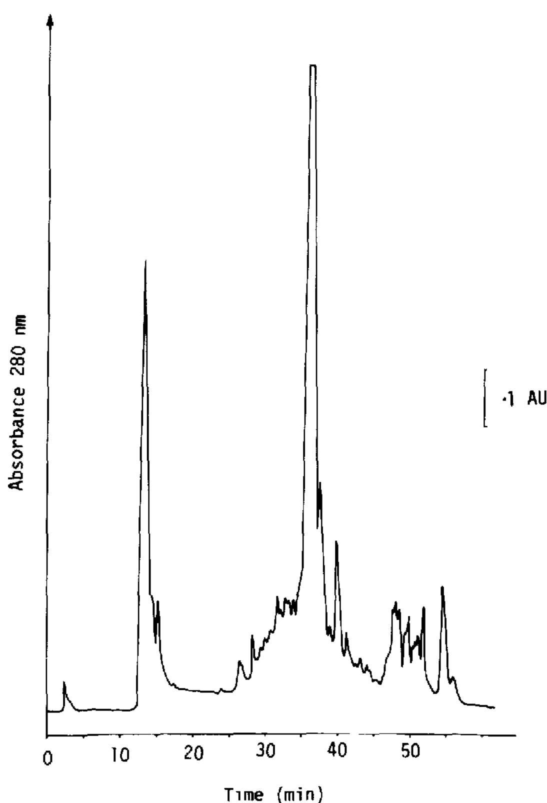

Fig. 3. Absorbance profile of a 20 to 60% acetonitrile minicolumn cut of mouse EGF eluted from C_{18} reversed-phase column. Conditions were as described in Fig. 2, except that the original extraction was performed with 0.155 *M* sodium chloride at pH 2.1. The major UV-absorbing peak contained the EGF activity.

activity as assayed by radioimmunoassay, by radioreceptor assay, or by bioassay on a mammary stromal cell line were observed (Fig. 2). A much cleaner extract was obtained if the initial extraction was performed with 0.155 *M* sodium chloride, adjusted to pH 2.1 with hydrochloric acid, and pepstatin added, rather than with 0.05 *M* acetic acid (Fig. 3). A similar result was obtained for the rat submaxillary gland, although the active peak eluted somewhat earlier (Fig. 4). The active fraction was reapplied to the column, and eluted with a shallower gradient (Fig. 4c). A number of minor UV absorbing contaminants were removed from the active peak at this stage. The overall recovery of highly purified EGF from the original extract was approximately 90%. Much of the remaining 10% could be recovered by rerunning fractions at the edges of the active peak, but this was not really worthwhile. Provided some purified material was available to act as a chromatographic standard, the whole procedure, involving the purification of EGF from 20 g of submaxillary gland could be completed within a working day.

Identification of mEGF

The mouse EGF, prepared as above, co-chromatographs with mouse EGF purchased from Collaborative Research on both C_{18} (ODS) and C_3 (RPSC)

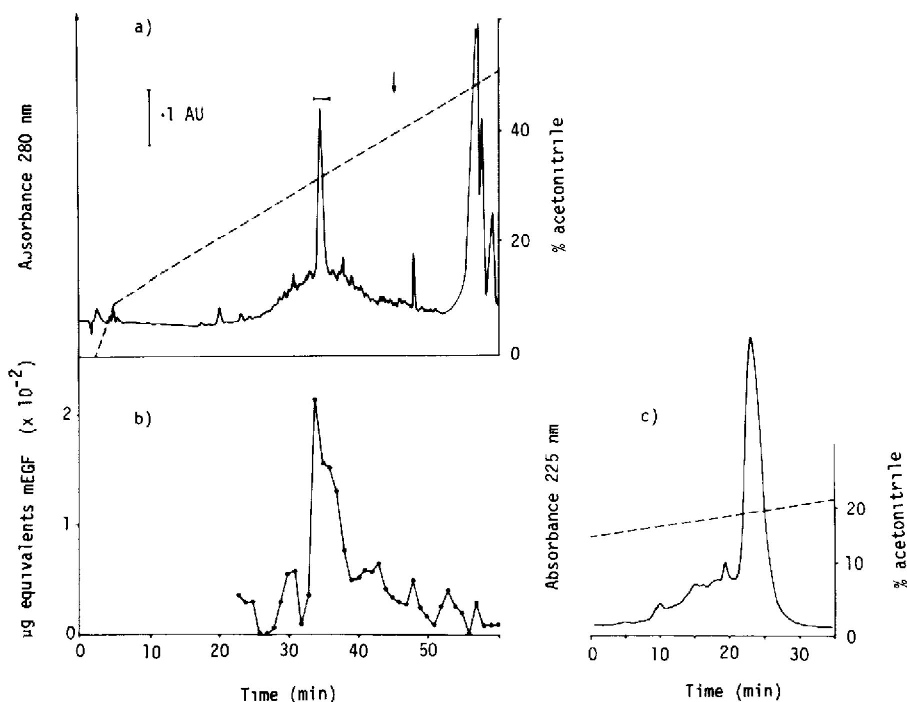

Fig. 4. Elution of rat EGF from C_{18} reversed-phase column Conditions were as described in Fig. 2 except that the rat preparation was eluted from the minicolumn in a cut of 15 to 40% acetonitrile before application to the gradient column. Arrow shows elution position of mouse EGF. (a) Absorbance profile at 280 nm and (b) radioimmunoassay are shown. (c) The active fraction from (a) and (b) was rechromatographed with a shallower gradient of acetonitrile 0.33% (v/v) per min and flow-rate of 1 ml/min. Absorbance profile at 280 nm is shown, and the major peak contained all EGF immunoactivity. The dashed lines show the gradient of acetonitrile applied to the column.

reversed-phase systems, by ion-exchange HPLC, and by gel chromatography on TSK 3000 SW. The HPLC-produced material and the commercial (conventionally purified) EGF also yielded the same peptides on digestion with trypsin, and on digestion of the performic acid-oxidised tryptic fragment with chymotrypsin, and had the same amino acid composition.

On all high-performance chromatographic systems, our HPLC-purified mouse EGF ran as a single peak unlike material prepared by conventional chromatographic methods [7, 9]. It chromatographed as α EGF on the systems of Matrisian et al. [9] and Burgess et al. [8] and was at least 99% homogeneous (Fig. 5 a and b). Peaks corresponding to the β-form described by these workers (and other components of the conventionally purified materials) were not seen (Fig. 5a). A small amount of a new product is, however, produced on prolonged storage. This elutes nearly 2 min earlier on the reversed-phase systems, and its content could be increased by treatment with an oxidizing agent (Fig. 5c). The single oxidation product was compatible with the existence of a single methionine residue per molecule. No separate products were observed under conditions which we know to be effective at engendering deamidated products in other proteins, although degradation of the molecule was observed on prolonged treatment with acid.

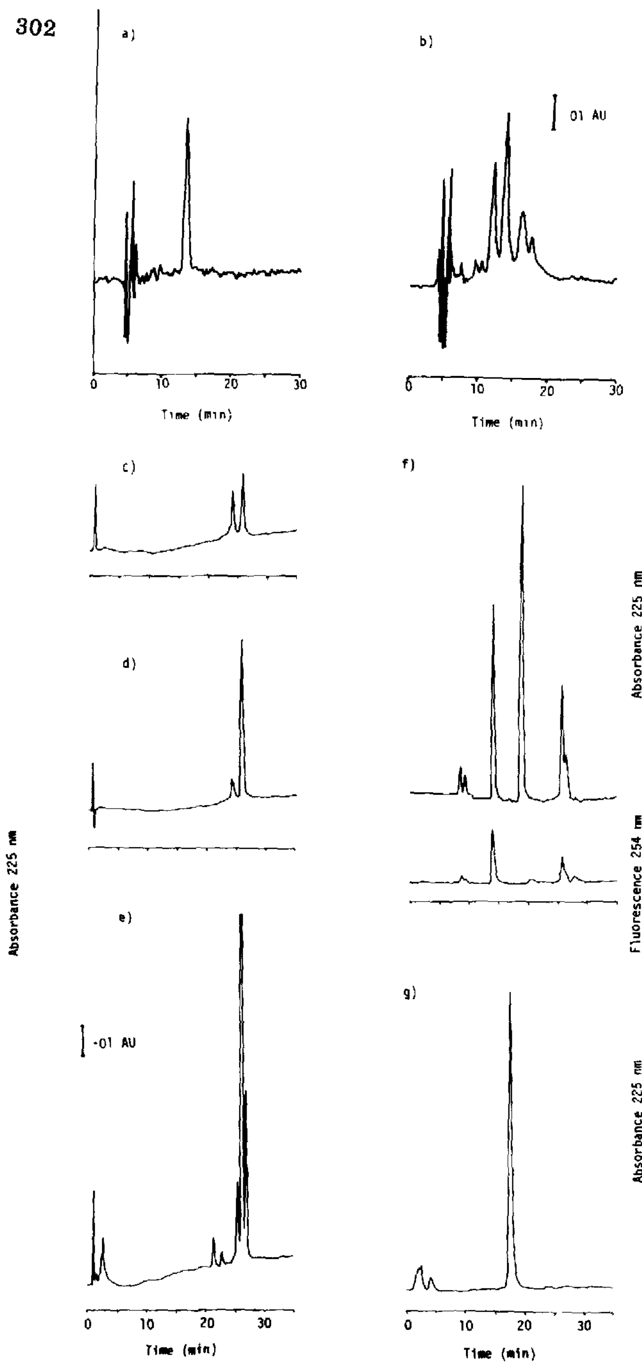

Fig. 5. Isocratic C_{18} reversed-phase chromatograhy, according to the method of Matrisian et al. [9] of (a) HPLC-prepared mouse EGF and (b) culture grade mouse EGF from Collaborative Research. The first peak of (b) eluting at 12 min is absent from receptor-grade EGF. (c—g) C_3 Reversed-phase chromatography of (c) mouse EGF oxidised as described in Experimental; (d) mouse EGF as found after preparation by HPLC and prolonged storage; (e) mouse EGF after limited digestion with chymotrypsin; (f) mouse EGF after digestion with trypsin; lower trace shows intrinsic fluorescence due to tryptophan (measured at 254 nm), which is present in the C-terminal pentapeptide, eluting at 14 min, and in the remaining intact EGF eluting at 26 min, but is absent from the T-EGF, eluting at 18 min; (g) rat EGF. Chromatography was performed using a gradient of acetonitrile in the presence of 0.1% (w/v) trifluoroacetic acid. Chymotrypsin digestion was performed as described by Savage et

A complex pattern similar to that seen in conventional preparations [7, 9] was observed if intact HPLC-purified EGF was briefly digested with chymotrypsin. These products were all eluted within 1 min of the major peak, with gradient elution (Fig. 5e); they may be due to the generation of molecules of EGF nicked at different sites by the protease, but still held together by disulphide bridges.

Isolation of rat EGF

Rat EGF was prepared in a similar manner to mouse EGF, except that the initial minicolumn cut was 15 to 40% in acetonitrile, as the rat activity eluted earlier from the reversed-phase column (at 20% acetonitrile compared with 26% acetonitrile). The lower hydrophobicity of the rat molecule is primarily due to its lack of tryptophan, as indicated by the absence of intrinsic fluorescence at 254 nm. In this respect, the rat EGF resembled T-EGF, the tryptic digestion product of mouse EGF, in which the C-terminal pentapeptide, containing both tryptophan residues, is absent [21]. Indeed, T-EGF chromatographed close to rat EGF on the reversed-phase HPLC (Fig. 5f and g). Mouse EGF, rat EGF and T-EGF were found to have a similar molecular weight by gel filtration (Fig. 6). However, if the organic modifier (acetonitrile) was omitted, the mouse EGF

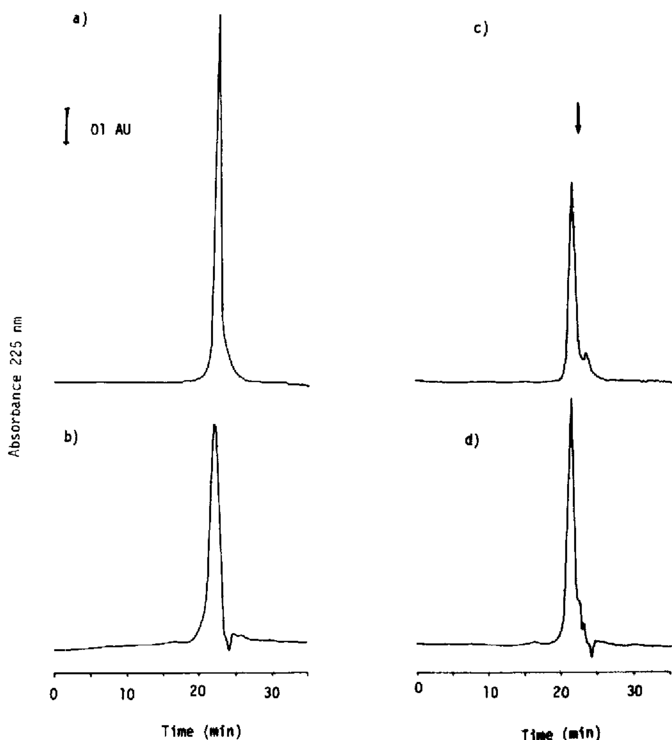

Fig. 6. Size exclusion chromatography on TSK 3000 SW of (a) mouse EGF prepared by HPLC; (b) mouse EGF obtained commercially; (c) rat EGF; and (d) T-EGF, obtained by C_3 reversed-phase chromatography of a trypsin digest of mouse EGF Conditions were as described in Experimental: flow-rate was 0.5 ml/min. Protein load was 5 μg. Arrow in (c) shows elution position of mouse EGF. Standard proteins eluted as follows: bovine serum albumin (68 Kd) 15.9 min; α-lactalbumin (14.2 Kd) 19 2 min; cytochrome C (11 7 Kd) 21.1 min; human calcitonin (3.4 Kd) 22.1 min; *l*-tryptophan (0.2 Kd) 25 6 min.

TABLE I

AMINO ACID ANALYSES OF EGF

Quantities are presented as number of residues per molecule EGF; the new data is normalised on the basis of Arg = 4.0. No correction has been made for losses of serine and threonine during hydrolysis, or for oxidation of the sulphur-containing amino acids.

	Mouse*	Mouse* sequence	Mouse (found)	Rat (found)	Rat**	Mouse* T-EGF
Asp	8—9	7	6.3	6.4	7	7
Thr	2	2	1.4	1.5	3	2
Ser	6—7	6	5 1	3.8	5	6
Glu	3—4	3	2.8	3 4	5	2
Pro	2	2	1.4	1.3	2	2
Gly	6—7	6	6.7	8.4		6
Ala	0	0	0	0	1—2	0
Cys***	6	6	4.2	4.0	6	6
Val	2	2	2 4	1.1	2	2
Met	1	1	0.9	0.5	1	1
Ile	2	2	1.8	1 3	2	2
Leu	4	4	3.8	3.0	4	3
Tyr	5	5	5 3	2.4	3	5
Phe	0	0	0	0	0—1	0
Lys	0	0	0	0	2—3	0
His	1	1	0.9	0.9	1—2	1
Arg	4	4	4.0	4.0	4	3
Tryp §	2	2	2	0	2	0

*From ref. 21.
**From ref. 4.
***Cysteine content was measured as cysteic acid.
§Tryptophan content was calculated from the intrinsic fluorescence at 254 nm.

was retained by the column, eluting near tryptophan (data not shown), while the rat EGF and T-EGF are unaffected; this was assumed to be due to hydrophobic interactions of the C-terminal end of the mouse EGF with the column packing in the absence of the organic solvent.

The amino acid analysis of the rat EGF showed it to be related to mouse EGF, notably by the absence of phenylalanine, alanine and lysine (Table I). This is in contrast with an earlier report of the composition of rat EGF [4], as is the observation, referred to above, of its lack of tryptophan. The contents of the other amino acids are similar in rat and mouse EGF, and to previously reported results; rat EGF has a significantly reduced amount of tyrosine. However, ion-exchange HPLC shows the rat EGF to be significantly less acidic than either mouse EGF, or its tryptic residue (Fig. 7). Similarly, chromatography of the chymotryptic digestion products of rat EGF and T-EGF did not show great homology (Fig. 8), probably because of the lower tyrosine content of the former. In its activity, however, the rat EGF is identical to mouse EGF, whether measured by stimulation of thymidine into DNA in rat mammary fibroblasts, by receptor binding, or by radioimmunoassay.

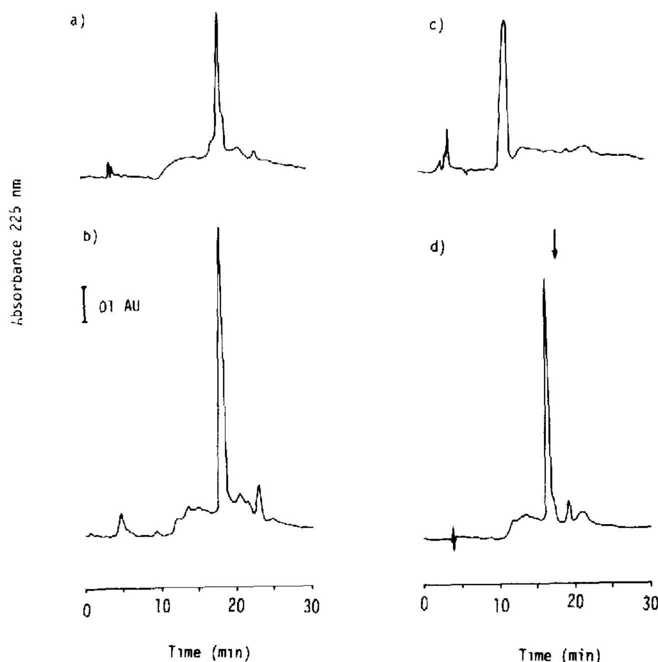

Fig. 7. Elution by ion-exchange chromatography (DEAE-TSK) HPLC of (a) mouse EGF obtained commercially; (b) mouse EGF prepared by HPLC; (c) rat EGF; and (d) T-EGF. Conditions were as described in Experimental, salt gradient was 10 m*M* per min, flow-rate 1 ml/min, protein load was 5 μg. No attempt has been made to correct for the variable background absorbance at 225 nm which is invariably encountered in the system at this sensitivity Arrow in (d) shows elution position of intact mouse EGF

DISCUSSION

The existence of more than one species of active mEGF has been known for some time; a well characterised proteolytic derivative was described by Savage and Cohen [3]. Reversed-phase HPLC revealed in addition a derivative which is separable by any of the systems used here [7], which appears to be due to the oxidation of the methionine residue, and a number of other derivatives which do not separate with 0.155 *M* sodium chloride—hydrochloric acid but which are resolved in the presence of the ion-pairing reagents trifluoroacetic acid and heptafluorobutyric acid [7, 8] and with a triethylamine—acetic acid solvent system [9]. These latter components separate within a narrow concentration range of organic modifier (0.75% acetonitrile), and behave similarly to derivatives generated by the action of chymotrypsin. Since they are not found in the mEGF prepared rapidly in the presence of pepstatin, the suggestion is that, when present, they have been formed by protease action during isolation. It is interesting to note in this regard that when electrophoresed under denaturing conditions the β EGF of Matrisian et al. [9] runs with a smaller apparent molecular weight than the α form. It appears, then, that only a single form of mEGF exists in the submaxillary gland in vivo.

Similarly, only one form of EGF was obtained from the rat submaxillary gland. The question remained whether the rat EGF as described above was the form found in vivo, or whether it was a natural digestion product related to

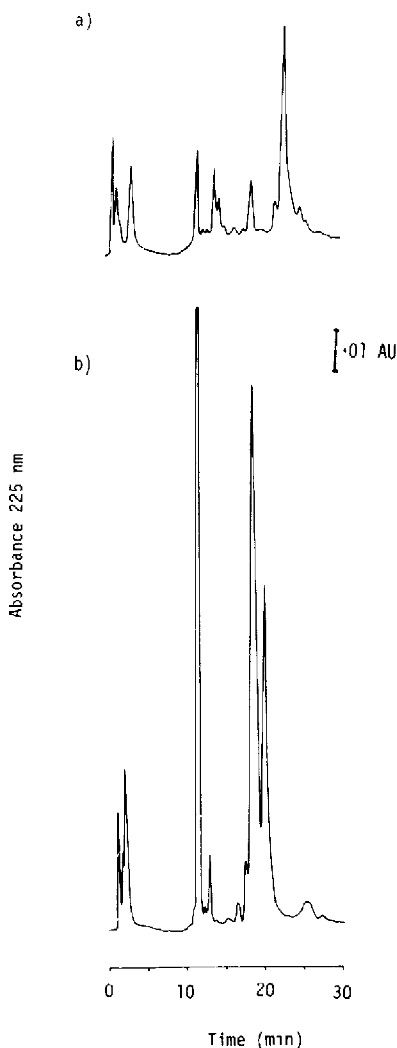

Fig. 8. Elution by C_3 reversed-phase HPLC of chymotryptic peptides of (a) rat EGF oxidised by performic acid; and (b) T-EGF oxidised by performic acid. Samples were prepared as described by Savage et al. [21]. Elution conditions were as described in Fig. 2 The following peptide allocations for the mouse were deduced from amino acid composition data; 2 min, mEGF residues 38—48; 11 min, mEGF residues 1—10; 18 min, residues 11—37 min; 20 min, mEGF residues 11—29

mouse T-EGF. No separate peak of activity from the rat preparations was found to elute at a time corresponding to greater hydrophobicity, and although the submaxillary gland is a rich source of proteases, the mouse EGF was not degraded under the conditions of isolation used here. Similar experience has been reported in the preparation of relaxin, where a family of molecules is obtained by the usual methods, but a single species is obtained when a reversed-phase system is used [11].

The rat EGF was obtained with a yield of 2 mg per 20 g of submaxillary gland from testosterone-treated males or females, and from untreated males

(untreated females were not used). The yield was comparable to that reported by Moore [4], and although substantially less than that obtained from mice, nevertheless represented an easy source of the growth factor. Previously, its purification has been lengthy, as the Biogel method used for mouse EGF was not appropriate, relying as it did on the hydrophobicity of the mouse factor. The present method allowed preparation of the EGF from either species in high yield and purity within a working day.

It is noteworthy that the apparently harsh conditions of reversed-phase HPLC did not inactivate EGF, which once purified, remained biologically and immunologically active on storage in the eluent solvent for more than 1 year at −20°C. A number of other growth factor activities have been chromatographed on the C_3 reversed-phase system to assess the general applicability of the method for their identification and purification. Thus fibroblast growth factor, ovarian growth factor, platelet-derived growth factor, and multiplication stimulating activity [22] chromatographed successfully, and had retention times of 3 min, 0 min, −2 and 1 min, and 0.5 min, respectively, relative to that of mouse EGF. In all of these cases activity was recovered without significant loss.

This makes it seem likely that HLPC methods, with their high resolving power for many polypeptides and small proteins [6], may be useful in the purification of novel growth factors, where their very low abundance precludes purification by conventional means alone. However, care will be required to ensure that they are not simply artefactual derivatives of existing growth factors. Of particular relevance, is the ability to compare and distinguish the EGF-related growth factors identified in cells from a number of normal and neoplastic tissues. They might be expected to be of interest in elucidating the relationship between tumours and their environment in host tissues. Regarding EGF itself, in addition to its proliferative action in vivo on the epidermis and related epithelial tissues, it also has an inhibitory effect on gastric acid secretion, and thus promotes ulcer healing [23]. Any clinical application of this effect to use EGF as an anti-ulcer agent, however, will require milligram quantities of EGF in a pure form. Reversed-phase HPLC as described here is a suitable means of producing such material.

ACKNOWLEDGEMENTS

We thank Messrs. M. Capp and F. Mitchell for excellent technical assistance, Dr. M. Warburton for the amino acid analyses, and Dr. M. Ellison for helpful discussion and also to Linda Adlam for typing the manuscript. We are also pleased to acknowledge the collaboration with Mr. E.C. Nice in the initial stages of this work.

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