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MACROSCALE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND INSTRUMENTAL IDENTIFICATION OF COMPONENTS OF DIETHYLAMINOETHYL MURINE EPIDERMAL GROWTH FACTOR

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SUMMARY

Murine epidermal growth factor (m-EGF), a polypeptide produced as a chromatographically homogeneous peak on diethylaminoethyl (DEAE) cellulose by the method of Savage and co-workers, and characterised as a single compound, has been shown by ourselves and several other groups to be a mixture. The present contribution extends our previously reported work and discusses the separation of this material, termed DEAE—m-EGF, into its components by preparative ion-pair reversed-phase high-performance liquid chromatography (RP-HPLC) on C_{18} μBondapak in quantities up to 50 mg per run. Isocratic elution was used and the mobile phase was acetonitrile—water (26:74, v/v, 0.04 *M* in triethylamine acetate); pH was 5.6, temperature 40°C, and detection was by ultraviolet absorption at 254 nm, and (for some runs) by differential refractometry. Seven significant peaks, four major, three minor, were detected. Of the major peaks, two designated α- and β-EGF, constituted 70% of the total mass and were the most important to our work. Each of the eluted peaks was recovered by lyophilisation, and this product checked for homogeneity by ion-pair RP-HPLC on a C_{18} μBondapak analytical column, with ultraviolet detection as before. All recovered peaks were found to be homogeneous by this criterion.

These chromatographically homogeneous compounds were investigated by modern physicochemical instrumentation to determine their structure. The molecular weight of each of the species was determined by fast atom bombardment mass spectrometry. High-field proton magnetic resonance at 270 MHz provided structural and conformational information. Polarimetry and ultraviolet absorption were also used to characterise the compound. α-EGF, for example, had a molecular weight of 6040 corresponding to the 53 amino acid residue peptide previously designated EGF; β-EGF had a molecular weight of 5930. This molecular weight differential of 110 suggested the hypothesis that β-EGF was a 52 residue peptide corresponding to α-EGF minus the terminal asparagine at position 1. Proton mag-

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netic resonance difference spectroscopy (β spectrum subtracted from α) provided powerful confirmatory evidence for this hypothesis.

All materials recovered from RP-HPLC were tested in the sheep and found to retain their biological activity.

INTRODUCTION

This paper presents the results of an investigation into a problem in biological chemistry, a field in which the compounds tend to be of large molecular weight and of uncertain stability, and thus difficult to separate and characterise. We are here stressing the characterisation of the separated compounds by sophisticated physicochemical (structure-determining) instrumentation such as high-field proton magnetic resonance (HF-PMR) and fast atom bombardment mass spectrometry (FAB-MS).

In addition to the intrinsic scientific interest in separating and characterising these compounds, we had a practical problem in mind in that the compound had physiological properties which we wished to utilise, and to do this we needed accurately measured doses, and this in turn required that each compound be pure and that its properties be known. Additionally, for this biological testing [1] the compound was required to be available in amounts up to 500 mg per experimental run.

The compound of interest to us was murine epidermal growth factor (m-EGF), a polypeptide obtained (largely) by the method of Savage and coworkers [2, 3] from the submaxillary glands of mice (Fig. 1). For the work reported here, the starting material, recovered from the diethylaminoethyl (DEAE) cellulose column as a single peak and therefore regarded as homogeneous, was DEAE—m-EGF. This product, characterised as a single compound [4, 5], has been reported to have 53 amino acid residues and a calculated molecular weight of 6040, and the molecule has three disulphide bonds (Fig.

Fig. 1. The amino acid sequence of m-α-EGF showing disulphide bonds [3, 5]. m-α-EGF is the 1—53 residue peptide (MW 6040); m-β-EGF is the 2—53 residue peptide (MW 5926).

1). For such a composition there are 10^{60} linear combinations alone, and this presents a potentially formidable problem in characterisation especially for the enzymatically synthesised or genetically engineered materials we subsequently expected to investigate. In the preliminary biological testing, prior to a tritium labelling experiment [6], the DEAE—m-EGF was checked for purity by reversed-phase high-performance liquid chromatography (RP-HPLC) on a C_{18} μBondapak column with acetonitrile—water (26:74) containing 10 g/l PIC D-4, a commercial ion-pair reagent (Waters Assoc.), at pH 3. It became apparent that the apparently homogeneous DEAE—m-EGF was a mixture [6]. Further work by ourselves [7, 8] and others [9—11] using a variety of RP-HPLC systems confirms this unequivocally.

At this stage in the investigation there were two broad areas of the problematic: (i) How can the DEAE peak be separated by HPLC into its components in at least 50-mg quantities per run with retention of biological activity? and, (ii) what are the characteristics of the compounds separated?

For the HPLC system ion-pair RP-HPLC was chosen because stable chemically bonded reversed-phase columns were available at relatively low cost, and concentrations of counter ion and organic modifier and ionic strength could all be easily changed to enhance separation selectivity. Additionally, aqueous samples, which were expected from a further phase of the biological investigation, are easily handled by this technique.

The decision to use RP-HPLC for the preparative separations, together with the further decision to recover the compounds by lyophilisation, ncessitated additional investigation of separation methods since, for example, the PIC D-4 reagent (above) was involatile. A further matter of concern was whether analytical methods (microgram amounts) could be scaled up to preparative (milligram) amounts, with adequate column resolution and loading. Since the compounds were required for subsequent biological investigations, tests on retention of biological activity were, ipso facto, part of the programme.

Since biological activity is dependent upon molecular structure, instrumental techniques, sensitive to changes in molecular structure, were used to characterise the compounds. FAB-MS, which has recently been extended to polar compounds larger than 6000 daltons [12], was used to determine the molecular weight of the recovered compounds. Changes in molecular structure (e.g. loss of an amino acid residue) are immediately detectable by such a technique. Some approach to the empirical formula, and thus the composition of the molecule, is also available by mass spectrometric techniques [13]. HF-PMR probes the environment of every hydrogen in the molecule, and yields spectra (usually overlapping for such complex compounds) which are especially sensitive to composition and conformation. Each of the recovered compounds was examined by HF-PMR as an aid to determining its structure (by peak—structure correlations) and to determine the difference between closely related compounds, e.g. by difference spectroscopy.

Other physical properties, such as ultraviolet absorption and optical activity, which are more characteristic of compound classes than individual compounds, were also determined.

EXPERIMENTAL

Chemicals and reagents

High-performance liquid chromatography. All water used in the project was deionised and then glass-distilled. Acetonitrile, orthophosphoric acid (85%) and acetic acid were AR grade from Ajax Chemicals (Sydney, Australia). Triethylamine (TEA) was from E. Merck (Darmstadt, F.R.G.).

PIC D-4 mobile phase modifier was from Waters Assoc. (Milford, MA, U.S.A.).

Mobile phase. This mobile phase, which consisted of an acetonitrile–water mixture (26:74), 0.04 *M* in triethylamine acetate and with a pH of 5.6, was used for all the preparative separations and also for the analytical runs unless otherwise noted. It was prepared by dissolving the triethylamine in water, adjusting the pH to 5.6 with acetic acid, filtering through a Millipore 0.5-μm PTFE filter and then degassing under vacuum. Acetonitrile was filtered (Millipore, 0.5 μm), degassed under vacuum, and the organic and aqueous phases were mixed.

For the alternative mobile phase, the PIC D-4 was dissolved in water, the solution degassed and mixed with degassed acetonitrile to yield a mobile phase of acetonitrile—water (26:74, v/v) and containing 10 g/l PIC D-4. The solution was adjusted to pH 3 using orthophosphoric acid. This mobile phase was used in some of the initial analytical work and in an alternative RP-HPLC procedure for checking the homogeneity of recovered peaks.

Proton magnetic resonance. The deuterium oxide was 99.75% ^2H_2O and was obtained from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, Australia). The sodium deuteroxide (NaO^2H) was 40% (w/v) in ^2H_2O and was supplied by Merck Sharpe & Dohme (Quebec, Canada).

Apparatus

High performance liquid chromatography. All chromatography data were collected isocratically using a Waters Assoc. HPLC system equipped with a Model 6000A solvent delivery system, a Model U6K universal injector (2 ml max.), and a Model 440 fixed-wavelength, dual-channel ultraviolet absorption detector.

The differential refractive index detector was a Perkin-Elmer Model LC-25 (Norwalk, CT, U.S.A.) equipped with a water jacket for temperature control. The prepacked columns (Waters Assoc.) were stainless steel, 30 cm in length and either 3.9 mm (analytical) or 7.8 mm (preparative) I.D. The reversed-phase column packing was C_{18} μBondapak (10 μm particle size), and a guard column was also packed with this material. The columns were water-jacketed and they and the differential refractive index detector were temperature-controlled to better than 0.5°C by a Braun Thermomix Model 1420 (Braun, Melsungen, F.R.G.). Control specification was ±0.02°C at 37°C.

The recorder was a Servoscribe 2 (Smiths, U.K.) with dual channels and switched range voltage input. It was operated on the 10 mV range. A Radiometer Model 26 pH meter (Copenhagen, Denmark) with glass—calomel electrodes was used and was calibrated by the two-buffer method.

Analytical samples were injected using a 25-μl SGE syringe (Melbourne,

Australia). Preparative samples were injected with a tuberculin (1 ml) syringe with a modified needle.

Nuclear (proton) magnetic resonance. The instrument used was a Bruker HFX-270 and was operated in the Fourier transform mode at 270 MHz to acquire proton spectra; 5-mm sample tubes were used (Bruker, Rheinstetten, F.R.G.).

Fast atom bombardment mass spectrometry. The instrument used was a VG analytical ZAB-HF mass spectrometer equipped with a fast atom bombardment source using xenon at 8 keV, a high-field magnet, and a mass marker calibrated against caesium iodide cluster ions [14] (VG Analytical, Manchester, U.K.).

Polarimeter. Optical activity was determined on a Perkin Elmer-Model 241 polarimeter (Bodenseewerk Perkin-Elmer, Überlingen, F.R.G.) equipped with a sodium and a mercury lamp.

Mercury lines were selectable by filter wheel. Accuracy of the polarimeter was 0.002°, repeatability 0.002°, and readout accuracy 0.001°.

Ultraviolet spectrophotometer. The instrument used was a Gilford Model 2600 (Gilford Instrument Labs., OH, U.S.A.) with a wavelength accuracy of 0.1 nm, a wavelength repeatability of better than 0.1 nm, and a photometric precision of better than 0.002 A. The instrument was equipped with automatic wavelength calibration using the 360.9-nm peak of holmium oxide. Matched quartz sample cells of 1 cm path length were used.

Lyophilisation apparatus. The lyophilisation apparatus was an Edwards Modulyo refrigerated unit equipped with a Pirani 10 vacuum gauge (Edwards, Crawley, U.K.).

Methods

High-performance liquid chromatography. Using the mobile phase containing TEA, all RP-HPLC separations, analytical and preparative, were carried out with the C_{18} μBondapak column (and the RI detector) at 40°C. Before initial use, and regularly during use, the plate count of each column was determined using uracil-acenaphthene and the 5-sigma peak measuring method of Waters Assoc. [15]. Initially, the analytical column had 2500, and the preparative column, 5900 theoretical plates. Before sample injection each column was equilibrated to the mobile phase for 60 min at the operating flow-rate.

For separations standardised conditions were used throughout. All samples were dissolved in the mobile phase. Analytical samples were made up to a concentration of 1 μg/μl and 25 μl were injected; column flow-rate was 1.5 ml/min. For preparative separations, 50 mg of DEAE—m-EGF was dissolved in 2 ml of the mobile phase, filtered through an 0.5-μm PTFE Millex-SR filter, transferred to the U6K injection loop in 2 × 1-ml injections and thence to the column. Isocratic elution was carried out at a constant flow-rate of 0.8 ml/min. These conditions had been established by previous experimentation (not reported here) to be optimal for the separation of α- and β-EGF with good separation efficiency and column loading. This method is largely a single-column version of the analytical method of Martrisian et al. [11] scaled up 1000-fold, and optimised to our requirements.

Detection of the eluted peaks was by ultraviolet (UV) at 254 nm and by dif-

ferential refractometry. Samples were collected from the detector flow cell outlet using 0.23-mm I.D. steel tubing to minimise dead volume, using the delay time (2.5 sec) previously established with methylene blue dye. To ensure minimal cross-contamination, peaks were collected after the rise from the baseline and before the return to it.

To recover the compounds the eluates were transferred to lyophilisation flasks, frozen in a dry ice--ethanol bath, and lyophilised on an Edwards refrigerated unit overnight. To check separation efficiency, a sample of each of the lyophilised products was dissolved in the mobile phase (concentration 1 $\mu g/\mu l$) and 4 $\times$ 25 μl were injected and chromatographed under identical conditions to the separation and on the same column. All products were found to yield a single peak. The products were also examined by HF-PMR and found to contain a coupled triplet—quartet (1.1 and 2.75 ppm) ascribed to TEA, hypothesised to be complexed to the EGF peptides since it was not removed by lyophilisation for four days. The TEA was removed by careful addition of 0.01 *M* sodium hydroxide to an aqueous solution of the product to a pH of 8.5, and the product recovered by further lyophilisation for 24 h. The products were again checked for homogeneity by RP-HPLC, and again run on HF-PMR. They were found to be homogeneous and free from complexed TEA. At the conclusion of each set of separation runs the material from each identical peak was composited, dissolved in water and lyophilised to provide uniform bulk batches. These bulk batches were checked by chromatography as before, and also examined by HF-PMR. It was on these bulk batches, stored under nitrogen in sealed containers, under refrigeration, that the biological testing and characterisations were carried out.

As an analytical back-up, RP-HPLC using the PIC D-4 mobile phase was carried out, under identical conditions to those described previously, except that ambient temperature was used. This alternative RP-HPLC confirmed that the peaks were homogeneous.

Proton magnetic resonance. For ^1H spectra, the compounds were dissolved in 0.4 ml of ^2H_2O, transferred to an 0.5-ml nuclear magnetic resonance (NMR) tube and the pH was adjusted in situ to 8—8.5 with NaO^2H.

For ^1H data acquisition, the spectrometer was operated in the Fourier mode at 270 MHz using a sweep window of 3600 Hz and collecting into 4 K of memory at an acquisition time of 0.54 sec. An 0.2-sec gated pulse was used to suppress the water resonance prior to data acquisition. Data processing involved the use of a resolution enhancement function (usually trapezoidal multiplication) followed by zero filling to 8 K of memory. The reference used was the internal H^2HO peak at 4.74 ppm, and all chemical shifts are in ppm. Output was to a chart recorder (X—Y plotter).

Difference spectrum. For the difference spectrum of α- minus β-EGF separate spectra of α-EGF and β-EGF were acquired sequentially, as above, with the spectrometer in the absorption mode. The samples were prepared and the spectra run with careful attention to detail so that all parameters and variables (pH, concentration, data acquisition) were identical. The separate spectra were then subtracted electronically using the elimination (approach to zero) of selected reference lines to monitor the process. The difference spectrum was then recorded as above.

Fast atom bombardment mass spectrometry. Samples were dissolved in 1 *M* hydrochloric acid, and a 1-μl aliquot was transferred onto a trace of glycerol on the stainless-steel stage of the mass spectrometer. Ionisation was by xenon atoms at 8 keV, and the scans were run at the reduced accelerating voltage of 4 kV to increase mass range [12, 16]. These scans were acquired under mass control from 6500 to 5000 a.m.u. and data output was obtained on an on-line oscillographic recorder. The mass marker was calibrated using caesium iodide cluster ions [14]. Mass assignments for the compounds were made on peak centroids.

Polarimetry. Optical activity (specific rotation) was determined in aqueous solution at a concentration of 1 mg/ml in a 1-dm tube at 589 nm (Na^2H line) and at 578 nm (Hg line). D-Glucose (stabilised with benzoic acid) was used to check the polarimeter (α_D + 50°).

The polarimetry was carried out on duplicate samples read in triplicate. The instrument was "re-zeroed" and checked with the standard glucose between each set of sample readings. Room temperature was 22°C.

Specific rotation = $[\alpha]_{\lambda}^{22^\circ} = \frac{100\alpha}{lc}$, where $[\alpha]$ is specific rotation, α is measured angle of rotation, l is path length of cell in dm, c is concentration in g per 100 ml, and λ is wavelength used.

Ultraviolet absorption. UV measurements were made on aqueous samples (pH 8—8.5) at a concentration of 0.2 mg/ml in matched quartz cells of 1 cm path length with water in the reference beam. Scans were between a wavelength of 220 and 320 nm, with step size of 0.100 nm and slit width of 0.22 mm.

$E_{1\,cm}^{1\%} = \frac{A}{bc}$ where A is the measured absorbance, b the path length of cell in cm, and c is the concentration in g per 100 ml.

RESULTS AND DISCUSSION

Separations

The separation achieved by RP-HPLC on C_{18} μBondapak using acetonitrile—water (26:74), 0.04 *M* in triethylamine acetate as the mobile phase, is shown in Fig. 2. Virtual baseline resolution has been achieved for the major components, α- and β-EGF, which were those of most interest to the biological programme. Of the two minor peaks of interest the one that eluted between 40 and 44 min was designated pre-α and the one that eluted between 58 and 63 min post-β.

Identical resolution was achieved during preparative runs with 50-mg column loading. This was monitored on the peak troughs of the UV trace though the UV detectors were saturated on the peak tops. Re-injection of a sample from each of the lyophilised peaks confirmed their homogeneity, and thus also confirmed that resolution was being maintained. Differential refractometry (as an approach to mass detection) indicated that no UV inactive peaks were being eluted. Of the DEAE—m-EGF injected 7% was recovered as pre-α, 49% as α, 20% as β, and 6% as post-β: this together with that in minor peaks gave a recovery of 95% by weight. This method of RP-HPLC has thus proved efficacious for the preparative separation of those components of DEAE—m-EGF of interest to our biological programme.

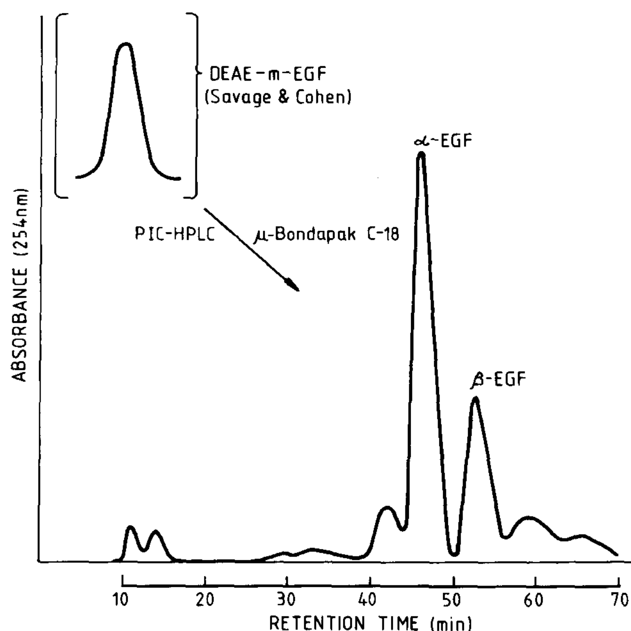

Fig. 2. Isocratic elution of DEAE—m-EGF on a C_{18} μBondapak column (300 × 7.8 mm). Conditions: flow-rate 0.8 ml/min; temperature 40°C; solvent acetonitrile—water (26:74), 0.04 *M* in triethylamine acetate, pH 5.6. Sample size was 100 μg injected in a total volume of 100 μl. Absorbance at 254 nm, 0.05 a.u.f.s.

The next problem to be addressed was whether the biological activity of interest to us had been retained, qualitatively and quantitatively, during RP-HPLC treatment. Possibilities for reactions resulting in such loss included: irreversible complexing of the TEA counter ion to the peptide; conformational changes and cleavage of labile amino acid residues, particularly those at terminal positions. Welinder et al. [17] reported that separation of iodinated insulin species from insulin (a polypeptide with similarities to EGF) on C_{18} columns by RP-HPLC resulted in loss of in vitro biological activity in that there was a loss of binding ability to isolated adipocytes. These authors suggested the possibility that the loss in activity was due to the extremely non-physiological conditions under which HPLC is performed: high pressure, shear forces, organic solvents, and the hydrophobic chromatographic matrix. They further suggested the possibility that the F_3CCOO^- ion from the triethylammonium trifluoracetate—acetonitrile buffer could irreversibly bind to the arginine residues, but no experimental investigation of this important hypothesis appears to have been undertaken. To the contrary, however, Smith et al. [18] reported, without citing supporting experimental evidence, that RP-HPLC on C_3 and C_{18} columns did not inactivate EGF which retained biological and immunological activity for more than one year at —20°C in the eluent solvent.

These findings may not be as conflicting as they appear, since biological activity is complex and for compounds such as insulin and EGF the mechanism of action is unknown, and though it is of course structure-related, it may involve only a part of the molecule. It seems likely that changes in biological activity depend on the nature of the test and on whether it is in vivo or in vitro.

In EGF we were interested in in vivo activity (defleecing action in the sheep) and extensive quantitative testing was carried out in a collaborative experiment using over 50 sheep on the compounds from RP-HPLC. DEAE—m-EGF from the same bulk batch, but not processed by RP-HPLC was used as a control. This investigation showed that, within the limits of experimental error, all materials were of the same potency, i.e. RP-HPLC did not deactivate the compound for this test. These results will be presented in detail elsewhere [1].

Characterisation

The difficulties associated with characterisation of polypeptides of the size of EGF is illustrated by the report of Johnson [19] on the characterisation of human insulin prepared by recombinant DNA technology. In all they used twelve discrete techniques including separation methods, instrumental techniques and biological (in vivo and in vitro) testing. For our purposes we used, principally, FAB-MS and HF-PMR. Both of these techniques are sensitive to changes in molecular structure. HF-PMR, in particular, was used to monitor the product at all stages of separation and recovery.

Proton magnetic resonance

PMR examination showed the product from RP-HPLC contained TEA after the first lyophilisation (Fig. 3). This assignment was made on the basis of the coupled triplet—quartet at 1.1 and 2.75 ppm (referenced to H^2HO at 4.74) which is typical of the ethyl group in amines. This TEA was found to be so stably bound to the peptide molecule that it could not be removed even by extended lyophilisation (four days). The complexed TEA was removed by treatment of an aqueous solution of the lyophilised material with 0.01 *M* sodium hydroxide to pH 8.5 and the compound recovered by a further lyophilisation (Figs. 4 and 5).

Because of its complex overlapping nature the proton spectrum constitutes

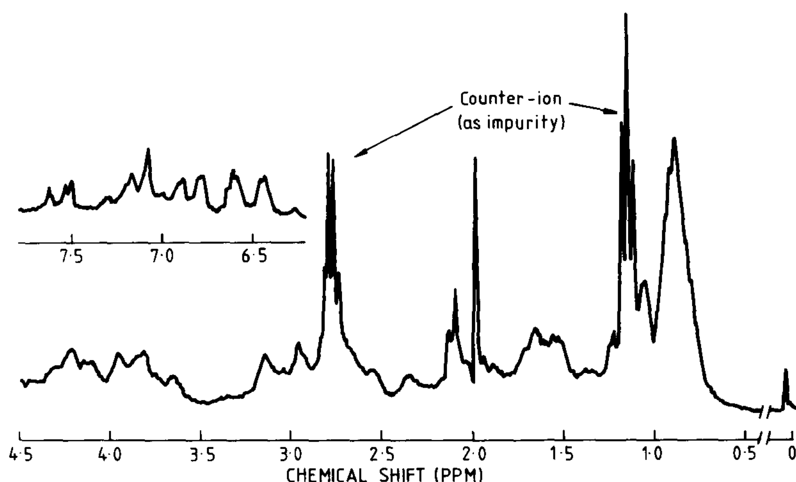

Fig. 3. PMR spectrum at 270 MHz of impure α-EGF ex stage 1 of lyophilisation (PIC-HPLC); 1 mg EGF in 0.5 ml ^2H_2O at pH 8.5. Coupled triplet—quartet at 1.1 ppm due to complexed TEA counter ion. Chemical shifts in ppm referenced to H^2HO at 4.74 ppm.

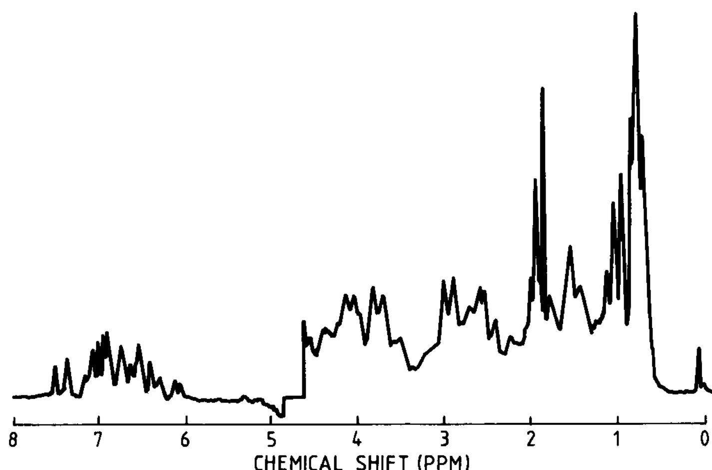

Fig. 4. PMR spectrum at 270 MHz of pure α-EGF ex PIC-HPLC from final stage of lyophilisation (desalted); 1 mg in 0.5 ml ^2H_2O at pH 8.5. Chemical shifts in ppm referenced to H^2HO at 4.74 ppm.

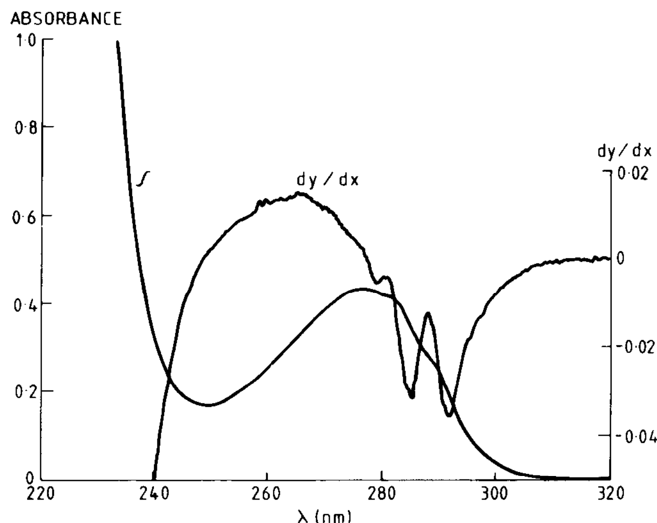

Fig. 5. The UV spectrum of m-α-EGF in water [ex (BBV) PIC-HPLC, C_{18}]. Concentration 0.2 mg/ml. Scanned range 200—320 nm. Cell path length 1 cm. Absorbance maximum value at 277.1 nm, 0.432 a.u. The first derivative (*dy*/*dx*) is also plotted for this sample.

a fingerprint of the molecule since it has a contribution from all hydrogens in the molecule except for those in readily exchangeable positions such as amide groups. Compound comparisons were facilitated by the peak—structure correlations which were undertaken concurrently [8, 20] and supplemented latterly by the report of De Marco et al. [21] who have assigned the peak—structure relationships in the aromatic regions (6—8 ppm). In our PMR studies we found no evidence that either the composition of the recovered material nor its conformation was altered by the separation and recovery processes.

Difference spectra ($\alpha - \beta$)

The only PMR absorption signal remaining in the difference spectrum of α- minus β-EGF was that in the region 2.55 ppm assigned [8, 20] to the terminal asparagine at position No. 1 of α-EGF (Fig. 1). The absorption signal was positive, thus indicating that asparagine was present in α-EGF but absent in β-EGF. Confirmatory evidence for the loss of this asparagine in β-EGF was given by the spectral dispersion lines for proline (No. 4) at 3.5 and 3.7 ppm and for tyrosine (No. 3) at 6.5 and 7.0 ppm indicating non-coincidence of these peaks in the α- and β-spectrum and thus indicating a greater degree of mobility in the moiety in the 2—4 region consequent on the loss of the asparagine terminal. The reduction to zero of all other peaks in the spectrum shows that in these regions, α and β are identical in composition and conformation. The hypothesis that α- and β-EGF differ by one amino acid (asparagine) was tested and confirmed by the molecular weight determined by FAB-MS.

Fast atom bombardment mass spectrometry

For the characterisation of a compound, the molecular weight is probably the single most important physical property. However, for polar, non-volatile, thermally unstable compounds of molecular weight in excess of 6000, such as the polypeptides of this study, it can only be obtained by the recently developed technique of FAB-MS [22]. For these compounds the instrument was equipped with a high-field magnet, and was run at reduced accelerating voltage to increase the mass range despite the resultant decreased sensitivity.

TABLE I

SUMMARY OF RESULTS

All compounds biologically active in the sheep.

Compound	Optical activity* 22°C	Absorptivity** (λ_{max} in nm)	Molar absorptivity	FAB-MS molecular weight (m/z)	Remarks
DEAE—m-EGF	—104.5° (mixture)	24.19 (276.6)	14611 (mixture)	6040 [Peaks at 6040 (α) and 5926 (β)]	
α-EGF	—88.2°	20.30 (277.1)	12261	6040	1—53 Residues (Fig. 1)
β-EGF	—88.4°	21.42 (277.0)	12693	5930	2—53 Residues (Fig. 1). Loss of asparagine terminal; confirmed by NMR
Pre-α-EGF	—95.5°	22.31 (277)	13475	6040	NMR spectrum similar to α-EGF
Post-β-EGF	—77.3°	21.56 (277)	12582	5836	3—53 Residues (Fig. 1). Possible loss of serine terminal from β-EGF

*$[\alpha]^{22°C}_{578\,nm}$.

**$E^{1\%}_{1\,cm}$.

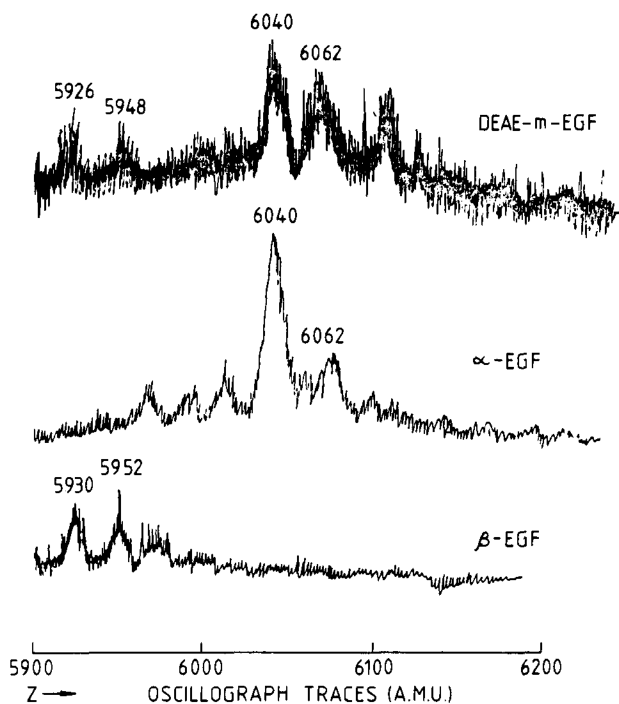

Fig. 6. Molecular ion for α-, β-, and DEAE—m-EGF obtained by FAB-MS on a VG ZAB-HF mass spectrometer (M-Scan, U.K.); ionisation by xenon at 8 keV, accelerating voltage 4 kV. Results: m/z for α-EGF, 6040 a.m.u.; for β-EGF, 5930 a.m.u.; DEAE—m-EGF was a mixture mainly of α- and β-EGF.

FAB-MS spectra were run on all four of the major compounds recovered and on the original DEAE—m-EGF (Table I). Fig. 6 shows the spectrum of DEAE—m-EGF together with that of the major components α- and β-EGF; the peaks 22 a.m.u. above the molecular ion is the sodiated adduct. The molecular weight of 6040 as determined by FAB-MS for the compound designated α shows this compound to be the 53 amino acid residue peptide of calculated molecular weight 6040 previously called m-EGF.

The molecular weight of 5930 for the β compound, a differential of 110 a.m.u. between it and α, confirms the hypothesis derived from difference HF-PMR that β-EGF differs from α-EGF only in the absence of the terminal asparagine (No. 1), i.e. β-EGF is the 2—53 residue compound (Fig. 1).

Other physical properties

Optical activity and UV absorption results on the compounds, shown to be homogeneous by RP-HPLC and characterised by FAB-MS and HF-PMR, are presented in Table I and Fig. 5. As would be expected from compounds of nearly identical structure, the values are also closely similar.

CONCLUSION

DEAE—m-EGF has been separated on a preparative scale, with retention

of biological activity, into four major and three minor components. The four major peaks have been characterized using FAB-MS and HF-PMR as well as by optical activity and UV absorption. No investigation, however, was made into the origin of the separated components which may, for example, be artifacts arising from proteolysis during recovery from the mouse as has recently been reported [18].

ACKNOWLEDGEMENTS

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