Human epidermal growth factor (hEGF) excreted by recombinant *Escherichia coli* K-12 has the correct N-terminus and is fully bioactive


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Abstract

The high stability and productivity of recently-developed *E. coli* JM101 strains expressing human epidermal growth factor (hEGF) facilitated scale-up of hEGF production, and a protocol to purify hEGF from bacterial culture supernatant was required. hEGF-containing supernatant from an induced *E. coli* JM101/lacUV5par8EGF culture was purified by (A) QAE Sephadex A-25 ion-exchange chromatography, (B) Sephadex G-25 desalting, (C) SP-Sepharose cation-exchange chromatography, and (D) reverse-phase HPLC. The hEGF obtained was pure by HPLC and SDS-PAGE. The N-terminus of the purified hEGF was authentic. Commercial pure hEGF, and hEGF purified as described, were assessed for bioactivity, and yielded superimposable curves. The recovery of hEGF with this protocol was 30% of original, while the purity was 97-100%.
Introduction

Human epidermal growth factor (hEGF), also termed human urogastrone (Hollenberg and Gregory 1977), is a small polypeptide (mol wt 6201), and contains 53 amino acid residues. hEGF promotes epithelial cell proliferation both in vitro and in vivo (Zoller and Smith 1982), and the hormone is also a potent inhibitor of gastric acid secretion (Cuatrecasas 1972). Several previous studies (Oka et al 1985; Shimizu et al 1991; Kim et al 1992; Ebisu et al 1992; Yadwad et al 1994) have attempted to achieve high volumetric hEGF productivity. It is desirable that recombinant product be expressed in the cell growth medium (Skipper et al 1985; Wong et al 1988; Wong and Sutherland 1993; Lam et al 1997; Wong W K et al 1998; Lam et al 1998; Wong D K-H et al 1998). Purification of the recombinant hEGF would then be simpler than would be the case with an intracellular protein as the product would not be contaminated with cytoplasmic components. Also, inclusion bodies would not be formed and possible toxic effects of the hEGF polypeptide product on the host cell would be reduced. An ampicillin (Amp)-resistant *Escherichia coli* K-12 strain, *E. coli* JM101[pETacEGF], has been engineered to produce extracellular hEGF (Wong and Sutherland 1993; Yadwad et al 1994), with hEGF secretion being directed by the OmpA signal sequence. In another approach, a synthetic gene for hEGF was fused with the signal peptide of *E. coli* alkaline phosphatase so that mature hEGF might be directed to the periplasm (Oka et al 1985). In *E. coli*, excreted proteins are usually stable (so are not significantly degraded), because of low levels of extracellular protease activity (Enfors 1992).

In 1998, the cost of commercially-available hEGF ranges from US$210 to US$1020 mg\(^{-1}\). Growing evidence that hEGF may be used to accelerate the healing of epithelial tissues, and the potential of hEGF for cosmetic applications (Hasegawa and Yamanoto 1992; Brown 1997) has created considerable excitement in this field. The expense of commercially available hEGF has led to a search for a new and economical source.
Recombinant *E. coli* JM101 strains harboring plasmids encoding human epidermal growth factor (hEGF), were earlier used in fermentations to optimize levels of excreted hEGF (Sivakesava et al 1998). Medium composition, inducer level, growth stage at induction, and culture conditions, were optimized with respect to volumetric production of the recombinant protein. A simple continuous fed-batch process for cultivation of a recombinant *E. coli* JM101 strain, expressing hEGF, was developed (Sivakesava et al 1998). In this paper, the purification of hEGF, in good yield, from the culture supernatant of such a culture is initially described. The excreted hEGF is shown to be authentic by N-terminal amino acid sequencing. Finally, a bioassay is used to show that the excreted hEGF is fully bioactive, as compared to a purified commercial sample.

**Materials and Methods**

**Materials**

Ampicillin and isopropyl-β-D-galactopyranoside were purchased from Sigma (St. Louis, MO). Pure commercial hEGF (catalog no. 100-15) was purchased from Pepro-Tech (London, England). Purity (SDS-polyacrylamide gel electrophoresis (SDS-PAGE)) was >98%.

**Strains and plasmids**

*E. coli* K-12 strain JM101 (Sambrook et al 1989) was the chosen host. Plasmid lacUV5par8EGF (Fig.1) contains the hEGF gene (preceded by the signal sequence of the *E. coli* *ompA* gene) under the control of the lacUV5 promoter, on a plasmid which also contains the *lacF*I gene. To obtain plasmid lacUV5par8EGF, an earlier pETacEGF construct (Wong and Sutherland 1993) was first mutagenized using the oligonucleotide-directed approach (Kunkel 1985), in which a 40-mer (5'−GGGGACATTCAGTCACATTTGCGCGCTGCTACGGTGAGC-3') was used to remove an artefactual sequence encoding the tetrapeptide ala-cys-ser-arg, lying between the *ompA* leader sequence and the coding sequence for hEGF. The resulting plasmid, designated tacIQpar8EGF, contained a precise linkage between the *ompA* leader sequence and the hEGF
gene. This fusion product was excised with \textit{NruI} and \textit{PstI}, and cloned to replace the \textit{cex} gene insert in the lacUV5par8cex construct (Lam et al 1998) restricted with the same enzymes. The resultant construct was designated lacUV5par8EGF (Fig.1), and was used for expression of extracellular hEGF.

Culture media

2xYT medium contained 16g/l Difco tryptone, 10g/l Difco yeast extract, and 10g/l NaCl, and was usually supplemented with 70μg/ml Amp. A modified MBL medium (MMBL medium) has been described (Sivakesava et al 1998).

Expression of extracellular recombinant hEGF

A fresh \textit{E. coli} K-12 JM101[lacUV5par8EGF] transformant was grown for 8h at 30°C in 30ml of Amp-supplemented 2xYT medium. The culture was inoculated into 300ml of MMBL medium with Amp, and growth at 30°C continued for a further 3h. Then, the 300ml culture was inoculated into 3l of MMBL medium with Amp, and growth continued until an A_{550} value of ca. 8.0 was attained. IPTG was then added to a final concentration of 0.2mM, and induction continued for a further 12h. The culture supernatant was obtained by low-speed centrifugation.

Assays of hEGF

The hEGF excreted by bacterial cultures was assayed directly from culture supernatants after removal of bacterial cells by centrifugation. The radioimmunoassay (RIA) kit (catalog no. IM. 1961) was purchased from Amersham Pharmacia (Buckinghamshire, England). Bioactivity of the purified hEGF was determined as described previously (Lam et al 1998) and compared with that of commercial hEGF - the assay involves measurement of increased [\textsuperscript{3}H]-thymidine incorporation, in the presence of hEGF, into epidermal cells in culture. hEGF exerts its biological activity in the concentration range 0.5-25.0 ng/ml.
SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were resolved on a 15% (w/v) tricine-SDS-polyacrylamide gel (Schagger and Von Jagow 1987), and stained with Coomassie Brilliant Blue R250.

Results

Purification of hEGF from culture supernatant

A batch of 400ml supernatant (ca. 65mg hEGF/l) from an induced E. coli JM101 [lacUV5par8EGF] culture was passed through a 0.45μm filter. The filtrate was adjusted to pH 7.4 with 1M HCl and then loaded to a QAE Sephadex A-25 (Amersham Pharmacia) ion-exchange column (50X300 mm) equilibrated with a phosphate buffer (PB; 1.44g Na₂HPO₄ and 0.24g KH₂PO₄ per l; pH 7.4) (Wong et al 1998). The column (equilibrated with 1.5l PB) was washed with 0.6l PB and the bound proteins were then eluted with a gradient (0 to 1M) of NaCl in PB, with the effluent collected in 10ml fractions at a rate of 2ml min⁻¹. The fractions were assayed for hEGF by RIA (Fig. 2A). The active fractions (60ml total) were passed (in 7 equal amounts) through a Sephadex G-25 (Amersham Pharmacia) desalting column (26X300mm) using 0.1xPB as the eluting agent (Fig. 2B). The RIA-active fractions (fractions 15-43; 2ml/fraction) were collected from each run. A total of 375ml of pooled eluates was subjected to SP-Sepharose (Amersham Pharmacia) (16X12mm column) cation-exchange chromatography, using 40mM ammonium acetate, pH 4.0 as the initial eluting agent (1ml/fraction) (Fig. 2C). After collection of 460 fractions, a gradient (to 1M ammonium acetate, pH 4.0) was applied over 50 ml, and a further 80x1ml fractions were collected and assayed for hEGF. Fractions 510-540 were pooled and lyophilized. The resuspended proteins were separated on a reverse-phase HPLC column (Delta-Pak C18 300Å°, 8X100 mm; Waters Chromatography, Milford, MA) (Fig. 2D). The bound proteins were eluted (with manual collection of the peak) with an acetonitrile gradient
of 0-80% in 0.1% v/v trifluoroacetic acid at a rate of 2ml min\(^{-1}\), and the hEGF peak confirmed by RIA.

Analysis of the purified hEGF

A sample of the purified hEGF was re-run on HPLC (Fig. 3). A single peak was obtained (as was also the case when the purified hEGF was co-chromatographed with authentic purified commercial hEGF - data not shown). The purified hEGF material was viewed on SDS-PAGE, in comparison with pure commercial hEGF (Fig. 4). No contaminating proteins were noted in either hEGF sample. Next, the N-terminal 20 amino acid residue sequence of 2 different preparations of the purified hEGF were determined by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center, Oklahoma City, OK, and the first 10 aa residues of the sequences were: Asn Ser Asp Ser Glu Cys Pro Leu Ser His, in agreement with that of authentic hEGF (Bell et al., 1986). Finally, the bioactivity of the purified hEGF was determined as described previously (Lam et al 1998) and compared with that of the commercial hEGF (Fig. 5). The bioactivity profiles were indistinguishable.

Yield of hEGF

Table 1 compares yield and purity values during hEGF preparation. After the HPLC step, the recovery was 30% of the original hEGF level, and the product was 97-100% pure.

Discussion

Rationale for use of E. coli JM101[lacUV5par8EGF] construct for hEGF expression.

In earlier work towards the expression of a different product, it was shown that the lacUV5 promoter functioned more effectively than the (stronger) tac promoter in the expression of the heterologous gene in E. coli JM101 (Lam et al. 1997). The high stability (Wong D K-H et al
1998) and productivity (Sivakesava et al 1998), of *E. coli* JM101 strains expressing hEGF would be expected to facilitate scale-up of production of hEGF. Despite the co-existence of quite a high background of contaminating proteins in the supernatant of an induced *E. coli* JM101 culture, the hEGF was readily purified by conventional chromatographic means. Importantly, the recombinant hEGF was shown to possess the expected N-terminus (thus: degradation from the N-terminus was absent or minimal after excretion of the protein). To confirm authenticity of the secreted recombinant product, a bioassay (involving the enhanced incorporation of labeled thymidine into DNA, by cultured epidermal cells, upon incubation with the hormone) was conducted, using pure commercial hEGF as a control. The bioassay curves were superimposable (Fig. 5). This confirms the integrity of the recombinant product, and augurs well for commercial production of fully-bioactive product upon scale-up to fermentation.

This high rate of production of hEGF by the *E. coli* JM101 clones has rendered them a better choice for use over other systems which have also been reported to express hEGF in the mg/l scale during the productive phase. Thus, the levels of hEGF produced by an engineered *Bacillus brevis* strain (Ebisu et al 1992) and by the *E. coli* JM101[pETacEGF] clone (Wong and Sutherland 1993), grown under optimized conditions (Yadwad et al 1994), were 1.1 g/l/6 days and 250 mg/l/32 h, respectively. These long growth times required for production of hEGF may be expected to adversely affect product bioactivity, and would also elevate the production cost and prolong the occupancy of the fermentation equipment. All these deficiencies may become even more difficult to tackle on large-scale production.

Acknowledgements

This work was supported by Industrial Support Fund AF/135/96 from the Hong Kong Government Industry Department and by Competitive Earmarked Research Grants HKUST 522/94 M and HKUST 639/96M from the Research Grants Council of Hong Kong. All of the experiments performed comply with the current laws of the Hong Kong Special Administrative Region.
References


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Legends to Figures

**Fig. 1.** Plasmid lacUV5par8EGF. In this plasmid, the lacUV5 promoter-operator region drives the transcription of the gene encoding hEGF, fused at the N-terminus, in-frame, to the signal sequence of the *E. coli* K-12 *ompA* gene. Transcriptional terminators occur after the gene encoding hEGF. For construction details of this lacUV5-cassette, see the text. The shaded area to the left of the *NruI* site is the *ompA* leader sequence, while the shaded area to the right of the *NruI* site is the *lacUV5* promoter. The arrow indicates the direction of expression of the hEGF gene.

- **lacIQ**: gene encoding the *lac* operon repressor
- **par**: plasmid stabilization region
- **Amp^R**: ampicillin-resistance gene
- **EGF**: gene encoding hEGF

**Fig. 2.** Purification of hEGF from a bacterial culture supernatant. The details are given in the text. hEGF-containing supernatant from an induced *E. coli* JM101/lacUV5par8EGF culture was purified by (A) QAE Sephadex A-25 ion-exchange chromatography, (B) Sephadex G-25 desalting, (C) SP-Sepharose cation-exchange chromatography, and (D) reverse-phase HPLC. Absorbance values are indicated by solid lines. Gradients are shown by dashed lines. hEGF activity (measured by RIA) is shown by dashed lines with filled circles (one circle/fraction). Buffer B (panel D) is 0.1% (v/v) trifluoroacetic acid in acetonitrile.

**Fig. 3.** The hEGF is pure by HPLC. A sample of purified hEGF (250μg) (see Fig. 1 above, and the text) was rechromatographed on a reverse-phase HPLC column. The bound proteins were eluted with an acetonitrile gradient of 0-80% (v/v) in 0.1% (v/v) trifluoroacetic acid at a rate of 2 ml min^{-1} and the hEGF assayed by RIA. A single peak, with elution time of 50.25 min., is noted.
**Fig. 4.** SDS-PAGE of hEGF. Commercial pure hEGF, and hEGF purified as described (see Fig. 2 and the text), were subjected to SDS-PAGE. Lane 1: See-Blue™ prestained markers; lane 2: commercial hEGF (5µg); lane 3: hEGF purified in this work (5µg). The positions of 2 mol. wt. standards are indicated at the left, and the hEGF band is arrowed on the right.

**Fig. 5.** Bioassay of hEGF. Commercial pure hEGF, and hEGF purified as described (see Fig. 2 and the text), were assessed for bioactivity, and yielded superimposable curves. The assay involves measurement of [³H]-thymidine incorporation into cultured human epidermal cells exposed to hEGF. Such incorporation, measured by counts per minute of label, increases with increasing levels of hEGF.
Table 1. Recovery, and purity, of hEGF during the purification protocol. For details of the purification steps, see the text.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% recovery</th>
<th>% purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation supernatant</td>
<td>100</td>
<td>001.3</td>
</tr>
<tr>
<td>From anion-exchange</td>
<td>057.2</td>
<td>036.4</td>
</tr>
<tr>
<td>From desalting</td>
<td>042.4</td>
<td>046.5</td>
</tr>
<tr>
<td>From SP-Sepharose</td>
<td>033.3</td>
<td>081.4</td>
</tr>
<tr>
<td>From HPLC</td>
<td>030.0</td>
<td>097.0-100</td>
</tr>
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