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Cloning, expression and purification of human epidermal growth factor using different expression systems

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Abstract

Epidermal growth factor (EGF) is a protein that belongs to the family of growth factors that bind the ErbB receptors, which play a prominent role in the development of carcinomas. We had demonstrated that potato carboxypeptidase inhibitor (PCI) acts as an EGF antagonist. Because of the low affinity of PCI for the epidermal growth factor receptor, it was decided to design EGF mutants with PCI abilities. In order to achieve this we have first cloned, expressed and purified the native protein, EGF. Different expression systems with different locations of the recombinant protein were designed and a purification protocol was designed with those which allowed expression of EGF. Finally, the sample needed folding. Differences in the amount of EGF obtained and its activity were observed depending on the expression system used.

Keywords: Cloning; Expression; Purification; Epidermal growth factor

1. Introduction

The epidermal growth factor (EGF) signal transduction pathway is a significant mediator of several cell functions and processes such as survival, motility, differentiation, proliferation and death. The epidermal growth factor receptor (EGFR) subfamily consists of four closely related tyrosine kinase receptors: ErbB-1 (also known as EGFR), ErbB-2 (or HER2/neu), for which no ligand has been described so far, ErbB-3 (or HER3), which is characterised by an impaired kinase function, and ErbB-4 (or HER4) [1–4]. The ErbB's receptors are transmembrane glycoproteins and signal transduction is initiated by ligand binding to the surface ectodomain that induces receptor homo or heterodimerization [5]. This dimerization activates the receptor intrinsic tyrosine kinase activity leading to receptor transphosphorylation that induces the recruitment of intermediary effectors [6]. These events initiate signalling pathways that form a complex cellular network.

We have recently described how potato carboxypeptidase inhibitor (PCI), a small protein that is a protease inhibitor, acts as an EGF antagonist and has antitumoral properties [7]. The molecular basis of these effects lies in the fact that PCI shows three-dimensional structural similarities with EGF [8]. Despite these properties of PCI, it has one great

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limitation: its low affinity for EGFR in contrast to EGF. It has other problems such as being citostatic but this can be solved by combining PCI with citotoxic agents.

Structural studies were carried out comparing the structures of PCI and EGF. The results showed that both molecules are very similar but they differ in their C-tail part: PCI lacks this tail. Some authors have suggested that two molecules of EGF stabilize EGFR dimer and that the part of the molecule that keeps the two receptors close is the C-tail part [9,10]. These results seemed obvious since our group had previously seen that PCI was not able to induce EGFR dimerization [11]. All these data suggested that the best way to solve PCI low affinity for EGFR was to modify both EGF and PCI structures. PCI had previously been cloned, expressed and purified in *Escherichia coli* by our group, but EGF cloning was required before mutants could be made. These EGF mutants would have modifications in the C-tail part and, like PCI, be unable to induce EGFR dimerization. This will be a long process, as the amino acids responsible for EGFR dimer stabilization are still unknown. Later on, it is expected that PCI structure will be modified to increase its EGFR affinity. In the present study, we focus on the cloning, expression and purification of EGF with the purpose of establishing a purification protocol for making truncated EGF forms in the future.

2. Materials and methods

2.1. Reagents

All chemical reagents used in this work were of analytical grade and, unless otherwise indicated, were purchased from Merck (New Jersey, USA), Serva (Heidelberg, Germany) or Sigma (St. Louis, MO, USA). Restriction enzymes were all purchased from Roche Diagnostics (Mannheim, Germany).

2.2. EGF cloning in pUC118 and pET vectors

Human EGF was a gift from Dr Masaharu Seno of Okayama University. He gave us the gene cloned in the pTB361 vector. The first step was to clone it in the pUC118 vector (expression vector with a lac promoter and ampicillin resistance) from Novagen (Madison, USA). Therefore specific primers were designed to obtain EGF from pTB361 vector. The primers purchased from Roche Diagnostics (Mannheim, Germany) were: EGF53: 5′ ATGAAAAAGACGAATTCTATGAACAGTGA 3′ (T_m 56.9 °C) and EGF35: 5′ TAACATTAAACGGATCCTCTGCAGCTATC 3′ (T_m 61.2 °C).

The pET vectors chosen were pET21b(+), pET22b(+) and pET32b(+) from Novagen (Madison, USA). The primers used to obtain EGF from pUC118 were: Forward Universal Primer (FUP) and Reverse Universal Primer (RUP) from Novagen (Madison, USA). Their sequences were: FUP24: 5′ CGCCAGGGTTTTCCCAGTCACGAC 3′ (T_m 55 °C) and RUP24: 5′ TCACACAGGAAACAGCTATGACCA 3′ (T_m 55 °C).

PCR reactions were carried out using DeepVent polymerase from New England BioLabs (Beverly, USA) which has correction activity 3′–5′ which is very important in cloning experiments. The program was: 2 min at 94 °C; then 30 cycles of: 1 min and 15 s at 94 °C, 1 min 30 s at annealing temperature, 1 min 15 s at 72 °C; followed by 2 additional minutes at 72 °C.

The restriction enzymes used during the cloning step were: *Eco*RI, *Pst*I, *Hin*dIII, *Apa*I and *Bgl*II. All restriction reactions were carried out at 37 °C for 2 or 3 h.

Usually 1.5% agarose gels were used in this work. For separating small DNA fragments, Metaphor agarose from BMA (New Jersey, USA) was used; otherwise normal agarose from BMA was used.

2.3. EGF expression in E. coli

The host used to express EGF was *E. coli* BL21(DE3). This is an expression strain that overproduces the lac repressor ($lacI^q$) and has the T7-RNA polymerase under the control of the lac operator. In some cases, a variant of this strain, *E. coli* BL21(DE3) *pLys*, was used, as it has lower expression of the recombinant protein preventing aggregation problems.

Media used to grow *E. coli* were either LB (10 g bactotriptone, 5 g yeast extract, 10 g NaCl, 15 g agar (if the medium had to be solid) and 1 l distilled water) or 2YT (16 g bactotriptone, 10 g yeast extract,

5 g NaCl and 1 l distilled water) from Scharlau Chemie (Barcelona, Spain). The antibiotic was ampicillin from Roche Diagnostics (Mannheim, Germany) at a final concentration of 100 μg/ml. When using the *E. coli* BL21(DE3) *pLys* strain, apart from ampicillin, cloramfenicol was used at a final concentration of 75 μg/ml.

Transformation of different plasmids into *E. coli* was carried out following the protocols described in the manual of Sambrook and Russell [12].

After transformation colonies were used to make a pre-culture that was grown overnight at 37 °C. An aliquot of this pre-culture (1:100) was used to start the culture. When bacteria were growing exponentially, IPTG was added at a final concentration of 50 μg/ml. Growth was stopped 4 or 5 h after induction.

2.4. Cellular fractionating

After cultures were grown they were harvested by centrifugation at 15 000 *g* for 15 min and immediately subjected to cellular fractionation to obtain the intracellular soluble and insoluble fractions. Two different methods were used depending on the vector.

2.4.1. pET21b(+) and pET22b(+)

After centrifugation, the pellet was resuspended in a 20 m*M* Tris–HCl pH 8.5, 2.5 m*M* EDTA solution, and sonicated for 10 min at 50 W. The sample was then centrifuged again at 22 000 *g* for 30 min. The supernatant corresponded to the intracellular soluble fraction. The pellet was resuspended in a 20 m*M* Tris–HCl pH 8.5, 2.5 m*M* EDTA, 2% solution Triton X-100, and immediately centrifuged at 22 000 *g* for 30 min. The pellet contained the intracellular insoluble fraction of the culture (also known as inclusion bodies).

2.4.2. pET32b(+)

Because the next step in the purification of the protein obtained from this construction had to be an affinity column, the protocol and the buffers were different from those described above. After centrifugation, the pellet was resuspended in binding buffer (40 m*M* imidazole, 4 *M* NaCl, 160 m*M* Tris–HCl, pH 7.9). The sample was then sonicated for 10 min at 50 W and again centrifuged at 20 000 *g* for 15 min. The supernatant corresponded to the intracellular soluble fraction of the culture. The pellet was then resuspended in binding buffer and again sonicated for 5 min at 50 W. Finally, the sample was centrifuged at 20 000 *g* for 15 min. The supernatant contained, as in the previous step, to the soluble fraction and the pellet contained the insoluble fraction (also known as inclusion bodies).

2.5. Solubilization of inclusion bodies

In both cases, using pET21b(+) and pET32b(+) vectors, it was necessary to solubilize the inclusion bodies. Urea and guanidinum chloride were used as denaturing agents. Urea was used for the purification of the fusion protein (thioredoxin–EGF) from the pET32b(+)–EGF construction as our group had previously seen that urea was better than guanidinum chloride when working with affinity columns.

Insoluble fractions were resuspended in 1/50 ml of the initial volume of: pET21b(+)–EGF: 5 *M* guanidinum chloride, 30 m*M* DTT, pH 8.5; or pET32b(+)–EGF: binding buffer with 6 *M* urea.

Samples were maintained for 5 or 6 h either at room temperature (pET21b(+)–EGF) or 4 °C (pET32b(+)–EGF). In the first case, after solubilization, the sample was dialysed against a 0.1 *M* Tris–HCl, pH 8.5 buffer for 24 h. In the second case, after solubilization, the sample was centrifuged at 39 000 *g* for 20 min; the supernatant contained the solubilized fraction.

2.6. Purification of recombinant human EGF

Protocols were different using the two expression systems. All chromatographic equipment used was purchased from Amersham Biosciences (Freiburg, Germany): ÄKTAbasic, RediFrac Fraction Collector and Recorder REC111.

2.6.1. pET21b(+)–EGF

After dialysis the sample was charged in a Vydac C_4 column from Waters (MA, USA) connected to an ÄKTA HPLC system from Amersham Biosciences (Freiburg, Germany). The elution gradient using acetonitrile was: 5 min 10%, 20 min 30%, 30 min 60% and 15 min 100%. An elution profile was

obtained and some peaks were analysed by EM MALDI–TOF (matrix assisted laser desorption ionisation–time of flight; BRUKER-BIFLEX spectrometer); the matrix used was sinapic acid in aqueous solution with 30% acetonitrile and 0.1% TFA, while the resin used was Bondapak C_{18} 125A from Waters (MA, USA). The mode was lineal, the ionisation was positive and the acceleration voltage was 19 kV (a 337-nm laser was used for ionisation). Western blot analyses were carried out as explained below and the antibody was the anti-EGF antibody (Z-12) from Santa Cruz Biotechnology (Santa Cruz, USA). Samples containing EGF were collected and lyophilised.

2.6.2. pET32b(+)–EGF

In this case recombinant protein was purified either from the soluble or the insoluble fractions. In both cases the protocol used was the same except for the solubilization step which was unnecessary for the soluble fraction. Samples were always filtered before being charged in the column. The column used was the HiTrap™ Chelating Sepharose® from Amersham Biosciences (Freiburg, Germany). The elution gradient was achieved by increasing imidazole concentration. When working with the insoluble fraction, buffers had to be supplemented with 6 *M* urea. The different fractions obtained were analysed using a polyacrylamide SDS–PAGE electrophoresis gel (16% acrylamide:bisacrylamide). Four four-fold buffers were prepared: apilation buffer (0.5 *M* Tris–HCl pH 6.8, 0.4% SDS), separation buffer (1.5 *M* Tris–HCl pH 8.8, 0.4% SDS), elution buffer (1.92 *M* glycine, 0.25 *M* Tris, 1% SDS, pH 8.3–8.5) and loading buffer for the samples (12 m*M* Tris–HCl pH 6.8, 8% SDS, 20% glycerol, 5% 2-mercaptoetanol and 0.02% bromofenol blue). To polymerise the gels, 15% persulfate and temed (*N*,*N*,*N*′,*N*′, tetramethiletilendiamine) were used. Gels were run at 60 mA for 90 min. Samples containing EGF were collected and lyophilised.

2.7. EGF folding using oxidised-reduced agents

To fold EGF, oxidised and reduced gluthathione that acted as chaperone was applied. Samples were resuspended in a folding buffer (0.1 *M* Tris–HCl pH 8.5, 2 m*M* GSSG, 4 m*M* GSH) and were maintained for 24 h at 22 °C. The samples were then dialysed against 10 m*M* acetic acid and quantified using the Bradford method. Finally, the samples were lyophilised and maintained either lyophilised at −20 °C or dissolved and aliquoted in 10 m*M* acetic acid, 0.2% BSA at −80 °C.

2.8. Cell lines

The human epidermoid tumoral cell line, A431, hyperproducing EGFR, was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 m*M* glutamine and 20 μg/ml gentamicine, unless otherwise indicated. All these products were purchased from Gibco BRL Life Technologies (Carlsbad, CA, USA).

2.9. EGF activity assay

The 50% confluent, serum-starved A431 cells growing in 100-mm dishes were washed twice with PBS, treated with recombinant EGF (from the three different protocols) and a commercial EGF from R&D Systems (Minneapolis, USA) (in DMEM) for 15 min. Cells were then lysed as follows. Cells were washed twice with cold phosphate-buffered saline (PBS), harvested in 0.5 ml of RIPA B lysis buffer (20 m*M* sodium phosphate pH 7.4, 150 m*M* NaCl, 1% Triton X-100, 5 m*M* EDTA, 5 m*M* PMSF, 10 μg/ml aprotinin and leupeptin, 250 μg/ml sodium vanadate) and lysed using a glass homogenizer, and protein concentration was determined by the Bradford method. Samples were then added to SDS–PAGE loading buffer with 5% β-mercaptoethanol, heated for 5 min at 100 °C and electrophoresed in an 8% gel. The level of tyrosine phosphorylation of the immunoprecipitated receptor was assessed by immunoblotting with anti-phosphotyrosine (PTyr) antibody RC-20 (Santa Cruz Biotechnology, USA). The blots were visualized by enhanced chemiluminescence.

After visualisation of phosphotyrosine level by enhanced chemiluminescence, antibodies were removed and the membranes were incubated for 30 min at 50 °C with 10 m*M* Tris–HCl (pH 7.5), 100 m*M* NaCl, 2% SDS and 100 m*M* β-mercaptoethanol.

Membranes were then immunoblotted with anti-EGFR antibody from Santa Cruz Biotechnology (USA), and visualised by enhanced chemoluminescence. The average ratio between tyrosine phosphorylation levels versus EGFR was assessed using Quantity One software from Bio-Rad (Philadelphia, USA).

3. Results

3.1. EGF cloning into three different expression systems

Cloning a protein in an expression system usually consists of several steps, which are sometimes delicate and difficult to achieve. Many authors have described different ways to obtain recombinant EGF using a great variety of bacterial systems [13–17]. To clone EGF in a bacterium, *E. coli*, we neither had to synthesise the gene nor make a DNA library, as a plasmid was given to us which contained the EGF gene, pTB361. As we were not familiar with this plasmid, it was decided to clone EGF in vectors of the pET family: pET21b(+), pET22b(+) and pET32b(+). To obtain EGF gene from pTB361 plasmid a PCR reaction was carried out using specific primers. The product of this reaction was then digested with the restriction enzymes *Eco*RI and *Pst*I. The three vectors chosen to clone EGF were: pET21b(+), pET22b(+) and pET32b(+).When ligating EGF DNA with these three vectors, a *Hin*dIII diane was needed, so EGF was first cloned in the pUC118 vector. From this vector it was possible to obtain EGF gene with a simple PCR reaction using the universal primers, FUP (Forward Universal Primer) and RUP (Reverse Universal Primer), and then digesting the product with the restriction enzymes *Eco*RI and *Hin*dIII. With this product and with the three plasmids also digested, a ligation was done. The different transformants obtained were then assessed to see if they were positive for EGF by carrying out digestion with restriction enzymes which would result in fragments of different lengths depending on whether the clone was positive for EGF or not (Fig. 1). Positive clones for each construction were obtained.

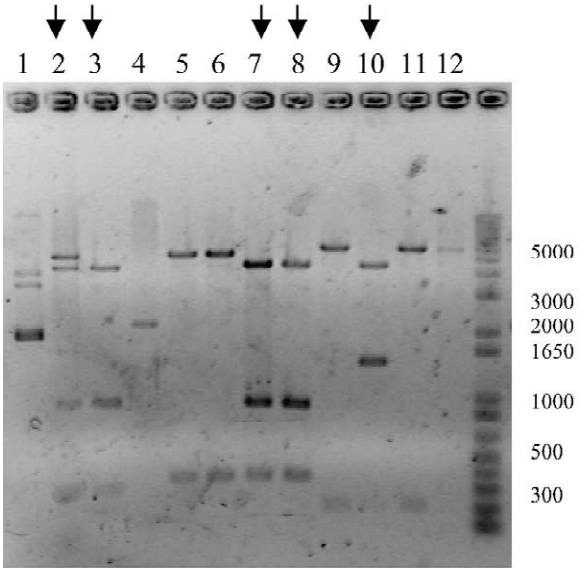

Fig. 1. Verification gel of transformants obtained after ligation between EGF gene and the three pET vectors. Samples were loaded on a 1.5% agarose gel. The different lanes are the digestions with *Apa*I and *Bgl*II of pET21b(+) (lanes 1–4), pET22b(+) (lanes 5–8) and pET32b(+) (lanes 9–12). The markers are also shown (1-kb Plus DNA Ladder). Positive clones are marked with an arrow.

3.2. Analysis of EGF expression in each expression system

Expression of the recombinant protein in each of the constructions was analysed (Fig. 2). It was shown that in both the pET21b(+)–EGF and pET32b(+)–EGF constructions the recombinant protein was totally located in the insoluble fraction of the cell as inclusion bodies whereas with pET22b(+)–EGF no expression could be detected. This resulted in the pET22b(+)–EGF system being abandoned because although it was possible that some EGF had been expressed, the amount should be so inappreciable that it was not worthy going on with this construction when trying to obtain recombinant EGF. The difference in the molecular weight of the EGF expressed using the pET21b(+)–EGF construction and that obtained using pET32b(+)–EGF is due to the fact that in the second case EGF is fused to thioredoxin increasing the molecular weight from 7900 to ~28 000. It has to be mentioned that

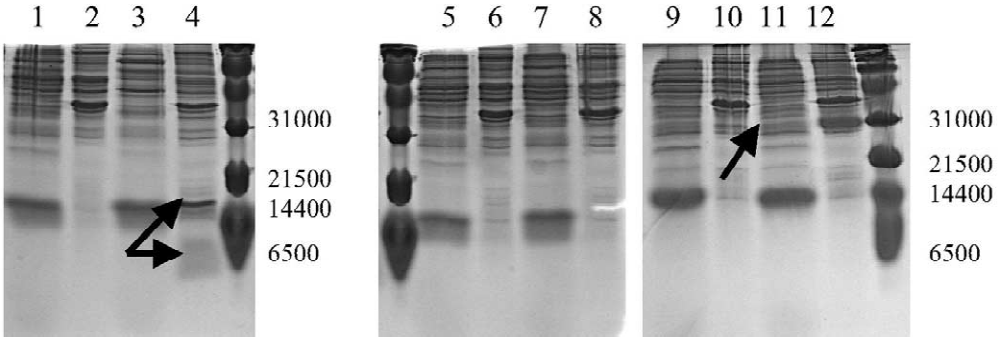

Fig. 2. Analysis of EGF expression in the three pET constructions. Soluble and insoluble intracellular fractions from the cultures of the different constructions and their controls are shown: lanes 1 and 2, pET21b(+) soluble and insoluble fractions; lanes 3 and 4, pET21b(+)–EGF soluble and insoluble fractions; lanes 5 and 6, pET22b(+) soluble and insoluble fractions; lanes 7 and 8, pET22b(+)–EGF soluble and insoluble fractions; lanes 9 and 10, pET32b(+) soluble and insoluble fractions; and lanes 11 and 12, pET32b(+)–EGF soluble and insoluble fractions. The arrows indicate the EGF expressed. Molecular mass markers are also shown (Bio-Rad).

EGF when expressed as a single protein dimerizes and so two bands appear in the gel.

The insolubility problem had to be faced. To try to solve this problem we tried three different strategies: decreasing the growth temperature of the culture from 37 to 28 °C [18], using an *E. coli BL21(DE3) pLys* strain, and not inducing cultures with IPTG. All three strategies had a common objective: to lower the amount of protein expressed in order to prevent aggregation of the protein and so insolubility. When decreasing temperature no changes were observed. Using the variant of *E. coli*, an increase in soluble EGF expressed using the construction pET32b(+)–EGF was observed whereas no changes were observed for the construction pET21b(+)–EGF. Finally, when cultures were not induced with IPTG the amount of protein expressed was the same as when they were not induced (none of these results have been included). In conclusion, with pET21b(+)–EGF neither of the strategies were successful whereas with pET32b(+) it will be necessary to work with *E. coli* variant in the future. In neither of the systems will it be any longer necessary to induce the cultures with IPTG.

3.3. EGF Purification protocols

Considering these results it was decided to start EGF purification from both the soluble and the insoluble fractions of the cells carrying the plasmid pET32b(+)–EGF, but only from the insoluble fraction of the cells carrying the plasmid pET21b(+)–EGF. Purification protocols had to be established for each of the two systems.

3.3.1. pET21b(+)–EGF

The insolubility of the protein expressed made a first step of solubilization necessary. This was carried out using guanidinum chloride. After solubilization the sample was dialysed and then charged in an RP-HPLC column (Fig. 3I). The elution profile showed a main peak with smaller secondary peaks. Three peaks were analysed by EM MALDI–TOF and it was shown that two of them contained EGF (Fig. 3II). Western blot analysis was carried out with the two samples collected (Fig. 3III). The purified protein was EGF and the purity was almost 100%. This sample was lyophilised and resuspended in a folding buffer containing oxidized and reduced glutathione. Finally it was dialysed against water and quantified.

3.3.2. pET32b(+)–EGF

Starting from the insoluble fraction a first step of solubilization was needed. In this case urea was used. After solubilization the next steps were the same for both the insoluble and the soluble fractions. The sample was charged in an affinity column that allowed the fusion protein to bind to the matrix charged with niquel carrying a histidine tag (Figs. 4I and 5I). SDS–PAGE electrophoresis gel was carried out to see whether the eluted protein was found

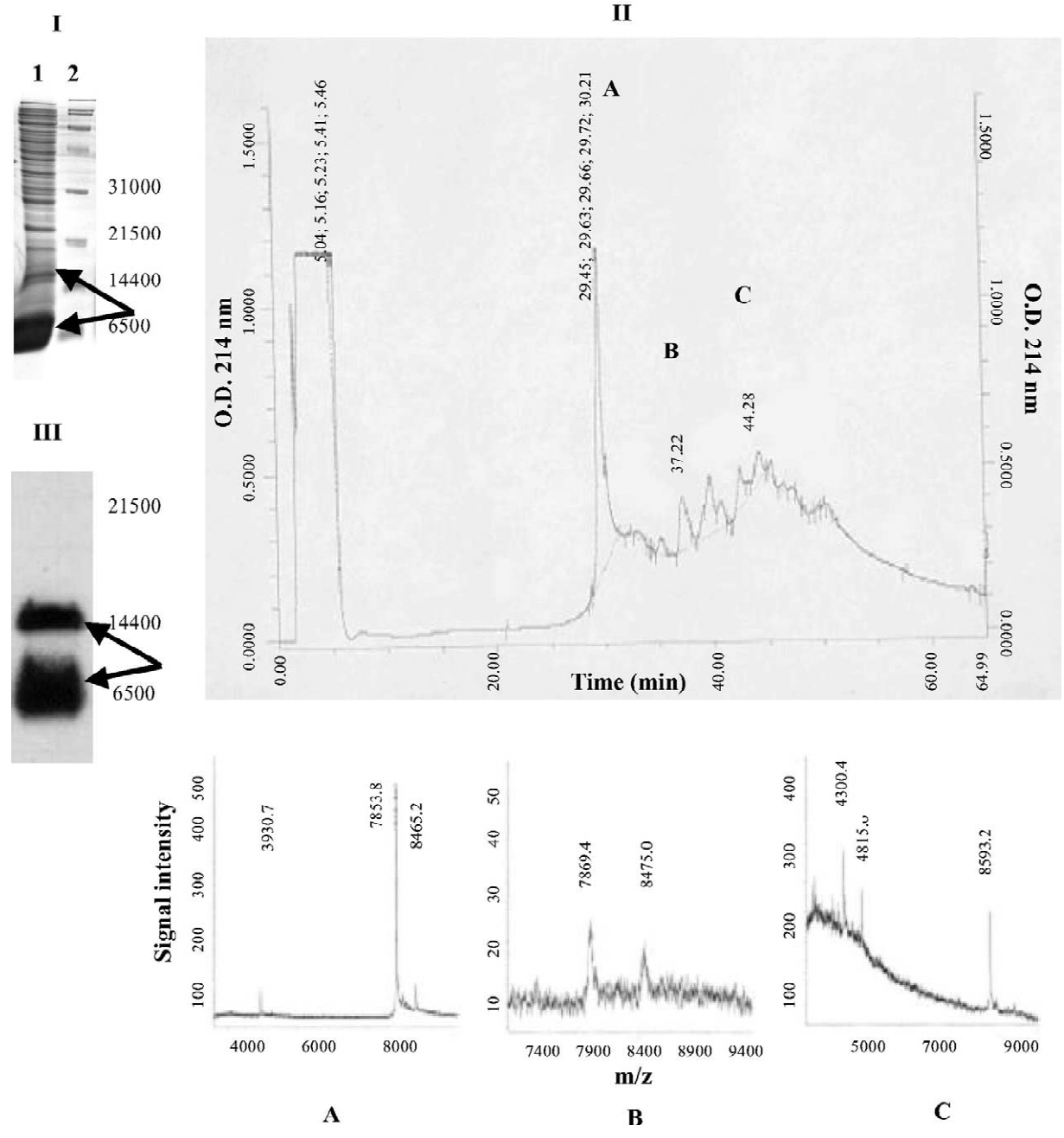

Fig. 3. Purification protocol of EGF obtained from the intracellular insoluble fraction of the culture transformed with pET21b(+)–EGF construction. (I) 16% SDS–PAGE electrophoresis gel of the sample after being dialysed and before charging it in the RP-HPLC column. (II) Elution profile of the protein at 214 nm in a Vydac C_4 column. A, B and C fractions were analysed in an EM MALDI-TOF; the graphics obtained are also shown. In each of these graphics molecular weight corresponding to every protein peak is shown. (III) Western blot analysis of EGF. Two bands appear: EGF monomer and EGF dimer.

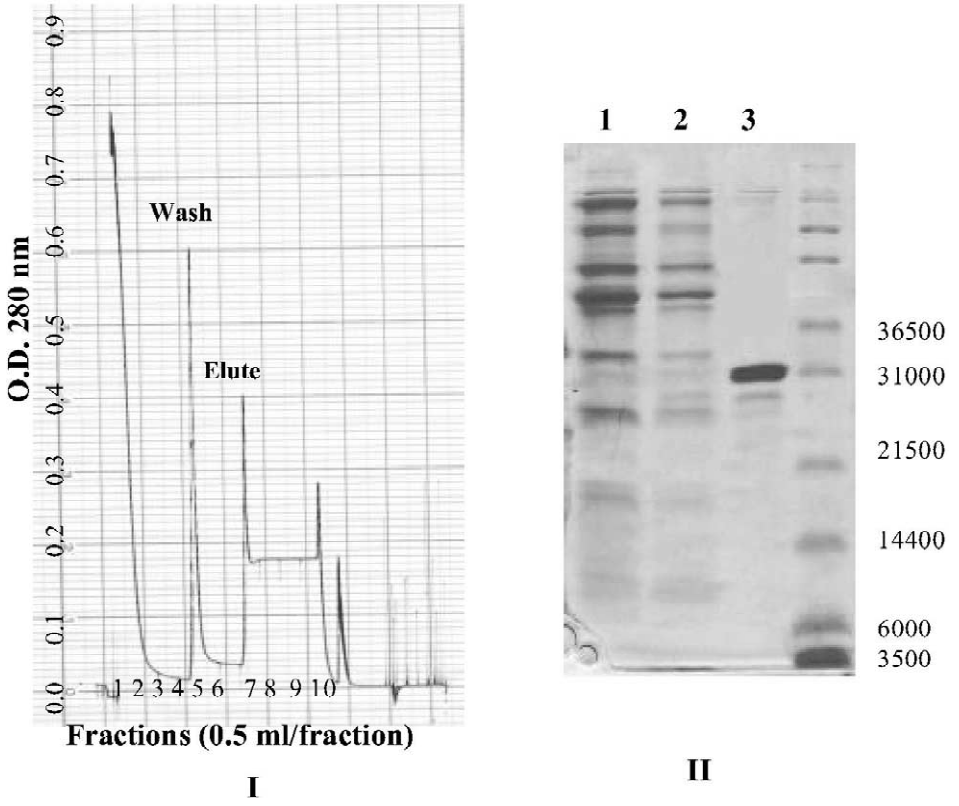

Fig. 4. Purification protocol of EGF obtained from the intracellular insoluble fraction of the culture transformed with pET32b(+)–EGF. (I) Elution profile of the protein at 280 nm obtained with the affinity chromatography in which the insoluble fraction of the pET32b(+)–EGF culture was charged. (II) Some wash and elute peaks were analysed in a 16% SDS–PAGE electrophoresis gel: wash 5 (lane 1), wash 6 (lane 2) and elute 8 (lane 3). Molecular mass markers are also shown (Novex).

(Figs. 4II and 5II). It was shown that it was mainly located in the elution peak as expected. In this case, antibodies against EGF did not work because of the thioredoxin that may block access of the antibody to EGF. So, the molecular weight of the protein was used to identify the recombinant protein. Note that using affinity columns it is very unlikely in this case that other proteins apart from EGF-thioredoxin would be able to bind the resin. Samples were then lyophilised against water and resuspended in a folding buffer containing oxidized and reduced glutathione. Finally, the sample was again dialysed against water and quantified.

3.4. Activity assay of recombinant EGF

To ensure that EGF had been properly folded and was therefore functionally active, we tested its ability to induce EGFR phosphorylation in the A431 tumor cell line which overexpresses EGFR (Fig. 6). The results showed that EGF obtained from the construction pET21b(+)–EGF was almost as active as a commercial EGF. On the other hand, the fusion protein (EGF and thioredoxin) obtained from the construction pET32b(+)–EGF was much less active than the commercial EGF.

4. Discussion

Human epidermal growth factor has been cloned, expressed and purified from different expression systems. In an initial attempt to express EGF from different systems, it was cloned in three pET vectors which allow three different kind of expression: intracellular, periplasmic and as a fusion protein. We

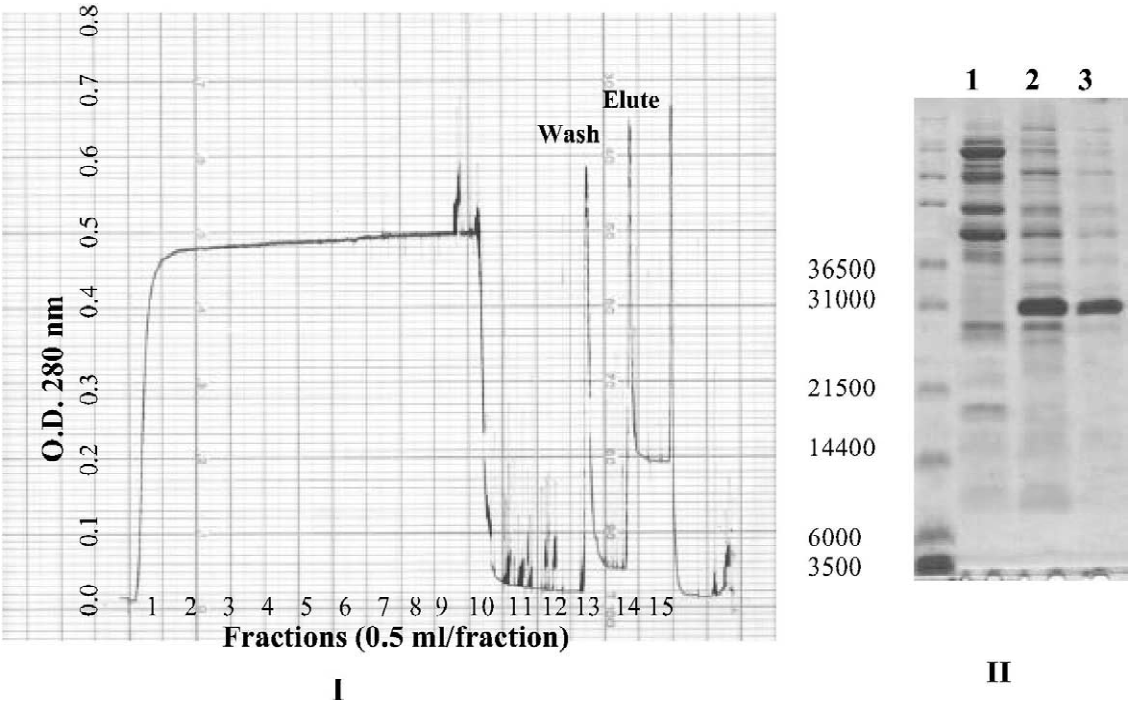

Fig. 5. Purification protocol of EGF obtained from the intracellular soluble fraction of the culture transformed with pET32b(+)–EGF. (I) Elution profile of the protein at 280 nm obtained with the affinity chromatography in which the soluble fraction of the pET32b(+)–EGF culture was charged. (II) Some fractions of the wash and elute peaks were analysed in a 16% SDS–PAGE gel: wash 13 (lane 1), elute 14 (lane 2) and elute 15 (lane 3). Molecular mass markers are also showed (Novex).

chose these strategies in order to assess which of the three different locations produced the greatest amount of protein. EGF was obtained with only two of these systems: pET21b(+)–EGF and pET32b(+)–EGF. Purification protocols were designed in order to purify the recombinant protein. These protocols are based on the location of the protein and its characteristics.

When working with the recombinant EGF obtained from the culture transformed with the pET21b(+)–EGF construction, reversed-phase chromatography was used since it had been described as a good method to separate EGF from other proteins [19]. It is expected that in the future we will test other chromatography columns to improve the yield of recovered protein. However, when working with the fusion protein an affinity column that allowed the histidine tag between EGF and thioredoxin to be bound was used. This system had been useful when working with other proteins in our laboratory since it has the advantage that the protein can be totally purified with only one step. With EGF fused to thioredoxin it has been shown that purification has been successful and that at the end of the process the protein has been obtained with almost 100% purity.

Compared to other methods, these purification protocols had one great advantage: a sufficient amount of protein was obtained in a short time and with few steps. This will allow us to obtain the different EGF truncated forms in a relatively short time, test them and see which is the best antagonist. In the future, when it is known which is the best mutant, it is expected the purification protocols will be improved to increase the yield of recombinant protein obtained.

As EGF is a small protein with three disulfide bonds it is almost impossible that is well folded in the intracellular environment of *E. coli* so it had to be folded using chaperones. To ensure proper folding a biological assay had to be carried out. EGF is a growth factor that is able to bind EGFR and induce its phosphorylation, dimerization and internalisation.

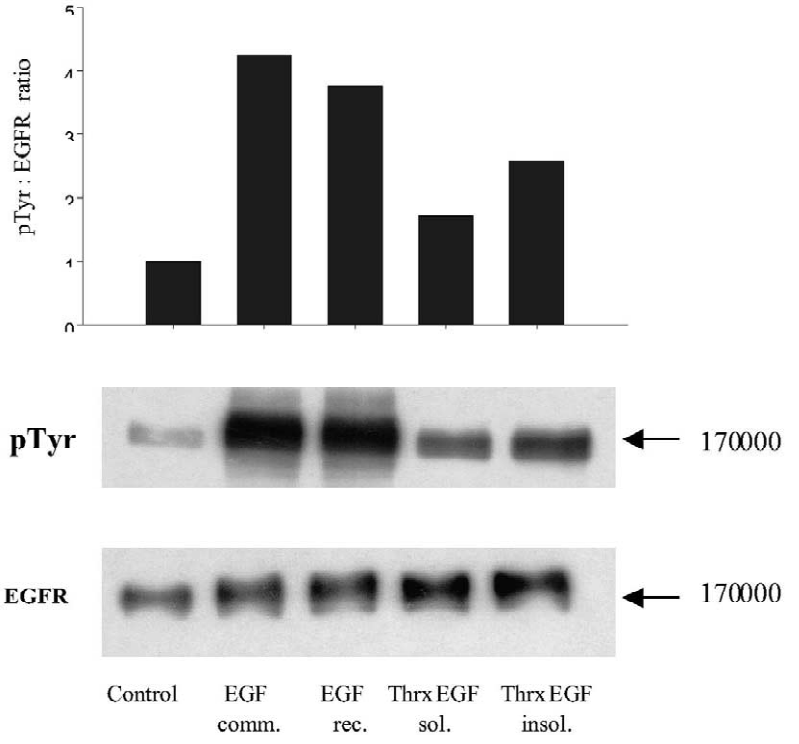

Fig. 6. EGF activity assay. Western blot analysis of EGFR phosphorylation induced by EGF. The different EGFs tested were: a commercial EGF that acted as a reference (EGF comm.), the recombinant EGF obtained from the pET21b(+)–EGF construction (EGF rec.), and the fusion protein obtained from either the soluble or the insoluble fractions of the culture transformed with the pET32b(+)–EGF construction (Thrx–EGF insol., and Thrx–EGF sol.). A control has been also included. Band intensity indicates the level of EGFR tyrosine phosphorylation (pTyr) and the amount of EGFR of every sample (EGFR). This intensity was calculated by densitometry and it represents the relationship between phosphorylation and amount of EGFR.

Therefore, we tested its ability to induce EGFR phosphorylation in a epidermoid tumoral cell line, A431, which overexpresses EGFR and allows easy detection. Activity results showed important differences between the three EGFs tested. Recombinant EGF obtained from pET21b(+)–EGF construction had almost the same activity as the commercial EGF. On the other hand, the fusion protein obtained from the pET32b(+)–EGF construction had much less activity than the commercial EGF. This decrease in the ability to induce EGFR phosphorylation could be due to thioredoxin, which could prevent EGF binding with high affinity to the EGF receptor.

Taken together the results relating to yield and activity show that the greatest amount of active recombinant human EGF is obtained from the pET21b(+)–EGF construction (Table 1). So, it has been decided to use it when making EGF mutants.

5. Conclusions

Epidermal growth factor has been cloned in an expression vector that allows intracellular expression, expressed in *E. coli* and purified. The purification protocol has been established in order to obtain protein as fast as possible and avoiding too many purification steps. The yield of recombinant protein obtained was sufficient to carry out the experiments in tumoral cell lines to test if the protein was active or not. As regards yield and activity of the recombinant protein it has been shown that the pET21b(+)–EGF construction is the best one to work with. This construction and purification protocol will allow us to produce some EGF truncated forms in order to make EGF more similar to PCI, an EGF antagonist, and so obtain an antagonist with high affinity for EGF receptor.

Table 1
Yield and activity of EGF obtained from the different expression systems: pET21b(+)–EGF and pET32b(+)–EGF soluble and insoluble fractions

Construction	EGF expressed (M_r)	mg/1 (mg of protein obtained per liter of culture)	% Activity[a]
pET21b(+)–EGF	EGF (~8000)	Between 5 and 10	94
pET32b(+)–EGF	Thrx–EGF (~28 000)/soluble fraction	3	42.5
pET32b(+)–EGF	Thrx–EGF (~28 000)/insoluble fraction	Between 3 and 5	67.5

[a] Using Quantity One software (Bio-Rad), activity was calculated by analysing the intensity of each band obtained with Western blot in relation to the control band.

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