Journal of Industrial Microbiology & Biotechnology (1998) 21, 208–214

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Pilot scale production of a recombinant human epidermal growth factor, secreted by *Bacillus brevis*, using expanded bed adsorption

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Recombinant *Bacillus brevis* which carried an expression plasmid encoding the human epidermal growth factor (EGF) gene on a cryptic high-copy number plasmid, pHT926, extracellularly produced EGF in its biologically active form at a concentration of over 1.5 g L^{-1} in the culture broth in a 30-L jar fermenter. The culture broth also contained some other EGF compounds, which mainly consisted of oligomeric and polymeric forms with disulfide bonds. We developed a simple purification method for EGF, without prior cell removal from the culture broth, comprising cation exchange expanded bed adsorption followed by ultrafiltration with UF 10 000 and 3000 membranes. The EGF compounds were efficiently separated from the EGF in its native form in the expanded bed adsorption step. With this purification method, only EGF in its native form was recovered from the culture broth, with a yield of nearly 80%, and 90% purity. This efficient and economic system has made it possible to use EGF as a pharmaceutical in the livestock industry.

Keywords: human epidermal growth factor; *Bacillus brevis* recombinants; expanded bed adsorption; fermentation

Introduction

Human epidermal growth factor (EGF) is a small polypeptide of 53 amino acid residues containing three disulfide bonds, with a molecular weight of 6216 daltons. This polypeptide has the ability to temporarily inhibit wool growth in Merino sheep [16], as well as activities inhibiting gastric acid secretion [8] and accelerating epithelial cell proliferation [9]. Therefore, this valuable wool can be easily harvested by hand from the bodies of Merino sheep administered EGF [17]. The use of EGF in this manner is called biological wool harvesting [17]. This system requires the production of high quality EGF in large quantities and at a low price.

The industrial production of recombinant proteins has been achieved using the well-studied bacterium, *Escherichia coli*, as a host. However, other potential host–vector systems, in which a heterologous protein is secreted into the culture medium, have been investigated. Protein secretion is attractive in order to obtain a high yield of the product, to simplify the purification scheme, and to eliminate the cell disruption step.

Udaka *et al* developed a host–vector system involving the protein hyper-producing bacterium, *Bacillus brevis*, as a host for efficient extracellular production of heterologous proteins [24,25]. *B. brevis* HPD31 [21], recently reclassified as *Brevibacillus choshinensis* [20], carrying EGF expression-secretion plasmids derived from pUB110 and pHY483 was able to produce EGF at 240 mg L^{-1} [28] and 1.1 g L^{-1} [6] in the culture medium, respectively. Sagiya *et al* [18] reported a new expression plasmid, pHT110tGH, which enables *B. brevis* HPD31 to secrete tuna growth hormone efficiently. This plasmid was derived from a cryptic multi-copy plasmid, pHT926 [5], found in *Brevibacillus borstelensis* HP926 [19], which belongs to the same genus as the HPD31 strain.

Even if an efficient secretion system is used, centrifugation and microfiltration are normally necessary to remove the cells from the fermentation broth in the first purification steps. The clarified fermentation broth is then subjected to several chromatographic steps for concentration and purification of the product. Recently, expanded or fluidized bed adsorption was developed as a downstream processing technique for the capture of proteins from unclarified fermentation broth or unclarified homogenates [7,10]. Expanded bed adsorption can reduce the number of operations in downstream processing, because this system has the ability of simultaneous clarification, concentration and purification. In general, fewer operations in the purification process will increase the yield, reduce waste, and decrease the capital cost. For these reasons, expanded bed adsorption is an attractive downstream processing technique. Therefore, we selected a cation exchange expanded bed adsorption system for the primary purification step in order to achieve efficient purification of EGF.

In this study, we found a considerable amount of high molecular weight EGF compounds in the culture broth, consisting of oligomeric and polymeric molecules with intermolecular disulfide bonds. We describe the production of EGF on a large scale, with *B. brevis* HPD31 as the host and plasmid pHT926 as the vector in a batch fermentation, and a purification method involving expanded bed adsorption which can efficiently separate the polymerized EGFs from the native form.

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Received 10 June 1998; accepted 10 September 1998

Materials and methods

Bacterial strain and plasmid

B. brevis HPD31-M3, which was obtained from the wild-type strain by means of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment as a methionine auxotroph, was used as the host strain. Plasmid pHT110EGF (Figure 1) was constructed as follows; the *Spe*1-*Pst*1 fragment of pNU200EGF [28] containing the 5′ region of the cell wall protein (CWP) gene [27] and the EGF gene was inserted into the *Spe*1-partial *Pst*I sites of pHT110R2L5tGH [18]. Transformation by electroporation was performed as described previously [22].

Media and cultivation conditions

2SL-ME medium, designed for EGF production, contains the following: 40 g L^{-1} of soybean polypeptone, 5 g L^{-1} of yeast extract, 1.23 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.24 g L^{-1} of $MnSO_4 \cdot 5H_2O$, 0.01 g L^{-1} of $FeSO_4 \cdot 7H_2O$, 20 g L^{-1} of glucose and 1 μg ml^{-1} of erythromycin.

Fermenter cultivation was performed as follows. One millilitre of an overnight culture in 2SL-ME medium at 30°C was inoculated into 100 ml of the same medium in a 500-ml flask, followed by incubation at 30°C for 6 h with shaking. The culture was then inoculated into 20 L of the same medium in a 30-L jar fermenter (Komatsugawa Chemical Engineering Co, Tokyo, Japan). Fermentation was carried out at 33°C, 1 vvm aeration, 200 rpm agitation, and 0.2 kg cm^{-2} inner pressure. The initial pH was adjusted to 7.2 but the pH was not controlled throughout the cultivation. Adekanol LG297 (Asahi Denka Kogyo Ltd, Tokyo, Japan) was used as an antifoaming agent.

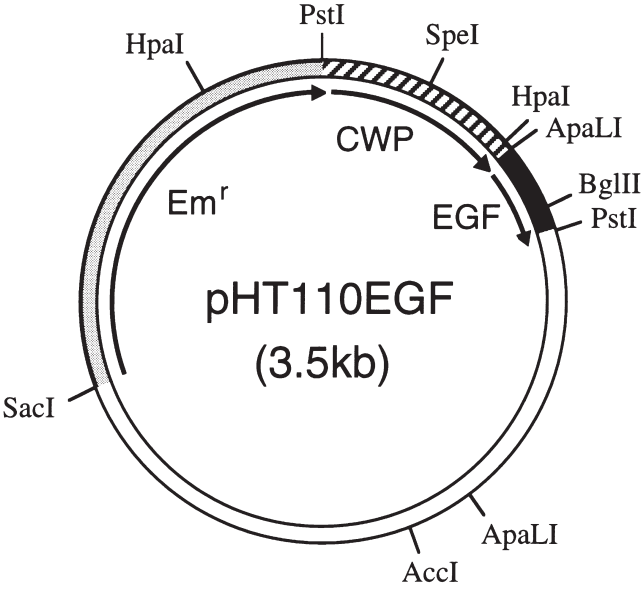

Figure 1 Structure of pHT110EGF, the expression vector used for hEGF production. The plasmid contains the 5′ region of the cell wall protein (CWP) gene of *B. brevis* HPD31 including the promoter region and signal sequence, the EGF gene, and the erythromycin resistance gene (Em^r). The sequence encoding the mature EGF is directly fused to the signal sequence.

Equipment for expanded bed adsorption

The column used was a STREAMLINE 50 (inner diameter, 50-mm) borosilicate glass one (Pharmacia Biotech, Uppsala, Sweden), containing the STREAMLINE SP adsorbent (Pharmacia Biotech) for cation exchange adsorption with the expanded bed procedure. The bed height in the sedimented configuration was 10 cm.

Purification process for EGF

The bed of sedimented adsorbent particles was expanded and equilibrated with an upward flow of 20 mM sodium acetate-HCl buffer, pH 3.0, at the flow rate of 300 cm h^{-1}. The resultant expanded bed height was 40 cm. Then the culture broth, which had been adjusted to pH 3.0 with 1N hydrochloric acid, was applied at a flow rate of 300 cm h^{-1}. Twenty millimolar sodium acetate-HCl buffer, pH 3.0, was then passed through the adsorbent in the expanded mode until the UV signal decreased to the baseline, this was done without stopping the flow through the column. Then the pump was stopped and the adsorbent was allowed to settle. The upper adapter of the STREAMLINE 50 column was lowered to the sedimented bed surface. Elution of the bed was performed with 50 mM sodium acetate-HCl buffer, pH 5.5, in the sedimented mode with downward flow at a flow rate of 200 cm h^{-1}. The UV-absorbing peak fractions containing EGF were pooled. The column was then cleaned with 20 mM sodium acetate buffer (pH 7.5) containing 2 M NaCl (cleaning buffer) in the sedimented mode.

The recovered EGF fraction was adjusted to pH 2.0 with 6N HCl. The solution was then filtered through a MW 10 000 ultrafiltration membrane (Biomax-10; Millipore, Bedford, MA, USA). Any EGF remaining in the retentate was obtained by diafiltration with a sufficient volume of 20 mM sodium acetate-HCl buffer, pH 2.0. The filtrate was then adjusted to pH 7.0 with 5N NaOH, and subsequently concentrated and dialyzed against sufficient pyrogen-free water using a MW 3000 ultrafiltration membrane (Omega 3; Pall Filtron Corp, Northborough, MA, USA) until the electric conductivity value of the filtrate solution reached the level of pyrogen-free water.

Preparation of standard EGF by HPLC

Standard EGF of 98% purity was prepared from the UF 3000-concentrated EGF by high performance liquid chromatography (HPLC). The column used was of CAPCELL PAK C18, 5 μm, 15 mm × 250 mm (Shiseido, Tokyo, Japan). Preparative HPLC was carried out as follows: a linear gradient of 20–40% acetonitrile in 0.1% trifluoroacetic acid was used for 120 min at a flow rate of 10 ml min^{-1}. Detection was with a 280-nm UV detector. The purity of EGF in each of the 10-ml fractions eluted from the column was determined by means of the analytical HPLC mentioned below. The fractions containing EGF of over 98% purity were pooled and lyophilized.

Analysis and activity of EGF

The amount and purity of EGF were determined by HPLC on a reverse phase column (C18 μBondasphere, 5 μm, 4.6 mm × 150 mm; Waters Co, Milford, MA, USA). The analysis was carried out with a linear gradient of 25–34% acetonitrile in 0.1% trifluoroacetic acid for 28 min at a flow

rate of 1.0 ml min^{-1}. Detection was with a 280-nm UV detector. The purity of EGF was calculated using the percentage of the EGF peak area per the total peak area.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis were performed as described by Laemmli [15], and Towbin *et al* [23], respectively. For immunoblotting, anti-EGF serum was obtained from a rabbit immunized with the standard EGF prepared above.

The NH_2-terminal amino acid was determined with a gas-phase protein sequencer (model 477A; Applied Biosystems, CA, USA). The COOH-terminal amino acid sequence was determined by the Klemm method [13] using carboxypeptidase Y.

The stimulatory effect of EGF on the initiation of Balb/c3T3 cell division was examined by the Carpenter and Cohen method [3]. The control EGF used in this experiment was purchased from Sigma (St Louis, MO, USA).

Other analytical methods

Cell concentrations were monitored by measuring the optical density at 660 nm (OD_{660}). The OD_{660} value was then converted into dry cell weight (DCW). One unit at OD_{660} corresponded to 0.31 g L^{-1} of DCW. The number of viable cells was determined by counting colonies on T2 plates containing 10 g L^{-1} of casein polypeptone, 2 g L^{-1} of yeast extract, 5 g L^{-1} of beef extract, 10 g L^{-1} of glucose and 15 g L^{-1} of agar, pH 7.0. The stability of the plasmid in the bacterium was defined as the ratio of the number of colonies on T2 plates with and without erythromycin at a concentration of 10 μg ml^{-1}.

Glucose concentrations were measured with a Blood Sugar Test kit (Boehringer Mannheim, Germany). The endotoxin content of the product solution was determined with a Limulus test kit (Wako Pure Chemical Industries, Osaka, Japan). The DNA content was determined with a silicon sensor-based system (threshold test) as described by Kung *et al* [14].

Results

Mass production of EGF by B. brevis

We designed 2SL-ME medium for both high-level production of EGF (Figure 2) and low accumulation of other extracellular proteins, as shown in Figure 3a. For efficient EGF production, we examined several fermentation parameters: pH, temperature, aeration, and agitation speed, in a 30-L fermenter. Figure 2 shows the time course of a fermentation of the strain under optimal conditions, as described in Materials and methods. Cell growth reached stationary phase at 16 h at a DCW concentration of 3.1 g L^{-1} and a viable cell count of 2.0×10^9 cells ml^{-1}.

The production of EGF in the culture medium increased markedly from the early stationary phase of growth. The production rate was constant until 54 h, and the amount of EGF in the culture supernatant reached 1.5 g L^{-1} at 64 h. SDS-PAGE (Figure 3a) indicated that EGF was the major extracellular protein secreted by *B. brevis* carrying pHT110EGF. The rate of glucose utilization was maximum at 16–42 h, being 0.5 g h^{-1}, while it was very low until the end of the log phase of growth. A small amount of glucose remained in the culture medium at the end of the fermentation. The pH of the culture broth changed from 7.2 to 8.0 in the early stationary phase of growth and from 8.0 to 8.5 after 48 h cultivation. The plasmid was maintained stably in over 95% of the cells until 64 h after inoculation.

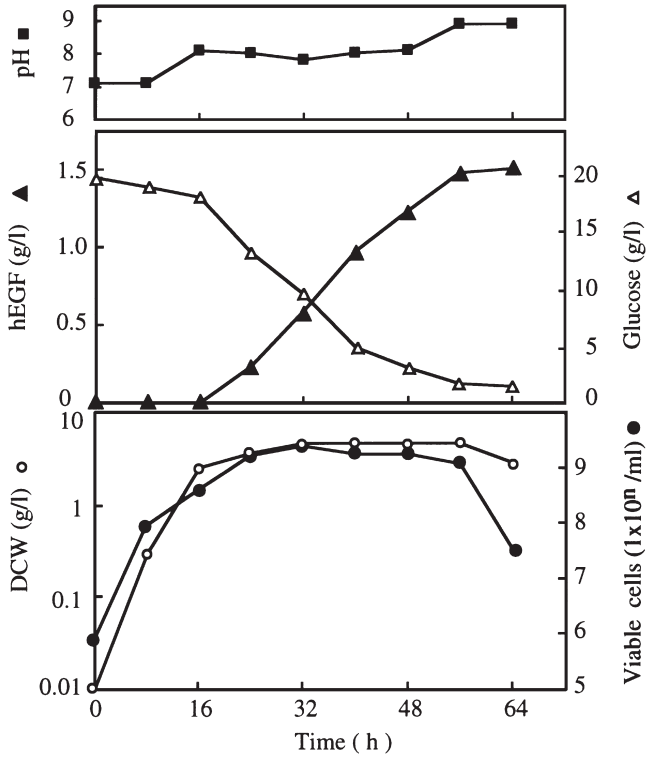

Figure 2 Time course of EGF production. *B. brevis* HPD31 containing pHT110EGF was grown in a 30-L jar fermenter under the conditions given under Materials and methods.

Characterization of EGF in the culture medium

The properties of EGF produced by *B. brevis* were determined by SDS-PAGE and Western blot analysis using anti-hEGF serum under reducing and non-reducing conditions (Figure 3). Many ladder bands, including high molecular ones, appeared on Western blotting of the culture supernatant under non-reducing conditions, as shown in Figure 3b, lane 2. The molecular weights of bands a, b, c and d in Figure 3b, lanes 2 and 4, were calculated to be 6000, 12 000, 18 000 and 24 000, respectively. Under reducing conditions, these ladder bands disappeared, only EGF with a molecular weight of 6000 remained. The results indicate that the proteins in the ladder bands might be oligomeric and polymeric EGFs with intermolecular disulfide bonds.

Both standard EGF and secreted monomeric EGF, which each gave a single peak with a retention time of 14 min on HPLC analysis (Figure 4), gave two separate bands on SDS-PAGE under a non-reducing condition.

We found that the polymerized EGF sedimented out of the culture supernatant when the pH was adjusted to 3.0, only the EGF in its native form remaining in the supernatant phase (Figure 3). The ratio of monomeric and polymeric EGF produced from HPD31 carrying pHT110EGF was 1:0.2, which was calculated from results obtained on

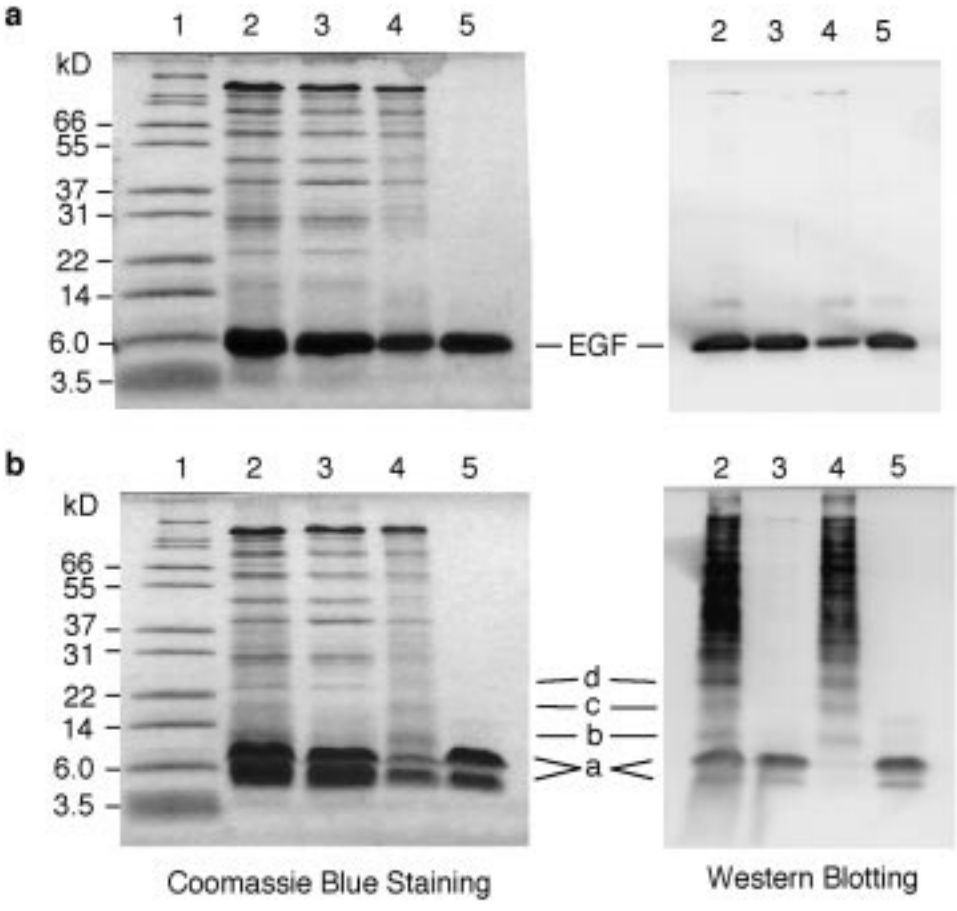

Figure 3 Coomassie brilliant blue (CBB) staining of SDS-PAGE gels (left), and Western blot analysis (right) of culture supernatants and acid-treated samples under reducing (a) and non-reducing (b) conditions. Molecular weight markers (lane 1) are indicated on the left-hand side of the gels. The culture supernatant at 64 h of fermentation (lane 2) was adjusted to pH 3.0. After acid treatment for 5 min, a supernatant (lane 3) was obtained by centrifugation. The precipitate was collected and solubilized with an equal volume of 20 mM Tris-HCl (pH 7.5) (lane 4). Lane 5 is the standard EGF purified (2.5 μg) by preparative HPLC. Each sample for CBB staining and Western blotting was diluted 2-fold and 20-fold, respectively. Five-microlitre aliquots of these samples treated with reducing and non-reducing SDS-PAGE sample buffer were applied to each gel. a, b, c and d indicate monomer and oligomeric EGF as described in the text.

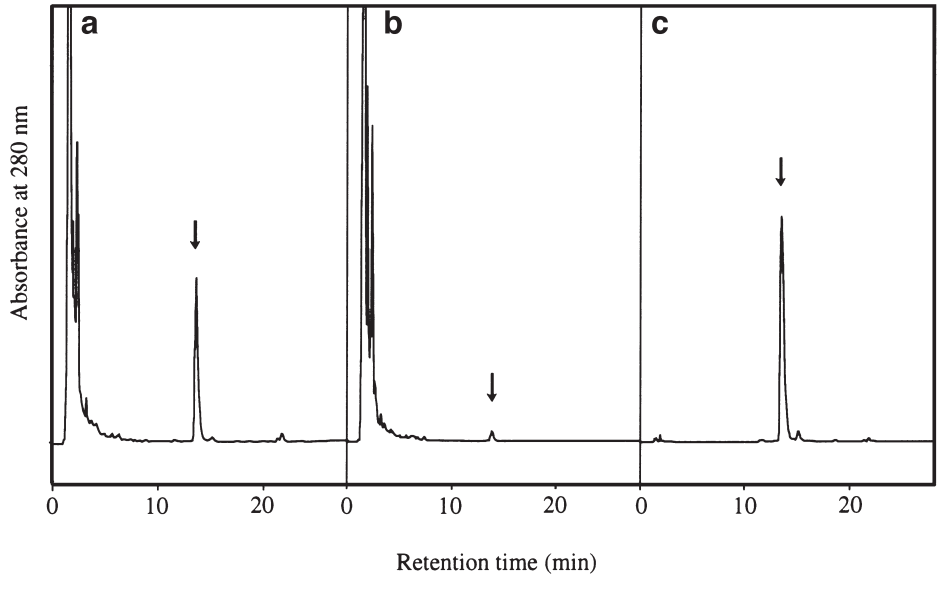

Figure 4 HPLC analysis of samples at different steps of the purification process. (a) Culture supernatant at the end of the fermentation; (b) supernatant of the flowthrough fraction on expanded bed adsorption; (c) fraction eluted from the expanded bed. The HPLC conditions are given in Materials and methods. The arrows indicate EGF.

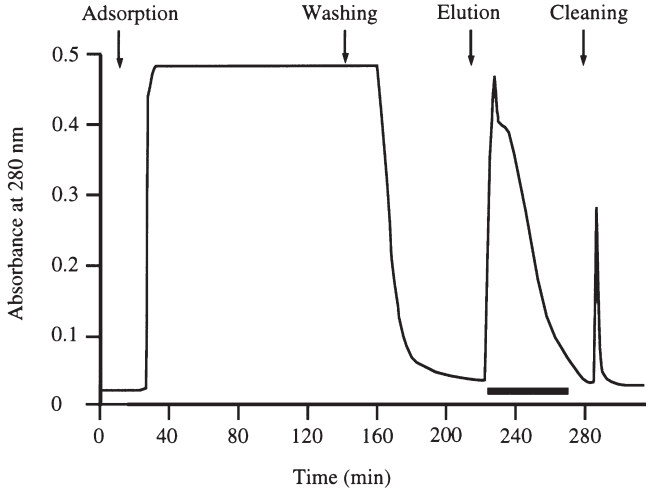

Figure 5 Chromatography of EGF from an unclarified *B. brevis* culture after expanded bed adsorption using STREAMLINE SP. The eluted fraction (from 225 min to 270 min), indicated by the bar, was collected.

SDS-PAGE under reducing conditions after acid treatment of the culture supernatant (Figure 3a, lanes 3 and 4).

Pilot scale purification of EGF from a batch culture

Figure 5 and Table 1 summarize the expanded bed adsorption procedure. The eluted fraction volume with the cationic exchange adsorbent of STREAMLINE was 8.5 L, which had an EGF concentration of 1.8 g L^{-1}. In this purification step, monomeric EGF was mainly found in the eluted fraction, as found on HPLC (Figure 4), while the EGF purity was increased to more than 90%. A yield of about 80% of the monomeric secreted EGF was obtained (Table 1). Although the sample applied to the expanded bed column contained viable cells, living cells were not detected in the eluted fraction (data not shown).

The protein content and the EGF quality were analyzed after each purification step by SDS-PAGE (Figure 6). EGF, which was the major product in the culture supernatant (lane 3), was efficiently recovered in the eluted fraction (lane 6). The polymerized EGF, which was insoluble at pH 3.0, was not adsorbed to the cationic resin under the conditions used (lane 6). The high molecular mass proteins found in the culture supernatant (lane 3) were almost totally removed in this step (lane 6), and a high level of purity was reached after the UF 10 000 step, as shown by the single band in lane 7 in Figure 6. Consequently, the EGF after the UF 3000 concentration step was more than 90% pure. High molecular weight proteins, endotoxins and DNA

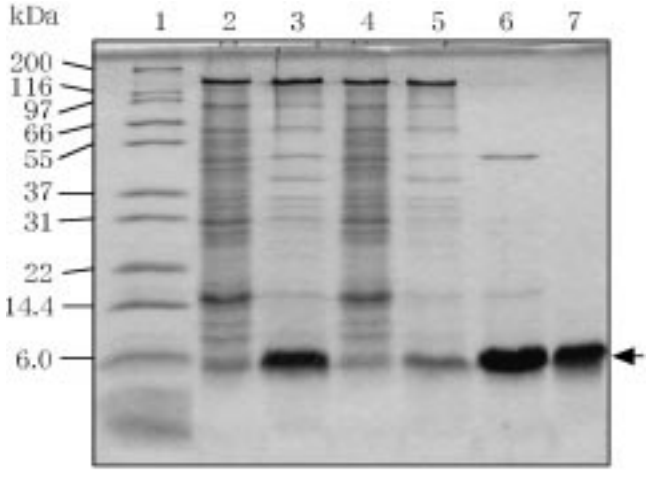

Figure 6 SDS-PAGE analysis under reducing conditions of samples at different steps of the purification process. Molecular weight markers (lane 1) are indicated on the left-hand side of the gel. Lanes 2 and 3 are the precipitate and supernatant of a culture at the end of the fermentation, respectively. Lanes 4 and 5 are the precipitate and supernatant of the flowthrough fraction with the expanded bed adsorption process, respectively. The culture and flowthrough fractions were adjusted to pH 7 prior to centrifugation, and each precipitate was suspended in an equal volume of water. Lane 6 is the fraction eluted from the expanded bed. Lane 7 is the filtrate of the UF 10 000 process. Then 5-μl aliquots of these samples treated with reducing SDS-PAGE sample buffer were applied to the gel. The arrow indicates EGF.

were detected at very low levels or not at all in the solution (Table 1).

The purification procedure demonstrated here gives a product yield in excess of 70% with effective and efficient removal of contaminants.

Structural analysis and biological activity of the purified EGF

The 20 residues of the NH_2-terminal amino acid sequence and the five residues of the COOH-terminal amino acid sequence of the EGF purified as described above were identical with those of authentic EGF (data not shown).

Purified EGF had a stimulatory effect on the initiation of Balb/c3T3 cell division. Its 50% effective dose (ED_{50}) was 0.7 ng ml^{-1}, which is almost the same as that of reagent grade EGF (ED_{50} = 0.6 ng ml^{-1}).

Discussion

In a 30-L jar fermenter, EGF production increased dramatically at the early stationary phase of growth (Figure 2). The

Table 1 Purification and characterization of EGF from *B. brevis* carrying pHT110EGF

Purification step	Volume (L)	Total EGF (g)	Endotoxin content (EU mg^{-1} product)	DNA content (pg mg^{-1} product)	Glucose content (mg mg^{-1} product)	Purity (%)	Yield (%)
Unclarified culture	15	19.6	–	–	3.6	–	100
STREAMLINE eluate	8.5	15.4	10	–	ND[a]	90	79
UF 10 000 filtrate	16	14.7	1	–	ND[a]	90	75
UF 3000 concentrate	0.5	14.0	1	<17	ND[a]	92	71

[a]ND: <0.1 μg mg^{-1}.

5′ region of the CWP gene, in which five tandemly arranged promoters exist, was utilized to express EGF. Since the P2 promoter leads to the efficient and constitutive expression of the CWP gene even in the stationary phase of growth in *B. brevis* [1], the P2 promoter may also act mainly to express the EGF gene in recombinant *B. brevis* cells.

B. brevis carrying pHT110EGF extracellularly produced over 1.5 g L^{-1} of EGF in a 30-L fermenter. This represents over 2.5- and 12.5-fold increases compared to *B. brevis* carrying pHY700EGF and pNU200EGF, respectively, as reported previously [6,28]. The expression plasmid, pHY7-00EGF, contains the replication origin of pWT481 [26] from *B. brevis* 481. It is stable, but exhibits a low copy number in *B. brevis* [6]. Plasmid pHT100, which was constructed by inserting the erythromycin resistance gene into plasmid pHT926 found in a strain of the same genus, was very stable in *B. brevis* over 260 generations in the absence of selective antibiotics with a high copy-number (80 copies per cell) [5]. In fact, the recombinant plasmid, pHT110EGF, was also stable in *B. brevis* up to 64 h after inoculation. The efficient production of EGF by *B. brevis* HPD31 cells carrying pHT110EGF seems to have resulted from a high gene dosage effect on EGF expression throughout fermentation.

The kinds of ingredients of the culture medium markedly influenced the production of EGF by *B. brevis*. In particular, a high concentration of soybean polypeptone in 2SL-ME medium caused higher productivity of EGF as compared with other peptones (data not shown). In addition, accumulation of other extracellular proteins was reduced under the optimal conditions. The amount of EGF secreted into the medium was calculated to be approximately 70% of the total extracellular proteins from the optical densities of the stained bands observed on SDS-PAGE in lane 2 in Figure 3a. On EGF production under optimal conditions, the machineries of protein synthesis and/or translocation seem to be primarily occupied with EGF gene expression, which may be 80-fold higher in gene dosage than the expression of other genes on the chromosome. Consequently, the amounts of other extracellular proteins from the chromosome may decrease. This is probably one of the important factors for the effective purification on expanded bed adsorption and the following processes. Purifying a gene product directly from the culture medium without cell disruption also has the advantage of minimizing the release of DNA, endotoxin and intracellular components.

The isoelectric points of EGF and cell wall layer protein, which is the major extracellular protein of *B. brevis* HPD31, are 4.5 and 3.8, respectively. By adjusting the pH of the culture broth to 3.0, many extracellular proteins along with oligomeric and polymeric EGFs were rendered insoluble (Figure 3). However, EGF in its native form remained soluble and stable in the acidified culture broth. Therefore, it was possible and suitable to perform the cationic exchange process at pH 3.0 in the expanded bed mode, where the majority of proteins other than the native EGF were not adsorbed and thus were effectively separated from the native EGF (Figures 4 and 6).

Not only EGF purified in this way but also the standard EGF as well as the control EGF purchased from Sigma gave a single band on reduced SDS-PAGE and HPLC. Nevertheless, these EGF molecules were detected as double bands with different mobilities under non-reducing conditions on SDS-PAGE (Figure 3). We have no evidence to explain this phenomenon. More investigation is needed on the structural alteration and modification of EGF under the present conditions.

A portion of the EGF secreted by *B. brevis* formed a high molecular weight complex with disulfide bonds. Chang *et al* [4] reported that several folding intermediates generated in the *in vitro* disulfide folding pathway from unfolded EGF to the native form were recognized. These intermediates contained non-native disulfide bonds, and the formation of the native EGF was accelerated by both redox agents and protein disulfide isomerase. In *B. brevis*, EGF is probably translocated through the cytoplasmic membrane in a reduced form. After secretion, continuous protein folding must occur spontaneously or with the aid of functional proteins which catalyze native disulfide formation *in vivo* such as Dsb A [2,12] found in *E. coli*, and Bdb protein [11] found in *B. brevis*. However, it is suggested that a portion of the reduced EGF or its intermediates might fold incorrectly on binding to each other through intermolecular disulfide bonds because of the locally high concentration of EGF around *B. brevis* cells.

This process, consisting of expanded bed adsorption, and ultrafiltration on UF 10 000 and UF 3000, is simple and efficient for the purification of a recombinant protein secreted by *B. brevis*. The process can be easily scaled up to industrial scale, and the EGF produced in this manner can be successfully used for biological wool harvesting.

Acknowledgements

We are grateful to Professor S Udaka of Tokyo University of Agriculture for valuable advice and fruitful discussion.

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