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Process development for production of recombinant human insulin-like growth factor-I in *Escherichia coli*

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Fed-batch cultures were carried out to overproduce human insulin-like growth factor I (IGF-I) in *Escherichia coli*. The effects of carbon sources (glucose or glycerol) and induction time on cell growth and IGF-I production were investigated in more detail. Glycerol was a better carbon source than glucose for IGF-I production in fed-batch culture. Induction at the mid-exponential phase with glycerol as a carbon source in the pH-stat fed-batch culture was optimal for IGF-I production. Under this condition, 2.8 g L^{-1} of fusion IGF-I was produced as inclusion bodies. We have also developed downstream processing for preparative scale purification of IGF-I from the fusion protein produced by the fed-batch culture using glycerol as a carbon source. After the fusion protein expressed was solubilized in 8 M urea and cleaved with hydroxylamine, the released IGF-I was purified by cation exchange chromatography, refolding and preparative scale reverse phase HPLC (rp-HPLC) to give recombinant IGF-I of >98% purity. The biological activities of the purified IGF-I were measured and found to be identical to those of commercial IGF-I. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 94–99.

Keywords: insulin-like growth factor I (IGF-I); fed-batch culture; downstream processing; *Escherichia coli*

Introduction

Insulin-like growth factor I (IGF-I), which is also known as somatomedin C, is a 70-amino acid polypeptide hormone with three disulfide bonds [3]. IGF-I was originally isolated from human plasma and characterized by Rinderknecht and Humbel [16]. It promotes cell growth and differentiation of various cell types [19], improves glomerular filtration and renal plasma flow [9] and is capable of lowering glucose levels *in vivo* [7]. Because of these biological activities, IGF-I is attracting considerable interest in the pharmaceutical industry and is currently being developed as a therapeutic agent for tissue reconstruction and for insulin-resistant diabetes remedy.

Recombinant human IGF-I has been produced via recombinant DNA technology using a variety of host systems including *E. coli* [6,14,17,18,20], mammalian cells [1,2] and yeast [8,21]. Among them, *E. coli* has been most widely used as a host to produce IGF-I because of its advantages over other hosts such as easy handling and culture, and high yields. More recently, we have developed a new expression system for IGF-I [10] by improving the previously reported expression plasmid [11] which was designed to express IGF-I as a fusion protein with the first 278 amino acid residues of β-galactosidase in *E. coli*. In this new expression system, 163 N-terminal residues of β-galactosidase were truncated, and consequently the expression yield was significantly improved. In this study we report the process development to produce large quantities of IGF-I using *E. coli* harboring our new expression plasmid. The fed-batch cultures were performed and optimized to produce the maximum amount of IGF-I through high cell-density culture, and downstream processing was also developed to obtain biologically active IGF-I of therapeutic grade.

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Materials and methods

Plasmid and strain

The plasmid pYKM-I1 for the expression of IGF-I in *E. coli* has been described previously [10]. *E. coli* W3110 harboring plasmid pYKM-I1 was used for overproduction of IGF-I.

Media and culture conditions

Frozen glycerol cell stock (100 μl) at –70°C was inoculated into a 50-ml tube containing 10 ml of Luria-Bertani (LB) medium supplemented with ampicillin at a concentration of 50 mg L^{-1}. After cultivation at 30°C and 250 rpm for 12 h in a shaking incubator, it was transferred into a 1-L flask containing 160 ml of LB medium supplemented with ampicillin (50 mg L^{-1}). The culture was then grown at 30°C and 250 rpm before it was used to inoculate a 2.5-L fermenter (Korea Fermentor Company, Incheon, Korea) containing 640 ml of modified R-medium [12]. The pH (InPro 3000/225 combination pH electrode, Mettler-Toledo GmbH, Greifensee, Switzerland) and the dissolved oxygen concentration (Ingold, Mettler-Toledo GmbH) were controlled at the set points by on-line monitoring. The composition of modified R-medium was (L^{-1}): $(NH_4)_2HPO_4$, 3 g; KH_2PO_4, 7 g; citric acid, 0.8 g; $MgSO_4 \cdot 7H_2O$, 1 g; glucose, 10 g or glycerol, 20 g; yeast extract, 1 g (for fermentation using glucose) or 2 g (for fermentation using glycerol); and trace metal solution, 3 ml. The trace metal solution con-

sisted of the following (L^{-1} of 5 M HCl): $FeSO_4 \cdot 7H_2O$, 10 g; $CaCl_2$, 1.35 g; $ZnSO_4 \cdot 7H_2O$, 2.25 g; $MnSO_4 \cdot 4H_2O$, 0.5 g; $CuSO_4 \cdot 5H_2O$, 1 g; $(NH_4)_6\ Mo_7O_{24} \cdot 4H_2O$, 0.106 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.23 g; and 35% HCl, 10 ml. Cells were cultured at 37°C and pH 6.8 (automatic feeding of 25% NH_4OH).

The dissolved oxygen level was maintained above 40% of air saturation by supplying air at a flow rate of 1.5 vvm and by controlling the agitation speed up to 1000 rpm. Pure oxygen was supplied when required. The composition of the nutrient feed used for the fed-batch culture was (L^{-1}): glucose or glycerol, 500 g; $MgSO_4 \cdot 7H_2O$, 15 g; yeast extract, 50 g. Two nutrient feeding strategies were employed, exponential and pH-stat feeding. Exponential feeding was done using an in-house program which was written in turbo C. When the carbon source (glucose or glycerol) was depleted in the initial medium, the feeding solution was exponentially fed into the fermentor using a computer-controlled pump. The feeding rate was predetermined using a material balance equation by assuming a constant cell yield on the carbon source (glucose or glycerol) [13]. Synthesis of IGF-I was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co, St Louis, MO, USA) to give the final concentration of 1 mM at the time of induction.

Purification and refolding of IGF-I

Inclusion bodies were isolated by centrifugation at $5000 \times g$ for 10 min after sonicating *E. coli* cells resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The resulting pellets were washed twice with a solution containing 3 M urea, 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA to remove contaminant proteins and then solubilized in a solution containing 8 M urea, 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The fusion protein was cleaved by adding hydroxylamine at a final concentration of 2.0 M (pH 9.0) and incubating the solution at 45°C for 4 h. The reaction mixture was dialyzed against 0.5 M acetic acid buffer (pH 2.85) containing 0.075 M NaCl. The truncated β-galactosidase fusion tag and uncleaved fusion proteins were precipitated during dialysis and then separated by centrifugation at $5000 \times g$ for 10 min. The supernatant containing soluble IGF-I was loaded on a HyperD cation exchange column (2.2×16 cm, Biosepra, St Christophe, France) equilibrated with 0.5 M acetic acid containing 0.075 M NaCl (equilibration buffer, pH 2.85). For elution of proteins, a linear gradient was applied by gradual mixing of the equilibration buffer and 0.2 M ammonium acetate buffer (pH 5.0).

The fractions containing IGF-I were collected, dialyzed against distilled water, and concentrated by ultrafiltration using an Amicon membrane (Mol wt cut-off = 3000, Amicon, Massachusetts, USA). Refolding of IGF-I was performed at room temperature for 72 h in 50 mM Tris-HCl buffer (pH 8.0) containing 1 M guanidine-HCl, 1 mM EDTA, 0.5 mM β-mercaptoethanol and 30% (v/v) ethanol. Refolding was terminated by addition of acetic acid to give a final pH of 4.0, and the solution was then loaded onto a preparative C8 RP-HPLC column (50×185 mm, 12 μm, Lichrosper RP select, Merck, Darmstadt, Germany). Elution was performed using a linear gradient of 25–35% acetonitrile over 30 min at a flow rate of 60 ml min^{-1} in the presence of 0.1% TFA. Fractions were collected and analyzed by an analytical RP-HPLC. Fractions containing correctly folded IGF-I were pooled and lyophilized to dryness.

Analytical methods

Growth was monitored by measuring the optical density at 600 nm. The concentrations of glucose, glycerol and acetate were determined by HPLC (Hitachi L-3300, Tokyo, Japan) equipped with an ion exchange column (Aminex HPX-87H, Hercules, CA, USA) using 0.01 N H_2SO_4 as the mobile phase. To determine the amount of total proteins, cells were washed and suspended in TE buffer (10 mM Tris-HCl, 1 mM, EDTA, pH 8.0), and disrupted by sonication. The cell lysate was centrifuged at $12\,000 \times g$ for 14 min at 14°C. The resulting supernatants and pellets containing soluble and insoluble protein fractions, respectively, were subjected to SDS-PAGE. The amount of fusion IGF-I was quantified by measuring the staining intensity of protein bands using a densitometer (ImageMaster, Pharmacia Biotech, Uppsala, Sweden). The total protein concentration was measured by the Bradford method [5]. The purity of correctly folded IGF-I was measured by RP-HPLC equipped with a YMC-Pack C8 column (5 μm, 4.6×250 mm, Beckman, System Gold, Fullerton, CA, USA). Elution was performed using a linear gradient of 25–35% acetonitrile containing 0.1% TFA at a flow rate of 1 ml min^{-1}.

Results and discussion

Fusion IGF-I production by fed-batch culture of recombinant E. coli with exponential feeding

Efficient production of fusion IGF-I was investigated through fed-batch cultures of recombinant *E. coli*. The first nutrient feeding strategy employed was an exponential feeding strategy with μ_{set} of 0.15 and 0.12 h^{-1} before and after induction, respectively [13]. Two carbon sources, glucose and glycerol, were compared for cell growth and fusion IGF-I production. The effect of induction time on fusion IGF-I production was also examined (Table 1). Induction at lower cell concentrations resulted in larger amounts of fusion IGF-I and higher maximum attainable dry cell weights than that obtained by induction at higher cell concentration. Glycerol was a better carbon source than glucose for production of fusion IGF-I by high-cell density culture. For fed-batch cultures using glucose, a low level of fusion IGF-I, accounting for $<0.5\%$ of total proteins, was produced when cells were induced at 28 g DCW L^{-1}. However, the fed-batch culture using glycerol produced a high level of fusion IGF-I (1.4 g L^{-1}) even when induction was done at 29 g DCW L^{-1}. Induction at lower cell concentration or mid-exponential phase with glycerol as carbon source was found to be better for IGF-I production. Under this condition, 1.6 g L^{-1} of fusion IGF-I was produced at the end of the culture. The time profiles of cell growth for four different fed-batch cultures are presented in Figure 1. Growth rates were similar for fed-batch cultures using different carbon sources, but higher cell concentrations were obtained using glycerol as carbon source. For induction at a high cell concentration (28 g DCW L^{-1}) using glycerol,

Table 1 Effects of carbon sources and induction time on the production of fusion IGF-I in fed-batch culture of recombinant *E. coli* with two nutrient feeding strategies

Carbon source	Feeding method	Induction cell conc. (g DCW L^{-1})	Maximum cell conc. (g DCW L^{-1})	Fraction of fusion IGF-I (%)	Fusion IGF-I produced (g L^{-1})	Total carbon source consumed (g)
Glucose	Exponential	14.5	70.3	6.1	1	297
		28	58.2	negligible	negligible	218
Glycerol	Exponential	17.7	75.7	8.4	1.6	233
		29.6	80.2	6.4	1.4	257
Glycerol	pH-stat	17.9	68.17	9.9	2.8	241
		32.3	63.23	7.0	2.0	214

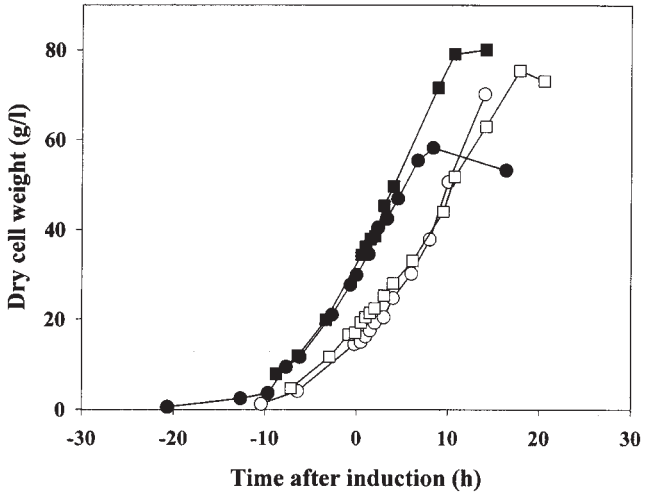

Figure 1 Time profiles of cell growth in fed-batch cultures performed with different induction times using glucose or glycerol as a carbon source. (○) glucose, induced at 14.5 g DCW L^{-1}; (●) glucose, induced at 28 g DCW L^{-1}; (□) glycerol, induced at 17.7 g DCW L^{-1}; (■) glycerol, induced at 29.6 g DCW L^{-1}.

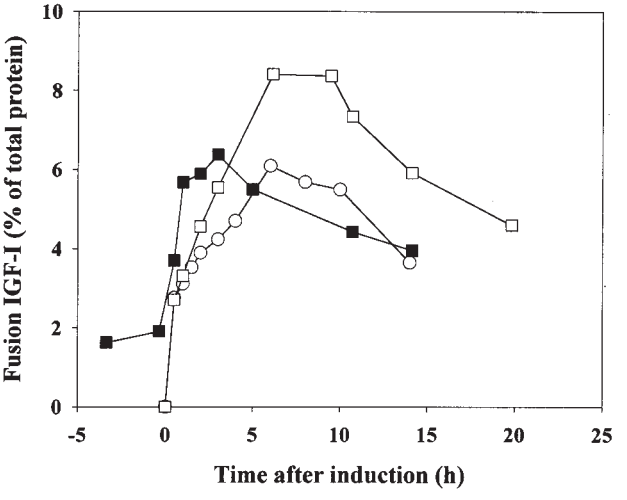

Figure 2 Time profiles of fusion IGF-I production in fed-batch cultures using glycerol or glucose as a carbon source. (○) glucose, induced at 14.5 g DCW L^{-1}; (□) glycerol, induced at 17.7 g DCW L^{-1}; (■) glycerol, induced at 29.6 g DCW L^{-1}.

the cells continued to grow for 8 h after induction. In contrast, cells stopped growing within 8 h after induction when glucose was used as a carbon source, resulting in a significant decrease in final cell concentration. For all fed-batch cultures, acetate concentration in the culture medium was below 1.5 g L^{-1} (data not shown). This concentration of acetate is much lower than the reported growth-inhibitory concentration (>5 g L^{-1}) of acetate [13], implying that acetate formation was not the reason for the termination of cell growth and IGF-I production.

Figure 2 shows the fraction of fusion IGF-I in total protein. When induction was done at lower cell concentrations (14.5 g DCW L^{-1} for fed-batch culture using glucose and 17.7 g DCW L^{-1} for fed-batch culture using glycerol), the rates of fusion IGF-I synthesis were similar during the first hour after induction. The fraction of fusion protein rapidly increased to approximately 3.5% of the total protein in the first hour. After that, the final attainable protein fraction varied depending on the carbon source used. In both cases, the maximum percentage of fusion IGF-I was obtained in about 6 h after induction. Different fusion IGF-I synthesis rates were observed when induction was done at higher cell concentrations. For induction at 29.6 g DCW L^{-1} using glycerol as carbon source, fusion IGF-I was produced even before induction. This suggests that basal induction occurred due to a prolonged growth phase prior to induction. The maximum percentage of fusion IGF-I was obtained after 3 h of induction, which was considerably earlier than the previous case when induction was done at lower cell concentrations. When glucose was used as carbon source, a very low level of fusion IGF-I (less than 0.5% of the total protein) was produced when induction was done at 28 g DCW L^{-1}. In all cases, the percentage of fusion IGF-I decreased after reaching the maximum level even though the cells continued to grow. Therefore, the time point showing the maximum total amount of fusion IGF-I did not correspond to the time point of maximum percentage of fusion IGF-I. The reduction of protein fraction in the cells may be due to reduced protein synthesis activity at high cell concentrations. It may also be caused by plasmid instability or protein degradation. Further study needs to be carried out to elucidate the reasons for the decrease of fusion IGF-I fraction during the late IGF-I production period.

Fusion IGF-I production by the pH-stat fed-batch culture

The second nutrient feeding strategy was the pH-stat with high cell density culture [13]. This strategy is based on the finding that pH increases when the carbon source is depleted. Since glycerol was superior to glucose, the feed-

ing solution containing glycerol was used in this study. A predetermined amount of nutrient feeding solution equivalent to 8 g glycerol was added when the pH became 0.1 unit higher than the set point [6,8].

Again, cells were induced with IPTG at two different cell concentrations. The percentage of fusion IGF-I increased rapidly upon induction (Figure 3 and Table 1). The maximum fusion IGF-I fraction was 99% of the total proteins when cells were induced at 17.9 g DCW L^{-1}. At this point, the fusion IGF-I concentration was as high as 2.8 g L^{-1}. The fusion IGF-I concentration obtained by inducing cells at higher cell concentration was lower than the above, but was higher than that obtained by exponential feeding. Again, the fraction of fusion IGF-I started to decrease after it reached a maximum, which suggested the importance of harvesting cells at the right time.

It is not clear why the pH-stat feeding strategy resulted in higher fusion IGF-I production. We recently demonstrated the importance of post-induction nutrient feeding strategy for the production of mussel glue protein by fed-batch cultures of recombinant *E. coli* [23]. It was concluded that the pH-stat feeding method generally results in good production of recombinant protein compared with exponential or non-optimal linearly increasing feeding.

For the production of fusion IGF-I, this finding seems to be true as well. Further increase of fusion IGF-I production may be possible by optimizing the post-induction nutrient feeding profile (eg optimized linearly increasing feeding profile instead of exponential feeding).

Development of downstream processing

Schemes for purification of IGF-I were first developed on a small scale, and then scaled-up to produce large amounts of IGF-I from cells cultured in the pH-stat fed-batch mode using glycerol as a carbon source. After the fed-batch culture, 1 L of the culture broth was harvested and used to purify IGF-I expressed as inclusion bodies in *E. coli*. Inclusion bodies isolated by centrifugation were washed carefully with 3 M urea to remove contaminant proteins, and then solubilized in 8 M urea. At this stage, the purity of fusion IGF-I was about 72% (Figure 4, lane 2). Since the plasmid used in this study was designed to express the fusion protein with the Asn-Gly linkage between the truncated β-galactosidase gene product and the IGF-I moiety, the fusion protein is easily cleavable by hydroxylamine treatment [4]. About 55% of the fusion protein was cleaved by hydroxylamine treatment, resulting in truncated β-galactosidase and IGF-I with molecular weights of 14 kDa and 7.2 kDa, respectively (Figure 4, lane 3). Truncated β-galactosidase and uncleaved fusion protein were precipitated under acidic conditions, whereas IGF-I was present in a soluble form. Using these properties, IGF-I was easily separated from the fusion partner and uncleaved fusion protein by dialyzing the mixture against 0.5 M acetic acid buffer (pH 2.85) and by collecting the supernatant after centrifugation. After this step, most of the contaminant proteins seemed to be removed, resulting in a single protein band on SDS-PAGE (Figure 4, lane 4). However, the sample volume was increased greatly, and not a few contaminant proteins were still observed on an analytical HPLC chromatogram. For concentration as well as further purification, therefore, the supernatant was subjected to cation exchange chromatography. The major fraction containing IGF-I was pooled, dialyzed against distilled water, concentrated and used for refolding.

It has been shown that IGF-I expressed in *E. coli* and *S. cerevisiae* consists of various misfolded forms with intramolecular disulfide bonds as well as a native form [8,22]. Raschdorf *et al* [15] identified two distinct three-disulfide-bonded forms, designated 'native IGF-I' (disulfides 6–48, 18–61 and 47–52, biologically active) and 'mismatched IGF-I' (disulfides 6–47, 18–61 and 48–52, lacks IGF-I receptor affinity). Refolding of IGF-I by a simple dilution method was successful for obtaining the correctly folded IGF-I [18], but a significant amount of misfolded IGF-I was also observed after refolding. More recently, Yamada *et al* [24] improved refolding efficiency of IGF-I greatly by the addition of water-miscible organic solvents such as ethanol, methanol, dimethylsulfoxide and acetonitrile. In the present

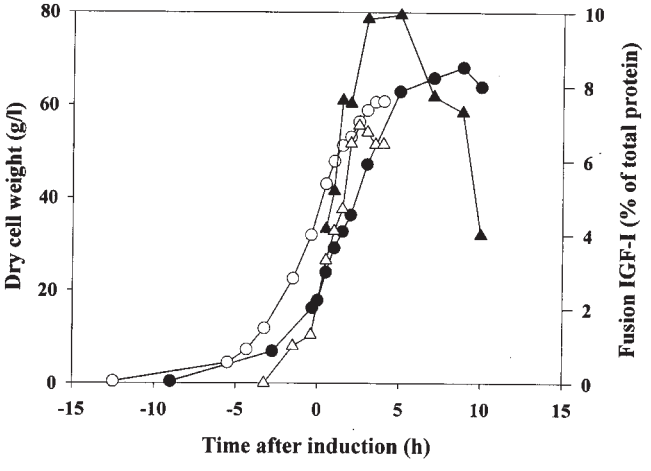

Figure 3 Time profiles of cell growth and fusion IGF-I production in the pH-stat fed-batch cultures using glycerol as a carbon source. (●) DCW, induced at 17.9 g DCW L^{-1}; (○) DCW, induced at 32.3 g DCW L^{-1}; (▲) fusion IGF-I production, induced at 17.9 g DCW L^{-1}; (△) fusion IGF-I production, induced at 32.3 g DCW L^{-1}.

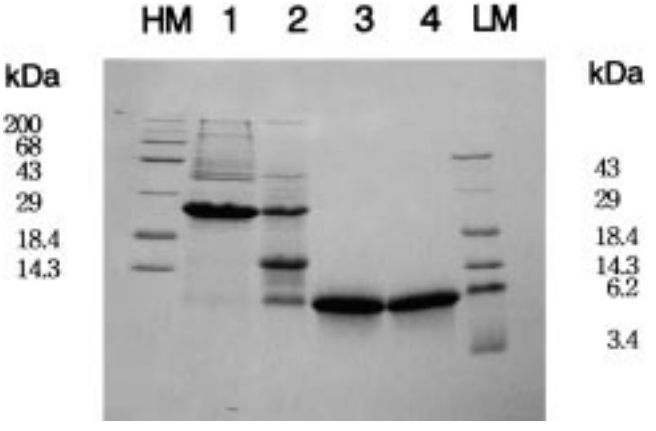

Figure 4 SDS-PAGE analysis of samples of IGF-I from different steps in the purification process. Lane HM: high molecular weight marker proteins. Lane 1: inclusion bodies washed with 3 M urea. Lane 2: after hydroxylamine treatment. Lane 3: after dialysis against 0.5 M acetic acid buffer (pH 2.85). Lane 4: after action exchange chromatography. Lane LM: low molecular weight marker proteins.

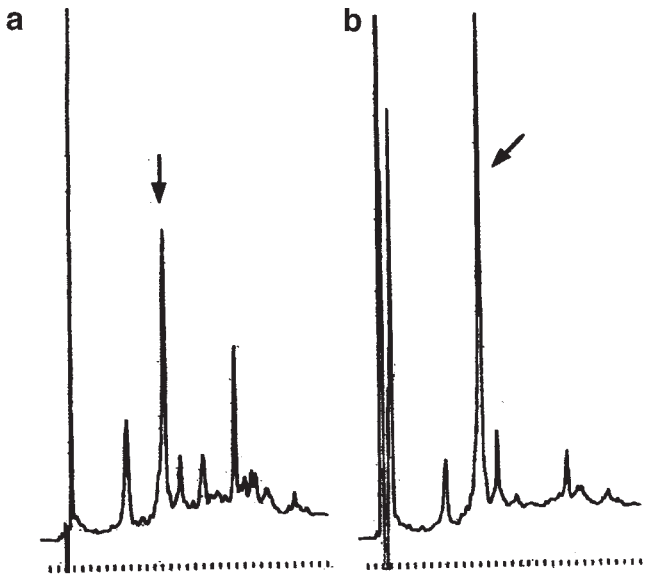

Figure 5 Reverse phase HPLC chromatogram before (a) and after (b) refolding. Arrows indicate the correctly folded IGF-I.

work, therefore, we carried out refolding of IGF-I in 50 mM Tris-HCl buffer (pH 8.0) containing 1 M guanidine-HCl, 0.5 mM β-mercaptoethanol, 1 mM EDTA and 30% (v/v) ethanol. Figure 5 shows the HPLC chromatograms prepared before and after refolding. Some correctly-folded IGF-I was observed before refolding, and its concentration was markedly increased after refolding. After 72 h of refolding, the solution was subjected to a preparative scale rp-HPLC, and the fractions containing only correctly-folded IGF-I were collected and lyophilized.

Purity and biological activities of the purified IGF-I

The purity of purified and lyophilized IGF-I was analyzed by an analytical rp-HPLC (Figure 6). Finally, 120 mg of native IGF-I with purity of >98% was obtained from 2.8 g of the fusion protein containing *ca* 945 mg of IGF-I. The purified protein was subjected to N-terminal amino acid sequencing. The sequence of the first five amino acids was Gly-Pro-Glu-Thr-Leu, which is identical to that of native IGF-I. We have also investigated the biological activities of the purified IGF-I as previously described [10]. Biological activities of the purified IGF-I to stimulate thymidine incorporation, protein biosynthesis and to bind IGF-I recepter were measured and compared to those of commercial IGF-I (Genzyme, Cambridge, USA). As a result, all the biological activities of the purified IGF-I tested were identical to those of commercial IGF-I (data not shown), offering the potential for therapeutic use of IGF-I produced by the process developed in this study.

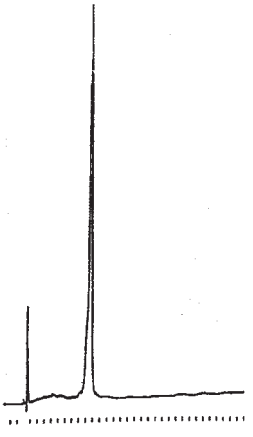

Figure 6 Reverse phase HPLC analysis of purified IGF-I.

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