Further studies on the chemical cleavage of an N-terminal extra methionine from recombinant methionylated proteins [1]

Masato Suenaga,[a] **Hiroaki Ohmae,**[a] **Norio Okutani,**[a] **Tomofumi Kurokawa,**[a] **Tsuneo Asano,**[a] **Takao Yamada,**[a] **Osamu Nishimura** *[b] and **Masahiko Fujino**[c]

[a] *Discovery Research Laboratories IV, Pharmaceutical Discovery Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532-8686, Japan*
[b] *Pharmaceutical Discovery Research Division, Takeda Chemical Industries, Ltd., Wadai-10, Tsukuba, Ibaraki 300-4293, Japan*
[c] *Takeda Chemical Industries, Ltd., Chuo-ku, Osaka 541-0045, Japan*

An additional methionine residue of recombinant human growth hormone (hGH) was converted into an oxoacyl form with glyoxylic acid, copper(II) sulfate and pyridine, and then cleaved from the rest of the protein with 3,4-diaminobenzoic acid in the presence of 1 M AcOH and 2 M HCO$_2$Na. The conditions for N-terminal methionine cleavage worked better than the previous conditions with 1,2-phenylenediamine. The same protocol was also applicable to the methionylated forms of recombinant human betacellulin (BTC), neurotrophin-3 (NT-3) and human interleukin-2 (IL-2). The conversion yield for hGH, BTC, NT-3 and IL-2 increased up to approximately 80, 70, 55 and 50%, respectively. These results indicate that non-methionylated recombinant proteins could be prepared from the methionylated derivatives by chemical methods.

Introduction

Numerous proteins of biological interest which cannot be obtained from natural sources in large quantities have instead been prepared owing to advances in DNA technology. However, there are some limitations to the prokaryotic cell expression system in the preparation of recombinant proteins. The problems are the addition of a methionine residue corresponding to the initiation codon (ATG)[2] at the N-terminus, the formation of inactive inclusion bodies,[3–5] post-translational modification,[6–8] heterogeneity[9,10] caused by endogenous proteases, and the difficulty of preparing small relative molecular mass biologically active peptides.[11–14] We have encountered and given some answers to these problems during the production of various kinds of recombinant proteins in our laboratories, such as NT-3,[5] interferon-*a*,[6] interferon-*γ*,[7,9] IL-2,[15] the basic fibroblast growth factor mutein,[8] prolactin-releasing peptides[14] and hepatitis B virus surface antigen.[10]

Recombinant proteins produced in *Escherichia coli* (*E. coli*) often possess an additional methionine at the N-terminus corresponding to the initiation codon (ATG).[2] The N-terminal methionine is hard to remove by *E. coli* methionine aminopeptidase when the side chain of the adjacent amino acid is bulky or charged.[16] For example, hGH, BTC and NT-3 produced in *E. coli* are fully methionylated because the N-terminal amino acid is phenylalanine,[2] aspartic acid[17] and tyrosine,[5] respectively. The N-terminal methionine is not completely removed even in the recombinant proteins in which the side chain of the neighboring amino acid is small and uncharged. Their incomplete cleavage of the N-terminal methionine could be due to the high-level expression and accumulation in inclusion bodies, presumably making the recombinant proteins inaccessible to *E. coli* methionine aminopeptidase. For example, the N-terminal amino acid of IL-2 is alanine and the recombinant IL-2 is accumulated as inclusion bodies in *E. coli* with partial removal of the N-terminal methionine.[15,18]

It is known that the physiological features such as biological activity, stability *in vivo* and antigenicity of methionylated recombinant proteins could be different from those of non-methionylated proteins.[19] Therefore, it seems to be very important to obtain recombinant proteins substantially free from their methionylated derivatives for clinical applications. In this regard, we have previously reported a procedure for the chemical cleavage of the additional methionine from recombinant methionylated human growth hormone (Met-hGH).[1] The method consists of two reaction steps. The first step transaminated the N-terminal methionine residue in the presence of glyoxylic acid, copper(II) sulfate and pyridine, resulting in an oxoacyl residue. The second step, the scission reaction, utilized 1,2-phenylenediamine in a buffer of 2 M AcOH and 2 M AcONa. The condition led to approximately 70% conversion. We have tested the scission conditions of Met-hGH in an attempt to improve the reaction yield, and found that 3,4-diaminobenzoic acid in 1 M AcOH and 2 M HCO$_2$Na worked much better than did the previously reported conditions.[1] Moreover, we have successfully applied the new conditions for scission to the removal of the N-terminal methionine residue from methionylated human betacellulin (Met-BTC), methionylated human neurotrophin-3 (Met-NT-3) and methionylated human interleukin-2 (Met-IL-2).

Here we describe the improved method for the chemical cleavage of the extra methionine at the N-terminus from the recombinant proteins produced in *E. coli*.

Results and discussion

Conversion of Met-hGH to hGH

hGH produced in *E. coli* has an additional methionine at the N-terminus. It has been reported that Met-hGH may play a major role in antibody formation in patients treated with the hormone.[19] This would indicate that it is important to prepare non-methionylated recombinant protein for therapeutic applications.

To obtain hGH from Met-hGH, first, we optimized the transamination reaction. For the concentration of CuSO$_4$, the maximum yield was achieved at 8 mM. Nickel, cobalt and zinc ions were also tested as the catalyst of transamination, but none

of them accelerated the reaction more than did cupric (Cu^{2+}) ion. For the concentration of glyoxylic acid and pyridine, 0.5 M glyoxylic acid and 10% pyridine gave the most favorable result.

Table 1 The conversion yields of hGH with various scission reagents in 2 M AcOH and 2 M AcONa for 96 h at 30 °C

| Scission reagent (40 mM)	Yield (%)
1,2-Phenylenediamine	67
3,4-Diaminobenzoic acid	69
4-Nitro-1,2-phenylenediamine	6
4-Chloro-1,2-phenylenediamine	47
3,4-Tolylenediamine	43
4,5-Dimethyl-1,2-phenylenediamine	37
2,3-Diaminotoluene	47
4-Methoxy-1,2-phenylenediamine	45
2,3-Diaminophenol	45
Benzene-1,2,4,5-tetramine	44
2-Aminobenzylamine	5
N-Methyl-1,2-phenylenediamine	37
2,3-Diaminopyridine	2

Table 2 The conversion yields of hGH with 3,4-diaminobenzoic acid in various buffer conditions for 96 h at 30 °C

Buffer	pH	Yield (%)
2 M AcOH and 2 M AcONa	4.7	69
2 M phosphate buffer	7.0	3
1 M citrate buffer	4.7	44
1 M AcOH and 2 M HCO$_2$Na	4.5	81
0.5 M HCO$_2$H and 2.5 M HCO$_2$Na	4.7	73

The scission reaction was also optimized. Various scission reagents in the presence of 2 M AcOH and 2 M AcONa were tested, and the results are summarized in Table 1. Among them, 3,4-diaminobenzoic acid was as effective as 1,2-phenylene-diamine for the scission reaction.

Other incubations were set up to select the best buffer conditions for the scission reaction with 3,4-diaminobenzoic acid (Table 2). The buffers with neutral pH such as 2 M phosphate buffer[20] hardly worked for scission of the oxoacyl-hGH, while 0.5 M HCO$_2$H and 2.5 M HCO$_2$Na was superior to 2 M AcOH and 2 M AcONa. 1 M citrate buffer did not give any improvement. The best yield was obtained by incubating the trans-aminated Met-hGH in 1 M AcOH and 2 M HCO$_2$Na with 3,4-diaminobenzoic acid. As for the scission reaction with 1,2-phenylenediamine, the yield with 1 M AcOH and 2 M HCO$_2$Na was similar to that with 2 M AcOH and 2 M AcONa. The results from these experiments indicate that 3,4-diamino-benzoic acid in 1 M AcOH and 2 M HCO$_2$Na is the best choice for the scission reaction. Based on the experimental results obtained here, the transamination (8 mM CuSO$_4$, 0.5 M glyoxy-lic acid, 10% pyridine, 4.8 M urea and 1.34 mg ml^{-1} Met-hGH) and scission reaction (40 mM 3,4-diaminobenzoic acid, 1 M AcOH, 2 M HCO$_2$Na and 4 M urea) were performed on Met-hGH (Scheme 1). Urea was added to the reaction mixture to prevent the aggregation of hGH protein, since the pH-value of the reaction is equal to the isoelectric point of hGH (pI 4.9–5.2).[21] The transamination and scission reaction were traced by ion-exchange chromatography (Fig. 1). The over-all conversion reached approximately 80% and the reaction product was purified by chromatography on Sephadex G-25, followed by DEAE-5PW to yield the purified non-methionylated hGH.
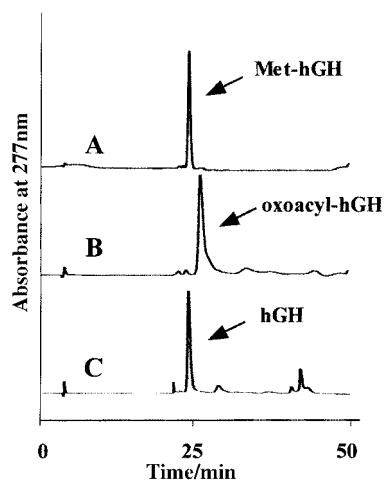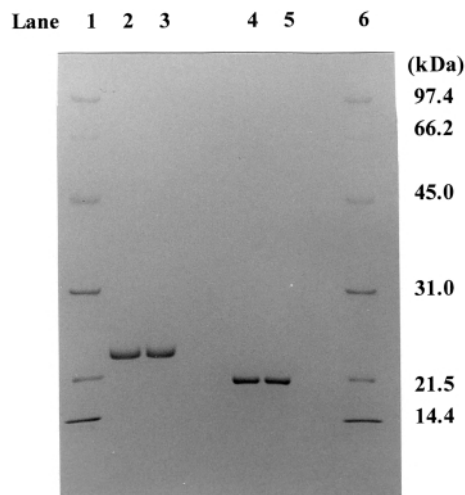
To confirm the structural identity of the purified hGH,
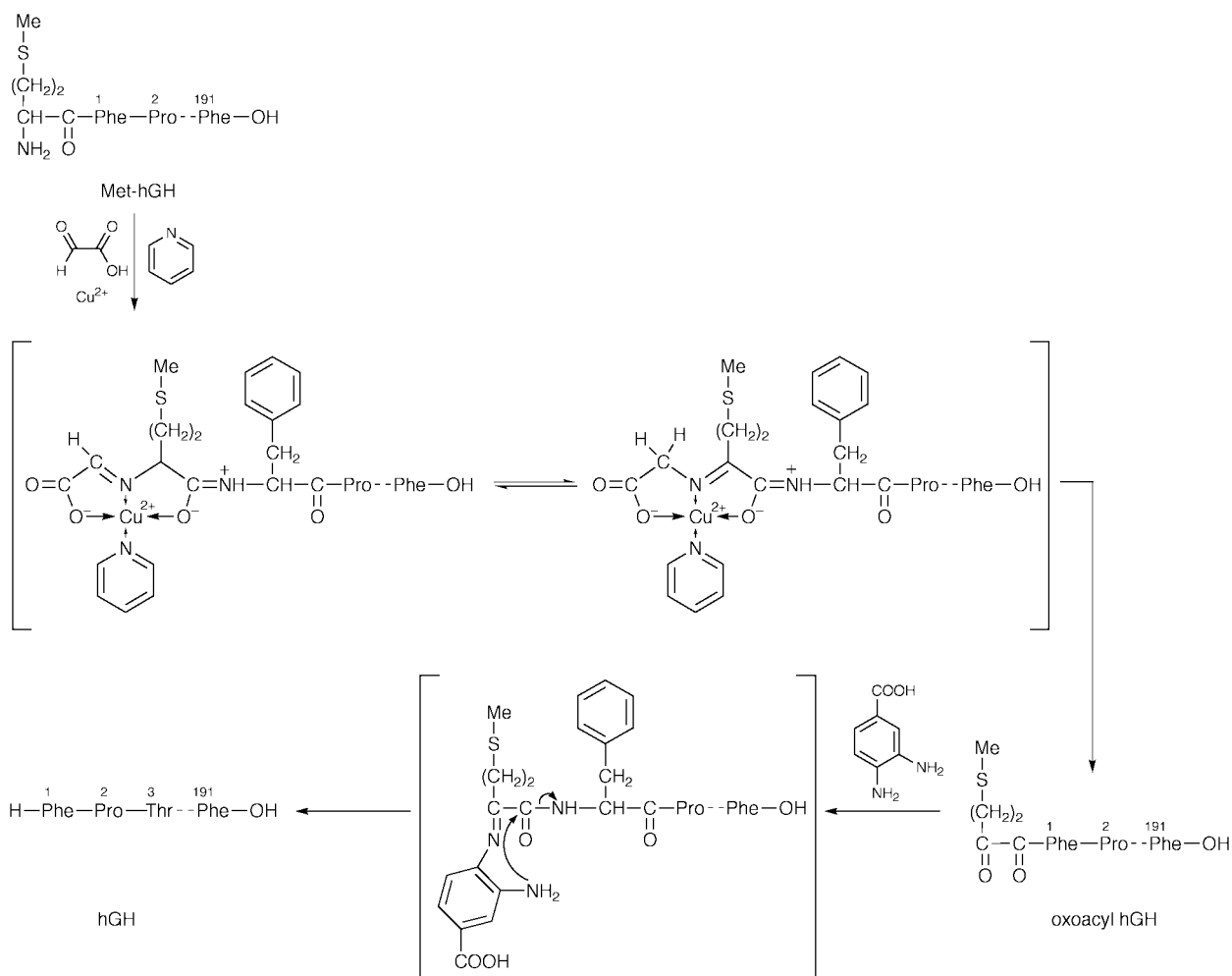
Scheme 1 Schematic representation of the transamination and scission reactions.

Table 3 Biological activity of hGH

Sample	Biological activity (IU mg^{-1})
Authentic hGH	3.00
hGH obtained from Met-hGH	3.29

Table 4 Biological activity of BTC

Sample	BTC (ng ml^{-1})	Cell number[a] ($\times 10^4$)
Control	0	3.6
Authentic BTC	3	7.0
BTC obtained from Met-BTC	3	7.0

[a] Promotion of Balb/c 3T3 cell proliferation was measured and the cell numbers were determined with a Coulter counter.
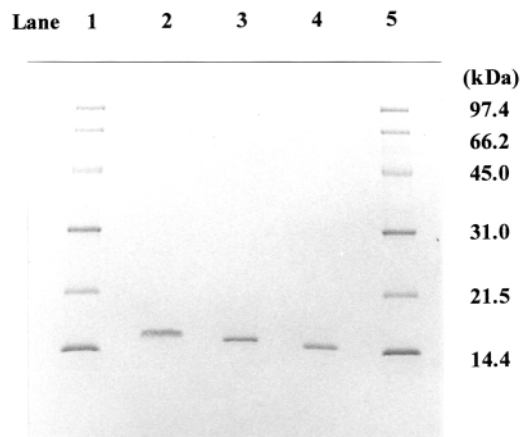

Fig. 1 HPLC analysis of the transamination and scission reactions. (A) Met-hGH, (B) transamination and (C) scission reaction mixtures.

Fig. 3 SDS-PAGE of non-methionylated recombinant proteins obtained from methionylated derivatives after transamination. Lanes 1 and 5, marker proteins. Lane 2, BTC. Lane 3, NT-3. Lane 4, IL-2.

Conversion of Met-BTC to BTC

BTC was initially identified in the condition medium of beta tumor cells established from transgenic mouse[24] as a mitogen for Balb/c 3T3 cells. Recently, Masashima *et al.*[25] reported that BTC and activin A converted amylase-secreting AR42J cells[26] into insulin-secreting cells. BTC synthesized by the *E. coli* system possesses an additional methionine at the N-terminus.[17] Also, the methionylated derivative was accumulated in inactive form in the cells. Met-BTC was renatured using L-arginine[5,27] from inactive protein at high yield and purified.

To obtain BTC from Met-BTC, we optimized the transamination and scission reaction. We found that the best conditions were: 6 mM CuSO$_4$, 0.5 M glyoxylic acid, 10% pyridine and 2.4 M urea for the former, and 40 mM 3,4-diaminobenzoic acid, 1 M AcOH, 2 M HCO$_2$Na and 2.5 M urea for the latter. The conversion reached approximately 70%, while the yield with 1,2-phenylenediamine in 2 M AcOH and 2 M AcONa was 40%. The reaction product was purified by chromatography on Sephadex G-25, followed by CM-5PW to give the purified non-methionylated BTC.

To confirm the structural identity of the purified BTC, protein chemical analysis was performed. The N-terminal amino acid sequence, the C-terminal amino acid and the amino acid analysis were all in good agreement with those predicted from the corresponding cDNA sequence. The purified BTC migrated as a single band on SDS-PAGE and showed the electrophoretic mobility of an approximately 18 kDa species under reducing conditions (Fig. 3), while the predicted molecular size was 9 kDa. Such discrepancies between the electrophoretic mobility and the predicted molecular size might be due to the low mobility in SDS polyacrylamide gel reflected by the primary structure characteristic to BTC.[17] In biological activity, the BTC obtained from Met-BTC was observed to be almost equivalent to an authentic BTC sample,[28] when assayed using Balb/c 3T3 cells (Table 4). These results indicate that biologically active BTC was obtained from Met-BTC by the chemical procedure.

Fig. 2 SDS-PAGE of Met-hGH and hGH obtained from Met-hGH after transamination. Lanes 1 and 6; marker proteins. Lanes 2 and 3; reducing conditions. Lanes 4 and 5; non-reducing conditions. Lanes 2 and 4; hGH. Lanes 3 and 5; Met-hGH.

protein chemical analysis was performed. The N-terminal sequence analysis of the first 20 amino acids of the protein corresponded with the expected sequence of hGH. The C-terminal amino acid and the amino acid analysis also agreed well with those predicted from the cDNA sequence of hGH. Sodium dodecyl sulfate-polyarylamide gel electrophoresis (SDS-PAGE) analysis of the purified hGH resulted in a single band with a mobility corresponding to a molecular mass of about 22 kDa under reducing conditions (Fig. 2). The copper ions were not detected in the purified hGH when checked by atomic absorption spectrometry. To obtain further structural information, the purified hGH was subjected to mapping analysis. The peptide map obtained was identical to that of authentic hGH. The purified hGH had the same order of biological activity as that of authentic hGH, when assayed using Nb$_2$ Node lymphoma cells[22,23] (Table 3). These results indicate that hGH obtained from Met-hGH has essentially the same chemical and biological identity as that of authentic hGH.

Table 5 Biological activity of NT-3

Sample	NT-3 (ng ml^{-1})	Viable cells[a] (%)
Control	0	2.5
Authentic NT-3	300	16.5
NT-3 obtained from Met-NT-3	300	16.6

[a] The biological activity in promoting the survival of neuronal cells was assayed with 8-day-old chick embryonic DRG.

Conversion of Met-NT-3 to NT-3

NT-3 is a family of growth factors that control the development and survival of certain neurons, but little is known about their function and regulation *in vivo*. To clarify their biological roles *in vivo*, it is necessary to prepare large amounts of recombinant proteins. In this regard, we have previously reported a method for preparing biologically active Met-NT-3 using the pro-karyotic cell expression system.[5]

To obtain NT-3 from Met-NT-3, we first optimized the transamination. This was achieved with 8 mM CuSO$_4$, 0.5 M glyoxylic acid, 10% pyridine and 2.4 M urea. After transamination, Met-NT-3 was converted into oxoacyl-NT-3 with high yield. The scission reaction was also optimized. NT-3 is inclined to form a dimeric structure in solution, as described previously.[29] HPLC analysis of the reaction products showed that much of the desired protein existed as heterodimers with by-products.[30] Thus, we estimated the conversion yield from the N-terminal sequence analysis data, and found that the scission reaction was optimized with 40 mM 3,4-diaminobenzoic acid, 1 M AcOH, 2 M HCO$_2$Na and 2.5 M urea. The over-all conversion yield reached approximately 55% while that under the previous conditions[1] was 30%. The reaction product, NT-3 homodimer, was purified by chromatography on Sephadex G-25, followed by CM-5PW with an over-all yield of 10%.

To confirm the structural identity of the purified NT-3, protein chemical analysis was performed. The N-terminal amino acid sequence, the C-terminal amino acid and the amino acid analysis were all in good agreement with those predicted from the corresponding cDNA sequence. The purified NT-3 migrated as a single band on SDS-PAGE and showed the electrophoretic mobility of an approximately 14 kDa species under reducing conditions (Fig. 3). The purified NT-3 had the same order of activity as that of authentic NT-3,[31] when assayed using dorsal root ganglia (DRG) (Table 5). We obtained the purified non-methionylated NT-3 from Met-NT-3 by the chemical procedure. The low yield of the purified NT-3 homodimer remains to be improved.

Conversion of Met-IL-2 to IL-2

IL-2 is a lymphokine produced by activated T-lymphocytes and plays important roles in the proliferation and differentiation of T-lymphocytes, as well as in the regulation of the immune system.[32–34] The N-terminal amino acid of IL-2 is alanine, and recombinant IL-2, composed of a methionylated derivative (Met-IL-2; 60%) and non-methionylated form (IL-2; 40%), is accumulated in *E. coli* cells. IL-2 is separated from Met-IL-2 by utilizing the difference in their isoelectric points[15] and the non-methionylated derivative (IL-2) is used for clinical applications. It is important to obtain IL-2 from Met-IL-2 for therapeutic purposes.

To obtain IL-2 from Met-IL-2, we optimized the transamination and scission reaction conditions. We found that the best conditions were: 4 mM CuSO$_4$, 0.5 M glyoxylic acid, 10% pyridine and 3.2 M urea for the former, and 40 mM 3,4-diaminobenzoic acid, 1 M AcOH, 2 M HCO$_2$Na and 3 M urea for the latter. The over-all conversion reached approximately 50%, while that under the previously reported condition[1] was 30%. The low yield might be due to the low solubility of IL-2

Table 6 Biological activity of IL-2

Sample	Biological activity (U mg^{-1})
Authentic IL-2	25 222
IL-2 obtained from Met-IL-2	25 091

in the reaction mixture and the addition of urea did not have much of an effect on the solubility. The reaction product was purified by chromatography on Sephadex G-25, followed by SP-5PW to give the purified IL-2.

To confirm the structural identity of the purified IL-2, protein chemical analysis was performed. The N-terminal amino acid sequence, the C-terminal amino acid and the amino acid analysis were all in good agreement with the values for IL-2. The purified IL-2 migrated as a single band on SDS-PAGE and showed the electrophoretic mobility of an approximately 14 kDa species under reducing conditions (Fig. 3). The biological activity of the IL-2 obtained from Met-IL-2 was consistent with that determined with authentic IL-2,[15,18] when assayed with NKC3 cells (Table 6). These results indicate that the IL-2 obtained here has the same characteristics as those of authentic IL-2.[15,18]

Conclusions

In the present study we have focused on a chemical method for removing an extra N-terminal methionine from the four recombinant proteins, hGH, BTC, NT-3 and IL-2 produced in *E. coli* cells. Various scission reagents and buffer conditions were tested, and we found that 3,4-diaminobenzoic acid in the presence of 1 M AcOH and 2 M HCO$_2$Na worked better than the conditions reported previously.[1] The conversion for hGH, BTC, NT-3 and IL-2 increased up to approximately 80, 70, 55 and 50%, respectively. This information may be useful for the preparation of non-methionylated recombinant proteins.

Experimental

Glyoxylic acid monohydrate, copper sulfate pentahydrate, nickel sulfate hexahydrate, zinc acetate dihydrate, cobalt sulfate heptahydrate, pyridine, 1,2-phenylenediamine, 3,4-tolylene-diamine, 4-chloro-1,2-phenylenediamine and 3,4-diamino-benzoic acid were all obtained from Wako Pure Chemical Industries, Ltd. (Japan). 2,3-Diaminophenol, 2-aminobenzyl-amine 4-methoxy-1,2-phenylenediamine, 4-nitro-1,2-phenylene-diamine, 2,3-diaminopyridine 4,5-dimethyl-1,2-phenylene-diamine, benzene-1,2,4,5-tetramine and 2,3-diaminotoluene were obtained from Aldrich Chemical Company, Inc. (U.S.). *N*-Methyl-1,2-phenylenediamine was obtained from Maybridge Chemical Co. Ltd. (U.K.).

Preparation of Met-hGH

Met-hGH was obtained as described previously.[35]

Transamination of Met-hGH

To 3.75 g of glyoxylic acid monohydrate were added 1.2 ml of 0.5 M CuSO$_4$ and 7.5 ml of pyridine and the total volume was adjusted to 15 ml with distilled water. Then 60 ml of Met-hGH (protein content 1.67 mg ml^{-1}) aqueous solution containing 6 M urea was added and the mixture was incubated for 1 h at 25 °C. The reaction mixture was applied to a Sephadex G-25 column (4.6 × 60 cm) (Pharmacia Biotech, Sweden) equilibrated with 20 mM Tris·HCl buffer (pH 8.0)–4 M urea at a flow rate of 10 ml min^{-1} and the sample was eluted with the same buffer. The main fraction (200 ml) was pooled.

Conversion of the oxoacyl-hGH to hGH

The protein solution (200 ml) was mixed with 200 ml of 2 M

AcOH, 4 M HCO_2Na, 4 M urea and 2.43 g of 3,4-diamino-benzoic acid, and incubated for 4 days at 30 °C. Then the reaction mixture was applied to a Sephadex G-25 column (11.3 × 80 cm) equilibrated with 20 mM Tris·HCl buffer (pH 8.0)–4 M urea at a flow rate of 30 ml min⁻¹ and the sample was eluted with the same buffer. The pooled fraction (1000 ml) was applied to a DEAE-5PW column (5.5 × 20 cm) (Tosoh Corporation, Japan) equilibrated with 50 mM Tris·HCl buffer (pH 8.0)–2.5 M urea at a flow rate of 15 ml min⁻¹. After adsorption, the protein was eluted with a linear pH gradient between 50 mM Tris·HCl buffer (pH 8.0)–2.5 M urea and 50 mM 2-morpholino-ethanesulfonic acid (MES) buffer (pH 4.0)–2.5 M urea. The main fraction (160 ml) (protein content 0.4 mg ml⁻¹) was pooled.

Analysis of transamination and scission reaction of hGH

The yield of the reaction was analyzed by HPLC using a DEAE-5PW column (7.5 × 75 mm). Elution was performed at a flow rate of 0.8 ml min⁻¹ with a linear pH gradient between 50 mM Tris·HCl buffer (pH 8.0)–2.5 M urea and 50 mM MES buffer (pH 4.0)–2.5 M urea.

Mapping analysis of hGH

hGH was dissolved in 0.2 M Tris·HCl buffer (pH 8.0) at a concentration of 2 mg ml⁻¹. The solution was incubated with TPCK-treated trypsin (Worthington Biochemical Corp., U.S.) at a substrate:enzyme ratio of 25:1 (w/w) at 37 °C for 18 h, followed by a second enzyme addition to give a final concentration of 12.5:1 (w/w). At the end of the digest (6 h), the pH was lowered to 3 with 1 M HCl. The tryptic peptides were analyzed by reversed-phase HPLC (RP-HPLC) using a C8P-50 column (4.6 × 300 mm) (Showa Denko, Japan) and eluted at a flow rate of 0.8 ml min⁻¹ with a linear gradient of 8–56% acetonitrile in the presence of 0.1% TFA.

An authentic sample of hGH was obtained from the National Institute of Health Sciences (Japan).

Biological assay of hGH

Biological assay of hGH was performed using Nb₂ Node lymphoma cells, as described previously.[22,23]

Construction of the expression plasmid for Met-BTC

E. coli strain DH-1 was used for plasmid construction. *E. coli* strain MM294 (DE3),[36] which carries the T7 RNA polymerase gene under the control of the lacUV5 promoter in its chromosome, was used as the host for gene expression. Plasmid pTB1515 contained the BTC gene.[28]

Plasmid pTB1515[28] was digested with *Eco*RI and *Bam*HI, and the DNA fragment (0.6 kbp) was obtained. The fragment was ligated with adaptors which were prepared with synthetic oligonucleotides (TATGGATGGG and AATTCCCATCCA). The resulting fragment was inserted into the *Nde*I–*Bam*HI site of pET-3C to obtain plasmid pTB1505. A 0.25 kbp fragment containing the hBTC gene was obtained by polymerase chain reaction using the plasmid pTB1505 as a template and two oligonucleotides (ATACATATGGATGGGAATTCCA and CCGGATCCTAGTAAAACAAGTCAACTCT) as a primer. The resulting 0.25 kbp fragment was ligated to pET-3c at the *Nde*I and *Bam*HI sites. The expression plasmid pTB1516 was obtained.

Expression of Met-BTC

Plasmid pTB1516 was introduced into *E. coli* MM294 (DE3) with plasmid pLysS and the cells were grown in 1 l of LB medium supplemented with 50 mg l⁻¹ ampicillin and 15 mg l⁻¹ chloramphenicol at 37 °C for 8 h. The culture was inoculated into 20 l of M9 medium supplemented with 1.5% glucose, 1.5%

Casamino acids, 5 mg l⁻¹ thiamine·HCl and 2.5 mg l⁻¹ iron(II) sulfate in a 50-l fermentor. When the Klett-value of the culture reached 500, isopropyl β-thiogalactopyranoside was added to the culture to a final concentration of 100 mg l⁻¹, and fermentation was continued for an additional 7 h. The cells were collected by centrifugation and stored at −80 °C until use (300 g wet weight).

Preparation of Met-BTC

Frozen cells (300 g wet weight) were suspended in 600 ml of 7 M guanidine hydrochloride, 0.1 M Tris·HCl buffer and 1 mM EDTA (pH 8.0). After centrifugation, the supernatant was diluted 30-fold with the refolding buffer, and incubated for 24 h at 4 °C. The final refolding solution consisted of 50 mM Tris·HCl buffer, 0.2 M L-arginine, 1.0 mM reduced glutathione and 0.5 mM oxidized glutathione (pH 8.0).

After renaturation, the refolding solution was applied to a SP-Sepharose column (5 × 50 cm) (Pharmacia Biotech, Sweden) equilibrated with 50 mM phosphate buffer (pH 6.0). After adsorption, the column was washed with the same buffer (pH 6.0) containing 0.2 M NaCl and the proteins were eluted with the same buffer (pH 6.0) containing 0.5 M NaCl. The desired fractions were pooled and applied to a SP-5PW column (5.5 × 30 cm) (Tosoh Corporation, Japan) equilibrated with 50 mM phosphate buffer (pH 6.0)–0.2 M NaCl. After adsorption, the protein was eluted at a flow rate of 35 ml min⁻¹ with a linear pH gradient between 50 mM phosphate buffer (pH 5.0)–0.2 M NaCl and 50 mM phosphate buffer (pH 8.0)–0.2 M NaCl. The main fraction was pooled. The pooled fraction was subjected to RP-HPLC on an ODS-120T column (5.5 × 60 cm) (Tosoh Corporation, Japan) and eluted at a flow rate of 50 ml min⁻¹ with a linear gradient of 16–40% acetonitrile in the presence of 0.1% TFA. The eluates were collected and lyophilized (Met-BTC 500 mg).

Transamination of Met-BTC

To 0.5 g of glyoxylic acid monohydrate were added 0.3 ml of 0.2 M CuSO₄ and 1 ml of pyridine and the total volume was adjusted to 2 ml with distilled water. Then 8 ml of Met-BTC (protein content 6.25 mg ml⁻¹) aqueous solution containing 3 M urea was added and the mixture was incubated for 1 h at 25 °C. The reaction mixture was applied to a Sephadex G-25 column (2.5 × 60 cm) equilibrated with 10 mM phosphate buffer (pH 6.0)–2.5 M urea at a flow rate of 4 ml min⁻¹, and the sample was eluted with the same buffer. The main fraction (50 ml) was pooled.

Conversion of the oxoacyl-BTC to BTC

The protein solution (50 ml) was mixed with 50 ml of 2 M AcOH, 4 M HCO₂Na, 2.5 M urea and 0.61 g of 3,4-diamino-benzoic acid, and incubated for 6 days at 25 °C. After the incubation, the reaction mixture was applied to a Sephadex G-25 column (4.6 × 60 cm) equilibrated with 10 mM phosphate buffer (pH 5.0)–2.5 M urea at a flow rate of 10 ml min⁻¹ and the sample was eluted with the same buffer. The pooled fraction (200 ml) was applied to a CM-5PW column (21.5 × 150 mm) (Tosoh Corporation, Japan) equilibrated with 50 mM phosphate buffer (pH 5.0)–0.1 M NaCl–2.5 M urea at a flow rate of 5 ml min⁻¹. After adsorption, the protein was eluted with a linear pH gradient between 50 mM phosphate buffer (pH 5.0)–0.1 M NaCl–2.5 M urea and 50 mM phosphate buffer (pH 8.0)–0.1 M NaCl–2.5 M urea. The main fraction (25 ml) (protein content 1.0 mg ml⁻¹) was pooled.

Analysis of transamination and scission reaction of BTC

The yield of the reaction was analyzed by HPLC using a CM-5PW column (7.5 × 75 mm). Elution was performed at a flow rate of 0.8 ml min⁻¹ with a linear pH gradient between 50

mM phosphate buffer (pH 5.0)–0.1 M NaCl–2.5 M urea and 50 mM phosphate buffer (pH 8.0)–0.1 M NaCl–2.5 M urea.

Biological assay of BTC

The Balb/c 3T3 cell mitogenic assay of BTC was performed as described previously.[37]

Preparation of Met-NT-3

Met-NT-3 was obtained as described previously.[5]

Transamination of Met-NT-3

To 0.5 g of glyoxylic acid monohydrate were added 0.4 ml of 0.2 M $CuSO_4$ and 1 ml of pyridine and the total volume was adjusted to 2 ml with distilled water. Then 8 ml of Met-NT-3 (protein content 6.25 mg ml^{-1}) aqueous solution containing 3 M urea was added and the mixture was incubated for 1 h at 25 °C. The reaction mixture was applied to a Sephadex G-25 column (2.5 × 60 cm) equilibrated with 10 mM phosphate buffer (pH 6.0)–2.5 M urea at a flow rate of 4 ml min^{-1}, and the sample was eluted with the same buffer. The main fraction (50 ml) was pooled.

Conversion of the oxoacyl-NT-3 to NT-3

The protein solution (50 ml) was mixed with 50 ml of 2 M AcOH, 4 M HCO_2Na, 2.5 M urea and 0.61 g of 3,4-diamino-benzoic acid, and incubated for 6 days at 25 °C. After the incubation, the reaction mixture was applied to a Sephadex G-25 column (4.6 × 60 cm) equilibrated with 10 mM phosphate buffer (pH 5.0)–2.5 M urea at a flow rate of 10 ml min^{-1} and the sample was eluted with the same buffer. The pooled fraction (200 ml) was applied to a CM-5PW column (21.5 × 150 mm) equilibrated with 50 mM phosphate buffer (pH 5.0)–0.2 M NaCl–2.5 M urea at a flow rate of 5 ml min^{-1}. After adsorption, the protein was eluted with a linear pH gradient between 50 mM phosphate buffer (pH 5.0)–0.2 M NaCl–2.5 M urea and 50 mM phosphate buffer (pH 8.0)–0.2 M NaCl–2.5 M urea. The main fraction (10 ml) (protein content 0.5 mg ml^{-1}) was pooled.

Analysis of transamination and scission reaction of NT-3

The reaction was analyzed by HPLC. After the reaction the eluate from Sephadex G-25 was loaded on a CM-5PW column (7.5 × 75 mm) equilibrated with 50 mM phosphate buffer (pH 5.0) containing 0.2 M NaCl–2.5 M urea and eluted at a flow rate of 0.8 ml min^{-1} with a linear pH gradient between 50 mM phosphate buffer (pH 5.0)–0.2 M NaCl–2.5 M urea and 50 mM phosphate buffer (pH 8.0)–0.2 M NaCl–2.5 M urea.

The conversion was calculated from the result of the amino terminal sequence analysis. After the scission reaction, the sequence analysis was performed with a gas-phase protein sequencer (Model 477A, Applied Biosystems, U.S.).

Biological assay of NT-3

The biological activity of NT-3 in promoting the survival of neuronal cells was assayed with 8-day-old chick embryonic dorsal root ganglia (DRG), as described previously.[5]

Preparation of Met-IL-2

Met-IL-2 was obtained as described previously.[15,18]

Transamination of Met-IL-2

To 2.5 g of glyoxylic acid monohydrate were added 2.5 ml of 0.1 M $CuSO_4$ and 5.0 ml of pyridine and the total volume was adjusted to 10 ml with distilled water. Then 40 ml of Met-IL-2 (protein content 1.25 mg ml^{-1}) aqueous solution containing 4 M urea was added and the mixture was incubated for 1 h at 25 °C. The reaction mixture was applied to a Sephadex G-25 column (4.6 × 60 cm) equilibrated with 10 mM phosphate buffer (pH 6.0)–2.5 M urea at a flow rate of 10 ml min^{-1}, and the sample was eluted with the same buffer. The main fraction (100 ml) was pooled.

Conversion of the oxoacyl-IL-2 to IL-2

The protein solution (100 ml) was mixed with 100 ml of 2 M AcOH, 4 M HCO_2Na, 2.5 M urea and 1.22 g of 3,4-diamino-benzoic acid, and incubated for 6 days at 25 °C. The reaction mixture was applied to a Sephadex G-25 column (4.6 × 60 cm) equilibrated with 10 mM phosphate buffer (pH 6.0)–2.5 M urea at a flow rate of 10 ml min^{-1} and the sample was eluted with the same buffer. The pooled fraction (300 ml) was applied to a SP-5PW column (21.5 × 150 mm) equilibrated with 25 mM phosphate buffer (pH 6.0) at a flow rate of 5 ml min^{-1}. After adsorption, the protein was eluted with a linear pH gradient between 25 mM phosphate buffer (pH 7.0) and 25 mM phosphate buffer (pH 8.0). The main fraction (40 ml) (protein content 0.5 mg ml^{-1}) was pooled.

Analysis of transamination and scission reaction of IL-2

The yield of the reaction was analyzed by HPLC using a SP-5PW column (7.5 × 75 mm). Elution was performed at a flow rate of 1.0 ml min^{-1} with a linear pH gradient between 25 mM phosphate buffer (pH 7.0) and 25 mM phosphate buffer (pH 8.0).

Biological assay of IL-2

IL-2 activity was determined by the ability to maintain an IL-2-dependent murine cell line, NKC3, as described previously.[38,39]

Amino acid analysis

The amino acid compositions of the proteins were determined following hydrolysis with 6 M HCl and 4% thioglycolic acid at 110 °C for 24 and 48 h with a Beckman model 6300E amino acid analyzer.

Amino-terminal sequence analysis

The amino-terminal sequence was determined with a gas-phase protein sequencer (Model 477A, Applied Biosystems, U.S.).

Carboxy-terminal amino acid analysis

The carboxy-terminal amino acid of the proteins was cleaved by hydrazinolysis,[40] and was analyzed with a Beckman model 6300E amino acid analyzer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli[41] using MULTI GEL 10/20 and 15/25 (Daiichi Pure Chemicals Co., Ltd., Japan) under reducing conditions. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250.

Acknowledgements

References

1 A part of this work has been published as a preliminary communication: O. Nishimura, M. Suenaga, H. Ohmae, S. Tusji and M. Fujino, *Chem. Commun.*, 1998, 1135.
2 K. C. Olson, J. Fenno, N. Lin, R. N. Harkins, C. Snider, W. H. Kohr, M. J. Ross, D. Fodge, G. Prender and N. Stebbing, *Nature*, 1981, **293**, 408.

3 S. Honda, H. Sugino, K. Nishi, K. Nara and A. Kakinuma, *J. Biotechnol.*, 1987, **5**, 39.
4 T. Yamada, A. Fujishima, K. Kawahara, K. Kato and O. Nishimura, *Arch. Biochem. Biophys.*, 1987, **257**, 194.
5 M. Suenaga, H. Ohmae, S. Tsuji, T. Itoh and O. Nishimura, *Biotechnol. Appl. Biochem.*, 1998, **28**, 119.
6 T. Takao, M. Kobayashi, O. Nishimura and Y. Shimonishi, *J. Biol. Chem.*, 1987, **262**, 3541.
7 S. Honda, T. Asano, T. Kajio and O. Nishimura, *Arch. Biochem. Biophys.*, 1989, **269**, 612.
8 M. Suenaga, H. Ohmae, S. Tsuji, Y. Tanaka, N. Koyama and O. Nishimura, *Prep. Biochem. Biotechnol.*, 1996, **26**, 259.
9 S. Honda, T. Asano, T. Kajio, S. Nakagawa, S. Ikeyama, Y. Ichimori, H. Sugino, K. Nara, A. Kakinuma and H. Kung, *J. Interferon Res.*, 1987, **7**, 145.
10 M. Kobayashi, T. Asano, M. Utsunomiya, Y. Itoh, Y. Fujisawa, O. Nishimura, K. Kato and A. Kakinuma, *J. Biotechnol.*, 1988, **8**, 1.
11 N. Koyama, M. Kuriyama, N. Amano and O. Nishimura, *J. Biotechnol.*, 1994, **32**, 273.
12 S. Nakagawa, Y. Tamakashi, T. Hamana, M. Kawase, S. Taketomi, Y. Ishibashi, O. Nishimura and T. Fukuda, *J. Am. Chem. Soc.*, 1994, **116**, 5513.
13 S. Nakagawa, Y. Tamakashi, Y. Ishibashi,, M. Kawase, S. Taketomi, O. Nishimura and T. Fukuda, *Biochem. Biophys. Res. Commun.*, 1994, **200**, 1735.
14 O. Nishimura, T. Moriya, M. Suenaga, Y. Tanaka, T. Itoh, N. Koyama, R. Fujii, S. Hinuma, C. Kitada and M. Fujino, *Chem. Pharm. Bull.*, 1998, **46**, 1490.
15 T. Yamada, K. Kato, K. Kawahara and O. Nishimura, *Biochem. Biophys. Res. Commun.*, 1986, **135**, 837.
16 A. Ben-Bassat, K. Bauer, S. Y. Chang, K. Myambo, A. Boosman and S. Chang, *J. Bacteriol.*, 1987, **169**, 751.
17 M. Seno, H. Tada, M. Kosaka, R. Sasada, K. Igarashi, Y. Shing, J. Folkman, M. Ueda and H. Yamada, *Growth Factors*, 1996, **13**, 181.
18 K. Kato, T. Yamada, K. Kawahara, H. Onda, T. Asano, H. Sugino and A. Kakinuma, *Biochem. Biophys. Res. Commun.*, 1985, **130**, 692.
19 K. Glasbrenner, *J. Am. Med. Assoc.*, 1986, **255**, 581.
20 J. Stevens and H. B. F. Dixon, *Biochim. Biophys. Acta*, 1995, 1252, 195.
21 C. H. Li, *Fed. Proc.*, 1957, **16**, 775.
22 R. L. Noble, B. C. Hochachka and D. King, *Cancer Res.*, 1975, **35**, 766.
23 T. Tanaka, R. P. C. Shiu, P. W. Gout, C. T. Beer, R. L. Noble and H. G. Friesen, *J. Clin. Endocrinol. Metab.*, 1980, **51**, 1058.
24 Y. Shing, G. Christofori, D. Hanahan, Y. Ono, R. Sasada, K. Igarashi and J. Folkman, *Science*, 1993, **259**, 1604.
25 H. Masashima, H. Ohnishi, K. Wakabayashi, T. Mine, J. Miyagawa, T. Hanafusa, M. Seno, H. Ymada and I. Kojima, *J. Clin. Invest.*, 1996, **97**, 1647.
26 J. Christophe, *Am. J. Physiol.*, 1994, **266**, G963.
27 R. Rudolph, in *Modern Methods in Protein and Nucleic Acid Analysis*, ed. H. Tchesche, Walter de Gruyter, Berlin and New York, 1990, pp. 149–171.
28 R. Sasada, Y. Ono, Y. Taniyama, Y. Shing, J. Folkman and K. Igarashi, *Biochem. Biophys. Res. Commun.*, 1993, **190**, 1173.
29 C. Radziejewski, R. C. Robinson, P. S. DiStefano and J. W. Taylor, *Biochemistry*, 1992, **31**, 4431.
30 H. B. F. Dixon and R. Fields, *Methods Enzymol.*, 1972, **25**, 409.
31 M. Iwane, T. Watanabe, A. Shintani, Y. Kaisho, S. Matsumoto, R. Sasada and K. Igarashi, *Appl. Microbiol. Biotechnol.*, 1994, **41**, 225.
32 D. A. Morgan, F. W. Ruscetti and R. Gallo, *Science*, 1976, **193**, 1007.
33 S. Gillis, M. M. Ferm, W. Ou and K. A. Smith, *J. Immunol.*, 1978, **120**, 2027.
34 K. A. Smith, *Annu. Rev. Immunol.*, 1984, **2**, 319.
35 S. Nakagawa, T. Yamada, K. Kato and O. Nishimura, *Biotechnology*, 1987, **5**, 824.
36 T. Watanabe, M. Seno, R. Sasada and K. Igarashi, *Mol. Endocrinol.*, 1990, **4**, 869.
37 R. Sasada, T. Kurokawa, M. Iwane and K. Igarashi, *Mol. Cell. Biol.*, 1998, **8**, 588.
38 K. Kato, K. Naruo, M. Koyama, K. Kawahara, S. Hinuma, H. Tada, H. Sugino and K. Tsukamoto, *Biochem. Biophys. Res. Commun.*, 1985, **127**, 182.
39 H. Tada, O. Shiho, K. Kuroshima, M. Koyama and K. Tsukamoto, *J. Immunol. Methods*, 1986, **93**, 157.
40 K. Narita, M. Murakami and T. Ikenaka, *J. Biochem.* (*Tokyo*), 1966, **59**, 170.
41 U. K. Laemmli, *Nature*, 1970, **227**, 680.