An efficient chemical method for removing N-terminal extra methionine from recombinant methionylated human growth hormone

Osamu Nishimura,*[a]† Masato Suenaga,[a] Hiroaki Ohmae,[a] Shinji Tsuji[a] and Masahiko Fujino[b]

[a] *Biotechnology Laboratories, Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532, Japan*

[b] *Takeda Chemical Industries, Ltd., Chuou-ku, Osaka 541, Japan*

Conversion of recombinant methionylated human growth hormone (Met-hGH) into its non-methionylated form (hGH) has been successfully achieved by a chemical process, which consists of a transamination reaction and phenylene-1,2-diamine treatment.

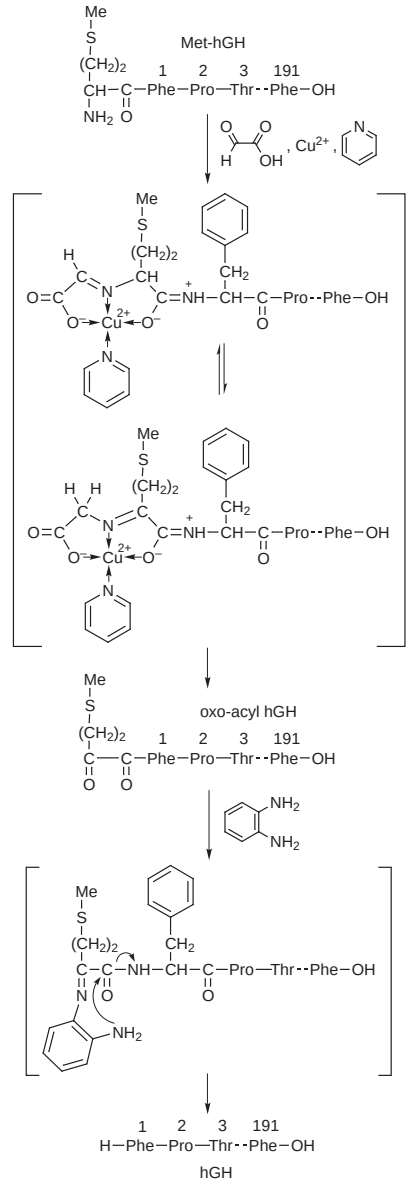
Production of recombinant proteins for clinical use has been increasing with the advent of genetic engineering technology. However, there are still numerous problems in the production of recombinant proteins. Typically, difficulties remain in the addition of methionine at the N-terminus corresponding to the initiation cordon (ATG),[1] refolding from inactive inclusion bodies,[2–4] post-translational modification[5–7] and heterogeneity[8,9] caused by endogenous proteases. We have encountered and given some answers to these problems during the production of various kinds of recombinant proteins in our laboratories, such as interferon-α,[5] interferon-γ,[6,8] interleukin-2[10] and the basic fibroblast growth factor mutein.[7]

Recombinant proteins are often produced with an additional methionine at the N-terminus because methionine aminopeptidase is strongly influenced by the kind of amino acid residue.[11] Thus, the N-terminal methionine is hard to cleave when the side chain of the adjacent amino acid is bulky or charged. Recombinant human growth hormone(hGH) produced in *Escherichia coli* is fully methionylated[1] because the N-terminal amino acid is phenylalanine. The removal of the additional N-terminal methionine is a great concern in the production of recombinant hGH for therapeutic applications since physiological features, such as biological activity, stability *in vivo* and antigenicity of methionylated derivatives, may be different from those of the natural species. In this regard, we have previously reported a procedure for cleavage by aminopeptidase M.[12]

Dixon and co-workers removed the N-terminal residue of *Pseudomonas* cytochrome c-551 after transamination[13,14] in order to study whether the N-terminal residue is essential for the function of the protein or not. However, this method has never been used to remove an additional methionine at the N-terminus of recombinant proteins. We took advantage of this method for the production of hGH from Met-hGH[12] produced in *E. coli*, and found it to be of great value as a versatile procedure for the preparation of non-methionylated hGH, as well as other non-methionylated recombinant proteins.

Here we describe our chemical procedure for removing extra-methionine at the N-terminus and show the first example of removing an additional methionine at the N-terminus of a recombinant protein using a chemical method.

To obtain hGH, we optimized both the transamination and the scission reaction and found that the best conditions were: 8 mM $CuSO_4$, 0.5 M glyoxylic acid and 10% pyridine for the former and 40 mM phenylene-1,2-diamine, 2 M NaOAc and 2 M AcOH for the latter. As shown in Scheme 1, Met-hGH[12] was converted into the oxo-acyl form‡ and then passed through a column of Sephadex G-25.§ The resulting product was cleaved with phenylene-1,2-diamine¶ to give crude hGH, in *ca.* 70% conversion yield, which was then purified by chromatography on Sephadex G-25, followed by DEAE-5PW.‖ Thus, about 60 mg of the purified hGH was obtained from 100 mg of Met-hGH. To confirm the structural identity of the purified hGH, 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 hGH was migrated as a single band on SDS-PAGE and showed the electrophoretic mobility of a *ca.* 22 kDa species under reducing conditions (Fig. 1). Copper ions were not detected in the



Scheme 1

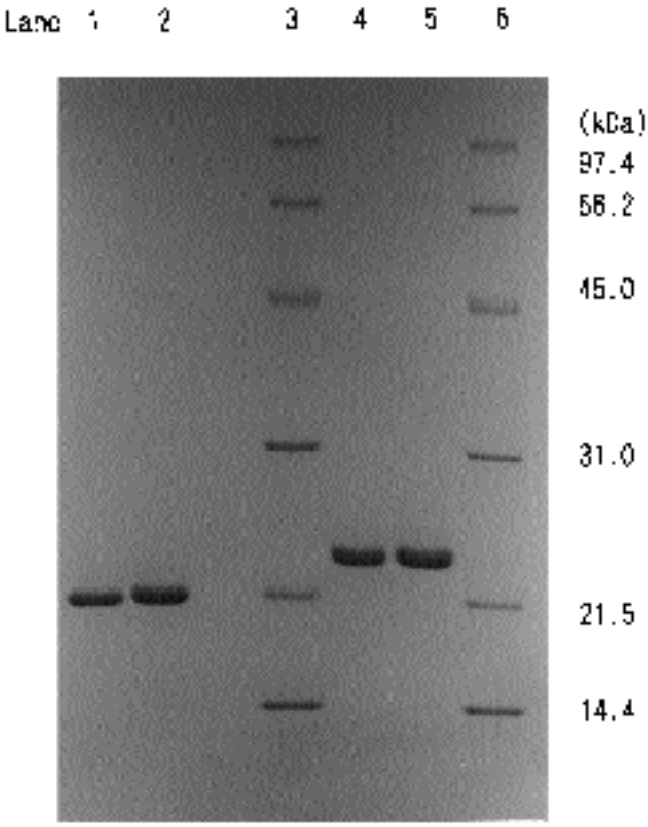


Fig. 1 SDS-PAGE of Met-hGH and hGH obtained from Met-hGH after transamination. Lanes 1 and 2: nonreducing conditions. Lanes 4 and 5: reducing conditions. Lanes 1 and 4: hGH. Lanes 2 and 5: Met-hGH. Lanes 3 and 6, marker proteins.

purified hGH when checked by AAS. To obtain further structural information, the purified hGH was subjected to mapping analysis.** As shown in Fig. 2, the peptide map obtained was identical to that of authentic hGH.†† The purified hGH also had the same order of activity as that of authentic hGH,†† when assayed using Nb2 Node lymphoma cells,[15] indicating that hGH obtained from Met-hGH has essentially the same chemical and biological identity as that of authentic hGH.††

In conclusion, we could obtain hGH from Met-hGH by a chemical method, and we could also apply the same procedure to other methionylated recombinant proteins including neurotrophin-3,[4] interleukin-2[10] and betacellulin,[16] to give the corresponding desired proteins having no methionine at the N-terminus. These results suggest that the present method might be widely applicable to the preparation of non-methionylated recombinant proteins for clinical use and show a new way for the production of recombinant proteins.

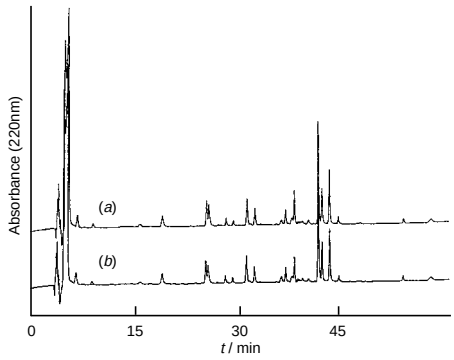


Fig. 2 Peptide maps of (*a*) authentic hGH and (*b*) hGH obtained from Met-hGH after transamination

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Notes and References

† E-mail: nishimura_osamu@takeda.co.jp

‡ 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. Then 60 ml of Met-hGH (protein content 1.67 mg ml^{-1}) solution containing 3 M urea was added and incubated for 1 h at 25 °C.

§ The reaction mixture was applied at a flow rate of 0.5 l h^{-1} to a Sephadex G-25 (Pharmacia Biotech, Sweden) column (4.6 × 60 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 2.5 M urea, and the sample was eluted with the same buffer. The main fraction (200 ml) was pooled.

¶ 200 ml of the protein solution, 200 ml of 4 M NaOAc, 4 M AcOH, 3 M urea and 1.73 g of phenylene-1,2-diamine were mixed and incubated for 3 days at 4 °C. In order to minimize oxidation, nitrogen was bubbled through the solutions to remove as much oxygen as possible and the incubations were carried out under nitrogen.

‖ The reaction mixture was applied at a flow rate of 2 l h^{-1} to a Sephadex G-25 (Pharmacia Biotech, Sweden) column (11.3 × 80 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 2.5 M urea and the sample was eluted with the same buffer. The pooled fraction (1000 ml) was applied at a flow rate of 1 l h^{-1} to a DEAE-5PW (Tosoh Corporation, Japan) column (5.53 × 20 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 2.5 M urea. After adsorption, the protein was eluted with a linear gradient of pH (8–4) between 50 mM Tris-HCl buffer (pH 8.0) containing 2.5 M urea and 50 mM MES buffer (pH 4.0) containing 2.5 M urea. The main fraction (150 ml) (protein content 0.4 mg ml^{-1}) was pooled.

** hGH was dissolved in 0.2 M Tris-HCl buffer (pH 8.0). The solution was incubated with TPCK-treated-trypsin (Worthington Biochemical Corp.) 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 (Showa Denko, Japan) column (4.6 × 300 mm) and eluted at a flow rate of 0.8 ml min^{-1} with a linear gradient of 8–56% MeCN in the presence of 0.1% TFA.

†† The hGH standard was obtained from the National Institute of Health Sciences (Japan).

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