Chemical Cleavage of Recombinant Fusion Proteins To Yield Peptide Amides

Shizue Nakagawa,† Yuri Tamakashi,† Takumi Hamana,† Masahiro Kawase,† Shigehisa Taketomi,† Yoshihiro Ishibashi,‡ Osamu Nishimura,§ and Tsunehiko Fukuda*,†

Pharmaceutical Research Laboratories II and Production Research Laboratories Takeda Chemical Industries, Ltd. Yodogawa-ku, Osaka 532, Japan Discovery Research Division Takeda Chemical Industries, Ltd. Wadai-10, Tsukuba-shi, Ibaraki 300-42, Japan

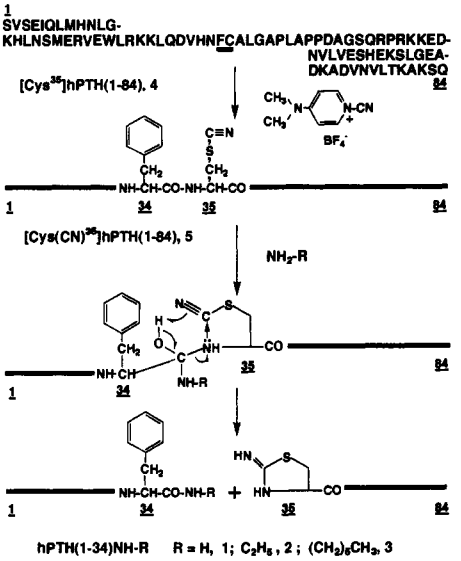
Received February 23, 1994

Recombinant technology has provided an alternative to chemical synthesis for the production of peptides.[1] Although there are many peptide hormones with a C-terminal α-amide structure, which is usually essential for biological activity, peptidyl amides cannot be produced by the usual expression system because prokaryotes do not have the C-terminal amidating enzyme, peptidylglycine-amidating monooxygenase.[2] The present communication describes a versatile method to yield peptidyl amides from recombinant products.

Several groups[3] have applied the amidating enzyme to the recombinant synthesis of α-amidated peptides, but the procedure requires the preparation of precursor peptides (glycine-extended objective peptide) and the enzyme is not readily available. Moreover, amidation of unprotected peptides by transacylation with amino acylamides or protected amine derivatives using proteases has been also tried.[4] However, it has not yet been applied to recombinant peptides.

Recombinant peptides are usually expressed as part of fusion proteins which protect them from endogenous enzymatic degradation.[5] Various enzymatic or chemical methods for site-specific cleavage of peptide bonds can be suitably employed to isolate the substrate peptides for amidation from the fusion proteins.[5] However, these methods are not always applicable because there are various limitations arising from the sequence of the desired peptide. Moreover, amidation by either amidating enzymes or enzymatic transacylation may be restricted by the amino acid sequence of the C-terminal portion of the objective hormone and the specificity of the enzyme.[4d,6]

Scheme 1[a]



[a] The amino acid sequence is written in single letter codes; those underlined are F, Phe; C, Cys.

It has been known that proteins are cleaved site-specifically at the amino–peptide bond of S-cyanylated cysteine residues under mild alkaline conditions, giving a peptide with a carboxylic acid at the C-terminal and a peptide with a (2-iminothiazolin-4-yl)-carbonyl residue at the N-terminal.[7]

We anticipated that amines, better nucleophiles than hydroxyl anion, would facilitate the cleavage giving α-amidated peptides. In fact, after extensive examination, we optimized the reaction conditions: the cleavage–amidation reaction was accomplished by treatment with 3 M ammonia at 0 °C for 15 min. The short exposure to the moderate concentration of amines, not prolonged exposure to diluted amines, prevented alkali-induced damage of the products. Another feature of the present method is the ability to generate alkylamide derivatives[8] which cannot be synthesized using the α-amidating enzyme. We applied this procedure to the synthesis of amide derivatives of human parathyroid hormone active fragment: hPTH(1–34)NH_2 (**1**),[9] hPTH(1–34)NHC_2H_5 (**2**), and hPTH(1–34)$NH(CH_2)_5CH_3$ (**3**) (Scheme 1).

Human parathyroid hormone, hPTH(1–84), is a peptide hormone consisting of 84 amino acid residues which regulates calcium and phosphate ion homeostasis. The N-terminal 34 amino acid fragment, hPTH(1–34), is biologically active[10] and thought to have promise in the treatment of bone diseases including osteoporosis.[11] A derivative with a C-terminal amide, **1**, has been

* Author to whom correspondence should be addressed.
† Pharmaceutical Research Laboratories II.
‡ Discovery Research Division.
§ Production Research Laboratories.

(1) Itakura, K.; Hirose, T.; Crea, R.; Riggs, A. R.; Heyneker, H. L.; Bolivar, F.; Boyer, H. W. *Science* **1977**, *198*, 1056–1063.
(2) Bradbury, A. F.; Finnie, M. D. A.; Smyth, D. G. *Nature* **1982**, *298*, 686–688.
(3) (a) Gilligan, J. P.; Lovato, S. J.; Jones, B. N.; Koehn, J. A.; LeSueur, L. F.; Sturmer, A. M.; Bertelsen, A. H.; Warren, T. G.; Birnbaum, R. S.; Roos, B. A. In *Advances in Gene Technology: Molecular Biology of the Endocrine System*; Puett, D., et al., Eds.; ICSU Press: Cambridge, U.K., 1986; pp 38–39. (b) Engels, J. W.; Glauder, J.; Mullner, H.; Tripier, D.; Uhllmann, E.; Wetekam, W. *Protein Eng.* **1987**, *1*, 195–199. (c) Jones, B. N. In *Fundamentals of Protein Biotechnology*; Stein, S., Ed.; Marcel Dekker, Inc.: New York, 1990; pp 171–179. (d) Tajima, M.; Iida, I.; Kaminuma, T.; Yanagi, M.; Fukushima, S. *J. Ferment. Bioeng.* **1991**, *72*, 362–367. (e) Ray, V. L.; Van Duyne, P.; Shields, P. P.; Charles, D.; Merkler, D. J.; Consalvo, A. P.; Gilligan, J. P.; Bertelsen, A. H.; Young, S. D. *Regul. Pept.* **1991**, *34*, 93. (f) Ray, M. V. L.; Van Duyne, P.; Bertelsen, A. H.; Jackson-Matthews, D. E.; Sturmer, A. M.; Merkler, D. J.; Consalvo, A. P.; Young, S. D.; Gilligan, J. P.; Shields, P. P. *Bio/Technol.* **1993**, *11*, 64–70.
(4) (a) Henriksen, D. B.; Breddam, K.; Møller, J.; Bruchardt, O. *J. Am. Chem. Soc.* **1992**, *114*, 1876–1877. (b) Green, J.; Margolin, A. L. *Tetrahedron Lett.* **1992**, *33*, 7795–7760. (c) Henriksen, D. B.; Breddam, K.; Buchardt, O. *Int. J. Pept. Protein Res.* **1993**, *41*, 169–180. (d) Breddam, K.; Widmer, F.; Meldal, M. *Int. J. Pept. Protein Res.* **1991**, *37*, 153–160.
(5) Uhlén, M.; Moks, T. *Methods Enzymol.* **1990**, *185*, 129–143.
(6) Tamburini, P.; Jones, B. N.; Consalvo, A. P.; Young, S. D.; Lovato, S. J.; Gilligan, J. P.; Wennogle, L. P.; Erion, M.; Jeng, A. *Arch. Biochem. Biophys.* **1988**, *267*, 623–631.
(7) (a) Catsimpoolas, N.; Wood, J. L. *J. Biol. Chem.* **1966**, *241*, 1790–1796. (b) Degani, Y.; Neumann, H.; Patchornik, A. *J. Am. Chem. Soc.* **1970**, *92*, 6969–6971. (c) Stark, G. R. *Methods Enzymol.* **1977**, *47*, 129–132. Recently, this procedure has been successfully applied to preparation of recombinant peptides from fusion proteins by Koyama et al. and us: (d) Koyama, N.; Kuriyama, M.; Amano, N.; Nishimura, O. *J. Biotechnol.* **1994**, *32*, 273–281. (e) Nakagawa, S.; Tamakashi, Y.; Ishibashi, Y.; Kawase, M.; Taketomi, S.; Nishimura, O.; Fukuda, T. *Biochem. Biophys. Res. Commun.*, in press.
(8) Fujino, M.; Shinagawa, S.; Obayashi, M.; Kobayashi, S.; Fukuda, T.; Yamazaki, I.; Nakayama, R.; White, W. F.; Rippel, R. H. *J. Med. Chem.* **1973**, *16*, 1144–1147.
(9) Tregear, G. W.; van Rietschoten, J.; Greene, E.; Keutmann, H. T.; Niall, H. D.; Parsons, T. A.; Potts, J. T., Jr. *Endocrinol., Proc. Int. Symp., 4th, 1973* **1974**, 1–15.
(10) Tregear, G. W.; Wan Rietschoten, J.; Greene, E.; Niall, H. D.; Keutmann, H. T.; Parsons, T. A.; O'Riordan, J. L. H.; Potts, J. T., Jr. *Hoppe-Seyler's Z. Physiol. Chem.* **1974**, *335*, 415–421.
(11) Reeve, J.; Meunier, P. J.; Parsons, J. A.; Bernat, M.; Bijvoet, O. L. M.; Courpron, P.; Edouard, C.; Klenerman, L.; Neer, R. M.; Renier, J. C.; Slovik, D.; Vismans, F. J. F. E.; Pott, J. T., Jr. *Br. Med. J.* **1980**, 1340–1341.

0002-7863/94/1516-5513$04.50/0

reported to be three times as active as the fragment with a C-terminal carboxylic acid.[9] These facts indicated that the α-amidation of hPTH(1–34) is a suitable target to show the usefulness of this reaction because the intact structure of the product can be evidenced by analytical methods used in peptide chemistry and by biological assays. Moreover, the fragment contains almost all protein-constituting amino acids, which also suggests the wide applicability of this synthetic method.

As a fusion protein, [Cys[35]]hPTH(1–84) (**4**), in which Val[35] of hPTH(1–84) is replaced with a Cys residue, was prepared by the recombinant procedure.[7e,12] The SH group of the Cys residue was site-specifically cyanylated using 1-cyano-4-(dimethylamino)-pyridinium fluoroborate (CDAP)[13] to give [Cys(CN)[35]]hPTH-(1–84) (**5**).[14] The modified protein **5** was treated with ammonia under the conditions described above.[15] Analytical reverse phase HPLC of the reaction mixture showed a simple elution profile. The integration of the peaks indicated the generation of the desired peptide amide **1** at a rate of 80% (Figure 1). Product **1** was isolated by preparative HPLC in a yield of 62%.[16]

The product **1** obtained showed a retention time identical to that of a chemically synthesized standard upon HPLC analysis, and it gave the expected results upon amino acid analysis and molecular weight measurement by fast atom bombardment mass spectrometry [MH^+ (monoisotopic), m/z observed 4114.74 *vs* theoretical 4115.15]. Additionally, the tryptic hydrolysate of **1** gave a peptide map on HPLC identical to that of the standard sample. The *in vitro* biological activity, stimulation of cAMP production in mouse MT-3T3 cells,[12] was in good agreement with that of the chemically synthesized compound and 2.8 times as potent as that of hPTH(1–34) with a C-terminal carboxylic acid as reported previously.[10] These data confirmed that the α-amidated product **1** had the intended intact structure.

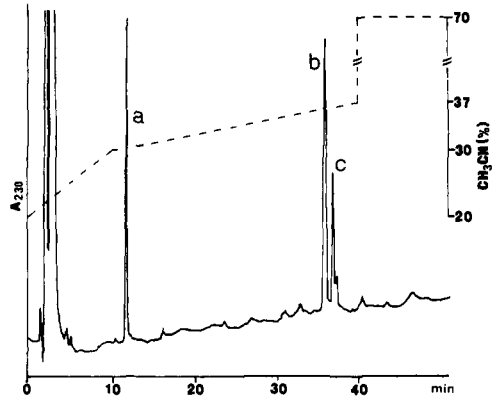


Figure 1. Reverse phase HPLC analysis of the reaction mixture of the cleavage–amidation reaction. The elution profile shown (—) is that for the reaction using ammonia. Eighty percent of the cyanylated protein was cleaved into (2-iminothiazolin-4-yl)carbonyl-hPTH (36–84) (peak a) and compound **1** (peak b), and 20% was converted to a byproduct, [dehydroalanine[35]]hPTH(1–84) (peak c). HPLC analysis was accomplished on a YMC C-18 reverse phase column eluting at 1 mL/min with a gradient of acetonitrile in 0.1% trifluoroacetic acid (- - -).

The ethylamide derivative **2** was synthesized in a similar yield by treatment with 3 M ethylamine solution at 0 °C for 20 min, and the hexylamide derivative **3** was synthesized in a yield of 30% by treatment with 1-hexylamine in 25% acetonitrile at 0 °C for 1 h. Derivatives **2** and **3** showed the same level and 60% of the *in vitro* biological activity of hPTH(1–34), respectively.

Thus, the method described here is an alternative to enzymatic methods for the synthesis of α-amidated peptides *via* the recombinant products. It does not require the isolation of the "precursor" peptide. Moreover, the method is widely applicable without regard to the sequences of the objective peptides, except Cys-containing peptides. Furthermore, it may provide a new procedure to obtain peptides with a C-terminal alkylamide or hydrazide *via* cysteine-extended peptides synthesized by the solid-phase method for which there is not yet generally applicable methodology.

Acknowledgment. We thank Drs. M. Fujino, S.Terao, K. Meguro, and M. Tsuda for their support and helpful discussion.

(12) Oshika, Y.; Yamada, T.; Nakagawa, S.; Fujishima, A.; Kawase, M.; Ishibashi, Y.; Fukuda, T. *Int. J. Pept. Protein Res.*, in press.

(13) Wakselman, M.; Guié-Jampel, E. *J. Chem. Soc., Chem. Commun.* **1976**, 21–22.

(14) Compound **4** (25 mg, 2.7 μmol) was dissolved in 0.1 M acetic acid (13 mL) containing 7 M urea, and the mixture was stirred gently for 20 min at room temperature under N_2 gas. CDAP (1.87 mg, 8.0 μmol) dissolved in the same solution (0.6 mL) was added all at once, and the mixture was stirred for an additional 15 min. The mixture was applied to a column of Sephadex G-25 previously equilibrated with 10% acetic acid. Lyophilization gave **5** (24 mg, 97%).

(15) Compound **5** (20 mg) was dissolved in 3 M ammonia (10 mL) and allowed to react at 0 °C for 15 min followed by neutralization with acetic acid (5 mL).

(16) The reaction mixture was applied to a column of Sephadex G-25 (26 × 68 cm); 3% acetic acid was used as the eluent. The fractions containing the desired material were collected and lyophilized. The product was further purified by reverse phase HPLC on a 2 × 30 cm YMC C18 column eluting at 5 mL/min over 30 min with a gradient between 30% acetonitrile/70% water/0.1% TFA and 40% acetonitrile/60% water/0.1% TFA.