CHEMISTRY LETTERS, pp. 1997-2000, 1994. © 1994 The Chemical Society of Japan

Synthesis of N-terminal Truncated Peptides of Human Epidermal Growth Factor (h-EGF)

Song Yub SHIN, Midori SHIMIZU, and Eisuke MUNEKATA*
Institute of Applied Biochemistry, University of Tsukuba, Tsukuba 305

The N-terminal truncated derivatives of h-EGF showed the correct three disulfide linkages in oxidative refolding process and equipotent activity in both the EGF-receptor binding and mitogenesis assay, as compared with authentic h-EGF. This results suggested that the N-terminus of h-EGF have no significant effect on the formation of the correct disulfide linkages and its biological activity.

Human epidermal growth factor (h-EGF) is a single chain polypeptide with 53 amino acid residues bearing three internal disulfide bridges.[1] EGF is a potent stimulator of the cellular proliferation and inhibitor of gastric acid secretion[2] and its biological effects are mediated through binding to cell-surface EGF receptor (EGFR) with intrinsic tyrosine-kinase activity.[3] Recently, there has been increased interest in assigning specific residues or regions of EGF in EGFR recognition and its biological activity. Elimination of 6 or 7 residue from C-terminus of h-EGF was known to cause a marked reduction in receptor binding, mitogenic activity and inhibition activity of gastric secretion.[4] Kohda *et. al.* reported that the C-terminal tail (residues 46-53) of mouse EGF (m-EGF) play an important role in binding to a putative hydrophobic pocket of the EGFR from two dimensional ^{1}H NMR study.[5] Rat-EGF (r-EGF), r-EGF(2-48), r-EGF(3-48) and r-EGF(4-48) isolated from rat submaxillary glands appeared equipotent in both receptor binding and mitogenic assays.[6] However, the possible importance of N-terminal sequence in EGF to its receptor binding affinity and mitogenic activity has not investigated in detail yet.

To elucidate the contribution of N-terminal sequence of h-EGF in its biological activity, h-EGF(2-53), h-EGF(3-53), h-EGF(4-53), h-EGF(5-53) and h-EGF(6-53) in which N-terminal amino acids (1-5 residue) were shortened one by one from h-EGF were synthesized by solid-phase method using the Fmoc strategy (Fig. 1) (Fmoc = 9-fluorenylmethoxycarbonyl). Since the six cysteines of h-EGF are linked by intramolecular disulfide bridges of pattern 1-3, 2-4 and 5-6, an important point in the synthesis of h-EGF derivatives is the oxidative refolding of cyteinyl residues for the formation of the correct disulfide pairs. In general, air oxidation in a highly diluted basic aqueous solution in the presence of reduced and oxidized glutathiones have been used to refold the reduced peptide, and m-EGF, h-EGF, h-TGF-α and several EGF-related peptides have successfully been synthesized by this method.[7-9] In this study, we investigated the influence of the five residues preceding the first cysteine residue in the h-EGF during oxidative refolding using air oxidation.

Preparation of the peptide-resins of all peptides was carried out by stepwise solid-phase method using Fmoc-Arg(Mtr)-hydroxymethylphenoxy (HMP)-resin (Mtr = 4-methoxy-2, 3, 6-trimethylbenzenesulfonyl) as a starting material. The Fmoc group, a protecting group for α-amino group of amino acids was removed with

20% piperidine in 1-methyl-2-pyrrolidone (NMP). Side-chain functional groups were protected by *tert*-butyl (tBu) for Asp, Glu, Ser and Tyr, trityl (Trt) for His, Asn, and Gln, Boc for Lys and Mtr for Arg. Coupling of

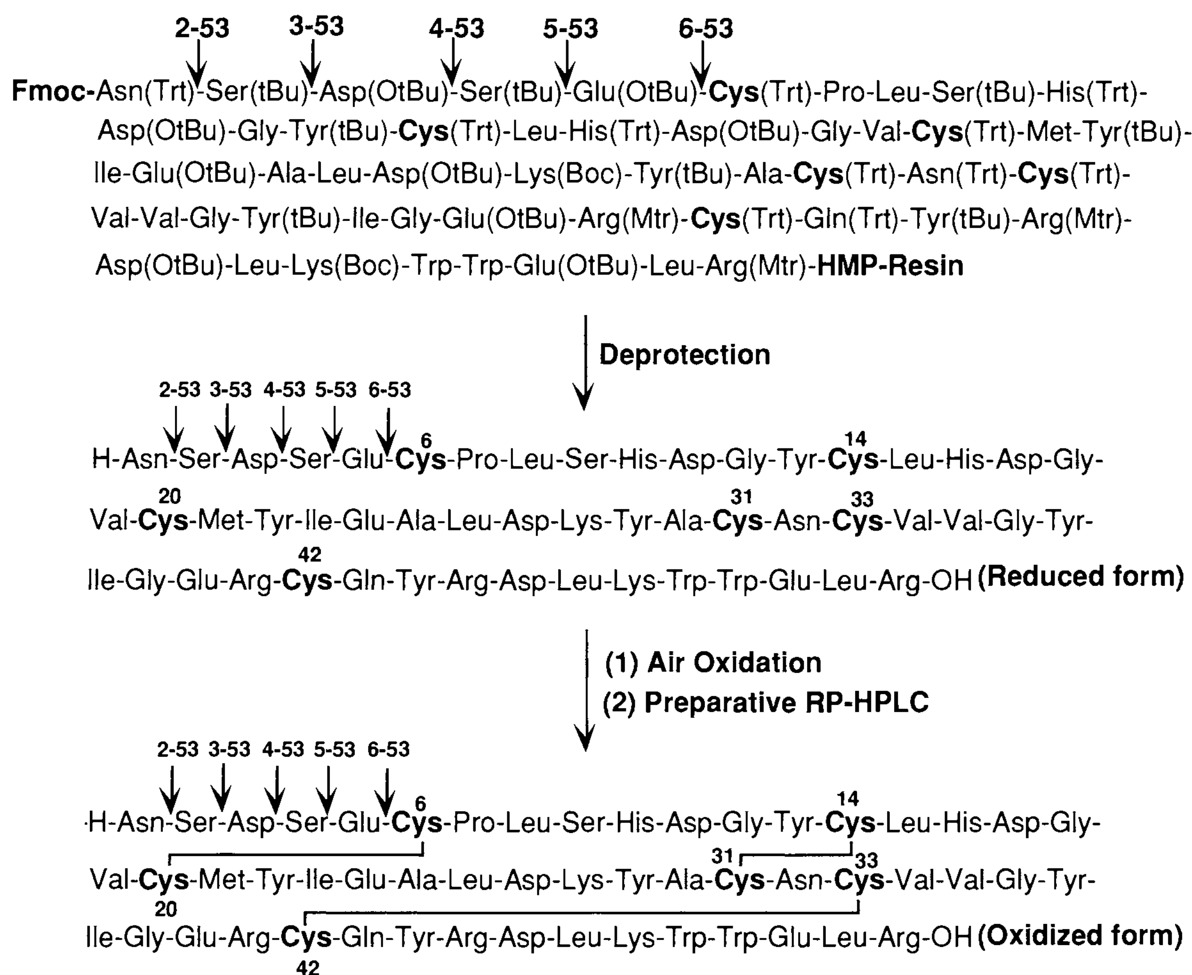

Fig. 1. Synthentic scheme of h-EGF and N-terminal truncated derivatives by solid phase method.

Fmoc-amino acids in each step was performed by either BOP / HOBt or HBTU / HOBt method in NMP [BOP = benzotriazole-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, HBTU = O-benzotriazole-1-yl-tetramethyluronium hexafluorophosphate]. Completeness of the coupling reaction was monitored by the Kaiser's ninhydrin test.[10] Incomplete coupling reaction were either recoupled until a negative Kaiser was obtained or capped by benzoic anhydride (20-fold) in DMF-CH_2Cl_2 (1:3, v/v). After completion of chain elongation, peptides were deprotected and cleaved from the resin by treatment with TFA-phenol-thioanisole-1, 2-ethanedithiol (EDT)-H_2O (82.5: 5: 5: 2.5: 5) (Reagent K)[11] at room temperature for 2 h and TFA-trimethylsilyl bromide (TMSBr)-thioanisole-EDT-m-cresol (69.1: 12.5: 11.1: 5.5: 1.8) at 0 °C for 1 h. To form the three disulfide linkages of synthetic peptides, the crude reduced peptides were oxidized in 0.1 M Tris-HCl buffer (pH 8.2) containing 0.1 mM EDTA, 0.30 mM oxidized and 0.15 mM reduced glutathione for 3 days at room temperature (final peptide concentration: 20

μM). In the air oxidation of the reduced peptides, presumably the most thermodynamically stable oxidized form of synthetic peptides appeared as a main peak in analytical reversed phase (RP)-HPLC (Intersil ODS-2, 4.6 × 150 mm). The crude oxidized peptides were purified by preparative RP-HPLC (Inertsil ODS-2, 20.0 × 250 mm) eluted with a H_2O-MeCN gradient containing 0.1% TFA, flow rate 5 ml / min. The purified oxidized peptides gave a single peak in analytical RP-HPLC, on a Inertsil ODS-2 column (4.6 × 150 mm) eluted with a H_2O-MeCN gradient containing 0.1% TFA, flow rate 1 ml / min, respectively. The amino acid analysis of synthetic peptides were in good agreement with the predicted composition. The molecular masses of synthetic peptides were determined by positive ion FAB-MS [h-EGF: $[M+H]^+$ = 6217.0 (Calc. = 6215.0), h-EGF(2-53): $[M+H]^+$ = 6101.1 (Calc. = 6102.9), h-EGF(3-53): $[M+H]^+$ = 6114.2 (Calc. = 6115.9), h-EGF(4-53): $[M+H]^+$ = 5899.6 (Calc. = 5900.8), h-EGF(5-53): $[M+H]^+$ = 5813.7 (Calc. = 5813.1), h-EGF(6-53): $[M+H]^+$ = 5684.2 (Calc. = 5684.5)].

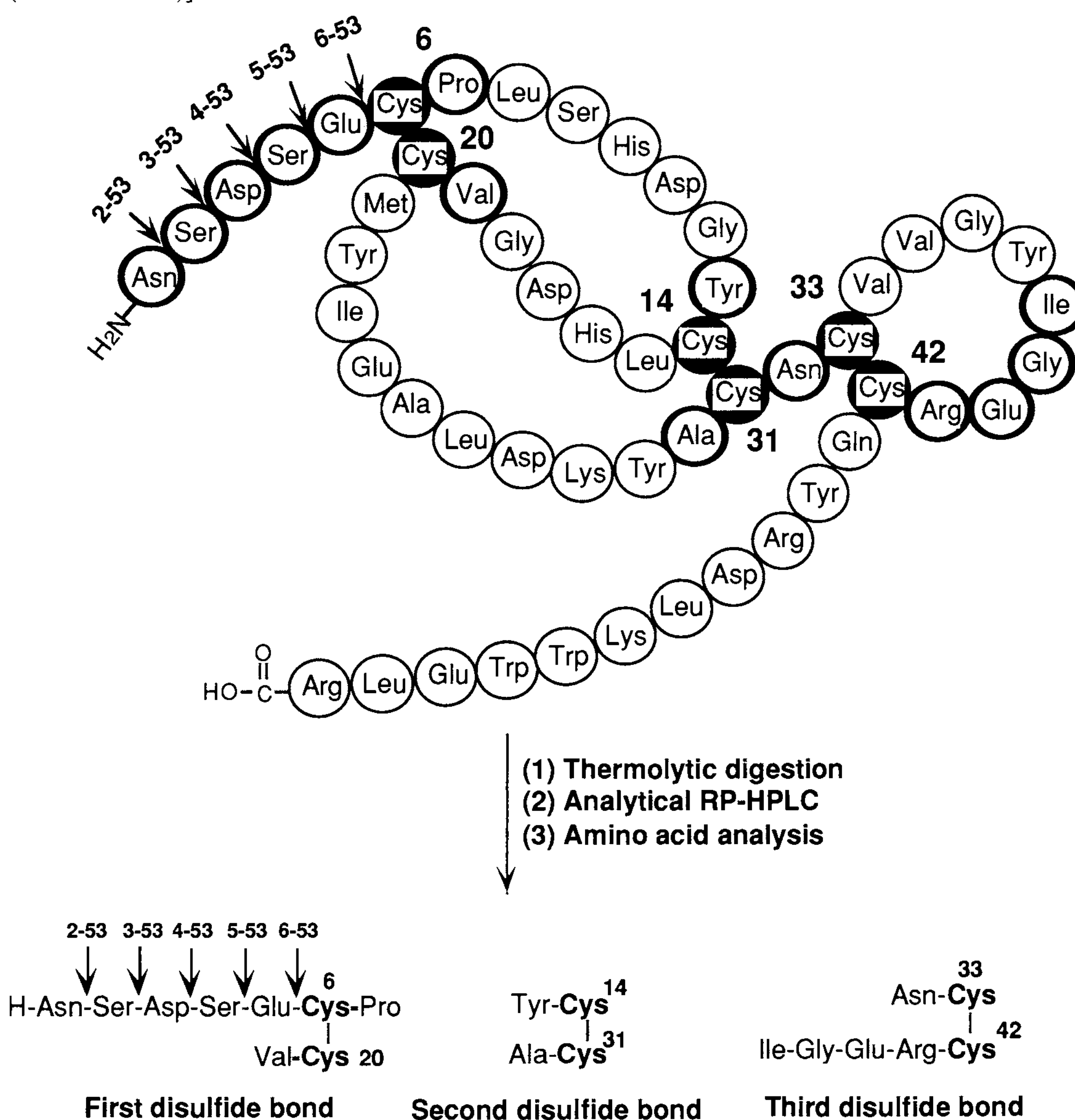

Fig. 2. Cystine-containing peptide fragments after thermolytic digestion of h-EGF and h-EGF derivatives.

To determine the pattern of three disulfide linkage of synthetic peptides, the purified oxidized peptide was subjected to thermolytic digestion. The digested fragments were separated by RP-HPLC and analyzed by amino acid analysis. The results indicated the first disulfide loop was paired by Cys-6 and Cys-20, the second was paired by Cys-14 and Cys-31 and the third was paired by Cys-33 and Cys-42 in the oxidized peptide of h-EGF fragments (Fig. 2). The pattern of such three disulfide linkages in synthetic peptides was consistent with that of h-EGF. In the air oxidation of the reduced peptides of all derivatives of h-EGF, the mispaired isoforms were not detected. The results suggested that the five residues of N-terminal sequence in h-EGF have no significant effect on the formation of the correct disulfide linkages in h-EGF.

Interestingly, ---$CX_7CX_{4-5}CX_{10-13}CX_1CX_8C$---, the structural motif of EGF-like domain has been identified in the EGF family include amphiregulin (AR), heparin binding EGF-like growth factor (HB-EGF), schawannoma derived growth factor (SDGF) and betacellulin (BTC).[12] We confirmed that the three disulfide bond pairings of HB-EGF (44-86) corresponding to the EGF-like domain of HB-EGF is similar to EGF.[13] The results suggested that the spacing pattern of six cysteines in EGF-like domain may play an important role in disulfide rearrangement of the EGF-family.

Each of the N-terminal truncated peptides of h-EGF appeared equivalent receptor binding and mitogenic activity in radioreceptor competition assay on A-431 epidermoid carcinoma cells[14] and [^{3}H]-thymidine incorporation assay on NIH-3T3 fibroblast cells,[4] compared with authentic h-EGF (data not shown). The results indicated that the five residues of N-terminus in the h-EGF do not exert a significant influence on their biological activity. We are grateful to Dr. T. Ohtaki, Dr. Y. Ishibashi and Miss H. Yoshida at Tsukuba Research Laboratory of Takeda Chemical Industries, Co. Ltd., for [^{3}H]-thymidine incorporation assay and FAB-MS.

References

1) C. R. Savage, T. Inagami, and S. Cohen, J. Biol. Chem., **247**, 7612 (1972).
2) H. Gregory, Nature, **257**, 325 (1975).
3) S. A. Buhrow, S. Cohen, and J. V. Staros, J. Biol. Chem., **257**, 4019 (1982).
4) H. Gregory, C. E. Thomas, J. A. Young, I. R. Willshire, and A. Garner, Regul. Peptides, **22**, 217(1988).
5) D. Kohda and F. Inagaki, Biochemistry, **31**, 677 (1992).
6) R. J. Simpson, J. A. Smith, R. L. Moritz, M. J. O'hare, P. S. Rudland, J. R. Morrison, C. J. Lloyd, B. Grego, A. W. Burgess, and E. C. Nice, Eur. J. Biochem., **153**, 629 (1985).
7) J. P. Tam, M. A. Sheikh, D. S. Solomon, and L. Ossowski, Proc. Natl. Acad. Sci. USA., **83**, 8086 (1986).
8) S. Y. Shin, Y. Kaburaki, M. Watanabe, and E. Munekata, Biosci. Biotech. Biochem., **56**, 404 (1992).
9) S. Y. Shin, M. Watanabe, K. Kako, T. Ohtaki, and E. Munekata, Life Sci., **55**, 131(1994).
10) E. R. Kaiser, R. Colescott, C. D. Bossinger, and P. I. Cooke, Anal. Biochem., **247,** 595 (1970).
11) D. S. King, C. G. Fields, and G. B. Fields, Int. J. Peptide Protein Res., **36**, 255 (1990).
12) S. A. Prigent and N. R. Lemoine, Prog. Growth Factor Res., **4**, 1 (1992).
13) S. Y. Shin, T. Takenouchi, T. Yokoyama, T. Ohtaki, and E. Munekata, Int. J. Peptide Protein Res. (in press).
14) D. Kuppuswamy and L. J. Pike, J. Biol. Chem., **264**, 3357 (1989).

(Received July 14, 1994)