Stability of Recombinant Human Epidermal Growth Factor in Various Solutions

Fujiko ARAKI,* Hiroko NAKAMURA, Naoto NOJIMA, Kenichi TSUKUMO, and Shunji SAKAMOTO

Institute for Biotechnology Research, Wakunaga Pharmaceutical Co., Ltd., 1624 Shimokotachi, Koda-cho, Takata-gun, Hiroshima 729-64, Japan.
Received August 1, 1988

The stability of recombinant human epidermal growth factor (hEGF) in various solutions was examined. hEGF degraded spontaneously and temperature-dependently to several degradation products in phosphate buffered saline or in 0.1 N acetic acid. The enzymatic degradation was observed in human serum or in pepsin/HCl solution. The structure and biological activities of these compounds were examined. The results suggest that the Asp^{11} and Trp^{50} residues are important for the receptor binding.

Keywords hEGF; stability; HPLC; α-β rearrangement; cyclic imide; degradation product

Epidermal growth factor (EGF) was first isolated by Cohen in 1962 from adult mouse submaxillary glands.[1] Subsequently, human epidermal growth factor (hEGF) was isolated by Gregory and Willshire in 1975 from urine.[2] It is a small polypeptide of 53 amino acid residues, and its molecular weight is 6216 daltons (Da). Because of its wide range of bioactivities, such as stimulation of cell proliferation[3] and inhibition of gastric acid secretion,[4] many studies had been done to clarify its role *in vivo*. If EGF is to be developed as a drug, however, its stability and metabolic clearance *in vivo* should also be determined.

Recently, the great progress in gene technology has made it possible to mass-produce peptide hormones such as hEGF. In this paper, we report the stability of recombinant hEGF in various solutions and the correlation between structure and activity.

Experimental

Materials hEGF was purified from a transformant *E. coli* strain[5] as previously described.[6] Acetonitrile (high performance liquid chromatography (HPLC) grade), trifluoroacetic acid (TFA) (sequencer grade), triethylamine (TEA) (GR grade) and ethyl *p*-hydroxybenzoate (GR grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Carboxypeptidase W was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), and human serum was from Miles Laboratories, Inc. (Naperville, U.S.A.). All reagents used in automatic sequencing were from Applied Biosystems Japan, Inc. (Tokyo, Japan).

Degradation Studies For stability studies in phosphate buffered saline (PBS) (pH 7.2) or in 0.1 N acetic acid (pH 3.0), hEGF was dissolved at 0.1 mg/ml in these solutions, and kept at −20 °C, 4 °C, room temperature, 37 °C, 60 °C, or 80 °C. At the indicated times, aliquots were removed for HPLC analysis.

In experiments on degradation of hEGF in serum, human serum was preincubated at 37 °C for 10 min. hEGF and ethyl *p*-hydroxybenzoate in PBS (pH 7.2) were added. The final concentrations of hEGF were adjusted to 0.001, 0.01, and 0.1 mg/ml. The mixed solution was incubated at 37 °C. At the indicated times, aliquots were removed and mixed with 1.5 volumes of cold methanol. After centrifugation, the supernatants were subjected to reverse-phase HPLC (RP-HPLC).

In experiments on degradation of hEGF in pepsin/HCl solution (pH 1.6), hEGF and pepsin were dissolved in 0.1 M Gly–HCl–NaCl buffer (pH 1.6) and preincubated at 37 °C. These solutions were mixed to start the reaction. The final concentrations of pepsin and hEGF were 0.5 and 0.05 mg/ml, respectively. The mixed solution was incubated at 37 °C. Aliquots removed at the indicated times were neutralized to pH 7.4 with 0.2 N NaOH and were analyzed by RP-HPLC.

Preparation of hEGF-Related Peptides T1 was prepared by tryptic digestion of hEGF as previously described.[6] P1b and P2 were purified from human urine as previously described.[6]

Structural Analysis Amino acid analysis was carried out as follows; 3 μg of peptide was hydrolyzed under vacuum at 110 °C for 20 h with 50 μl of constant-boiling HCl. For tryptophan determination, hydrolysis was performed with 50 μl of constant-boiling HCl containing 3% thioglycolic acid. Cystine was determined after conversion to cysteic acid. Hydrolyzates were analyzed on an HLC-825AA system (TOSOH Co., Ltd., Tokyo, Japan).

Automated NH_2-terminal sequence analysis was performed as previously described.[6]

The COOH-terminal sequence was analyzed by using carboxypeptidase W on 2.5 nmol of peptide containing 5 nmol of norleucine as an internal standard with an enzyme/peptide (w/w) ratio of 1/50. Released amino acids were determined on the HLC-825AA system (TOSOH Co., Ltd., Tokyo, Japan).

Radio Receptor Assay The assay was performed using human KB cells as previously described.[7]

Bioassay Mitogenic activity was assayed with mouse 3T3 fibroblasts as previously described.[7]

Results and Discussion

Stability of hEGF in PBS or in 0.1 N Acetic Acid Purified hEGF, which gave a single peak on RP-HPLC (Fig. 1-a), was dissolved in PBS (pH 7.2) and its stability was analyzed by RP-HPLC. Gradually, the peak of hEGF decreased and that of D1 (PBS), a degradation product, appeared near it (Fig. 1-b). This change was accelerated at higher temperature (Fig. 2). However, hEGF was quite stable at −20°C. These results suggested that some structural change occurred depending on temperature. The same deterioration was observed in 0.1 N acetic acid solution, and

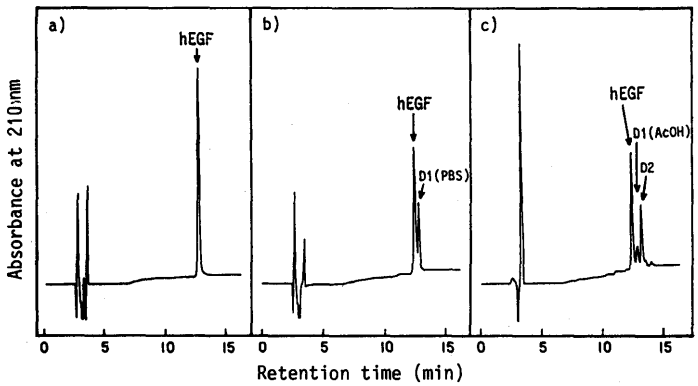

Fig. 1. RP-HPLC of Purified hEGF (a) and Incubated hEGF in PBS (pH 7.2) at 37 °C for 7 d (b) or in 0.1 N Acetic Acid (pH 3.0) at 37 °C for 7 d (c)

Separation was achieved on a TSK gel ODS-120T column (0.46 × 25 cm, TOSOH Co., Ltd.) with linear gradient elution from 30 to 40% B over 10 min. Elution solvents were 1% acetonitrile containing 0.05% TFA and 0.025% TEA (solvent A) and 80% acetonitrile containing 0.05% TFA and 0.025% TEA (solvent B). Flow rate was 1 ml/min.

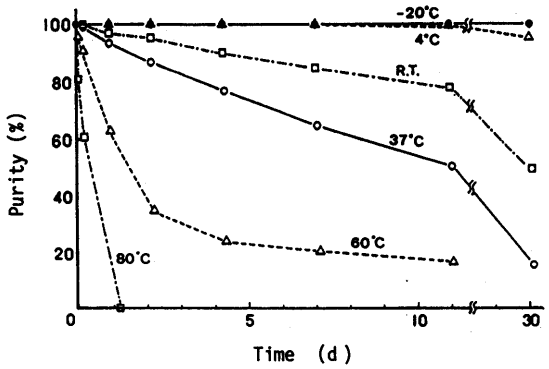

Fig. 2. Degradation Curves of hEGF in PBS (pH 7.2) at Several Temperatures

The purity is given as the percentage of hEGF in total proteins.

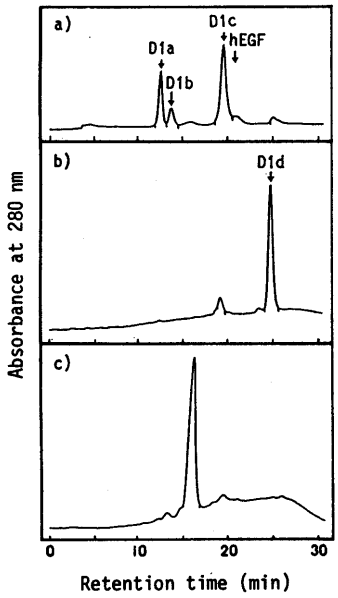

Fig. 3. IEX-HPLC of D1 (AcOH) (a), D1 (PBS) (b), and D2 (c)

The fractions from RP-HPLC were lyophilized, dissolved in 30 mM ammonium acetate buffer containing 1% acetonitrile (pH 6.0), and applied to IEX-HPLC. Separation was achieved on a TSK gel DEAE-5PW column (0.75 × 7.5 cm, TOSOH Co., Ltd.) with linear gradient elution from 5 to 65% B over 20 min. The elution solvents were 30 mM ammonium acetate buffer containing 1% acetonitrile (pH 6.0) (solvent A) and 300 mM ammonium acetate buffer containing 1% acetonitrile (pH 6.0) (solvent B). Flow rate was 1 ml/min.

D1 (AcOH) and D2 were detected as degradation products (Fig. 1-c).

These products were purified to homogeneity on RP-HPLC. Subsequently, they were subjected to ion exchange HPLC (IEX-HPLC), and D1 (AcOH) was separated into three peaks; D1a, D1b, D1c (Fig. 3-a). On the other hand, D1 (PBS) was separated into D1d and a minor product (Fig. 3-b). D2 gave a single peak (Fig. 3-c). These five products were purified to >95% purity.

Stability of hEGF in Serum hEGF was incubated with human serum *in vitro*, and its stability was analyzed by RP-HPLC (Fig. 4). Exponential disappearance of hEGF and appearance of SR17 were observed. The disappearance rate half-time for hEGF was 17 min. SR17 was stable for at least a few hours. On the other hand, when serum was preheated at 60°C for 30 min, hEGF was fully recovered even 2 h thereafter (data not shown). It was suggested that hEGF might be degraded by some proteases in serum which were inactivated by heating.

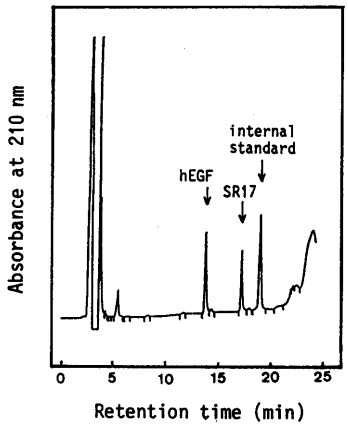

Fig. 4. RP-HPLC of hEGF Incubated with Human Serum at 37 °C for 10 min

Conditions were as described in Fig. 1, except that linear gradient was 30 to 50% B over 20 min.

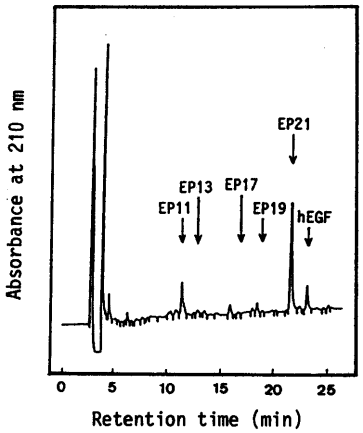

Fig. 5. RP-HPLC of hEGF Incubated with Pepsin in 0.1 M Gly–HCl–NaCl Buffer (pH 1.6) at 37 °C for 3 min

Conditions were as described in Fig. 1, except that linear gradient was 20 to 40% B over 20 min.

Stability of hEGF in Pepsin/HCl Solution hEGF was incubated with 0.5 mg/ml pepsin in 0.1 M Gly–HCl–NaCl buffer (pH 1.6) to simulate the environment in the stomach. Rapid disappearance of hEGF and appearance of EP21 and EP11 were detected on RP-HPLC (Fig. 5). After 10 min, EP21 reached a maximum, and thereafter EP21 decreased and several minor compounds appeared. The disappearance rate half-times for hEGF and EP21 were 1.4 and 50 min, respectively. On the other hand, the content of EP11 reached a plateau after 10 min. These results suggested that the oral administration of hEGF might result in its rapid degradation in the stomach and the physiological activities might be attributed to EP21.

Structures and Properties of hEGF Derivatives The structures of hEGF derivatives were proposed to be as shown in Table I on the basis of amino acid analysis and sequence analysis. The NH_2-terminal three residues of hEGF were lost in D1a. Edman degradation was unsuccessful at the positions of Asn^1 (D1d), Asp^3 (D1b), Asp^{11} (D1c, D2). These positions corresponded to an aspartyl or aspar-

Table I. The Binding Activities of hEGF Derivatives

		Receptor binding activity (% of control)[a]
hEGF		100.0
D1a	hEGF (4—53)	98.2
D1b	#3—4 cyclic imide	100.0
D1c	#11 $^{\beta}$Asp	17.9
D1d	#1 $^{\beta}$Asp	93.1
D2	#11—12 cyclic imide	41.5
D4	#3 $^{\beta}$Asp	96.4
SR17	hEGF (1—52)	96.4
P1b	hEGF (1—51)	90.0
P2	hEGF (1—50)	94.7
EP21	hEGF (1—49)	54.5
T1	hEGF (1—48)	11.3
EP11	hEGF (50—53)	N.D.

a) Percent of control $= \frac{C_{50}\ (\text{hEGF})}{C_{50}\ (\text{peptide})} \times 100$.

aginyl linkage followed by glycine or serine. In general, the rearrangement of an α-aspartyl peptide bond to β-form at these sequences has been well documented.[8] D1b and D2 were selectively formed by heating hEGF in the dry state, which was previously reported to promote the formation of cyclic imides.[9] D1b and D2 were immediately transformed to more acidic molecules (D4 and D1c, respectively) by incubation in 0.1 M ammonium solution at 37°C, which was reported to promote α–β rearrangement.[10] D1d was selectively formed from hEGF under the same conditions. These results suggested that the cyclic imide formation took place in D1b and D2, and α–β rearrangement took place in D4, D1c and D1d. On the other hand, SR17 was found to lack the Arg^{53} residue of hEGF. EP21 was identified as hEGF (1—49), and EP11 was identified as hEGF (50—53).

In addition to these nine peptides, T1: hEGF (1—48) which was prepared by tryptic digestion of hEGF, and P2: hEGF (1—50) and P1b: hEGF (1—51), which were purified from human urine,[6] were examined for binding affinity to the receptor. As shown in Table I, D1c, D2, EP21, and T1 had lower affinities to the receptor than the others. Moreover, these four compounds had decreased mitogenic activities; the relative activities were 52.9, 71.3, 79.9 and 54.1% with respect to hEGF. These activities were slightly higher than the binding activities. This might result from the two different assay procedures; the binding activities were examined by the competitive assay, and the mitogenic activities were examined by direct bioassay without hEGF. Makino *et al.* proposed the formation of a cluster of aromatic residues[11] composed of His^{10}, Tyr^{22}, Tyr^{29}, Trp^{49}, and Trp^{50}. These results suggested that Asp^{11}, Trp^{49} and Trp^{50} are close to each other in the tertiary structure and are important for the receptor binding.

Stability studies using HPLC made it possible to clarify the changes in structure which could not be detected by an assay based only on the biological activities. It was concluded that not only bioassay but also structural analysis is important for stability studies in the field of formulation or drug design of peptide hormones.

References

1) S. Cohen, *J. Biol. Chem.*, **237**, 1555 (1962).
2) H. Gregory and I. R. Willshire, *Hoppe-Seyler's Z. Physiol. Chem.*, **356**, 1765 (1975); H. Gregory, *Nature* (London), **257**, 325 (1975).
3) G. Carpenter and S. Cohen, *Annu. Rev. Biochem.*, **48**, 193 (1979); M. D. Hollenberg, *Vitam. Horm.*, **37**, 69 (1979); E. D. Adamson and A. R. Rees, *Mol. Cell. Biochem.*, **34**, 129 (1981).
4) J. M. Bower, R. Camble, H. Gregory, E. L. Gerring, and I. R. Willshire, *Experientia*, **31**, 825 (1975).
5) T. Oka, S. Sakamoto, K. Miyoshi, T. Fuwa, K. Yoda, M. Yamasaki, G. Tamura, and T. Miyake, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 7212 (1985).
6) K. Tsukumo, H. Nakamura, and S. Sakamoto, *Biochem. Biophys. Res. Commun.*, **145**, 126 (1987).
7) S. Sumi, A. Hasegawa, S. Yagi, K. Miyoshi, A. Kanezawa, S. Nakagawa, and M. Suzuki, *J. Biotech.*, **2**, 59 (1985).
8) K. Weber and W. Konigsberg, *J. Biol. Chem.*, **242**, 3563 (1967); W. R. Groskopf, J. W. Holleman, E. Margoliash, and I. M. Klotz, *Biochemistry*, **5**, 3783 (1966); F. Wold, *Trends Biochem. Sci.*, **10**, 4 (1985); R. P. Diaugustine, B. W. Gribson, W. Aberth, M. Killy, C. M. Ferrua, Y. Tomooka, C. F. Brown, and M. Walker, *Anal. Biochem.*, **165**, 420 (1987).
9) S. Luo, C. Liao, J. F. McClelland, and D. J. Graves, *Int. J. Peptide Protein Res.*, **29**, 728 (1987).
10) B. A. Johnson, E. D. Murray, Jr., S. Clarke, D. B. Glass, and D. W. Aswad, *J. Biol. Chem.*, **262**, 5622 (1987).
11) K. Makino, M. Morimoto, M. Nishi, S. Sakamoto, A. Tamura, H. Inooka, and K. Akasaka, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7841 (1987).