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Stabilization of Human Epidermal Growth Factor (hEGF) in Aqueous Formulation

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INTRODUCTION

Human epidermal growth factor (hEGF) is a single chain polypeptide containing 53 amino acid residues (MW = 6045) and three disulfide bridges. The polypeptide can stimulate the proliferation of a wide range of epidermal and epithelial cells (1) and also inhibit gastric acid secretion (2,3). It can be formulated into ophthalmic and topical preparations for faster and better healing of wounds and burns (4–6).

In order to use EGF as a therapeutic agent, it is necessary to develop a physically and chemically stable formulation during storage. Chemical and physical stability of proteins were well defined and reviewed in ref. 7. The most prevalent chemical reaction occurring in EGF was deamidation, a process in which the side chain amide group in glutaminyl or asparaginyl residues was hydrolyzed to form a free carboxylic acid (8,9). hEGF contained three such residues, Asn^1, Asn^{32} and Gln^{43}, of which the first asparagine residue was be the most labile site in neutral or alkaline pH, high temperature and high ionic strength (10). It was also reported that physical instability of EGF came from polymerization of monomer into dimer and trimer by disulfide exchange (11), which may change biological activity or immunological properties (12). The purpose of this study was to prepare a stable hEGF formulation using potential stabilizing agents which retard or inhibit hydrolysis (deamidation and peptide bond cleavage) and aggregation during storage.

MATERIALS AND METHODS

Materials

hEGF (>98% purity) was obtained from Pepro Tech. Inc. (Rocky Hill, NJ). Tris base and Tris-HCl, Na Phosphate, Na Borate, Na Citrate, NaCl, Triton X-100, Tween 20 and $ZnCl_2$ were purchased from Fisher. Hydroxypropylmethylcellulose (HPMC) from Union Carbide (Danbury, CT). Chondroitin sulfate, hyaluronic acid, sucrose monocaproate, and fibronectin were purchased from Sigma.

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Methods

Chemical Stability Test. 0.5 μg of hEGF was dissolved in 1 ml of neutral or alkaline solution with or without additive(s) and then incubated at 60°C at the predetermined time. The samples were subjected to HPLC analysis and mitogenic assay. Peak remained was calculated as follows: peak remained (%) = (peak area of hEGF solution stored under the given conditions × 100)/{peak area of hEGF solution freshly made from lyophilized hEGF (>98% purity)}. Peptide bond cleavage (%) was expressed as 100 − (% peak I + % peak II). Mitogenic activity remained was calculated as follows: mitogenic activity remained (%) = {mitogenic activity of hEGF solution stored under the given conditions − mitogenic activity of control (no hEGF)} × 100/{mitogenic activity of hEGF solution freshly made from lyophilized hEGF (>98% purity)}. The results shown in Tables I, II, and III were statistically evaluated using Student's t-test and expressed as mean (n = 3): the significant figures were all within 5–10%.

α-EGF and β-EGF, two closely related forms of EGF, were separated as the major peaks on reversed-phase HPLC (13). Later, DiAugustine *et al.* (14) demonstrated that α-EGF and β-EGF were native and deamidated (isoaspartyl or desasparaginyl) forms of EGF, respectively. Since HPLC methods used to separate these two forms (13,14) were too time-consuming for our stability studies, we performed reversed-phase HPLC according to DiAugustine *et al.* (14) and Araki *et al.* (15) with a slight modification. The Waters Associates HPLC system consisted of a Model 510 Solvent Delivery System, a Model 486 Turnable Absorbance Detector and a Model 746 Data Module. The samples were separated isocratically at ambient temperature over 5 μm C_{18} TSKgel ODS-120T column (4.6 mm ID × 15 cm, TOSOH Corp., Tokyo, Japan). The mobile phase used was acetonitrile-10 mM diethylenetriamine phosphate, pH 6.0 (22:78) at a flow rate of 0.5 ml/min. Detection was monitored at 214 nm.

Measurement of Biological Activity. The assay was performed according to Riemen *et al.* (16): It was assessed by its ability to stimulate DNA synthesis in NRK cells *in vitro*.

Measurement of Aggregation. This was performed according to Chawla *et al.* (17) with a slight modification. 2 ml of control hEGF or hEGF-surfactant solution was placed in a glass vial and shaken in a water bath (Precision Scientific) for 14 days (37°C, 100 rpm). Aggregation was determined turbidimetrically by measuring the increase in A_{600} using a Pharmacia LKB Spectrophotometer.

RESULTS AND DISCUSSION

As shown in Fig. 1, peaks I and II were deamidated (RT = 22.94 min) and native hEGF (RT = 26.72 min), respectively. Each peak was confirmed by performing reversed-phase HPLC according to DiAugustine *et al.* (14). There were no other peaks present in the chromatogram. Since deamidation is a chemical reaction involving the hydrolysis of Asn and Gln residues, it yields a new chemical entity. The deamidation of hEGF was determined as % increase in peak I on reversed-phase HPLC. hEGF was heated

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Table I. The Effect of Buffer Ions (50 mM) on Deamidation of hEGF in Neutral Solution (pH 7.0) Stored at 60°C for 2 and 14 Days

Buffer ion	% peak remained in 2 days			Mitogenic activity remained in 2 days (%)	% peak remained in 14 days			Mitogenic activity remained in 14 days (%)
	I	II	I + II		I	II	I + II	
Tris-HCl	37.5	62.5	100	98	11.4	0	11.4	15.2
Na phosphate	62.8	37.3	100	101	0	0	0	0
PBS	83.1	16.9	100	99	0	0	0	0
Na borate	51.7	48.3	100	100	0	0	0	0
Na acetate	49.1	50.9	100	96	0	0	0	0
Na citrate	50.7	49.3	100	103	0	0	0	0

All numbers represent mean (n = 3). See "Methods" for the details.

to 60°C, the condition that accelerated the deamidation (10), in physiological buffer (pH 7.0) for 2 and 14 days. As shown in Table I, when hEGF was stored for 2 days to see the hydrolytic deamidation only, the formation of peak I occurred to a much lesser extent in Tris-HCl (pH 7.0) than any other buffers (pH 7.0) such as Na phosphate, PBS, Na borate, Na acetate or Na citrate. However, the biological activity of each sample remained unchanged under the same conditions for 2 days, indicating that deamidation was not critical for mitogenic activity of hEGF as reported previously (14). When heated to 60°C for 14 days, hEGF was completely degradated since the peaks disappeared from the chromatogram. Surprisingly, about 11.4% of hEGF in Tris-HCl solution (pH 7.0) still remained and eluted as a deamidated form. Mitogenic activity of the polypeptide remained approximately 15% in Tris buffer but, in other buffers, was completely lost. The high dependence of deamidation on the anion used in the buffer preparation is an indication of a general base catalysis and possibly related to the conformational restraints on imide formation and thus the pronounced influence by tertiary and quaternary interactions (18).

hEGF was subjected to heating at 60°C in alkaline solution (50 mM Na phosphate, pH 8.0), in order to accelerate condition for deamidation (10), for 1 day. We chose to take the samples at day one because the samples stored for 2 days under the same conditions gave rise to such extensive degradation of hEGF that we were unable to observe the stabilizing effect of each additive. Samples stored for 14 days were completely degraded. As shown in Table II, hEGF was deamidated and/or degraded. Deamidation was inhibited to a larger extent with 0.5% fibronectin, 0.2% hyaluronic acid, 1% sucrose monocaproate or surfactants such as Triton X-100 (0.02%) or Tween 20 (0.01%). However, all of these additives and Zn ions contributed to the prevention of hEGF peptide bond cleavage which is also a chemical reaction involving hydrolysis, yielding a new chemical entity. Unexpectedly, HPMC and chondroitin sulfate did not stabilize the polypeptide at all. The additives tested were chosen because (i) polyhydric alcohol and sugars have been used for many years as stabilizing agents of proteins (19), (ii) synthetic and natural polymers such as polysaccharides are protective colloids which are able to protect proteins and peptides against physical and chemical insult, (iii) the EGF receptor is known to be a glycoprotein, and (iv) small cationic molecules may stabilize the structure of proteins: Hyaluronic acid (noncovalent binding to peptide) and chondroitin sulfate (covalent binding to peptide) are proteoglycans (95% of polysaccharide and 5% of protein), having viscoelastic properties.

Table II. The Effect of Additives on Deamination and Peptide Bond Cleavage of hEGF in Alkaline Solution (50 mM Na Phosphate, pH 8.0) 60°C for 1 Day

Additive	% peak remained			Peptide bond cleavage (%): 100 − (I + II)	Mitogenic activity remained (%)
	I	II	I + II		
—	27.1	20.5	47.7	52.4	49.3
0.02% Triton X-100	46.1	35.2	81.3	18.7	84.3
0.01% Tween 20	44.9	27.7	72.6	27.4	75.2
0.1 M NaCl	22.3	15.3	37.6	62.4	38.8
0.4 M NaCl	13.2	—	13.2	86.8	13.5
0.2% HA[a]	28.1	45.0	73.1	26.9	80.0
1.0% SM[b]	37.9	43.4	81.3	18.7	84.3
0.05% FN[c]	31.7	49.8	81.5	18.5	84.5
6 mM $ZnCl_2$	49.3	24.4	73.7	26.3	76.4
0.5% HPMC[d] + 1% CS[e]	25.3	17.9	43.3	56.7	44.9

[a] Hyaluronic acid.
[b] Sucrose monocaproate.
[c] Fibronectin.
[d] Hydroxypropylmethylcellulose.
[e] Chondroitin sulfate.
All numbers represent mean (n = 3). See "Methods" for the details.

Table III. The Effect of Surfactants on the Tubidity of hEGF Solution Stored at 37°C for 2 Weeks

Surfactant	A_{600}	Mitogenic activity remained (%)
—	0.201	62
0.02% Triton X-100	0.008	102
0.01% Tween 20	0.018	98

All numbers represent mean (n = 3). See "Methods" for the details.

HPMC is a polysaccharide and is used as a viscosity enhancing agent. Sucrose monocaproate is sugar ester. Fibronectin is a glycoprotein found in the matrix. $ZnCl_2$ is a cationic molecule stabilizing insulin (20). Triton X-100 and Tween 20 were added since they inhibited aggregation (Table III). NaCl was tested since ionic strength affected deamidation (10). Biological activities of each sample were not affected by the degree of deamidation but by the peptide bond cleavage. Particularly, the addition of high salt, 0.4 M NaCl, destabilized hEGF, resulting in almost 100% loss in the native polypeptide and about 85% loss in biological activity (15% activity remaining came from approximately 13% of the deamidated form). It was observed that the degree of degradation did not depend on the concentration of the additive(s) added in the formulation (not shown). However, the products produced by cleavage of peptide bond did not exhibit similar biological activity as a mitogen to deamidated EGF. Increase in the breakage of the peptide bond markedly reduced the potency of hEGF as expected (15). This can be prevented by the stabilizer(s) used to prevent deamidation (nonionic surfactants and polymers) or by Zn ions. This finding may suggest that the charge and size of the side chains also contributed to these chemical reactions.

Two nonionic surfactants used for preventing hydrolysis (Table II) were added as stabilizing additives to prevent aggregation. As shown in Table III, hEGF became turbid during storage at 37°C for 14 days with shaking, in order to accelerate aggregation. As expected, the increase in turbidity reduced the biological activity of the polypeptide. This result indicated that hEGF aggregated into higher molecular weight polymers with reduced mitogenic activity (Table III), possibly leading to an abnormal immune response as found from aggregated insulin (21). It is not clear if the aggregation is a result of noncovalent self-association from hydrophobic interaction or of polymerization by disulfide exchange. However, the presence of Triton X-100 or Tween 20 inhibited the increase in A_{600}. Therefore, this problem can be solved by adding nonionic detergents as previously reported (17), leading to complete stabilization.

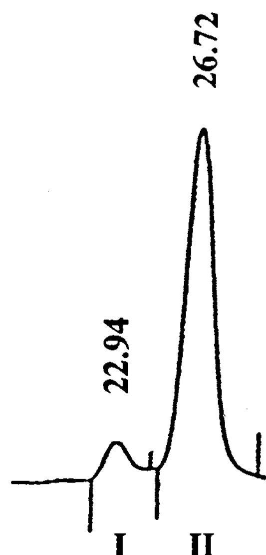

Fig. 1. Reversed-phase HPLC of 10 ng hEGF. Equipment and column conditions were described under Materials and Methods. Eluant was monitored at A_{214}. Peak I and Peak II corresponded to deamidated- and native-EGF, respectively.

In conclusion, chemical stability of hEGF is determined by hydrolysis (deamidation and cleavage of peptide bond), which was inhibited (i) in neutral solution (Tris buffer) and (ii) by adding nonionic surfactants (0.01–0.1%) or polymers (0.1–1.0%), at low salt concentrations. Aggregation can also be prevented by nonionic surfactants (0.01–0.1%).

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