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PURIFICATION AND CHARACTERIZATION OF HIGH MOLECULAR WEIGHT HUMAN EPIDERMAL GROWTH FACTOR FROM HUMAN URINE

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SUMMARY: High molecular weight human epidermal growth factor (HMW-hEGF) was purified to homogeneity from human urine. The purification was achieved exclusively by the use of immunoaffinity chromatography and reverse phase high performance liquid chromatography (HPLC). The purified HMW-hEGF is composed of a single polypeptide chain with an apparent molecular weight of 30,000 and has pI of 4.0. Its N-terminal sequence was determined as Val-Ser-Asp-Gln-Asp-Asp-( )-Ala-Pro-Val-Gly-( )-Ser-Met-Tyr-Ala-Arg-( )-Ile-Ser-. The trypsin treatment of this protein gave hEGF like fragments. These results suggest that the obtained HMW-hEGF may play an important role in biosynthetic precursor of hEGF.

Epidermal growth factor (EGF) was first described in the male mouse submandibular gland (1). It is a polypeptide hormone composed of 53 amino acids (2), which stimulates growth and differentiation of various cells from many tissues (3). Human EGF (hEGF) was isolated from human urine (4) and appears to be identical to β-urogastrone, an inhibitor of stimulated gastric acid secretion (5). It is also known that a high molecular weight hEGF, which is supposed to play a precursor role in the biosynthesis of hEGF, exists in human body fluids, although the mechanism of processing and molecular properties are poorly understood(6-9).

The recombinant hEGF has been successfully produced in Escherichia coli by Oka et al. (10) and we have prepared the rabbit antiserum against the highly purified recombinant hEGF. Because of the importance of clarifying the biological role and the biosynthetic mechanism of hEGF, we have attempted to purify the hEGF, its fragments and HMW-hEGF effectively using the immunoaffinity chromatography. In this report, we describe the first

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purification of the HMW-hEGF to homogeneity and the determination of its N-terminal sequence.

MATERIALS AND METHODS

Purification of recombinant hEGF: The hEGF used in this study was expressed in Escherichia coli by the technique of gene technology, and was secreted to the fermentation broth. Then it was purified to homogeneity by following six sequential steps: removal of bacteria with membrane filter, concentration by ultra-filtration with hollow fiber, salting out, ethanol precipitation, ion exchange chromatography (DEAE-Toyopearl,Toyo Soda Mfg.Co.), and reverse phase HPLC (ODS-120T,Toyo Soda Mfg.Co.).

Preparation of the immunoaffinity column: The antiserum against highly purified recombinant hEGF was raised in a rabbit. The immunoglobulin G (IgG) in this antiserum was purified by affinity chromatography on Protein A-Sepharose CL-4B. According to the general procedure, about 200 mg of IgG was coupled with 20 ml of wet cyanogen bromide activated agarose.

Purification of the HMW-hEGF: Twenty liters of adult male human urine was acidified with 1 liter of acetic acid. 250 ml of Bio-Rex 70 ion exchange resin (Bio-Rad Laboratories), which was activated with 5 % acetic acid, was added to the 20 liters of acidified urine and the mixture was stirred for 18 hr at 4 °C. The resin was collected on a glass filter, washed with 3.6 liters of 0.001 N HCl and then adsorbed protein fractions were eluted with 1 liter of 1 M ammonium acetate buffer (pH 8.0). The eluate was diluted with two volumes of distilled water and applied to a column (2.5 x 7.0 cm) of the anti-hEGF-Sepharose, equilibrated with 0.1 M phosphate buffer, pH 7.0 , containing 0.15 M NaCl. The column was washed with the same buffer and then the adsorbed immunoreactive materials were eluted with a 1 M acetic acid. The eluate was lyophilized and dissolved in 5.0 ml of 1 M acetic acid. The solution was applied to a column (1.6 x 100 cm) of Sephadex G-50, equilibrated with 1 M acetic acid. The eluted HMW-hEGF fractions were lyophilized and dissolved in 1.5 ml of 30 mM phosphate buffer, pH 7.2, containing 0.15 M NaCl and 2.5 mM KCl (PBS). The solution was subjected to gel filtration on a column of Sephacryl S-200 (1.0 x 120 cm), equilibrated with the same buffer. The obtained HMW-hEGF fractions were concentrated and finally purified to homogeneity by reverse phase HPLC with a TMS-250 column (0.42 x 7.5 cm, Toyo Soda Mfg.Co.) using a linear gradient elution from 20 % to 36 % acetonitrile containing 0.05 % trifluoroacetic acid in 20 min.

Radioimmunoassay for hEGF: All dilutions were performed with an assay buffer, which consisted of 10 mM phosphate buffer, pH 7.2, containing 0.5 % BSA and 0.1 % NaN_3. Standards and unknown samples were incubated with approximately 10,000 cpm of [^{125}I]-hEGF (100 µl) and anti-hEGF antibody at a dilution of 1:100,000 (100 µl) overnight at 4°C. To separate bound hEGF from a free one, 100 µl of goat antiserum against rabbit IgG (Miles Laboratories,Inc.), a dilution of 1:20, 100 µl of normal rabbit serum (diluted at 1:100) and 100 µl of 15 % polyethyleneglycol 6000 were added and incubated overnight at 4 °C. The mixtures were centrifuged and the precipitates were counted in a well-scintilating gamma counter.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed on 12.5 % polyacrylamide gels by the method of Laemmli (11) and the gel was stained by a silver method. Ovalbmine (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400) and lysozyme (14,300) were used as an internal molecular weight marker (Bethesda Research Laboratories).

Isoelectric focusing: The isoelectric point (pI) was determined by isoelectric focusing on 7.5 % polyacrylamide disc gels containing carrier ampholytes (Ampholine, LKB Instruments,Inc.), pH 3.5-5.0 and 3.5-10.0. (12) After isoelectric focusing, the gels were cut into 0.25 cm slices and the HMW-hEGF was eluted by overnight incubation in 300 µl of assay buffer at 25 °C.

Digestion with trypsin: 9.0 μg of purified HMW-hEGF was incubated with 45 μg of trypsin (Worshington Biochemical Corp.) in 100 μl of PBS at 37 °C for 1 hr. A control incubation was performed under the same condition without trypsin. Each incubation mixture was immediately analyzed by reverse phase HPLC with a TMS-250 column using a linear gradient elution from 20 % to 36 % acetonitrile containing 0.05 % trifluoroacetic acid in 20 min.
Sequence Analysis: Automated Edman degradations were performed on a Model 470A protein sequencer (Applied Biosystems) according to a standard protocol (13,14).(See figures 1 and 2).

RESULTS AND DISCUSSION

Purification scheme of the HMW-hEGF from adult male human urine is summarized in Table 1. Twenty liters of urine contained a total of 370 μg of hEGF as determined by the radioimmunoassay. The step of the Bio-Rex 70 ion exchange chromatography in acidic condition gave 95-100 % recovery of the activity. Then the clear brown eluate was subjected to a column of the anti-hEGF-Sepharose at a flow rate of 70 ml/hr. While the bulk of protein, salts and pigments passed through the column, hEGF and hEGF-like compounds were retained on the column. This procedure improved specific activity by 580-fold. The fractions containing the HMW-hEGF were separated from those of hEGF(s) by following gel-filtration on a column of Sephadex G-50. The results from the chromatogram of Sephasex G-50 revealed that five per cent

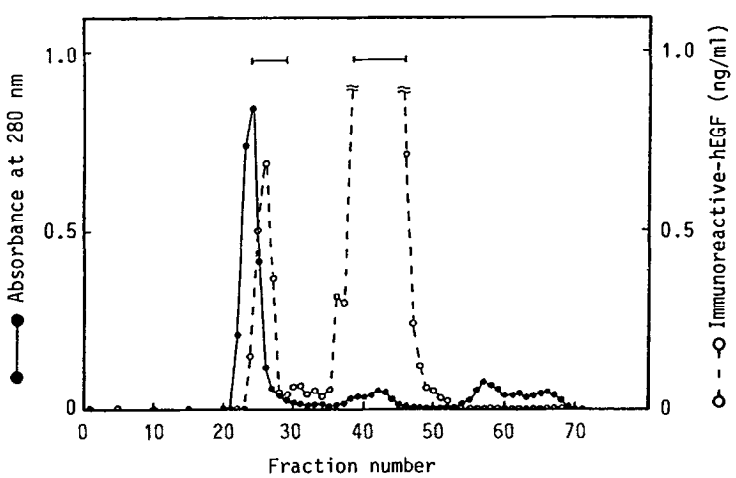

Fig. 1. Sephadex G-50 gel-filtration chromatogram of adsorbed proteins from anti-hEGF-Sepharose. The column (1.6 x 100 cm) was equilibrated with 1.0 M acetic acid. Absorbance was measured at 280 nm (●——●) and the activity was determined by radioimmunoassay as described under MATERIALS AND METHODS (○- -○). The flow rate was 85 ml/hr; 2.4 ml fractions were collected.

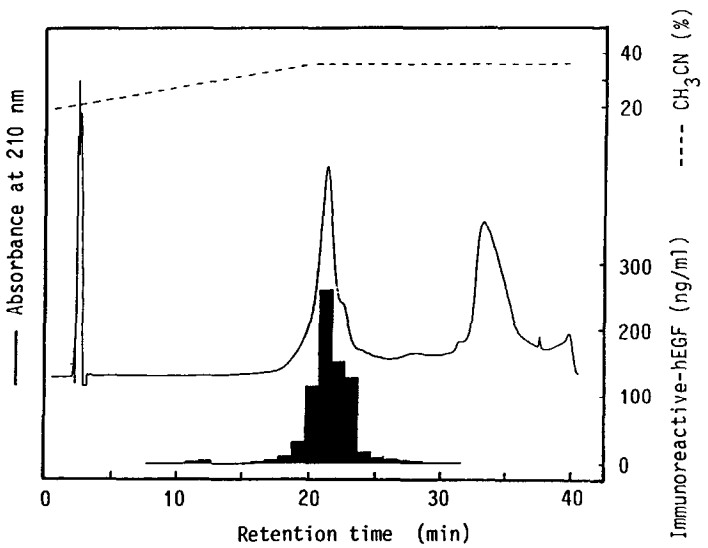

Fig. 2. The first reverse phase HPLC (TMS-250) of the HMW-hEGF. The fractions containing the HMW-hEGF from Sephacryl S-200 were lyophilized and subjected to reverse phase HPLC with a TMS-250 column using a linear gradient elution from 20 % to 36 % acetonitrile (----) containing 0.05 % trifluoroacetic acid in 20 min. The flow rate was 0.6 ml/min; 0.6 ml fractions were collected.

of the total immunoactivity in the eluate referred to the HMW-hEGF. The small molecule fractions containing such peptides as hEGF, hEGF(1-52), hEGF(1-51) and hEGF(1-50), were applied to an ion exchange chromatography. Each compound of these was finally purified to homogeneity by the C18 reverse phase HPLC. On the other hand, the HMW-hEGF fractions were further purified by gel-filtration on a column of Sephacryl S-200. The specific

Table 1. Purification of HMW-hEGF from human urine

Step	Volume (ml)	Total protein[a] (mg)	Total activity[b] (μg)	Specific activity (μg hEGF/mg protein)	Yield (%)
Urine	20000	1260000	370.0	2.94×10^{-4}	100
Bio-Rex 70	1000	25000	364.5	1.45×10^{-2}	98.5
IgG-Sepharose	150	39.0	330.0	8.46	89.2
Sephadex G-50	28.8	11.9	12.9	1.08	3.5
Sephacryl S-200	7.2	0.189	9.0	47.6	2.4
RP-HPLC (TMS-250)	11.0	0.017[c]	6.3	370.6	1.7
RP-HPLC (TMS-250)	2.0	0.013[c]	5.0	384.6	1.4

a) Determined by measuring the absorbance at 280 nm.

b) Determined by radioimmunoassay; the dilution curve of HMW-hEGF was parallel to that of standard hEGF.

c) Determined by the Bio-Rad dye binding assay using BSA as standard.

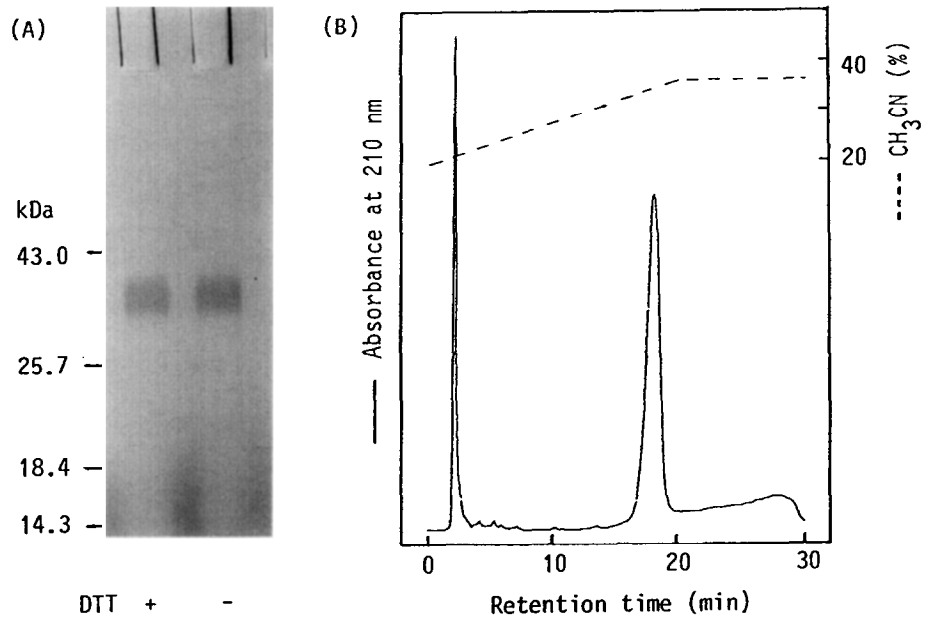

Fig. 3. SDS-polyacrylamide gel electrophoresis (A) and reverse phase HPLC (B) of the purified HMW-hEGF. (A) The purified HMW-hEGF (0.1 μg) was incubated in the absence (-) or presence (+) of 10 mM dithiothreitol and separated on a 12.5 % SDS-polyacrylamide gel. (B) The purified HMW-hEGF (2.0 μg) was applied to a column of TMS-250 with the same gradient elution conditions as described in Fig.2.

activity was increased from 1.08 to 47.6 by this step. Further purification was achieved effectively by reverse phase HPLC on a C1 column. Since the active fraction was contaminated with small amounts of impurities by the first reverse phase HPLC on a C1 column, it was subjected to rechromatography under the same conditions to get a finally purified HMW-hEGF. The purified HMW-hEGF was verified to be homogeneous by reverse phase HPLC and also by SDS-PAGE (Fig. 3). Thus, it was proved that the HMW-hEGF consists of a single polypeptide chain with an apparent molecular weight of 30,000.

The HMW-hEGF appeared to have a pI of approximately 4.0 (Fig. 4). Both the molecular weight and the pI of HMW-hEGF determined in this study were similar to those of high molecular weight hEGFs derived from human urine by Hirata et al. (6) and those from platelets by Oka et al. (9). As shown in Fig. 4, a small peak with a pI of approximately 4.5 was observed beside the HMW-hEGF peak on isoelectric focusing. This minor peak appeared to be

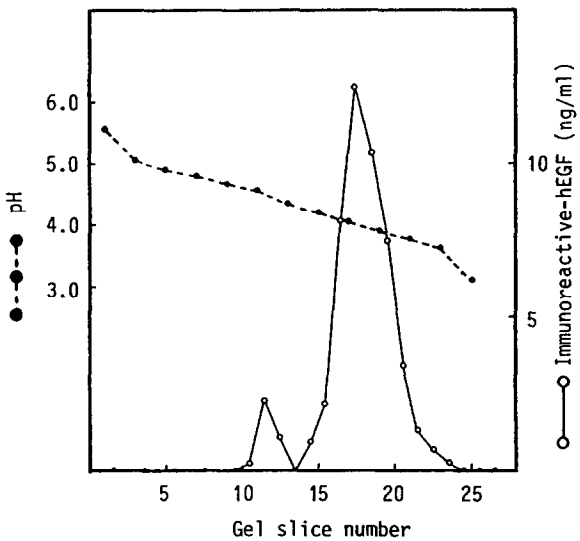

Fig. 4. Isoelectric focusing of the purified HMW-hEGF on 7.5 % polyacrylamide gel. About 1.0 μg of the HMW-hEGF was analyzed by isoelectric focusing. The activity eluted from sliced gels was measured by radioimmunoassay (o—o). The pH gradient was measured after soaking sliced gels in degassed HPLC-grade water overnight at room temperature (●--●).

released from the HMW-hEGF in the isoelectric focusing procedure and its pI of 4.5 was similar to that of hEGF (5). This result might be important to suggest that HMW-hEGF has the biosynthetic relationship with hEGF.

The N-terminal sequence of the HMW-hEGF was determined as Val-Ser-Asp-Gln-Asp-Asp-( )-Ala-Pro-Val-Gly-( )-Ser-Met-Tyr-Ala-Arg-( )-Ile-Ser- by the automated Edman degradation method. Recently, complementary DNA clones encoding the hEGF precursor were isolated and sequenced, and the amino acid sequence of prepro EGF was predicted (15). Comparison of the N-terminal sequence of the HMW-hEGF with prepro EGF sequence reported by Bell et al. revealed complete homogeneity between residues 829-848. It is presumed that the HMW-hEGF, when considered with its molecular weight, contains EGF moiety and following transmembrane domain.

Hirata and Orth (7) have reported that trypsin and arginine esterase are capable of cleaving urinary high molecular weight hEGF to yield a EGF-like molecule. However, it is still unknown whether or not the newly formed small molecular weight hEGF is intact hEGF. In this study, we attempted to identify tryptic fragments of the purified HMW-hEGF as intact hEGF by

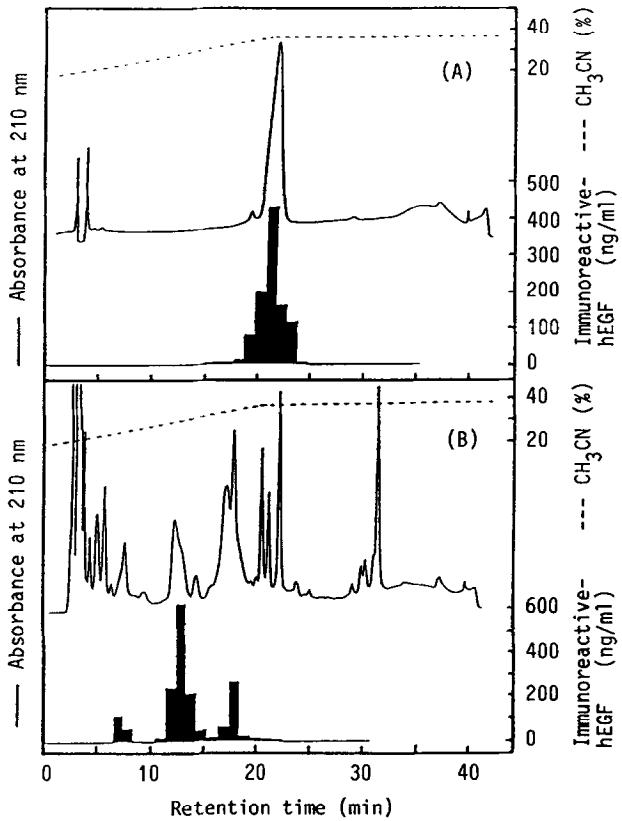

Fig. 5. Reverse phase HPLC of the trypsin treated HMW-hEGF on a TMS-250 column. The purified HMW-hEGF was incubated without enzyme (A) or with trypsin (B). Each incubation mixture was immediately analyzed by reverse phase HPLC with a TMS-250 column using a linear gradient elution from 20 % to 36 % acetonitrile containing 0.05 % trifluoroacetic acid in 20 min.

chromatographic comparision on reverse phase HPLC with authentic peptides. As shown in Fig. 5, after control incubation, the HMW-hEGF was eluted at a retention time of 21.0 min. On the other hand, after incubation with trypsin, three immunoactive peaks were newly observed at a retention time of 7.0 min, 14.0 min and 18.0 min. In this HPLC system, authentic recombinant hEGF was eluted after 18.0 min and hEGF(1-48) that was formed from hEGF with trypsin treatment (E/S=1/10 W/W, 5hr.) was eluted after 7.0 min. Thus, we demonstrated that the HMW-hEGF was capable of converting to hEGF and hEGF(1-48) by trypsin digestion. These results suggest that the HMW-hEGF purified from human urine may play a role in the biosynthetic precursor of hEGF. Furthermore, if the HMW-hEGF might have a much longer half-life than hEGF, it is possible that the HMW-hEGF might not only play a

precursor role, but it might exert hEGF-like effect by itself, therefore making its physiological significance more important. Generally, at the proteolytic processing site of large precursor protein to a corresponding hormone, the pair of basic amino acid residues, such as Lys-Arg, or Arg-Arg or Lys-Lys, occurred. In the case of hEGF, the peptide bonds between Arg-Asn at the N-terminus and Arg-His at the C-terminus are cleaved for the processing. It is curious that the processing of prepro EGF to the HMW-hEGF appears to occur between Met-Val at the N-terminus. It is difficult to understand why this site is cleaved and what kind of enzyme will hydrolyze it. Now antibodies against the HMW-hEGF are being prepared in our laboratory to make a further investigation about its characters. For example, to know the distribution of the HMW-hEGF in the human body using this antibody will serve to understand the physiological function and metabolism of hEGF.

REFERENCES

1. Cohen,S. (1962) J.Biol.Chem. 237, 1555-1562.
2. Savage,C.R., Jr., Inagami,T. and Cohen,S. (1972) J.Biol.Chem. 247, 7612-7621.
3. Carpenter,G. and Cohen,S. (1979) Annu.Rev.Biochem. 48, 193-216.
4. Cohen,S. and Carpenter,G. (1975) Proc.Natl.Acad.Sci.U.S.A. 72, 1317-1321.
5. Gregory,H. (1975) Natuer 257, 325-327.
6. Hirata,Y. and Orth,D.N. (1979) J.Clin.Endocrinol.Metab. 48, 673-679.
7. Hirata,Y. and Orth,D.N. (1979) J.Clin.Endocrinol.Metab. 49, 481-483.
8. Gregory,H., Walsh,S. and Hopkins,C.R. (1979) Gastroenterol. 77, 313-318.
9. Oka,Y. and Orth,D.N. (1983) J.Clin.Invest. 72, 249-259.
10. Oka,T., Sakamoto,S., Miyoshi,K., Fuwa,T., Yoda,K., Yamasaki,M., Tamura,G. and Miyake,T. (1985) Proc.Natl.Acad.Sci.U.S.A. 82, 7212-7216.
11. Laemmli,U.K. (1970) Nature, 227, 680-685.
12. Righetti,P. and Drysdale,J.W. (1971) Biochim.Biophys.Acta. 236, 17-28.
13. Hunkapiller,M.W., Hewick,R.M., Dreyer,W.J. and Hood,L.E. (1983) Methods in Enzymol. 91, 399-413.
14. Tsunasawa,S., Kondo,J and Sakiyama,F. (1985) J.Biochem. 97, 701-704.
15. Bell,G.I., Fong,N.M., Stempien,M.M., Wormsted,M.A., Caput,D., Ku,L., Urdea,M.S., Rall,L.B. and Sanchez-Pescador,R. (1986) Nucleic Acid Res. 14, 8427-8446.