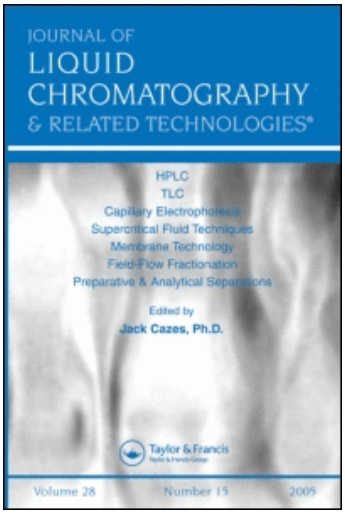

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FREE-SOLUTION CAPILLARY ELECTROPHORESIS OF TRYPTIC DIGEST FRAGMENTS OF A RECOMBINANT PORCINE PRO-GROWTH HORMONE RELEASING HORMONE (2–76)OH

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ABSTRACT

The application of free-solution capillary electrophoresis (FSCE) to the separation of peptide fragments produced by tryptic digestion of a new recombinant porcine pro-growth hormone releasing hormone (2-76)OH (rpGHRH) composed of 75 amino acid residues is presented. It was found that 11 digest peptide fragments of rpGHRH could be separated by FSCE using an uncoated fused silica capillary with 0.1 M phosphate buffer, pH 3.3, used as the separating electrolyte. Individual peptide fragments prepared by solid phase synthesis method were spiked into the tryptic digest mixture to verify structure and fragment assignments of the polypeptide in electrophoresis. Optimal separation conditions were obtained by studying the effect of pH in the separating electrolyte and applied voltage. An excellent

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correlation was found for the electrophoretic migration time, t_m, of the digest peptides versus $q/MW^{2/3}$, where q is the calculated charge, and MW is molecular weight of peptides.

INTRODUCTION

Characterization of the identity and purity of recombinant peptides used as drugs is necessary for manufacturing and recovery processes. Tryptic digest mapping of proteins has been used extensively to provide information about protein structure and purity. One of the useful approaches to characterization is reversed phase high performance liquid chromatography (RP-HPLC) (1,2). Recently, capillary zone electrophoresis (CZE) has been widely employed to separate and characterize biomolecules due to its simplicity, high resolving power, and ability to automate the analyses (3,4). Some papers have reported the use of CZE in separation of peptide mixtures containing 19 peptide fragments produced from the tryptic digest of biosynthetic human growth hormone (5,6). Nielsen et al. (7) compared capillary zone electrophoresis and RP-HPLC to characterize tryptic digests of recombinant human growth hormone. Peaks were identified and the differences in selectivity in the two techniques were reported.

In this paper, an application of free-solution capillary electrophoresis (FSCE) to the separation of the tryptic digest peptides of the recombinant porcine pro-growth hormone releasing hormone (2-76)OH (rpGHRH) (8), composed of 75 amino acid residues, is reported. Phosphate buffer and acidic pH were selected as the separating electrolyte because of its low ultraviolet absorbance at 200 nm and low osmotic flow. Optimal separation conditions have been found by studying the effect of pH and applied voltage in electrophoresis. Correlation of electrophoretic migration time of digest peptides with $q/MW^{2/3}$ was studied.

EXPERIMENTAL

Instrument

High performance capillary electrophoresis was performed on the P/ACE system 2000 (Beckman Instruments, Palo Alto, California, USA) in an uncoated fused silica capillary (75 μm i.d.x 57 cm long to the window) mounted in a cartridge with on-column flow cell for optical detection. Another coated capillary CElect-P175 (57 cm long to the window x 75 μm i.d.) (Supelco Inc., Bellefonte, PA, USA) was mounted in a Beckman cartridge. P/ACE system 2000 version 1.5 software was used to collect data and the System Gold chromatography data system (Beckman) was used to analyze results. The peptides were monitored by UV absorbance at 214 nm.

RP-HPLC was performed on a Waters 625 LC System equipped with a 991+ photodiode array detector (Waters Associates, Bedford, MA, USA) and a Brownlee Aquapore OD-300 column (250 x 7 mm i.d., 7 μm particle size).

Reagents and Materials

rpGHRH was produced at Eli Lilly & Company (Indianapolis, IN, USA). TPCK[L-(tosylamido-2-phenyl)ethylchloromethyl ketone] treated trypsin was purchased from Fluka Chemie AG (Switzerland). Reagent-grade water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA) and was used to prepare all solutions. All other reagents were analytical grade without further purification. All amino acids and resins for solid phase peptides synthesis were purchased from Applied Biosystems, Inc. (Foster City, CA, USA) and Peptides International (Louisville, KY, USA).

Tris-acetate buffer used in the tryptic digestion was prepared by adjusting the pH of 0.1 M Tris solution to 8.6 using acetic acid. Phosphate buffer solutions of different pH were prepared by mixing 0.1 M phosphoric acid and 0.1 M

sodium monobasic phosphate in different ratios. A 0.6 M phosphate buffer (pH 2.4) was used as a rinsing buffer in electrophoresis. Borate buffer solution used as a running electrolyte was purchased from Beckman (Palo Alto, CA, USA).

Method

Synthesis of polypeptide fragments. Peptide fragments were prepared by solid phase peptide synthesis using an Applied Biosystems 430A peptide synthesizer (Applied Biosystems Inc., Foster City, CA, USA). Protected amino acids used in the synthesis were of the L-configuration and the alpha amino groups were exclusively Boc-protected. Side chain functionalities were protected as follows: benzyl for threonine and serine, 2-bromobenzyloxy carbonyl for tyrosine, p-toluene-sulfonyl for arginine, cyclohexyl for aspartic and glutamic acids, benzyloxymethyl for histidine, and formyl for tryptophan. The side chains of asparagine and glutamine were unprotected. All amino acids were singly coupled except for arginine, asparagine, glutamine, and histidine which were doubly coupled. The N-terminal Boc group of each peptidyl resin was removed on treatment with trifluoroacetic acid in dichloromethane and neutralized with N,N-diisopropylethyl-amine in dichloromethane. Each peptidyl resin was dried and treated with 1 mL m-cresol and 10 mL anhydrous hydrogen fluoride per gram of resin. 1,2-ethanedithiol was added to the cleavage mixture for fragment 9. Each cleavage-deprotection was stirred in an ice bath for 60 min. After removal of the hydrogen flouride in vacuo, the free peptides were precipi-tated with ether, filtered, washed with ether, extracted with aqueous acetic acid, and lyophilized. Most products (solubility permitting) were desalted over a Sephadex G-10 column. All peptides fragments were analyzed on a 0.45 x 15 cm Vydac C18 column by gradient elution, and characterized by amino acid analysis (6N hydrochloric acid hydrolysis) and by fast atom bombardment mass spectrometry.

Trypsin digest. One mg of polypeptide rpGHRH was dissolved in 0.5 mL water and then mixed with 0.5 mL Tris-

acetate buffer. Periodically, the solution became cloudy because of poor solubility of the polypeptide in this buffer solution, but digestion results were not affected. A 40 µL of TPCK-treated trypsin solution (1 mg /mL in tris-acetate buffer) was added in the solution and vortexed 1 min. The mixture was then incubated for 4 hrs at 37°C. Aliquots of the digest mixture were frozen (-20°C) for use at a later time.

Capillary zone electrophoresis. The digested peptide mixture was electrophoresed in an uncoated fused silica capillary. Peaks in the peptide map were identified by electrophoresis using a sample solution spiked with relative peptide fragment prepared by solid phase synthesis. Before each run, the capillary was rinsed for 1 min with 0.6 M sodium phosphate buffer, pH 2.4, and then for 1 min with separating electrolyte, 0.1 M phosphate, at the appropriate pH. At the end of each run the capillary was rinsed again with 0.6 M phosphate buffer and conditioned over night with this buffer. A pressure injection was used to apply the sample into the capillary, and the temperature of the capillary cartridge was kept at 22°C.

Reversed phase HPLC. Reversed-phase chromatographic analysis of the digest peptides was performed using a Brownlee Aquapore OD-300 column at room temperature. To enhance separation efficiency, a 7.5 mm I.D. column at a flow rate of 2.3 mL/min was used in this study. Mobile phase consisted of 0.1% aqueous TFA (v/v) (A solvent) and 0.1% TFA in 60% acetonitrile (B solvent). A gradient profile, 0 to 85% B in 50 min, hold 85% B for 5 min, and return to initial condition immediately, was applied. The injection volume was 100 µL and the peptides were detected at 215 nm.

RESULTS AND DISCUSSION

The sequence and molecular weight of 12 tryptic digest peptide fragments of polypeptide rpGHRH are shown in Table 1.

Table 1

TRYPTIC DIGEST OF BIOSYNTHETIC POLYPEPTIDE

Fragment	Amino acid residues	Sequence	MW	Calculated charge
1	10	ADAIFTNNYR	1184.3	1.06
2	1	R	174.2	1.00
3	8	VLTQLSAR	887.1	1.44
4	1	R	174.2	1.00
5	8	LLQDILSR	957.2	1.06
6	5	QQGER	616.6	1.38
7	7	NQEQGAR	801.8	1.38
8	2	VR	273.3	1.44
9	3	LGR	344.4	1.44
10	10	QVDSLWADQR	1217.3	0.67
11	16	QLALESILATLLQEHR	1835.2	2.32
12	4	NSQG	404.4	0.44

MW = Molecular weight

The digest mixture containing all of the peptide fragments were separated by FSCE. It was found that poor resolution and fewer peaks were obtained in electrophoresis using pH 9 borate buffer as a separating electrolyte (Fig. 1), but the digest peptides could be completely separated by FSCE when a 0.1 M phosphate buffer (pH 3.3) was used (Fig. 2).

All separations in this study were performed on an uncoated capillary using acidic phosphate buffers. Use of low pH buffer significantly reduces the magnitude of the electroosmotic flow through the silica capillaries since silanols on the capillary wall become more protonated. In addition, low pH values minimized wall interaction because of

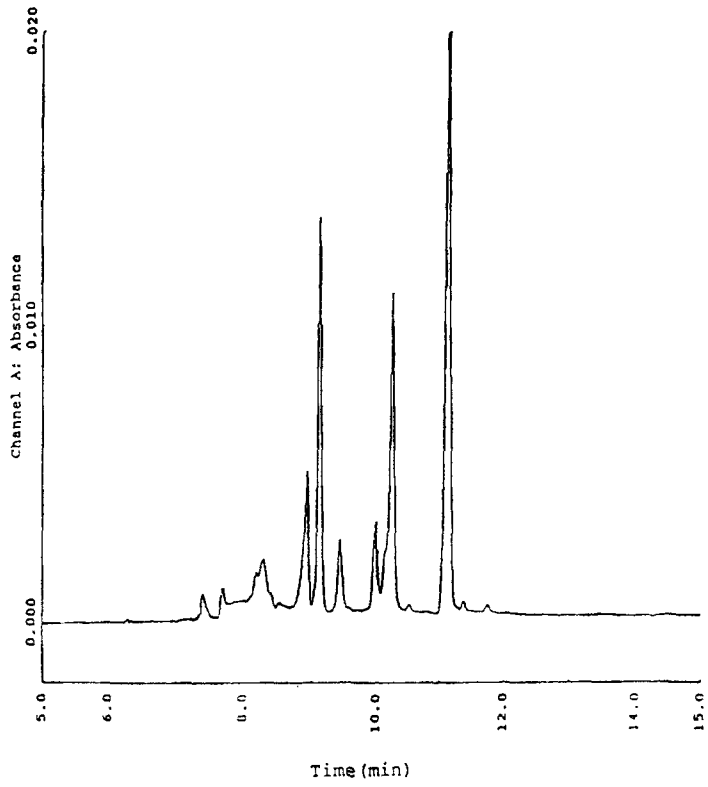


Fig. 1
Electropherogram of peptide fragments from tryptic digestion of polypeptide rpGHRH. Electrophoresis condition: field, 350V/cm; current, 9 μA; Injection, 3 seconds by pressure; separating electrolyte, 0.1 M borate pH 8.6; other conditions given in text.

proteins with higher positive charge (9). Phosphate has also been reported to reduce the interaction of proteins with polysilicic acid (10). A study was conducted to determine the optimal pH for separation of the digest peptides in 0.1 M phosphate buffer at 15 kV applied voltage. Five pH values ranging from 2.0 to 3.3 were selected for this study. As

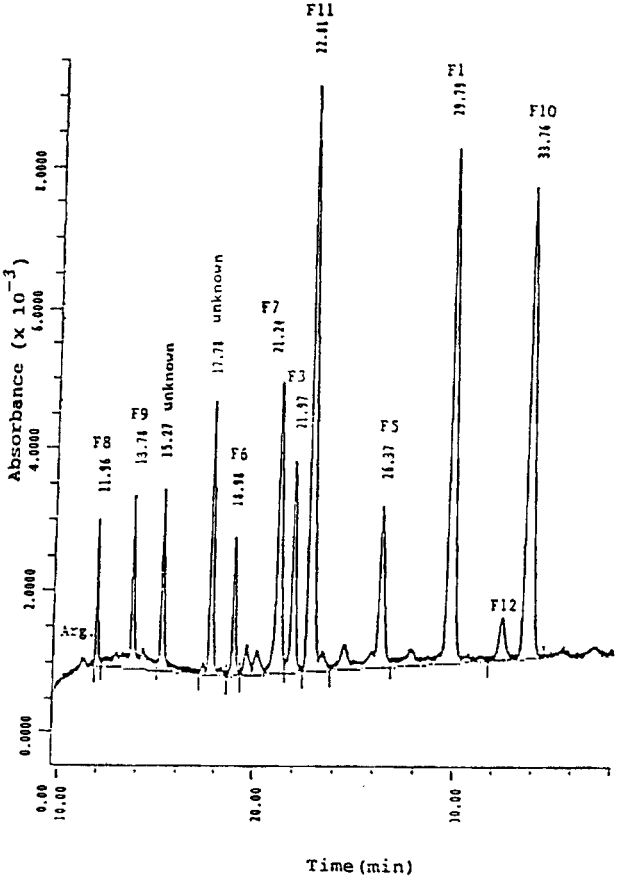


Fig. 2
Electropherogram of peptide fragments from tryptic digestion of polypeptide rpGHRH. Electrophoresis conditions: same as in Figure 1 except field, 263 V/cm; current, 100 μA and separating electrolyte, 0.1 M phosphate, pH 3.3.

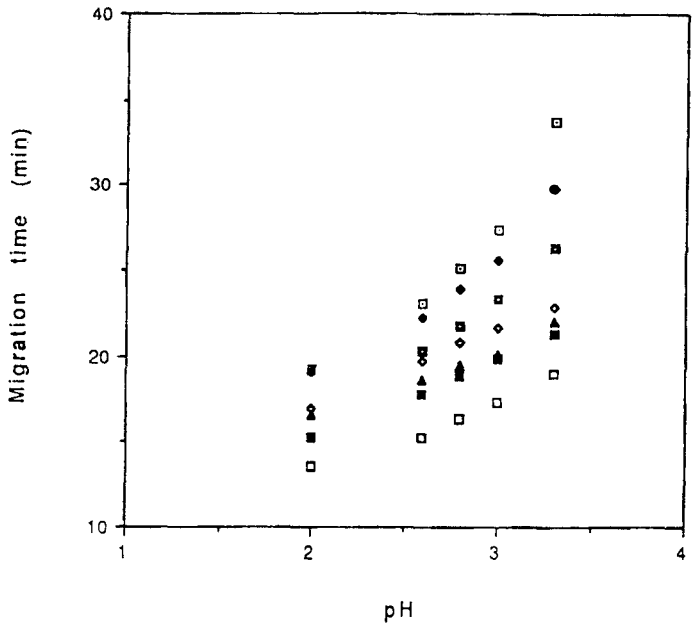


Fig. 3
Effect of pH on the migration time of digest peptide fragments. Electrophoresis conditions: same as in Figure 2 except pH was changed. ⊡–F10; ◆–F1; ◘–F5; ◇–F11; ▲–F7; ■–F6; □–F3.

shown in Fig. 3, the migration times t_m of digest peptides increased with increasing pH of separating electrolyte. It was observed that poor resolving power for fragments F1 and F10; F11 and F5; F6, F8, and F9 was obtained at pH 2.0. The best separation was reached in the separating electrolyte of 0.1 M phosphate buffer at pH 3.3. Under these conditions, all 11 peptides can be excellently resolved as shown in Fig. 2. The peak assignment was accomplished by spiking individual synthesized fragments. There are two unknown peaks on the electropherogram which will be studied in future work.

The effect of applied voltage in capillary electrophoresis has been studied. From Fig. 4, it can be seen that the resolution of peptide separation at four different voltages is similar, but F12 can only be resolved with F10 under the condition of 15kV applied voltage.

In order to improve the reproducibility of migration time in electrophoresis, a capillary rinsing procedure (11) was used in this study. The capillary was rinsed with 0.6 M phosphate buffer (pH 2.4) prior to run and stored overnight in the same buffer. Results of reproducibility in migration time are shown in Table 2.

In most cases, polymer-coated capillaries for capillary electrophoresis can reduce electroendosmosis and eliminate electro-interaction between silanols on the wall surface and proteins or peptides. In this work, we have compared the resolution of peptide fragments on the uncoated and the capillary coated with hydrophilic layer on the wall surface under the identical experiment conditions. From Fig. 5, it can be seen that a comparable result in the separation of digest peptide mixture was obtained on the coated capillary, but fragments F3 and F7 could not be resolved.

Comparing the tryptic digest peptide map by FSCE with that obtained from RP-HPLC, some peptides such as F6, F12, F2, F4 (Arginine) were co-eluted on RPHPLC (Fig. 6), but these peptides can be completely separated by FSCE. Assignment of peptide fragments was carried out by spiking individual peptides prepared by solid phase synthesis into the unseparated digest mixture under the same separation conditions.

Differences in peak elution order between RP-HPLC and FSCE are apparent. In RP-HPLC, the smaller the more hydrophilic peptide, generally, the lower the retention time is. For example, F2 and F4 are arginine, a single amino

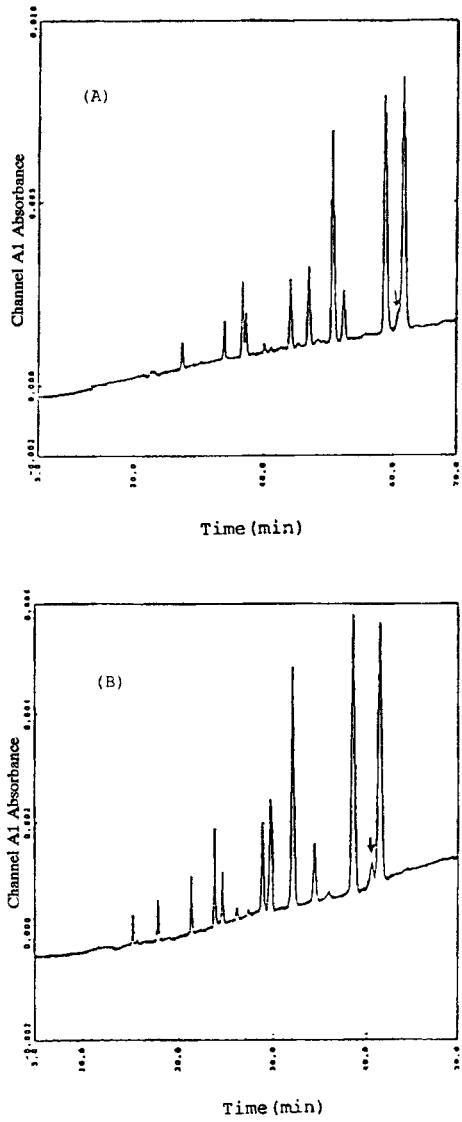
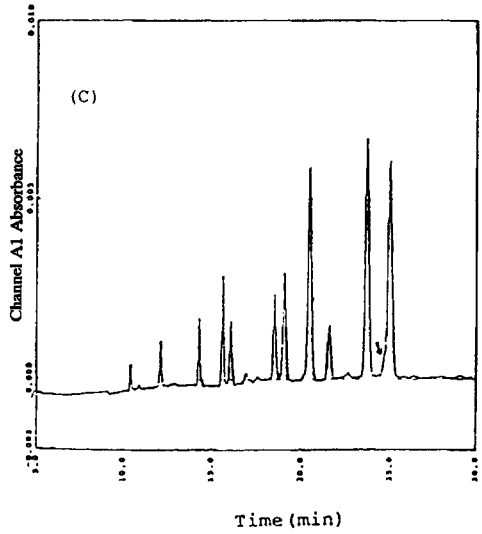


Fig. 4
Effect of field voltage on resolution of digest peptide fragments in FSCE. Electrophoresis conditions: same as in Figure 2, field applied voltage (A) 10kV; (B) 15 kV; (C) 20 kV; (D) 25 kV.



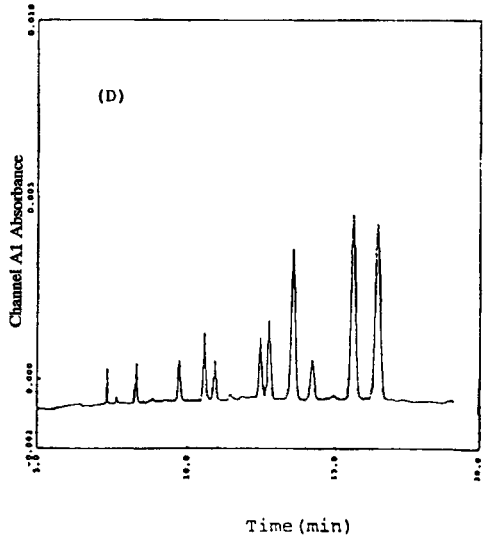


Fig. 4 (continued)

Table 2

REPRODUCIBILITY OF MIGRATION TIME t_m

Fragment	N	Average t_m	RSD %
F8	5	8.22	0.46
F9	5	9.81	0.53
F6	5	12.63	0.57
F7	5	14.34	0.57
F3	5	15.05	0.53
F11	5	15.82	0.56
F5	5	16.23	0.60
F1	5	17.66	0.57
F10	5	18.18	0.62

acid; consequently, it was eluted early in the void volume. F1 and F10 are decapeptides and F11 is a hexadecapeptide which are more hydrophobic than arginine; therefore, those peptides were eluted later in RPHPLC. In CZE, under the same experimental condition, the electrophoretic mobility depends on the net charge and molecular size of the analyte. From Fig. 2 and Table 1 we can see that the migration time of F10 is smaller than that of F1 due to a lower charge of F10 (0.67) even though the molecular weight of both peptides are similar. F11 has the largest molecular weight, 1837, and more charge, 2.32, so that it shows a short migration time. F12 , a tetrapeptide, has the least calculated charge (0.44) among digested peptides. As a consequence, it shows a longer migration time in electrophoresis. The values of calculated charge in Table 1 were calculated by using the same approach reported in the literature (12).

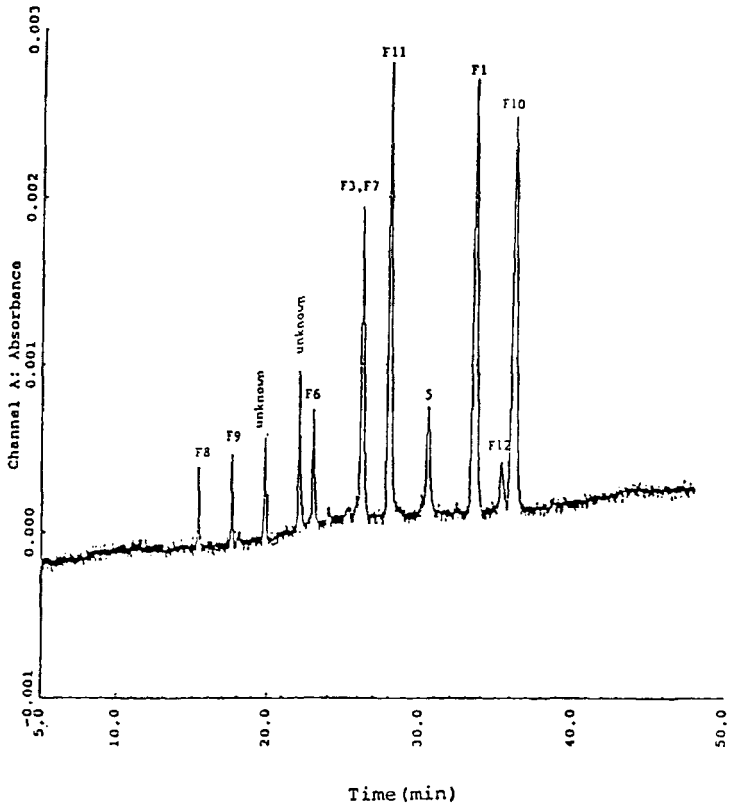


Fig. 5
Electropherogram of tryptic digest peptides of rpGHRH on the coated fused silica capillary. Conditions: same as in Figure 2.

Recently, a linear correlation between the electrophoretic mobility measured by FSCE and the ratio of the net charge to molecular weight of peptides has been reported (12). These results indicated that the mobility of peptides is proportional to the surface area (2/3 power of the molecular weight) of peptides. Based upon that result, the reciprocal of migration time, $1/t_m$, of peptide fragments,

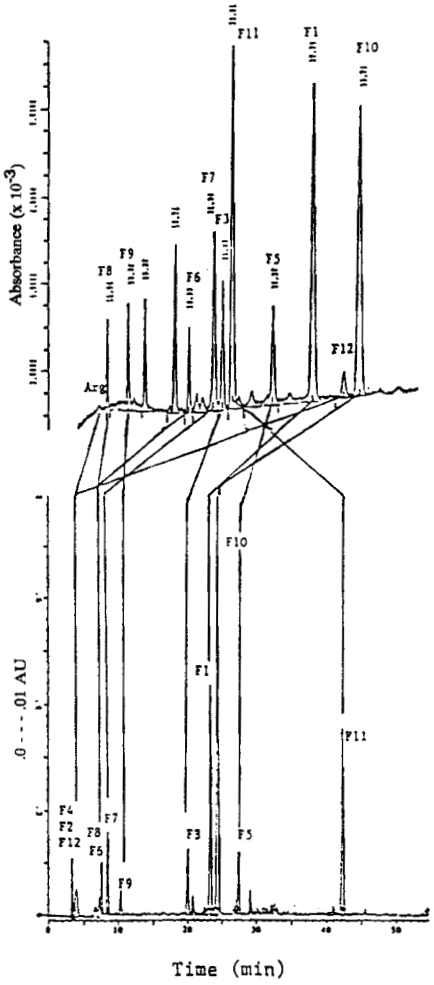


Fig. 6

Comparison of FSCE (A) and RPHPLC tryptic digest peptide maps. Electrophoresis conditions: same as in Figure 2. Chromatographic conditions: as described in the text.

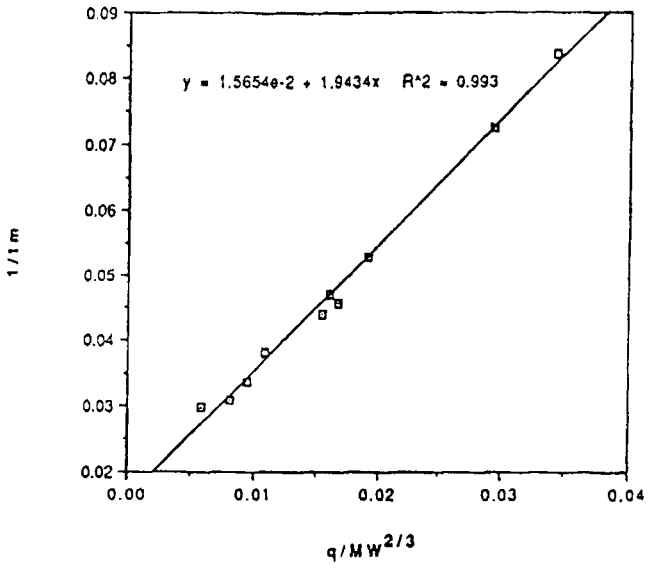


Fig. 7
Linear correlation curve of the reciprocal of electrophoretic migration time $1/t_m$ vs calculated charge to molecular size for digest peptides separated in pH 3.3, 0.1 M phosphate buffer. Equation and correlation coefficient for the linear least squares line are given.

obtained from FSCE in this study, were correlated with the ratio of q/MW, where q is the calculated charge (see Table 1), and MW is molecular weight of peptide fragments. A similar conclusion as in the literature (12) was reached from the results in this study. An excellent linear correlation was found for the $1/t_m$ in electrophoresis versus $q/MW^{2/3}$ (Fig. 7). The correlation coefficient (r) was 0.993. A slightly lower correlation coefficient (r = 0.944) was obtained in the correlation with $MW^{-1/2}$. The correlation coefficient for the Stoke's radius ($MW^{-1/3}$) of peptide molecule was only 0.780.

CONCLUSION

CZE is a valuable method for primary structure determination and uses as a "finger-print" to identify a recombinant peptide rpGHRH. FSCE in a system of the uncoated capillary-acidic phosphate medium provides high efficiency for the tryptic peptide separation with resolving power comparable to that on the coated capillary. The separation obtained in this study demonstrated that the variation of pH may provide optimal efficiency and resolution for the CZE of tryptic peptides of rpGHRH. The resolving power in the CZE separation is slightly better than that in the RPHPLC.

An excellent correlation for the $1/t_m$ versus $q/MW^{2/3}$ was found in this study. It is consistent with the results reported in the literature.

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