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Journal of Chromatography B, 656 (1994) 99–106

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Application of capillary and free-flow zone electrophoresis and isotachophoresis to the analysis and preparation of the synthetic tetrapeptide fragment of growth hormone-releasing peptide

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Abstract

The use of high-performance electromigration separation methods, capillary zone electrophoresis (CZE) and capillary isotachophoresis (CITP) and continuous free-flow arrangements of these two separation principles, free-flow zone electrophoresis (FFZE) and free-flow isotachophoresis (FFITP), was investigated in the analysis and purification of the synthetic C-terminal tetrapeptide fragment (H_2N–Ala–Trp–D-Phe–Lys · NH_2) of the growth hormone-releasing peptide. CZE and CITP were used for microanalysis of peptide preparations after different steps of their purification. The homogeneity of the peptide preparations, including fractions of preparative separations, was quantified by relative zone length (CITP) and/or relative peak height (CZE). In addition, the data obtained by CZE and CITP (electrophoretic and electroosmotic flow migration velocities) were utilized for conversion of micro-scale capillary separations (nano- to picomole level) into the preparative separations realized by FFZE and FFITP with a capacity from tens to hundreds of milligrams per hour.

1. Introduction

High-performance electromigration separation methods, capillary zone electrophoresis (CZE) and capillary isotachophoresis (CITP), are being used increasingly as highly sensitive and effective tools for analyses at nano- to picomole levels of synthetic biologically active peptides [1–4]. They serve as a complement or counterpart to the most commonly used separation technique for the analysis and preparation of synthetic peptides, reversed-phase high-performance liquid chromatography (RP-HPLC).

Whereas for analytical purposes the advantages of high-performance capillary electrophoresis (HPCE) over HPLC are apparent (10–100 times higher separation power, achieving 10^5–10^7 theoretical plates per metre, high sensitivity in the nano- to femtomole range, carrierless

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SSDI 0378-4347(94)00042-4

biocompatible separation medium, low running costs), the application potential of HPCE for the preparation of synthetic peptides is substantially lower than that of HPLC. This is due not only to the more complicated adaptation of analytical HPCE systems to preparative systems [5] than in HPLC, but also to the relatively low capacity of the capillary systems.

Because of the small dimensions of capillaries (typical I.D. 0.050–0.100 mm for CZE and 0.2–0.5 mm for CITP), the preparative capacity of HPCE is limited to nano- to microgram amounts of peptides. An alternative way to extend the preparative capacity of electrophoretic separations is to realize ZE or ITP separations in a continuous thin-layer free-flow arrangement in a flow-through electrophoretic chamber [6–8]. As both capillary and free-flow (FF) separations are performed in a free solution and in the same electrolyte systems, the data obtained by CZE and CITP, namely electrophoretic and electroosmotic flow migration velocities, can be utilized for the development and optimization of separation conditions for FFZE and FFITP. The theoretical background for the procedure was described in previous papers [9,10].

The aim of this work was to show the applicability of CZE and CITP to the analysis of the synthetic C-terminal tetrapeptide of the growth hormone-releasing peptide, to determine the purity of the peptide preparation after different steps of its purification and to include FFZE and FFITP in the preparative purification of this peptide.

2. Experimental

2.1. Instrumentation and methods

CZE was performed on the experimental device developed in the Institute of Organic Chemistry and Biochemistry (Prague, Czech Republic) [9]. It consists of an untreated fused-silica capillary with an outer polyimide coating (I.D. 0.05 mm, O.D. 0.120 mm, effective length 500 mm, total length 650 mm) and a UV spectrophotometric detector set at 206 nm. The separations were performed in the constant-current mode (8 μA, 16.5–17 kV) at ambient temperature (22–24°C) without active cooling of the separation compartment. The sample was introduced manually, forming a hydrostatic pressure (50 mm of water column) for 5–15 s at the injection end of the capillary. Acetic acid (0.5 mol/l) was used as the background electrolyte (BGE) and the peptide samples were dissolved in this BGE in concentrations of *ca.* 1 mg/ml.

CITP was performed with a CS-ITP analyser (Institute of Radioecology and Applied Nuclear Techniques, Spišská Nová Ves, Slovak Republic; this apparatus is now commercially available from Villa, Spišská Nová Ves, Slovak Republic), in the column-coupling configuration of the separation unit [11] and equipped with two conductivity and one UV spectrophotometric detector. The characteristics of the device and the experimental conditions of CITP analyses are summarized in Table 1.

FFZE and FFITP experiments were carried out with a VaP 22 free-flow electrophoresis system (Bender-Hobein, Munich, Germany; now commercially available from Dr. Weber GmbH, Ismaning, Germany). The core of this system is a flow-through electrophoretic chamber with the dimensions of height (direction of hydrodynamic flow) 500 mm, width (direction of electric field) 100 mm and depth (thickness of electrolyte layer) = 0.5 mm. The chamber wall material is mirror glass (rear side) and plastic [poly(methyl methacrylate)] (front side). Joule heat is removed by rear-side water cooling. At the outlet side of the chamber the solution is collected in 96 fractions. The chamber is equipped with an online moving-spot UV spectrophotometric detector set at 254 nm. The experimental conditions for the FFZE and FFITP separations are summarized in Table 2.

2.2. Chemicals

All chemicals were of analytical-reagent grade. Sodium hydroxide and acetic acid (AcOH) were obtained from Lachema (Brno, Czech Republic); acetic acid was distilled before use. β-Alanine (BALA) was purchased from Koch-

Table 1
Characteristics of CITP device (CS-ITP analyser) and experimental conditions of CITP analyses

Characteristic	Compartment	
	Preseparation	Analytical
Capillary		
Material	FEP[a]	FEP[a]
I.D./O.D. (mm)	0.8/1.2	0.3/0.7
Effective length (mm)	190	150; 180[b]
Total length (mm)	230	230
Detection	a.c. conductivity	Spectrophotometric (254 nm); a.c. conductivity
Current (constant) (μA)	250	50
Voltage (kV)	2.5–5	4–9
Temperature (ambient) (°C)	22–24	22–24
Leading electrolyte (LE)	0.01 *M* NaOH–AcOH (pH 4.8)	
Terminating electrolyte (TE)	0.01 *M* BALA–AcOH (pH 4.2)	
Sample		
Application	Manual, microsyringe (1–10 μl)	
Solvent	LE	
Concentration (mg/ml)	1	

[a] Fluorinated ethylene–propylene copolymer.
[b] Effective lengths to the spectrophotometric and conductivity detector, respectively.

Table 2
Experimental conditions for FFZE and FFITP

Parameter	FFZE	FFITP
Background electrolyte (BGE)		
Chamber	0.5 *M* AcOH	–
Electrode	1 *M* AcOH	–
Leading electrolyte (LE)		
Chamber	–	0.01 *M* NaOH–AcOH (pH 4.8)
Electrode	–	0.03 *M* NaOH–AcOH (pH 4.8)
Terminating electrolyte (TE)		
Chamber	–	0.01 *M* BALA–AcOH (pH 4.2)
Electrode	–	0.03 *M* BALA–AcOH (pH 4.2)
Sample solvent	BGE	LE
Sample concentration (mg/ml)	15	15
Flow-through time (s)	350	790
Sample flow rate (ml/h)	1	10
Coolant temperature (°C)	4	10
Voltage (constant) (V)	700	650
Current (mA)	108	22

Light (Colnbrook, UK). The C-terminal peptide fragment of growth hormone-releasing peptide (GHRP) was synthesized in the Institute of Organic Chemistry and Biochemistry (for more details, see Results and Discussion).

3. Results and discussion

3.1. Analysis of synthetic GHRP fragment

CZE and CITP were used for the microanalysis of the synthetic C-terminal tetrapeptide of growth hormone-releasing peptide (GHRP). GHRP hexapeptide with the sequence H_2N–His–D-Trp–Ala–Trp–D-Phe–Lys · NH_2 represents a new class of drugs that selectively stimulate the release of growth hormone but not other pituitary hormones [12]. GHRP or its analogues and fragments may have an important application in agriculture (stimulation of growth and/or milk production) and in medicine (treatment of growth hormone deficiency syndromes) [13]. GHRP fragment, C-terminal tetrapeptide of GHRP, *i.e.*, with the sequence H_2N–Ala–Trp–D-Phe–Lys · NH_2, was synthesized by the solid-phase method on 4-methylbenzhydrylamine resin (1% cross-linked divinylbenzene, 0.9 mequiv./g; Peptides International, Louisville, KY, USA) [14]. The crude product of GHRP fragment was desalted on a Sephadex G-10 column, eluted with 0.2 mol/l acetic acid and lyophilized. The lyophilizate was dissolved in background electrolyte (0.5 mol/l acetic acid) for CZE analysis and in leading electrolyte (LE) (see Table 1) for CITP analysis. CZE analysis of the tetrapeptide is shown in Fig. 1 and CITP analysis in Fig. 2.

For quantitative evaluation of peptide purity, the so-called ITP degree of purity and CZE degree of purity were used [15]. The ITP degree of purity of peptide P, p_{ITP} is defined as the ratio of zone length of the peptide P itself, l_P, to total zone length, l_T, of all UV-positive zones on the isotachopherogram of a preparation of peptide P. The total zone length l_T is given by the sum of all (n) zones present on the isotachopherogram (including the zone of peptide P itself):

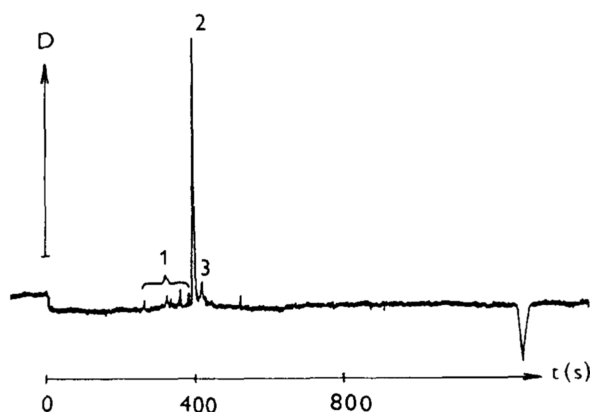

Fig. 1. CZE analysis of GHRP tetrapeptide. Crude synthetic product was desalted on Sephadex G-10 and lyophilized; 0.5 mg of the lyophilizate was dissolved in 0.4 ml of 0.5 mol/l acetic acid and applied to analysis. The other experimental conditions are given in the text. Peaks: 2 = main synthetic product; 1 and 3 = contaminants with (1) higher and (3) lower electrophoretic mobility than the main component. D = Absorption (optical density) at 206 nm; $D = 1 - T$, where T is transmittance; t = time.

$$p_{ITP} = l_P/l_T = l_P \Big/ \sum_{i=1}^{n} l_i \qquad (1)$$

The CZE degree of purity, p_{CZE}, was determined as the ratio of the peak height of peptide P itself, h_P, to the sum of heights of all (n) peaks present on the CZE electropherogram of the given preparation of peptide P:

$$p_{CZE} = h_P \Big/ \sum_{i=1}^{n} h_i \qquad (2)$$

The results of CZE and CITP analyses (see Figs. 1 and 2 and Table 3) show a relatively high content of the main synthetic product in the desalted crude synthetic preparation. However, the presence of contaminants, by-products of the peptide synthesis, is also evident. The results of CZE and CITP are in qualitative agreement, *i.e.*, in both instances admixtures with both higher and lower mobilities than the main synthetic product were found. On the isotachopherogram the individual admixtures are less resolved than on the CZ electropherogram because of direct neighbouring of short ITP zones.

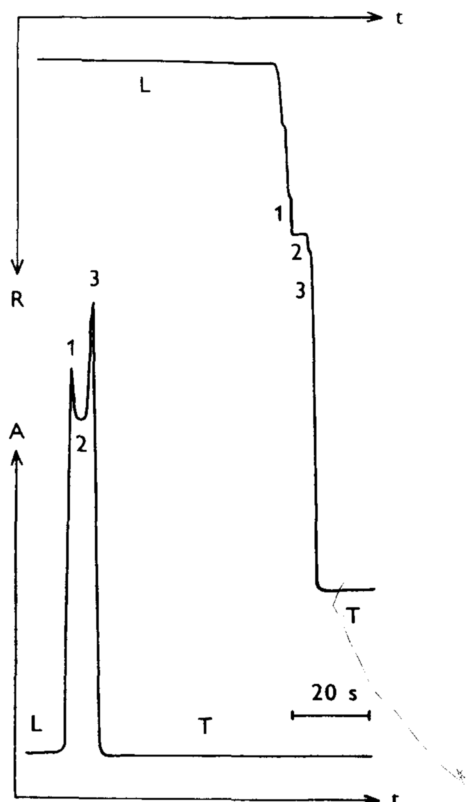

Fig. 2. CITP analysis of GHRP tetrapeptide. The sample is of the same origin as in Fig. 1; 0.5 mg of the lyophilizate was dissolved in 0.1 ml of leading electrolyte; applied sample volume, 2 μl; other experimental conditions as in Table 1. Peaks: 2 = main synthetic product; 1 and 3 = contaminants. A = Absorbance at 254 nm; R = resistance of conductivity detector; t = time; L = leading electrolyte; T = terminating electrolyte.

3.2. Preparation of GHRP fragment

The successful separations of the contaminants from the main synthetic product by ITP and ZE separation principles in the capillary systems led us to the idea of including these separation principles also in the preparative purification of GHRP fragment.

As tens to hundreds of milligrams of peptide need to be purified, it is not possible to realize this purification in capillary systems. Based on the previously described simple mathematical model of the correlation between CZE and FFZE [10] and utilizing the results of CZE and CITP, the analytical micro-scale CZE and CITP separations were converted into preparative FFZE and FFITP separations.

From the experimental conditions of CZE and CITP and from CZE data (electrophoretic velocities and electroosmotic flow velocities calculated from migration times of charged components and from the migration time of an uncharged electroosmotic flow marker), the main parameters of FFZE and FFITP experiments, *e.g.*, mean flow-through time, clamp voltage, sample flow-rate and compositions of the BGE, LE and TE, were derived (see Tables 1 and 2).

The lyophilizate of the desalted crude product of GHRP tetrapeptide was preparatively separated by FFZE and FFITP. The record of FFZE separation, *i.e.*, off-line-measured absorption of individual FFZE fractions, is shown in Fig. 3. The record of the FFITP separation (see Fig. 4) was obtained by on-line measurement of the absorption inside the flow-through chamber through a quartz window with a moving-spot UV spectrophotometric detector at 254 nm.

Comparison of Figs. 1 and 3 shows the qualitative similarity of the CZE and FFZE separation patterns. In addition to the main peak, representing the GHRP tetrapeptide, the contaminants with higher and lower mobilities than that of the GHRP fragment are present. Because

Table 3
Degree of purity of different GHRP fragment preparations and preparative capacity of FFZE and FFITP separations

GHRP fragment preparation	p_{CZE}	p_{ITP}	q (mg/h)
Crude product	0.69	0.57	–
FFZE preparation	0.96	–	15
FFITP preparation	0.83	–	150

p_{CZE} = CZE degree of purity; p_{ITP} = ITP degree of purity; q = capacity of preparative separation.

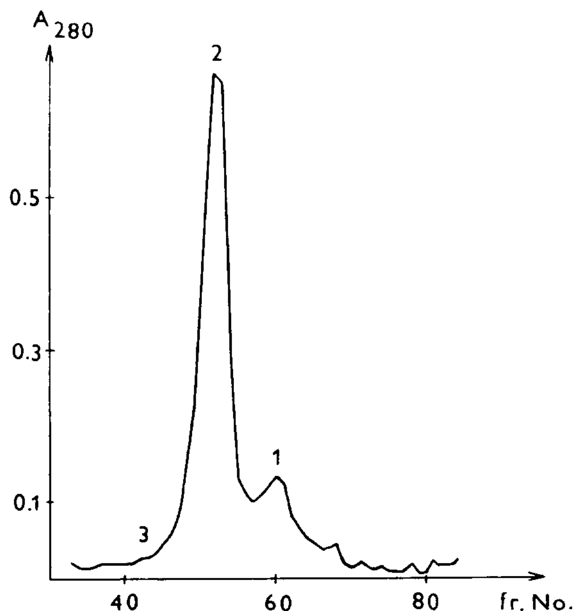

Fig. 3. FFZE separation of the desalted crude synthetic product of GHRP tetrapeptide. Peaks: 2 = main synthetic product; 1 and 3 = contaminants. A_{280} = Absorbance at 280 nm; fr. No. = number of fraction. For experimental conditions, see Table 2 and section 2.1.

of the much higher separation power of CZE than that of FFZE [9,10], these contaminants are much better resolved in CZE than in FFZE.

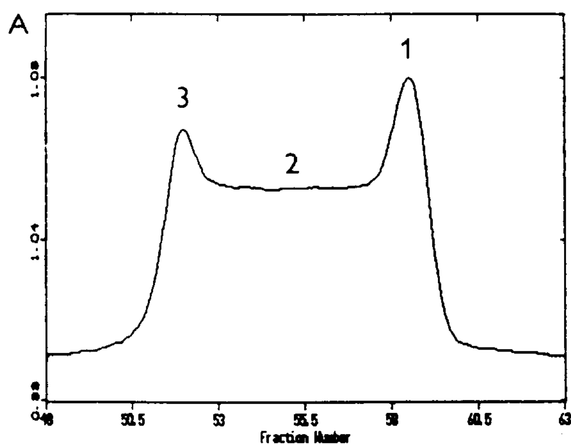

Fig. 4. FFITP separation of the desalted crude synthetic product of GHRP tetrapeptide. Peaks: 2 = main synthetic product; 1 and 3 = contaminants. A = absorbance at 254 nm. For experimental conditions see Table 2 and section 2.1.

Whereas in CZE individual admixture components are separated, in FFZE only a "global" and not complete separation of slower and faster components from the main product is achieved.

Similarly to CZE and FFZE, CITP and FFITP also show relatively close agreement (see Figs. 2 and 4). The UV absorption pattern of the FFZE and FFITP fractions gives only an approximate estimation of the FFZE and FFITP separations. More precise data about the purity of the substances in individual fractions can be obtained by their direct analysis. For this reason all UV-positive fractions from FFZE and FFITP were subjected to CZE analysis. Examples of such two analyses, the CZE analysis of FFZE fractions 52 and 53, *i.e.*, from the top of the peak of the FFZE trace (see Fig. 3) are demonstrated in Fig. 5.

From the CZE analyses of all fractions a more precise reconstruction of FFZE and FFITP separations with respect to the distribution of individual sample components can be obtained (see Figs. 6 and 7). In Figs. 6 and 7 the peak heights of three classes of components (main product, contaminants with higher mobility and contaminants with lower mobility than that of the main product) are plotted against fraction number of FFZE and FFITP separations.

From the peak heights of CZE analyses of FFZE and FFITP fractions, the degree of purity, p_{CZE}, of the main synthetic product of GHRP-frag. was calculated according to Eq. 2 for different fractions of FFZE and FFITP separations (see Fig. 8).

From Figs. 6–8 it follows that the purest preparation of GHRP fragment was obtained from the top of the peak of the FFZE separation where only minority admixtures were revealed by CZE analysis (see Fig. 5 and Table 3). FFITP seems to be, at least under the given experimental conditions, less convenient for the preparative purification of GHRP fragment, as part of the admixtures remains in all fractions after FFITP separation of the crude synthetic product. Although a plateau of the main component can be observed between two adjacent admixture zones (see Fig. 4), the real ITP steady-state apparently was not established. However, even

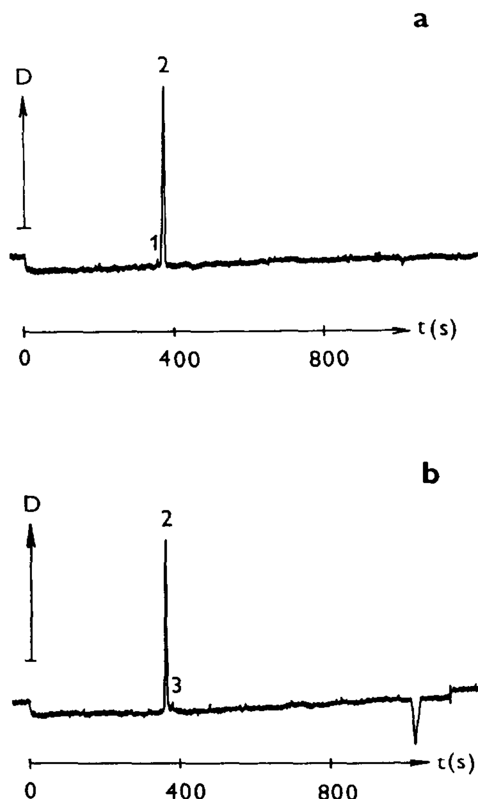

Fig. 5. CZE analysis of (a) fraction 52 and (b) fraction 53 from FFZE separation of GHRP fragment presented in Fig. 3. Nanolitre aliquots of FFZE fractions were analysed under the same conditions as in Fig. 1. Peaks: 2 = main synthetic product; 1 and 3 = contaminants. D = absorption (optical density) at 206 nm; t = time.

in this instance an evident increase in the degree of purity of GHRP fragment was achieved (see Table 3). The advantage of FFITP separation is about a one order of magnitude higher preparative capacity in comparison with FFZE (see Table 3). For better separability of the contaminants, the separation conditions should be further optimized.

4. Conclusions

It can be concluded that the combinations CZE–FFZE and CITP–FFITP provide efficient methods for the analysis and preparation of synthetic biologically active peptides. Their ap-

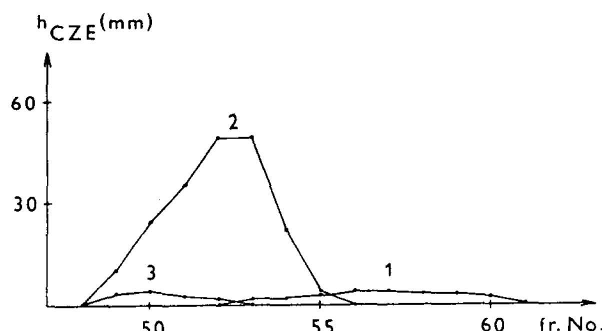

Fig. 6. FFZE separation of GHRP fragment (see Fig. 3) evaluated by CZE analysis of FFZE fractions. For CZE conditions see Fig. 1. h_{CZE} = Peak height of sample components of FFZE fractions; fr. No. = fraction number. 2 = Peak height of main synthetic product; 1 (3) = sum of peak heights of sample components with higher (lower) mobility than GHRP fragment.

plication is recommendable especially when interactions of the separated peptides with the solid-phase material of the HPLC column cause a loss of the peptide and/or a loss of its biological activity. Unlike HPLC, in these electrophoretic techniques the separation is performed in a free solution, in a carrierless medium and

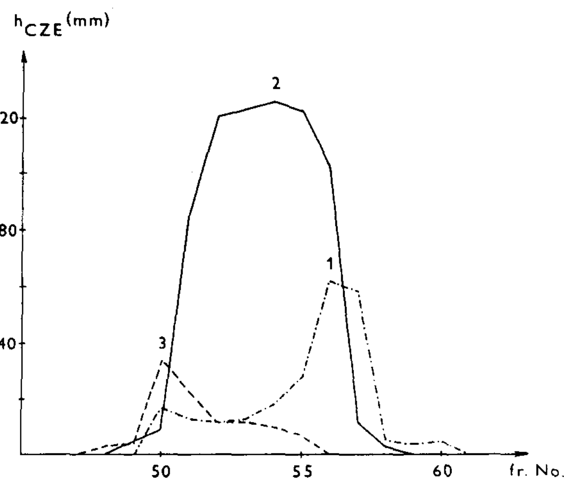

Fig. 7. FFITP separation of GHRP fragment (see Fig. 4) evaluated by CZE analysis of FFITP fractions. For CZE conditions see Fig. 1. h_{CZE} = Peak height of sample components of FFITP fractions; fr. No. = fraction number. 2 = Peak height of main synthetic product; 1 (3) = sum of peak heights of sample components with higher (lower) mobility than GHRP fragment.

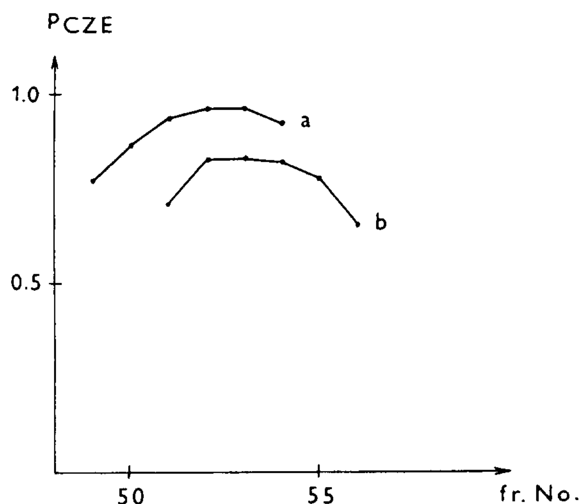

Fig. 8. Degree of purity, p_{CZE}, of the GHRP fragment in the different fractions of (a) FFZE and (b) FFITP separations.

under mild conditions, where the biological activity of the peptide is retained and where the losses of the separated peptides are minimal.

5. Acknowledgements

Mrs. V. Lišková is thanked for skilful technical assistance. This work was supported by the Grant Agency of the Czechoslovak Academy of Science, Grant No. 45511.

6. References

[1] M.V. Novotny, K.A. Cobb and J. Liu, *Electrophoresis*, 11 (1990) 735.
[2] V. Kašička and Z. Prusík, *J. Chromatogr.*, 569 (1991) 123.
[3] W.G. Kuhr and C.A. Monnig, *Anal. Chem.*, 64 (1992) 389R.
[4] M.H.J.M. Langenhuizen and P.S.L. Janssen, *J. Chromatogr.*, 638 (1993) 311.
[5] N. Banke, K. Hansen and I. Diers, *J. Chromatogr.*, 559 (1991) 325.
[6] Z. Prusík, in Z. Deyl (Editor), *Electrophoresis, Part A: Techniques*, Elsevier, Amsterdam, 1979, p. 229.
[7] K. Hannig and H.G. Heidrich, *Free-Flow Electrophoresis*, GIT, Darmstadt, 1989.
[8] H. Wagner and J. Heinrich, in H. Tschesche (Editor), *Modern Methods in Protein and Nucleic Acid Research*, Walter de Gruyter, Berlin, 1990, p. 69.
[9] Z. Prusík, V. Kašička, P. Mudra, J. Štěpánek, O. Smékal and J. Hlaváček, *Electrophoresis*, 11 (1990) 932.
[10] V. Kašička, Z. Prusík and J. Pospíšek, *J. Chromatogr.*, 608 (1992) 13.
[11] F.M. Everaerts, Th.P.E.M. Verheggen and F.E.P. Mikkers, *J. Chromatogr.*, 169 (1979) 21.
[12] C.Y. Bowers, F.A. Momany, G.A. Reynolds and A. Hong, *Endocrinology*, 114 (1984) 1537.
[13] B.E. Ilson, D.K. Jorkasky, R.T. Curnow and R.M. Stote, *J. Clin. Endocrinol. Metab.*, 69 (1989) 212.
[14] J. Hlaváček, O. Smékal, J. Pospíšek and T. Barth, *Collect. Czech. Chem. Commun.*, in press.
[15] V. Kašička and Z. Prusík, *J. Chromatogr.*, 470 (1989) 209.