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Direct analysis of peptides and amino acids from capillary electrophoresis

Tomas Bergman[1], Birgitta Agerberth[2] and Hans Jörnvall[1]

Departments of [1] *Chemistry I and* [2] *Biochemistry II, Karolinska Institutet, S-104 01 Stockholm, Sweden*

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Capillary zone electrophoresis of peptides on a preparative scale and direct analysis of both amino acid sequences and compositions are demonstrated. Using native and synthetic peptides prepared by repetitive runs and appropriate collections, sufficient material for direct sequence analysis is obtained after just a few runs, while single runs suffice for total compositions. Electrophoretic direct amino acid analysis without derivatization is also possible and gives especially high sensitivity for aromatic residues (< 200 fmols) plus convenient distinction of amidated versus free C-terminal residues after carboxypeptidase digestions.

Capillary zone electrophoresis; Amino acid sequence analysis; Amino acid composition; Carboxypeptidase digestion

1. INTRODUCTION

Capillary electrophoresis in different separation modes, here capillary zone electrophoresis, is a powerful method for separation of small peptides [1,2]. The high sensitivity (fmol level), small volumes (nl) and rapid separation (minutes) can be used to conveniently test the purity of peptide fractions separated by conventional HPLC (cf. [3]). However, the small amounts and volumes have been considered a difficulty for use of the method in preparative work, although the high resolution would make it extremely powerful for peptide purifications in structural analyses. We therefore tested capillary zone electrophoresis for preparative purposes and direct analysis for both amino acid sequences and compositions. We also found it useful for further structural analyses, such as direct amino acid determination of, for example, carboxypeptidase-liberated residues.

Results show that repetitive separations are highly reproducible, allowing direct use for preparative purposes. Four to five repeat runs can easily be collected and give more than sufficient material for ordinary sequence analysis in gas-phase or solid-phase instruments, while single runs suffice for reliable total compositions utilizing phenylthiocarbamyl (PTC) amino acid analysis. Finally, underivatized amino acids can be analyzed directly at fmol levels for detection of C-terminal residues and distinction of amidated and free residues after carboxypeptidase digestions. All 3 applications demonstrate the usefulness of capillary electrophoresis for direct peptide analysis.

2. MATERIALS AND METHODS

Peptides were obtained from natural sources as indicated or prepared by solid phase synthesis [4]. Reagents and solvents were of analytical grade. Bovine insulin (Sigma) A and B chains, were obtained by reduction, [^{14}C]carboxymethylation [5], and exclusion HPLC in 30% acetic acid on Ultropac G 2000 at 0.5 ml/min. Peptide digestions were performed with the Glu C endoprotease from *Staphylococcus aureus* and with carboxypeptidase Y [6], both obtained from Boehringer. For HPLC separation, the Glu C digest was analyzed on Vydac C$_4$, developed with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid at 1 ml/min.

For capillary zone electrophoresis, a Beckman P/ACE system 2000, equipped with an IBM PS/2 (Windows-based control including System Gold software) was used. Capillaries were of fused silica (Beckman), with inner diameter 75 μm and total length 57 cm (UV detector at 50 cm). As electrolyte, a 50 mM phosphate buffer, pH 2.5, was employed. Samples were injected by pressurization for 5–30 s. In preparative runs, separated peptides were recovered by a programmed positioning at the capillary outlet of conically shaped microvials containing 5–10 μl electrolyte buffer.

Acid hydrolysis of peptides and amino acid analysis of PTC derivatives were performed as described [7]. Sequencer degradations were carried out with an Applied Biosystems 470A instrument, utilizing PTH analysis by reverse phase HPLC with a sodium acetate/acetonitrile gradient in a Hewlett Packard 1090 instrument [8].

3. RESULTS AND DISCUSSION

3.1. *Direct amino acid sequence analysis*

Resolution by capillary zone electrophoresis and conventional reverse phase HPLC of a Glu C protease digest of [^{14}C]carboxymethylated insulin B chain is shown in Fig. 1. The patterns demonstrate the advantage of capillary zone electrophoresis for analytical pur-

Correspondence address: H. Jörnvall, Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden. Fax: (46) (8) 337462.

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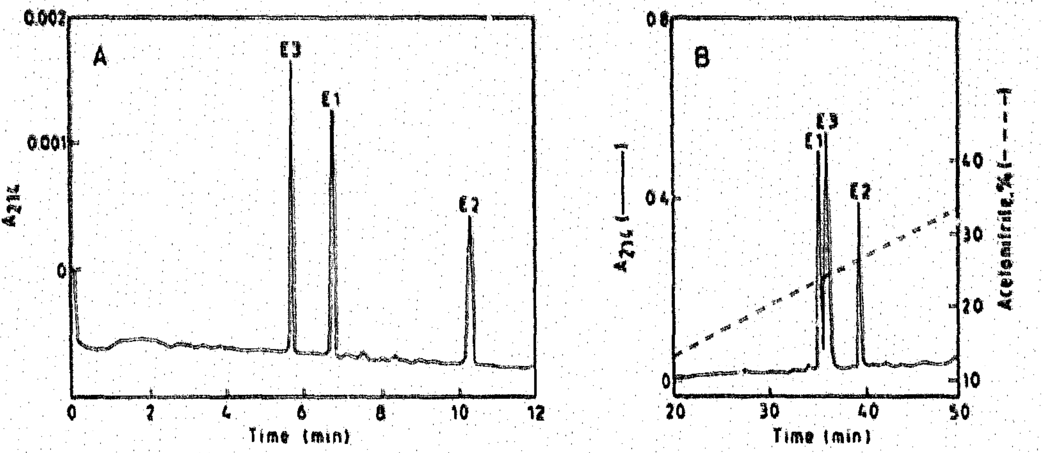

Fig. 1. Separations by capillary zone electrophoresis (A) and reverse phase HPLC (B) of a Glu C endoprotease digest of [^{14}C]carboxymethylated insulin B chain. The peaks correspond to: E1, residues 1-13; E2, residues 14-21; E3, residues 22-30. Amount in (A) was 200 fmol, while in (B) a 5000-fold larger amount (1 nmol) was injected. Conditions for electrophoresis: 30 s pressure injection, 25 kV and 25°C. HPLC: Vydac C_4, developed with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid.

poses, giving considerably shorter separation time (10 instead of 40 min), a higher sensitivity (only 200 fmol peptide in Fig. 1A), and a better resolution (cf. peaks E1 and E3, Figs. 1A,B). However, as now demonstrated, capillary zone electrophoresis can also be used for direct analysis. Thus, after a 24-fold concentration of the digest by freeze-drying and resolubilization in less volume of electrolyte buffer (from initially 1.5 pmol/μl to 36 pmol/μl final solution), peptides E2 and E3 (Fig. 1A) were collected in 4 and 5 preparative runs, respectively. The isolated peptides were directly applied to sequencer degradation and the results are given in Table I. Interpretation up to the C-terminus was possible for both peptides. We can thus conclude that it is feasible to obtain sequence data on peptides recovered from a limited number of capillary zone electrophoresis runs, provided an adequate sample concentration can be obtained (about 40 pmol/μl).

3.2. *Amino acid analysis for total composition*

Capillary zone electrophoresis of a sample containing 3 synthetic hexapeptides (at 0.4 nmol/μl in water/acetic acid/formic acid, 94:5:1, by vol.) is shown in Fig. 2A. To recover peptide 1, it was collected in 4 preparative runs. The amount generated allowed amino acid analysis on just a fraction (cf. legend Table II), showing that single runs at this concentration appear to be sufficient for compositional analysis. The original sample was not concentrated, the only parameter changed was the injection volume that was doubled (from 45 to 90 nl). After this purification, the peptide sample recovered in 10 μl phosphate buffer, was checked for homogeneity by capillary zone electrophoresis. As shown in Fig. 2B, the preparation was pure and the previously closely migrating components (cf. Fig. 2A) have been removed. Peptide 1 thus isolated, was dried and subjected to acid hydrolysis and amino acid analysis. Table II shows the results from 2 different preparations. An excellent reproducibility is demonstrated, and a perfect agreement with the known total composition. Consequently, reliable total compositions can be obtained from samples separated and prepared by capillary zone electrophoresis.

3.3. *Detection of amino acids and derivatives*

A further application now shown, is the separation and direct detection of underivatized amino acids. An unmodified mixture of amino acids was analyzed by

Table I

N-terminal sequence analysis of peptides E2 and E3 (Fig. 1) prepared by capillary zone electrophoresis

Peptide E2			Peptide E3		
Cycle	residue	amount recovered (pmol)	Cycle	residue	amount recovered (pmol)
1	Ala	10	1	Arg	4
2	Leu	11	2	Gly	6
3	Tyr	13	3	Phe	7
4	Leu	8	4	Phe	7
5	Val	7	5	Tyr	7
6	Cys	3[a]	6	Thr	2
7	Gly	3	7	Pro	4
8	Glu	3	8	Lys	3
			9	Ala	2

Peptide E3 was collected in 5 runs and peptide E2 simultaneously in 4 of these. Before preparative runs, the digest (cf. legend Fig. 1) was concentrated 24-fold by volume reduction. Conditions for capillary zone electrophoresis were as described (legend Fig. 1). The total amount from each preparation (19-24 pmol) was applied to a gas-phase sequencer.

[a] Value from radioactivity of [^{14}C]carboxymethyl-cysteine PTH derivative

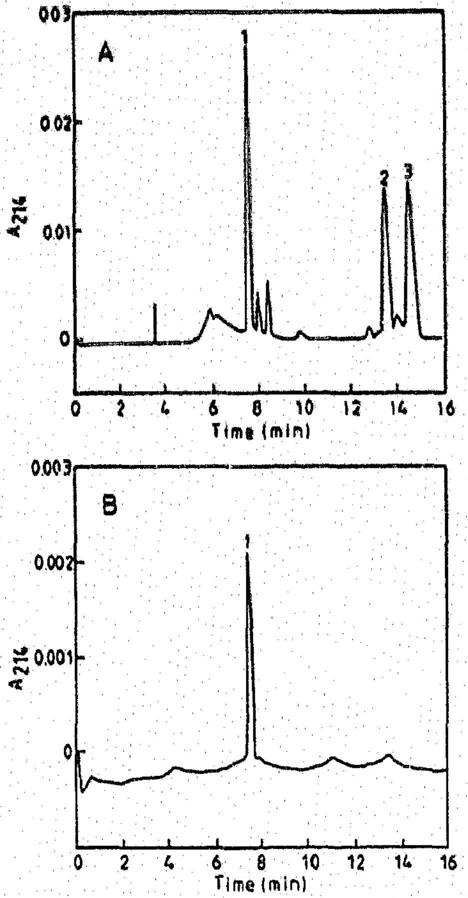

Fig. 2. Separation of synthetic peptides on a preparative scale by capillary zone electrophoresis. Components 1, 2 and 3 represent three acetylated hexapeptides: 1, Ac-ANEVIK; 2, Ac-ADQVIR; 3, Ac-ADEVIK, in amounts of 20 pmol (peptide 1) and 10 pmols (peptides 2 and 3) (A). Component 1 was collected from 4 runs, and the re-separation by capillary zone electrophoresis is shown in (B) with an amount of 1 pmol. The applied potential was 25 kV at 25°C. Pressure injections were performed for 10 s in (A) and 20 s in (B).

Table II

Amino acid analysis of peptide 1 (Fig. 2) prepared by capillary zone electrophoresis

Residue	Known	Preparation	
		1	2
Asp	1	1.2	1.1
Glu	1	1.2	1.2
Ala	1	0.9	0.9
Val	1	0.9	0.9
Ile	1	0.8	1.0
Lys	1	1.1	0.9

Sample was collected 4 times (preparation 1) and 7 times (preparation 2). Each preparation (140 and 250 pmols, respectively) was subjected to acid hydrolysis and PTC derivatization as described [7]. One tenth of this mixture was sufficient to give the results, showing that single electrophoretic runs generate enough material for analysis. Pressure injections for 20 s were utilized; running conditions for electrophoresis were as described in the legend to Fig. 2.

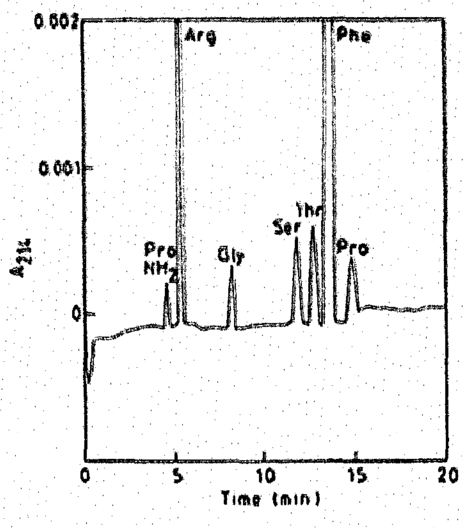

Fig. 3. Capillary zone electrophoresis of unmodified amino acids. Amounts: ProNH$_2$, 5 pmol; Phe, 20 pmol; remaining amino acids, 40 pmols. Conditions: 20 s pressure injection, 25 kV and 25°C. Several different amino acids are separated and identified at high sensitivity.

capillary zone electrophoresis at 214 nm. The electropherogram is shown in Fig. 3. It reveals a sensitivity in the fmol range for strongly absorbing, aromatic amino acids. Separation time is short, about 15 min, and structurally similar amino acids (cf. Ser/Thr) or amino acids with modifications not always easily distinguished with conventional methods (cf. Pro/ProNH$_2$) are well resolved. This direct analysis of amino acids and amides can be utilized for fast and inexpensive analysis of protein and peptide structures. It is particularly useful in rapid interpretations of results from carboxypeptidase digestions for characterization of polypeptide C-termini. This is demonstrated in Fig. 4, where 0.5 nmol of a 39 amino acid porcine intestinal peptide was digested with carboxypeptidase Y. Aliquots were lyophilized and redissolved in electrolyte buffer (4.5 pmol/μl) followed by capillary zone electrophoresis (400 fmol per injection). Previous analysis of the peptide total composition and amino acid sequence, suggested tests for the presence of Pro, ProNH$_2$ or Phe in the C-terminal region of the peptide. After mixing of the aliquots with standards and re-separation, Phe was identified (Fig. 4).

In short, these results establish that capillary zone electrophoresis is useful not only for analytical purposes as shown before, but also for direct analyses in preparative applications. Total compositions and N-terminal amino acid sequences can be directly determined by existing methods, and in addition capillary zone electrophoresis can be used for amino acid analysis. All these approaches offer high sensitivity and short analytical times at low costs.

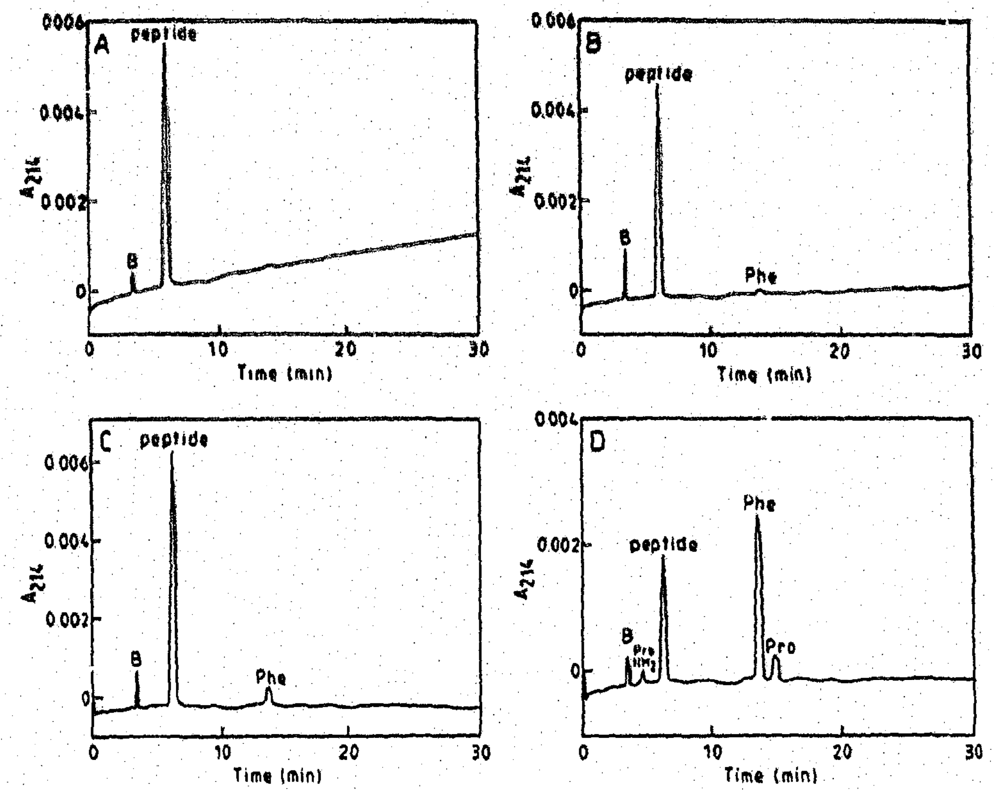

Fig. 4. Analysis of aliquots from carboxypeptidase Y digestion of a 39-residue peptide (400 fmol) by capillary zone electrophoresis. (A) before digestion; (B) after 30 min, and (C) after 4 h of digestion; (D) aliquot in (C) mixed with ProNH$_2$, Phe and Pro. Conditions: 20 s pressure injection, 25 kV and 25°C. Component B is a buffer constituent (probably pyridine) present in blank runs.

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