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Improved peptide mapping using phytic acid as ion-pairing buffer additive in capillary electrophoresis

Troels Kornfelt[a,*], Anders Vinther[a], George N. Okafo[b], Patrick Camilleri[b]

[a]Novo Nordisk A/S, Niels Steensens vej 1, DK-2820 Gentofte, Denmark

[b]SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Hertfordshire Al6 9AR, UK

Abstract

By digestion of the highly basic polypeptide aprotinin or bovine pancreatic trypsin inhibitor (BPTI) with endoproteinase Lys-C after unfolding, reduction and pyridylethylation, five fragments are obtained. These fragments are separated by free solution capillary electrophoresis using a phosphate buffer at neutral pH. The effect of the ion-pairing buffer additive phytic acid on the separation was investigated. It is shown that phytic acid through ion-pair formation influences the mobility of only those peptide fragments having a net positive charge at the pH of the separation buffer. The affinity of phytic acid to the peptides correlates with their isoelectric point and the charge to mass ratios. Hence, by changing the concentration of phytic acid, it is possible to manipulate the migration order and the separation of the peptides.

Keywords: Peptide mapping; Buffer composition; Capillary electrophoresis; Ion-pairing reagents; Phytic acid; Peptides

1. Introduction

Peptide mapping has for many years been used for the fundamental characterization of the primary structure of proteins. For protein chemists working in the biotechnological field in pharmaceutical companies, peptide mapping has also become an indispensable tool for the documentation of the batch-to-batch integrity of the primary sequence of recombinant proteins. Owing to its tremendous separation potential, RP-HPLC has become the separation technique of choice for peptide mapping. Nevertheless, peptide mapping using a technique complementary to RP-HPLC is required both for checking the purity of the RP-HPLC peptide fractions and for proteins so large that the capacity of RP-HPLC is insufficient. Several papers [1–11] have demonstrated that capillary electrophoresis (CE) is very useful for the second dimension of peptide mapping of proteins. Whereas the separation in RP-HPLC is based on differences in hydrophobicity, separation in free solution capillary electrophoresis takes place according to differences in the mass and charge of the peptides [1]. The resolving power of CE has been demonstrated by the separation of peptides differing only in neutral amino acid composition or different amino acid sequence [2].

Use of acidic separation buffers for peptide mapping by CE possesses several advantages. At pH values below the pK_a of the free silanol

* Corresponding author.

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groups on the fused-silica wall, their dissociation will be significantly suppressed. The negative charge on the fused-silica surface will be reduced correspondingly. The electroosmosis is accordingly negligible, whereby the peak capacity is greatly increased. Low pH also means that the majority of the peptides will carry a positive charge. Consequently, they will have a positive electrophoretic mobility, which ensures that they will pass the detector window at the cathodic end of the capillary. The low negative charge on the capillary wall also means that the coulombic interaction between the fused-silica surface and the peptides will be markedly diminished. Thereby the most important reason for peak broadening is removed. In accordance with these theoretical considerations, 10–100 m*M* sodium phosphate (pH 2–3) is frequently used as a background electrolyte in peptide mapping by CE [3,6,9–11]. The prevalent use of phosphate buffers as background electrolytes in CE is connected with their high UV transparency even below 200 nm and their interaction with both the fused-silica surface and the peptides.

However, satisfactory separation is not always obtained using acidic buffer conditions. As the charge of the peptides plays a pivotal role in their separation by CE, optimization of the pH of the background electrolyte is the most important parameter for improvement of the selectivity [2]. We report here a study of peptide mapping by CE at neutral pH of the very basic polypeptide aprotinin ($pI \approx 10.5$, 58 amino acids, three disulphide bridges) digested with endoproteinase Lys-C. The study shows how the separation can be manipulated by using phytic acid as a buffer additive. The study also indicates that the rank order of the individual peptide fragments with respect to pI can be suggested by studying their migration behaviour in buffers with increasing concentration of phytic acid. In recent reports [12–15], the potential of the sodium salt of phytic acid [myoinositol hexakis-(dihydrogenphosphate)] as a buffer additive in buffers of neutral pH for the CE separation of peptides and proteins has been demonstrated. It has been shown that the enhanced resolution obtained by addition of phytic acid is due to ion-pair formation between the strongly negative polyanionic phytic acid and positive charged amino acids, particularly lysine and arginine [13]. As the traditionally used ion-pair reagents possess both a hydrophobic and an ionic group (e.g., tetraalkylammonium salts and alkylsulphonic acids), they may change both the hydrophobicity and the charge of the analyte. Phytic acid, on the other hand, is of polyanionic nature and has virtually no hydrophobic character. The ionic interaction with positively charged species will therefore primarily result in a change in their net charge in a negative direction. Thereby the peak broadening observed by CE of basic proteins, which is due to coulombic interaction with the negatively charged fused-silica surface, can be effectively eliminated by addition of a sufficient amount of phytic acid to the separation buffer [15].

2. Experimental

2.1. Materials

Tris, EDTA, trifluoroacetic acid, phosphoric acid, sodium hydroxide and acetonitrile were purchased from Merck, guanidine hydrochloride and dithiothreitol from Sigma, phytic acid, dodecasodium salt hydrate from Aldrich and endoproteinase Lys-C from Boehringer Mannheim.

2.2. Preparation of digest with endoproteinase Lys-C

Aprotinin (1 mg/ml) in 20 m*M* Tris–2 m*M* EDTA (pH 8.0) was unfolded by mixing with 6 *M* guanidinine hydrochloride dissolved in the same buffer. The mixing ratio was 1:9 (v/v) so that the final concentration of guanidine hydrochloride was 5.4 *M*. The disulphide bridges were subsequently reduced by addition of an excess of dithiothreitol (DTT), followed by a nitrogen purge for 30 s and finally left for 2 h at 30°C to complete the reaction. The cysteine residues were finally alkylated by addition of 4-vinylpyridine in the molar ratio 3:1 with respect to

dithiothreitol. The reaction mixture was left in the dark for 90 min at room temperature. The process was stopped by acidification with 4 *M* acetic acid and addition of dithiothreitol. Prior to digestion, the reduced and alkylated aprotinin was desalted on a 50×4 mm I.D. LiChrospher C_{18} column using 0.05% (v/v) trifluoroacetic acid as buffer A and 0.05% (v/v) trifluoroacetic acid–60% (v/v) acetonitrile as buffer B. The column was eluted with a linear gradient from 0 to 100% buffer B in 15 min, followed by isocratic elution with 100% buffer B for 5 min. The flow-rate was 1 ml/min and the detection wavelengths were 215 and 260 nm. After desalting, the solution was evaporated to near dryness using a nitrogen purge and the residue was dissolved in 20 m*M* Tris–2 m*M* EDTA (pH 8.0), making a solution of 0.1 mg/ml aprotinin. Endoproteinase Lys-C (Lys-C) was dissolved in water making a solution of 0.1 mg/ml. The enzyme and substrate were mixed in the ratio of 1:100. Digestion was accomplished by incubation of the mixture at room temperature for 2 h. The reaction was stopped by addition of 4 *M* acetic acid.

2.3. Preparation of samples of the individual peptide fragments

The individual peptide fragments of Lys-C-digested aprotinin were collected from an RP-HPLC peptide map of the digest. The column was LiChrospher C_{18} (50×4 mm I.D.), buffer A was 0.05% (v/v) trifluoroacetic acid and buffer B was 0.05% (v/v) trifluoroacetic acid–60% (v/v) acetonitrile. The HPLC instrumentation was obtained from Waters (Milford, MA, USA). The fractions were concentrated to obtain an appropriate response. The fragments were characterized by automatic amino acid sequence analysis using Edman degradation and electrospray ionization mass spectrometry.

2.4. CE conditions

A fused-silica capillary from LC Packings (total length $L_T = 95$ cm, effective length $L_E = 87$ cm, I.D. 75 μm) with built-in Z-shaped detector window (3 mm path length) was used throughout the study. The capillary was mounted in a Waters Quanta 4000 CE instrument. Sample introduction was effected by hydrostatic injection (siphoning) at the anode. UV detection at 214 nm took place at the cathodic end. The separation buffers containing 0–5 m*M* phytic acid were prepared by mixing two stock solutions: 100 m*M* phosphoric acid and 100 m*M* phosphoric acid–5 m*M* phytic acid. The mixtures were titrated to pH 7.0 with sodium hydroxide. Prior to use, the buffer solution was filtered (0.2 μm) directly into the buffer vials.

3. Results and discussion

Initially, peptide mapping of pyridylethylated aprotinin digested with endoproteinase Lys-C using free zone electrophoresis in phosphate buffer and also micellar elektrokinetic chromatography at pH 2 and 7 was attempted. Surprisingly, virtually no separation was obtained at pH 2 with any of the buffers tested. The most satisfactory results were achieved using a 0.1 *M* sodium phosphate buffer (pH 7.0). By addition of 1–5 m*M* phytic acid to the phosphate buffer,

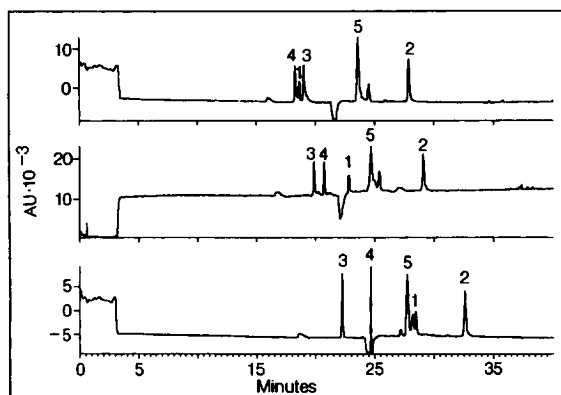

Fig. 1. CE map of Lys-C digested aprotinin using phytic acid as ion-pairing buffer additive. Background electrolyte: 100 m*M* sodium phosphate (pH 7.0). The separations were performed in a fused-silica capillary (LC Packings), $L_T = 95$ cm, $L_E = 87$ cm, I.D. 75 μm; Z-shaped detector window; detection wavelength, 214 nm; voltage, 13–14 kV at ca. 90–95 μA; injection 50 s (siphoning), ca. 5 ng. For peak numbers, see Table 1. Phytic acid concentration: top, 0; middle, 1; bottom, 2 m*M*.

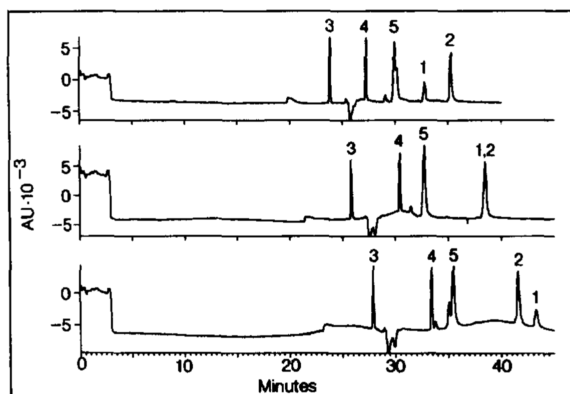

Fig. 2. CE map of Lys-C digested aprotinin using phytic acid as ion-pairing buffer additive. Experimental conditions as in Fig. 1. Phytic acid concentration: top, 3; middle, 4; bottom, 5 m*M*.

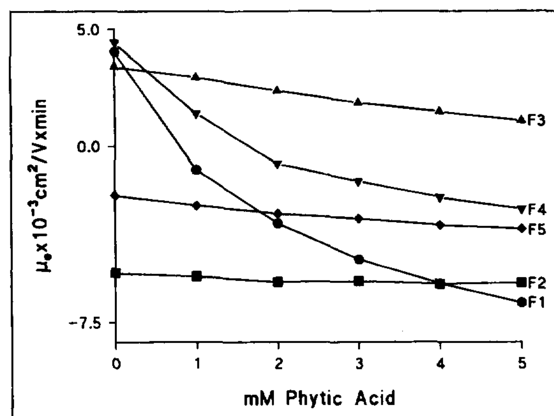

Fig. 3. Correlation between the electrophoretic mobility, μ_E, of the five Lys-C fragments of aprotinin (F1–F5) and the concentration of phytic acid in the separation buffer. The figure is a graphical presentation of the data in Table 1.

marked changes in the migration order of the five peptides were introduced.

The separations by CE of the peptide fragments obtained by Lys-C digestion of aprotinin in 100 m*M* sodium phosphate buffer (pH 7.0) containing 0, 1, 2, 3, 4 and 5 m*M* phytic acid are shown in Figs. 1 and 2. The electrophoretic mobilities of the five fragments in the six different buffers are shown in Table 1 together with the different μ_{EOF} values related to the variable concentration of phytic acid. Fig. 3 gives a graphical presentation of the data in Table 1 except those for μ_{EOF}. Obviously phytic acid interacts with the five fragments in the order $1>4>3>5>2$. Whereas the mobilities of fragments 1, 4 and 3 clearly decrease with increasing concentration of phytic acid in the running buffer, the mobilities of fragments 5 and 2 show little or no change.

To interpret the way in which phytic acid interacts with the peptides, their p*I* values and relative charges were calculated (Table 2). By comparison of these numbers with the effect of phytic acid on the mobilities of the five fragments

Table 1
Electrophoretic mobility ($\mu_e \times 10^{-3}$ cm^2/V·cm) of the five peptide fragments (F1–F5) obtained by digestion of aprotinin (reduced and alkylated) with Lys-C, and mobility of the electroosmotic flow, μ_{EOF}

Fragment No.[a]	Phytic acid (m*M*)					
	0	1	2	3	4	5
*F*1	4.05	−1.01	−3.28	−4.81	−5.82	−6.64
*F*2	−5.38	−5.53	−5.77	−5.73	−5.85	−5.80
*F*3	3.41	2.95	2.37	1.86	1.48	1.09
*F*4	4.43	1.41	−0.75	−1.50	−2.18	−2.68
*F*5	−2.10	−2.53	−2.87	−3.08	−3.35	−3.51
μ_{EOF}	23.9	22.2	21.3	19.5	18.9	17.0

[a] Amino acid sequences (one-letter codes) of the five peptide fragments: F1 = RNNFK; F2 = SAEDCMRTCGGA; F3 = AGLCQTFVYGGCRAK; F4 = ARIIRYFYNAK; F5 = RPDFCLEPPYTGPCK.

Table 2
Amino acid sequences (one-letter codes) and position numbers of the five peptide fragments obtained by digestion of aprotinin with Lys-C

Fragment No.	Sequence and position in protein	M_r[a]	z[b]	z/M_r[c] $\times 10^{-3}$	pI[d]
F1	RNNFK; 42–46	677.8	1.7	2.5	11.4
F2	SAEDCMRTCGGA; 47–58	1410.3	−1.4	−1.0	4.4
F3	AGLCQTFVYGGCRAK; 27–41	1783.8	1.6	0.9	9.2
F4	ARIIRYFYNAK; 16–26	1414.7	2.7	1.9	11.3
F5	RPDFCLEPPYTGPCK; 1–15	1933.0	−0.4	−0.2	5.9

Cysteine residues were identified as the pyridylethylated derivatives. The charge and pI values were calculated using the means of the pK_a values of the amino acids listed in Table 1.2 in Ref. [16].
[a] M_r = molecular mass of the reduced and 4-VP alkylated fragments.
[b] z = Net charge of the peptides at pH 7, calculated using Table 1.2 in Ref. [16].
[c] z/M_r = relative charge.
[d] pI calculated using Table 1.2 in Ref. [16].

(Table 1), it is observed that fragments 1, 4 and 3 all have a pI above the buffer pH of 7.0, indicating a positive net charge at this pH value, whereas fragments 5 and 2 have pI values below pH 7.0 and zero or negative net charge. Further, by ranking the five fragments according to their pI values and their charge to mass ratios, we obtain the same order as for the interaction with phytic acid. It is therefore concluded that by including phytic acid in the running buffer the mobility of those peptides which have pI values above the pH of the running buffer is decreased. This effect is due to ion-pair formation between the strongly negative phytic acid and the positively charged peptide. The affinity of phytic acid to the peptides correlates with their basicity. This example suggests that simply by changing the phytic acid concentration, it may be possible to rank the individual fragments according to their pI values. This approach may be generally applicable when peptides with different pI values are to be separated. It has been found previously [15] that a similar correlation does not exist for the proteins that have been investigated. This suggests that the interaction between phytic acid and proteins is more complex.

In agreement with the earlier reports, we observe a significant decrease of the electroosmotic flow on addition of phytic acid. From Fig. 4, it is seen that within the range studied, μ_{EOF} decreases linearly with increasing concentration of phytic acid. The polyanionic nature of phytic acid means that the addition of even small amounts of the substance increases the ionic strength of the background electrolyte substantially. The electroosmotic flow is decreased correspondingly. Although the decreased electroosmotic flow means a longer separation time, the beneficial effect is a larger peak capacity.

To summarize our results, this study has shown that it is possible not only to optimize the separation of peptides but also to obtain some knowledge of the mutual pI values of the in-

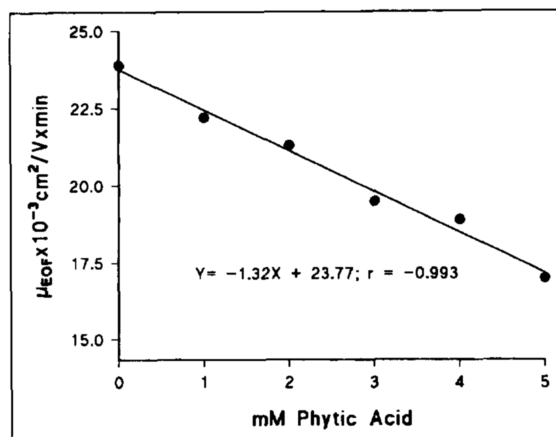

Fig. 4. Effect on the electroosmotic flow, μ_{EOF}, of addition of phytic acid to the separation buffer. See Fig. 1 for experimental details.

dividual peptides by CE analysis using phytic acid as an ion-pair-forming buffer additive.

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