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Application of capillary zone electrophoresis to the characterization of multiple antigen peptides

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ABSTRACT

Capillary zone electrophoresis (CZE) was applied to the analysis of a recently described class of synthetic branched peptides, multiple antigen peptides (MAPs). In comparison with high-performance liquid chromatography (HPLC), CZE showed superior resolution of minor impurities in samples of MAPs obtained by preparative chromatography. MAPs that differed only in the substitution of uncharged residues (Ala–Ser) could be separated by the addition of organic solvents (acetonitrile, methanol, ethanol) to the aqueous background electrolyte.

INTRODUCTION

Multiple antigen peptides (MAPs) are branched molecules in which a branching core of lysine residues, with both α- and ε-amide bonds between themselves, form the skeleton for the assembly of multiple copies of a given peptide sequence (see Fig. 1). These peptides, first described by Tam [1], are finding widespread application as candidate synthetic vaccines [2–4], as antigens for solid-phase immunoassays [5,6] and to raise antibodies for immunocytochemistry [7]. Moreover, a MAP-like structure has recently been described as a prototype synthetic enzyme [8].

MAPs are assembled by solid-phase synthesis [9] either by the Boc–polystyrene strategy [1] or by the flow polyamide method [10].

Until now analytical characterization of MAPs has been performed by high-performance size-exclusion chromatography (SEC) [1,2,5,10] and reversed-phase high-performance liquid chromatography (RP-HPLC) [5,8,10]. SEC has also been used for the purification of the MAPs [1,2,5,8].

Capillary zone electrophoresis (CZE) has been recently applied to the separation of protein and peptide mixtures [11–18]. Some desirable features of this tech-

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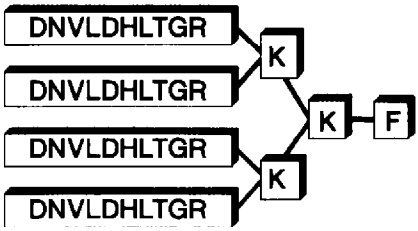

Fig. 1. Structure of MAPs. The figure depicts the structure of MAP^4 (DNVLDHLTGR), *i.e.*, compound 1 in Table I. In MAP nomenclature, the superscript refers to the number of branches and the sequence in parentheses is that assembled on each amino group of the lysine residues. The one-letter code for the amino acids is used throughout (D = Asp, N = Asn, V = Val, L = Leu, T = Thr, H = His, G = Gly, R = Arg, P = Pro, A = Ala, S = Ser).

nique, namely high efficiency and resolution and short analysis time, suggested that it should be well suited for the analytical characterization of MAPs. In this paper we describe the application of CZE to the analysis of a series of MAPs produced by the flowpolyamide method.

EXPERIMENTAL

Apparatus and procedures

The electrophoretic experiments were performed in a Bio-Rad Lab. (Richmond, CA, USA) HPE 100 unit equipped with an on-column UV detector. The separations were done in a 20 cm × 0.025 mm I.D. coated fused-silica capillary (Bio-Rad) at room temperature. The sample was introduced into the capillary by electromigration. The capillary was washed after each run with the appropriate background electrolyte. Lyophilized peptides were dissolved in doubly distilled water, filtered through a 0.45-μm filter and used without further treatment. Electropherograms were recorded with an LKB Model 2210 line recorder at a chart speed of 10 mm/min.

For analytical HPLC, a Merck–Hitachi Model 655A liquid chromatograph was used equipped with a Rheodyne Model 7161 injection valve, a Jasco Model 875 UV detector and a Merck–Hitachi Model D-2000 integrator. Other conditions are given in the legend to Fig. 3.

Chemicals

Sodium dihydrogenphosphate, ammonium acetate, acetic acid and methanol were purchased from Carlo Erba (Milan, Italy) and phosphoric acid, ethanol and acetonitrile (ACN) from Merck (Darmstadt, Germany); trifluoroacetic acid (TFA), also from Merck, was distilled before use. The background electrolytes (BGE) used were a 0.1 *M* solution of phosphate buffer (pH 2.5 and 3.5) and a 0.1 *M* solution of acetate buffer (pH 4.5). For the separations in aqueous–organic mixtures, a stock solution of 0.1 *M* phosphate buffer (pH 2.5) was mixed with the appropriate amounts of organic solvent and water; the final concentration of the electrolyte was 0.05 *M*.

Peptides

The peptides used were prepared by the flow polyamide method as described

[10]. Details of the synthesis and of the purification of MAP 1 in Table I have been reported elsewhere [19].

RESULTS AND DISCUSSION

The peptides used are listed in Table I. Compound 1 is a well characterized molecule, whose synthesis, purification and 2D-NMR conformational analysis have been reported [19]. Preparative purification by reversed-phase sample displacement chromatography (SDC) [20] had made available the purified main product in the crude mixture (fraction A), and a pool of the more closely related impurities (fraction B). Therefore, this product was an ideal test case to compare the resolving power of CZE, when applied to MAPs, with that of HPLC. Compounds 2 and 3 represent another difficult test case: the sequences of these high-molecular-weight MAPs differ only by an uncharged amino acid. As the antigenic sequence is a tandem repeated dimer, $(PGTHLPA/SLP)_2$, the substitution takes place twice.

Moreover, to examine how the multi-branched structure affects the electrophoretic behaviour, these MAPs were compared with the corresponding linear peptides (compounds 4 and 5 in Table I).

The CZE experiments were carried out in a relatively short, coated capillary tube (Bio-Rad); this allowed short analysis time and minimized adsorption of the analytes on the capillary walls.

The synthetic peptides studied all possess positive charge at acidic pH. Therefore, various BGEs at pH 2.5, 3.5 and 4.5 were tested to find the optimum separation conditions, which must be established for each peptide.

The migration time of all the components in the mixture increased with increase in pH, and the BGE at pH 3.5 represents the optimum compromise between selectivity, efficiency and duration of the analysis. Fig. 2 shows the electropherogram of compound 1, fraction B at pH 3.5.

Fig. 3 shows the RP-HPLC of the crude mixture of compound 1 (Fig. 3a), fraction A (purified product, Fig. 3b) and fraction B (more closely related impurities,

TABLE I

PEPTIDES USED IN THIS STUDY

The MAP nomenclature is explained in the legend to Fig. 1.

Peptide	Sequence	Sample
1	MAP^4 (DNVLDHLTGR)	Crude[a]
		Fraction A[b]
		Fraction B[c]
2	MAP^8 $(PGTHLPALP)_2$	Crude
3	MAP^8 $(PGTHLPSLP)_2$	Crude
4	$(PGTHLPALP)_2$-NH_2	Crude
5	$(PGTHLPSLP)_2$-NH_2	Crude

[a] Crude means peptide cleaved from the resin after the solid-phase synthesis without further purification, apart from desalting (Sephadex G-10).

[b] Purified main product from SDC (see text).

[c] Pool of the more related impurities, also from SDC (see text).

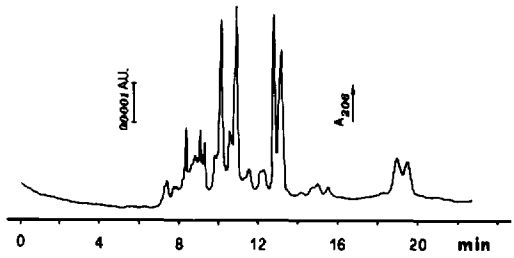

Fig. 2. Electropherogram of MAP[4] (DNVLDHLTGR), fraction B, using BGE at pH 3.5. Sampling, 4 kV, 5 s; sample concentration, 0.6 mg/ml; electrophoresis at 8 kV (constant), 16–20 μA.

Fig. 3c). The corresponding electropherograms of these peptides are reported in Figs. 2 (fraction B), 4a (crude mixture) and 4b (fraction A). HPLC was performed at 230 nm, to minimize drifting of the baseline during gradient elution, due to the organic phase modifier (TFA). This is common with peptide samples, and does not alter the qualitative picture, as absorption in the range 206–230 nm is mainly due to the amide chromophore. The absorption maximum of the peptide chromophore at 206 nm was instead chosen for CZE.

The overall pictures given by the two techniques are very similar, indicating the successful purification of the main peak from several impurities. CZE reveals, however, that at least ten minor peaks are present in the "pure" preparation (fraction A), which HPLC shows as essentially a single peak. The impurities collectively account for only a small fraction of the total material present in the sample, which can be classified as >90% pure; this is consistent with a series of deleted and truncated sequences, each one present in a very small amount, as often found in peptides prepared by solid-phase synthesis [21]. The multi-chain nature of the MAP may explain the difficulty encountered in their separation from the desired sequence. It therefore

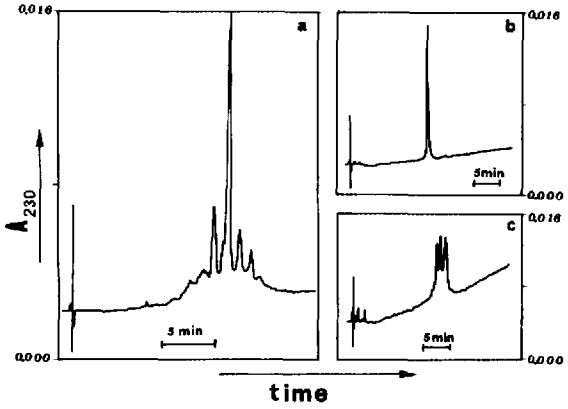

Fig. 3. Analytical HPLC of MAP[4] (DNVLDHLTGR). (a) Crude mixture from solid-phase synthesis; (b) purified main product; (c) pool of the more closely related impurities. Column, Vydac 218TP (5 μm); eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in acetonitrile; linear gradient from 25 to 40% B over 20 min; flow-rate 1.5 ml/min; injection, 5 μl; sample concentration, 1 mg/ml.

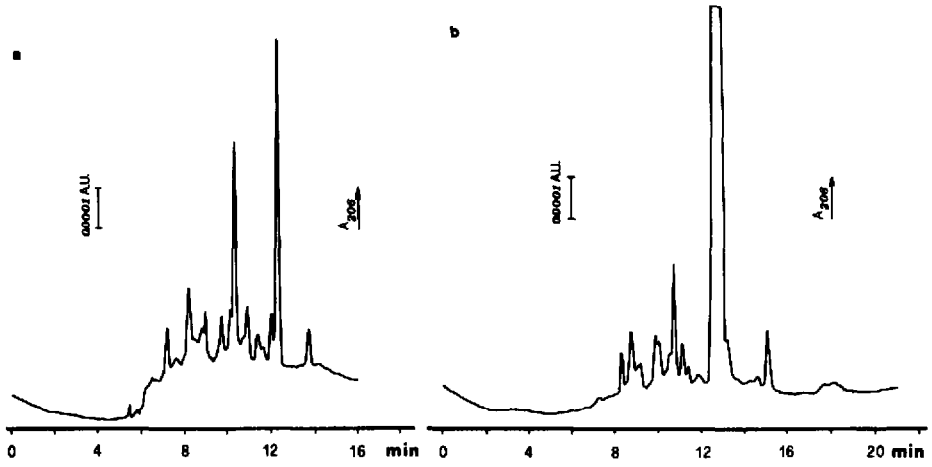

Fig. 4. Electropherograms of MAP[4] (DNVLDHLTGR). (a) Crude; (b) fraction A. Sampling: (a) 6 kV, 7 s; (b) 4 kV, 5 s. BGE pH, 3.5. Electrophoresis at 17 μA (constant), 8.5 kV. Sample concentration: (a) 0.6 mg ml; (b) 0.6 mg/ml.

seems necessary to include CZE among the routine controls applied to this class of peptide.

Samples 2–5 were injected separately and electrophoresis was performed at pH 2.5, 3.5 and 4.5. With all the BGEs used, a short migration time (less than 5 min) of the peptides was complemented by a good separation of several minor impurities from the main peak. For these compounds, the best results were obtained with the BGE at pH 2.5. At every pH, the mobility of each MAP was higher than that of the corresponding linear peptide.

However, no variation in BGE led to the separation of the pairs of compounds that differ only in the Ala–Ser substitution (*i.e.*, compounds 2 and 3 and compounds 4 and 5).

It has been reported that the addition of organic solvents to an aqueous BGE can influence the effective mobility of the analytes [22,23]. Therefore, we studied the influence of various organic solvents (methanol, ethanol and acetonitrile) on the resolution of mixtures 2–3 and 4–5.

Methanol, ethanol and acetonitrile were added, separately, to the BGE at different concentrations ranging from 10 to 50% (v/v) and these aqueous organic mixtures were used for the separation of peptide mixtures 2–3 and 4–5. With all the aqueous–organic BGEs used the migration times of all the four peptides analysed increased owing to a decrease in their effective electrophoretic mobilities. For all solvents an optimum percentage could be found that maximized the separation of the two components. For both mixtures, acetonitrile yielded the best resolution, albeit at different percentages: 40% (v/v) for mixture 2–3 and 50% (v/v) for mixture 4–5.

Fig. 5a and b show the separation obtained by using the same BGE in aqueous and aqueous–acetonitrile mixture, respectively.

Grossman *et al.* [13] recently observed that in a series of short, linear peptides that differed in a single neutral amino acid, the electrophoretic mobility in an aqueous

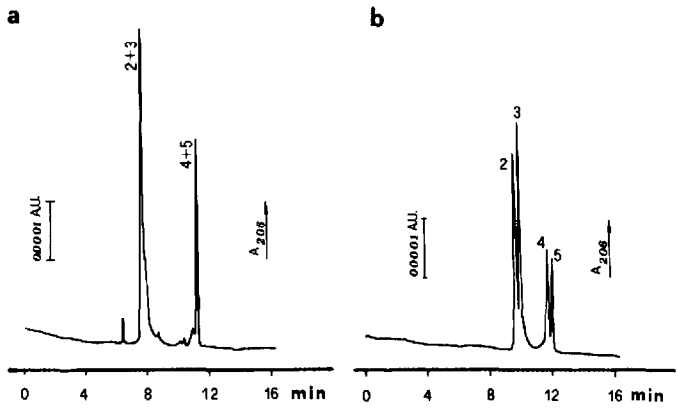

Fig. 5. Electrophoretic separation of peptides differing only in the Ala–Ser substitution. Sample: mixture of compounds 2, 3, 4 and 5, 0.1 mg/ml each. (a) BGE, 50 m*M* phosphate buffer (pH 2.5); sampling, 4 kV, 6 s; electrophoresis at 7 kV (constant), 11 μA; (b) BGE, 40% ACN in 50 m*M* phosphate buffer (pH 2.5); electrophoresis at 7 kV (constant), 7 μA.

solvent decreased with increase in the hydrophobicity index of the amino acid. In our case the Ser peptides (3 and 5) always moved towards the cathode with lower velocity than the Ala peptides, despite the lower hydrophobicity of Ser. The influence of the organic solvents, which appeared to be the same for the linear and the branched compounds, was probably due to the different solvation of the peptide chains. Further studies are planned to ascertain whether this is a general effect.

CONCLUSIONS

The results indicate that CZE is a powerful technique for the characterization of MAPs. The known advantages of CZE, *e.g.*, short analysis time, low electrolyte consumption and high sensitivity, are complemented by the superior resolution and efficiency with respect to HPLC. By addition of organic solvents to the BGE, it is possible to separate MAPs that differ only in neutral amino acids, such as the Ala–Ser pair. The combined use of preparative HPLC and analytical CZE therefore offers a good potential to solve the purification problems with this important class of pharmaceutical peptides.

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