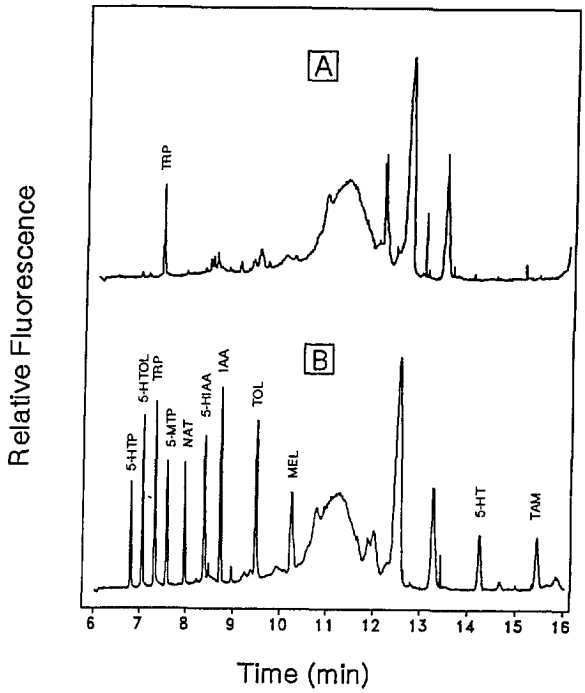

Fig. 6. Electropherograms for the MEKC of (A) serum, and (B) serum fortified with 90 ng/ml of each indole standard. The serum was diluted 200-fold with water before injection. Running conditions were the same as in Fig. 4A.

Studies involving pathological samples and quantitation are being investigated.

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The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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JOURNAL OF CHROMATOGRAPHY A

Use of capillary zone electrophoresis in an investigation of peptide uptake by dairy starter bacteria☆

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Abstract

A capillary zone electrophoresis (CZE) method to separate the peptide series val-gly$_n$, where n is 1 to 4, has been evaluated and compared to separation by reversed-phase high-performance liquid chromatography (RP-HPLC). The method was able to quantitate peptides present at very low concentrations (down to 0.05 mM) with high reproducibility and accuracy and was capable of separating peptides differing in size by only a single glycine residue. It could also separate the peptides val-gly and leu-gly which differed in only a single side-chain methylene group. The method was fast, required small sample volumes, and proved to be superior to RP-HPLC. The suitability of the CZE method to analyze peptide uptake by dairy starter bacteria is discussed.

1. Introduction

In the manufacture of cheese from milk, starter bacteria (*Lactococcus lactis*) convert large amounts of lactose to lactic acid, acidifying the milk and facilitating the formation of milk curds. For these starter bacteria to grow to the high cell densities required to sustain this lactic acid production they must obtain peptides and amino acids from the hydrolysis of the casein milk protein [1]. The metabolites are acquired by the activity of a complex proteolytic system, a key step of which is the transport of the casein-derived peptides into the bacterial cell [2,3]. It is presently not known whether the relatively large peptides (average length 11 residues) produced by the initial degradation of casein by a cell-wall-associated proteinase can be transported into the cell intact or only after further degradation by extracellular peptidases.

The rate of peptide transport into whole cells is generally calculated by measuring the uptake of synthetic peptides by the starter bacteria. Whilst reversed-phase high-performance liquid chromatography (RP-HPLC) has been used previously for the analysis of changes in the levels of these peptides [4–6] this method is slow, requires relatively large sample volumes and often cannot resolve closely related peptides.

The present paper reports the use of a capillary zone electrophoresis (CZE) method to separate and measure synthetic peptides belonging to the homologous series val-gly$_n$, where n is from 1 to 4. The peptide series was based on valine as this amino acid is essential to the growth of the *L. lactis* strain used in this study [7]. For the

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purposes of this study on peptide uptake, the analytical method used was required to resolve peptides from background interference, to differentiate between peptides differing in size by only one amino acid residue and to differentiate between peptides differing by only one side-chain group. The ability of a CZE method to fulfil these criteria was examined.

2. Experimental

2.1. Chemicals

All buffers, glucose and trifluoroacetic acid (TFA) were of analytical grade or better and were obtained from either BDH Chemicals (Poole, UK) or Sigma (St. Louis, MO, USA). Water was purified by reverse osmosis followed by deionization (Milli-Q, Millipore, Bedford, MA, USA). The peptides val-gly, val-gly$_2$ and leu-gly were obtained from Sigma; the remaining peptides (val-gly$_3$ and val-gly$_4$) were synthesized using an Applied Biosystems (Foster City, CA, USA) 430A peptide synthesizer.

2.2. Peptide uptake experiment

Peptide uptake experiments were performed by growing bacterial cells (*Lactococcus lactis*) in a chemically defined medium until the mid-exponential phase (4 h) and then suspending them in phosphate buffer solution. After maintaining the cells in buffer for 10 min, glucose was added to a final concentration of 0.2%. After a further 15 min the peptide(s) of interest was added (final concentration 1 m*M*). Samples were taken over a 60 to 120 min time course and TFA (final concentration 1%) was added to each sample to stop further peptide uptake. The cells were then removed by centrifugation (MSE Microcentaur bench centrifuge at 11 600 *g* for 5 min) and the supernatant frozen until analyzed by HPLC or CE.

2.3. Capillary electrophoresis

Capillary zone electrophoresis (CZE) was performed on an Applied Biosystems 270A-HT CE system (Foster City, CA, USA) using a PE Nelson 900 series interface and a PE Nelson TurboChrom 3.3 software package (Cupertino, CA, USA) for data acquisition and analysis, respectively. The uncoated capillary (72 cm total length, 50 cm effective length and 50 μm internal diameter) was supplied by Applied Biosystems. Filtered samples were injected at the anode using vacuum (17 kPa) for 2.5 s. The buffer system was 20 m*M* sodium citrate, pH 2.5, at 30°C and the separation voltage was 30 kV with detection by absorbance at 200 nm. Between injections the capillary was flushed for 2 min (68 kPa) consecutively with 0.1 *M* NaOH, Milli-Q water and buffer to retain separation reproducibility.

2.4. HPLC

Reversed-phase HPLC (RP-HPLC) was performed on a Philips PU4100 HPLC (Philips, Eindhoven, Netherlands) with a Vydac (Hesperian, CA, USA) 218TP C_{18} column (250 × 4.6 mm I.D.; pore size, 10 μm; pore diameter, 30 nm). The eluate was monitored by absorbance at 220 nm using a Philips PU4110 UV–Vis detector. Samples were separated using an isocratic gradient of 0.1% TFA in Milli-Q water.

3. Results and discussion

3.1. Comparative analysis of val-gly$_3$ using RP-HPLC and CZE

The RP-HPLC chromatogram of the separation of val-gly$_3$ from the other components present in the supernatant of the bacterial cell suspension is shown in Fig. 1a. Due to its low hydrophobicity the val-gly$_3$ peptide interacted weakly with the reversed-phase matrix and eluted soon after the non-retained material. Whilst the val-gly$_3$ peak was resolved from the other peaks, there was considerable background noise which reduced the sensitivity. There was also interference from other peptides leaked from the bacterial cells and, when other val-gly$_n$ peptides were used, some overlap with these other peptides (data not shown).

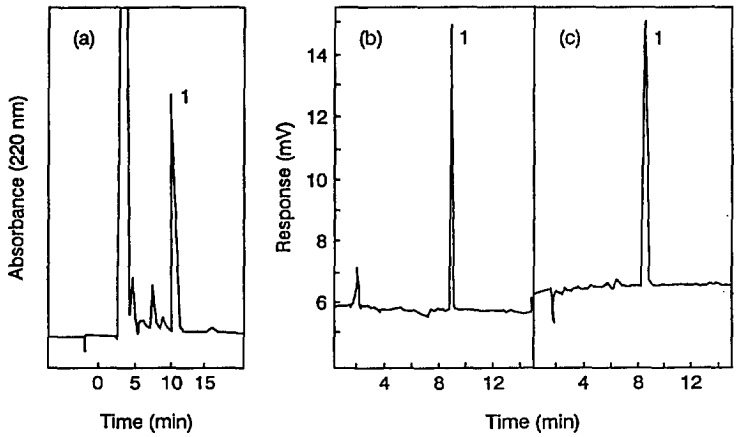

Fig. 1. Separation of val-gly$_3$ by (a) RP-HPLC, (b) CZE and (c) CZE in the presence of 1% TFA. Conditions for chromatography and CE were as in the Experimental section. Peak 1 was val-gly$_3$.

In contrast to the RP-HPLC chromatogram, the CZE electropherogram (Fig. 1b) showed that migration of the val-gly peptide through the capillary was retarded by the separation conditions such that it was well resolved from other material found in the cell suspension and that it was a very sharp peak. When 1% TFA was included in the samples taken from the bacterial cell suspension to inhibit further peptide uptake, the separation of the val-gly$_3$ peptide was not seriously affected (Fig. 1c). Whilst the val-gly$_3$ peak was not as sharp as in the absence of TFA, there was still good resolution and reproducibility.

The CZE method, therefore, gave excellent separation of the tetrapeptide val-gly$_3$ from contaminating material both in the presence and absence of 1% TFA. It also had the advantages over the RP-HPLC method of having a total assay time, including washing and regenerating the capillary, of 20 min compared with 60 min for the RP-HPLC method, and of requiring only small amounts of sample (20 μl for CZE versus 100–200 μl for RP-HPLC).

Overall these results reinforce previous studies that have compared the use of CZE and RP-HPLC to separate peptides [8,9]. The two methods are best described as orthogonal with the CZE selectivity based on peptide charge and mass and the RP-HPLC separation based on the relative hydrophobicity of the peptides. Whilst this provides a degree of complementarity between the two techniques, in the present case the CZE method was deemed to be superior to RP-HPLC due to its quicker separation, greater resolution and higher sensitivity.

3.2. Separation of peptides differing in size by one amino acid

When determining what size of peptides could be transported into the bacterial cell it was deemed to be essential that the assay method should be able to accurately separate peptides which differed in size by as little as one amino acid residue. Using the CZE method (Fig. 2) there was baseline separation of a series of val-gly$_n$ peptides where $n = 1$ to 4. This series was generated during the synthesis of the pentapeptide val-gly$_4$ with the peptides val-gly, val-gly$_2$ and val-gly$_3$ appearing as contaminants that resulted from the incomplete coupling to the solid-phase. As neither val nor gly have a charged side-chain, the separation was presumed to be based on both the change in the charge-to-size ratio of the peptide and changes in the local hydrophobic environment as successive gly residues are introduced.

3.3. Separation of peptides differing by one amino acid

The ability of dipeptides structurally related to val-gly to inhibit the uptake of val-gly was