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Characterization of cheese proteolysis by capillary electrophoresis and reverse-phase HPLC analyses of peptides

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Abstract The possibilities of reverse-phase high performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE) when used for separation of cheese peptides are discussed. A CE method using a coated capillary column and a low pH buffer was developed to analyze the water-soluble fraction of a 6-month-old cow's milk cheese. The CE patterns were compared with the chromatograms obtained by RP-HPLC using a C18 column and a gradient of acetonitrile in water. The CE method gave shorter analysis times but RP-HPLC provided lower coefficients of variation of the retention times and better detection limits. In addition, the elution behavior of peptides in CE strongly depended on the sample matrix. The results show that both techniques provide complementary information for the analysis of cheese peptides.

Key words Capillary electrophoresis · Reverse-phase high performance liquid chromatography · Cheese peptides · Water-soluble nitrogen

Introduction

The water-soluble fraction (WSF) of cheese is considered to make a major contribution to flavor, owing to the presence of low molecular weight peptides, free amino acids, free fatty acids and their breakdown products [1]. Although the specific role of peptides is still not clear, differences in the peptide pattern are considered to be related to differences in cheese flavor, and it is generally accepted that certain peptides are directly responsible for the development of bitterness in cheese [2].

Reverse-phase high performance liquid chromatography (RP-HPLC) has been widely used for peptide analyses because of its versatility, short analysis times and high resolution [3]. During the past few years, capillary electrophoresis (CE) has gained considerable acceptance as a useful technique for the analysis of biopolymers [4]. CE has been proved as a powerful technique for the separation of milk proteins [5–7] and peptides [8], because of its high efficiency and resolving power, small sample and buffer requirements, ease and speed of separations [9]. The complementary use of these two techniques, owing to their different mechanisms of separation (mainly hydrophobicity in RP-HPLC and charge/mass ratio in CE) may solve many problems related to the analysis of biopolymers. However, despite the fact that CE is a very promising alternative technique for food analysis, there are very few reports on its application to the study of cheese peptides [10, 11].

The objective of this work was to demonstrate the potential of CE for the separation of peptides in cheese and to establish the electrophoretic behavior and elution characteristics of these peptides. For this purpose, a CE method using a coated capillary column and a low pH buffer was developed to analyze the WSF of cheese. This CE method was compared with a well-established RP-HPLC method using a C18 column and a gradient of acetonitrile in water [12]. In order to simplify the interpretation of results and improve resolution, the water-soluble extract was further fractionated using centrifugal filtration and Sep-Pak C18 chromatography.

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Materials and methods

Extraction and fractionation of the cheese samples. Cheese was made from cow's milk pasteurized at 72 °C for 15 s with the addition of a direct vat inoculation starter culture and animal rennet as previously described [13]. To obtain the water-soluble nitrogen fraction (WSNF), 10 g of the 6-month-old grated cheese was mixed with 50 ml deionized

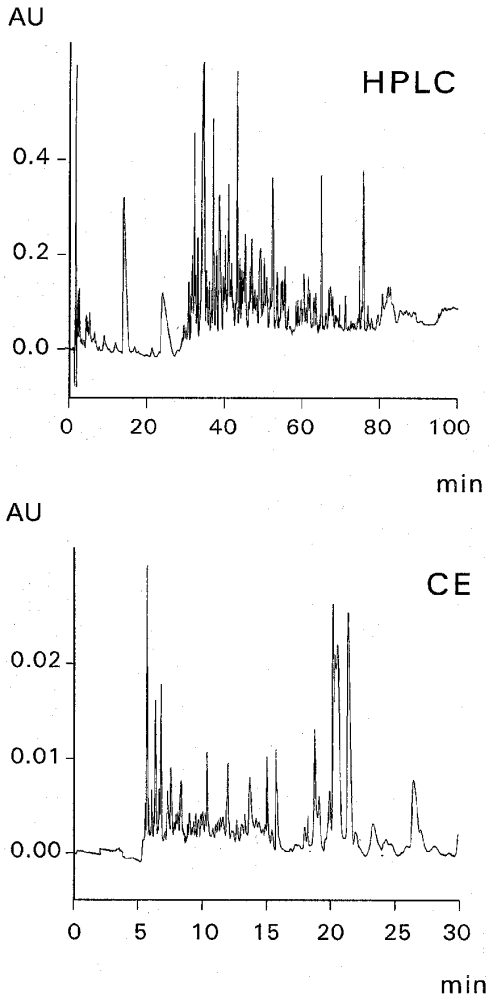

Fig. 1 Reverse-phase high performance liquid chromatography (*RP-HPLC*) and capillary electrophoresis (*CE*) analyses of the water soluble nitrogen fraction containing compounds with masses lower than 10 000 daltons (WSNF <10 000) of a 6-month-old cow's milk cheese (detection at 214 nm). *AU* absorbance units

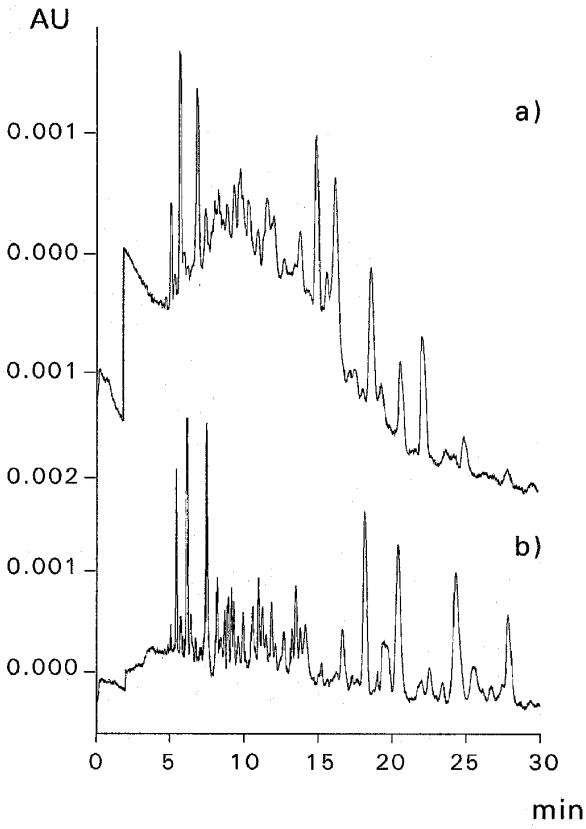

Fig. 2 CE analyses of the WSNF <10 000 of a 6-month-old cow's milk cheese eluted from the Sep-Pak C18 cartridge with **a** 30% acetonitrile and **b** the same fraction reconstituted in water after removal of the acetonitrile by lyophilization (detection at 214 nm)

water and homogenized for 5 min at 40 °C using an Ultraturrax (Ika-Werk, Staufen, Germany). The homogenates were then kept at 40 °C for 10 min and filtered through glass wool.

Fifteen milliliters of the WSNF was separated into two different molecular weight fractions by filtration with Centriprep units (Amicon, Beverly, Mass., USA), with a cut-off point of 10 000 daltons, and centrifugation at 3000 *g* for 45 min at 25 °C.

One milliliter of the WSNF with a mass lower than 10 000 daltons (WSNF <10 000) was then loaded onto a Sep-Pak C18 cartridge (Millipore, Bedford, Mass., USA) and separated into six different fractions. Cartridge conditioning prior to use was as described by Herraiz and Casal [14]. After the first, unretained, fraction was recovered, the cartridge was washed with 1 ml of 0.1% trifluoroacetic acid (TFA) in water, and peptides were eluted with a stepwise gradient of 1 ml portions of 0.1% TFA in 10%, 20% and 30% acetonitrile in water and, finally, with 1 ml of 0.1% TFA in 100% acetonitrile.

Peptide analysis by HPLC. A Beckman System Gold HPLC equipped with a diode array detector was used together with a System Gold Software data acquisition system version 711 (Beckman Instruments, Fullerton, Calif., USA). Separations were performed on a C18 Nucleosil 5 µm, 300 Å column (250 mm × 4.6 mm) (Macherey Nagel Düren, Düren, Germany) at 40 °C. Solvent A was 0.1% TFA (Merck, Darmstadt, Germany) in Milli-Q water (Millipore) and solvent B was 0.1% TFA in acetonitrile (HPLC grade obtained from Scharlau, Barcelona, Spain) and water (60:40 v/v). Separations were performed following the method described by Gonzalez de Llano et al. [12]. A flow rate of 1 ml/min was employed with simultaneous detection at 214 nm and 280 nm. The samples were filtered through a Durapore 0.45 µm filter (Millipore) before analysis.

Commercial standards of the aromatic amino acids tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp) (Sigma, St. Louis, Mo., USA) were run on the HPLC to identify their retention times.

Peptide analyses by CE. CE was carried out using a Beckman P/ACE System 2050 controlled by the System Gold Software (Beckman). Separations were performed using a hydrophilic-coated fused-silica capillary column, Supelco CElect P1 (Bellefonte, Pa., USA), 47 cm in total length (40 cm effective length) × 50 µm internal diameter. The running electrolyte was 0.3 M acetic, 0.3 M formic acid, at pH 2.5, previously filtered through an Acrodisc 0.22 µm filter (Gelman Sciences, Mich., USA). Sample injection was by pressure for 2 s. All separations were run at 20 kV. Detection was performed both at 214 nm and 280 nm.

As above, commercial standards of the aromatic amino acids Tyr, Phe and Trp were also run on CE to identify their migration times.

Results and discussion

The RP-HPLC and CE analyses of the WSNF showed complex peptide patterns (results not shown). Since the WSF of cheese consists of a heterogeneous mixture of

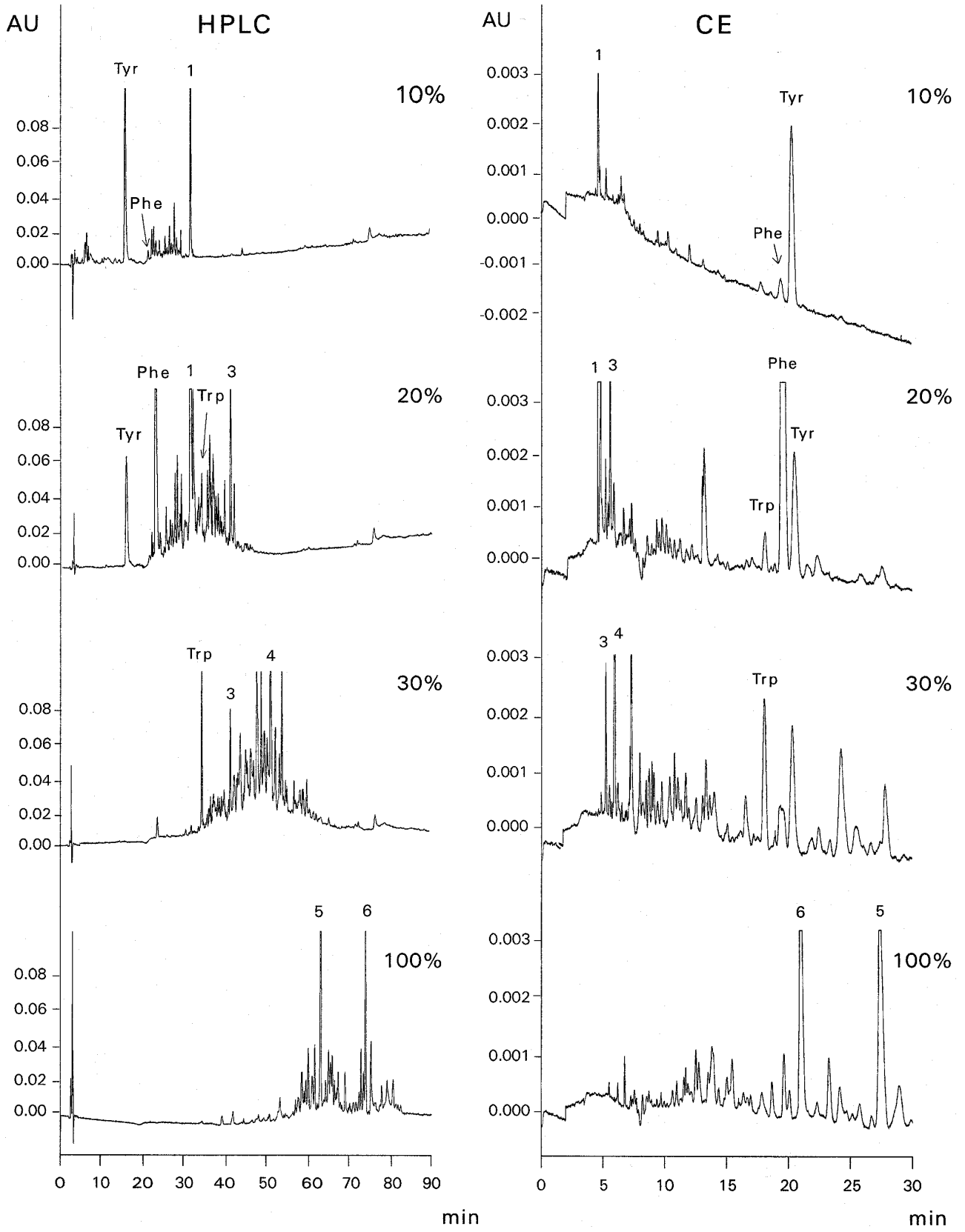

Fig. 3 RP-HPLC and CE analyses of the WSNF $<10\,000$ of a 6-month old cow's milk cheese eluted from a Sep-Pak cartridge with 10%, 20%, 30% and 100% acetonitrile (detection at 214 nm)

peptides, amino acids and whey proteins, with the aim of simplifying the analysis, proteins and peptides of high molecular weight were separated using centrifugal filtration units with a cut-off point of 10 000 daltons.

The RP-HPLC and CE separations of the amino acids and peptides with WSNF <10000 of the 6 month-old cow's milk cheese are shown in Fig. 1. A first comparison between these techniques showed that both methods provided efficient separations. The study of the run-to-run analysis time reproducibility demonstrated that the coefficients of variation (CV) calculated from the CE data were higher than those obtained by RP-HPLC (CV $<9\%$ in CE versus CV $<4\%$ in RP-HPLC), while the detection limit (signal-to-noise ratio = 3) was three times better using HPLC (0.15 in CE versus 0.06 in RP-HPLC). However, the CE method was much faster than the RP-HPLC one (30 min versus 90 min). These results on reproducibility, sensitivity and speed agree with previous reports regarding the application of CE and HPLC to the separation and quantification of milk proteins and peptides [5, 8].

CE separations were carried out using a coated capillary column to minimize adsorption of the peptides on the capillary wall. At the pH of the separation buffer (2.5), all the peptides are positively charged and, therefore, migrate in the same direction towards the cathode. Although, according to the manufacturer, very low values of pH could affect the coating of the capillary, it should be noted that approximately 200 injections were made with only slight variations in the migrations times, and negligible loss of the separation efficiency.

To allow an easier interpretation of the complex RP-HPLC and CE patterns of the WSNF <10000, a further fractionation was carried out using Sep-Pak C18 cartridges. Direct analyses of the Sep-Pak C18 fractions by CE (Fig. 2a) revealed worse resolution and higher variations in the migration times, as compared with the electropherograms of the WSNF. This was probably caused by the presence of different ions and acetonitrile in the sample matrix. Indeed, removal of the organic eluent by lyophilization, followed by redispersion of the residues in water, before CE analyses, greatly improved resolution and baseline quality and eliminated the variations in the migration times (Fig. 2b). This is probably attributable to the diminishing effect of acetonitrile on the conductivity and dielectric constant of the sample plug, which modified the charge of peptides during the separation and brought about the observed undesired effects. Other authors have also shown that the presence of an organic modifier in the sample solution can affect the migration behavior of analytes [15].

No peaks appeared in the unretained fraction or in the fraction eluted with 0.1% TFA in water. The elution order from the C18 cartridge of the fractions obtained with 10%, 20%, 30% and 100% acetonitrile was the same as that from the RP-HPLC analyses, showing that, as expected, the fractionation of the peptides depended on their relative hydrophobicity [14]. Corresponding analyses by CE indicated that the mass and charge characteristics of peptides with similar hydrophobicities were quite different from each other (Fig. 3).

The chromatographic and electrophoretic patterns of the different Sep-Pak C18 fractions detected at 214 nm and 280 nm were compared. The amino acids Tyr and Phe, as well as a hydrophilic peak which did not absorb at 280 nm (identified by the number 1 in Fig. 3) were the main components present in the fraction eluting from the Sep-Pak C18 cartridge with 10% acetonitrile. With 20% acetonitrile, in addition to the ones previously mentioned, the main peaks corresponded to Trp and another compound which did not absorb at 280 nm, identified by the number 2. When separated by RP-HPLC, the components of the fraction eluted with 30% acetonitrile had retention times between 34 min and 60 min. However, the migration times of these components, when separated by CE, were highly heterogenous. One of the main peaks in these fractions was detected at 280 nm and is identified by the number 3 in Fig. 3. Finally, in the fractions more tightly bound to the cartridge that were eluted with 100% acetonitrile, there were two main peaks (identified by the numbers 4 and 5 in Fig. 3). The compound represented by peak number 5 absorbed at 280 nm and, under these experimental conditions, showed very similar mobility to that of Tyr.

Undesirable bitter flavors in cheese have traditionally been associated with late-eluting, hydrophobic peptides from RP columns [16]. The removal of bitterness has been achieved, for example, by adding peptidases, which increase the number of early-eluting hydrophilic components [17]. Although the elution order of peptides in RP-HPLC can give an indication of their hydrophobicity, other parameters, such as size and conformation, strongly influence the elution sequence, and it should be noted that recent studies have demonstrated that both hydrophilic and hydrophobic peptides can be bitter [18]. In addition, the most savory compounds of cheese are likely to be very hydrophilic and of molecular sizes corresponding to tripeptides or smaller [1], which probably elute too early in a RP-HPLC separation, whereas CE would allow an easier separation based on their charge/mass ratio. The preliminary results shown in this paper show that the complementary use of CE and RP-HPLC seems to be a good approach for the characterization of cheese peptides. The CE method developed provided efficient separations in a third of the time required for a RP-HPLC analysis, while, on the other hand, RP-HPLC offered the advantages of better sensitivity and higher reproducibility.

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