Journal of Chromatography, 623 (1992) 345–355
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 454

Analytical and micropreparative separation of peptides by capillary zone electrophoresis using discontinuous buffer systems

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(First received April 8th, 1992; revised manuscript received June 10th, 1992)

ABSTRACT

The tiny injection volumes that are usually necessary to maintain the high efficiency of capillary zone electrophoresis present a major problem if only limited sample amounts are available. To increase the sample load, discontinuous buffer systems were developed that allow the on-column concentration of dilute samples. Injection volumes can be increased in this way by at least a factor of 30. These stacking systems were applied to the analysis of tryptic peptides, to the purity checking of high-performance liquid chromatographic fractions and for the micropreparative separation of peptides with subsequent amino acid sequence analysis.

INTRODUCTION

Owing to its high efficiency and ease of automation, capillary zone electrophoresis (CZE) has found much interest in the field of biochemistry. In capillary gel electrophoresis of oligonucleotides more than 10^7 theoretical plates have been reported [1]. In the analysis of peptides, owing to its lower charge [2], the theoretical plates obtained are of the order of several hundred thousand. To maintain the extremely high efficiency of CZE, care has to be taken that extra-column effects do not contribute to peak dispersion. For the injection volume it follows that it may not exceed a few nanolitres for a capillary of 100 μm I.D. Although these very tiny sample volumes can be of advantage in specific applications, *e.g.*, single cell analysis [3], in most instances the limited sample load presents a major drawback as high sample concentrations are required. Using a UV detector the detection limit for, *e.g.*, peptides, is of the order of several tens of femtomoles. As the volume necessary for injection is usually 5–10 μl, a sample amount of several tens of picomoles is needed for an analytical separation. The detection limit can be improved by several orders of magnitude by using a laser-induced fluorescence detector, but as only a small number of analytes exhibit native fluorescence, derivatization has to be carried out. This leads to all the problems already known from high-performance liquid chromatography (HPLC) such as derivatization of highly diluted samples or the formation of more than one defined product.

In peptide and protein chemistry, in most instances further structural information on the separated components, such as amino acid sequence or amino acid composition, is desired, *i.e.*, a micropreparative separation has to be carried out. To be able to separate 10 pmol of a peptide, the initial sample amount has to be about 10 nmol dissolved in 5–10 μl under the usual injection conditions. This presents no problem with synthetic peptides, but in the analysis of biological samples such high concentrations are unrealistic.

If CZE is to be coupled with mass spectrometry

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0021-9673/92/$05.00

(MS), higher sample concentrations are also needed. For a single ion electropherogram only several tens of femtomoles are necessary, but if the whole mass range is scanned or if even MS–MS experiments are to be carried out, sample amounts of several picomoles have to be separated.

Many of these problems can be overcome by increasing the injection volume and performing a concentration step prior to the separation. This can be achieved by applying a gradient in the electric field strength. The easiest approach for concentrating a sample zone is by injecting a sample that is dissolved only in water without any ionic matrix present [4–6]. Owing to the low conductivity in the sample zone, the electrical field strength in this zone is increased, which results in concentration of the analytes at the front. However, the requirement that the conductivity of the sample has to be much lower than that of the carrier electrolyte also represents the major drawback of this concentration technique, as realistic samples are very seldom free from ionic contaminants. Therefore, in these instances isotachophoresis can be used for sample stacking. Experimentally this preconcentration can be realised in a single- or a dual-coulmn mode. In the coupled column arrangement the analytes are isotachophoretically concentrated in the first column and are then transferred to the second column, where they are separated zone electrophoretically [7,8]. In this way a sample load of about 10 μl is easily reached. As these systems are not available commercially, discontinuous buffer systems may be applied for sample stacking, as generally used in classical sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [9,10]. So far these systems have been described in CZE only for the separation of proteins at high pH [11,12]. In this work discontinuous buffer systems at low pH were introduced for the analysis of peptides, but which are also applicable for the separation of proteins and many other substances at that pH.

The concentration of the dilute sample zone in a discontinuous system is caused by the fact that the buffer electrolytes are chosen such that at the beginning of the separation isotachophoretic conditions are created. In this way the concentration of the sample zone is adapted to the concentration of the preceding zone according to Kohlrausch's regulating function [13]. After the concentration step the analytes are separated zone electrophoretically as the buffer composition changes and the isotachophoretic conditions no longer hold for the sample components.

EXPERIMENTAL

Instrumentation

CZE measurements were carried out on a P/ACE System 2100 instrument (Beckman, Palo Alto, CA, USA) equipped with a UV detector. The absorbance of the peptides was detected at 200 nm in all instances. The separation capillary made of fused-silica (CS, Chromatographie Service, Langerwehe, Germany) had an I.D. of 100 μm and an effective length of 50 cm (57 cm total length). The inner wall of the capillary was coated with linear polyacrylamide according to the procedure described by Hjertén [14] in order to suppress electroosmosis and adsorption of the analytes. Injection was carried out by pressure, an injection time of 1 s corresponding to a volume of about 25 nl. Separation was carried out at a constant voltage of 20 kV, the capillary being thermostated at 30°C.

The different electrolyte solutions forming a discontinuous buffer system were filled into the capillary by applying a low pressure in the same way as hydrodynamic injection is carried out. A volume of about 25 nl is introduced when a low pressure is applied for 1 s at the inlet of a capillary of the above dimensions. The different volumes of the individual buffer solutions are shown in Fig. 1.

Fractions were collected electrophoretically by changing the outlet vials, which were filled with 10 μl of the separation buffer. The time windows for collecting the single fractions were calculated from the migration velocities determined in a previous run as described [15].

N-Terminal amino acid sequence analysis was carried out on a Model 477A gas-phase sequencer (Applied Biosystems, Foster City, CA, USA) as published [16].

Chemicals

All reagents were of analytical-reagent grade except β-alanine (for biochemistry) and ε-aminocaproic acid (for synthesis) (E. Merck, Darmstadt, Germany). β-Casein (from bovine milk) was obtained from Sigma (Deisenhofen, Germany), the

five standard peptides from Bachem (Heidelberg, Germany) and trypsin (sequencing grade) from Boehringer (Mannheim, Germany). As standard peptides the following basic, neutral and acidic peptides were used: (1) Leu–Trp–Met–Arg; (2) Leu–Trp–Met–Arg–Phe; (3) Leu–Trp–Met–Arg–Phe–Ala; (4) Tyr–Gly–Gly–Phe–Leu; and (5) Val–Leu–Ser–Glu–Gly. They were dissolved in water or the corresponding buffer electrolyte at concentrations ranging from 1 to 50 ng/ml.

The tryptic digest of β-casein was carried out in 50 m*M* Tris–HCl (pH 8.5). Water was purified in a Milli-Q Plus system (Millipore, Bedford, MA, USA). The buffer solutions in the electrode vessels were refilled after every 2–3 runs.

RESULTS AND DISCUSSION

"Three-buffer" stacking system

The classical stacking system used in SDS-PAGE applies a pH gradient and an amphoteric buffer component [9], which first acts as a terminating electrolyte but then overtakes the sample ions as the pH changes. Analogously a stacking system was developed for CZE for the separation of cationic peptides at an acidic pH of about 4.8. Its composition and arrangement of the electrolytes in the separation system is shown in Fig. 1a. It consists of three buffer electrolytes: a leading electrolyte and a stacking electrolyte, having the same constituents but different pH, and a terminating electrolyte. In this system tris(hydroxyethyl)aminomethane (Tris) acts as the leading ion whereas ε-aminocaproic acid (EACA) acts as the terminating ion when it is in a zone of high pH (>6), as formed by the stacking electrolyte. Under these conditions the mobility of EACA is very low, as it is almost a zwitterion at this pH. Hence, if the sample ions have a mobility between those of the leading and terminating electrolytes, its concentration is adapted to the concentration of the leading electrolyte, that is, concentration of the dilute sample occurs. As EACA enters a zone of lower pH, the dissociation of the carboxylic group is suppressed and it gains a higher positive charge and therefore a higher electrophoretic mobility. Hence it can overtake the analytes, which are then separated by zone electrophoresis in the EACA buffer.

In Fig. 2a and b the separation of three basic peptides dissolved in buffer electrolyte is shown without stacking and in the above stacking system. In the discontinuous system the sample is diluted tenfold, but at the same time the injection time is increased by a factor of ten compared with the non-stacking system, where injection was carried out for only 3 s. It can be clearly seen that in the discontinuous system, in spite of the high injection volume of 750 nl, a higher efficiency and a higher sensitivity

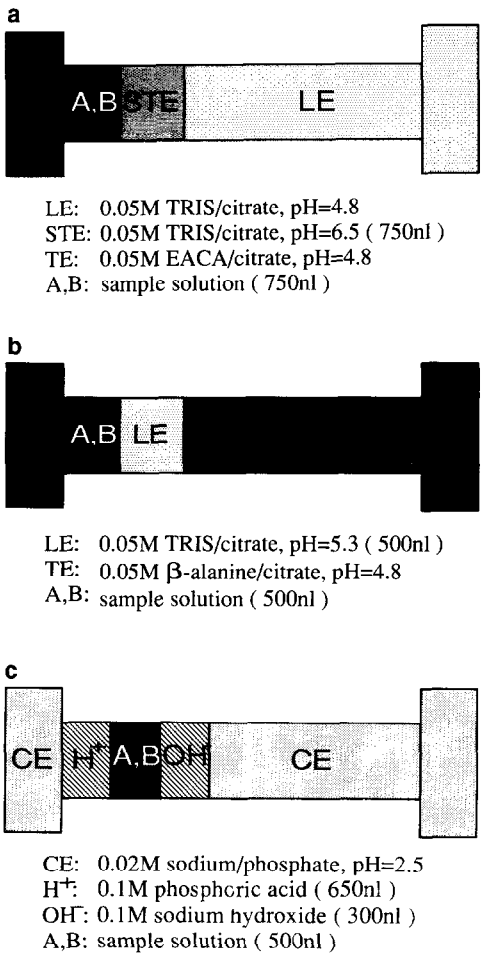

Fig. 1. Composition and arrangement of the buffer solutions of the different discontinuous systems in the separation unit. In parentheses the approximate volumes of the different zones for a 100-μm capillary, having a volume of about 4.5 μl, are given. LE = Leading electrolyte; STE = stacking electrolyte; TE = terminating electrolyte; CE = carrier electrolyte. (a) "Three-buffer" stacking system; (b) "two-buffer" stacking system; (c) "one-buffer" stacking system.

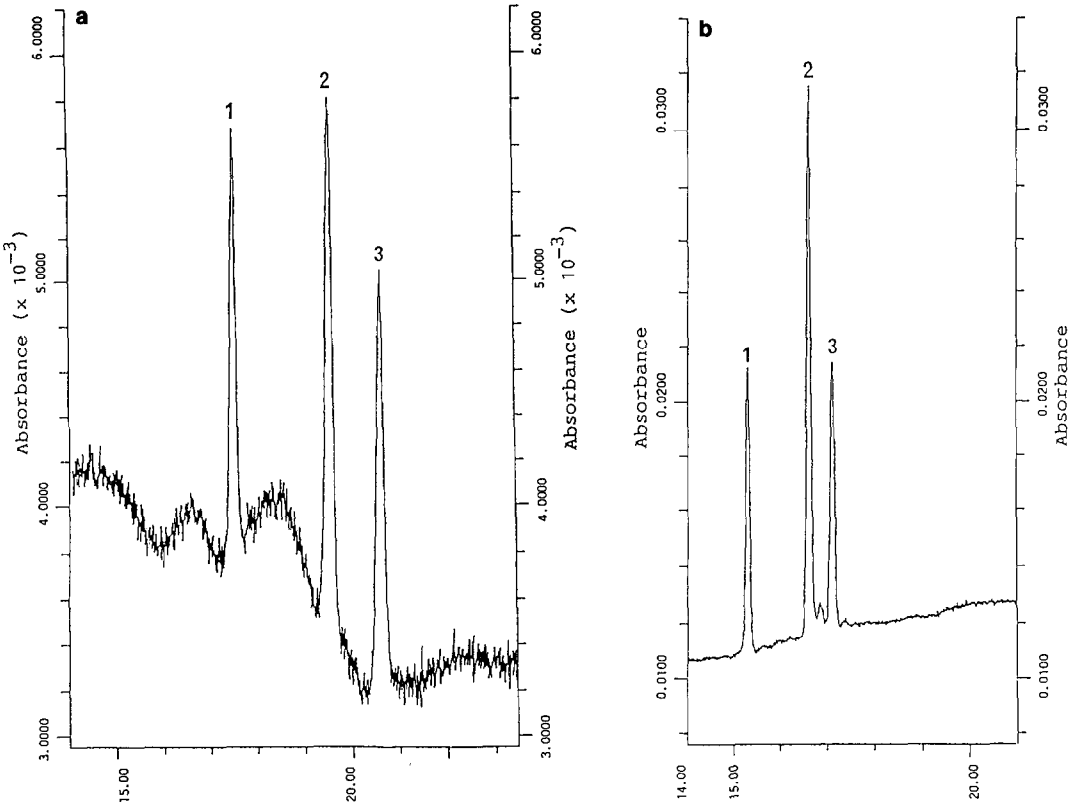

Fig. 2. Separation of three basic peptides: (a) without stacking in the EACA buffer of pH 4.8; (b) under stacking conditions in a three-buffer system. Concentrations of the peptides that were dissolved in the EACA buffer: (a) 10 ng/μl; (b) 1 ng/μl. Injection time: (a) 3 s; (b) 30 s. The sequence of the peptides is given under Experimental. For details of the electrolyte system, see Fig. 1a.

are obtained compared with the system without stacking.

The precision of the quantification under stacking conditions is comparable to that without stacking. The migration times obviously change in the discontinuous systems and are dependent on the length and conductivity of the injection zone.

To compare the different modes of injection, in Fig. 3 the peak width, expressed by the standard deviation, σ_z, based on length, is shown as a function of the injection time for the above separation of the three basic peptides. If a sample dissolved in buffer electrolyte is injected without stacking for 1 s, corresponding to a volume of about 25 nl, the maximum efficiency is not reached, *i.e.*, the contribution of injection to peak broadening is not negligible. For even higher injection times a steep increase in peak width, that is, a tremendous loss of efficiency, is observed. If injection is carried out from a sample dissolved only in water, about ten times the volume can be loaded without a decrease in efficiency. With the sample dissolved in buffer and using a discontinuous system, at least a 30 times higher sample load can be achieved compared with the non-stacking system. It can be seen that the peak widths remain constant over the whole injection range studied. In this way about 750 nl of sample can be introduced into the capillary, the volume being mainly limited by the dimensions of the capillary.

This kind of classical stacking system has the slight disadvantage that the choice of the terminating electrolyte sometimes presents a problem, as its mobility has to be lower than that of all the analytes at one pH and at the other pH it has to be higher, so

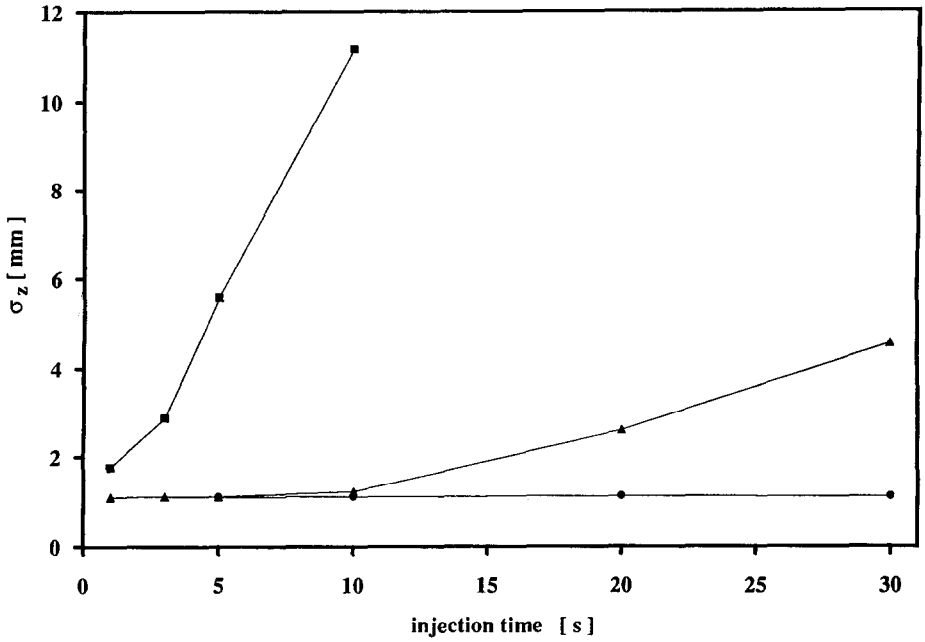

Fig. 3. Dependence of the peak width, expressed by the standard deviation based on length, σ_z, on the injection time for the different modes of injection. ■ = Injection of a sample dissolved in buffer electrolyte without stacking; ▲ = injection of a sample dissolved in water; ● = injection of a sample dissolved in buffer electrolyte under stacking conditions. The peak widths were calculated from the separation of basic peptides as shown in Fig. 2.

it can overtake the analytes. Especially with peptides, covering a wide range of mobilities, some problems may arise if other pH values are chosen. However, for the separation of basic peptides in the above system good stacking and destacking are observed.

"Two-buffer" stacking system

To facilitate the buffer selection, a stacking system, that requires no pH gradient, consisting of only two electrolytes, was developed as shown in Fig. 1b. The capillary is filled with an electrolyte of very low mobility, acting as a terminator, and just in front of the sample is placed a zone having a very high mobility, which will act as a leading electrolyte. The sample concentration is now increased as it is adapted to the concentration of the leading zone. Because this leading electrolyte is placed behind an electrolyte of lower mobility, it itself migrates zone electrophoretically in the terminating electrolyte and moves away from the sample ions. The analytes then are also separated by zone electrophoresis in the terminating electrolyte. In Fig. 4a and b the separations of three basic peptides without a leading electrolyte and in the presence of a leading electrolyte placed in front of the sample zone are shown. Injection was carried out for 20 s (*ca.* 500 nl). Without stacking the efficiency is lost and no resolution is observed. With the stacking system, high efficiency, sensitivity and resolution are obtained.

This stacking effect is also observed if a sample containing a high concentration of a mobile salt is separated in a buffer of very low mobility. Under these conditions the highly mobile salt ions may act as a leading electrolyte, resulting in sample concentration [17].

The reverse arrangement of the two electrolytes, the capillary being filled with leading electrolyte and a zone of a terminating electrolyte being placed behind the sample zone, may also lead to concentration of the analytes.

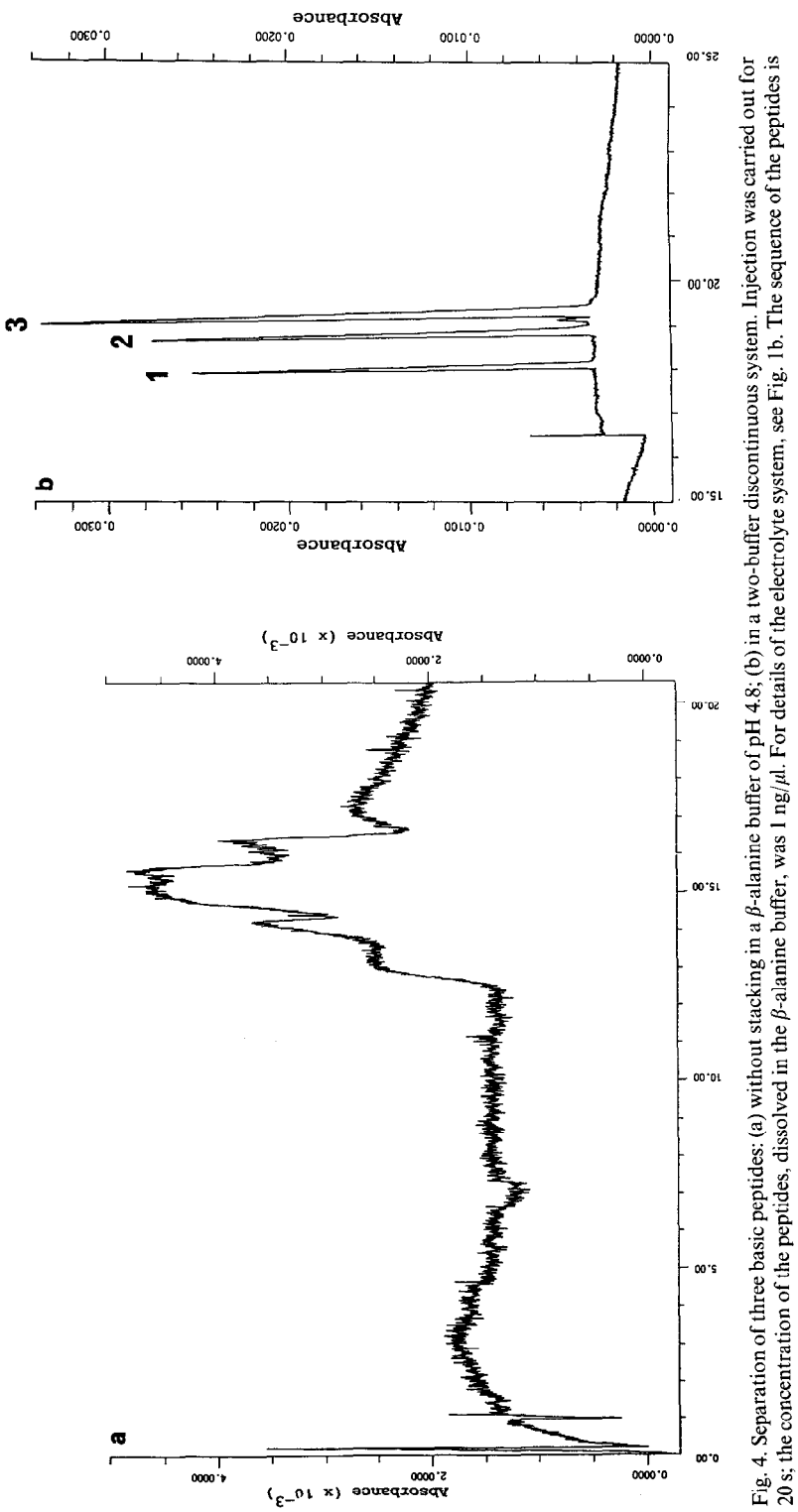

Fig. 4. Separation of three basic peptides: (a) without stacking in a β-alanine buffer of pH 4.8; (b) in a two-buffer discontinuous system. Injection was carried out for 20 s; the concentration of the peptides, dissolved in the β-alanine buffer, was 1 ng/μl. For details of the electrolyte system, see Fig. 1b. The sequence of the peptides is given under Experimental.

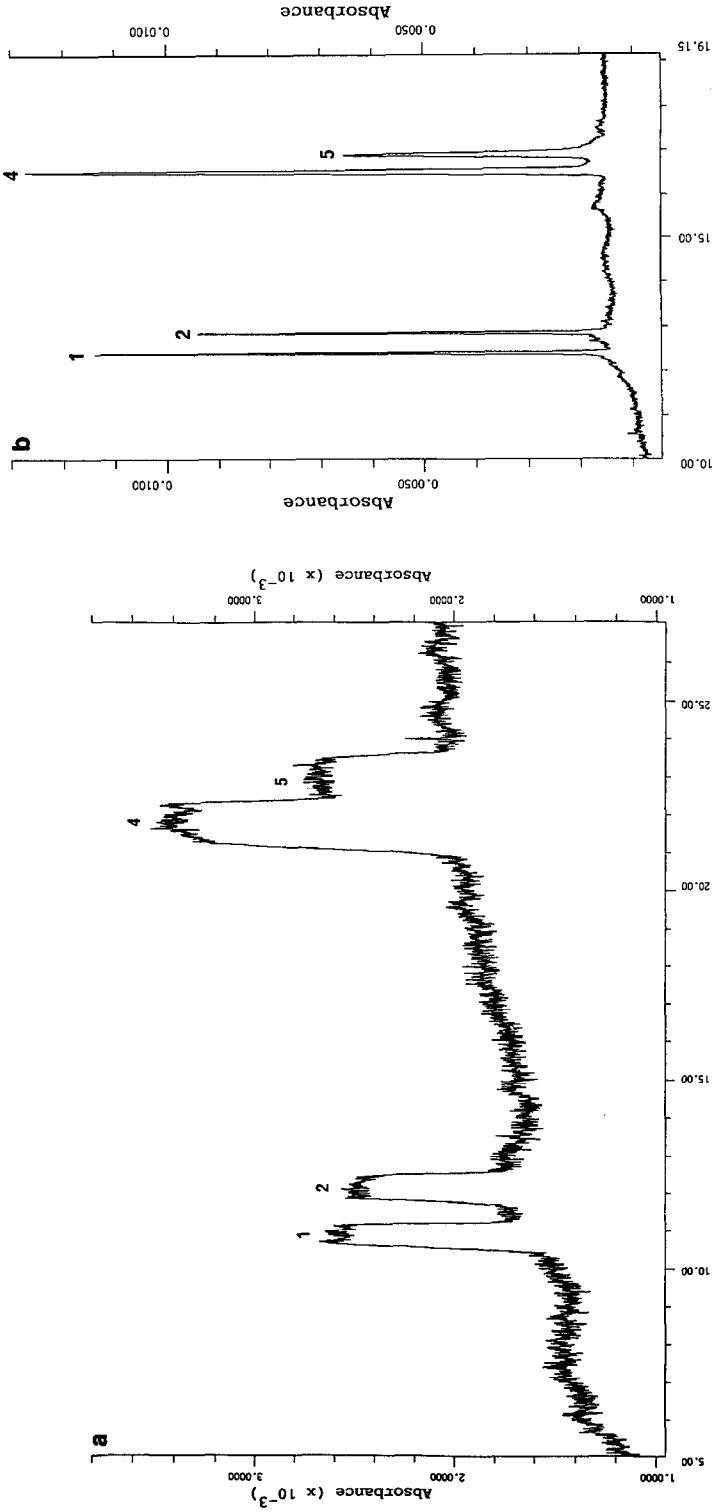

Fig. 5. Separation of four standard peptides in a phosphate buffer of pH 2.5; (a) without stacking; (b) sample sandwiched between a zone of H^+ and OH^-. Injection was carried out for 20 s; the concentration of the peptides, dissolved in the running buffer, was 2 ng/μl. The peptide code is explained under Experimental. For details of the electrolyte system, see Fig. 1c.

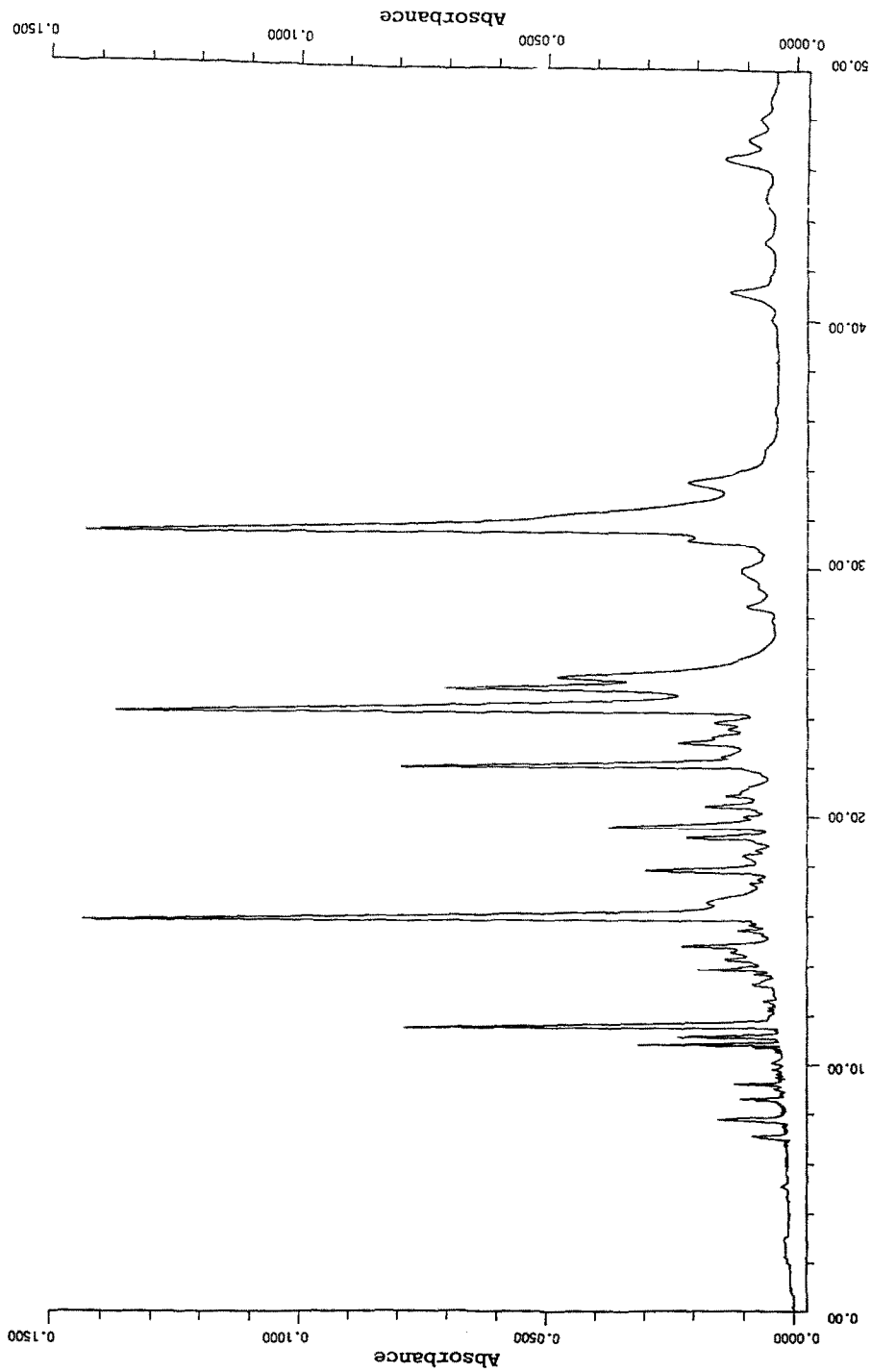

Fig. 6. Electropherogram of a tryptic digest of β-casein under stacking conditions at pH 2.5. Injection was carried out for 20 s; the concentration of the digested protein was 10 pmol/μl. For details of the electrolyte system, see Fig. 1c.

"One-buffer" stacking system

A disadvantage of the above stacking systems is that they do not work at extreme pH values. If peptides are to be analysed at a very low pH of about 2.5, so that all peptides migrate cationically, another stacking method has to be developed. Stacking of the analytes is achieved by sandwiching the sample between a zone of OH^- and H^+ as shown in Fig. 1c. As OH^- and H^+ are migrating towards each other, a zone of low conductivity is formed, as can be observed by a decrease in the electric current for a certain time at the beginning of the separation. Further OH^- and H^+ may act as a terminator, leading to isotachophoretic concentration of the analytes. Care has to be taken that the OH^- and H^+ zones are not too long, as overheating due to the high electric field strength in the low-conductivity zone formed may become a problem. In Fig. 5a and b the separation of four standard peptides (two basic, one neutral and one acidic) is shown in phosphate buffer of pH 2.5. Stacking leads to highly increased sensitivity and efficiency compared with a separation without stacking conditions.

This stacking system is not only applicable for the concentration of selected standard peptides but also for realistic samples, *e.g.*, a tryptic digest of β-casein as shown in Fig. 6. Injection was carried out for 20 s (*ca.* 500 nl) from a solution containing about 10 pmol/μl of digested protein. The peptide pattern obtained under stacking conditions is identical with that observed when a smaller volume of a more concentrated sample is injected without stacking. This means that with the described stacking system all peptides are recorded and none are lost.

With this system peptide fractions collected from HPLC can also be checked for purity with increased sensitivity. Fig. 7 shows an electropherogram obtained under stacking conditions of an HPLC fraction collected from a separation of tryptic peptides of β-casein. The fractions obtained from the RP-HPLC separation of 100 pmol of digested protein in an acetonitrile gradient containing 0.1% trifluoroacetic acid were directly injected into the CZE system without any preconcentration. Owing to the orthogonal separation principles of HPLC and CZE, a single peak in the chromatogram is resolved by CZE into several components.

A further aim with these stacking systems is to increase the sample load such that micropreparative

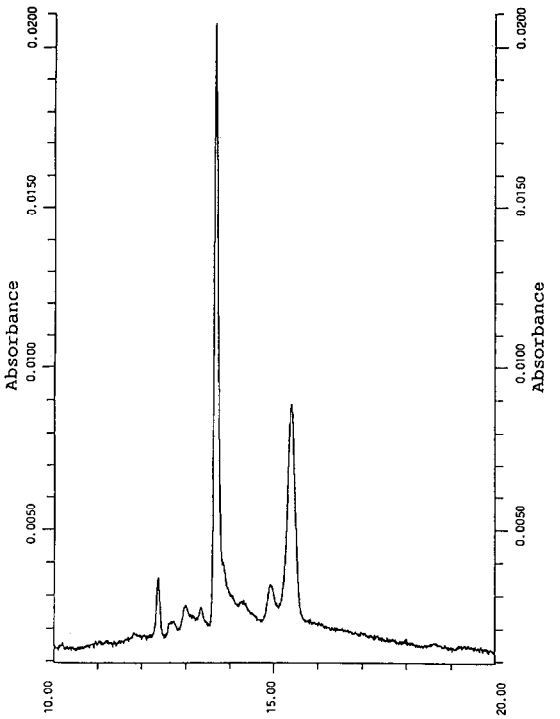

Fig. 7. Electropherogram of an HPLC fraction collected from the separation of digested β-casein. About 100 pmol of digest were separated by HPLC. Injection was carried out for 20 s. For details of the electrolyte system, see Fig. 1c.

separations also become possible with limited sample amounts. In Fig. 8 the separation of four standard peptides in phosphate buffer of pH 2.5 under stacking conditions is shown. The concentration of the analytes was 50 ng/μl (less than 100 pmol/μl). Injection was carried out for 20 s. From this electropherogram time windows were calculated for fraction collection of the four peptides. During the two following separation runs fractionation of the four peptides was carried out. To check the purity, the single fractions were reinjected also under stacking conditions as shown in Fig. 9. It can be seen that the first three peptide fractions were obtained almost pure, whereas the last fraction still contained a significant amount of the preceding peptide. This is probably caused by the fact that the calculation of the migration time from the detector to the end of the capillary is impeded by gradients in the electric field strength present in the separation system. This is the case when stacking systems are used for sam-

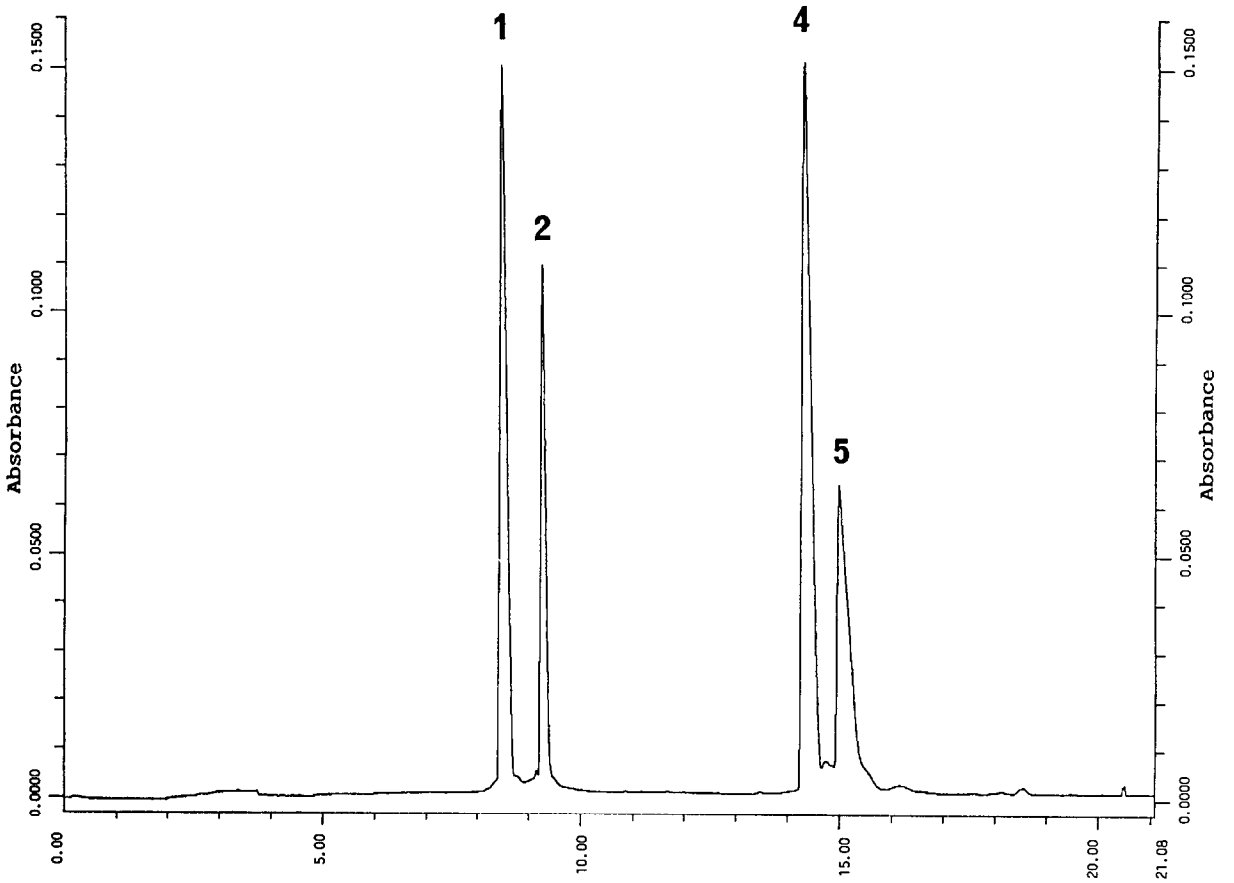

Fig. 8. Micropreparative separation of four standard peptides in phosphate buffer of pH 2.5 under stacking conditions. Injection was carried out for 20 s; the concentration of the peptides was 50 ng/μl. The peptide code is explained under Experimental. For details of the electrolyte system, see Fig. 1c.

ple concentration, or even if larger volumes of a low-conductivity sample are injected. More exact mobility values could be obtained using a double-detector system [18]. The purity of the single fractions may also be improved using other methods of collection [19].

From the re-injection, the sample amount collected was determined. It corresponded to a concentration of about 5 ng/μl, that is, about 50 ng of the single peptides were collected. The three pure peptide fractions were used for amino acid sequence analysis. An initial yield of about 40 pmol was found, corresponding well with the amount collected.

CONCLUSIONS

Discontinuous systems were introduced that allow the analysis of peptides and other analytes with an increased sample load of at least a factor of 30. These stacking systems can be applied for the concentration of dilute samples in commercially available instruments, making a micropreparative separation of moderately concentrated samples possible. Fractions can be collected from these separations and can be further characterized, *e.g.*, by amino acid sequence analysis, as was successfully demonstrated.

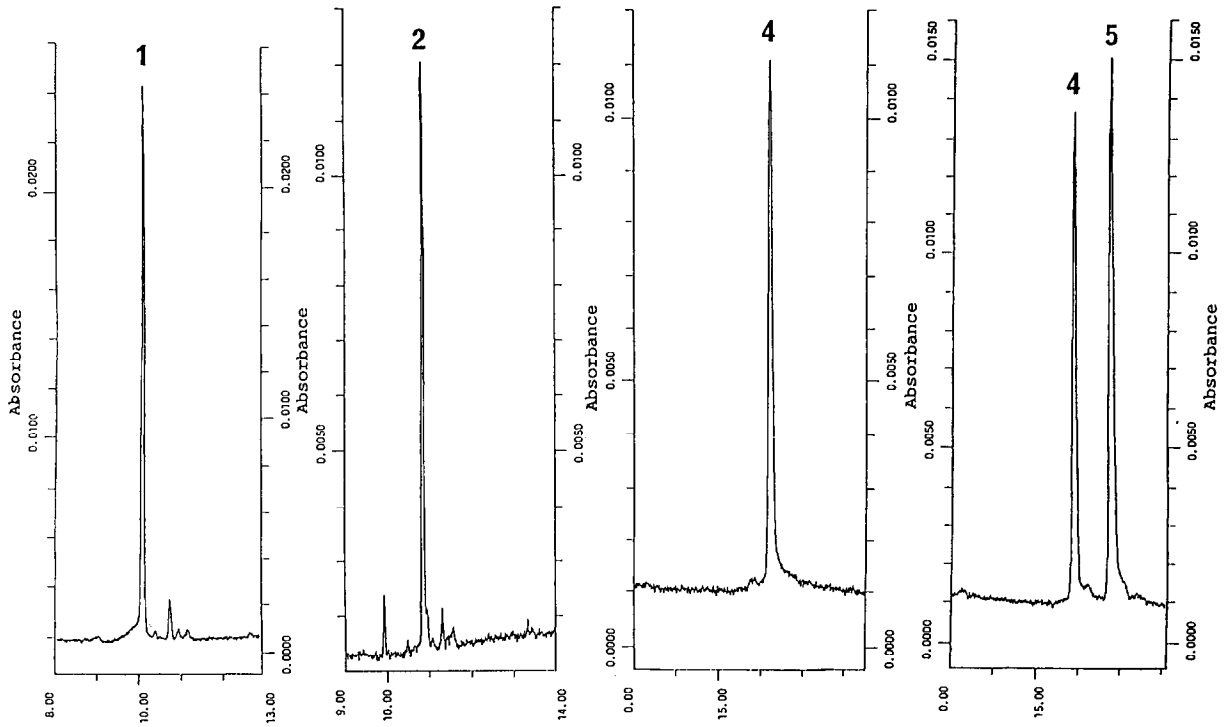

Fig. 9. Purity check of the fractions collected from the separation of four standard peptides as shown in Fig.8. The experimental conditions were the same as for the micropreparative run.

ACKNOWLEDGEMENT

The authors thank Beckman Instruments for providing the P/ACE 2100 CZE instrument. This work was supported by a bursary from the Commission of the European Communities.

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