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Separation of a bioactive cyclic peptide and its oligomeric forms by micellar electrokinetic chromatography

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Abstract

The monomer and the oligomers of a bioactive cyclic peptide were separated using micellar electrokinetic chromatography (MEKC) in order to determine if oligomeric forms of the peptide were present or not. Producing cyclic peptides involves the formation of an intramolecular disulphide bridge. During cyclisation, intermolecular disulphide bonds may be formed, thus resulting in unwanted oligomeric forms of the peptide. The cyclic peptide studied was shown by size-exclusion chromatography to be capable of forming a monomer, dimer, trimer and tetramer. The higher resolution MEKC provides was needed to separate further components of the oligomers. In phosphate buffer pH 7.5 containing sodium dodecyl sulphate 20 species could be separated in 18 min. Both linear and cyclic oligomeric isomers appeared to be present.

Keywords: Peptides

1. Introduction

An increasing number of peptides are becoming available for biomedical research and for therapeutic applications. In producing them in the pharmaceutical industry nowadays, high resolution separation and analysis techniques are essential since extensive purification and characterisation, not only of the peptides themselves but also of the impurities resulting from the processing steps, is necessary. Various analytical techniques for characterising peptides are in use. The different modes of high-performance liquid chromatography are the most prominent [1,2], but capillary electrophoresis (CE) is rapidly gaining in acceptance.

Cyclic peptides, which may be required for biological activity, can be produced by the formation of an intramolecular disulphide bridge. During cyclisation, intermolecular disulphide bonding may occur. This can result in unwanted oligomeric forms. The cyclic bioactive peptide investigated in the present study is one made synthetically. The monomer has a molecular mass ~1000. At low or neutral pH values the peptide is positively charged since it contains the basic amino acid arginine. Different oligomers of the peptide have been separated by size-exclusion chromatography (SEC) [3]. Oligomers up to tetramer were identified by off-line electrospray ionisation (EI) mass spectrometry (MS) of the fractions col-

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lected. The separation took approximately one hour, some of the oligomeric isomers being only partially resolved. Since increased resolution would have been needed for further oligomeric isomers to be detected, the peptide monomer and its oligomeric forms were analysed by CE, known for its high efficiency and wide applicability [4–6]. CE has been utilised for monitoring the disulphide bond formation occurring when homo- and heterodimers are formed during the co-oxidation of synthetic peptides in a 20 m*M* citrate buffer pH 2.5 [7].

In micellar electrokinetic chromatography (MEKC) an additional mechanism for selectivity is introduced through analyte interaction with surfactant micelles [8].

In the present study the monomer and the oligomers of a cyclic peptide were investigated. Since the size-to-charge ratios of these are similar, a surfactant was used to resolve the different oligomeric forms. The migration order was established by analysis of the fractions collected from SEC.

2. Experimental

2.1. Chemicals

Unless stated otherwise, chemicals were of analytical-reagent grade. The water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA). Acetonitrile (ACN; for chromatography), orthophosphoric acid (85%), sodium hydroxide solution (Combi-Titrisol 5 *M*), sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate anhydrous and disodium tetraborate cryst. were obtained from Merck (Darmstadt, Germany). Boric acid was from Riedel-de Haën (Seelze, Germany). Sodium dodecyl sulphate [SDS; approx. 99% (GC)] and DL-dithiothreitol (DTT; Sigma grade) were from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

To ensure an oligomer-rich sample, the synthesis conditions were deliberately chosen to promote the production of oligomers. The peptide sample, containing both the monomer and the oligomers, was then freeze-dried. The peptide stock solution (2 mg/ml) was prepared in ACN–water (3:7) and stored at 4°C for less than a week. Isolated fractions from SEC were freeze-dried and then dissolved to the concentrations 1 mg/ml of fraction A (mainly tetramers), 2 mg/ml of fraction B (mainly trimers), 0.25 mg/ml of fraction C (dimers) and 0.5 mg/ml of the monomer in ACN–water (3:7). Prior to analysis, the peptide solutions were diluted (1:3) in water or in a 5 m*M* solution of DTT (1:9) so as to reduce the disulphide bonds.

2.3. Electrophoresis buffer preparation

The phosphate buffers used as electrolyte solutions were prepared by mixing either stock solutions of 20 m*M* Na_2HPO_4 and 20 m*M* H_3PO_4 to pH 2.5 or solutions of 20 m*M* Na_2HPO_4 and 20 m*M* NaH_2PO_4 to pH 7.5. SDS was then dissolved in the pH 7.5 buffer to a final concentration of 50 m*M* (the critical micelle concentration for SDS in water is 8.1 m*M*). The borate buffer was prepared by mixing 50 m*M* H_3BO_3 and 12.5 m*M* $Na_2B_4O_7$ to pH 9.0. The SDS was dissolved to 100 m*M* in the borate buffer. Prior to use, the buffers were filtered through a 0.2-μm DynaGard filter tip (Microgon, Laguna Hills, CA, USA) and were degassed for 5 min by ultrasonication.

2.4. CE instrumentation

The equipment used for the majority of the measurements was a Beckman P/ACE 2050 CE instrument controlled by P/ACE Series 3.0 software (Beckman, Fullerton, CA, USA). The absorbance was measured at 200 nm on the cathode side of the capillary. A P/ACE system equipped with diode-array detection (DAD) was also employed. The capillary was of polyimide-coated fused-silica, 58.0 cm (51.2 cm to the detector)×50 μm I.D.×375 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA). The detection window was created by glowing off the polyimide coating using a match. The capillary was fixed in a cartridge with a 100×200 detection aperture.

2.5. CE separation conditions

Before each injection, the capillary was rinsed by

pressure (20 p.s.i.; 1 p.s.i.=6894.76 Pa) with 0.1 *M* sodium hydroxide, water and the electrolyte solution of current interest. The sample was introduced on the anode side of the capillary by pressure injection (0.5 p.s.i.) for 5 s. The capillary cartridge was thermostatted at 25°C. A constant separation voltage of 25 kV was applied, generating a current of 45 μA for the MEKC experiments.

2.6. Size-exclusion chromatography

A Kontron HPLC system Model 322 equipped with a Kontron variable-wavelength detector Model 432 was used for the SEC (Kontron Instruments, Zürich, Switzerland). Two Superdex Peptide HR 10/30 columns (300×10 mm) were coupled in series (Pharmacia Biotech, Uppsala, Sweden). The eluent consisted of 30% ACN and 0.1% trifluoroacetic acid with the flow-rate set at 0.6 ml/min. 20 μl of a 1 mg/ml peptide sample dissolved in ACN–water (3:7) was injected, detection being performed at 223 nm. 100 μl of a 62.5 mg/ml peptide solution was used for each preparative run in which the fractions were collected.

3. Results and discussion

The cyclic peptide sample and the SEC fractions collected were analysed by MEKC. SEC was performed according to Ref. [3]. A chromatogram from a SEC analysis is presented in Fig. 2, inset. It indicates there to be a larger number of components than the four molecular masses detected by the EI-MS, consistent with a tetramer, trimer, dimer and monomer [3]. In particular, isomers of the trimer seem to be present. A possible explanation of this may be that the oligomers occur in both cyclic and linear forms, although this would be difficult to confirm by EI-MS analysis without extensive optimisation. Both the high efficiency possible with CE, and separation based on a different range of selectivities than SEC provides would be required for separating further oligomeric isomers.

The isoelectric point was calculated to be 11.3 for the cyclic monomer and 8.3 for the linear using a locally constructed software based on Ref. [9]. In order to minimise the adsorption of basic peptides on the silica surface of the capillary, low pH buffers are normally used. When the oligomeric peptide sample was analysed in the 20 m*M* phosphate buffer pH 2.5, no resolution was achieved. This can be explained by the monomer and the oligomers having similar size-to-charge ratios, making their electrophoretic mobilities virtually identical.

Surfactant was then added to enhance selectivity through hydrophobic and electrostatic interactions with the micelles being introduced. The anionic surfactant SDS was dissolved to 50 m*M* in the 20 m*M* phosphate buffer pH 7.5 and to 100 m*M* in the 50 m*M* borate buffer pH 9.0. Better resolution was achieved in the phosphate buffer pH 7.5 than in the borate buffer pH 9.0 despite the higher SDS concentration used in the borate buffer (Fig. 1). Using these buffers, a separation voltage of 25 kV could be applied without excessive Joule heating. That such a high number of components was detected can be explained by the presence of both linear and cyclic oligomeric forms. The oligomers may also be formed with the asymmetric monomer coupled in different ways, thus forming isomers. They may be rather similar in hydrodynamic size and are consequently difficult to resolve by SEC.

As a first step towards classification of the species resolved in the MEKC analysis, DAD was performed. The DAD spectra obtained at the apexes were all very similar, indicating all the components separated to be peptides.

In order to distinguish the monomer and the oligomeric forms, fractions were collected in preparative SEC runs and were analysed by MEKC. The SEC fractions isolated were first analysed by MEKC to check the contents, Fig. 2. The SEC fractions were also added to the original sample. The migration order was established by comparing migration times and, if minor changes in the migration times were observed, by recording changes in relative peak heights when fractions were mixed with the original sample. The last component eluted was identified as the monomer and the second to last as the dimer. Both of these SEC fractions, C and D, seemed to be homogeneous when analysed by MEKC, only one component being detected in each albeit the peak representing the dimer was asymmetric. When the SEC fraction B, mainly containing trimers, was analysed, all but two of the components detected in

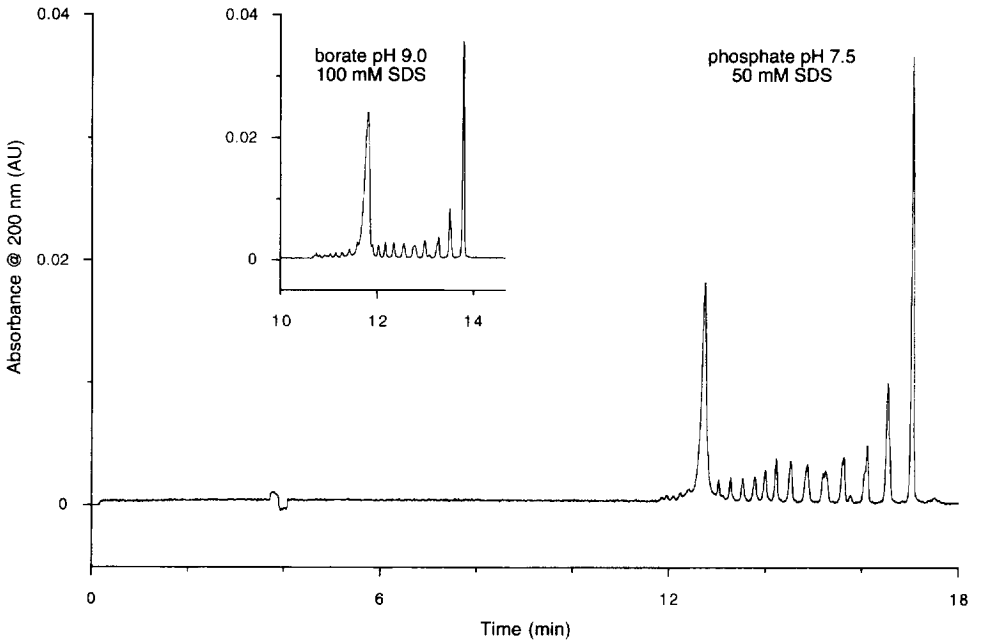

Fig. 1. Separation of the monomer and oligomers of the peptide by MEKC. 50 m*M* SDS added to a 20 m*M* phosphate buffer pH 7.5 and 100 m*M* SDS added to a 50 m*M* borate buffer pH 9.0 (inset) were used as the electrolyte solutions. Capillary 58 cm×50 μm I.D., voltage 25 kV, current 45 μA in phosphate buffer and 47 μA in borate buffer, injection 5 s×0.5 p.s.i., temperature 25°C, detection wavelength 200 nm.

the original sample were present. Only the two slowest migrating species, identified as the monomer and dimer, were absent. This result agrees with the MS analysis, the molecular masses of both the trimer and the tetramer being found in fraction B. This SEC fraction was inhomogeneous due to the poor resolution of the tetramer and the trimer in the preparative SEC runs. For this reason, it may be difficult to point out clearly which of the components separated in the MEKC analysis are trimers and which tetramers or possibly higher oligomers. When the SEC fraction A was analysed, the fastest migrating components from the original sample were found to be present. This SEC fraction contained small amounts of other peptide oligomers as well. The irregular peak shape may be unresolved components, derived from the number of isomers tremendously increasing as the oligomers get larger. There may also be interactions between the different isomers resulting in aggregation.

The migration order, clearly established by the monomer and the dimer and strongly indicated by the larger oligomers, was found to be tetramer, trimer, dimer and monomer. The SDS micelles thus appears to have interacted more effectively with the small peptides than with the larger peptides. This does not agree with the behaviour of many other peptides. The hydrophobicity of most peptides increases as their size increases. The migration normally found is in the order of increasing size. Large peptides are generally considered to interact too strongly with the micelles to be separable by MEKC without the addition of organic solvents [10].

Another approach taken to demarcate the oligomeric forms linked by disulphide bonds, an approach independent of SEC, was to reduce the disulphide bridges in the original sample by the addition of DTT. After complete disulphide bond reduction, separation should result in a single peak, representing the linear monomer. DTT was also added to a monomer sample for comparison. The result indicated the monomer to be the component that was eluted last (Fig. 3). Incomplete reduction, when too short a reaction time was used, resulted in more than

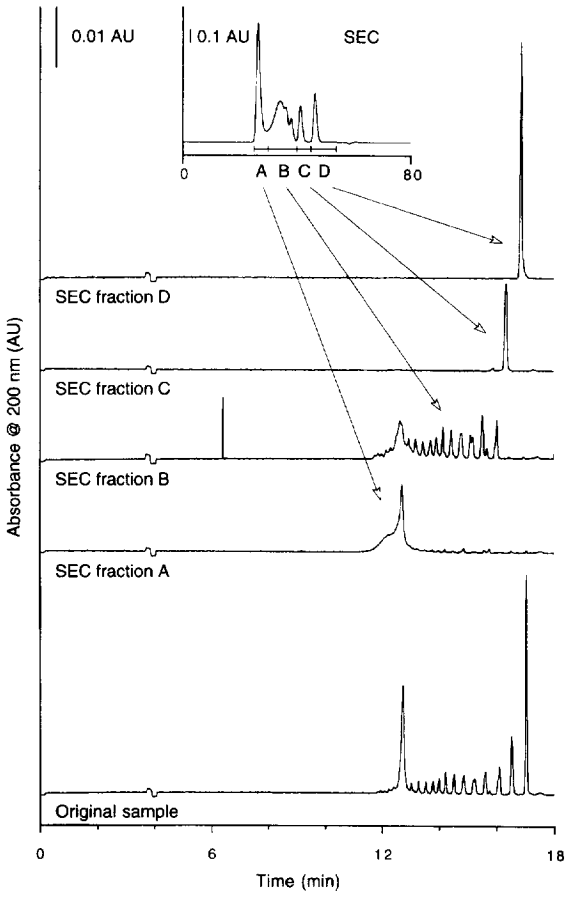

Fig. 2. Separation of the SEC fractions A, B, C and D by MEKC. Capillary 58 cm×50 μm I.D., electrolyte 50 m*M* SDS added to a 20 m*M* phosphate buffer pH 7.5, voltage 25 kV, current 45 μA, injection 5 s×0.5 p.s.i., temperature 25°C, detection wavelength 200 nm. Inset: Separation of the peptide monomer and oligomers by SEC. The tetramer was eluted in the void volume. 20 μl of a 1 mg/ml peptide sample was injected. In the preparative runs, four fractions were collected (A: 25–30, B: 30–40, C: 40–45 and D: 45–54 min). A MS analysis indicated that fraction A contained tetramer, fraction B tetramer and trimer, fraction C dimer and fraction D monomer.

one component, but fewer components nevertheless, than were detected in the original sample. Large oligomers were reduced very rapidly.

Since the peptide monomer and its oligomeric forms were difficult to dissolve in pure water, the peptide stock solution was dissolved in an ACN–water mixture. This was a useful step since the ACN marked the electroosmotic flow then through a disturbance in the baseline. If quantitative results are of interest, care should be taken in using fresh samples or a cooled tray, since the peak height ratios changed during the day when the sample was left in a non-cooled tray.

4. Conclusions

MEKC was used for the separation of a complex bioactive cyclic peptide sample containing both the monomer and oligomers. In a phosphate buffer pH 7.5 containing SDS, 20 species could be separated within 18 min. The peak shapes indicated further components, not yet resolved, to be present. The peptide sample of interest contained isomers that only could be disclosed by MEKC, but not by SEC. The size information obtained by SEC combined with the high resolving power of MEKC provided a tool for characterising the peptide sample. The MEKC migration order was tetramer, trimer, dimer and monomer. Various isomers of the trimer were separated by MEKC. The oligomeric isomers are formed when the asymmetric monomers are linked in different ways. The isomers can also occur in both linear and cyclic forms. Differences in the distribution of charged and hydrophobic sites thus strongly influence separation. MEKC represents a fast fingerprinting method for process analysis of the cyclic peptide monomer and its oligomers.

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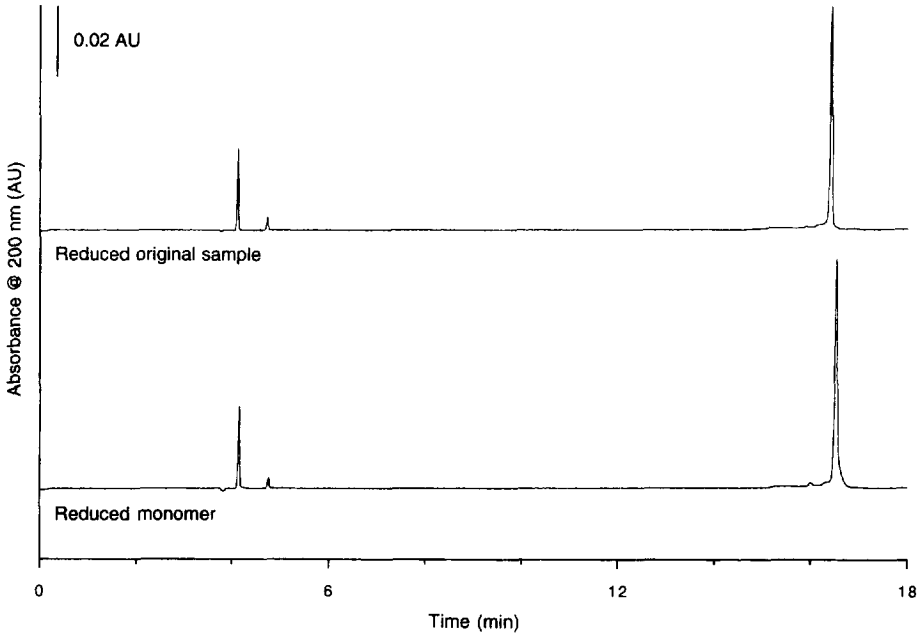

Fig. 3. Analysis of the reduced original sample and the reduced monomer of the peptide by MEKC. All the oligomeric forms have disappeared. The two peaks between 4 and 5 min originate from DTT since both appeared when a DTT solution was analysed. Capillary 58 cm×50 μm I.D., electrolyte 50 m*M* SDS added to a 20 m*M* phosphate buffer pH 7.5, voltage 25 kV, current 45 μA, injection 5 s×0.5 p.s.i., temperature 25°C, detection wavelength 200 nm.

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