Journal of Chromatography, 515 (1990) 313–320
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1751

Automated high-resolution two-dimensional liquid chromatographic system for the rapid and sensitive separation of complex peptide mixtures

KUNIE MATSUOKA, MASATO TAOKA, TOSHIAKI ISOBE* and TSUNEO OKUYAMA
Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Setagaya-ku, Tokyo 158 (Japan)
and
YOSHIO KATO
Central Research Laboratory, TOSOH Manufacturing Co., Ltd., Sinnanyo-shi, Yamaguchi 753 (Japan)

ABSTRACT

An automated two-dimensional liquid chromatographic system for the rapid and sensitive separation of complex peptide mixtures is presented. The method presents an application of the column-switching technique, and performs sequential anion-exchange and reversed-phase chromatography under a programme directed by a computer-assisted controller. To facilitate rapid and sensitive separations, short analytical columns (3.5 cm in length) packed with non-porous packing materials of small particle size (2.5 μm) were selected for both dimensional separations, and the dead volumes of the flow system were reduced to the minimum. With this system, complex peptide mixtures such as a crude peptide fraction prepared from brain extracts were resolved into *ca.* 150 peaks within 80 min, with a detection limit of 10 ng. The method can be used for the systematic analysis of biologically active peptides and for the micro-scale separation of peptide fragments in the strategy of protein and gene sequence analysis.

INTRODUCTION

Two-dimensional high-performance liquid chromatographic (2D HPLC) is an application of the column-switching technique, which performs sequential chromatography on two different columns. The automated system for this method developed by Takahashi and co-workers[1,2] and by us[3] consists of two columns with different separation modes connected in tandem through a tee-tube or a column-switching valve, two independent flow systems for each column and a computer-assisted controller for regulation of the total system (see refs. 4 and 5 for reviews). Both of these systems employ ion-exchange and reversed-phase chromatography for the first- and second-dimensional separations, respectively, and separate peptides and proteins by

0021-9673/90/$03.50

two independent parameters, first by charge and second by hydrophobicity, which is correlated strongly with the molecular weight[6]. Hence the separation principle of the 2D-HPLC technique resembles two-dimensional electrophoresis, which is widely used for the systematic separation of complex protein mixtures.

Like two-dimensional electrophoresis, the automated 2D HPLC system exhibits excellent resolution and reproducibility when applied to the systematic separation of proteins in crude tissue or cell extracts[4,5] and to the total separation of peptides produced by enzymatic digestions of very large proteins such as ceruloplasmin[1] or genetic variants of human serum albumin[2]. Other advantages of this system are easy operation and quantification and the simultaneous recovery of isolated proteins or peptides. The system is flexible, and various types of separation are possible depending on the purpose and the complexity of the sample mixture, *e.g.*, by modification of the elution conditions and number of separation cycles, or by replacing the columns with larger or smaller diameter columns or with columns having different separation specificites.

In our standard system, focused particularly on the separation of acidic proteins in the brain, we used a polymer-based TSK-gel DEAE-5PW column (75 mm × 7.5 mm I.D.; particle size 10 μm, pore diameter 1000 Å) and a polymer-based TSK-gel Phenyl-5PWRP column (75 mm × 4.6 mm I.D.; particle size 10 μm, pore diameter 1000 Å) for the first- and second-dimensional separations, because of their weak hydrophobic nature and excellent durability in both acidic and alkaline conditions. This system separates crude tissue or cell extracts into *ca.* 200 proteins by a single operation of chromatography. Takahashi and co-workers[1,2] constucted their peptide separation system with convensional silica-based DEAE and octadecyl columns of 15–25 cm × 4.6 mm I.D., and separated tryptic digests of ceruloplasmin or serum albumin into *ca.* 300 peaks. Both systems required about 12 h for the total analysis.

Here, we present a 2D HPLC system for the more rapid and sensitive separation of complex peptide mixtures. The system can separate more than 100 peptides within 80 min, using micro-amounts of samples.

EXPERIMENTAL

Materials

Calmodulin and D59 protein were purified from bovine brain extracts essentially as previously described[7,8]. A crude mixture of brain peptides was obtained by passing the brain extract through a molecular sieve ultrafiltration membrane (Ultrafree C3-LGC, molecular cut 10 000; Millipore, Bedford, MA, U.S.A.).

The sources of enzymes and reagents were as follows: trypsin, treated with 1-(tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) (Cooper Biomedical, Malvern, PA, U.S.A.); lysylendopeptidase (Wako, Osaka, Japan); chymotrypsin (Sigma, St. Louis, MO, U.S.A.); trifluoroacetic acid (TFA) (Sequanal grade) and urea (biochemical grade) (Wako); 4-vinylpyridine (Tokyo Kasei Industries, Tokyo, Japan); and acetonitrile (chromatography grade) (Merck, Darmstadt, F.R.G.). All other reagents were of analytical-reagent grade from Wako. Water was distilled, passed through a mixedbed ion-exchange resin and redistilled before use.

TSK-gel DEAE-NPR (3.5 cm × 4.6 mm I.D.) column and TSK-gel octadecyl-NPR (3.5 cm × 4.6 mm I.D.) column were products of TOSOH (Yamaguchi, Japan).

Proteolytic digestion

Before digestion, D59 protein was reduced and pyridylethylated in 2 *M* Tris–acetate buffer (pH 7.6) containing 8 *M* urea and 1% (v/v) ethylenediaminetetraacetic acid[9]. The pyridylethylated protein was then digested with lysylendopeptidase or chymotrypsin (enzyme-to-substrate ratio = 1:100) in 0.1 *M* ammonium hydrogencarbonate solution (pH 8.0) at 37°C for 7 h (lysylendopeptidase) or 20 h (chymotrypsin). Tryptic digestion of calmodulin was performed at 37°C for 5 h using TPCK-treated trypsin (enzyme-to-substrate ratio = 1:100).

Apparatus and performance of the 2D HPLC system

The automated 2D HPLC system is illustrated in Fig. 1. The system was constructed from Model 8010 HPLC assemblies provided by TOSOH. An anion-exchange DEAE-NPR column (C1) is connected in tandem with a reversed-phase octadecyl-NPR column (C2) through a six-way electrical column-switching valve (Model MV-8010). For each column, programmed elution is performed with a Model SC-8010 controller (SC). The controller regulates two dual-plunger pumps (Model CCPM; P11–P12 and p21–P22) to perform a series of stepwise elutions for C1 and to perform repetitive gradient elution for C2. The stepwise elution of the first-dimensional anion-exchange chromatography and the gradient elution of the second-dimensional reversed-phase chromatography are synchronized by time-dependent control of the flow system.

The 2D HPLC was performed as follows. On starting the programme, columns C1 and C2 are equilibrated with B1 (25 m*M* Tris–HCl buffer, pH 7.5) and B3 [0.1% trifluoroacetic acid (TFA)] respectively at a flow-rate of 1.0 ml/min. After 10 min P2 is stopped and the column switching valve moves to connect C1 and C2. Analysis is started by applying a sample mixture through a sample injecter (I) followed by elution of C1 with B1 for 3 min. During this time the eluent flows directly into C2. P1 is stopped; simultaneously, P2 starts pumping at a flow-rate of 1.0 ml/min with a linear gradient from B3 to B4 (0 to 50% acetonitrile in 0.1% TFA unless mentioned

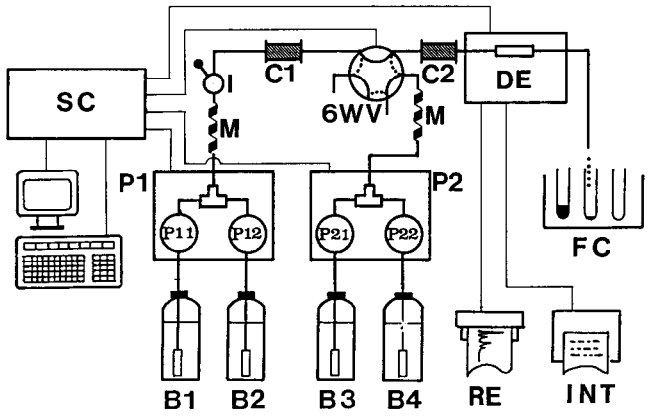

Fig. 1. Schematic diagram of the 2D HPLC system for micro-scale separation of peptides. The system is similar in principle to that described previously[4,5], except for columns C1 and C2 and some modifications to the flow system for micro-scale separation. Details are explained in the text.

otherwise in the figure legends) in 10 min. When the gradient elution has finished, C2 is equilibrated again with B3 for 3 min and then P2 is stopped. At this stage the first cycle of the 2D HPLC is completed. P1 then starts to elute peptides stepwise again from C1 by introducing and mixing a portion of buffer B2 (0.4 *M* Tris–HCl buffer, pH 7.5) into B1. After applying the eluent to C2, the second reversed-phase chromatography is repeated exactly as in the first cycle. These procedures are repeated for a programmed number of cyles, changing the mixing ratio of B1 and B2.

The eluent was monitored at 210 nm with a 1-cm light path (cell volume 12 μl) with a Model UV-8010 detector (DE) connected to an FBR-2 recorder (RE) and a PR-8010 integrator. When necessary, the eluent was collected in an FC-8000 peak collecter (FC). In order to reduce the dead volumes of the system, a conventional 2-ml volume dynamic mixer was replaced by an on-line coil solvent mixer (M; 1 m × 0.6 mm I.D.) and the tube connecting the outlet of the second column and the detector was reduced in volume to 1.2 μl to avoid diffusion of the eluent.

Amino acid analysis and nomenclature of peptides

The amino acid composition was determined on a Model 800 amino acid analyser (JASCO, Tokyo, Japan) after hydrolysis of peptides with 6 *M* hydrochloric acid containing 5% (v/v) phenol for 20–24 h in evacuated, sealed tubes. The calmodulin peptides are numbered by the fragmentation method used (T for trypsin) followed by the residue numbers starting from the N-terminus of the complete amino acid sequence[10].

RESULTS AND DISCUSSION

Separation of tryptic peptides of bovine calmodulin

Calmodulin is an acidic calcium-binding protein found in all eukaryotic cells. It contains 148 amino acids and has the potential to yield 12 tryptic peptides[10]. The micro 2D HPLC separation of the tryptic digest of calmodulin using a 10-μg sample (600 pmol) resulted in seventeen well resolved peptide fragments (Fig. 2). Of these, eight major fragments were collected from HPLC, subjected to amino acid analysis after hydrolysis with 6 *M* hydrochloric acid (see Experimental) and their positions in the primary sequence were identified on the basis of the amino acid composition and the published sequence. The identified peptides (shown in Fig. 2) included all of the peptides expected from tryptic cleavage of calmodulin, except for very small peptides T31–37, T76–77, T78–86, T87–90 and a free lysine. Two peptides, T1–30 and T107–148, were the fragments generated by incomplete tryptic cleavage.

Determination of the hydrophobicity by the method of Sasagawa *et al.*[11] indicated that all peptides identified in Fig. 2 had a hydrophobicity (ln *H*) greater than 1.9, whereas others had a value of less than 1.4. Therefore, we expected that the four small peptides mentioned above and a free lysine would not be retained on the reversed-phase column during the first-dimensional ion-exchange chromatography. The retention time of each identified peptide was linearly related to its hydrophobicity (correlation coefficient = 0.97), suggesting that the present system could separate peptides having hydrophobicities of ln $H > 1.5$.

Fig. 3 shows a 2D HPLC profle in which 1 μg of the same digest of calmodulin was analysed by increasing the detector sensitivity 10-fold over that for standard

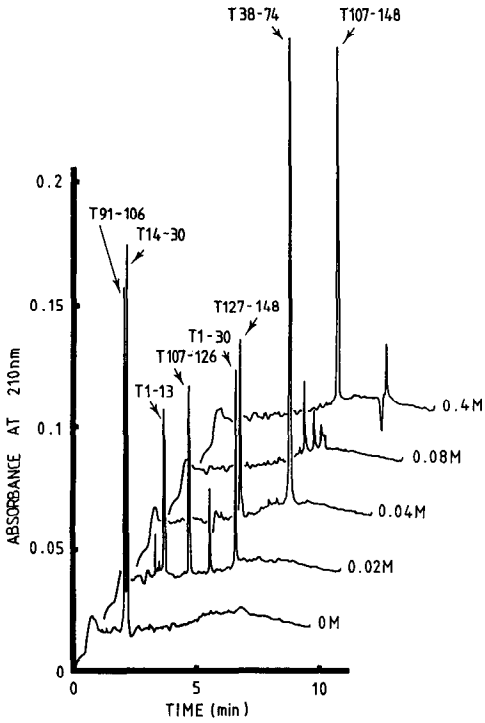

Fig. 2. Tryptic peptide map of bovine brain calmodulin obtained by automated 2D HPLC. The tryptic digest (10 μg) was applied to the system and eluted under the standard conditions described in the text. Each horizontal profile represents the result of the second-dimensional reversed-phase chromatography of a peptide fraction eluted from the first-dimensional anion-exchange column. The sodium chloride concentration for the stepwise elution of the anion-exchange column is shown on the right side of each profile. Peptides are identified according to the nomenclature described under Experimental.

conditions. At this sensitivity, the signal-to-noise ratio was considerably reduced by the appearance of many small peaks detected at an early stage of gradient elution and a very broad peak observed around 5 min. However, the peptide fragments that were identified in the standard analysis (Fig. 2) were still clearly distinguishable from the baseline. From this profile, the detection limit of the present system was estimated to be 10 ng. Peptide separation at this level of sensitivity is valuable for many biologically interesting proteins that are available in only limited amounts for identification, characterization and comparison with other proteins on the basis of primary structure. The method should be applicable to a wide range of such proteins, because the amount required (1–10 μg) is compatible with the currently available, most efficient protein separation techniques such as 2D electrophoresis or 2D HPLC and also other micro protein chemical techniques such as the *in situ* protease digestion of a protein blotted onto a nitrocellulose membrane[12].

Separation of proteolytic fragments of D59 protein

D59 protein is an acidic protein (pI = 4.1) with an apparent molecular weight of 59 000 daltons as estimated by 2D electrophoresis in the presence of sodium dodecyl

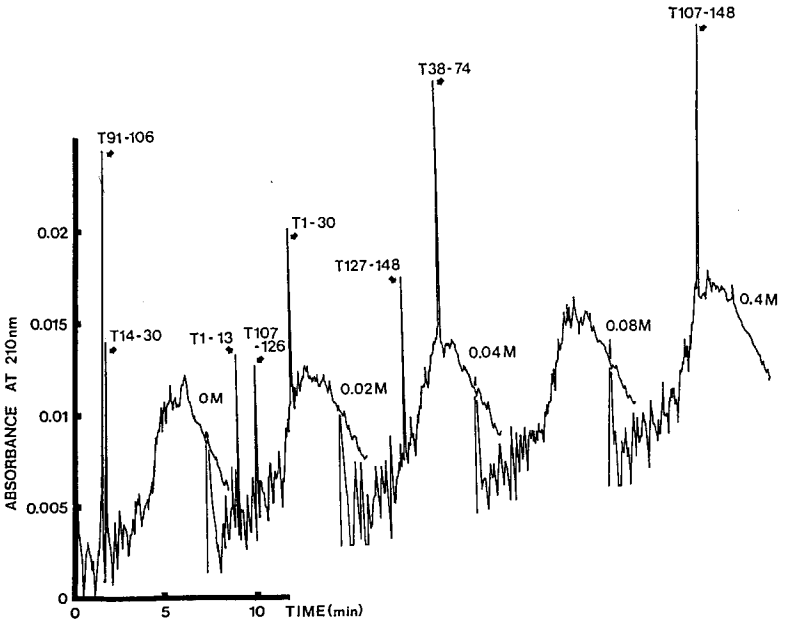

Fig. 3. High-sensitivity analysis of the tryptic digest of calmodulin. A 1-μg amount of the digest was separated under the same conditions as in Fig. 2, except that the detector sensitivity was set at 0.02 a.u.f.s. at 210 nm. To avoid complication the time-axis is indicated only for the first chromatogram.

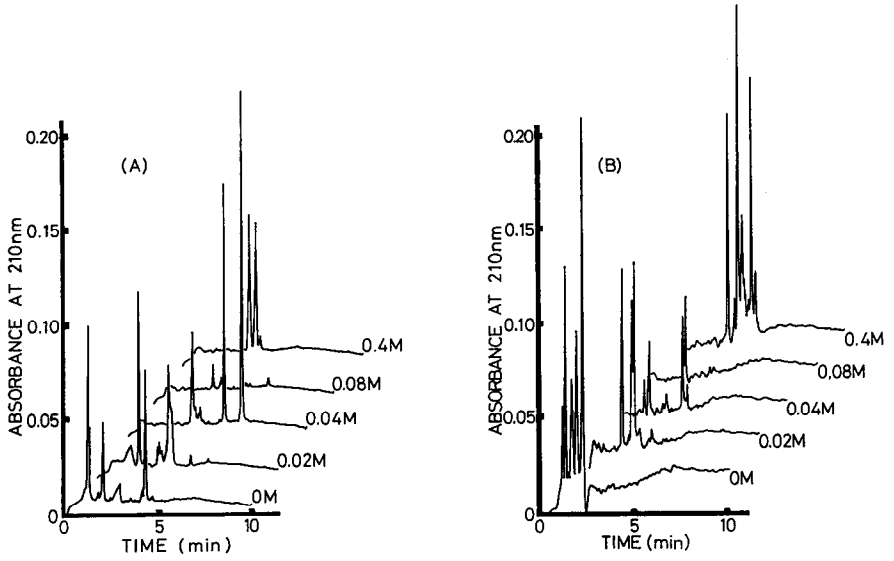

Fig. 4. Elution profile of peptide fragments of bovine brain D59 protein produced by cleavage with (A) lysylendopeptidase and (B) chymotrypsin. The digests (20 μg each) were separated by 2D HPLC under the conditions described in the text.

sulphate. This protein has been isolated during systematic studies of brain proteins but its biological function is unknown. In order to characterize this protein in further detail, the pyridylethylated D59 was cleaved by lysylendopeptidase and chymotrypsin and the peptide fragments generated were separated by 2D HPLC. As shown in Fig. 4, 20–30 fragments were detected for each digest using 20 μg (300 pmol) of protein. The number of fragments detected was less than that expected from the estimated molecular weight of this protein, probably because the large number of lysine or hydrophobic residues in D59 led to the generation of large numbers of very small fragments on lysylendopeptidase or chymotryptic digestion that did not bind to the reversed-phase column.

Subsequent protein chemical analysis proved that most of the fragments isolated by 2D HPLC had sufficient purity for the determination of their amino acid composition and sequence. Because computer-assisted searches of sequence databases showed that the determined peptide sequences of D59 did not show significant homologies with any protein with a known amino acid sequence, we assumed that D59 was one of the brain proteins that had not been described. The determined sequences were used as probes for gene cloning of this protein.

Separation of a crude peptide mixture prepared from brain

The brain tissue contains many biogenic peptides important for neural function and homeostatis. Because of the complexity of the brain extract, however, the isolation

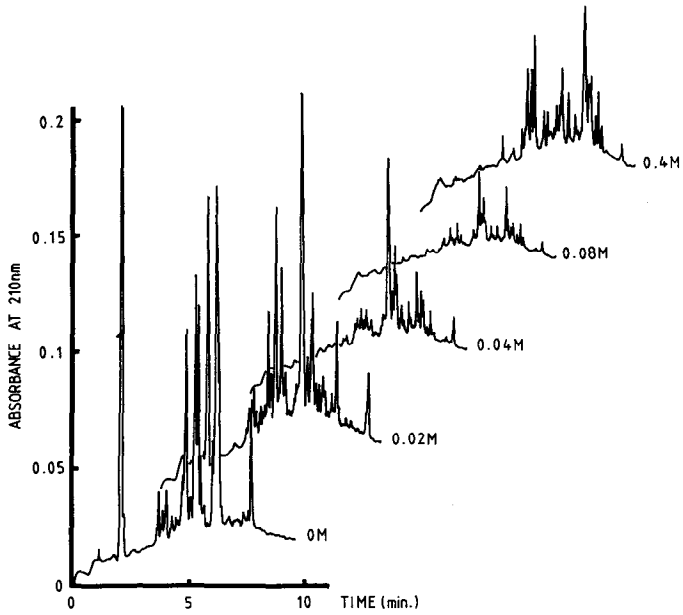

Fig. 5. Separation of crude peptide mixture extracted from brain tissue. A crude peptide fraction was prepared from bovine brain extracts as described under Experimental and an aliquot (40 μg of peptide) was analysed directly with the 2D HPLC system. The analytical conditions were as described in the text, except that the second-dimensional reversed-phase chromatography was performed with a gradient from 20% to 60% acetonitrile in 0.1% TFA.

of such components has generally been achieved by tedious multi-step purification procedures that often result in low recoveries. To test the resolution of the 2D HPLC technique, a crude peptide fraction was prepared by filtration of the brain extracts through a molecular sieve membrane (see Experimental) and an aliquot of filtrate was applied directly to the automated 2D HPLC system. The separation was achieved by repeating both the ion-exchange and reversed-phase chromatograhic processes five times within 80 min. As shown in Fig. 5, the peptide fraction was resolved into *ca.* 150 peaks by a single operation of chromatography. Although these peaks were not characterized in detail, this suggests that the system presented should be useful for the isolation of biologically important peptides which are present in small amounts in the brain and other tissue extracts or in physiological fluids such as serum and urine.

ACKNOWLEDGEMENTS

This work was supported in part by a research grant from the Ministry of Education, Science and Culture, Japan.

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