Automated two-dimensional liquid chromatographic system for mapping proteins in highly complex mixtures

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ABSTRACT

An automated two-dimensional liquid chromatographic system was developed for systematic protein separations which could serve for analytical mapping and preparative separations of proteins. The system applies the principles of the column-switching technique, and consists of two different columns connected in tandem through an electrical column switching valve, two pumping systems to operate each column independently and a system controller to perform sequential chromatography on the two columns. A protein mixture is applied to the first-dimensional anion-exchange column and is separated by stepwise elution with an increasing sodium chloride concentration. The eluent is introduced directly to the second-dimensional reversed-phase column, and is further separated by gradient elution with an increasing acetonitrile concentration. The two elution stages are synchronized by a computer program. By this system, very complex protein mixtures such as crude cerebellar extracts were resolved reproducibly into *ca.* 200 peaks within 12 h. The method can be used for the total analysis of proteins in various tissues and cells without complicated premanupulation of samples, and allows the simultaneous analysis of a protein isolated by chromatography. The isolated protein is most suitable for use in the strategy of protein and gene sequence analysis.

INTRODUCTION

With recent advances in biochemical and gene cloning techniques, the need for rapid, systematic and universal methods for purifying and analysing proteins has become increasingly important. The development of such methods will serve, for instance, to reduce the time and labour of purifying proteins and to elucidate the molecular basis of various biological events accompanying qualitative or quantitative changes in proteins. Two-dimensional (2D) electrophoresis meets many of these requirements [1–3] and is widely used for both analytical and preparative purposes [4–6], but it has limitations in several respects: multi-step manual handling, small sample capacity, possible contamination of impurities from the gel matrix, etc.

In general, the purification of biological substances such as proteins has been achieved by multi-step chromatography operated manually, *e.g.*, the effluents from a first column are collected in a number of fractions, each fraction is desalted or/and concentrated when necessary and the concentrated fractions are applied to a second column of different specificity, and so on. Although such an approach has been extensively applied to the separation not only of proteins but also of peptides [7] and small urinary substances [8], a problem often encountered is low reproducibility through accumulation of experimental errors due to multi-step manual handling.

In the course of systematic studies of brain proteins, we have developed an automated two-dimensional high-performance liquid chromatographic (2D-HPLC) system that is applicable to systematic separations of complex protein mixtures such as crude tissue/cellular extracts. The system is an application of the column-switching technique, and is

a modification of the system of Takahashi and co-workers [9,10] and its microscale version described by Matsuoka *et al.* [11], both of which were designed for the separation of complex mixtures of peptides. The modification includes the instrumentation of the system, where we employed one HPLC assembly with a valve control mechanism instead of two independent HPLC assemblies, and the utilization of polymer-based columns for both dimensional separations. The former modification simplified the apparatus and made the system more specific for the 2D-HPLC technique, and the latter approach was important in adapting the system for the separation of crude protein mixtures (described below). The system employs an ion-exchange and a reversed-phase column, and separates proteins first by charge [12] and second by hydrophobicity [13,14], which correlates with molecular weight [15]. These columns are connected in tandem through an electrical column-switching valve, and are eluted sequentially with a computer-assisted, time-dependent control of the flow system.

The automated 2D-HPLC technique shows reasonably high resolution and reproducibility, and has several advantages over the electrophoresis technique with respect to ease of sample handling, recovery, quantification and flexibility. A general feature of the 2D-HPLC technique has been described briefly [16,17]. This paper presents details of the protein separation system and its application to the separation of crude tissue/cellular extracts.

EXPERIMENTAL

Chemicals and proteins

Acetonitrile (chromatography grade) was obtained from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA) (Sequanal grade) from Wako (Tokyo, Japan). All other reagents were of analytical-reagent grade from Wako, unless mentioned otherwise. Water was distilled, passed through a mixed-bed ion-exchange resin and redistilled before use.

Bovine serum albumin was purchased from Seikagaku Kogyo (Tokyo, Japan). Haemoglobin was prepared from bovine erythrocyte lysate by repeated crystallization. Other proteins were purified from bovine brain essentially as described [18,19].

Apparatus

The system is illustrated in Fig. 1. It consists of two HPLC columns (C1 and C2) connected in tandem through an electrical column-switching valve (4WV; Mode EIE010, Senshu Kagaku, Tokyo, Japan) and two independent flow systems each equipped with a high-pressure pump (P1 and P2; Model LC6A, Shimadzu, Kyoto, Japan), two pairs of solenoid valves (V1/V2 and V3/V4; Model MTV2-M6, Takasago Electric, Tokyo, Japan) and a coil solvent mixer (M; 1.2 m × 0.25 mm I.D.). A system controller, composed of an eight-bit microcomputer (MC; Model PC-8801, NEC, Tokyo, Japan) connected to electrical relays through an interface, controls the pumps 1 and 2 (on/off), the column-switching valve (connect/disconnect C1 and C2) and the two pairs of solenoid valves to perform a series of stepwise elutions for C1 and to perform repetitive linear gradient elution for C2. Two wavelength-tunable UV detectors (DE; Model SPD-6A, Shimadzu, and Model UVIDEC-100E, Jasco, Tokyo, Japan), a recorder (RE; Model R-112, Shimadzu) and an integrator (INT; Model C-R6A, Shimadzu) are set to monitor the eluent and to quantify the peaks obtained by HPLC. The system is also equipped with a fraction collector (FC; Model 203, Gilson, Worthington, OH, USA) for peak collection. For our standard system focused on the separation of brain acidic proteins, we selected an anion-exchange TSK-gel DEAE-5PW column (7.5 cm × 0.75 cm I.D.) (Tosoh, Tokyo,
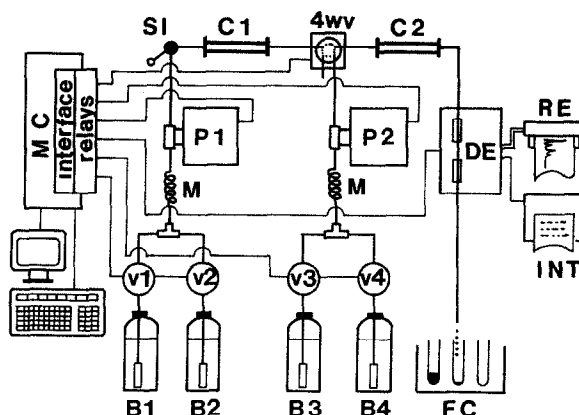

Fig. 1. Schematic diagram of the 2D-HPLC system for the separation of complex protein mixtures.

Japan) and a reversed-phase TSK-gel Phenyl 5PWRP column (7.5 × 0.46 cm I.D.) (Tosoh) for C1 and C2, respectively.

Performance of 2D-HPLC

On starting the program, columns C1 and C2 are equilibrated with B1 (25 mM Tris–HCl buffer, pH 7.5) and B3 (20% acetonitrile in 0.1% TFA), respectively, at a flow-rate of 1.0 ml/min. After 40 min, pump 2 is stopped and the column-switching valve (4WV) moves to connect C1 and C2. A sample mixture is applied to C1 through the sample injector (SI) and eluted with B1 for a time t_1 (20 min as standard) at a flow-rate of 1.0 ml/min. Pump 1 is stopped and, simultaneously, the second chromatography begins as pump 2 starts to pump at a flow-rate of 1.0 ml/min with a linear gradient from B3 to B4 (20 to 60% acetonitrile in 0.1% TFA) during the time t_2 (40 min as standard). Column C2 is equilibrated again with B3 for 15 min after the linear gradient elution is finished, then pump 2 is stopped. After this step, the first cycle of the 2D-HPLC is completed. Pump 1 then starts again to elute proteins stepwise from the anion-exchange column C1 by introducing and mixing a portion of buffer B2 (0.4 M NaCl in 25 mM Tris–HCl buffer, pH 7.5) into B1. After applying the eluent to C2, the second chromatography is repeated exactly as described above. These procedures are repeated for a number of cycles (*n*), changing the mixture ratio of B1 and B2 with time-dependent control of solenoid valves V1 and V2. In our standard procedure, the mixing ratio of B2 was increased as follows: 0, 5, 10, 15, 20, 25, 30, 40, 50 and 100% (*n* = 10). For versatility, however, the computer program has been made open for the elution times of C1 and C2 (t_1 and t_2), the cycle number (*n*) and the B1/B2 mixing ratio in each cycle for possible modification of these parameters depending on the complexity and ionic distribution of the sample mixture.

Preparation of soluble proteins from bovine cerebellum and from mouse myeloma cell

Bovine brain was obtained fresh from a slaughter house. The cerebellum was resected and was homogenized with a fourfold volume of 0.1 M potassium phosphate buffer (pH 7.1) containing 1.6 M ammonium sulphate and 1 mM EDTA. The homogenate was centrifuged at 10 000 g for 30 min and the

soluble extracts were precipitated by addition of solid ammonium sulphate to 85% saturation at pH 4.7. After centrifugation, the precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.1) containing 1 mM EDTA and dialysed against buffer B1 for 2D-HPLC. The preparation was divided into 0.5-ml portions and stored frozen at −80°C until use.

Mouse myeloma cells (P3U1) were cultivated in a 24-well plastic plate in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% (v/v) of calf serum (Gibco Labs., Scotland, UK). The cells (*ca.* 10^6) were collected in a 1.5-ml plastic tube by centrifugation and washed twice with 10 mM sodium phosphate buffer (pH 6.8) containing 0.15 M NaCl. Soluble proteins were obtained by freezing and thawing the cells twice in distilled water, followed by centrifugation at 15 000 g for 5 min.

RESULTS AND DISCUSSION

Separation of standard proteins

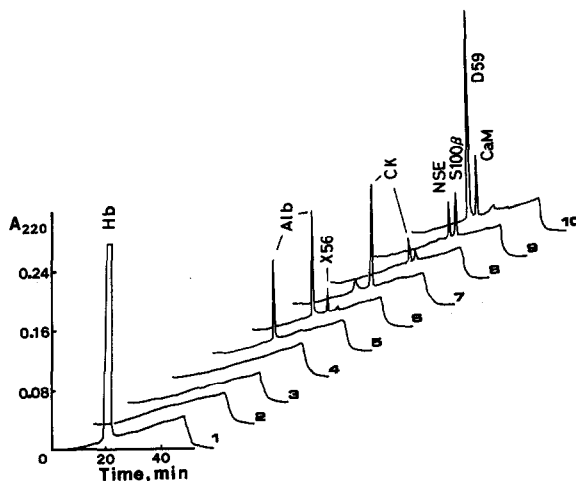The performance of the 2D-HPLC system was tested by applying a mixture of seven proteins puri-

Fig. 2. Three-dimensonal representation of the 2D-HPLC of a mixture of haemogobin (Hb), serum albumin (Alb), X-56 protein (X-56), creatine kinase (CK), neuron-specific enolase (NSE), S100 protein β-chain (S100β), D59 protein (D59) and calmodulin (CaM). The chromatography was performed under the standard conditions described in the text. Each horizontal profile represents the result of the second reversed-phase chromatography of a protein fraction eluted from the first anion-exchange column. The cycle number is shown at the right of each profile.

fied in our laboratory or obtained from a commercial source (see legend to Fig. 2). Although a long, continuous elution profile was obtained as a result of the chromatography, the profile is represented in Fig. 2 by three-dimensional drawings of a series of elution profiles, each profile corresponding to one cycle in the chromatography. Thus, the separation cycle of the first anion-exchange and the second reversed-phase chromatography was repeated ten times, which required a total analysis time of 12 h.

Under these conditions, all of the tested proteins were separated from each other and were eluted at the positions indicated in Fig. 2. Here, five proteins appeared in a single peak, and two proteins, serum albumin and creatine kinase, were found in duplicated peaks eluted in the adjacent, first-dimensional chromatography. Because the elution profile was reproducible for these standard proteins and for the tissue extract as described below, we considered that the double peaks observed might be due to charge heterogeneity of the albumin and creatine kinase preparations used. It has been shown that commercial albumin exhibits charge heterogeneity during 2D-electrophoresis [2], probably because it contains several molecular species such as fatty acid-binding albumin and mercaptoalbumin, and microheterogeneity is known also for creatine kinase [20]. The profile also indicates that the proteins are eluted from the first-dimensional column in increasing order of acidity, as expected from the specificity of the anion-exchange column, and that those having similar acidities, such as neuron-specific enolase ($pI = 4.7$) and S100b protein ($pI = 4.6$), are eluted together from the first column and are subsequently separated on the second-dimensional, reversed-phase column.

Separation and analysis of bovine cerebellar extracts

To examine the resolution of the 2D-HPLC technique, the soluble extract of bovine cerebellum was injected directly into the system and eluted under the same conditions as in Fig. 2. The cerebellar extracts were resolved into *ca.* 200 peaks (Fig. 3) by applying 6 mg of proteins. Quantitative analysis indicated that each peak contained 0.5–60 μg of protein, suggesting that our system could detect proteins present at a level of more than 0.01% of total soluble proteins in bovine cerebellum with the detector sensitivity used (220 nm, 0.64 a.u.f.s.).

The number of peaks resolved by the separation of the cerebellar extracts increased to *ca.* 250 when the cycle number of the chromatography was increased to 20 and conversely decreased to *ca.* 75 with a cycle number of 4. A plot of the observed peak number *versus* the number of cycles (Fig. 4) suggests that the present system could separate the cerebellar extract into a maximum of about 280 peaks under the conditions employed. On the other hand, *ca.* 400 protein spots were detected by 2D electrophoresis of this preparation followed by Coomassie blue staining [21].

To test the purity of proteins separated by the 2D-HPLC technique, fourteen major peaks were collected as indicated in Fig. 3, and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In spite of the condition that these peaks were obtained directly from the crude tissue extracts, many of the peaks analysed contained proteins with sufficient purity for subsequent analysis, and several peaks contained a number of minor proteins or contained some impurities giving rise to a smeared band in the stained gel (Fig. 5). This demonstrates a reasonably high efficiency of the 2D-HPLC technique as a preparative method for proteins. In fact, most of these major cerebellar proteins were obtained in pure form by a single 2D-HPLC operation or by an additional reversed-phase chromatography with elution using a gentle gradient of acetonitrile or using 0.08% heptafluorobutyric acid in place of TFA. The purified proteins were characterized by one or more of the following analyses: (i) 2D electrophoresis, (ii) dot–blot immunochemical analysis, (iii) analysis of amino acid composition and (iv) analysis of amino-terminal sequence or internal amino acid sequence and, as a result of these analyses, we have so far identified fifteen brain proteins in the protein map of bovine cerebellum [16,17].

Reproducibility and stability

The reproducibility of the method is illustrated in Fig. 3. The profile was obtained by repeating the chromatography of the same extracts of bovine cerebellum. Between the analyses shown in Fig. 3a and b, the system was used twenty times mainly for separation of crude tissue extracts. In spite of these conditions and of the complexity of the protein mixture analysed, the separation patterns of the two
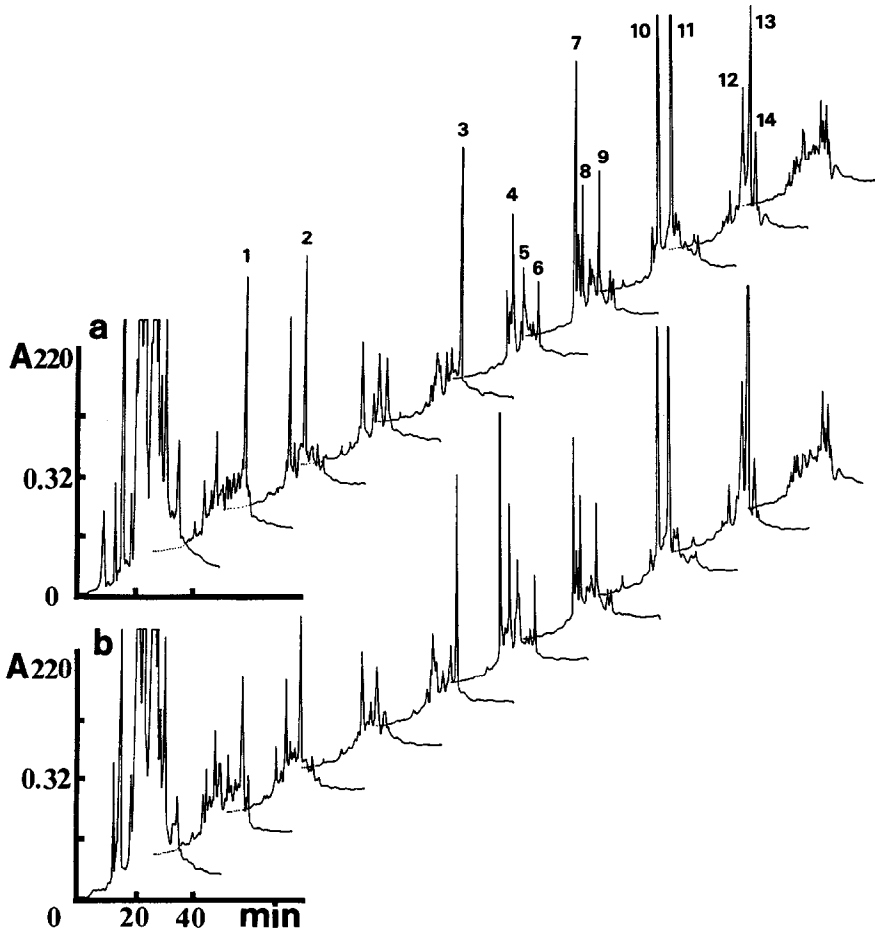
Fig. 3. Protein map of bovine cerebellum. The soluble extracts were applied to the 2D-HPLC system and eluted as described in the text; (a) and (b) are two separate experiments that illustrate the reproducibility of the method. The fourteen numbered peak were collected for SDS–PAGE.
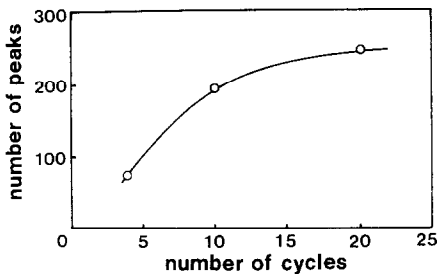

Fig. 4. Relationship between the number of stepwise elutions in the first anion-exchange chromatography, i.e., the cycle number, and the number of peaks observed in the 2D-HPLC. The data were collected by applying crude cerebellar extracts (6 mg of protein).

chromatograms are highly reproducible, although there are small but noticeable changes in peak height for a number of peaks, including peaks 1, 4 and 7.

This 2D-HPLC system has currently been used more than 200 times for preparative and analytical protein separations. Repeated application of crude protein mixtures such as tissue extracts tended to increase the column pressure from 20 to more than 80 kg/cm^2, probably owing to the accumulation of lipids, nucleic acids and very large proteins of low solubility, etc., and this resulted in a gradual decrease in peak resolution. However, these columns could be regenerated by washing with 20 ml of 0.5
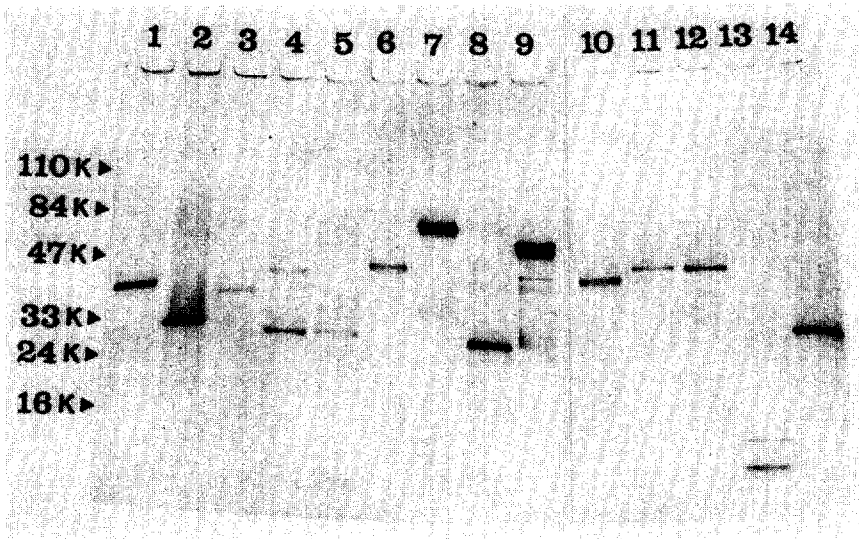
Fig. 5. SDS-PAGE of bovine cerebellar proteins collected from the 2D-HPLC. The numbers at the top of each lane correspond to the peak numbers in Fig. 3. A portion of the eluates was analysed in a 4–20% gradient of polyacrylamide gel containing 0.1% SDS (Coomassie blue staining). The molecular weight values indicated were measured with a prestained molecular weight standard mixture obtained from Bio-Rad Labs. (Richmond, CA, USA); K = kilodalton.

M sodium hydroxide solution without detectable deterioration in peak resolution. Hence the chemical stability of polymer-based columns is important for the maintenance of the system and to perform reproducible protein mapping.

Sensitivity

Our standard 2D-HPLC system equipped with a 7.5 mm I.D. anion-exchange column and a 4.6 mm I.D. reversed-phase column has a protein loading capacity of about 10 mg. Application of this
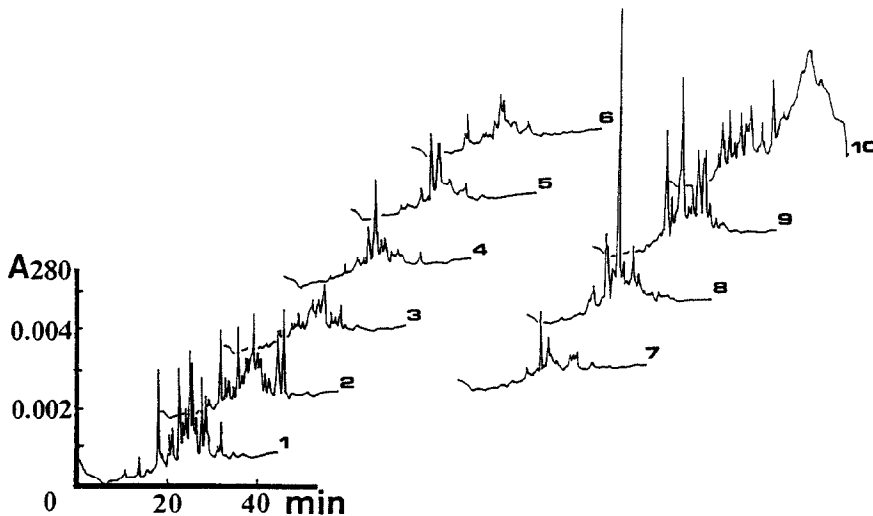

Fig. 6. Protein map of mouse myeloma cell, P3U1. Soluble proteins prepared from 10^6 cells (see Experimental) were analysed by the automated 2D-HPLC system. The detector sensitivity was set at 0.01 a.u.f.s. at 280 nm.

amount of the cerebellar extract enabled *ca.* 1–100 μg each of protein to be isolated by a single chromatography, and this sufficed for various biochemical and protein chemical analyses.

For analytical purposes, the sensitivity of this system could be increased 200-fold simply increasing the detector sensitivity without introducing a significant baseline problem. This allowed us to perform analytical protein mapping with 100 μg of extracts derived from 10 mg of bovine cerebellum and, likewise, to perform protein mapping of cells cultivated on an experimental scale. Fig. 6 shows the separation of soluble proteins in mouse myeloma cells (1 × 10⁶ cells) grown in a single well of a conventional 24-well plastic plate. Here, *ca.* 210 peaks are detected in a clearly different pattern from that of the bovine cerebellum.

Applicability

Because of the high resolution and reproducibility, and because of the small amount of sample required, the method should be useful for analytical mapping of proteins in various tissues and cells. For instance, the system could detect differences in protein composition among various bovine tissues. Fig.

7 illustrates a partial comparison of the protein maps of (a) the cerebellum (b) the cerebrum and (c) the adrenal medulla, in which differences are apparent despite the close ontogenetic relationships among the tissues.

We have also applied this technique to the protein mapping of rat cerebellum at various developmental stages and searched for proteins that may be related to the maturation of this tissue. We analyzed the developmental profiles of more than 120 proteins resolved by the 2D-HPLC technique, and detected several proteins that were expressed transiently at an early stage of the postnatal development. One of these proteins, termed V-1, were isolated by HPLC and characterized in detail by direct protein sequence analysis and by cloning its complementary DNA [22]. In addition to such an application, the method should also be useful for the general purification of proteins that occur naturally or are produced by recombinant DNA techniques. Because the system is flexible, various types of separation are possible, depending on the purpose and the complexity of the protein mixture, *e.g.*, by modification of the elution conditions and number of cycles or by replacing the columns with larger or
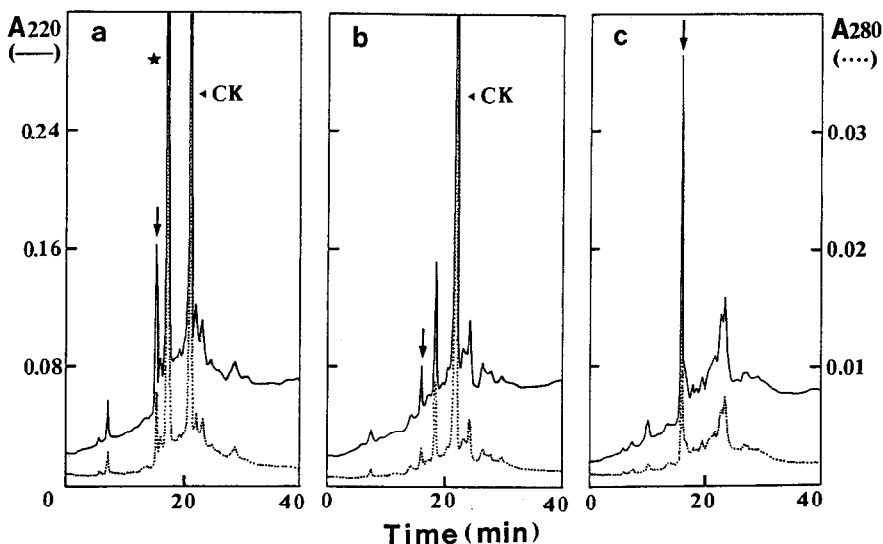

Fig. 7. Partial comparison of the protein maps of (a) bovine cerebellum, (b) cerebrum and (c) adrenal medulla. The profiles obtained at cycle 8, the reversed-phase separation of a protein fraction eluted from the first anion-exchange column with 40% B2, are shown. To facilitate comparison, a protein peak with a retention time of 17 min is indicated by an arrow. CK refers to the brain-type creatine kinase which is found abundantly in the cerebellum and the cerebrum, but is not detected in the adrenal medulla. A protein peak found in the cerebellum (indicated by an asterisk) is much less significant in the cerebrum and adrenal medulla.

smaller columns or columns having different specificities.

Limitations

Although the 2D-HPLC technique is useful for systematic protein separations, as could be demonstrated by the protein mapping of the bovine cerebellum and of the cultivated myeloma cells, it has some practical limitations, mainly due to the separation mode employed. We selected an anion-exchange column for the first separation, because our initial purpose in developing the 2D-HPLC system was the systematic separation of proteins in the brain, a tissue characterized by a high content of acidic proteins. Based on the analysis of a series of proteins with known acidity, this anion-exchange column adsorbs proteins having an isoelectric point below 6.5 under the solvent condition employed [12]. Thus, more basic proteins elute together in the first cycle of the chromatography. Complementary to the present system would be the use of a cation-exchange column, and in fact a cation-exchange TSK-gel CM-5PW column (Tosoh) or a ceramic hydroxyapatite column [23] was found to be more effective than the present system in separating basic proteins. It is advantageous, therefore, to select either an anion- or a cation-exchange column depending on the charge distribution of the protein mixture. Alternatively, the use of tandem anion- and cation-exchange columns or a mixed-bed ion-exchange column for the first-dimensional separation will decrease the number of proteins eluted in the first cycle.

The second reversed-phase separation introduces two limitations. First, very hydrophobic proteins will bind to the column too tightly and may be recovered in low yields, or may not be recovered during the chromatography. The reversed-phase column selected is the one which, in our experience, has the lowest hydrophobicity among the commercial columns tested and is able to separate relatively large proteins such as serum amine oxidase (subunit $M_r = 95\ 000$). However, we still cannot exclude the above possibility when the method is applied to very hydrophobic and poorly soluble proteins. Probably further optimization process of the chromatographic conditions, including column parameters such as the type of packing material and the size of column and also the mobile phase composi-

tion, as discussed by Burton and co-workers [24,25], will reduce this limitation. Second, the reversed-phase separation will reduce the biological activities of proteins which are labile at high acetonitrile concentrations and acidic pH. In general, small proteins can easily be renatured after the chromatography; however, the renaturation of large protein molecules may be relatively difficult. Therefore, as a tool for preparative purposes, the 2D-HPLC system presented here is thought to be most suitable for use in protein chemical studies such as microscale sequencing or the purification of a protein to produce a specific antibody. Such methods are needed in various biological studies, including the protein and gene analyses.

ACKNOWLEDGEMENTS

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