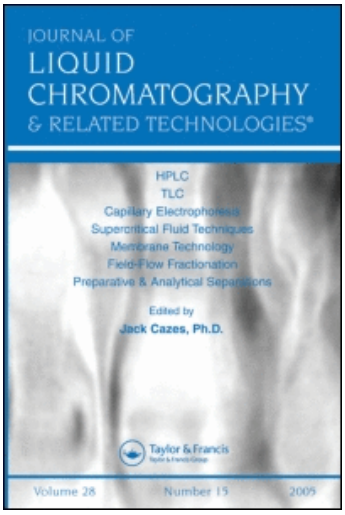

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Toshiaki Isobe[a]; Yasuyuki Kurosu[a]; Yan-Il Fang[a]; Noriaki Ishioka[a]; Hiroshi Kawasaki[a]; Nobuharu Takai[b]; Tsuneo Okuyama[a]

[a] Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Tokyo [b] Institute of Industrial Science, University of Tokyo, Tokyo, Japan

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF LOW MOLECULAR WEIGHT PROTEINS ON A NON-IONIC MACRORETICULAR POLYSTYRENE RESIN

Toshiaki Isobe, Yasuyuki Kurosu, Yan-Il Fang,*
Noriaki Ishioka, Hiroshi Kawasaki, Nobuharu Takai*
and
Tsuneo Okuyama
Department of Chemistry, Faculty of Science,
Tokyo Metropolitan University, Setagaya-ku, Tokyo 158
and
*Institute of Industrial Science,
University of Tokyo, Minato-ku, Tokyo 106, Japan

ABSTRACT

A high performance liquid chromatography system is presented for analytical and preparative separation of proteins. The method utilizes a macroreticular polystyrene resin having no specific functional groups, and proteins are eluted by the use of linear gradient of acetonitrile (20% - 75%, v/v) in 0.1% (v/v) trifluoroacetic acid. In this standard elution system, twenty proteins having a molecular weight of 4,200 - 58,000 and an isoelectric point of 3.9 - 11.0 have been chromatographed successfully within 80 min. The method allows a rapid, sensitive, and high resolution separation of relatively low molecular weight proteins, where the isolated proteins can be used for subsequent biochemical determinations.

INTRODUCTION

Purification of proteins is often an essential step in molecular studies of biological events, and this has been tried by various physical and chemical means. Among these methods, the chromatography technique is one of the most common and convenient approach with respect to simple operation, considerable

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resolution, and easy recovery of separated proteins. However, classical chromatography procedures utilizing conventional dextran or agarose based supports are time consuming, and require relatively large quantity of starting material essentially because the efficiency of such a column is usually low. For more impact approach to the biology field, it seems necessary to devise more rapid, sensitive, and high resolution methods for the purification and analysis of proteins.

Recent advances of high performance liquid chromatography (HPLC), such as development of reverse phase HPLC using silica-based supports, have significantly improved this underlying problem. It has been shown that the reverse phase HPLC is powerful for the separation of polypeptides such as opioid peptides (1,2) and peptides derived from limited proteolysis (3-5). This method has been also applied for the purification of a number of proteins such as interferons (6), insulin-like growth factors (7), histons (8), and trypsin and chymotrypsin (9). However, the reverse phase separation of proteins seems to be not yet widely accepted as a substitute for the "conventional" chromatography procedures, presumably because the reverse phase supports, available so far, often bind proteins nonspecifically and tightly. On the other hand, HPLC of the gel permeation type has been widely used for the separation of proteins (10), but only for group separation of proteins or separation of rather simple protein mixtures.

In the previous papers (11-13) we have demonstrated that the macroreticular ion-exchange polystyrene resin have many advantageous characteristics in the chromatography of peptides. It is comparable to the silica-based reverse phase supports in high resolution, with added advantages in chemical stability and wide sample capacity range. In these studies we have noticed that relatively large peptides such as those produced by CNBr cleavage or partially degradated proteins are eluted from the resin column in a considerable recovery. This observation has

led us an attempt to apply a macroreticular resin for the separation of proteins.

This paper describes an application to the separation of proteins of the macroreticular polystyrene resin, having no specific functional groups other than the styrene group. The method showed remarkable peak resolution for twenty tested proteins having a molecular weight of 4,200 - 58,000 daltons, where the separation occured in less than 80 min with manogram to milligram quantities of samples.

MATERIALS AND METHODS

Proteins: Calmodulin, S100a protein, micro glutamic acid-rich protein, neuron specific enolase, and DEK protein were purified from bovine brain by ammonium sulfate fractionation of brain soluble extracts and following column chromatography procedures. The purification of calmodulin, S100a protein and micro glutamic acid-rich protein has been described (14-16), and that of other proteins is described elsewhere. Bence-Johns protein, NIG-64, was purified from urine of a patient of primary amyloidosis (17). Streptomyces subtilisin inhibitor was kindly donated by Dr. T. Kainosho of Tokyo Metropolitan University, ion-type superoxide dismutase of Pseudomonus ovalis by Dr. F. Yamakura of Juntendo University (Narashino, Japan), phospholipase C (α-toxin) of Clostridium perfringens by Dr. Y. Yamakawa of National Institute of Health, Japan, cancer cachexia specific protein by Dr. O. Oda of the Bio-dynamic Research Institute of the Sinseikai Foundation (Nagoya, Japan). Synthetic parathyloid hormone (residues 1 - 34) and C-reactive protein were the products of Toyo Jozo Co. Ltd. Other proteins were obtained from sources indicated in parentheses; L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Millipore Corp.), α-chymotrypsin (Worthington Biochemicals Corp.), thermolysin (Sigma Chemicals), lysozyme (egg white, Seikagaku Kogyo Ltd.), cytochrome c (horse heart, Seikagaku Kogyo Ltd.), and insulin (Calbiochem-Behring Corp.).

Chemicals for chromatography; A macroreticular polystyrene resin, Hitachi-Gel 3013, with a particle size distribution of 5 - 7 μm and a cross-linkage of 35%, was obtained from Hitachi Ltd. (Tokyo, Japan). Acetonitrile (chromatography grade) and trifluoroacetic acid (sequanal grade) were purchased from Wako Pure Chemical Industries (Tokyo). Water was distilled, passed through a mixed-bed ion-exchange resin, and redistilled before use.

Apparatus; A Shimadzu Corporation (Kyoto, Japan) Model LC-3A liquid chromatograph, equipped with a dual wavelength u. v. detector (8 mm light path), was employed. The Hitachi-Gel 3013 resin was made in a slurry in 50% acetonitrile in water and packed into a stainless-steel column (250 mm x 4.6 mm ID) at a pressure of 30 kg/cm^2. The solvent used was 50% acetonitrile, which was then replaced by 20% acetonitrile for most compact packing.

Chromatographic procedure; Sample proteins were introduced onto a column of Hitachi-Gel 3013 and eluted at 40 °C by the use of linear gradient from 20% (v/v) to 75% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min. The gradient was formed by placing 20 ml of the initial solvent in a gradient mixer, into which the final solvent was added at a constant flow rate of 0.25 ml/min. Where necessary, column effluent was collected either manually or automatically and aliquots were removed for subsequent measurements. The removed effluent was lyophilized after dilution with water (for measurements of enzymatic activities), or dried up under N_2 stream (for amino acid compositional analyses). After completion of one analytical run, the column was reequilibrated for 30 - 60 min with the initial solvent for the next analysis.

Chemical modification and amino acid analysis of proteins; Oxidation with performic acid and S-aminoethylation with ethylene imine were performed essentially as described (17-19). Amino acid analysis was performed with a modified Hitachi Model KLA-5 analyzer as described (16). Proteins were hydrolized with 250 μl of 6 M HCl for 24 h at 110 °C in evacuated, sealed tubes.

RESULTS AND DISCUSSION

Application of the method

In order to evaluate the present chromatography system, we have applied twenty test proteins having different physicochemical properties in terms of molecular weight (4,200 - 58,000) or isoelectric point (pI 3.9 - 11.0)(Table 1). Upon mobile phase gradient of acetonitrile in 0.1% trifluoroacetic acid, all these proteins were eluted from the resin column within 80 min. The chromatograms are shown in Fig. 1, a to t, and the retention time for each protein is incorporated in Table 1. Some comments on the chromatograms are described below.

[1] micro glutamic acid-rich protein (microGluP); MicroGluP is a brain protein having a novel amino acid composition; there are abundant glutamic acid (51% of the total composition) and no hydrophobic amino acids such as Val, Met, Ile, Leu, Tyr, Phe, and Trp (16). This protein exhibits no absorption at 280 nm due to the lack of aromatic residues, and has the smallest retention time among the proteins tested (Fig. 1, c).

[2] streptomyces subtilisin inhibitor (SSI); The preparation of SSI was separated into at least two major peaks in our chromatography system (Fig. 1, e). This corresponds to an argument that the SSI molecule may be heterogeneous in the amino-terminal region (20)

[3] calmodulin; Calmodulin, a calcium binding protein having a repetitive amino acid sequence of an "EF-hand" (21,22), gave rise to a major peak at 52.5 min with a slight shoulder at the leading edge (Fig. 1, g). This would be due to des(Ala-Lys)calmodulin which was present in the calmodulin preparation in almost 10% quantity (12). The oxidation of nine methionyl residues in the protein reduced significantly the retention time of calmodulin (Fig. 1, h), suggesting that the oxidation caused considerable structural changes on the surface of molecule.

[4] S100a protein; S100a protein is a dimer of non-covelently associated subunits, α and β, which have highly homologous amino acid sequence (18,19). Under the solvent conditions employed,

Table 1. A list of proteins subjected to HPLC

Protein	Source	Molecular Weight	Isoelectric point	Retention time (min)
parathyroid hormone (1-34)	chemical synthesis	4,200	9.7*	31.5
insulin	bovine pancreas	5,700	5.3*	36.0
micro glutamic acid-rich protein	bovine brain	10,000	3.9	18.5
cytochrome c	horse heart	11,000	6.5	33.5 34.0
streptomyces subtilisin inhibitor	*Streptomyces subtilis*	11,500	4.5*	40.5 42.0
lysozyme	chicken egg	14,300	10.5-11.0	37.5
calmodulin	bovine brain	16,700	4.0	52.5
oxidized calmodulin	(modification)	17,000	> 4.0	45.0
S100a protein	bovine brain	10,400(α) 10,500(β)	4.2 4.5	60.0 64.0
C-reactive protein	human urine	20,900	5.8	47.0
superoxide dismutase	*Pseudomonus ovalis*	23,000(x2)	4.5	51.5
S-aminoethyl superoxide dismutase	(modification)	23,000	< 4.5	52.0
S-aminoethyl Bence-Jones protein NIG-64	human urine (modification)	23,400	7.7*	34.5
trypsin	bovine pancreas	23,900	10.1	37.5 38.0
cancer cachexia specific protein	human serum	25,000	6.5	51.5
α-chymotrypsin	bovine pancreas	25,100	8.1-8.6	44.5
thermolysin	*Bacillus thermo-proteolyticus*	34,400	4.6*	52.0
phospholipase C	*Clostridium perfringens*	43,000	5.5	40.5
neuron-specific enolase	bovine brain	50,000(x2)	4.5	49.5
DEK-protein	bovine brain	58,000	4.3	42.5

*Values are estimated from the amino acid compositions by the micro computer method of Manabe (32).

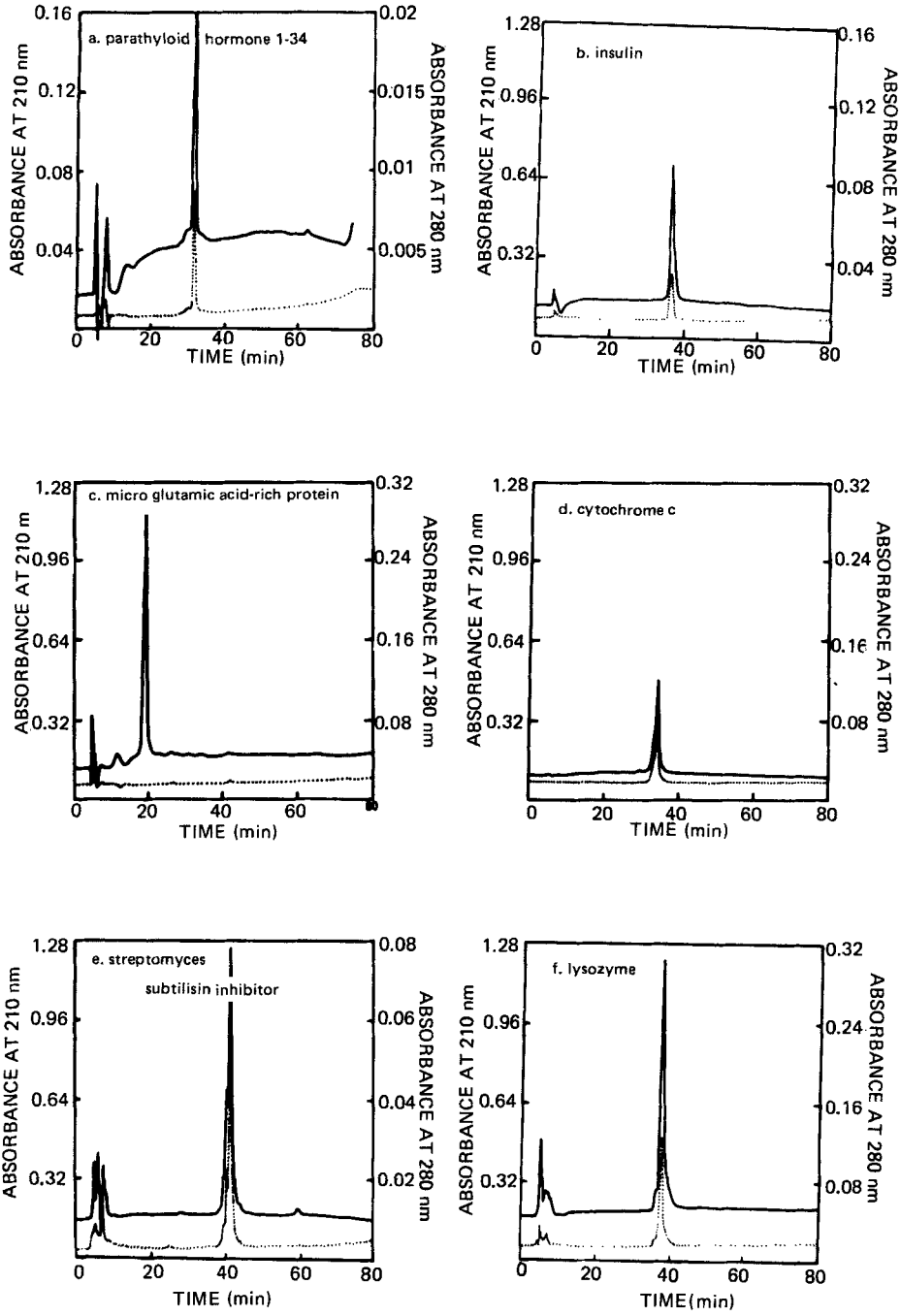


FIGURE 1

High performance liquid chromatography of proteins on a non-ionic macroreticular polystyrene resin. The proteins (1 - 50 µg) were applied to a column (25 x 0.46 cm i.d.) of Hitachi-Gel 3013, and eluted by the use of linear gradient of acetonitrile (20% - 75%) in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Other conditions are given under Methods.

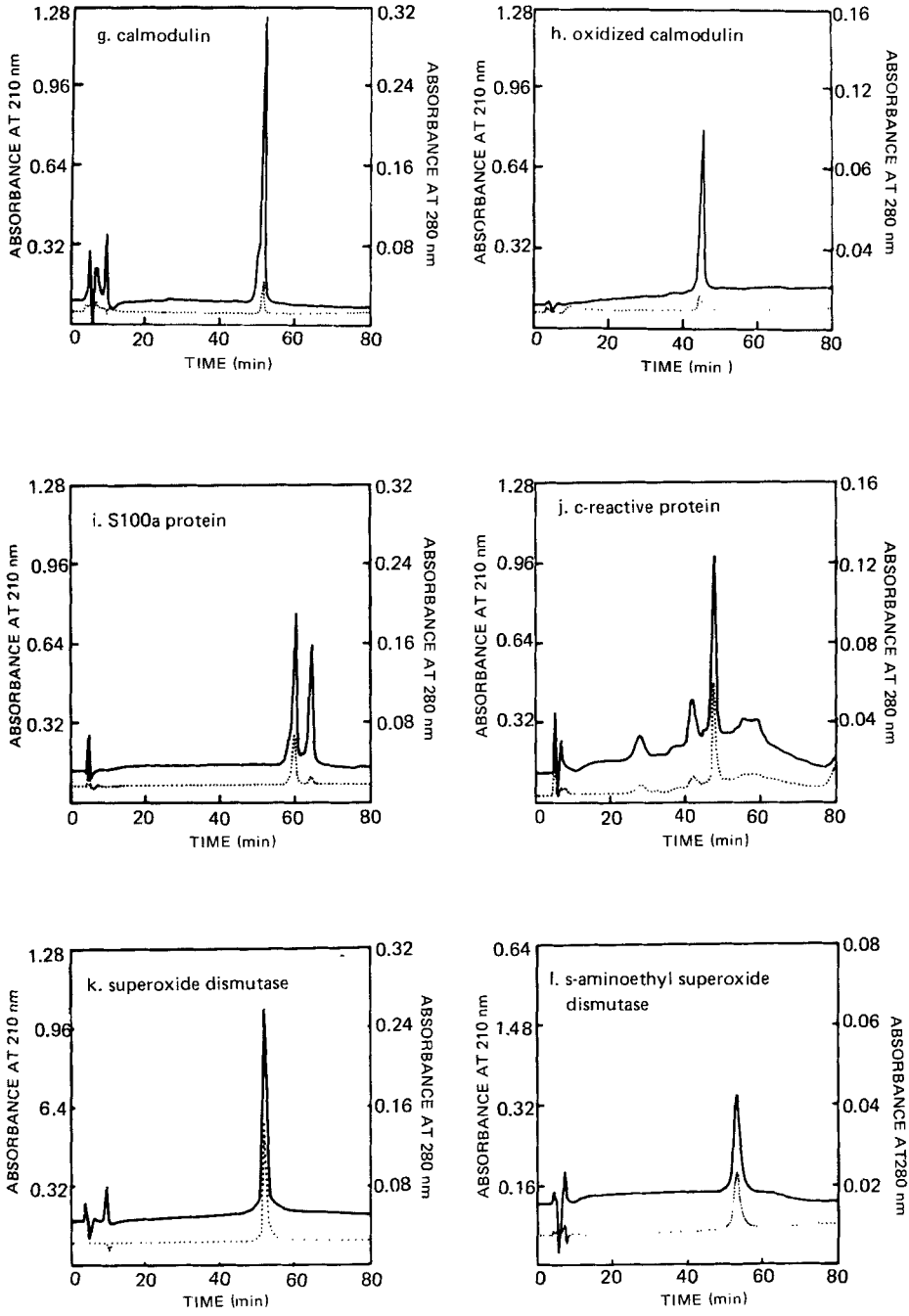
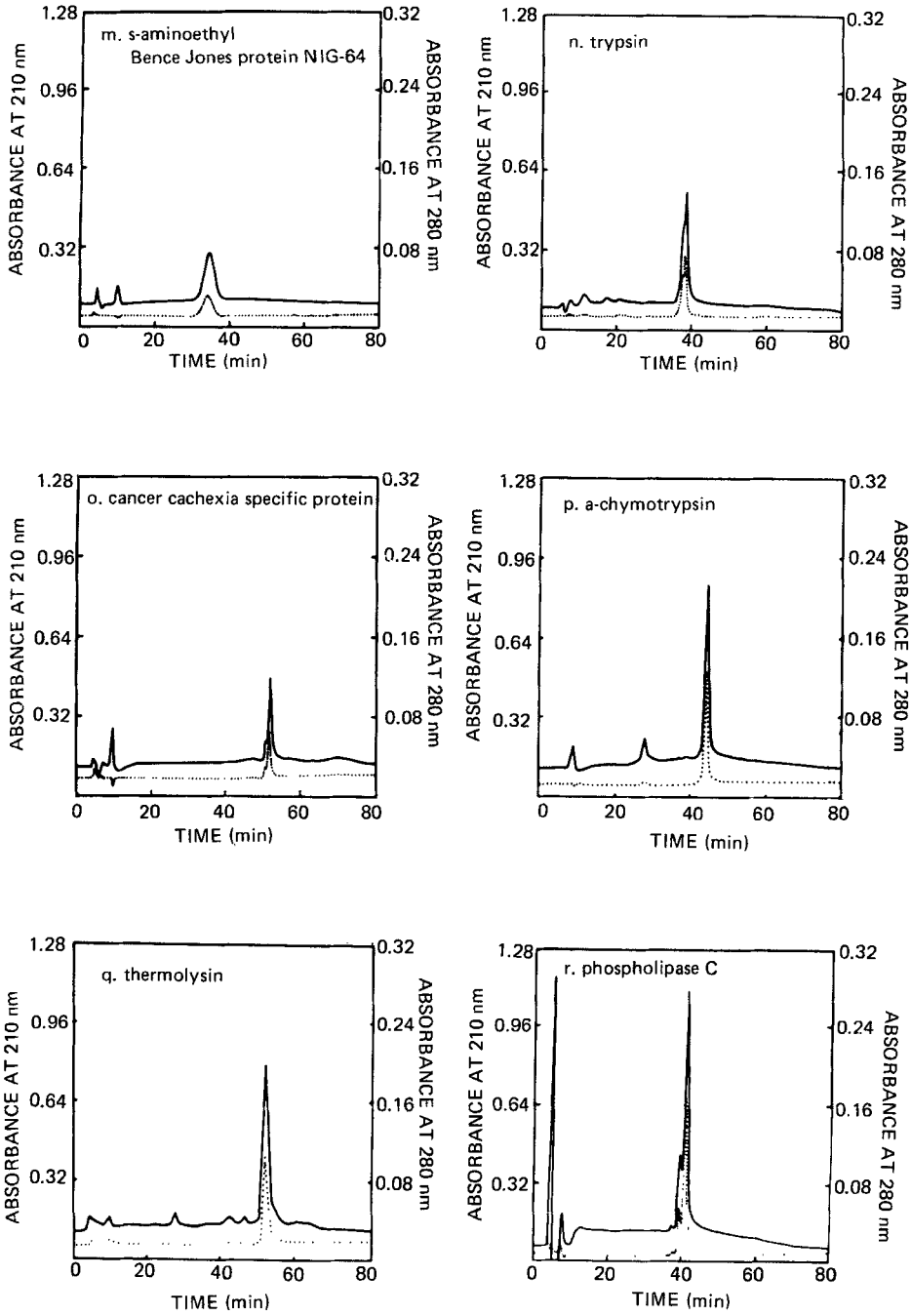


FIGURE 1 (continued)



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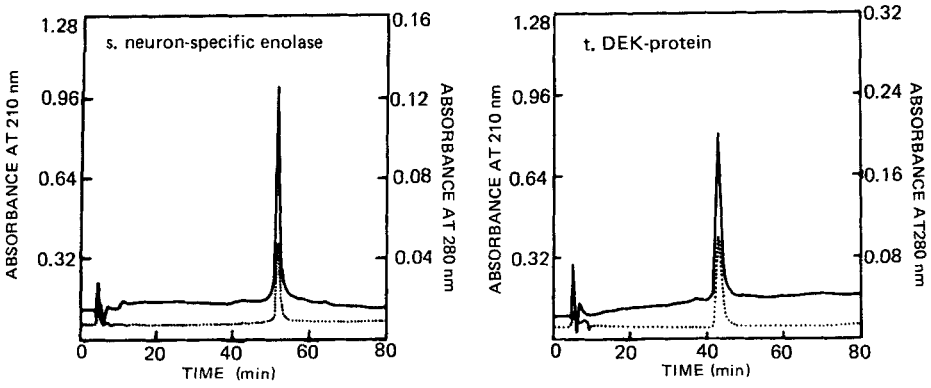

FIGURE 1 (continued)

these subunits of S100a protein were dissociated, and separated from each other as shown in Fig. 1, i. This chromatography procedure could be therefore applied successfully to the estimation of subunit compositions of a series of S100 protein species purified from bovine brain, as well as from other vertebrate sources (23).

[5] superoxide dismutase; Ion-type superoxide dismutase from Pseudomonus ovalis (24) gave rise to a single peak at a retention time of 51.5 min (Fig. 1, k). Aminoethylation of four cystein residues in the protein under reduced, denaturing condition (in 8 M urea) resulted in broadning of the protein peak without significant alteration in the retention time (Fig. 1, l).

[6] trypsin; Commercial trypsin gave rise to two partially-separated peaks (Fig. 1, n). These peaks should be due to α and β forms of trypsin respectively, where α-trypsin was derived from the β form by an internal cleavage at Lys_{131}-Ser_{132} (25). The present chromatography system enables to separate trypsin from α-chymotrypsin (Fig. 1, p) which is often present in trypsin preparations, although we are not able to detect this contaminated chymotrypsin at this scale of experiment.

[7] neuron-specific enolase; Neuron-specific enolase is a dimer

of two homogeneous, non-covalently linked subunits with a molecular weight of about 50,000 (26). The chromatography of this protein showed a single symmetrical peak at 49.5 min (Fig. 1, s).

[8] DEK protein; The brain DEK protein has a monomer molecular weight of 58,000, and is the largest molecule among the proteins tested. This protein was eluted in a single peak at a retention time of 42.5 min (Fig. 1, t).

Separation principle

The present method utilizes a macroreticular resin of the styrene-divinylbenzene type, where proteins are eluted by a mobile phase gradient of acetonitrile in 0.1% trifluoroacetic acid at pH 2. Since this resin has no functional group other than the styrene matrix, the separation should have occured on the basis of interaction between proteins and the styrene matrix. This type of interaction is called "matrix effect" by Regnier et al (27), and is frequently observed in the "ion-exchange" chromatography using polystyrene resins with ionic groups. Thus, the present method is analogous, in separation principle, to the reverse phase HPLC using silica-based supports.

Such a separation principle is particularly clear when the separation of peptides is concerned; where the retention time correlates to the hydrophobicity of peptides estimated from the hydrophobic fragmental constant provided by Recker (28), or modified by Sasagawa et al (29)(Kurosu et al, manuscript in preparation). For the protein molecules, however, this correlation was ambiguous by relatively large deviation. This indicates that proteins are in partially folded conformation even under rather denaturing conditions employed, so that only a portion of the residues is available to interaction with the polystyrene resin. It should be noted that the "intact" proteins appeared in sharp, and narrow peaks as compared to the proteins modified after denaturation (Fig. 1, h, l, m), suggesting that a conformational factor, in fact, contributed to the separation.

It is also noted that the S100a protein has been dissociated into subunits during the chromatography (Fig. 1, i). This could

be attributed to the solvent conditions employed, high organic solvent concentration and acidic pH, which would desrupt both electrostatic and hydrophobic interaction between the subunits. We assume that the subunit separation has also occured for other dimeric proteins such as superoxide dismutase and neuron-specific enolase, although we can not evaluate this assumption as the subunits are identical in both these cases.

Recovery of proteins

We have estimated the recoveries of some proteins tested (c.f. calmodulin, S100a protein, superoxide dismutase, thermolysin, and neuron specific enolase) by means of amino acid analysis of proteins before and after the chromatography, and obtained the values of 85%, 90%, 55%, 60%, and 56% for these proteins, respectively. These results suggest that the recoveries are considerable high for small proteins, and are within an acceptable extent even for relatively large proteins.

Recovery of activities of proteins

Titani et al (9) purified commercial trypsin and chymotrypsin by mean of reverse phase HPLC with almost complete recovery (70 - 104%) of their proteolytic activity. Since the separation conditions employed in this study and that of Titani et al are analogous, a similar extent of activity recovery could be expected for these enzymes.

We found that the trypsin, recovered after this chromatography step, was sufficiently active for peptide mapping studies of proteins. Phospholipase C was also active towards p-nitrophenyl-phosphorylcholine (Y. Yamakawa, personal communication), and two calcium binding proteins, calmodulin and S100a protein, retained their calcium binding abilities. We anticipate therefore that the procedure could be useful for purification of any acid-stable proteins and enzymes.

Maintenance of the column

As noted in the previous reports (11,13,30,31), the macroreticular resin is definitely stable both chemically and mechanically. This means that practically any solvents can be used for

elution. For instance, 2 M NaOH containing acetone or isopropanol was effective in regeneration of the column, which might be necessary after repeated application of crude protein mixtures. However, introduction of large volume of aqueous solution, without organic solvent, should be avoided because this causes rapid increase in back pressure. Note that the polystyrene resin without ionic group has very low affinity against water. We are including 20% acetonitrile in the initial solvent for routine maintenance of the resin column. In our experience, all proteins are adsorbed to the column under this solvent condition.

In conclusion, the chromatography using a macroreticular polystyrene resin appears to be a useful tool for rapid, sensitive, and high resolution separation of proteins. The method is applicable either for analytical or preparative separation of proteins because the resin column has large sample capacity due to its large surface area and porocity (11,13). However, application of this method is limited so far for relatively low molecular weight proteins, apparently because large protein molecules have generally high hydrophobicity and little solubility in the elution solvent. Therefore, the problem remains to devise an equivalent technique that is effective in separation of larger protein molecules.

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