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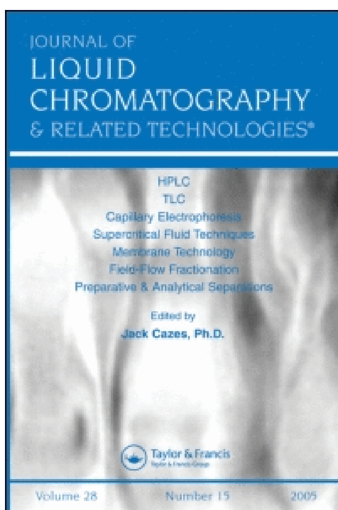

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HIGH PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF PROTEINS

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ABSTRACT

High performance anion-exchange chromatography using an aqueous solvent system is presented for the analysis and preparation of proteins. Ten purified proteins, having a molecular weight of 10,000 - 190,000 daltons and an isoelectric point of 3.9 - 8.5, were applied to a diethylaminoethyl (DEAE) polymer-based column and eluted within 60 min by a linear salt gradient of NaCl in 0.05 M Tris-HCl buffer at pH 7.5. The retention time of protein increases linearly with a decreasing order of the pI value of the protein in this system. By the application of this method, neuron-specific enolase and ceruloplasmin were purified from partially-purified preparations of these proteins respectively, and a series of isoforms of brain S100 protein were separated from each other. This column is capable of separating proteins in high speed, high resolution, large capacity, and in considerably high recovery of proteins without losing the biological activity.

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INTRODUCTION

It has been shown that the reverse-phase, or hydrophobic, HPLC using alkyl-bonded silica-based supports (1-6) or a non-ionic macroreticular polystyrene resin (7) is powerful for the separation of peptides as well as for the separation of relatively small (~50kDa) proteins. The reverse-phase separation of proteins, however, seems to be less effective for high molecular weight proteins because large protein molecules generally have high hydrophobicity and little solubility in organic solvents; i.e., the mobile phase modifier in the reverse phase system. Further, the organic solvent shows adverse effects towards the native conformation of proteins and cause low recovery of their biological activity in some cases. The ion-exchange HPLC has an apparent advantage that the separation can be achieved solely in aqueous buffer solution.

Among the column packings for ion-exchange HPLC, surface-modified silica gels are most commonly used for protein separation. Although such columns are useful with respect to resolution (8-11), there are several weak points from practical viewpoint; relatively short column life, low sample loading capacity, tight adsorption of large proteins, and chemical instability in alkaline solution. In order to improve these inconvenience, various organic polymer-based supports have recently been developed (12,13).

In this communication, we describe the application of a hydrophilic polymer-based anion-exchange column to the separation of relatively high molecular weight proteins. The chromatography in an aqueous buffer solution showed remarkably high resolution and high protein recoveries for numbers of proteins having molecular weights of 10,000 - 190,000 daltons. The activities of enzymes were well preserved during the chromatography.

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MATERIALS AND METHODS

Proteins; Calmodulin, S100 protein, neuron-specific enolase and micro glutamic acid-rich protein were purified from bovine brain by ammonium sulfate fractionation of brain soluble extracts and following column chromatography procedures. The purification of these proteins has been described.(14-17) Serum amine oxidase was prepared from bovine serum by ammonium sulfate fractionation and following DEAE-Sephadex A-50 column chromatography (18). The NIG-65 protein, human myeloma immunoglobulin D, was isolated from the serum of a patient with multiple myeloma by ammonium sulfate fractionation, DEAE-Sephadex A-50 column chromatography and Bio-Gel A-5m gel permeation (19). Other proteins were obtained from sources indicated in parentheses; bovine serum albumin (ICN Pharmaceuticals INC.), human transferrin (Sigma Chemicals Co.), ovalbumin (Sigma Chemicals Co.), human immunoglobulin G (Green Cross Inc.).

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Reagents; Tris(hydroxymethyl)aminomethane, hydrochloric acid and sodium chloride were purchased from Wako Pure Chemical Industries (Tokyo). Water was distilled, passed through a mixed-bed ion-exchange resin, and redistilled before use.

Column and Apparatus; The separation was carried out on a 75 mm x 7.8 mm I.D. column of TSK-gel DEAE-5PW(Toyo Soda Manufacturing Co.Ltd, Tokyo), with a precolumn of 10 mm x 4.6 mm I.D. packed with TSK-guardgel DEAE-5PW. Japan Spectroscopic Co.(Tokyo) HPLC system consisted of a twincle pump, Reodyne model 7125 injector and a Model UVIDEC-100-III uv spectrophotometer(10 mm light pass) connected to a model R-111 recorder(Simadzu Co.,Kyoto), was used.

Chromatographic procedure; Sample proteins(2 μg- 20 mg proteins) were introduced onto a column of TSK-gel DEAE-5PW and eluted with a sodium chloride linear gradient method or an isocratic method in 0.05 M Tris-HCl buffer(pH 7.5) at a flow rate of 1.0 ml/min. The gradient was formed by placing 30 ml of the initial

solvent in a gradient mixer into which the final solvent was added at a constant flow rate of 0.5 ml/min. All separations were conducted at room temperature. The effluent was monitored at 280 nm or 210 nm. Where necessary, column effluent was collected into a test tube. After completion of one analytical run(60min), the column was reequilibrated for 40 min with an initial solvent for the next analysis.

Recovery of proteins and enzyme activity; Recovery of proteins was estimated by measuring UV absobance at 280 nm before and after chromatography. The enzyme assay of serum amine oxidase was carried out spectrophotometrically as described by Yamada and Yasunobu.(20) The activity of neuron-specific enolase was assayed by the method of Baranowski et al(21) using sodium 2-phospho-D-glycerate as substrate.

Estimation of isoelectric point(pI) of proteins; The pI values of proteins were measured by two-dimensional electrophoresis in the absence of denaturing agents as described previously.(22) The isoelectric focusing and the polyacrylamide gradient(4-17%) gel electrophoresis were employed for the first or second dimension, respectively.

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RESULTS AND DISCUSSION

Anion-exchange HPLC of proteins

In order to evaluate the present method, ten purified proteins (listed in Table 1) were applied to the column and eluted by a 60 min-linear gradient from 0 to 0.4 M sodium chloride in 0.05 M Tris-HCl buffer(pH 7.5). Under this standard condition all the tested proteins were eluted from the column with almost complete recoveries (more than 90 %). The recovery of each protein (incorporated in Table 1) appeared to be independent on the molecular weight of proteins, suggesting that there were no "non-specific" interaction between proteins and the resin matrix.

Table 1. A list of proteins subjected to the anion-exchange HPLC

ein	Source	Molecular Weight	Isoelectric Point	Retention Time (min)
in G	human serum	160,000	6.0-8.5	3.0
	human serum	80,000	5.5-5.9	13.0, 14.5, 15.5 16.0, 17.5
in D	human serum	170,000	5.0-5.5	20.0
in	bovine serum	68,000	4.7	21.0
	chicken egg	45,000	4.6,4.7	17.5, 20.0
oxidase	bovine serum	190,000	4.7	30.0
ific	bovine brain	50,000(x2)	4.5	39.0
in	bovine brain	20,900(a) 21,000(b)	4.5 4.2	38.5 40.0
	bovine brain	16,700	4.0	43.5
mic rotein	bovine brain	10,000	3.9	45.0

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The chromatograms for relatively large molecular weight proteins (more than 45,000 daltons) are shown in Fig. 1, a to f. Most of the tested proteins gave rise to a symmetrical single peak respectively, while several proteins showed multiple peaks in this chromatography system. These include transferrin (a), ovalbumin (d), and bovine serum albumin (e). Among these proteins, bovine serum albumin has been shown to be heterogeneous in containing fatty-acid binding albumin and mercaptoalbumin (23), and ovalbumin in containing several molecular species that are different in their content of phosphate group (24). The third protein, transferrin, is also a mixture of isoproteins as demonstrated by two-dimentional electrophoresis without denaturing agents, where a series of spots having different isoelectric point (pI; 5.5 - 5.9) are observed (22). Thus, all the examples mentioned above suggest that the multiple peaks are due to intrinsic heterogeneity of the protein preparation used. This would, in turn, indicate that the resolution of the present method is considerably high from a practical view point.

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It has been also demonstrated that the biological activity of proteins, such as activities of enzymes, are well preserved during the chromatographic procedure as assayed by activity measurements of serum amine oxidase (MW;180,000) and neuron specific enolase (MW; 110,000) before and after chromatography (recoveries of more than 90 %).

Relation between retention time and pI values of proteins

Figure 2 shows the plots of the retention time against pI values of the proteins. The retention time of the protein increases linearly with a decreasing order of the pI value of the protein. This correlation indicates that the major separation mode of the present system is an ionic interaction, and that hydrophobic interaction between resin matrix and proteins contributes little on the separation. In this experiment, we have used pI values as a tentative scale for ionic

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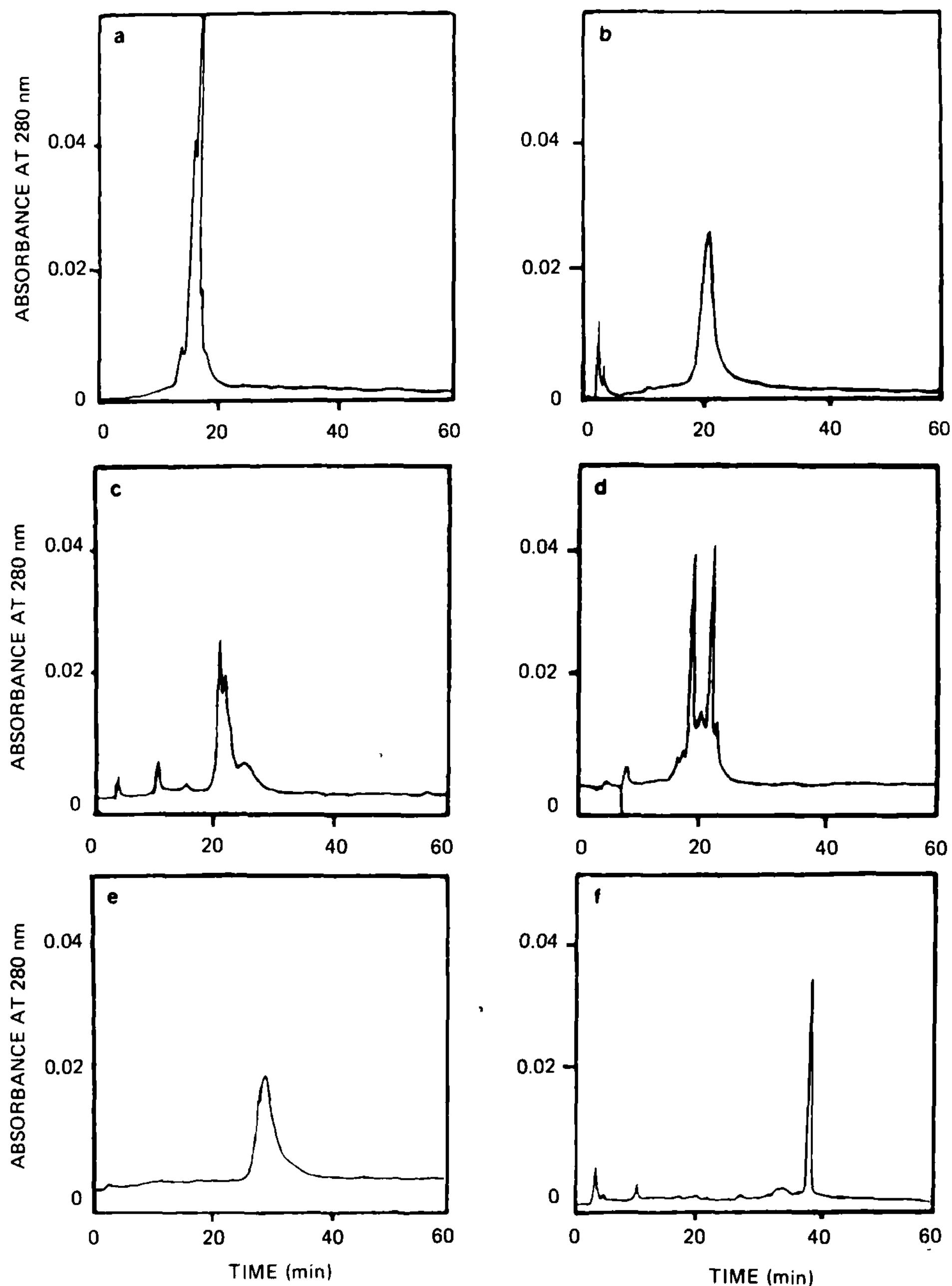

Fig. 1. High performance anion-exchnge chromatography of proteins on TSK-gel DEAE-5PW. The proteins were applied to the column (75 x 7.8 mm I.D.) and eluted by a linear gradient of sodium chloride (0-0.4 M) in 0.05 M Tris-HCl(pH 7.5) at a flow rate of 1.0 ml/min. Other conditions are given under MATERIALS AND METHODS. a;transferrin, b;NIG-65, c;bovine serum albumin, d;ovalbumin, e;serum amine oxidase, f;neuron-specific enolase.

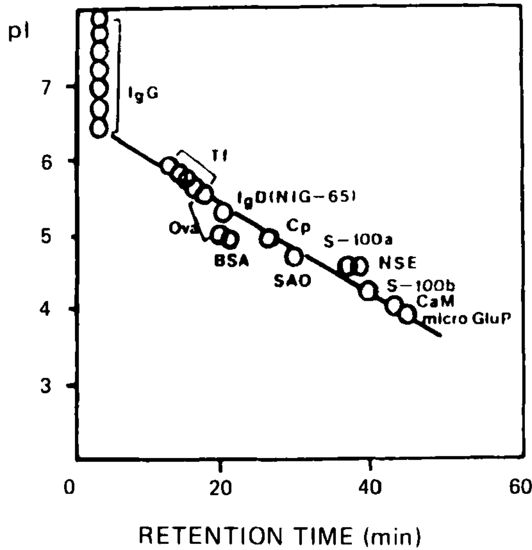

Fig. 2. Plots of the retention time against isoelectric points of proteins. The pI values were measured by two-dimensional electrophoresis in the absence of denaturing agents. IgG;immunoglobulin G, Tf;transferrin, Ova;ovalbumin, BSA;bovine serum albumin, Cp;ceruloplasmin, SAO;serum amine oxidase, NSE;neuron-specific enolase, CaM;calmodulin, micro GluP;micro glutamic acid-rich protein.

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characteristics of proteins for convenience, although the electric charge at a given pH should give more theoretical account (25). From the result in Fig. 2, the elution site, or the capacity factor (k'), of proteins can be easily predicted from their isoelectric point.

Application of the method to the crude preparation

[1] Separation of neuron-specific enolase

Neuron-specific enolase was purified from a partially purified preparation obtained by ammonium sulfate(35-85% saturation) fractionation of bovine brain soluble proteins and following DEAE-Sephadex A-50 column chromatography performed as

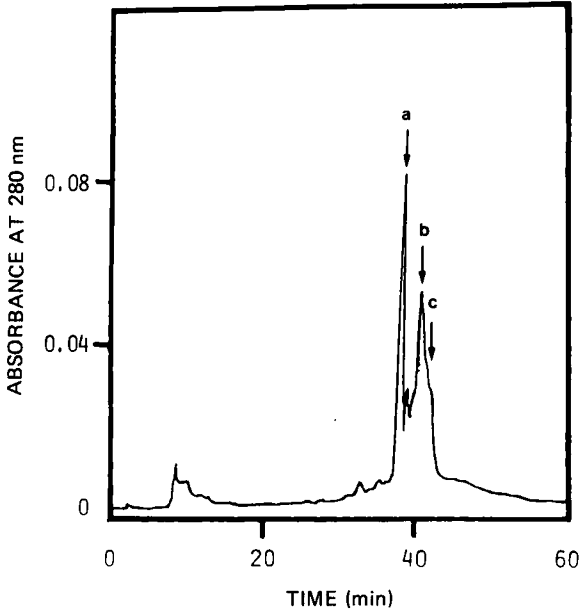

Fig. 3. (A) Separation of neuron-specific enolase on TSK-gel DEAE-5PW and (B) SDS-PAGE of the eluted proteins. Lanes 1;molecular weight marker (Bovine serum albumin, ovalbumin, chymotrypsinogen A), 2;the crude preparation of neuron-specific enolase, 3;peak a, 4;peak b, and 5;peak c.

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described (16). The preparation (320µg proteins) was applied to the column and eluted by the gradient method. The column effluent was collected for SDS-polyacrylamide gel electrophoresis. The chromatogram and SDS-electrophoretogram of eluted proteins are shown in Fig.3. Neuron-specific enolase appeared in a sharp peak at the same retention time as the purified enolase(Fig.1,f). Following the enolase, two other proteins having molecular weights of about 24,000 daltons were eluted.

[2] Separation of ceruloplasmin

Ceruloplasmin, a copper-binding glycoprotein having a molecular weight of 130,000 and an isoelectric point of 4.9, was

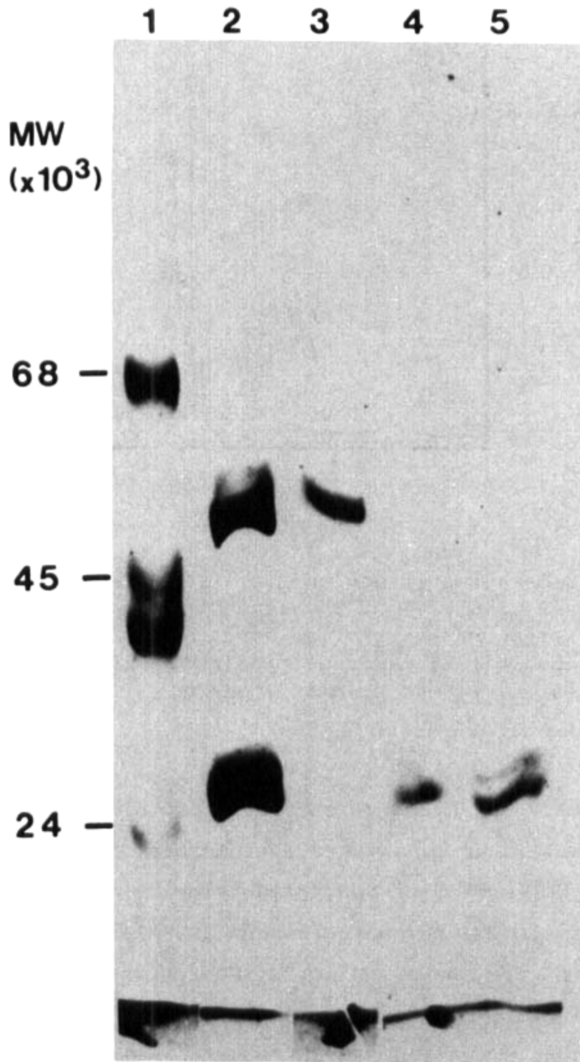

Fig. 3. (B)

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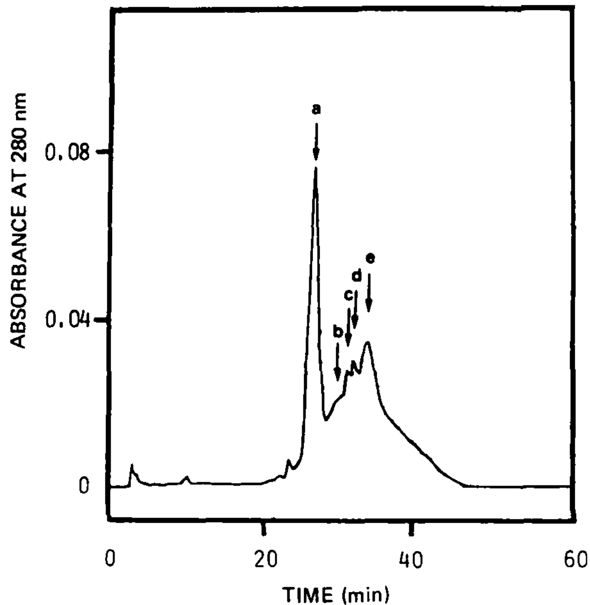

Fig. 4. (A) Separation of ceruloplasmin on TSK-gel DEAE-5PW,and (B) SDS-PAGE of the eluted proteins. Lanes 1;molecular weight marker, 2;the crude preparation of ceruloplasmin, 3;peak a, 4;peak b, 5;peak c, 6;peak d, and 7;peak e.

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partially purified from bovine serum by ammonium sulfate(35-55%) fractionation and following DEAE-Sephadex A-50 column chromatography using a step-wise elution. This preparation (560 μg proteins) was applied to the column. The chromatogram and SDS-electrophoretogram of eluted proteins are shown in Fig.4. Ceruloplasmin was eluted at a retention time of 27 min as expected from the result in Fig.2.

[3] Separation of S100 protein isoforms

Bovine brain S100 protein contains a number of molecular species that have different subunit compositions; αα ($S100a_0$), αβ (S100a), α'β (S100a'), and ββ (S100b)(26). The each subunit has highly homologous amino acid seaquence, and the separation of these isoforms on a conventional DEAE-Sephadex A-50 column

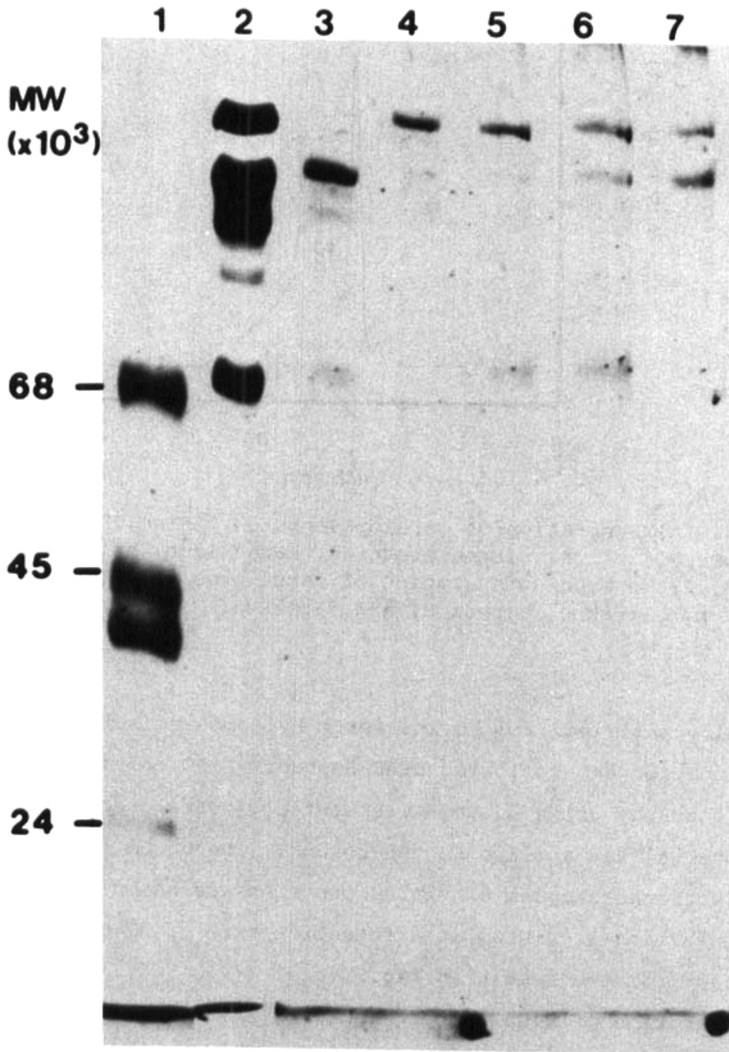

Fig. 4. (B)

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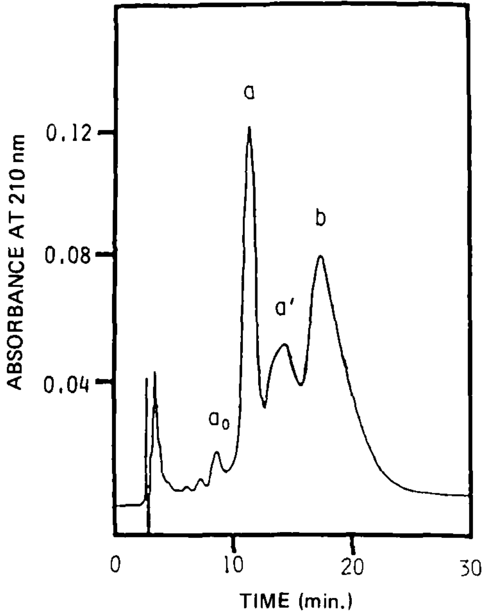

Fig. 5. Separation of S-100 protein isoforms on TSK-gel DEAE-5PW. Bovine brain S100 protein (100 ug) was chromatographed using an isocratic elution with 0.05 M Tris-HCl(pH 7.5) containing 0.2 M NaCl.

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requires two weeks under highly restricted chromatographic conditions (14). With the new ion-exchange column, however, the same separation has been achieved within 30 min by an isocratic elution with 0.05 M Tris-HCl buffer(pH 7.5) containing 0.2 M NaCl (Fig. 5). The purified S100a protein was separated into two peaks of α and β subunit by the subsequent reverse phase HPLC in a trifluoroacetic acid-acetonitrile solvent system (7), suggesting that the quarternary structure of S100 protein was preserved during the anion exchange HPLC.

Stability of the column

This polymer-based column is chemically stable in wide range of pH, which allows chromatography in alkaline conditions. We

have employed a Tris-HCl buffer(pH 7.5) throughout the experiments, but the use of elution buffer of higher pH will extend the applicability of this method for more basic proteins. In addition, we have noticed that a strongly alkaline solution, such as 0.2 M NaOH, is effective in regeneration of the column which might be neccessary after repeated application of crude protein mixture.

In conclusion, the high performance anion-exchange chromatography using an aqueous solvent system seems to be a useful and powerful technique for the biochemical study of various proteins including enzyme, because the method shows excellent resolution in high speed with considerably high recovery of proteins or of the enzyme activity. The method can be applied either for analytical or preparative separation of proteins because the resin column has large sample loading capacity.

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Abbreviations used: HPLC; high performance liquid chromatography, Tris; Tris(hydroxymethyl)aminomethane, DEAE; diethylaminoethyl.

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