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NEW HIGH-PERFORMANCE CATION EXCHANGER FOR THE SEPARATION OF PROTEINS

YOSHIO KATO*, KOJI NAKAMURA and TSUTOMU HASHIMOTO
Central Research Laboratory, Toyo Soda Mfg. Co., Ltd., 4560 Tonda, Shinnanyo-shi, Yamaguchi-ken (Japan)
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SUMMARY

The new cation exchanger TSKgel SP-5PW was found to be very useful for protein separations. Neutral to basic proteins and also some acidic proteins could be separated with high resolution. Protein subunits could be separated in a buffer containing concentrated urea. This cation exchanger could also be applied to peptide separations.

INTRODUCTION

We previously reported that TSKgel DEAE-5PW is an ideal anion exchanger for the separation of biopolymers such as proteins and nucleic acids[1]. It was developed by introducing diethylaminoethyl (DEAE) groups into G5000PW, which is a hydrophilic polymer-based material of large pore size for high-performance gel filtration, of particle diameter 10 μm. Recently, a cation exchanger containing sulphopropyl groups and prepared from the same base material has become commercially available under the trade-name of TSKgel SP-5PW (Toyo Soda, Tokyo, Japan). We have now evaluated it for the separation of proteins.

EXPERIMENTAL

TSKgel SP-5PW was titrated with 0.5 *M* sodium hydroxide as described previously[1].

The adsorption capacity for γ-globulin, haemoglobin and lysozyme was determined by the static method. TSKgel SP-5PW (3 ml) and 10 ml of a 2% solution of each protein were mixed, and 0.02 *M* sodium phosphate buffer (pH 6.0) was added to give a total volume of 50 ml. After the mixture had been left at 25°C for 30 min with occasional swirling, it was filtered through filter-paper and washed repeatedly with *ca.* 10 ml of 0.02 *M* phosphate buffer (pH 6.0). The filtrates were pooled to a total volume of 100 ml and the protein content was determined by spectrophotometry at 280 nm. The adsorption capacity for proteins was determined as the difference between the protein contents in the initial mixed solution and in the filtrate.

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The recovery of proteins from TSKgel SP-5PW was evaluated by injecting proteins (0.4 mg) into a stainless-steel column (75 × 7.5 mm I.D.) equilibrated with 0.02 *M* phosphate buffer (pH 6.0) at a flow-rate of 1 ml/min and washing out the adsorbed proteins by linear gradient elution to the same buffer containing 0.5 *M* sodium chloride in 1 min. The column effluent was collected for 20 min after sample injection. The protein contents in the effluents were determined spectrophotometrically at 225 nm.

Ion-exchange chromatographic measurements were carried out on a stainless-steel column (74 × 7.5 mm I.D.) with a high-speed liquid chromatograph Model SP8700 (Spectra-Physics, San Jose, CA, U.S.A.) equipped with a variable wavelength UV detector Model UV-8 (Toyo Soda). The proteins and peptides were separated by linear gradient elution with two buffers at a flow-rate of 1 ml/min at 25°C and detected at 280 nm, unless otherwise stated. In the separation of some enzymes the column effluent was collected and the enzymatic activity was determined.

All proteins and peptides employed were commercial products, except crude β-amylase. Haemoglobin, trypsinogen, ribonuclease, α-chymotrypsinogen, cytochrome *c*, lysozyme and lipoxidase were purchased from Sigma (St. Louis, MO, U.S.A.). γ-Globulin was obtained from Miles Labs. (Elkhart, IN, U.S.A.), human haemoglobulin standard was from Nippon Chemipher (Tokyo, Japan) and fibrinogen was from Wako (Osaka, Japan). Crude β-amylase was prepared by homogenizing sweet potato in a mixer, squeezing the homogenate with cloth, centrifuging the filtrate for 10 min at 10,000 *g*, adding cold acetone (−20°C) to the supernatant (1:1) and collecting the precipitate by centrifugation for 10 min at 10,000 *g*.

RESULTS AND DISCUSSION

Basic properties

Fig. 1 shows a titration curve, indicating that TSKgel SP-5PW contains *ca.* 0.12 mequiv./ml ionic groups with $pK_a \approx 2.3$. Owing to the low pK_a value, TSKgel SP-5PW can be applied not only to neutral to basic proteins but also to some acidic

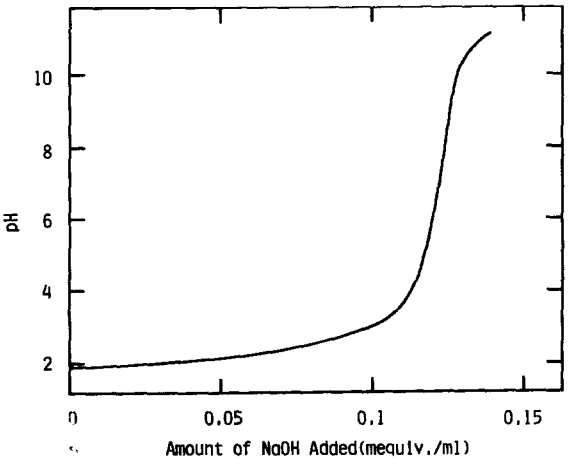

Fig. 1. Titration curve of TSKgel SP-5PW.

TABLE I

ADSORPTION CAPACITY OF TSKgel SP-5PW FOR PROTEINS IN 0.02 *M* PHOSPHATE BUFFER (pH 6.0) AT 25°C

Protein	*Adsorption capacity (mg/ml)*
γ-Globulin	40
Haemoglobin	43
Lysozyme	54

proteins. This is advantageous compared with carboxymethyl type cation exchangers. The ion-exchange capacity is comparable to those of many conventional ion exchangers[2,3]. Therefore, it is expected that proteins will be separated under similar eluting conditions as for conventional ion exchangers.

The adsorption capacity for proteins is summarized in Table I. TSKgel SP-5PW has a high adsorption capacity even for large proteins like γ-globulin, as expected from its large pore size. Therefore, it can be applied to proteins with high molecular weights.

Table II summarizes the recovery of proteins. All proteins investigated were recovered almost quantitatively. In addition, the recovery of enzymatic activity was also high (>80%).

Applications to the separation of proteins and peptides

Cation exchangers are typically used for the separation of neutral to basic proteins. Fig. 2 shows an example of such separations. Basic proteins of comparatively low molecular weights were well separated. Fig. 3 presents another example of typical separations, where human haemoglobin standard was separated. Cation-exchange chromatography has been widely employed to analyse glycosylated haemoglobin in human blood. The peak eluted in *ca.* 15 min is probably glycosylated haemoglobin A_{1c}.

Figs. 4 and 5 illustrate the separations of acidic proteins, lipoxidase and β-

TABLE II

RECOVERY OF PROTEINS FROM TSKgel SP-5PW IN 0.02 *M* PHOSPHATE BUFFER (pH 6.0) AT 25°C

Sample loading: 0.4 mg.

Protein	*Recovery (%)*
γ-Globulin	98
Haemoglobin	96
Trypsinogen	101
α-Chymotrypsinogen	98
α-Chymotrypsin	104
Myoglobin	88
Lysozyme	95
Ribonuclease	100
Cytochrome *c*	103

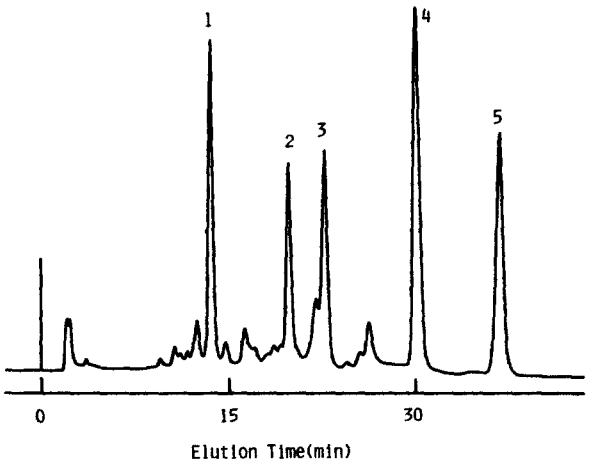

Fig. 2. chromatogram of a mixture of basic proteins pbtained by high-performance ion-exchange chromatography on TSKgel SP-5PW with a 60-min linear gradient of sodium chloride from 0 to 0.5 *M* in 0.02 *M* phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. Samples: 1 = trypsinogen (0.2 mg); 2 = ribonuclease (0.4 mg); 3 = α-chymotrypsinogen (0.2 mg); 4 = cytochrome *c* (0.2 mg); 5 = lysozyme (0.1 mg).

amylase, which are eluted in *ca.* 25 and *ca.* 28 min, respectively. Although these two proteins have isoelectric points of 5.4 and 4.7, they could be successfully separated by using eluents of low pH, 4.5 and 4.0. Also, these two proteins were eluted as sharp peaks although they have fairly high molecular weights (102,000 and 197,000). This is probably due to the large pore size of TSKgel SP-5PW. The column effluents between two vertical lines in Figs. 4 and 5 were fractionated, and the enzymatic activity was determined. Enzymatic activities as high as 84 and 88% were recovered for lipoxidase and β-amylase, respectively.

Fig. 6 shows a chromatogram of fibrinogen as an example of the separation in a buffer containing concentrated urea. It is possible to separate protein subunits with such buffers. In Fig. 6, six peaks are observed in addition to the solvent peaks

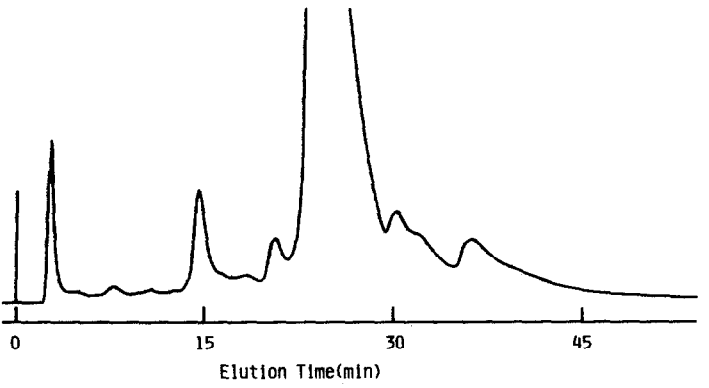

Fig. 3. Chromatogram of human haemoglobin standard (1 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on TSKgel SP-5PW with a 60-min linear gradient of sodium perchlorate from 0 to 0.1 *M* in 0.05 *M* phosphate buffer (pH 6.0) containing 2% 2-propanol at a flow-rate of 1 ml/min.

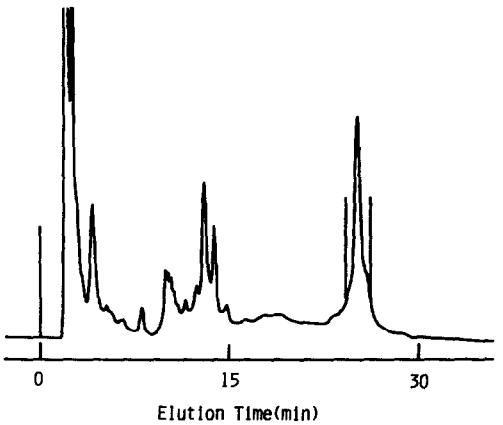

Fig. 4. Chromatogram of lipoxidase (1 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on TSKgel SP-5PW with a 60-min linear gradient of sodium sulphate from 0 to 0.5 *M* in 0.02 *M* acetate buffer (pH 4.5) at a flow-rate of 1 ml/min.

at elution times of *ca.* 6 min. These peaks are believed to be fibrinogen subunits, although they have not been identified.

TSKgel SP-5PW could also separate peptides with high resolution. Fig. 7 shows a chromatogram of a mixture of peptides which exist in the human brain and have molecular weights between several hundreds and several thousands. The resolution seems to be comparable to that attained by reversed-phase chromatography, which has been widely adopted to separate peptides. The separation shown in Fig. 7 was performed with an eluent containing acetonitrile. This is because some peptides were eluted very slowly as broad peaks in the eluents not containing organic solvents, probably due to hydrophobic interactions between the peptides and ion exchangers. This abnormal elution behaviour was eliminated by the addition of 20–40% acetonitrile in the eluent. Also, the relative elution positions of some components changed with the content of acetonitrile. This can be utilized to improve their separations.

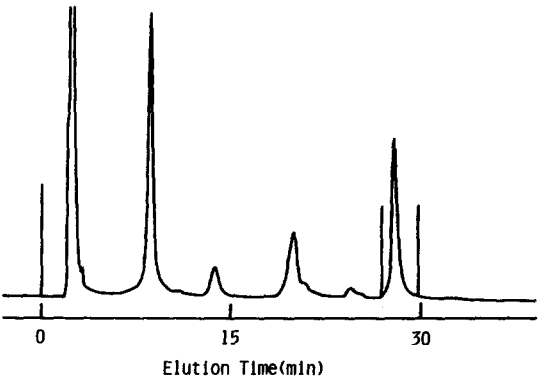

Fig. 5. Chromatogram of β-amylase (0.4 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on TSKgel SP-5PW with a 60 min linear gradient of sodium perchlorate from 0 to 0.5 *M* in 0.02 *M* acetate buffer (pH 4.0) at a flow-rate of 1 ml/min.

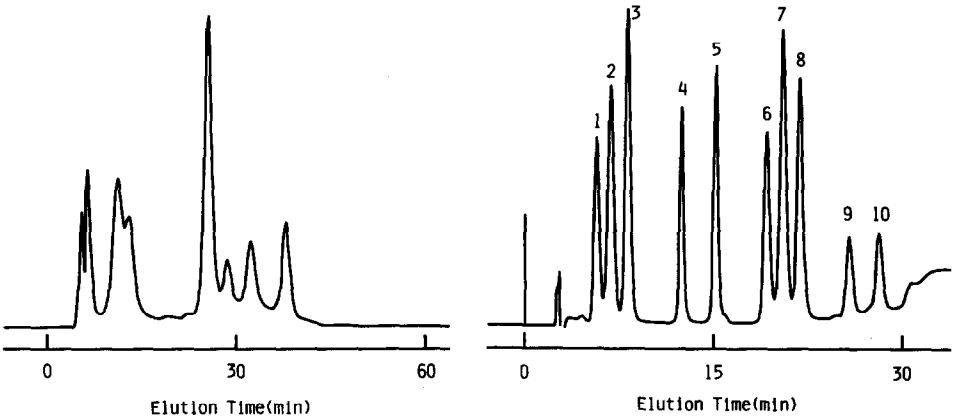

Fig. 6. Chromatogram of fibrinogen (0.2 mg in 0.1 ml) obtained by high-performance ion-exchange chromatography on TSKgel SP-5PW with a 100-min linear gradient of sodium perchlorate from 0.1 to 0.5 *M* in 0.05 *M* acetate buffer (pH 4.5) containing 7 *M* urea at a flow-rate of 0.5 ml/min.

Fig. 7. Chromatogram of a mixture of peptides obtained by high-performance ion-exchange chromatography on TSKgel SP-5PW with a 30-min linear gradient from a mixture of 0.02 *M* phosphate buffer (pH 3.0) and acetonitrile (70:30) to a mixture of 0.5 *M* phosphate buffer (pH 3.0) and acetonitrile (70:30) at a flow-rate of 1 ml/min. Detection was made at 220 nm. Samples: 1 = oxytosin; 2 = met-enkephalin; 3 = thyrotropin-releasing hormone; 4 = α-endorphin; 5 = luteinizing hormone-releasing hormone; 6 = neurotensin; 7 = α-MSH (melanocyte-stimulating hormone); 8 = angiotensin II; 9 = substance P; 10 = β-endorphin. Sample loading was 2 μg of each peptide, except LHRH (1 μg), in 0.05 ml.

The addition of organic solvent to the eluent was useful also in the separation of some proteins. The separation of glycosylated haemoglobin (Fig. 3) was slightly improved by the addition of 2% 2-propanol.

The loading capacity was evaluated with a mixture of equal amounts of ribonuclease and α-chymotrypsin in a similar manner to that employed with TSKgel DEAE-5PW[1], and was estimated to be *ca.* 0.5 mg for each component. Therefore, the sample loading should be kept below this level in order to achieve the highest resolution. This loading capacity is equivalent to that observed for TSKgel DEAE-5PW.

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