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Note

High-performance metal chelate affinity chromatography of proteins

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Metal chelate affinity chromatography of proteins was introduced in 1975 by Porath *et al.*[1]. Since then, it has been applied successfully to many kinds of proteins[2] and is becoming a standard tool for the isolation of proteins. It has been especially useful for group separation between two classes of proteins with and without affinity for heavy metal ions. However, it seems difficult to achieve good separation among the proteins having affinity for heavy metal ions. Furthermore, most separations have been performed at low speed using agarose derivatives. If these aspects were to be improved, this technique would become much more useful.

Very recently, a new support for metal chelate affinity chromatography has become commercially available under the trade-name of TSKgel Chelate-5PW (Toyo Soda, Tokyo, Japan). According to the manufacturer, it is prepared by introducing iminodiacetic acid into TSKgel G5000PW[3], which is a hydrophilic resin-based material of large pore size (particle diameter 10 μm) employed for high-performance gel filtration. Therefore, the schematic structure is believed to be as shown in Fig. 1. The amount of immobilized iminodiacetic acid is about 20 μmol per ml swollen gel. We have evaluated this new support for protein separation and the results are now described.

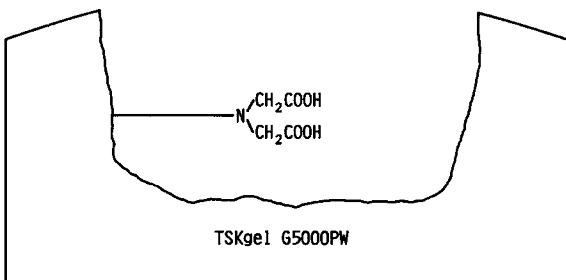

Fig. 1. Schematic structure of TSKgel Chelate-5PW.

EXPERIMENTAL

All chromatographic measurements were performed on 75 × 7.5 mm I.D. stainless-steel columns with a high-speed liquid chromatograph Model SP8700 (Spectra-Physics, San Jose, CA, U.S.A.) equipped with a variable wavelength UV detector

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Model UV-8 (Toyo Soda). Proteins were usually separated on a zinc-loaded column by a 60-min linear gradient of glycine from 0 to 0.2 *M* in 0.02 *M* Tris–HCl buffer (pH 8.0) containing 0.5 *M* sodium chloride and at a flow-rate of 0.5 ml/min at 25°C; detection was at 280 nm, unless stated otherwise. Before each separation, the column was washed with *ca.* 10 ml of 0.05 *M* ethylenediaminetetraacetic acid, tetrasodium salt containing 0.5 *M* sodium chloride. Then, the column was equilibrated with *ca.* 5 ml of the initial buffer and 2 ml of 0.2 *M* zinc chloride were applied to the column by using a sample injector. This amount of zinc chloride was enough to saturate the column with zinc ion. After the column had been re-equilibrated with *ca.* 15 ml of the initial buffer, the sample dissolved in the initial buffer was applied. The gradient of glycine was started at the same time as sample injection.

The recovery of proteins was determined from the areas of the eluted peaks. As controls, we used areas obtained when the column was replaced by empty 1 mm I.D. stainless-steel tubing of 1 ml total inner volume and the elution was performed isocratically at the eluent compoositions at which each protein was eluted in gradient elution. Amounts of 50 μg of each protein were injected. The peak areas were measured with a data processor Model CP-8000 (Toyo Soda).

Ribonuclease A (bovine pancreas), transferrin (human), carbonic anhydrase (bovine erythrocytes), myoglobin (horse skeletal muscle), lactate dehydrogenase (rabbit muscle), alcohol dehydrogenase (yeast), L-glutamic dehydrogenase (bovine liver) and carboxypeptidase A (bovine pancreas) were purchased from Sigma (St. Louis, MO, U.S.A.). Lipoxidase (soya bean) was obtained from P-L. Biochemicals (Milwaukee, WI, U.S.A.) and malate dehydrogenase (yeast) from oriental Yeast (Osaka, Japan).

RESULTS AND DISCUSSION

Fig. 2 shows the separation of an artificial mixture of rather pure proteins which have affinity for zinc ion. All the proteins were eluted as fairly sharp peaks. It may be concluded that metal chelate affinity chromatography on TSKgel Chelate-5PW has a resolution comparable to that of high-performance ion-exchange chromatography, reversed-phase chromatography or hydrophobic interaction chro-

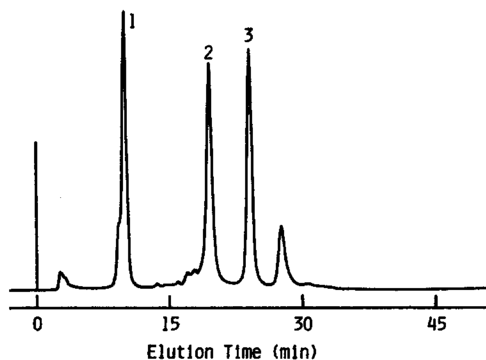

Fig. 2. Chromatogram of a mixture of ribonuclease A (1), transferrin (2) and carbonic anhydrase (3) obtained on Zn^{2+}-Chelate-5PW. The flow-rate was 1 ml/min.

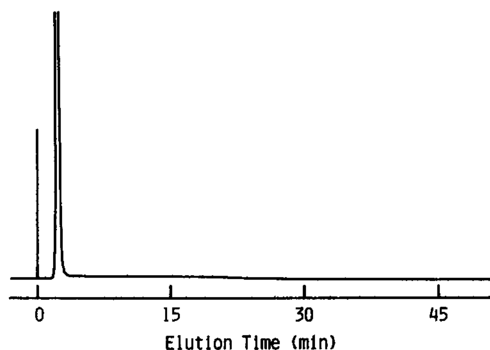

Fig. 3. Chromatogram of the same mixture as in Fig. 2 obtained without loading zinc chloride on the column. The flow-rate was 1 ml/min.

matography. The same protein mixture was chromatographed without loading zinc ion on the column (Fig. 3). All the proteins were eluted in the void volume of the column. Consequently, it seems that the proteins were retained by interaction with chelated zinc ion in the separation shown in Fig. 2. Other proteins (myoglobin, lactate dehydrogenase, alcohol dehydrogenase, L-glutamic dehydrogenase and carboxypeptidase A) which were retained on Zn^{2+}-Chelate-5PW were also eluted in the void volume of the column in the absence of zinc ion. Therefore, non-specific adsorption of proteins on TSKgel Chelate-5PW must be minimal and this support whould be very useful to separate selectively proteins which have affinity for heavy metal ions.

The recoveries of proteins from Zn^{2+}-Chelate-5PW are listed in Table I. All proteins tested were recovered in greater than 80% yield.

TABLE I

RECOVERY OF PROTEINS FROM Zn^{2+}-CHELATE-5PW COLUMN

Protein	*Recovery (%)*
Ribonuclease A	93
Myoglobin	93
Carbonic anhydrase	86
Transferrin	100
Lactate dehydrogenase	95

Fig. 4 shows an example of the application of metal chelate affinity chromatography on TSKgel Chelate-5PW to protein purification. Commercial crude lipoxidase was separated. Many components were retained on Zn^{2+}-Chelate-5PW in 0.02 *M* Tris–HCl buffer (pH 8.0) containing 0.5 *M* sodium chloride, and they were eluted separately by increasing the glycine concentration from 0 to 0.2 *M*. Because the peak eluted at *ca.* 30 min was confirmed to be lipoxidase by an enzymatic activity test, the column effluent was collected between the two vertical lines shown in Fig. 4 and examined for the recovery of enzymatic activity and the degree of purification. The

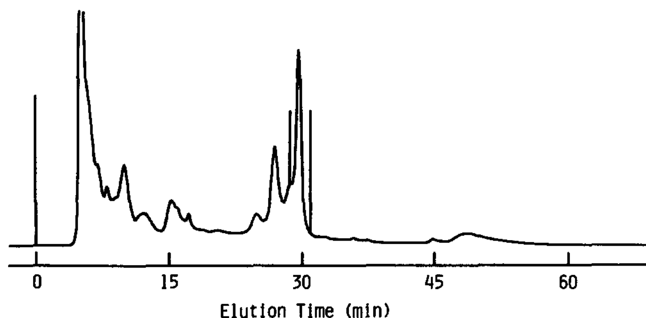

Fig. 4. Separation of commercial lipoxidase (0.5 mg) on Zn^{2+}-Chelate-5PW.

recovery of enzymatic activity was 90%, and the degree of purification based on the specific activity was 7.5-fold. The fraction was also subjected to high-performance ion-exchange chromatography and reversed-phase chromatography (Figs. 5 and 6, respectively). One main peak and a few very small peaks are seen in each chromatogram of the fraction. The main peak in the ion-exchange chromatogram exhibited enzymatic activity, suggesting that the main peaks in the chromatograms of the fraction correspond to lipoxidase. Consequently, it can be said that fairly pure lipoxidase was obtained from a commercial sample containing many impurities in a single step by metal chelate affinity chromatography on TSKgel Chelate-5PW.

Fig. 7 shows the separation of commercial malate dehydrogenase. The peak eluted at *ca.* 25 min was identified as malate dehydrogenase by an enzymatic activity test, and hence the column effluent between two vertical lines was collected. Of the applied enzymatic activity, 95% was found in the fraction. The degree of purification was 3.9-fold. Malate dehydrogenase recovered in the fraction was of high purity according to high-performance liquid chromatography.

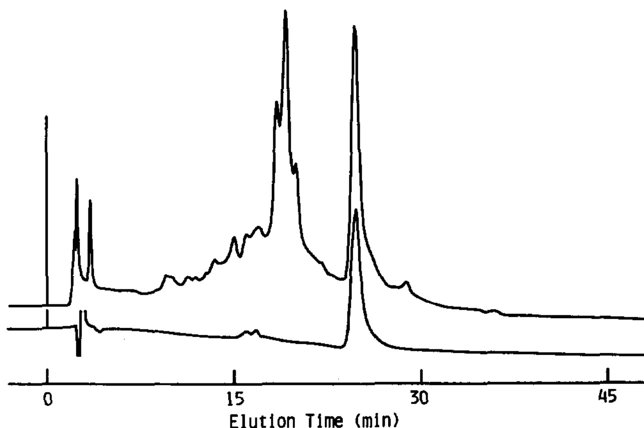

Fig. 5. Ion-exchange chromatograms of a crude lipoxidase sample (upper curve) and the fraction in Fig. 4 (lower curve). The samples were separated on a TSKgel DEAE-5PW column (75 × 7.5 mm I.D.) with a 60-min linear gradient of sodium chloride from 0 to 0.5 *M* in 0.02 *M* Tris–HCl buffer (pH 8.0) at a flow-rate of 1 ml/min at 25°C and detected at 280 nm.

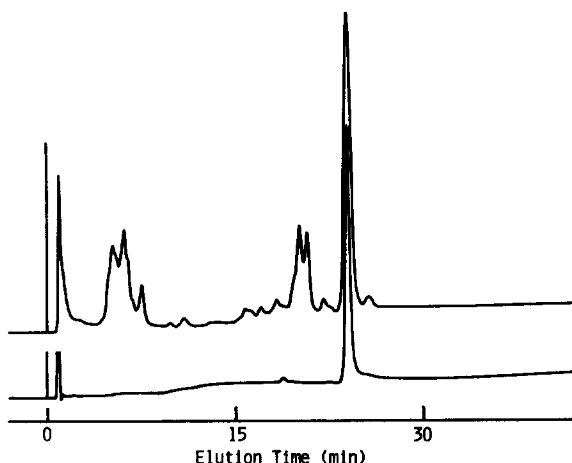

Fig. 6. Reversed-phase chromatograms of a crude lipoxidase sample (upper curve) and the fraction in Fig. 4 (lower curve). The samples were separated on a TSKgel Phenyl-5PW RP column (75 × 4.6 mm I.D.) with a 2-min linear gradient of acetonitrile from 5 to 20% followed by a 48-min linear gradient of acetonitrile from 20 to 80% in 0.05% trifluoroacetic acid at a flow-rate of 1 ml/min at 25°C and detected at 220 nm.

Fig. 8 shows the effect of the flow-rate and gradient time on resolution. Higher resolution was obtained at longer gradient times. However, this effect was diminished at gradient times longer than 120 min, except in the case of very low flow-rates. Because a longer gradient time results in a longer separation time, gradient times of 60–120 min, corresponding to 0.1–0.2 *M* glycine per h, seem to be a good compromise between resolution and separation time. Using these times, the highest resolution was obtained at flow-rates of *ca.* 0.5 ml/min. Although an increase in flow-rate results in a slight decrease in separation time, it also results in more dilution of the sample during separation. Therefore, the optimum flow-rate is around 0.5 ml/min.

The effect of eluent pH was investigated by separating a mixture of transferrin and carbonic anhydrase in 0.02 *M* Tris–HCl buffers of pH 7.0–9.0 (Fig. 9). Proteins were more strongly retained at lower pH, although the affinity of proteins and amino acids for heavy metal ions should be diminished[1,4,5]. This is probably because the affinity of glycine for zinc ion decreased more than that of proteins when the eluent pH was decreased. The peak width and peak interval of the two proteins were also dependent on the eluent pH. Although both characteristics became broader as the eluent pH decreased, the resolution was maximal at pH 7.5–8.0.

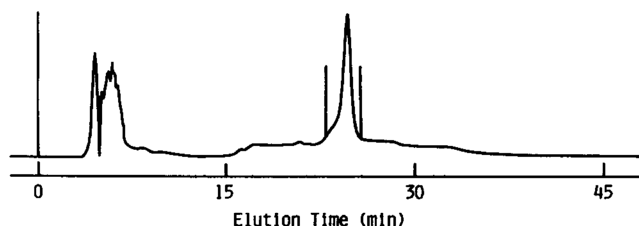

Fig. 7. Separation of commercial malate dehydrogenase (0.1 mg) on Zn^{2+}-Chelate-5PW.

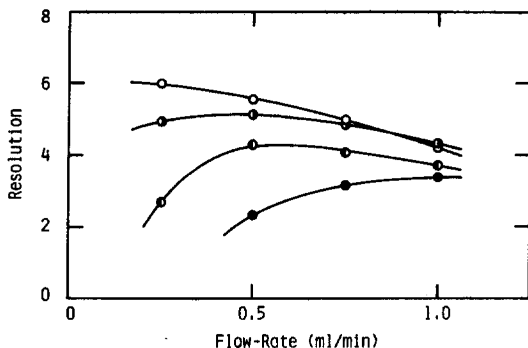

Fig. 8. The effect of the flow-rate and gradient time on resolution. A mixture of transferrin and carbonic anhydrase was separated on Zn^{2+}-Chelate-5PW at flow-rates of 0.25–1 ml/min and gradient times of 30–240 min. The resolution was calculated from the peak widths and elution volumes of the two proteins. Gradient times: 30 (●—●), 60 (◐—◐), 120 (◑—◑) and 240 min (○—○).

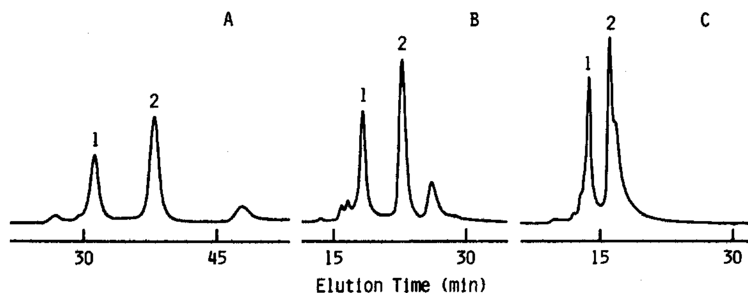

Fig. 9. Chromatograms of a mixture of transferrin (1) and carbonic anhydrase (2) obtained on Zn^{2+}-Chelate-5PW at pH 7.5 (A), 8.0 (B) and 8.5 (C). The flow-rate was 1 ml/min.

The support capacity was studied by separating various amounts of commercial crude lipoxidase. Almost identical separations were obtained with sample loadings up to 2 mg as shown in Fig. 10, but the peaks gradually became broader with higher sample loadings. Therefore, the maximum sample loading resulting in the highest resolution is about 2 mg for this sample, although it is believed to depend on

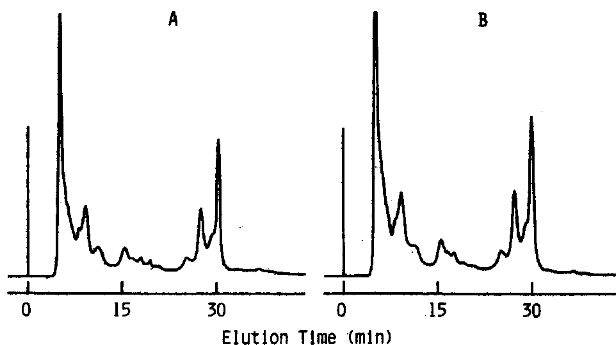

Fig. 10. Chromatograms of commercial crude lipoxidase obtained on Zn^{2+}-Chelate-5PW with sample loadings of 0.2 (A) and 2 mg (B).

the sample, as observed in other modes of high-performance liquid chromatography of proteins[6–8].

As demonstrated above, TSKgel Chelate-5PW is very useful for metal chelate affinity chromatography of proteins. Proteins which have affinity for zinc ion could be separated rapidly with high resolution and recovery. Moreover, it should be possible to apply TSKgel Chelate-5PW to very high-molecular-weight proteins because it has pores with a diameter of several thousand ångströms. In addition, it is chemically very stable. No change in the iminodiacetic acid content was observed when it was suspended in 0.5 *M* sodium hydroxide or 20% acetic acid for 10 days at 25°C. Therefore, metal chelate affinity chromatography on TSKgel Chelate-5PW should become an important technique for the separation of proteins.

REFERENCES

1 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598.
2 E. Sulgowski, *Trends Biotechnol.*, 3 (1985) 1.
3 T. Hashimoto, H. Sasaki, M. Aiura and Y. Kato, *J. Polym. Sci., Polym. Phys. Ed.*, 16 (1978) 1789.
4 E. S. Hemdan and J. Porath, *J. Chromatogr.*, 323 (1985) 247.
5 E. S. Hemdan and J. Porath, *J. Chromatogr.*, 323 (1985) 255.
6 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 292 (1984) 418.
7 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 333 (1985) 93.
8 K. Nakamura and Y. Kato, *J. Chromatogr.*, 333 (1985) 29.