The Bulletin of Aichi Univ. of Education, 56 (Natural Science), pp. 45～49, March, 2007

Techniques for the separation of proteins by isoelectric point column chromatography

Yoko SAKAKIBARA* and Hiroshi YANAGISAWA**

*Course of Science Education, Aichi University of Education, Igaya, Kariya 448-8542, Japan

**Department of Biology, Aichi University of Education, Igaya, Kariya 448-8542, Japan

Abstract

The common protein streptavidin, and the n-type glycoproteins ovalbumin and transferrin were eluted at their respective isoelectric points from CM-Sephadex, CM-Sepharose, SP-Sephadex, and SP-Sepharose by 0.1 M Tris succinate buffer. No tailing occurred with a linear gradient where the ionic strength was gradually decreased. Although streptavidin and a large proportion of ovalbumin were not absorbed by CM-Sephadex equilibrated with a 0.2 M buffer, transferrin and some of the ovalbumin were eluted at their isoelectric points.

This method should be called isoelectric point column chromatography because compounds are eluted from the column at their isoelectric points. This method may be suitable for use with various proteins. In particular, glycoproteins, which are considered to be difficult to purify, can be easily eluted at their isoelectric points under high ionic strength.

Key words: Isoelectric point; Chromatography; Glycoprotein; Protein; Ionic strength; Separation

1. Introduction

In ion-exchange chromatography, the resin absorbs an oppositely-charged target substance in a buffer. After the resin is thoroughly washed, the target substance is removed from the resin by desorption. Generally, ion-exchange chromatography is not suitable for use under conditions of high ionic strength: under such conditions, since there is intense competition for the dissociating group in the resin it is difficult for the resin to hold the target substance. Desorption methods involve increasing the buffer concentration or changing the pH[1].

Proteins are ampholytes, and they have a certain isoelectric point at which they have no net charge. Proteins are strongly charged by pH conditions far from their isoelectric points, and are weakly charged at a pH close to their isoelectric points. Although a protein is electrically absorbed onto a resin, depending on its isoelectric point, the buffer or coexisting substances can also affect the isoelectric point of the protein. As a result, the isoelectric point of the protein can vary. There have been no previous reports on ion-exchange (column) chromatography in which nothing influenced the isoelectric point of a target substance. In our method, a protein is easily eluted from a resin at a particular isoelectric point.

In this report, we describe our method in detail and discuss differences with the choice of the resin and the effects of the buffer concentration.

2. Materials

2. 1. Chemicals

Ovalbumin, streptavidin, transferrin and α1-acid glycoprotein were purchased from Sigma Chemicals. CM-Sephadex, CM-Sepharose, SP-Sephadex, SP-Sepharose and QAE-Sephadex were obtained from Amersham Biosciences. All other chemicals were of analytical reagent grade.

2. 2. Standard system

Isoelectric point column chromatography was performed as follows. One mg of ovalbumin, streptavidin, and transferrin were dissolved in a total volume of 3 mL of 0.1 M Tris succinate buffer, pH 4.0. The protein solution (3mL) was applied to a cation-exchange column (1.2 x 10 cm) equilibrated with the same buffer. After the column was washed with the same buffer

(4 times the column volume), proteins were eluted with a linear gradient of 150 mL of the same buffer and 150 mL of 0.1 M Tris solution. CM-Sephadex, CM-Sepharose, SP-Sephadex, and SP-Sepharose were used as cation-exchange columns. The eluate was fractionated into ca 5.1-mL volumes with a Bio-Rad Model 2110 fraction collector. The optical density at 280 nm and the pH of each fraction were measured by a Shimadzu UV-180 Spectrophotometer and TOA HM-20E pH Meter, respectively.

2．3．Effect of the buffer concentration

One mg of ovalbumin, streptavidin, and transferrin were dissolved in a total volume of 3 mL of 0.2 M Tris succinate buffer, pH 4.0. The protein solution (3mL) was applied to a column (1.2 x 10 cm) of CM-Sephadex equilibrated with the same buffer. After the column was washed with the same buffer (15 times the column volume), the proteins were eluted with a linear gradient of 150 mL of the same buffer and 150 mL of 0.2 M Tris solution. The eluate was fractionated into ca 5.1-mL volumes with a Bio-Rad Model 2110 fraction collector. The optical density at 280 nm and the pH of each fraction were measured as described above.

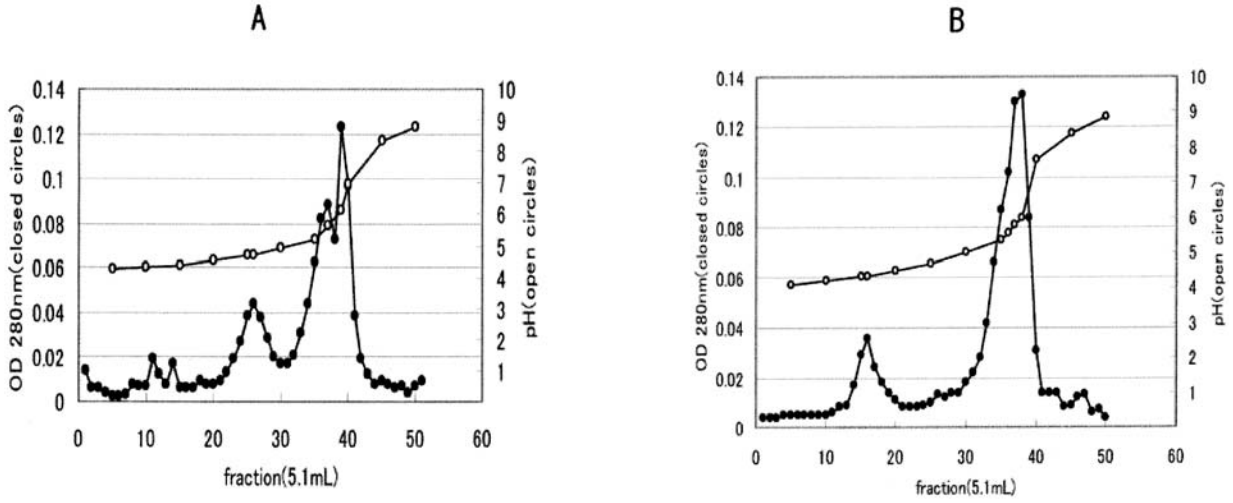
2．4．Elution in anion-exchange column chromatography

One mg of ovalbumin and α1-acid glycoprotein were dissolved in a total volume of 3 mL of 0.1 M Tris succinate buffer, pH 8.5. The protein solution (3mL) was applied to a column (1.2 x 10 cm) of QAE-Sephadex equilibrated with the same buffer. After the column was washed with the same buffer (4 times the column volume), the protein was eluted with a linear gradient of 150 mL of the same buffer and 150 mL of 0.1 M Tris succinate buffer, pH 4.5. Furthermore, column was also washed with 0.1 M Tris succinate buffer, pH 4.5 containing 0.2 M NaCl.

3．Application

3．1．Elution in cation-exchange column chromatography

The following resins were used in cation-exchange columns: CM-Sephadex, CM-Sepharose, SP-Sephadex, and SP-Sepharose. Separation was inadequate on CM-Sepharose because the pH curve increased rapidly. Similar results were seen even if the volume of CM-Sepharose was increased. Figure 1 shows that ovalbumin, streptavidin and transferrin were eluted at pH 4.5, 5.0 and 6.2, respectively. These values indicate the respective isoelectric points. SP-Sepharose was the best resin with regard to yield and separation of the proteins. Desorption was achieved with a linear gradient where the ionic strength was gradually decreased while the pH slowly increased. Interestingly, streptavidin was eluted from the cation-exchange column at pH5.0: streptavidin has been reported to have an isoelectric point of 5.0-5.5 for the biotin-binding face, but 6.3 for the net protein[2]. Streptavidin might have an isoelectric point of 5.0 for its cation-exchange-binding face. Fetuin, an o-type glycoprotein, was absorbed onto CM-Sephadex equilibrated with 0.1 M Tris succinate buffer, pH 4.0, but was not eluted from the column by 0.1 M Tris. This method may not be suitable for use with an o-type glycoprotein.



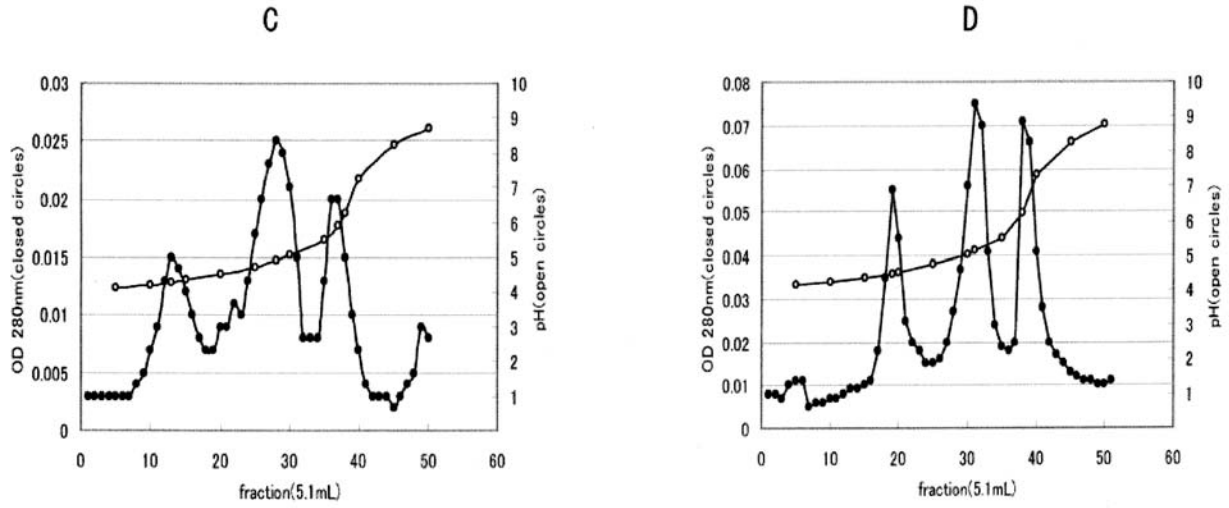


Figure 1. Elution in cation-exchange column chromatography
Resins A, B, C, and D are CM-Sephadex, CM-Sepharose, SP-Sephadex, and SP-Sepharose, respectively. Elution occurred in the order ovalbumin, streptavidin and transferrin.

3．2．Effect of the buffer concentration

Although streptavidin was not absorbed onto CM-Sephadex equilibrated with 0.2 M Tris succinate buffer, pH 4.0, transferrin and a portion of ovalbumin were eluted from column at their respective isoelectric points (Figure 2). This shows that CM-Sephadex does not absorb a common protein or an n-type glycoprotein with a weak bond under conditions of high ionic strength.

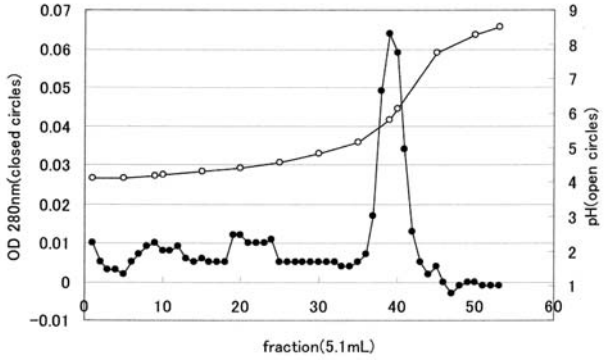


Figure 2. Elution profile of ovalbumin and transferrin from CM-Sephadex equilibrated with 0.2 M Tris succinate buffer, pH 4.0
Ovalbumin and transferrin show a small peak in the neighborhood of pH 4.5 and a large peak near pH 6.2, respectively.

3．3．Effect of the buffer composition

To investigate the effect of the buffer composition, we experimented with CM-Sephadex equilibrated with 0.1 M Tris acetate buffer, Tris citrate buffer or Tris phosphate buffer instead of Tris succinate buffer. The same methods for cation-exchange columns were used except the buffer. Ovalbumin, streptavidin, and transferrin were adsorbed onto the CM-Sephadex column, but since the pH curve for elution was not appropriate, all three proteins were eluted at once (Figure 3 A, B). Ovalbumin and streptavidin were eluted at their isoelectric points from a CM-Sephadex column equilibrated with 0.1 M Tris acetate buffer pH 4.2 (results not shown). If the pH curve is adequate, these buffers should allow separation based on the isoelectric point. On the other hand, although ovalbumin, streptavidin, and transferring were each absorbed onto a CM-Sephadex column equilibrated with 0.1 M Tris phosphate buffer, pH 4.0, all three proteins were eluted regardless of the isoelectric point, even though the pH curve was suitable (Figure 3C).

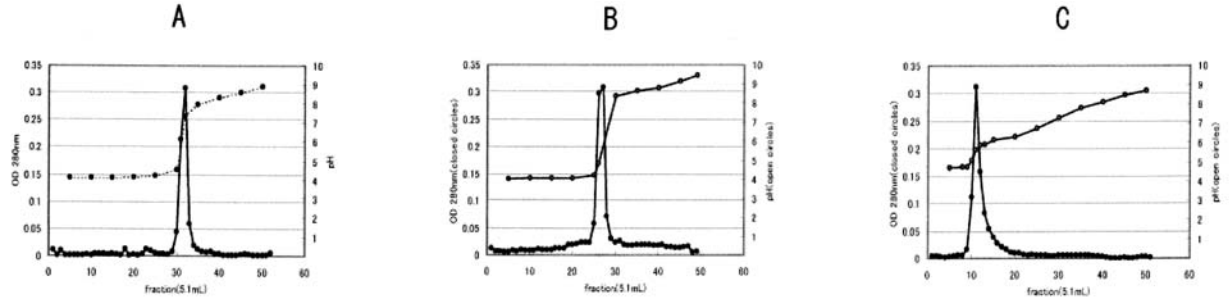


Figure 3. Effects of different buffers
A, B, and C show 0.1 M Tris acetate buffer, 0.1 M Tris citrate buffer, and 0.1 M Tris phosphate buffer, respectively.

3．4．An n-type glycoprotein and a common protein with a weak bond: an example of the separation of an n-type glycoprotein（peroxidase）and a common protein（carbonic anhydrase）

Under the condition of a weak bond between the protein and the column, we performed isoelectric point chromatography at a low buffer concentration. Although peroxidase and carbonic anhydrase were not absorbed onto a CM-Sephadex column equilibrated with 0.1 M Tris succinate buffer, pH 4.5, they were absorbed onto a CM-Sephadex column equilibrated with 20 mM Tris succinate buffer, pH 4.5. The same methods for cation-exchange columns were used except the buffer.

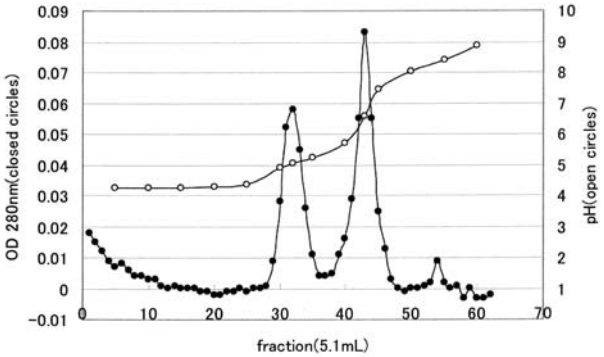


Figure 4. Elution profile of carbonic anhydrase and peroxidase from CM-Sephadex equilibrated with 20 mM Tris succinate buffer, pH 4.5 Elution occurred in the order carbonic anhydrase and peroxidase.

Figure 4 shows the separation of peroxidase and carbonic anhydrase based on their isoelectric points. Although carbonic anhydrase and transferrin were absorbed onto a CM-Sephadex column equilibrated with 20 mM Tris succinate buffer, pH 4.5, the peak of carbonic anhydrase overlapped that of transferrin and was eluted at the isoelectric point of transferrin. We have demonstrated that an n-type glycoprotein with an exceptionally weak bond and a common protein can be separated based their isoelectric points with a CM-Sephadex column under conditions of low ionic strength. However, we must be careful of coexisting substances, especially glycoproteins, in isoelectric point column chromatography under a low ionic strength.

3．5．Elution in anion-exchange column chromatography

Since isoelectric point column chromatography with a cation-exchange column was successful, we considered the use of an anion-exchange column. Ovalbumin and α1-acid glycoprotein were absorbed onto QAE-Sephadex equilibrated with 0.1 M Tris succinate buffer, pH 8.5. Ovalbumin was eluted from the column at pH 6.0. Furthermore, α1-acid glycoprotein was eluted from the column with 0.1 M Tris succinate buffer, pH4.5, containing 0.2 M NaCl. The trend in the elution pattern was similar to that in general ion-exchange column chromatography rather than isoelectric point column chromatography. The different binding sites for protein in the anion-exchange column might have caused the different results.

4．Concluding remarks

We have established a new method for the separation of an n-type glycoprotein and a common protein by isoelectric point column chromatography. We observed no tailing with a linear gradient where the ionic strength was gradually decreased. With the latest high-speed liquid chromatography system, it may be possible to separate proteins even better. Users should determine which resin to use by trial and error. Depending on the resin, a common protein may be absorbed quite strongly. Better results in the separation of a common protein and glycoprotein may be possible with the highest ionic strength, which can be determined by trial and error. Strong basic and acidic conditions are rarely found in vivo. The buffer solution in isoelectric point column chromatography must use a weak base-weak acid system. When the physical data of a glycoprotein equilibrated with a weak base-weak acid buffer system are studied further, we may be able to obtain useful data

To test the applicability of this method, we carried out two experiments: the one-step purification of ovalbumin from egg white and the one-step purification of Cu-containing amine oxidase from pea epicotyls. Both ovalbumin and Cu-containing amine oxidase are n-type glycoproteins[3,4]. We obtained each homogeneous protein in high yield by SDS polyacrylamide gel electrophoresis. The details will be published elsewhere.

This isoelectric point column chromatography can be used to purify glycoprotein and common protein. For example, it can be used for the separation, detection, and recovery of a useful glycoprotein from a living sample. Moreover, this method may be useful in the production of an allergy-free vaccine. This method can also be used to determine the isoelectric points of glycoproteins and common proteins. Finally, this method may be useful for separating a glycoprotein from glycoforms that differ only with regard to the oligosaccharide portion. There are also likely to be applications beyond those mentioned here.

References

［1］G.V. Samsonov, *Ion-Exchange Sorption and Preparative Chromatography of Biologically Active Molecules*. Plenum Press NY. 1986. pp. 1-

123.
[2] S. Sivasankar, S. Subramaniam, and D. Leckband, *Proc. Natl. Acad. Sci. USA* 95(1998). pp. 12961-12966
[3] V. Pacakova, S. Hubena, M. Ticha, M. Madera, and K. Stulik, *Electrophoresis* 22(2001). pp. 459-463
[4] A. J. Tipping , M. J. McPherson, *J. Biol. Chem.* 270(1995). pp. 16939-16946

（平成18年９月５日受理）