Hydrophobic chromatography of proteins using a nitrocellulose membrane

Kazuyuki Nakamura, Tatehiko Tanaka and Kazusuke Takeo

SUMMARY

A technique of chromatography using a nitrocellulose (NC) membrane was established for the separation of proteins. The proteins were developed on the NC membrane with an aqueous solution containing a non-ionic detergent, polyoxyethylene sorbitane (Tween 20). The rate of flow of the proteins correlated to the strength of hydrophobicity of the proteins which was theoretically calculated from the hydrophobicity of side chains of amino acid residues composed of the proteins, but neither to the molecular weight nor to the isoelectric point of the proteins. The detailed examinations on the effects of pH, temperature, and chemicals such as urea, neutral salts, methanol, and an ionic detergent (SDS) on the chromatography strongly suggested that hydrophobic interactions between the proteins and nitrocellulose play a major role in the protein binding to nitrocellulose membrane. So that, this technique may be useful for the separation of a very small amount of membrane bound proteins such as enzyme complexes, receptors, and ribosomes into their components.

Key words: chromatography, protein, hydrophobic interaction, nitrocellulose membrane, non-ionic detergent.

INTRODUCTION

Nitrocellulose (NC) membrane was originally designed as a surface filter which was prepared with nitrate esterified cellulose in a thin film of 150 μm thickness. Recently, a blotting technique using the NC membrane has been developed for identification and quantitation of proteins with a high sensitivity because of practically irreversible binding of proteins to the NC membrane through the hydrophobic interaction between them. However, it has been reported that significant differences in the affinity of binding proteins to the NC membrane in the presence of detergents.

On the other hand, Shaltiel et al. have reported a technique of hydrophobic chromatography using a series of different length of hydrocarbon chains immobilized to insoluble matrices for separation of histidine binding protein J, glycogen synthetase, and glycogen phosphorylase. The interactions between glycogen phosphorylase and the hydrocarbon chains was also analysed by using a technique of affinity electrophoresis.
This type of electrophoresis has been also applied to analyse the interactions between serum albumin and a long chain aliphatic ligand. We applied the principle of these techniques to establish a technique of chromatography using a nitrocellulose membrane for the separation of a tiny amount of proteins by development with a non-ionic detergent, polyoxyethylene sorbitane. We will discuss here the parameters affecting this chromatography and the nature of the binding of the proteins to the NC membrane matrices.

EXPERIMENTAL

Materials

Nitrocellulose membrane (pore size; 0.22 μm) was obtained from Millipore Corporation (Bedford, Mass.). Polyoxyethylene sorbitane (Tween 20), sodium dodecylsulfate (SDS), urea, serum albumin (bovine), trypsin inhibitor (soybean), β-lactoglobulin (bovine milk), carbonic anhydrase-B (human erythrocyte), L-lactate dehydrogenase (rabbit muscle), catalase (bovine liver), concanavalin A, ceruloplasmin (human), fibrinogen (human), and ferritin (horse spleen) were purchased from Sigma Chemical Company (St. Louis, Mo.). Phosphorylase a was purchased from Boehringer Mannheim GmbH (Darmstadt, West Germany). Ponceau 3R (CI 16155) was obtained from Nakalai Tesque (Kyoto, Japan). Ammonium sulfate and methanol was purchased from Wako Pure Chemicals (Osaka, Japan). All of other chemicals were of the highest grade commercially available.

Methods

Chromatography

(1) Sample application

Protein solutions were prepared with 10 mM phosphate buffer, pH 7.2 containing 150 mM NaCl (P buffer) in final protein concentration of 1 mg/ml. The protein concentration was determined by Lowry's method and by protein microassay on the NC membrane using protein dye-staining procedure. Three microliter of the protein solutions were aspirated into a small tip connected with Pipet Man (volume; 20 μl, Gilson Medical Electronics, Middleton, Wi.), and then the protein solutions were carefully spotted at the position of 2 cm height from the bottom of a rectangular NC membrane filter.

(2) Development

The NC membrane was then immersed its bottom of 0.5 cm into a developer consisted of P buffer or P buffer containing a series of different concentration of non-ionic detergent, Tween 20 in a cylindrical glass bottle which has been equilibrated at 25°C. The top of the NC membrane was fixed to the sealed cap of the bottle. Then, development was carried out until the front of the developer ascended about 10 cm from the origin of the protein spots. The development was terminated by removing the NC membrane from the bottle.

(3) Detection of the protein spots and determination of the rate of flow (Rf) of the protein spots

The NC membrane was incubated with 0.5% (w/w) Ponceau 3R in 7% (v/v) acetic acid for 5 min at room temperature. Then, the excess dye was removed by washing the membrane with continuous shaking in 7% (v/v) acetic acid until the background become colorless. The rate of flow, Rf, of the stained protein spots was determined by dividing the distance from the center of the spot to the origin with the distance from the front of the developer to the origin.

Examination of effects of chemicals on the chromatography

The effects of urea, an anionic detergent (SDS), and a non-ionic detergent (Tween 20) were examined by using the developer consisted of P buffer containing a series of different concentration of those chemicals. The effects of those chemicals were estimated from the magnitude of the increase in Rf of the protein spots.

The effects of ammonium sulfate and methanol were examined by using the developer consist-
ed of P buffer containing a series of different concentration of those chemicals with 1.6% of Tween 20. The effects of those chemicals were estimated from the magnitude of the change in \( R_f \) of the protein spots.

**Examination of effects of pH and temperature on the chromatography**

The effect of pH on the chromatography was examined by using the developer consisted of three different buffer systems with 0.8% Tween 20: 1) 50 mM acetate buffer, pH 4.0 containing 150 mM NaCl, 2) P buffer, and 3) 50 mM borate buffer, pH 10.0 containing 150 mM NaCl.

The effect of temperature was examined by using the developer consisted of P buffer containing 0.8% Tween 20 at 7°C, 25°C, and 37°C. The effects of pH and temperature were estimated from the magnitude of the changes in \( R_f \) of the protein spots.

**RESULTS AND DISCUSSION**

**Chromatography of proteins using a nitrocellulose (NC) membrane**

Twelve proteins; bovine serum albumin (BSA), carbonic anhydrase B (CH), catalase (CT), concanavalin A (ConA), ferritin (FT), fibrinogen (FB), \( \alpha \)-lactate dehydrogenase (LDH), beta-lactoglobulin (LG), phosphorylase a (PL), and trypsinogen (TP) were retained at the origin with a developer consisted of 10 mM phosphate buffer, pH 7.2 containing 150 mM NaCl (P buffer). Ceruloplasmin (CR) and trypsin inhibitor (TI) seemed to be developed slightly and their spots became blurry as shown in Fig. 1. Even though 1 M urea was added into the developer, no significant change was observed in the chromatogram (data not shown). On the contrary, Tween 20 (0.4%) strikingly changed the chromatogram in which the spots of the proteins except LDH, ConA, PL, and TP were developed toward the top of the NC membrane (Fig. 2). However, the chromatogram obtained by using Tween 20 showed the big differences in \( R_f \) and the shape of the spots of BSA and ConA from that obtained by using SDS. BSA was developed to form an arrayal spot without any retention at the origin with 0.4% Tween 20, and the spot was composed of two fractions having \( R_f \) values of 0.38 and 0.1. On the other hand, the protein was developed to form a short rocket spot continued from the origin with 0.4% SDS (lane 12 in Fig. 2). ConA was not developed at all with 0.4% Tween 20 while the protein was developed to form a tall rocket spot continued from the origin with 0.4% SDS (lane 4 in Fig. 2). All of the proteins tested here were developed to form the rocket shape spots continued from the origin with SDS. This may indicate that SDS form the stable complexes with the proteins\(^{16}\) which tightly bind to the NC mem-
brane. So that, SDS is not available for the separation of the proteins by chromatography using the NC membrane.

In the contrast, Tween 20 developed TI and LG to form clear round spots without any retention of the spots at the origin (Fig. 2). The proteins proportionally increased their Rf values to the concentration of Tween 20 (Fig. 3), and the concentration of Tween 20 (T0.5) which gave them the Rf value of 0.5 were 1.2% for TI and 1.5% for LG, respectively (Fig. 4). T0.5 of other proteins were determined as summarized in Table 1. These data indicate that Tween 20 interfere with the binding of the proteins to the NC membrane, the proteins are separated according to the strength of their affinity of binding to the NC membrane through hydrophobic interactions between them. Furthermore, Tween 20

---

Fig. 2. The chromatography of protein using a nitrocellulose membrane in the presence of detergents.

A. The chromatogram in the presence of 0.4% Tween 20. B. The chromatogram in the presence of 0.4% SDS. The number of the protein spots indicates the proteins: 1, ferritin; 2, ceruloplasmin; 3, l-lactate dehydrogenase; 4, concanavalin A; 5, catalase; 6, fibrinogen; 7, phosphorylase a; 8, carbonic anhydrase-B; 9, ß-lactoglobulin; 10, trypsin inhibitor; 11, trypsinogen; 12, bovine serum albumin. △ indicates the front of the developer, and ▼ indicates the origin of the protein spots.

Fig. 3. Effects of the concentration of Tween 20 on the chromatography of proteins.

The concentrations of Tween 20 were: A, 0%; B, 0.4%; C, 0.8%; D, 1.2%; E, 1.6%; and F, 2.0%. The number of the protein spots indicates the proteins: 1, ß-lactoglobulin; 2, trypsin inhibitor; 3, trypsinogen; and 4, bovine serum albumin. △ indicates the front of the developer, and ▼ indicates the origin of the protein spots.
has a low potency to denature the proteins. So that, Tween 20 is available for separation of the proteins by chromatography using the NC membrane. $T_{0.5}$ is also a good indicator for determining the strength of the binding affinity of the proteins (Table 1). The relationships between $T_{0.5}$ and protein molecular weight, isoelectric point, and hydrophobic index which was theoretically calculated from the free energy changes of side chains of amino acid residues composed of the proteins for transfer from ethanol to water at 25°C as reported by Levitt$^{14}$ or Tanford$^{15}$ as shown in parenthesis. The smaller values of the index calculated as described by Levitt indicate the stronger hydrophobicity of the proteins, and the larger values of the index calculated as described by Tanford indicate the stronger hydrophobicity of the proteins.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>M. W. (Kb)</th>
<th>pI</th>
<th>Hydrophobic* $T_{0.5}$b index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin</td>
<td>448</td>
<td>4.6</td>
<td>25.8 (99.5)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>341</td>
<td>5.5</td>
<td>— ( — )</td>
</tr>
<tr>
<td>Catalase</td>
<td>225</td>
<td>5.5</td>
<td>15.7 (114.1)</td>
</tr>
<tr>
<td>Phosphorylase a</td>
<td>198</td>
<td>5.8</td>
<td>24.8 (122.4)</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>160</td>
<td>4.3</td>
<td>3.7 (105.0)</td>
</tr>
<tr>
<td>LDH-5</td>
<td>140</td>
<td>8.9</td>
<td>3.9 (111.9)</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>100</td>
<td>5.5</td>
<td>3.7 (105.0)</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>67</td>
<td>4.9</td>
<td>24.5 (104.0)</td>
</tr>
<tr>
<td>Carbonic anhydrase-B</td>
<td>30</td>
<td>5.3</td>
<td>11.1 (98.1)</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>24</td>
<td>3.3</td>
<td>1.2 (90.4)</td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>23</td>
<td>4.3</td>
<td>96.8 (65.9)</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>18</td>
<td>4.7</td>
<td>15.9 (116.4)</td>
</tr>
</tbody>
</table>

* The hydrophobic indices were calculated by using the free energy changes in calories per mole of side chains of amino acid residues composed of those proteins from ethanol to water at 25°C as reported by Levitt$^{14}$ or Tanford$^{15}$ as shown in parenthesis. The smaller values of the index calculated as described by Levitt indicate the stronger hydrophobicity of the proteins, and the larger values of the index calculated as described by Tanford indicate the stronger hydrophobicity of the proteins.

b The values of $T_{0.5}$ were determined at 15°C as described in EXPERIMENTAL.

* The $R_f$ value for the slow fraction of bovine serum albumin.

** The $R_f$ value for the fast fraction of bovine serum albumin.

Effects of chemicals, pH, and temperature on the chromatography

As shown in Fig. 5, a high concentration of ammonium sulfate caused a small decrease in the $R_f$ of the proteins, and the $R_f$ values of LG in the presence of 0, 5, 10, and 20% ammonium sulfate became 0.54, 0.50, 0.41, 0.31, respectively. Higher concentrations of ammonium sulfate than 10% caused the retention of the protein spot of TI at the origin. This may be due to the decrease of the solubility of the protein.

The results obtained from the examination of the effect of methanol on the chromatogram were summarized in Table 2 in which only a small
A decrease in the Rf of LG, TI, and BSA was observed by increasing the concentration of methanol.

The effect of pH on the chromatography with CR, ConA, LG, and TI was examined by using the different buffer systems, at pH 4.0, 7.2, and 10.0 as shown Fig. 6. Little changes in the Rf of LG were observed by changing the pH from 4.0 to 10.0, and the Rf values were 0.52 at pH 4.0, 0.52 at pH 7.2, and 0.50 at pH 10.0. In the case of TI and CR, a small decrease in their Rf was observed by decreasing the pH from 10.0 to 4.0, and a part of protein spot of TI was retained at the origin at pH 4.0. ConA was not developed at any pH, and retained at the origin.

The effect of temperature on the chromatography was examined with the proteins, LG, TI, TP, and BSA at 7, 25 and 37°C as shown in Fig. 7. Those proteins remarkably decreased their Rf as the temperature was elevated, and the Rf value of LG was 0.06 at 37°C which was one sixth of that at 7°C. In the case of BSA, the changes of temperature severely affected Rf of the protein spot, and the Rf values of both fractions of the protein spot at 37°C became less than one tenth of that at 7°C. The entity of the two fractions of the arrayal spot of BSA is still unknown. But it may be due to the different molecular structure of BSA such as monomeric form and dimeric form or the protein conjugated with lipid.

These results obtained from the examinations of the effects of neutral salt, pH, and temperature on the chromatography of the proteins strongly suggested that hydrophobic interactions between...
the proteins and the NC membrane matrices play a major role in the protein binding to the NC membrane.

It is conclusive that: 1) Chromatography of proteins using a nitrocellulose (NC) membrane in which the development was carried out by using aqueous solution containing a non-ionic detergent, polyoxyethylene sorbitane (Tween 20) is available for convenient separation of a tiny amount of proteins according to their strength of hydrophobicity. 2) So that, this type of chromatography may be useful for the separation of protein components consisting enzyme complexes, receptors, and ribosomes which are bound to the cell membrane by the difference of their hydrophobicity. 3) Furthermore, this technique offers the entire informations to optimization of the condition for extraction of proteins from the NC membrane to analyse the amino acid compositions and sequences of the proteins in micro scale after blotting the proteins to the NC membrane.

REFERENCES

(312) 生物物理化学


要 旨

ニトロセルロース膜を支持体に用いたクロマトグラフィーによりタンパク質の分離を試みた。分子量や等電点の異なる12種類のタンパク質をニトロセルロース膜にスポットした後、非イオン性界面活性剤、ツイーン20を用いて展開すると、タンパク質は膜への結合親和性の強さの違いに従ってそれぞれの展開速度を与え、この展開速度はタンパク質を構成するアミノ酸残基の側鎖の疎水度から理論的に求めたそれぞれのタンパク質の疎水度に相関を示し、タンパク質の分子量や等電点には相関を示さなかった。また、このクロマトグラフィーのpHおよび温度依存性や尿素、中性塩、メタノールなどの影響を調べた結果は、タンパク質がニトロセルロース膜と疎水性の結合力の違いにより分離されることを強く示唆した。このニトロセルロース膜を用いたクロマトグラフィー法は、微量のタンパク質（たとえば、腫瘍結合タンパク質）をその疎水性の差によって簡便に分離できる方法として有用と思われる。