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LIMITATIONS OF HIGH-SPEED REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OBSERVED WITH INTEGRAL MEMBRANE PROTEINS

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SUMMARY

The use of high-speed reversed-phase high-performance liquid chromatography was evaluated for membrane proteins. Using the photosynthetic reaction centre of *Chromatium tepidum* as a model, parameters such as flow-rate, gradient steepness and contact time of the proteins with the stationary phase were investigated. The results demonstrate that for optimum recovery and resolution, the gradients applied should not be too steep and that very long gradient times may cause protein loss.

INTRODUCTION

In recent years, reversed-phase (RP) high-performance liquid chromatography (HPLC) has been established as an indispensible tool for separation of peptides and soluble proteins. However, today the RP-HPLC of very hydrophobic peptides and membrane proteins is still an extreme technical challenge. Because of solubility problems and the close hydrophobic interaction of these substances with reversed stationary phases, unacceptably low mass recoveries are often obtained. Membrane proteins are often insoluble in common solvents and require detergents with or without high salt concentrations to keep them in solution. Therefore, only a limited number of successful separations by RP-HPLC have been reported[1–18]. Mostly mobile phases containing strong acids of various concentrations were used with organic modifiers such as acetonitrile, ethanol, 2-propanol, butanol or mixtures of various organic compounds.

Recently, high-speed HPLC using high flow-rates, elevated temperatures and short columns was introduced[19]. The efficiency of the columns is based on specially designed micropellicular sorbents exhibiting favourable mass transfer characteristics and the fast diffusion rates and sorption kinetics at elevated temperatures. So far, these commercially available, specialized columns have proved well suited to the RP-HPLC of hydrophilic proteins and peptides but, in general, are too hydrophobic for application to integral membrane proteins. However, short columns packed with the stationary phases commonly used in protein chemistry can also be operated in a high-speed mode with ordinary modern HPLC equipment.

In this work, the influence of the contact time of the proteins with the stationary

phase, the flow-rate and gradient steepness on the recovery and separation of integral membrane proteins using high-speed RP-HPLC was investigated. A partially purified reaction centre of the thermophilic photosynthetic purple bacterium *Chromatium tepidum* was used as a model system of integral membrane proteins[20]. This reaction centre is composed of four polypeptides, termed L (25 kDa), M (30 kDa), H (34 kDa) and cytochrome *c* subunit (44 kDa). It is structurally similar to the reaction centre of *Rhodopseudomonas viridis,* in which the subunits H, L and M are integral membrane proteins with several membrane spanning helices and the cytochrome subunit is a membrane-associated protein due to covalently bound fatty acids[21-23].

EXPERIMENTAL

The reaction centre of *C. tepidum* was kindly provided by Dr. R. Nozawa (Chemical Research Institute of Non-aqueous Solutions, Tohoku University, Sendai, Japan). The sample contained about 2.7 mg/ml of reaction centre in 20 mM Tris (pH 8.5), containing at least 0.05% (w/v) of lauryl-N,N-dimethylamine N-oxide (LDAO) and sodium chloride.

Chromatography was performed using an HP 1090 HPLC system (Hewlett-Packard, Waldbronn, F.R.G.) equipped with an HP 1040 diode-array detector and an autosampler.

An Aquapore RP-300 reversed-phase column (30 × 2.1 mm I.D.) (Applied Biosystems, Weiterstadt, F.R.G.), was used for the separations. The injection volume was 5 μl, which corresponded to approximately 12 μg of protein.

Gradient elution was performed using as solvent A 0.1% trifluoroacetic acid (HPLC/Spectro grade, Sequenal quality; Pierce, Rockford, IL, U.S.A.) in water and as buffer B 0.1% trifluoroacetic acid in acetonitrile (LiChrosolv, gradient grade; Merck, Darmstadt, F.R.G.). In all experiments a gradient from 5 to 95% of solvent B in solvent A was applied. The gradient time and flow-rate were changed in the different experiments. The oven temperature was kept at 50°C. Routinely after each separation two blank runs were done. The chromatograms were monitored at 206 nm, and the fractions were collected manually in Eppendorf vials. Fractions were further analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Minigel system; Biometra, Göttingen, F.R.G.) using standard conditions as described by Laemmli[24].

RESULTS AND DISCUSSION

The aim of this investigation was the evaluation of the use of high-speed RP-HPLC for the separation of integral membrane proteins. This separation is usually difficult because these proteins have many hydrophobic amino acid residues which interact with the hydrophobic stationary phase ligands, resulting in a requirement for high concentrations of organic solvent to elute the proteins. Usually membrane proteins can only be kept in solution in the presence of detergents. During chromatography when the detergents are separated from the protein, the proteins may precipitate on the column. An additional problem is that the detergents themselves may interact with the reversed-phase material and modify its surface, consequently changing the selectivities and causing irreproducible results.

For this work, the photosynthetic reaction centre of *C. tepidum* was chosen as a model protein system. The four subunits differ in size and hydrophobicity, allowing one to evaluate changes in the resolution and recovery of different integral membrane proteins. The reaction centre is structurally similar to that of *R. viridis*, and conclusions relating to hydrophobicity can be drawn from our previous experience with the structural studies on this complex molecule. In addition to this current work, the samples from this study will be further used for the structural analysis of the reaction centre of *C. tepidum*. Therefore, a volatile solvent system was chosen that allowed the samples to be applied directly to amino acid composition analysis and amino acid sequence analysis (data not shown). The amount of available starting material was approximately 3 mg. Owing to the limited amount of starting material and the further structural analyses, UV detection of the fractions was performed at 206 nm with high sensitivity using the trifluoroacetic acid–acetonitrile solvent system. This solvent system additionally leaves the samples better suited for analysis by SDS-PAGE than systems containing formic acid, where removal of the acid is difficult.

In preliminary experiments, several column materials, previously used for successful separations of hydrophilic proteins, were examined for the recovery of the L and M subunits of the reaction centre of *R. viridis* as tested by UV absorption and SDS-PAGE. Aquapore RP-300 (30 × 2.1 mm I.D.) (Molnar, Berlin, F.R.G.), TSK-C_{18}-NPR (35 × 4.6 mm I.D.) (Hewlett-Packard), Vydac C_4 and Vydac C_8 (25 × 4 mm I.D.) (Chrompack, Müllheim, F.R.G.) were all tested. Even though the separation and recovery of the more hydrophilic subunits (H and cytochrome subunits) of the reaction centre were the same with all the columns (data not shown), the recovery of the more hydrophobic subunits L and M was best with the Aquapore RP-300 column. This column was, therefore, chosen for the remainder of the investigations.

Fig. 1. shows different chromatographic conditions that were used for the evaluation of the influence of flow-rate, gradient steepness and contact time of the proteins with the stationary phase. Increasing gradient steepness is indicated by increasing shading of the circle. The chromatograms described by the horizontal lines represent a constant gradient time with different flow-rates which represent the different gradient steepness. The chromatograms with identically shaded circles represent

gradient time (min)	Flow rate (ml/min)			
	3	1.5	0.75	0.375
0.8	●	●	●	●
1.6	●	●	●	●
3.2	○	●	●	●
6.4	○	○	●	●

Fig. 1. Conditions used for high-speed RP-HPLC of the reaction centre of *C. tepidum*. Increasing shading of the circles corresponds to increasing gradient steepness.

separations using the same gradient. These were obtained by maintaining a constant gradient volume with changes in the gradient times and flow-rates. The vertical columns indicate chromatograms where the flow-rate was kept constant but the gradient time was changed, leading to different gradient shapes and different contact time of the proteins on the column.

In Fig. 2 a set of original experimental chromatograms are shown. In this instance a constant gradient time of 1.6 min was used, which corresponds to the conditions and results indicated in the second horizontal line of Fig. 1. The HPLC fractions were further analysed by SDS-PAGE. As an example, the results for the SDS gel corresponding to the uppermost chromatogram are shown in the lower right corner of Fig. 2. In the first fraction small amounts of hydrophilic proteins unrelated to the reaction centre were seen. The H subunit (34 kDa) was mostly found in fraction 2, but in fraction 3 a small amount of both H and cytochrome subunit were detected. The cytochrome subunit (44 kDa) was mostly found in fraction 4. Fraction 5 contained a mixture of subunits L and M (25 and 31 kDa, respectively). Both hydrophobic subunits (L and M) could be seen only as faint bands. Both of these subunits are difficult to analyse by SDS-PAGE owing to aggregation or precipitation of the proteins on top of the gel. This behaviour is generally observed in electrophoresis of membrane proteins (*e.g.*, reaction centre starting material, Fig. 2, lane RC). In the chromatograms using the steepest gradient conditions in the SDS gels only the two less hydrophobic subunits could be detected. The resolution using different chromatographic conditions resulting in high recovery was not significantly altered as evaluated by SDS-PAGE. However, it is obvious that both the resolution and the mass recovery are drastically diminished with the steepest gradient at the lowest flow-rate.

A comprehensive graphical presentation of the data obtained is shown in Fig. 3. At short gradient times a decrease in the recoveries of the proteins was observed. This may be caused either by the influence of the flow-rate or by the low gradient volume which corresponds to a very steep gradient.

The lowest flow-rate tended to give slightly lower recoveries when the chromatograms exhibiting the same gradient steepness were compared (similar shaded circles seen in the diagonals in Fig. 1). The conditions for these chromatograms are, for example, a gradient time of 1.6 min at a flow-rate of 0.375 ml/min and a gradient time of 0.8 min at a flow-rate 0.75 ml/min. However, the effect of flow-rate was not pronounced and may actually be caused by the longer contact time of the protein with the stationary phase.

The change in the gradient steepness had a drastic effect on the recoveries. Very clearly, a gradient volume that was too small (*i.e.*, a gradient which was too steep) caused severe protein loss, as seen in Fig. 3. However, if the gradient volume exceeded 1.2 ml this loss became negligible. With very small gradient volumes the change in the organic modifier content in the mobile phase was probably too rapid to allow the protein to migrate through the whole column. For proteins the graph of log *k'* *versus* percentage of organic modifier follows a steep parabolic curve[25]. Increasing the organic solvent concentration will cause only a slow migration of the protein along the column, or it will stick to the top of the column. As the organic solvent concentration is increased there is a small concentration window in which the protein will migrate efficiently. If the organic solvent concentration is increased further, the proteins will again be strongly retarded.
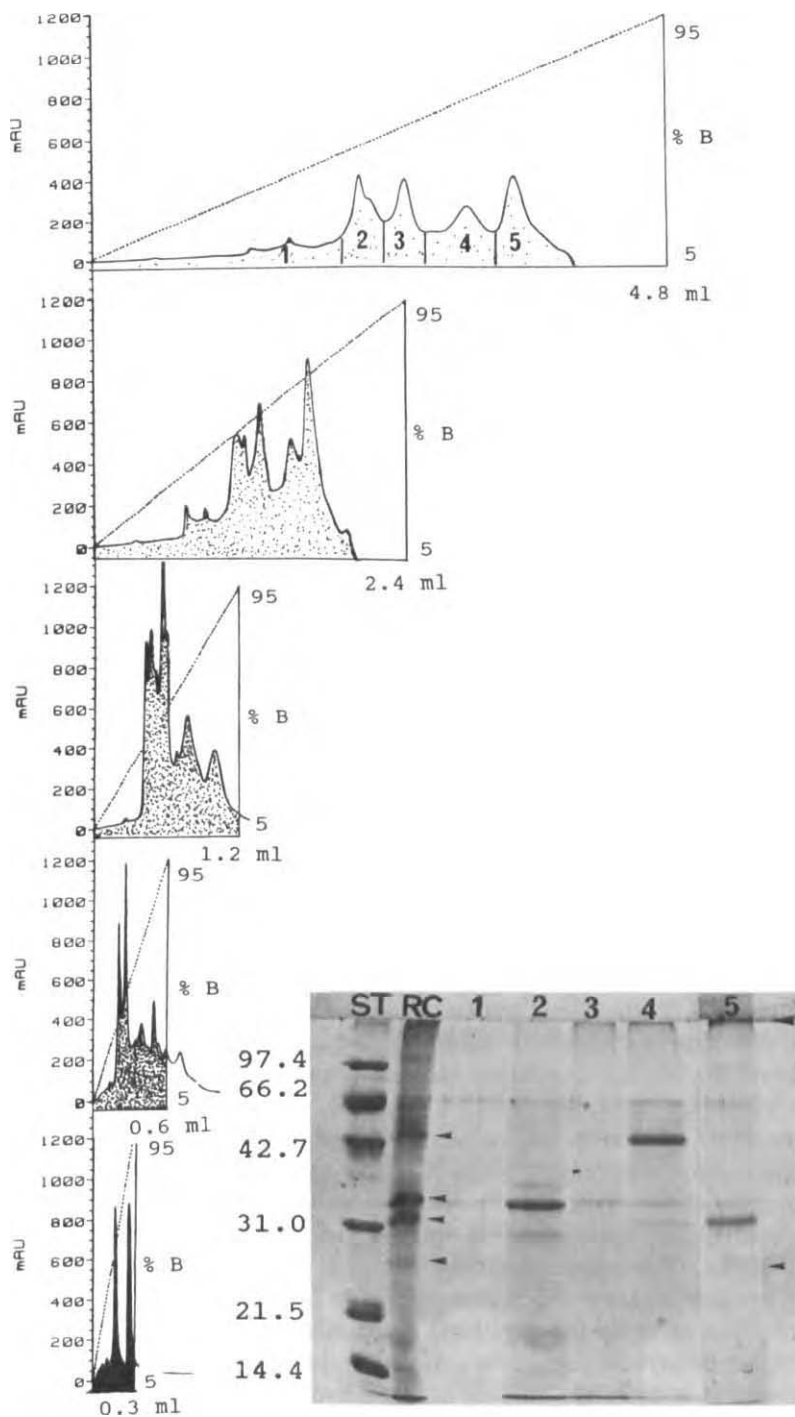
Fig. 2. Set of chromatograms using a constant gradient time of 1.6 min and flow-rates of 3, 1.5, 0.75, 0.375 and 0.187 ml/min (from top to bottom). For other conditions, see Experimental. In the right lower corner the Commassie Brilliant Blue-stained, SDS polyacrylamide gel of the fractions obtained from the top chromatogram is shown. RC, reaction centre; ST, reference proteins; molecular masses are given in kDa. The arrows indicate the main components of the RC, cytochrome c (44 kDa), H (34 kDa), M (30 kDa), L (25 kDa), and the protein precipitation on the top of the gel.
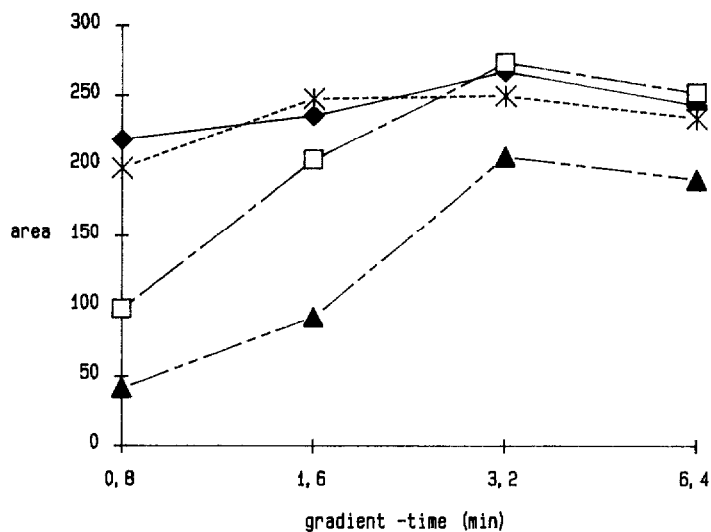
Fig. 3. Graphical presentation of the protein recovery obtained with various flow-rates (◆, 3; *, 1.5; □, 0.75; ▲, 0.375 ml/min) at different gradient times. Chromatographic conditions as given in Fig. 1 and Experimental.

There is a tendency for proteins with longer contact time with the stationary phase to elute with lower recoveries. This can been seen in Fig. 3, where there is a slight decrease in the recoveries at the longest gradient times. This observation was also previously made for other proteins[26]. However, all the chromatograms shown here were obtained in a high-speed mode and used a short gradient time to see a more marked effect of analysis time on recovery. Hence the influence of the contact time of membrane proteins with the stationary phase should be analysed in more detail using longer gradient times.

Exact quantification of the proteins recovered was difficult. The starting material was not pure, and some UV-absorbing byproducts interfered with quantification. The reaction centre complex is difficult to solubilize for application on to a SDS polyacrylamide gel, and the fractions collected after chromatography are more difficult to solubilize because they had to be dried for removal of the acid. Additionally, the hydrophobic subunits, L and M, precipitated, to a large extent, on top of the gel. Nevertheless, an approximate recovery of 50–80% of the total protein could be obtained under optimum conditions (see Fig. 3).

In conclusion, we feel that there is a clear optimum for resolution and recovery in the high-speed RP-HPLC of integral membrane proteins. The gradients should not be too steep and the gradient volume should exceed 1 ml. This is also advantageous for the resolution and circumvents the problems connected with the collection of small fractions. Very low flow-rates should be avoided and long gradient times may cause protein loss owing to irreversible binding of these hydrophobic proteins on to the column.

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