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Stimuli-responsive separation of proteins using immobilized liposome chromatography

Toshinori Shimanouchi, Seiichi Morita, Hiroshi Umakoshi, Ryoichi Kuboi*

Department of Chemical Science and Engineering, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama-cho, Toyonaka, Osaka 560-8531, Japan

Abstract

The possibility of the stimuli-responsive separation of proteins was investigated using immobilized liposome chromatography (ILC) as novel aqueous two-phase systems. The specific capacity factor (k_s) of β-galactosidase, obtained by analysis of ILC, was varied by changing the pH of the solution and was maximized at the specific pH of 5 ($k_{s,max} = 5.57$). The k_s values were found to correspond well with their local hydrophobicities, which can be determined by the aqueous two-phase partitioning method. The variation of k_s, therefore, indicates a change in the surface properties of a protein during conformational change under pH stimuli. A similar phenomenon is observed in the case of other proteins (α-glucosidase, $k_{s,max} = 11.3$ at pH 4; carbonic anhydrase from bovine, $k_{s,max} = 6.53$ at pH 4). The difference in the height and/or the position of the peaks of the k_s–pH curves of each protein suggests a difference in their pH denaturation in the ILC column. Based on these results, the mutual separation of the above proteins at pH 4 could be successfully performed by selecting their specific capacity factor as a design parameter.

Keywords: Stimuli-responsive separation; Immobilized liposome chromatography; Proteins

1. Introduction

Many useful biomaterials, such as proteins and polypeptides, have been produced by utilizing bacterial cells [1–3]. In order to separate the target protein from impurities, various kinds of bioseparation processes have been reported: (i) separation using gel permeation chromatography based on the size exclusion effect [4]; (ii) affinity separation using the specific interaction between targets and immobilized ligand [5–7]; and (iii) protein separation based on their surface properties using aqueous two-phase systems (ATPS) [8–11]. Generally, there are some restrictions, such as (i) the yield, (ii) the purity of the final product, and (iii) the multi-step operations in the above conventional bioseparation processes. It is considered that these restrictions are caused by the similarity of the surface properties (e.g., size, net charge, hydrophobicity, and ligand affinity) among the target and other proteins under the operational conditions, which are optimized in conventional bioprocesses. Although a common methodology for protein separation has gradually been established, the present methods still have operational and economic drawbacks.

The stimuli-mediated bioprocess, which can be defined as a bioprocess exploiting various stimuli (such as pH, temperature, salt, etc.), has recently been introduced in a series of reports [12–14], where the target protein could be separated with higher yield and selectivity by utilizing the stimuli-induced

*Corresponding author. Tel./fax: +81-6-6850-6285.
E-mail address: kuboi@cheng.es.osaka-u.ac.jp (R. Kuboi)

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transition of the conformation and surface properties of the target protein. It has also been reported that the surface properties of proteins can easily be controlled by exposing the proteins to a specific stimuli and their stimuli-responsive behaviors are identical to the type of protein [15]. Actually, the target protein has been selectively separated using ATPS [16] and immobilized liposome chromatography (ILC) [14], both of which are similar in relation to the use of a non-specific interaction. The mutual separation of proteins can be effectively and selectively achieved in comparison with the conventional bioprocesses by utilizing the stimuli-induced change of the surface properties of proteins.

Liposomes, which are self-assemblies of closed phospholipid bilayers, have been widely studied as a model of the biological membrane for many purposes [17–20]. Liposomes consist of inner and outer aqueous phases, which are clearly separated by a stimuli-responsive functional phospholipid membrane, and are considered to be a kind of ATPS. The liposome system is, therefore, very attractive for the use as novel ATPS, which have a stable but stimuli-responsive functional boundary. In our series of studies using liposomes as an aqueous two-phase partitioning method, we have reported that the interaction between protein and liposome membrane was enhanced with a specific stimuli [12,13]. Based on translocation phenomena, the possibility of the selective separation of target proteins from a protein mixture was investigated and the selective separation of specific proteins was accomplished using suitable heating conditions [12]. Recently, we introduced immobilization techniques for liposomes on the gel support of a chromatograph [21–24]. The possibility to utilize this immobilized liposome chromatography (ILC) for the bioprocess has also been investigated, regarding the immobilized liposome as an ATPS immobilized on a gel support [14]. It is expected that this novel bioseparation method can be developed by the combined use of both (i) the techniques of immobilized liposome chromatography and (ii) a method to control the protein–lipid membrane interaction induced by environmental stimuli.

The objective of the research reported in this article was to investigate the possibility of designing a stimuli-responsive separation process for proteins using ILC which has two aqueous phases at the exterior and interior of the phospholipid membrane. The elution profiles of some proteins (such as β-galactosidase, α-glucosidase, and carbonic anhydrase from bovine) in the ILC were first characterized by exposing the protein to a pH stimuli. The possibility of the mutual separation of proteins using ILC was then investigated based on the characterized elution profiles of the proteins.

2. Experimental

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and egg yolk phosphatidylethanolamine (EPE) were purchased from Avanti Polar Lipids Inc. (Birmingham, UK). The glass column (HR5/5) was purchased from Pharmacia Biotech (Uppsala, Sweden). TSK G6000PW gel was purchased from Tosoh (Tokyo, Japan). Tetrameric protein, β-galactosidase (β-gal), was purchased from Sigma (New York, USA). Monomeric proteins, α-glucosidase (α-glu) and carbonic anhydrase from bovine (CAB), were purchased from Sigma. Poly(ethylene glycol) (PEG 4000; 3000 average molecular mass) and dextran 100–200k (Dex, 100 000–200 000 average molecular mass), which were used as phase-forming polymers, were obtained from Wako Pure Chemicals (Osaka, Japan). Nitrophenyl chloroformate was obtained from Aldrich (Milwaukee, WI, USA). All other reagents were of analytical grade.

2.2. Immobilized liposome chromatography (ILC)

The immobilization of liposomes on the gel beads (TSK G6000PW) was performed as previously reported [14,25]. Briefly, small unilamellar vesicles of POPC containing 1 mol% EPE of about 30 nm diameter, prepared by sonication [14], were stirred with TSK G6000PW (denoted TSK) gel activated by nitrophenyl chloroformate as described by Wilchek and Miron [26]. The mixture was washed with buffer T (0.1 *M* Tris–HCl buffer, pH 7.5) on a glass filter to remove non-immobilized liposomes. The immobilized liposome gel was packed into a gel bed (5 mm diameter×50–55 mm) in a glass column (HR5/5). The non-reacted ligands were further blocked by

circulating buffer T containing 20 m*M* ethanolamine on the gel bed overnight at 23°C. The immobilized liposome column was connected to a HPLC system (AKTA, Pharmacia Biotech) with a pump, a detector set at 280 nm, and a fraction collector interfaced to a personal computer. This column was sustained in the water bath so that the inter-column temperature was constant in the higher temperature range.

Retardation of the proteins on the immobilized liposome gel bed was expressed as the apparent specific capacity factor, k_s, which is defined as

$$k_s = (V_S - V_N)/M \quad (1)$$

This is derived from the equation described by Beigi et al. [27]. V_S (ml) is the retention volume of proteins under the stimuli (pH and salt concentration) and V_N (ml) is the elution volume of native proteins in the absence of salts. *M* (mmol) is the apparent amount of immobilized liposomes and can be estimated as $M = V_N \times C_T$, where C_T is the concentration of immobilized liposomes in the gel bed (mmol/ml). The amounts of immobilized liposomal phospholipids were determined using methods described previously [28,29] and were 26 μmol lipid/ml gel [14].

2.3. Evaluation of the local hydrophobicity of proteins

The local hydrophobicity (*LH*) of proteins was analyzed using the aqueous two-phase partitioning method [30]. When the pH is selected at the p*I* in the low ionic strength condition, the partition coefficients of proteins mainly depend on the hydrophobic effect. When Triton X-405 is added to PEG/Dex two-phase systems, Triton X-405 preferably partitions to the top (PEG) phase. The protein which has hydrophobic binding sites with Triton X-405 is likely to partition to the top phase [31]. The difference between the partition coefficients of the proteins in PEG4000 (9 wt%)/Dex 100–200k (9 wt%) two-phase systems with and without 1 m*M* Triton X-405 gives the local hydrophobicity of the protein (*LH*) [30]. The *LH* value of the protein was defined as

$$LH = \ln K_{\text{with ligand}} - \ln K_{\text{without ligand}} \quad (2)$$

where $K_{\text{with ligand}}$ and $K_{\text{without ligand}}$ are the partition coefficients of the protein in aqueous two-phase systems with and without Triton X-405, respectively. The local hydrophobicity of enzymes was determined at various temperatures and at various pH.

3. Results and discussion

3.1. Variation of elution profiles of β-galactosidase on immobilized liposome chromatography (ILC) under stimuli

The effect of pH on the elution profile of β-galactosidase (β-gal) in ILC was first investigated in order to characterize the interaction between β-gal and liposomes under pH stimuli. Fig. 1 shows the elution profiles of β-gal in ILC under various pH conditions. At neutral pH (7.5), the peak of the curve was detected at a retention time of 2.3 min. When the pH of the solution was changed to 5.0, the peak of the elution profile was retarded and was detected at a retention time of 3.5 min. On the other hand, retardation of the peak was not detected at extremely low pH values (pH 2–4). The elution behavior of

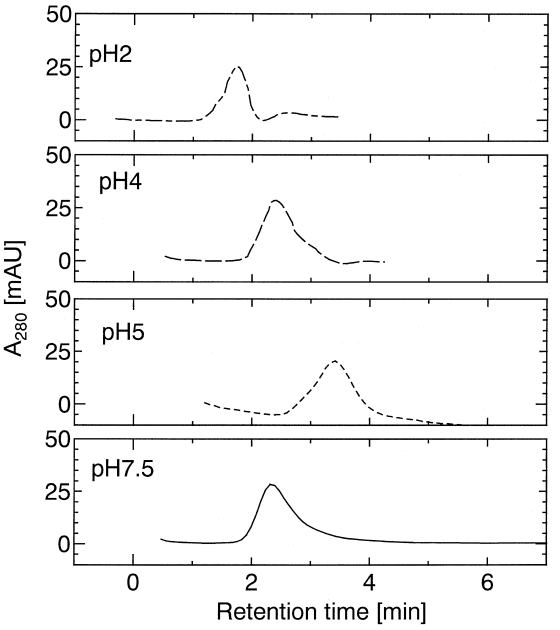

Fig. 1. Elution profiles of β-galactosidase under various pH conditions. The concentration of β-galactosidase was 0.5 μ*M* and 10 μl of this solution was injected into the ILC column with 0.25 ml/min flow-rate.

β-gal in the ILC column is thought to be affected by the surface properties of the gel and/or the liposomes immobilized on the gel. The peak derived from native β-gal (pH 7.5) should be retarded with respect to that at other pH because of the size exclusion effect of the gel [32], considering both the exclusion size of the TSK gel (50 000 molecular mass) and the size of β-gal (464 000 molecular mass). The above results show that the retardation of the peak was not induced by the size exclusion effect, but by other kinds of effects. In our previous study [14], it has been reported that the elution peak of carbonic anhydrase from bovine (CAB) in a partly unfolded state was retarded in an ILC experiment because of the hydrophobic interaction between the protein and the lipid membrane. Similarly, retardation of the peak is thought to be caused by the increase of the hydrophobic interaction between the immobilized liposome and β-gal, followed by conformational change at pH 5.

The specific capacity factor (k_s), obtained from the elution profiles of β-gal, is plotted against pH in Fig. 2a. The value of k_s was maximized at pH 5. The local hydrophobicity (*LH*) of β-gal, which was evaluated using the aqueous two-phase partitioning method [30], is also plotted versus pH in Fig. 2b. A similar curve, showing the maximal value at pH 5, could be obtained depending on the type of protein. It has been reported that the k_s values of GuHCl-denatured CAB in ILC correspond well with the *LH* value, where the partly denatured state indicating higher *LH* values has been shown to be most interactive with the surface of the immobilized liposomes [14]. The *LH* values of β-gal have already been shown to increase when the conformation changes to the partly damaged state under specific heat stimuli [12,13]. It was concluded that retardation of the elution peak of β-gal at pH 4–5 (Fig. 1) was due to the increase of the hydrophobic interaction between the liposomal membrane and β-gal, followed by a conformational change under pH stimuli.

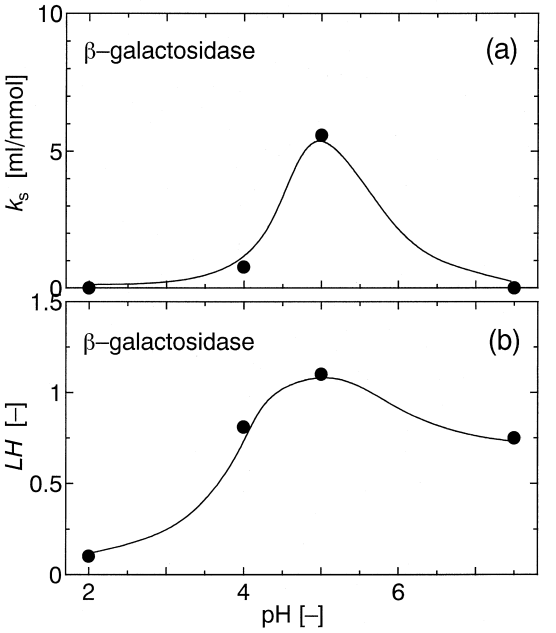

Fig. 2. pH dependence of the specific capacity factor of β-galactosidase and its local hydrophobicity. The specific capacity factor is obtained from retardation of its elution volume by normalizing to the amount of immobilized liposome on the gel (see Eq. (1)). Local hydrophobicity was estimated using the ATPS method (details in Section 2.3). PEG 4k (9 wt%)/Dex 100–200k (9 wt%) was used as the ATPS. The final concentration of Triton X-405 used was 1 m*M*.

3.2. Elution profiles of other proteins in ILC at various pH

The elution profiles of other proteins were also characterized using ILC in order to investigate the possibility to apply the retardation of elution peaks for the design of bioseparation processes. The pH dependence of the k_s values of some proteins is shown in Fig. 3. The k_s values of both α-glu and CAB were maximized at pH 4 and were different from that of β-gal. Generally, the pH denaturation process of proteins depends on the pK_a of charged amino acid residues [32] and, therefore, the difference in the pH values to give peaks in the k_s–pH curves is thought to be caused by the variation of the composition of the charged amino acid sequence. In addition, the maximal $k_{s,\,max}$ value of α-glu was higher than that of CAB at pH 4, indicating that α-glu strongly interacts with liposomes. This difference in the maximal k_s value between CAB and α-glu may be due to the difference in the intensity of the local hydrophobicity of hydrophobic amino acid residues, which are exposed to the protein surface after pH denaturation.

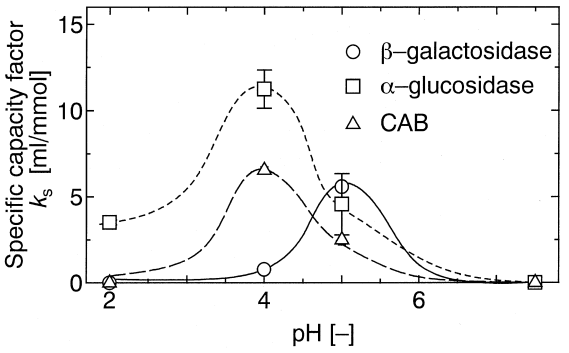

Fig. 3. pH dependence of the specific capacity factors of various proteins. Three proteins α-glucosidase (□), β-galactosidase (○), and CAB (△) were used in these experiments. The concentration of β-galactosidase was 0.5 μ*M*. Those of CAB and α-glucosidase were 5.0 μ*M*. Ten microliters of these solutions were injected into the ILC column with a 0.25 ml/min flow-rate.

Based on the above results, it is expected that (i) the pH value for the maximization of the k_s value and (ii) the maximal k_s value in the k_s–pH curves of various proteins may be utilized as quantitative parameters for the design of a protein separation process utilizing ILC, together with the quantitative characteristics of the pH denaturation of the proteins.

3.3. Stimuli-mediated separation of proteins using ILC

The possibility of the pH stimuli-responsive separation of three proteins (β-gal, CAB, and α-glu) was investigated on the basis of their specific capacity factors (k_s). After a solution of the protein mixture was prepared at a specific pH, the protein solution was injected into the ILC column, equilibrated by the eluent at the same pH. Mutual separation can be carried out based on the difference in retardation of the proteins, caused by variation of the hydrophobic interaction of the protein and the immobilized liposomes under specific stimuli.

A protein mixture containing 5 μ*M* α-glu, 5 μ*M* CAB, and 0.5 μ*M* β-gal was first prepared at neutral and optimal pH. In this case, pH 4 was selected as the optimal pH for mutual separation because the difference in k_s, representing retardation of the elution behavior of the proteins, was the largest among the pH values tested. Ten microliters of protein solution was injected into the ILC column and, also, conventional gel permeation chromatography (GPC). Fig. 4 shows the elution profiles of ILC and GPC at pH 4.0 and 7.5. In the elution profile of GPC, two peaks can be observed at both pH 4.0 and 7.5 (Fig. 4a,b). It is considered that the first and second peaks are derived, respectively, from β-gal with the largest molecular weight (464 000 molecular mass) and from a mixture of α-glu (68 000 molecular mass) and CAB (28 800 molecular mass), judged from the exclusion size of the TSK gel (50 000 molecular mass) tested here. Similarly, two peaks were also observed in the case of ILC at pH 7.5 (Fig. 4c), although the resolution of the peaks

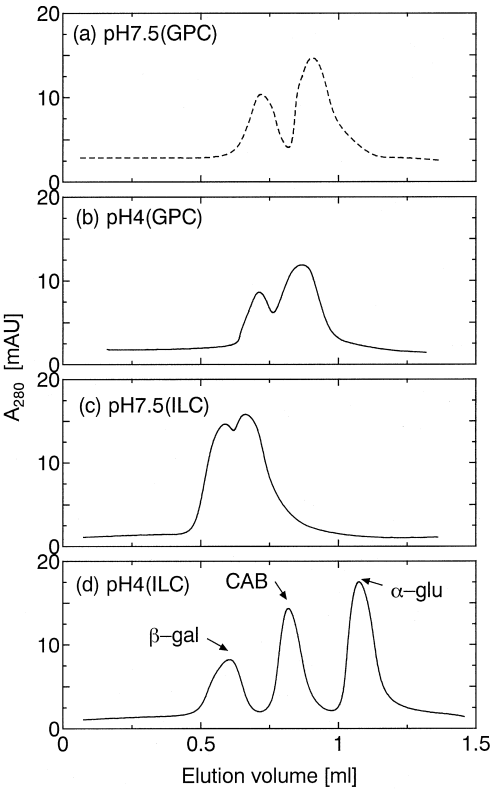

Fig. 4. Chromatogram of a protein mixture of β-gal, CAB, and α-glu in ILC at various pH. TSK6000PW was used in gel permeation chromatography (GPC) as a control. The immobilized, liposome gel and gel alone packed into a 5 mm×50–55 mm gel bed in a glass column (HR5/5) was connected to a HPLC system. A 10 μl volume of protein solution containing three enzymes ([β-gal]=0.5 μ*M*, [α-glu]=[CAB]=5.0 μ*M*) was applied to the column with a flow-rate of 0.25 ml/min. 50 m*M* of Tris–HCl (pH 4 and 7.5) was used as eluent.

was relatively low in comparison with that in the case of GPC (pH 7.5 and 4). On the other hand, three distinct peaks were obtained when the protein mixture was applied to the ILC column at pH 4.0, showing that the three proteins were clearly separated (Fig. 4d). Based on an analysis of the samples fractionated at each peak, the first to third peaks can be attributed to three proteins β-gal, CAB, and α-glu. The effectiveness of the stimuli-responsive bioprocess using immobilized liposome chromatography is clearly presented.

In our previous report on the selective separation of β-gal from a protein mixture based on the translocation phenomena across the liposomal membrane, it was reported that the recovery yield of β-gal was only 10% at the optimal specific temperature of 45°C, resulting in incomplete separation [12]. Since each protein has a particular stimuli-responsive behavior, as shown in Fig. 3, the mutual separation of a protein solution with high yield (95%) and high selectivity can be achieved with a more simple operation by selecting the adequate stimuli to maximize the difference in the k_s value. It is also expected that the resolution of the separation can be further improved by dynamic control of the applied stimuli (such as the application of a stimuli gradient on the ILC column) based on the dynamic change of the protein–liposome interaction. Recently, it was reported that on-line analysis of the protein–liposome interaction can be achieved using dielectric spectroscopy techniques, which represents the interfacial states on the surface of liposomes and proteins [33,34], where the dielectric parameters were found to be correlated with both the local hydrophobicity and the protein–liposome interaction [34]. With a combination of permittivity measurement and the ILC method it is possible to achieve not only on-line detection of the protein–liposome interaction, but also on-line control of the protein separation process.

4. Conclusion

The possibility of the stimuli-responsive separation of proteins was investigated using immobilized liposome chromatography (ILC) as an immobilized ATPS. It was found that the pH denaturation of proteins could be evaluated using the specific capacity factor (k_s), obtained from the ILC, as an indicator. The k_s values of proteins in ILC at various pH corresponded well with the *LH* values at each pH. Since the k_s value represents the retardation of the protein elution behavior through the ILC column, these values can be used as the design parameters for protein separation processes. The present separation method based on both (1) the conformational change of the protein and (2) the control of the protein–lipid membrane interaction under stimuli can be utilized for the separation of other proteins.

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