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Pressure-induced retention of the lysozyme on reversed-phase liquid chromatography

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Abstract

This study investigated the effect of pressure on the retention behavior of a model protein, lysozyme, on octadecylsilica (C_{18}) stationary phase under various equilibrium conditions. It is demonstrated that the retention time of the lysozyme was increased by as much as two to three times as the absolute pressure on the viewing window was increased from 23 to 318 bar. This pressure-induced retention was likely to be reversible and the corresponding volume change ($\Delta V = V_{sta} - V_{mob}$) was found to be on the order of minus tens to hundreds of mL/mol. Moreover, the pressure-induced retention was also observed for a homologous series of hydrophobic poly-L-phenylalanine, which do not have the secondary structure, and the volume change was determined to be around minus 10 mL/mol per phenylalanine. Perturbations in solute ionization and conformational change are predicted to have a minor impact under the investigated conditions. It is believed that the pressure-induced shift of the equilibria regarding hydrophobic ad–desorption is the major cause of the observed increase of protein retention. About ten phenylalanine-equivalent residues on the lysozyme surface were involved in the hydrophobic association with the chromatographic ligands.

Keywords: Retention behaviour; Pressure-induced retention; Lysozyme; Proteins

1. Introduction

Although liquid solution is normally considered to be incompressible, recent studies [1–7] indicate that, even in the LC pressure regime (<350 bar), solute retention in different modes of chromatographic methods can be significantly altered. These influences are achieved through shifts in many interaction equilibria that govern the solute retention such as ionization, complexation, and hydrophobic interactions [1–7]. The primary mechanism determining the solute retention in reversed-phase liquid chromatography depends mainly on the hydrophobic interactions. For small molecules, a partitioning mechanism, in which the solute is continuously distributed between two bulk phases, is normally assumed. Because of the negative volume change ($\Delta V = V_{sta} - V_{mob}$), the pressure increase favors the smaller volume and leads to an increase of the solute retention. The ΔV value for the retention equilibrium of the methylene homologues, typical model solutes to mimic the partitioning mechanism on reversed-phase liquid chromatography, ranges from -0.76 to -17 mL/mol per ethylene group [5]. For proteins, the retention process can be considered as adsorption of the solute at the hydrophobic stationary surface. The retardation is based on a hydrophobic association

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between the solute and the hydrophobic ligands of the surface and is described by the solvophobic theory, advocated by Horváth and co-workers [8]. By increasing the organic solvent composition of the mobile phase in a gradient elution, the attraction force is weakened and the solute is eluted. At a constant temperature, the pH value of the mobile phase controls the ionization of carboxylic acid and amine groups on the protein surface; the pairing agents such as trifluoroacetic acid (TFA) form ion pairs with the proteins to increase the solute retention on the hydrophobic stationary phase [9,10]. Therefore, protein retention is very sensitive with the variation of both parameters [9,10]. On the other hand, because of the folding structure of proteins, conformational changes of proteins may be induced during the chromatographic separation and lead to a different retention mechanism [11–17]. For example, a non-polar environment, such as a hydrophobic stationary phase, may induce helical structures in potentially helical molecules [18]. If a molecule becomes helical on binding and contains a preferred binding domain, as in the case of an amphipathic α-helix, then obviously some residues may not be interacting with the reversed-phase sorbent [18]. Moreover, in a gradient elution, an organic solvent-induced conformational change may promote the elution of the adsorbed proteins from the hydrophobic stationary phase [17,19]. Moreover, pressure-induced denaturation of proteins has been observed and the reaction volume has been reported to be on the order of minus tens to hundreds of mL/mol [20]. However, the perturbed changes reported in the literature occur only at high pressures (>1 kbar) [21–28] rather than the medium pressure range for chromatographic separation. Among the pressure studies of chromatographic separation, insulin is the only protein that has been investigated and found to show a pressure-induced change under chromatographic conditions but detailed characterizations are lacking [7].

In this study, we applied the reversed-phase liquid chromatographic approach for the systematic studies of the pressure effect on retention behaviors of a model protein, lysozyme. A homologous series of hydrophobic poly-amino acids containing $2-5$ mers of L-phenylalanine was also judiciously chosen for the investigation to provide useful information for comparisons. These solutes do not have the secondary structures and their retention is mainly due to the hydrophobic interaction that is a function of the solute chain length.

2. Experimental

2.1. Chemicals and reagents

Lysozyme (chicken egg white), tyrosine, and di-, tri-, tetra-, penta-L-phenylalanine were of the highest available grade purity purchased from Sigma (St. Louis, MO, USA) and were used as received. HPLC-grade acetonitrile (ACN) and methanol (MeOH) were obtained from Labscan (Labscan, Ireland), phosphoric acid was from Fisher (Fisher Scientific, Japan), and TFA was from Lancaster (Lancaster, UK). Water was deionized to 18 MΩ with a Barnstead NANOultrapure water system.

2.2. Chromatography and detection systems

Although similar studies may be conducted using a conventional column [7], the transparent characteristics of the capillary column allows the detection window to be made directly on the packed bed and should be more accurate for observation and for pressure calculations. Moreover, the column temperature can be better controlled due to the fast heat dissipation of capillary tubing. The temperature was kept at 25 ± 1°C throughout the experiment by controlling the room temperature and a thermometer was placed near the capillary column for temperature monitoring. The instrumentation is similar to those described in the literature [1–6]. A 57-cm fused-silica tubing (200 μm I.D.$\times$350 μm O.D.; Polymicro Technologies) was packed under 350 bar with a slurry of stationary phase in methanol. The stationary phase is polymeric bonded octadecysilica (Vydac 218, $d_p=5$ μm) with 300 Å pore size, $8-9$% carbon loading, and endcapped. A high-pressure syringe pump (Model LC-10AD, Shimadzu, Japan) was used to deliver the mobile phase at a constant flow-rate mode and the effluent was subsequently split (1:100) between the microcolumn and a splitting capillary (Fig. 1), resulting in a nominal flow-rate of 1.0 μl/min throughout the experiment. Sample injection

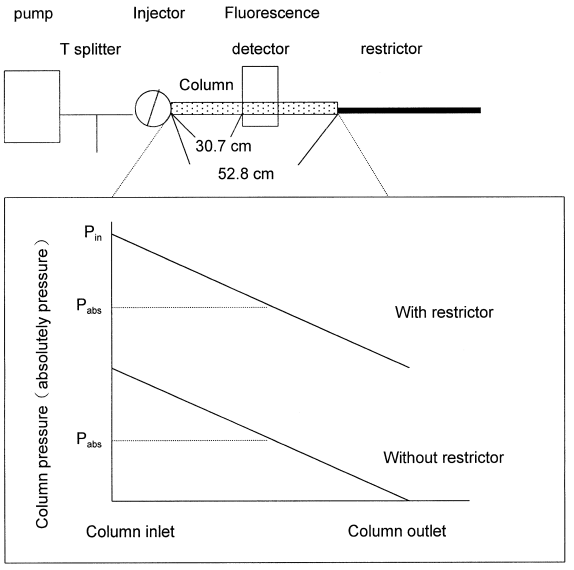

Fig. 1. Schematic diagram of the experimental setup and the column pressure as a function of the column length.

was accomplished by means of a 60-nL injection valve (Valco, Houston, TX, USA). The lysozyme (2500 ppm) was dissolved in 0.1% of TFA or phosphoric acid depending on whichever was used in the mobile phase. The mixture of di-, tri-, tetra-, and penta-L-phenylalanine was prepared by dissolving the poly-amino acids in 0.1% TFA containing 50% aqueous ACN with a final concentration of 250 ppm for each mer. The mobile phase was composed of 0.1% TFA or phosphoric acid containing specified composition of organic solvent (ACN or MeOH) with a final pH value around 2.3. The experiments were designed to maintain a constant column flow-rate while varying the absolute pressure on the column [1–6]. As depicted in Fig. 1, a restricting capillary was attached to the column outlet. The fluorescence detector (Model 920, Jasco, Japan) with a cell holder for capillary tubing was positioned on the packed bed at a distance of 34.5 cm from the column head. A cut-off filter (305 nm) was placed in front of the entrance slit of photomultiplier tube to eliminate the scattering light. For the separation of polyphenylalanines, the fluorescence detector was replaced by UV detector (Model UV3000, Thermo Separation Products, San Jose, CA, USA) at the wavelength of 214 nm. During the course of experiments, the lengths of the restricting and splitting capillaries were simultaneously decreased, so that the mobile phase velocity and split ratio remained constant while the inlet pressure was varied from 350 bar to 53 bar. Upon the change of the column pressure, no injection was performed until the system was stabilized. Assuming a linear pressure drop along the column (0.93 bar/cm), the absolute pressure on the detection window could be calculated (Fig. 1): $P_{abs} = P_{in} - 0.93$ bar/cm $\times 34.5$ cm. For the investigation of retention equilibrium, two identical fluorescence detectors were placed along the column to monitor the solute retention time.

2.3. Calculations of capacity factor (k')

The capacity factor (k') was calculated based on the method of statistical moments because it requires no assumptions concerning the mathematical form of the zone profile [29]. In this study, tyrosine served as the void marker and was proven to have no retention as long as the mobile phase contained more than 10% of ACN or 30% of MeOH at all pressures. Throughout the experiments, tyrosine was constantly injected in order to obtain the void time (t_0) values at each condition as well as to confirm the stability of the flow-rate. The relative standard deviations of the k' values were within 3% based on the triplet injections under each condition.

2.4. Calculations of volume change

Pressure effects are governed by Le Chatelier's principle, which states that, at equilibrium, a system tends to minimize the effect of any external factor by which it is perturbed. Consequently, an increase in pressure favors reduction of the volume of a system. When the solute concentrations in the mobile phase (P_m) and in the stationary phase (P_s) reach an equilibrium,

$$P_m \rightleftharpoons P_s \quad (1)$$

the following general expressions hold:

$$K = [P_s]/[P_m] \quad (2)$$

$$\Delta G = -RT \ln K = \Delta E + p\,\Delta V - T\,\Delta S \quad (3)$$

where ΔG, ΔE, ΔV, and ΔS are the changes in free energy, internal energy, volume, and entropy; K is the equilibrium constant of the process, T, p and R are temperature, pressure, and the gas constant respectively. The effect of pressure on the equilibrium may then be predicted from the pressure dependence of the change in free energy:

$$(\partial \Delta G/\partial p)_T = -RT\,(\partial \ln K/\partial p)_T = \Delta V = V_s - V_m \quad (4)$$

where ΔV is the volume difference between partial molar volume of the solute (protein) in the stationary phase V_s and the mobile phase V_m. The measured k' value can be further related to K:

$$K' = [P_s]V_s/[P_m]V_m = K\beta \quad (5)$$

where β is the volumetric ratio of the stationary and mobile phase (V_s/V_m). By combining Eqs. (4) and (5), The pressure dependence of k' is related to ΔV:

$$-RT\,(\partial \ln k'/\partial p)_T + RT\,(\partial \ln \beta/\partial p)_T = \Delta V = V_s - V_m \quad (6)$$

Assuming that β is constant with the pressure, i.e. $(\partial \ln \beta/\partial p)_T = 0$ [4–6], the ΔV can be determined from the first derivative of the ln k' versus pressure plot. In this study, both the linear and quadratic equations were attempted for the fittings using a commercial software Statistica. The ΔV values were calculated from the slope of the linear regression or from the first derivative of the quadratic curves at specified pressures as shown in the following:

For linear regression, $\ln k' = ap + b$

$$\Delta V = -RT(\partial \ln k'/\partial p)_T = -aRT \quad (7)$$

For quadratic regression, $\ln k' = ap^2 + bp + c$

$$\Delta V = -RT\,(\partial \ln k'/\partial p)_T = -(2ap + b)RT \quad (8)$$

3. Results and discussion

3.1. Chromatographic conditions

In this study, the local pressure around the detection point was carefully controlled and isolated from all other parameters by maintaining a constant flow-rate. Other chromatographic parameters were also carefully considered to exclude the possible complications of the observed results. Since the dissociation of a neutral molecule into two ions induces a contraction of about −10 to −20 mL/mol [20], the pressure-induced ionization is possible and may lead to the variation of pH value or deformation of ion pairs. As mentioned above, these variations could result in changes of solute retention. For pressure-induced ionization, Eq. (7) needs to be modified [1–3]:

$$\Delta V = -RT(\partial \ln K_a/\partial p)_T + \Delta n\,RT\,\kappa_s \quad (9)$$

in which $\Delta n\,RT\,\kappa_s$ term accounts for the increase in molar concentration with pressure based on the change in the number of moles of the product from the reactant (Δn) and the solvent compressibility (κ_s). The ionization constant at elevated pressure K_a^p can be determined as a function of the ionization constant at 1 atm (=101 325 Pa), K_a^0:

$$K_a^p = K_a^0 \exp(\partial \ln K_a/\partial p)_T\,(P-1) = K_a^0 \exp[-(\Delta V - \Delta n\,RT\,\kappa_s)(P-1)/RT] \quad (10)$$

Assuming ΔV value to be −15 mL/mol [20] and the κ_s value to be 10^{-5} bar^{-1} [3], the K_a^p value at P=311 bar is 1.2 K_a^0. The hydrogen ion concentration under 311 bar can then be calculated as a function of K_a^0. For 0.1% (v/v) of TFA and phosphoric acid, the pressure-induced ionization, $([H]_{311} - [H]_0)/[H]_0$, was estimated to be 0.4% and 5%, respectively. The corresponding decrease of pH value is less than 0.002 and 0.03 for TFA and phosphoric acid, respectively. Since the protonated bases have a near zero or positive ΔV value [20,21], the pressure-induced ionization of the protonated amine group on the protein surface is expected to be small. Although the ΔV and K_a^0 values for the dissociation of ion pairs are not available, the pressure-induced perturbation on ion-pair formation is also expected to be small since the pairing interaction is one kind of acid–base interaction which has been estimated in the above. On the other hand, the mobile phase composition is likely to affect the solute retention as well as the solute conformation. Preliminary studies were conducted to find out the appropriate mobile phase conditions for the investigation. Normally, chromatographic separation of proteins is accomplished by a gradient

elution and protein molecules are eluted as a sharp peak with very little interaction with the hydrophobic stationary phase, as long as the organic modifier composition reaches a certain critical value that is usually characteristic of individual proteins. However, an isocratic elution was performed in this study to maintain a constant pressure as well as a thermodynamic equilibrium throughout the elution process. The retention of lysozyme molecules on octadecylsilica (C_{18}) stationary phase was first examined by using different organic solvent compositions for the elution. As shown in Fig. 2, when TFA was used as the pairing agent, the retention time decreased instantly at the organic solvent composition between 35–38% and 65–68% for ACN and MeOH, respectively. When phosphoric acid was used as the pairing agent, the retention time also decreased instantly at a lower ACN composition range, 29–32%, due to the less hydrophobic nature of phosphoric acid compared to TFA [30]. Two organic solvent compositions that represent retained and eluted conditions for each system were judiciously chosen for the investigations: TFA–ACN (36:64) (retained) and (55:45) (eluted); TFA–MeOH (33:67) (retained) and (25:75) (eluted); phosphoric acid–ACN (71:29) (retained) and 36% (eluted). In order to ensure that equilibrium retention was obtained under the chosen conditions, particularly under the retained conditions, the variation of k' was monitored at two locations along the column. The variation of k' along a 40-cm distance of the column was found to be less than 3% under all retained conditions, which was within the error range of the k' measurement. Although these measurements were not performed at high pressures due to technical difficulties, the investigated retention was believed to be under an equilibrium or quasi-equilibrium state. We did not study the buffer system of phosphoric acid containing MeOH because an equilibrium condition could not be obtained.

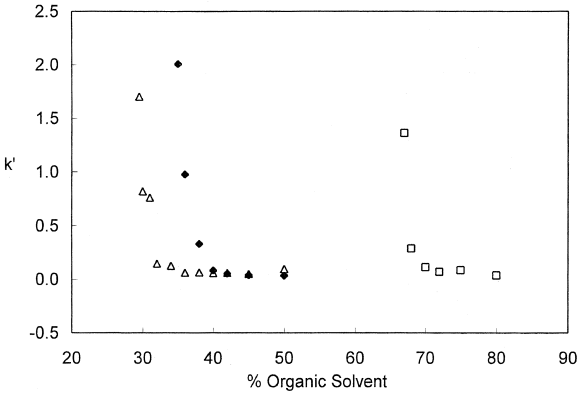

Fig. 2. Effect of organic solvent composition on the capacity factor of the lysozyme. ♦: TFA–ACN; □: TFA–MeOH; △: phosphoric acid–ACN.

3.2. Effect of pressure on k′

The effect of pressure on the protein retention was examined under the chosen conditions. As shown in Fig. 3 of the typical chromatograms under the retained conditions, the increase of the local pressure caused the retention time to increase by as much as 3 times with a concomitant increase of the bandwidth. It is also noticeable that when the injection was

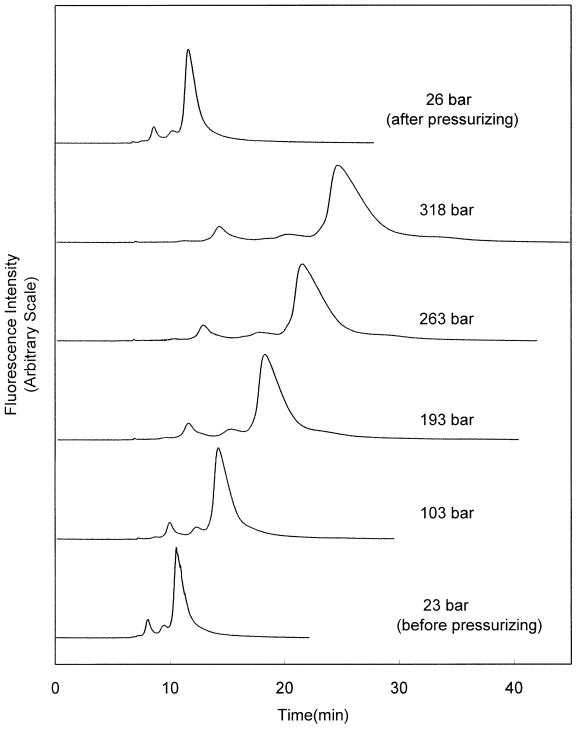

Fig. 3. Typical chromatograms showing effect of pressure on the retention time of the lysozyme under the retained conditions. The mobile phase is composed of 0.1% aqueous TFA–ACN (64:36) (pH 2.3). The acquired sequence of the chromatograms was from the bottom to the top and the absolute pressures on the viewing window were indicated.

performed after the column was returned to low pressure by disconnecting the restrictor, the obtained retention time was the same as that obtained before the column pressurization. This indicates two things: first, the observed pressure effects were not due to column fouling; secondly, the influence of the pressure on the stationary phase if it existed was reversible. Furthermore, when the fractions eluted under 292 bar (Fig. 4A) were collected and re-injected into the column under a low column pressure 20 bar without the restrictor (Fig. 4B), the retention time was similar to that obtained by injecting fresh solutes with a small offset (Fig. 4C). Such offset may indicate a conformational change but most likely it is due to the variation of injections. This indicates that the pressure influence on the solute was likely to be reversible and also further supports that the observed retention was likely to be an equilibrium condition. Under the eluted mobile-phase conditions, the peak profiles were not influenced much by the pressure since the equilibrium of Eq. (1) lies dominantly on the left. The investigation was also conducted under a shallow gradient from 35–45% ACN at 2%/min using TFA as the pairing agent and the retention time was found to increase by 25% as the pressure was raised to 350 bar. This magnitude is much smaller compared to the isocratic elution because the solute was rapidly desorbed by the gradient so that the hydrophobic interaction was greatly reduced. For pressure effect on the retention of 2–5 mers of poly-L-phenylanaline which do not have the secondary structure were shown in Fig. 5. The plot of logarithm of the capacity factor versus the number of phenylanaline is linear, indicating that the solute retention is mainly dependent on the hydrophobic interaction, which is linearly related to the chain length. Moreover, the capacity factors of the solutes were increased as the pressure was increased. Since the pressure-induced ionization and ion pairing were small and the conformational effect did not exist, the pressure-induced perturbation observed for these solutes must be associated with the equilibria of hydrophobic partitioning. For lysozyme molecules, under isocratic conditions where a quasi-equilibrium of hydrophobic adsorption and desorption is reached, pressure-induced perturbations on such equilibria are suspected to be the major cause of the observed increase of protein retention. Interestingly, for the interaction of hen egg white lysozyme with two monoclonal antibodies, the complex with the greater electrostatic character did not exhibit pressure sensitivity, whereas, the more hydrophobic complex exhibited a strong

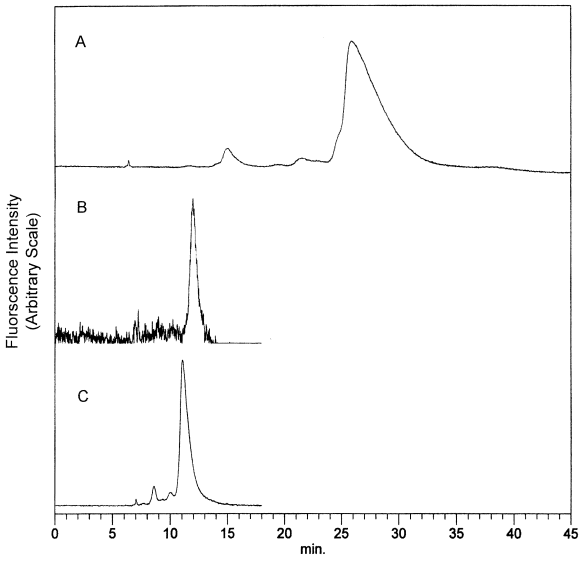

Fig. 4. Reversibility of the effect of pressure on the lysozyme retention. Chromatograms of the lysozyme eluted by the mobile phase composed of 0.1% aqueous TFA–ACN (64:36) (pH 2.3) under the following conditions: (A) Injection of the sample under an absolute column pressure of 292 bar; (B) Re-injection of the collected fraction from (A) under an absolute column pressure of 20 bar; and (C) Injection of the fresh sample under an absolute column pressure of 20 bar.

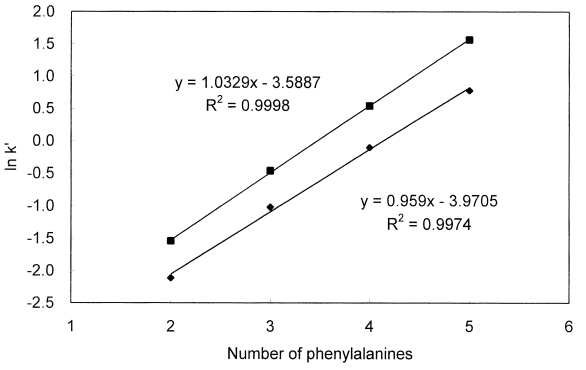

Fig. 5. The change of ln k' of poly-L-phenylalanines as a function of the number of chain length and the column pressure. ♦: 24 bar, ■: 312 bar. The elution was under an isocratic condition using 0.1% TFA buffer containing 40% ACN.

pressure sensitivity [27]. On the other hand, the aggregation of protein molecules will also alter the solvation structure of the solute and increase the retention. However, it is less likely to happen here since the pressure tends to promote the protein dissociation rather than aggregation [20,21].

3.3. Volume change (ΔV)

The pressure-induced retention was further quantified from the calculation of the corresponding ΔV values based on Eqs. (7) and (8). As shown in Fig. 6, the plots of ln k' versus pressure were fitted with both linear and quadratic forms and all the obtained R^2 values were greater than 0.9 (Table 1). The calculated ΔV values were listed in Table 1. Generally speaking, the obtained ΔV values were on the order of minus tens to hundreds of mL/mol, which are in the same range of magnitudes as those observed for another protein, insulin [7]. For poly-phenylalanines, the corresponding ΔV values (Table 2) become more negative with the chain length and the volume change per phenylanaline ($\Delta\Delta V$) is around -10 mL/mol. The small positive $\Delta\Delta V$ value (Table 2) deduced from di-L-phenylalanine and tri-L-phenylalanine could be due to the approximately equal length and width of di-L-phenylalanine, that allows the solute to be inserted into the space between octadecyl chains from either orientation, making the contact area for di-L-phenylalanine and tri-L-phenylalanine less distinguishable. It has been reported that at high pressures (350 bar) the octadecylsilica stationary phase becomes solid-like and behaves more like a self-assembly membrane [4,5] than a bulk solvent. Based on the deduced $\Delta\Delta V$ values and ΔV values of the lysozyme, it could be indicated that about tens of phenylanaline-equivalent residues on the protein surface were involved in the hydrophobic association with the chromatographic ligands. This is consistent with the general belief that only part of the residues on the protein surface are adsorbed and then desorbed from the stationary phase during the chromatographic elution.

It is also noticeable from Table 1 that for mobile phases containing TFA as the pairing agent, the ΔV value obtained from quadratic fittings decreases substantially as the pressure is increased, with an average value close to that obtained from the linear

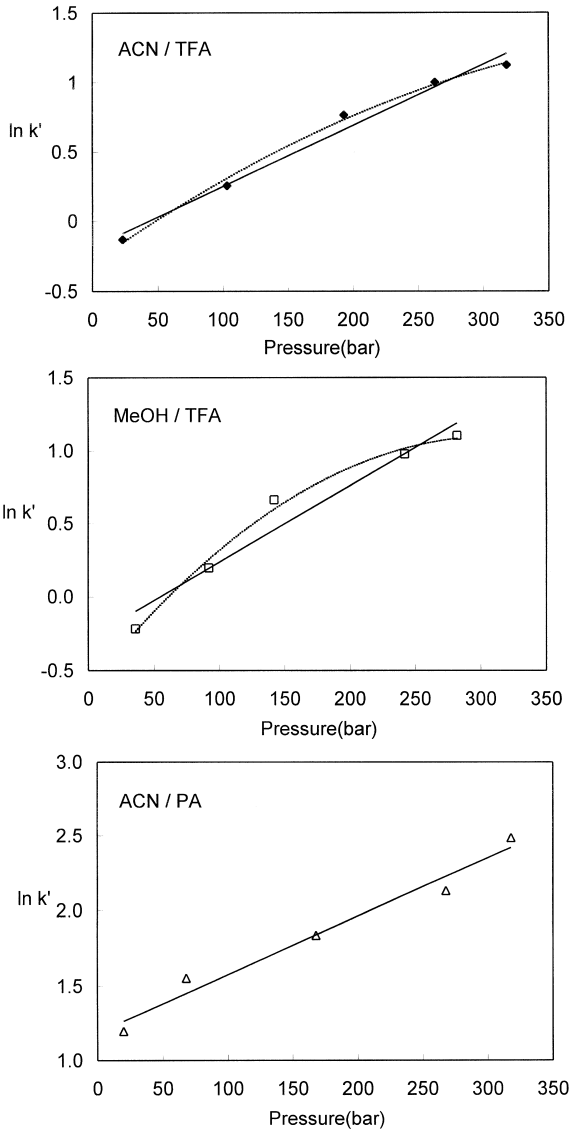

Fig. 6. Regressions of the plot of ln k' of the lysozyme versus the absolute column pressure under the following mobile phase conditions; ♦: TFA–ACN, □: TFA–MeOH, △: phosphoric acid–ACN and — represents linear fitting; ... represents quadratic fitting. For ACN–phosphoric acid buffer system, both the linear and quadratic fittings were coincident.

fitting. Moreover, the R^2 values are slightly greater for the quadratic form compared to the linear form. For the phosphoric acid–ACN system, however, the ΔV values obtained from quadratic fittings are similar to those obtained from the linear fitting and do not

Table 1
Reaction volumes

	ACN–TFA				MeOH–TFA				ACN–phosphonic acid			
	R^2	P (bar)[a]	k'[b]	ΔV (mL/mol)	R^2	P (bar)[a]	k'[b]	ΔV (mL/mol)	R^2	P (bar)[a]	k'[b]	ΔV (mL/mol)
Linear fittings	0.9809	23–318	2.02[c]	−110	0.9470	36–282	1.92[c]	−130	0.9712	20–318	6.95[c]	−97
Quadratic fittings	0.9956	23	0.88	−157	0.9919	36	0.80	−242	0.9712	20	3.31	−96
		103	1.29	−131		92	1.22	−193		68	4.73	−96
		193	2.15	−102		142	1.94	−148		168	6.27	−97
		263	2.72	−79		242	2.65	−60		268	8.42	−97
		318	3.08	−62		282	3.01	−24		318	12.01	−97

[a] Relative standard deviations were estimated to be less than 5%.
[b] Relative standard deviations based on three replicate injections were less than 3%.
[c] Average value of five pressure measurements.

vary significantly with the pressure. Moreover, the obtained R^2 values for both linear and quadratic fittings do not appear to differ. Clearly, the absolute value of ΔV decreased as the pressure was increased when using TFA as the pairing agent but not when using phosphoric acid, indicating that the compressibility of the solute decreased with the pressure when TFA was used. The cause of the change of the solute compressibility can be very complicated with the involvement of the mobile phase, stationary phase, and the solute. From our fluorescence and fluorescence quenching studies, it was found that under both the retained (36% ACN and 67% MeOH) mobile phase conditions of TFA buffer, the lysozyme conformation is rather aromatic functional group-exposed, whereas, the conformation becomes non-exposed under the retained (29% ACN) condition of phosphoric acid buffer. Therefore, it is suspected that the exposed conformation may become less compressible at high pressures. Moreover, TFA is more hydrophobic and has a greater tendency to modify the surface of the octadecylsilica stationary phase compared to phosphoric acid [30], which may result in a different pressure-induced change of the stationary phase property, leading to a different solvation environment. However, detailed characterizations are not possible with the present experimental approach since chromatographic measurements are an average of all contributions towards the solute retention.

Table 2
ΔV values of polyphenylalanines

	ΔV (mL/mol)	$\Delta\Delta V$ (mL/mol)
Di-L-phenylalanine	−49.34	
Tri-L-phenylalanine	−46.87	2.47
Tetra-L-phenylalanine	−56.74	−9.87
Penta-L-phenylalanine	−66.61	−9.87

4. Conclusions

The above observations suggest that substantial volume change on the order of minus tens to hundreds of mL/mol is associated with the protein/stationary phase interaction. These findings have several indications: First, when a great pressure drop is produced along the column, pressure fluctuations during chromatographic separation of proteins could lead to significant impact in maintaining high quality-control standards. This impact is expected to be more pronounced for peptides since these solutes are slowly desorbed under a gradient elution and significant retention remains throughout the elution. Secondly, such large volume change perturbed by the pressures within a moderate range ($<$350 bar) could indicate a potential value for using pressure studies to assess other separation mechanisms such as affinity chromatography. Combined with the temperature studies, many thermodynamic approaches can be implemented. These studies should lead to fundamental understandings of protein interactions as

well as practical uses of high pressures in protein manipulations.

Acknowledgements

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