Journal of Chromatography A, 1217 (2010) 7393–7400

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Effects of urea induced protein conformational changes on ion exchange chromatographic behavior

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ARTICLE INFO

Article history:
Received 8 July 2010
Received in revised form
16 September 2010
Accepted 20 September 2010
Available online 29 September 2010

Keywords:
Ion exchange
Urea
Protein conformation
Steric Mass Action

ABSTRACT

Urea is widely employed to facilitate protein separations in ion exchange chromatography at various scales. In this work, five model proteins were used to examine the chromatographic effects of protein conformational changes induced by urea in ion exchange chromatography. Linear gradient experiments were carried out at various urea concentrations and the protein secondary and tertiary structures were evaluated by far UV CD and fluorescence measurements, respectively. The results indicated that chromatographic retention times were well correlated with structural changes and that they were more sensitive to tertiary structural change. Steric Mass Action (SMA) isotherm parameters were also examined and the results indicated that urea induced protein conformational changes could affect both the characteristic charge and equilibrium constants in these systems. Dynamic light scattering analysis of changes in protein size due to urea-induced unfolding indicated that the size of the protein was not correlated with SMA parameter changes. These results indicate that while urea-induced structural changes can have a marked effect on protein chromatographic behavior in IEX, this behavior can be quite complicated and protein specific. These differences in protein behavior may provide insight into how these partially unfolded proteins are interacting with the resin material.

1. Introduction

Ion exchange chromatography (IEX) is widely employed for protein purification at the manufacturing scale. In IEX, protein separations are primarily due to electrostatic interactions. Several investigators have examined the possibility that certain favorable charged regions on the protein surface promote protein–resin interactions as compared to other areas on the protein surface. Gill and co-workers have shown that only a small fraction of the charged residues on the protein surface undergoes ion exchange with the resin surface [1,2]. Chicz and Regnier have found that a single mutation had a significant impact on the retention behavior of a protein variant [3]. We have recently employed a library of cold shock protein B (CspB) mutant variants to study protein binding affinity and preferred orientations in cation exchange chromatography [4]. In that work we found that mutants with the same net charge exhibited significant changes in retention time, indicating that the location and microenvironment of charge regions on the protein surfaces plays an important role [4]. That work along with other papers from the literature [4–8] have established that charge distribution on the protein surface plays an important role in protein retention in IEX. Clearly, protein structure will have a significant effect on charge distributions on proteins, thus potentially affecting retention.

IEX can also be used to facilitate protein refolding [6,9]. In this process, unfolded proteins are adsorbed in the presence of high concentrations of urea. After all the contaminants are eluted, the bound proteins are refolded by appropriate changes in the mobile phase composition. Finally, the native proteins are eluted from the column. While this process has potential advantages over other approaches, the effects of urea on protein IEX behavior are not well understood, making methods development challenging. A recent study has also shown that the presence of urea can improve the selectivity and efficiency of protein separations in IEX systems [10].

Urea, as a well known denaturant, can cause protein conformational changes. In urea-induced protein denaturation studies, two main models have been proposed and often used in MD simulations [11–23]. One is called the “direct interaction model”, in which urea interacts with polar side chains and backbones via hydrogen bonds and other electrostatic interactions, as well as through van der Waals attractions [11–14]. The other model is the “indirect model”,

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doi:10.1016/j.chroma.2010.09.052

where urea affects the hydrogen bonds of water, altering the solution environment by hydrophobic interactions, thus changing the solvation of proteins [15–19]. While recent theoretical studies seem to support the direct interaction model [14,20–22], others argue that both mechanisms are equally relevant [19,23].

Yamamoto and et al. [24] have demonstrated that the retention behavior of two model proteins changed in ion exchange systems in the presence of the denaturations urea and dithiothreitol. Size exclusion chromatography was employed in that work to evaluate the change in protein radius induced by the denaturants. The current study builds upon this preliminary work by carrying out a detailed examination of how urea-induced protein conformational changes can affect protein IEX chromatographic behavior. Linear gradient experiments with the proteins human serum albumin, bovine α-lactalbumin, and cellulase as well as the kinetically stable proteins glucose oxidase and invertase are carried out at various urea concentrations. The protein secondary and tertiary structures are evaluated by far UV CD and fluorescence measurements, respectively, and DLS analysis of changes in protein size is conducted. Finally, the Steric Mass Action (SMA) model [25,26] developed in our group is employed to characterize the protein chromatographic behavior under different urea conditions.

2. Materials and methods

2.1. Materials and equipment

2.1.1. Materials

Strong anion exchange Q Sepharose FF was donated by GE Health care (Uppsala, Sweden). The following proteins were purchased from Sigma (St. Louis, MO): human serum albumin (Cat. no. A1887), cellulase (Cat. no. 22173), calcium-depleted bovine alpha-lactalbumin (Cat. no. L6010), glucose oxidase (Cat. no. G7141) and invertase (Cat. no. I4504). Sodium phosphate (mono- and dibasic), sodium chloride and urea were also purchased from Sigma.

2.1.2. Equipment

Analytical linear gradient chromatographic experiments were carried out using a GE Healthcare Akta explorer 100 controlled by Unicorn 5.0 chromatography software.

2.2. Procedures

2.2.1. Linear gradient chromatographic experiments

The linear gradient chromatographic experiments were carried out on 1 ml Q Sepharose FF column at pH 6. Linear gradient elution were carried out from 100% buffer A (10 mM sodium phosphate, pH 6.0) to 100% buffer B (10 mM sodium phosphate containing 500 mM of sodium chloride, pH 6.0) in 32, 40, 48, 60 and 80 min at a flow rate of 0.5 ml/min and the column effluent was monitored at 280 nm. 25 μl of protein samples were injected to the column at a concentration of 2 mg/ml in the buffer A. (*Note*: the exception was α-lactalbumin which used a concentration of 1 mg/ml in buffer A containing 0.5 mM EDTA to avoid calcium binding.) For experiments run under urea conditions, the same protocol was used except 3 M or 7 M urea was added to the buffers. All samples were incubated in the urea buffer for at least 5 h before column experiments. All of the linear gradient experiments were carried out in duplicate.

2.2.2. SMA parameter determination

The previously developed Steric Mass Action (SMA) formalism and determination of parameters were described in detail elsewhere [25–27]. In this model, protein binding to the resin is described as a function of three parameters: the SMA equilibrium constant (K_{SMA}), the characteristic charge (υ) and the steric factor (σ). The SMA isotherm was used to evaluate protein chromatographic behavior in the presence of urea. In order to obtain the SMA parameters, five different linear gradient slopes were employed to determine the protein elution time at each urea condition. The retention volumes obtained from the linear gradient experiments were substituted into the following equation [27] and the linear SMA parameters (υ and K_{SMA}) were obtained by a local optimization fitting method using Matlab R2008:

$$Vg = \left[\left(x_i^{\upsilon+1} + \frac{V_m K_{SMA} \beta \Lambda^{\upsilon} (\upsilon+1)(x_f - x_i)}{V_G} \right)^{\left(\frac{1}{\upsilon+1}\right)} - x_i \right] \cdot \frac{V_G}{(x_f - x_i)} \quad (1)$$

where V_g is the solute retention volume; x_i and x_f are the initial and final salt concentrations, respectively; V_G is the total gradient volume; V_m is the dead volume; β is the phase ratio; and Λ is the total ionic capacity of the stationary phase.

2.2.3. Circular dichroism

Far-UV circular dichroism (CD) spectra were determined using a dual beam DSM CD spectrophotometer (OLIS, Bogart, GA). Urea-induced protein denaturation experiments were measured at 20 °C in a cylindrical 1 mm cuvette. Samples for the urea denaturation studies were prepared at each urea concentration in 10 mM sodium phosphate buffer (pH 6.0) 5 h prior to the measurements. Scans were carried out over the range of 190–260 nm, each with the buffer signal subtracted. The average for each urea condition was then determined from three separate scans. The measured ellipticity was also determined at 222 nm, baseline corrected and converted to molar ellipticity. The data were also normalized to the apparent fraction of unfolded protein F_u using the following expression [28]:

$$F_u = \frac{Y_0 - Y_n}{Y_U - Y_n} \quad (2)$$

where Y_0 is the signal at a given urea concentration and Y_n and Y_u are the observed values for the native and unfolded protein.

2.2.4. Fluorescence

Intrinsic fluorescence measurements were performed on a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) at 20 °C using a 0.1 cm path length cuvette. Full wavelength scans were taken from 300 to 400 nm using an excitation wavelength of 295 nm. The excitation and emission slits were set at 5 and 10 nm, respectively. The samples were prepared in the same manner as the CD experiments.

2.2.5. Dynamic light scattering

The dynamic light scattering (DLS) experiments were performed on a DynaPro titan dynamic light scattering system from Wyatt Technology Corp (Santa Barbara, CA) controlled by DYNAMICS software for acquisition and analysis of the data. A rectangular cuvette containing 100 μL of the same sample used in the chromatography experiments was maintained at 20 °C for at least 10 min prior to measurement. Data was collected for 15 min for each sample and each analysis was carried out in duplicate. The method of cumulants [29] was used to analyze the data and the effective diameter was obtained by averaging the intensity-weighted diameters. The average value of all data at the 10 s integration time for each correlation function was recorded as the final result.

3. Results and discussion

In order to examine the effects of urea on the chromatographic behavior of proteins in IEX, five model proteins with different size, charge, surface areas and stability were examined. This included

Table 1
Summary of HSA retention time and SMA parameters at various urea concentrations.

Urea conc.	Retention time (min)			SMA parameters		
	40 min	60 min	80 min	υ	K_{SMA}	R^2 [a]
0 M	18.63	27.49	33.89	5.48	0.0053	0.986
3 M	18.18	24.08	29.19	4.27	0.023	0.962
7 M	15.11	18.16	21.15	2.22	0.80	0.983

[a] R^2 is for the SMA parameters determined from Eq. (1).

the proteins human serum albumin, bovine α-lactalbumin and cellulase as well as the kinetically stable proteins glucose oxidase and invertase. A series of experiments were carried out including linear gradient experiments at various urea concentrations, protein secondary and tertiary structural determinations using CD and fluorescence spectroscopy, and the estimation of SMA isotherm parameters.

3.1. Urea effects on unstable proteins in IEX

3.1.1. Human serum albumin

Human serum albumin (HSA) is a monomeric multi-domain protein with a molecular weight (Mw) of 66.5 kDa. It consists of three homologous domains, each of which displays specific structural and functional characteristics [30]. As described in Section 2, linear gradient ion exchange experiments were carried out with HSA in the presence of 0, 3 and 7 M urea concentrations. The resulting retention times for HSA using linear gradients of 40 min, 60 min and 80 min are given in Table 1. For all three gradients, there was a small decrease in retention time going from 0 to 3 M urea and a more significant decrease observed at 7 M. As expected, the longer the gradient the more significant the reduction in retention time. Clearly, the presence of urea resulted in a decreased retention for this protein. In order to examine this chromatographic behavior in more detail, the linear SMA parameters (i.e. characteristic charge (υ) and equilibrium constant (K_{SMA})) were determined from the gradient retention data for each urea condition and are listed in Table 1. As can be seen in the table, there was a small decrease in the characteristic charge (υ) going from 0 to 3 M urea and a more significant decrease observed at 7 M. On the other hand, the equilibrium constant was observed to increase with increasing urea concentration. These results indicate that the observed decrease of retention time was mainly reflected in the accompanying decrease of the characteristic charge. Since the characteristic charge is a measure of the number of interaction sites of the protein with the ion exchange resin material, these changes may be due to possible conformational changes induced by the presence of urea.

In order to examine the urea-induced unfolding of HSA, secondary and tertiary structural changes were measured by CD and fluorescence spectroscopy under various urea conditions at pH 6.0. Fig. 1a shows the urea-induced unfolding transition curve of HSA as monitored by CD at 222 nm, which corresponds to its α-helix content. As seen in the figure, the midpoint of the transition for CD was 5.78 M urea, which is close to the value 5.74 M reported by Gonzalez-Jimenez and Cortijo [31] at pH 7.4. Fig. 1b shows the unfolding transition curve of HSA as monitored by intrinsic fluorescence intensity at 341 nm after exciting the protein at 295 nm, which is characteristic of its tryptophan residues. The midpoint of the transition for fluorescence measurement was approximately 4.5 M urea, which is less than the 5.71 M value reported by Gonzalez-Jimenez and Cortijo [31] at pH 7.4 monitoring at 335 nm. Since the native structure of HSA is pH dependent [32], it is not surprising that the tertiary structure transition curve (which is more sensitive than the secondary structure) results would be different at these various pH.

As seen in Fig. 1, while the tertiary structure of HSA exhibited a measurable change at 3 M urea (Fig. 1b), minimal change of secondary structure (Fig. 1a) was observed. This indicates that the decrease of characteristic charge (υ) value from 5.48 (no urea) to 4.27 (3 M urea) shown in Table 1 may be due primarily to tertiary rather than secondary structural changes. On the other hand, at 7 M urea, there were significant changes in both the tertiary and secondary structure of HSA (Fig. 1). This indicates that the significant reduction in characteristic charge (2.22) observed in 7 M urea may have been due to both tertiary and secondary structural changes.

Dynamic light scattering was also carried out to examine the effect of urea on the hydrodynamic radius of HSA. As shown in Fig. 2, the hydrodynamic radius (R_H) of HSA increased from 3.6 nm to 8.3 nm when going from 0 to 7 M urea. It is interesting to speculate on how this increase in protein size may affect the interaction of the protein with the ion exchange material. On the one hand, an increased size could result in a larger interacting region of the protein with the resin. This could result in an increased number of interaction sites (or characteristic charge) between the unfolded protein and the resin. However, the chromatographic results indicated the opposite trend, with a decreased characteristic charge at elevated urea concentrations. One possible reason for this may be that when the protein undergoes a conformational change, the charge density on the protein surface may be reduced due to more spreading between the charged residues. This reduced electrostatic potential may then result in lower affinity for the resin material.

3.1.2. α-Lactalbumin

Bovine α-lactalbumin (BLA) is a protein with a large α-subdomain and a small β-subdomain with a Ca^{2+}-binding site at the junction of the two subdomains [33]. The Ca^{2+} ion in BLA plays

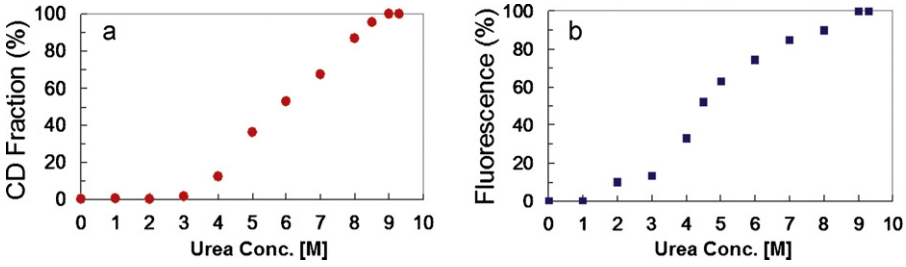

Fig. 1. (a) Urea-induced changes in the secondary structure of HSA monitored by following changes in ellipticity at 222 nm obtained from the far-UV CD curves. (b) Changes in fluorescence of HSA monitored by fluorescence emission at 341 nm and excitation at 295 nm. Samples were prepared at 10 mM phosphate buffer, pH = 6.0 at 20 °C.

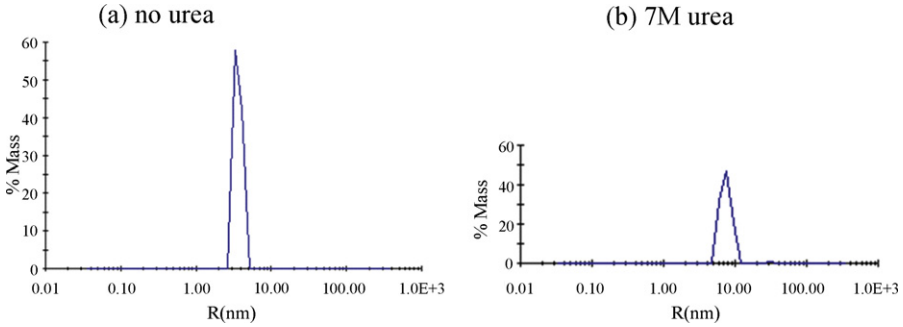

Fig. 2. Dynamic light scattering results with HSA: (a) no urea, the hydrodynamic radius R_H = 3.6 nm, polydispersity (Pd%) = 11.8% and (b) 7 M urea, R_H = 8.3 nm, Pd% = 16.6%. Samples were prepared at 10 mM phosphate buffer, pH = 6.0 at 20 °C.

a structural role in stabilizing the protein [34]. In the current study, BLA was used in the absence of Ca^{2+} in order to examine the less stable form of the protein. Linear gradient experiments were carried out in the presence (or absence) of urea as described in Section 2 and the resulting retention times for BLA using linear gradients of 40 min, 60 min and 80 min are given in Table 2. For all three gradients, there was a significant decrease in retention time going from 0 to 3 M urea and a further decrease observed at 7 M. As was the case with HSA, the presence of urea resulted in a decreased retention for this protein. In order to examine this chromatographic behavior in more detail, the linear SMA parameters (i.e. characteristic charge (υ) and equilibrium constant (K_{SMA})) were determined from the gradient retention data for each urea condition and are also listed in Table 2. As was observed for HSA, the characteristic charge (υ) decreased while the equilibrium constants (K_{SMA}) increased with increasing concentrations of urea. However, in contrast to HSA, the characteristic charge of BLA exhibited a significant reduction in both 3 and 7 M urea. Again, these results indicated that the observed decrease of retention time was mainly reflected in the accompanying decrease of the characteristic charge.

In order to examine the urea-induced unfolding of BLA, secondary and tertiary structural changes were measured by CD and fluorescence spectroscopy in the presence of 0.5 mM EDTA. Fig. 3 shows the urea-induced unfolding transition curve of BLA as monitored by CD at 222 nm. As seen in the figure, the midpoint of the curve was around 4 M urea, which is in close to the literature value of 3.4 M urea that was obtained using a disulfide scrambling method under different conditions (pH 8.4, containing 0.02–0.25 mM 2-mercaptoethanol) reported by Chang and Li [35]. Thus, the secondary structural change of BLA appears to occur at a lower urea concentration as compared to HSA which had a midpoint of 5.78 M.

The unfolding of BLA as monitored by intrinsic fluorescence was also carried out and the results using the transition fraction curve at 333 nm are presented in Fig. 4a. The midpoint of the transition curve for fluorescence was 3.5 M urea. In order to compare these results with the literature, the maximum emission wavelength (λ_{max}) data for BLA was also examined (Fig. 4b). The λ_{max} of native BLA emission occurred at 333 nm, which is very close to the previously reported λ_{max} value of 332 nm obtained at different conditions (pH 8.7, 1 mM

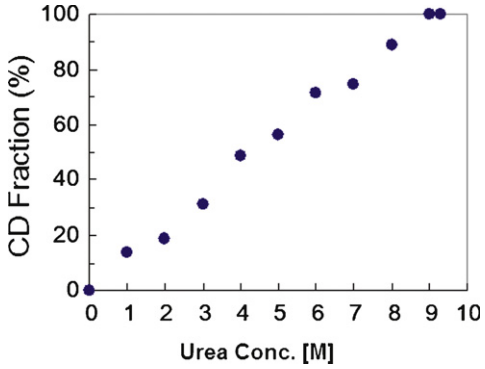

Fig. 3. Urea-induced changes in the secondary structure of BLA monitored by following changes in ellipticity at 222 nm obtained from the far-UV CD curves. Samples were prepared at was 1 mg/ml in 10 mM phosphate buffer, 0.5 mM EDTA, pH = 6.0 at 20 °C.

EDTA, excitation wavelength of 280 nm) by Ewbank and Creighton [36]. As seen in Fig. 4b, the fluorescence emission maximum exhibited a significant quenching of the red shift in the range of 0–2.5 M urea, followed by a gradual shift at higher concentrations of urea.

From these CD and fluorescence results, the significant change in the characteristic charge of BLA at 3 M urea was possibly due to both secondary and tertiary structural changes of BLA, which caused modification in the orientation of surface charged residues on the protein surface.

Dynamic light scattering was also carried out to examine the effect of urea on the hydrodynamic radius of BLA. As shown in Fig. 5, the hydrodynamic radius (R_H) of BLA at 0 and 7 M urea was 1.3 and 1.5 nm, respectively. This suggests that the effective size of BLA during unfolding did not change significantly, in contrast to the results obtained with HSA. In fact, this is in agreement with prior work in the literature which indicates that BLA forms a molten globule (MG) state in the presence of urea [37–41], where the size of the protein is only 10–20% larger than the native structure [37,42], even though the tertiary structure has changed. Thus, while the significant change in the characteristic charge of BLA at 3 M urea was correlated with both secondary and tertiary structural changes, it was not correlated with significant changes in the size.

Table 2
Summary of BLA retention time and SMA parameters at various urea concentrations.

Urea conc.	Retention time (min)			SMA parameters		
	40 min	60 min	80 min	υ	K_{SMA}	R^2 [a]
0 M	18.60	23.82	28.63	2.64	0.82	0.999
3 M	14.08	17.59	20.36	1.83	1.93	0.995
7 M	12.33	14.48	16.16	1.25	5.41	0.999

[a] R^2 is for the SMA parameters determined from Eq. (1).

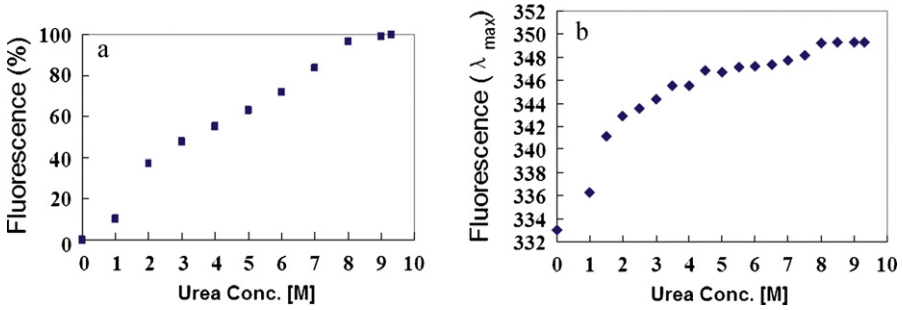

Fig. 4. (a) Urea-induced changes of BLA monitored by fluorescence emission at 333 nm and excitation at 295 nm. (b) Changes in the wavelength maximum (λ_{max}) of BLA monitored by fluorescence excitation at 295 nm. Samples were prepared at was 1 mg/ml in 10 mM phosphate buffer, 0.5 mM EDTA, pH = 6.0 at 20 °C.

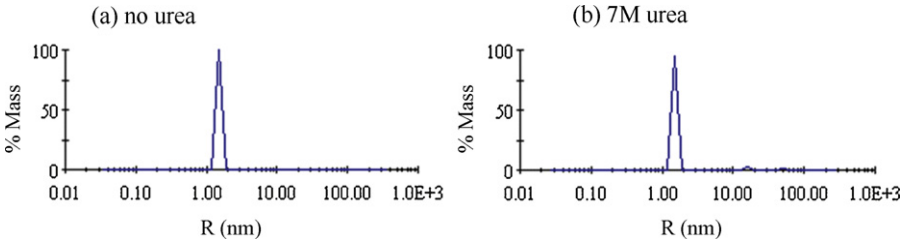

Fig. 5. Dynamic light scattering results of BLA: (a) no urea, the hydrodynamic radius R_H = 1.3 nm, Pd% = 0% and (b) 7 M urea, R_H = 1.5 nm, Pd% = 0%. Samples were prepared at was 1 mg/ml in 10 mM phosphate buffer, 0.5 mM EDTA, pH = 6.0 at 20 °C.

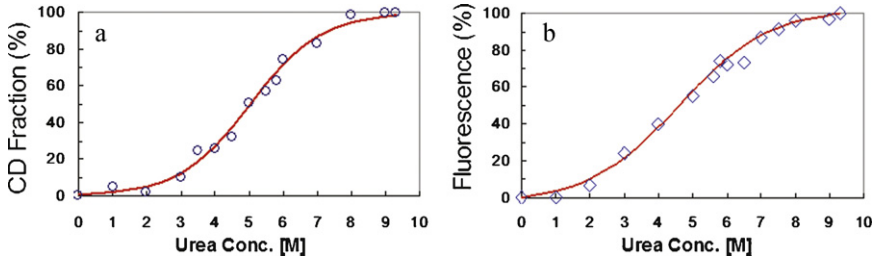

Fig. 6. (a) Urea-induced changes in the secondary structure of cellulase monitored by following changes in ellipticity at 222 nm obtained from the far-UV CD curves. (b) Changes in fluorescence of cellulase monitored by fluorescence emission at 340 nm and excitation at 295 nm. Samples were prepared at 10 mM phosphate buffer, pH = 6.0 at 20 °C. The solid lines represent the fits assuming two-state unfolding.

3.1.3. Cellulase

Another model protein, cellulase, was tested at the 0, 3 and 7 M urea conditions and the retention data and fitted SMA parameters are listed in Table 3. As was observed for the other proteins, the retention time decreased with increasing urea concentration. However, in contrast to the results obtained with HSA and BLA, the characteristic charge of cellulase increased with increasing urea concentration. In addition, the equilibrium constant decreased for this protein at higher urea concentrations, again in contrast to the results with the other two model proteins. Clearly, this protein is acting differently in response to the presence of urea. One possible explanation for this may be that as this particular protein unfolds, a larger binding face of the protein becomes available to interact with the resin, resulting in an increase in the characteristic charge.

Secondary and tertiary structural changes of cellulase were measured by CD at 222 nm and intrinsic fluorescence intensity at 340 nm after excitation at 295 nm and the results are presented in Fig. 6a and b, respectively. The solid lines on both graphs represent the fits to the data assuming two-state unfolding (*note*: a two state unfolding model [43] was employed in order to obtain a good fit of the data and to facilitate analysis). As seen in the figures, the secondary structural change (~10%) was less than the tertiary structural change (~20%) at 3 M urea. However at 7 M urea, both secondary and tertiary structures exhibited greater than 80% change. This data on structural change agrees qualitatively with the retention time and SMA parameter data which exhibited some changes at 3 M and significant changes at 7 M urea.

Table 3
Summary of cellulase retention time and SMA parameters at various urea concentrations.

Urea conc.	Retention time (min)			SMA parameters		
	40 min	60 min	80 min	υ	K_{SMA}	R^2 [a]
0 M	28.38	37.67	47.02	4.11	0.42	0.999
3 M	25.46	34.47	42.89	4.56	0.12	0.999
7 M	20.79	27.59	34.19	5.59	0.004	0.996

[a] R^2 is for the SMA parameters determined from Eq. (1).

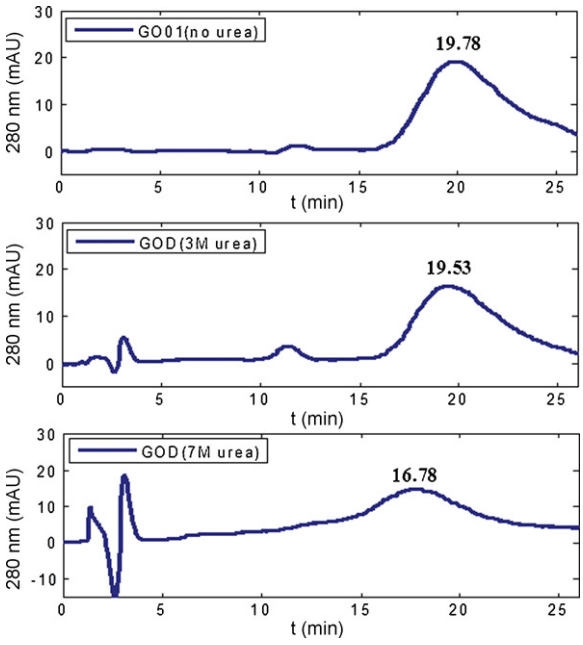

Fig. 7. Comparison of GO retention time at no urea, 3 M and 7 M urea with 40 min linear gradient length.

3.2. Urea effects on kinetically stable proteins in IEX

The chromatographic and structural properties of the kinetically stable proteins glucose oxidase and invertase were also examined. Kinetically stable proteins are proteins that have a very high transition energy state between the native folded and the unfolded state. This high transition energy state can result in extremely slow unfolding rates [44].

3.2.1. Glucose oxidase

Glucose oxidase (GO) is an acidic protein which shows resistance to SDS denaturation at pH 6.0 even after prolonged incubation [45]. Fig. 7 presents the chromatograms for a 40 min linear gradient with 0, 3 and 7 M urea concentration, respectively. The corresponding SMA parameters are listed in Table 4. The results show that GO is quite stable in terms of its chromatographic behavior. For example, when going from 0 to 3 M urea, the retention time is almost identical. The characteristic charges at these two conditions are also essentially unchanged. At 7 M urea, the retention time was observed to decrease as well as the characteristic charge, albeit to much less an extent than the three protein discussed above. These results indicate that the number of interaction sites between GO and the charged ligands on the ion-exchange material are similar at 0 and 3 M urea and are only moderately lower at 7 M Urea. In addition, the equilibrium constants for all three conditions underwent only minimal change as compared to the three proteins discussed above.

The urea-induced structural changes of GO upon unfolding have been extensively studied by Akhtar et al. [46]. Since their experimental conditions (e.g. buffer, pH and temperature) were very similar to ours, their results will be used here to interpret our chromatographic data. The urea concentration C_m associated with a 50% tertiary structural change was 6 M urea, when the experiment was carried out in the presence of 0.5 M NaCl. In addition, their data indicated that there was negligible unfolding of GO at 3 M urea in the presence of salt in agreement with our chromatographic data. Again, these results indicate that there is a strong correlation between urea induced structural changes and chromatographic behavior. The minor reduction in retention time observed at 7 M urea could also be due to the increase in dielectric constant in the presence of urea which would result in a minor reduction in electrostatic interactions.

Table 4
Summary of SMA parameters for GO and invertase at various urea concentrations.

Urea conc.	Glucose oxidase			Invertase		
	υ	K_{SMA}	R^2 [a]	υ	K_{SMA}	R^2 [a]
0 M	5.43	0.011	0.998	2.74	0.17	0.993
3 M	5.46	0.006	0.999	2.89	0.11	0.998
7 M	4.71	0.007	0.997	4.12	0.004	0.986

[a] R^2 is for the SMA parameters determined from Eq. (1).

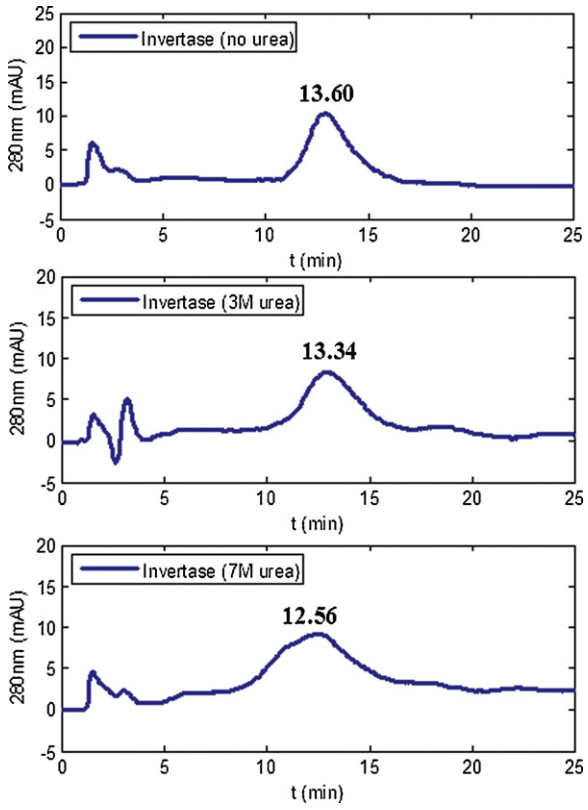

Fig. 8. Comparison of invertase retention time at no urea, 3 M and 7 M urea with 40 min linear gradient length.

3.2.2. Invertase

Invertase is another kinetically stable protein. It is quite resistant to urea unfolding, with the midpoint of the fluorescence transition curves reported to be 7.2 M urea at pH 5 [47]. The chromatographic behavior at 0, 3 and 7 M urea with a 40 min gradient length are plotted in Fig. 8 and the SMA parameters are listed in Table 4. At 3 M urea, minimal changes were observed in retention and the SMA parameters. At 7 M urea there was a small change in the chromatographic behavior and some changes in the SMA parameters. The characteristic charge was seen to increase from 2.74 (native) to 4.12 (7 M urea) and the equilibrium constant decreased from 0.17 to 0.004. It is interesting to note that the peak shape at 7 M urea was broader than at 0 and 3 M urea. This could be due to the presence of multiple species of the protein in various stages of unfolding.

The CD full wavelength signals for no urea and 3 M urea were found to be almost identical (Fig. 9) (*note*: the full wavelength signals were used for this protein instead of the transition curve due to the minimal changes observed in the signal). For fluorescence, the maximum emission wavelength (λ_{max}) excited at 295 nm for native

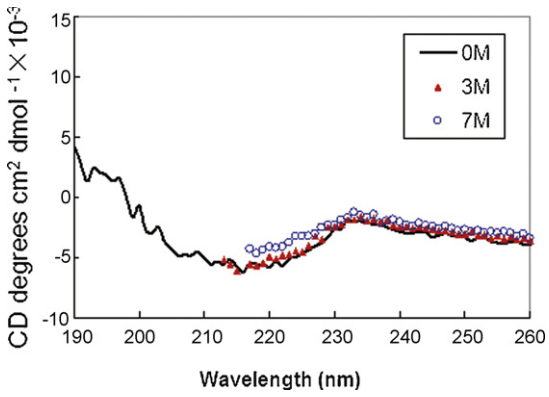

Fig. 9. CD results of invertase at no urea, 3 M and 7 M urea. Samples were prepared at 10 mM phosphate buffer, pH = 6.0 at 20 °C.

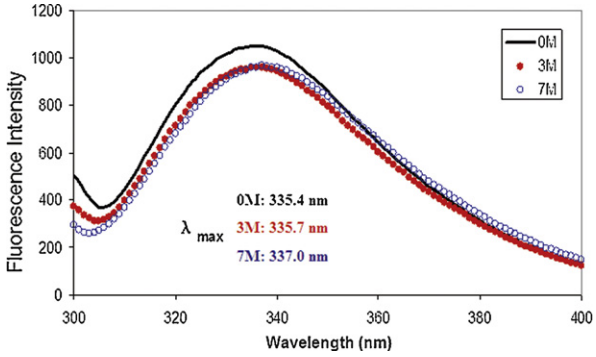

Fig. 10. Fluorescence emission of invertase at no urea, 3 M and 7 M urea, excitation at 295 nm.

and 3 M urea was found to be 335.4 nm and 335.7 nm, respectively (Fig. 10). These results indicate that there was negligible change in the protein structure at 3 M urea which is in agreement with the chromatographic behavior.

On the other hand, at 7 M urea, there were some subtle changes in both the tertiary and secondary structure of invertase. The fluorescence measurement at 7 M urea (Fig. 10) resulted in a maximum emission wavelength of 337.0 nm, which was different from the native (335.4 nm) and 3 M urea signal (335.7 nm). However, while there were some changes in the structure of invertase at 7 M urea, these changes were not as significant as those observed for the unstable proteins, such as calcium depleted BLA.

These results with the kinetically stable proteins indicated that they exhibited negligible change in chromatographic behavior in 3 M urea and minor changes in 7 M urea. This chromatographic behavior was reflected both in the SMA parameters as well as in the protein structural determinations. This is in contrast to the results that were obtained with the other proteins. If these changes in chromatographic behavior were simply due to mobile phase effects induced by the presence of urea (e.g. minor changes in dielectric constant), it would not be expected to see such qualitative differences between these two classes of proteins. Thus, it appears that the changes are due primarily to protein structural changes induced by the presence of urea.

4. Conclusions

In this work, five model proteins were used to examine the chromatographic effects of protein conformational changes induced by urea in ion exchange chromatography. Linear gradient experiments were carried out at various urea concentrations and the protein secondary and tertiary structures were evaluated by far UV CD and fluorescence measurements, respectively. The results indicated that the protein retention time was more sensitive to tertiary structure than secondary structure. SMA isotherm parameters were also examined and the results indicated that urea induced protein conformational changes could affect both the characteristic charge and equilibrium constants in these systems. DLS analysis of changes in protein size due to urea-induced unfolding indicated that the size of the protein was not correlated to SMA parameter changes. For example, the size of HSA increased in 7 M urea while the characteristic charge decreased. In contrast, for BLA, the size of the protein did not change much in 7 M urea, however, the characteristic charge was still observed to decrease. Clearly, more work is required to understand this behavior in more depth. For stable proteins (e.g. GO and invertase), the structures were relatively stable at 3 M, and the retention and SMA parameters were similar to the no urea condition. At 7 M urea, for these stable proteins, only minor changes in retention were observed, and this was reflected both in the SMA parameters as well as in the protein structural determinations. Therefore, the effects of urea-induced structural changes on protein chromatographic behavior in IEX can be quite complicated and protein specific. MD simulations will be conducted in future work to determine protein structures in the presence of urea and to provide more insight into the relationship between protein structure and SMA parameters.

Acknowledgements

The authors thank Professors Wilfredo Colón and Linda McGown for providing access to the CD and fluorescence equipment. The authors also thank Yuehua Yu for assistance in the CD analysis of cellulase. This work was financially supported by Novo Nordisk A/S.

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