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Use of molecular crowding for the detection of protein self-association by size-exclusion chromatography

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ABSTRACT

The feasibility of employing molecular crowding cosolutes to facilitate the detection of protein self-association by zonal size exclusion chromatography is investigated. Theoretical considerations have established that although the cosolute-induced displacement of a self-association equilibrium towards the oligomeric state invariably occurs in the mobile phase of the column, that displacement is only manifested as a decreased protein elution volume for cosolutes sufficiently small to partition between the mobile and stationary phases. Indeed, the use of a crowding agent sufficiently large to be confined to the mobile phase gives rise to an increased elution volume that could be misconstrued as evidence of cosolute-induced protein dissociation. Those theoretical considerations are reinforced by experimental studies of α-chymotrypsin (a reversibly dimerizing enzyme) on Superdex 200. The use of cosolutes such as sucrose and small polyethylene glycol fractions such as PEG-2000 is therefore recommended for the detection of protein self-association by molecular crowding effects in size exclusion chromatography.

1. Introduction

The realization that protein self-association equilibria can be displaced towards the oligomeric state(s) by supplementing the solution with a high concentration of an inert cosolute such as polyethylene glycol or dextran [1–4] has attracted considerable attention to the possibility that the macromolecular state of a protein deduced from dilute solution studies may not apply in the highly crowded physiological environment [5–7]. In that regard the use of such non-absorbing cosolutes to promote this effect of thermodynamic nonideality has the advantage that conventional column monitoring with a near-UV detector can be retained to study the cosolute effect on protein size by size-exclusion chromatography (SEC). In this communication we explore theoretical aspects of this approach with a view to maximizing its effectiveness for the detection of protein self-association by virtue of a decreased elution volume effected by molecular crowding.

These effects of inert polymeric cosolutes have been attributed to the excluded volume phenomenon [8–10] whereby their occupancy of space within the protein solution enhances the thermodynamic activity (effective concentration) of protein monomer and hence displaces the self-association equilibrium position towards the larger oligomeric state (s) in accordance with the law of mass action [1–4]. Realization that the phenomenon of protein solvation [11–16] may also be rationalized on the statistical-mechanical basis of excluded volume [17–19] has led to the subsequent use of small cosolutes such as sucrose [20–23] and other osmolytes [24,25] as molecular crowding agents for the displacement of self-association equilibria. Here we show that the selection of a relatively small cosolute is essential for molecular crowding to become a useful tool for the detection of protein self-association by size-exclusion chromatography.

1.1. Theoretical considerations

In the absence of cosolute the elution volume (V_e) of a non-associating protein (species 2) in SEC is described in terms of a partition coefficient σ_2 [26,27] as

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$$V_e = V^\alpha + \sigma_2 V^\beta \quad (1)$$

where V^α and V^β denote the respective volumes of the mobile and stationary phases of the column. The fact that the elution volume is an equilibrium (not migration) parameter [28,29] justifies rationalization of the partition coefficient as an experimental measure of c_2^β / c_2^α, the ratio of protein concentrations in the stationary and mobile phases, albeit a purely operational description of the situation. Furthermore, theoretical considerations [30–32] of that operational situation establish that the partition coefficient also reflects the corresponding ratio of thermodynamic activities. In conventional (zonal) SEC the protein concentration is sufficiently small to justify assumed thermodynamic ideality and hence the essential identity of protein concentrations and their thermodynamic activities.

That situation changes in situations where a high concentration c_3^α of cosolute (species 3) is included in the buffer being used to equilibrate the SEC column. In that regard there are two situations to consider: that in which the cosolute also partitions between the mobile and stationary phases of the column, and that in which the cosolute is sufficiently large to be confined to the mobile phase. For simplicity we shall assume that the SEC matrix is sufficiently rigid to withstand any osmotic shrinkage as the result of different cosolute concentrations in the two phases [33,34] – a situation that is likely to be met with the SEC matrices used in FPLC.

1.2. Chromatography in the presence of a partitioning cosolute

Consider initially the situation in which protein partition between the mobile and stationary phases is being monitored under conditions where the partition of buffer components (species 1) and a partitioning cosolute (species 3) between mobile and stationary phases ensures measurement the chemical potential of protein (species 2) under the constraints of constant temperature and constant chemical potential of all partitioning species (cosolute as well as buffer components and water), which can then be regarded as the solvent. On the grounds that the protein then becomes the sole solute component, the expressions for the protein chemical potential in the mobile (α) and stationary (β) phases become [35].

$$[\mu_2^\alpha]_{T,\mu_1,\mu_3} = \left[(\mu_2^0)^\alpha\right]_{T,\mu_1,\mu_3} + RT \ln z_2^\alpha \quad (2a)$$

$$[\mu_2^\beta]_{T,\mu_1,\mu_3} = \left[(\mu_2^0)^\beta\right]_{T,\mu_1,\mu_3} + (\mu_2^\beta)_{T,\mu_1,\mu_3} + RT \ln z_2^\beta \quad (2b)$$

where $[(\mu_2^0)^\alpha]_{T,\mu_1,\mu_3}$ and $[(\mu_2^0)^\beta]_{T,\mu_1,\mu_3}$ are the standard-state protein chemical potentials under those constraints, and where z_2^i denotes the molar thermodynamic activity of protein in the appropriate phase ($i = \alpha$ or β): R is the universal gas constant and T the absolute temperature. The term $(\mu_2^\beta)_{T,\mu_1,\mu_3}$ is included to emphasize the fact that there is a constant contribution to the chemical potential of protein in the stationary phase arising from the presence of the matrix. A case can therefore be made for its inclusion as an offset in the standard-state chemical potential, $[(\mu_2^0)^\beta]_{T,\mu_1,\mu_3}$ [35]. However, an alternative practice has been adopted whereby the protein − matrix term $(\mu_2^\beta)_{T,\mu_1,\mu_3}$ is incorporated as a thermodynamic nonideality contribution to z_2^β [30–32].

On the basis of identical solute chemical potentials in the two phases at partition equilibrium it then follows that

$$z_2^\beta/z_2^\alpha = \left[\exp\left\{[(\mu_2^0)^\alpha]_{T,\mu_1,\mu_3} - [(\mu_2^0)^\beta]_{T,\mu_1,\mu_3}\right\}\right]/(RT) \quad (3)$$

which establishes that the ratio of molar solute thermodynamic activities is a constant defined by the difference between the standard-state chemical potentials of solute (protein) in the two phases [30,31]. On the other hand the experimental partition coefficient, measured as $\sigma_2 = (V_e - V^\alpha)/V^\beta$ [Eq. (1)], is the ratio of the corresponding molar concentrations of solute ($C_2 = c_2/M_2$, the ratio of weight concentration to molar mass), which varies because of the different concentration dependencies of the molar activity coefficients γ_2 (ratio of molar thermodynamic activity to molar concentration) in the two phases. In other words

$$\sigma_2 = C_2^\beta/C_2^\alpha = c_2^\beta/c_2^\alpha = (z_2^\beta/z_2^\alpha)\,(\gamma_2^\alpha/\gamma_2^\beta) \quad (4)$$

Provided that effects of thermodynamic nonideality can be described adequately in terms of a virial expansion truncated at quadratic terms in concentration, the molar activity coefficient of solute can be expressed [36] as

$$\gamma_2 = \exp[2B_{22}C_2^\alpha + \Sigma B_{2j}C_j] \quad (5)$$

where B_{22} is the osmotic second virial coefficient for physical protein self-interaction and B_{2j} is the corresponding parameter for protein interaction with other species contributing to the thermodynamic nonideality. In the present context the expressions for the activity coefficients of protein in the two phases become

$$\gamma_2^\alpha = \exp(2B_{22}C_2^\alpha) \quad (6a)$$

$$\gamma_2^\beta = \exp(2B_{22}C_2^\beta + B_{2X}C_X^\beta) \quad (6b)$$

where Eq. (5) has been used to incorporate the $(\mu_2^\beta)_{T,\mu_1,\mu_3}$ contribution to protein themodynamic activity in the stationary phase. B_{2X} is the nominal second virial coefficient accounting for physical interaction between protein and matrix species X, which has been assigned a corresponding nominal concentration C_X^β. The relationship for the partition coefficient may then be written as

$$\sigma_2 = (z_2^\beta/z_2^\alpha)\exp[2B_{22}(C_2^\alpha - C_2^\beta) - B_{2X}C_X^\beta] \quad (7)$$

Because of the low protein concentrations employed in conventional SEC the terms in C_2 are effectively zero, whereupon the expression for the partition coefficient simplifies to

$$\sigma_2 = (z_2^\beta/z_2^\alpha)\exp(-B_{2X}C_X^\beta) = \sigma_2^0 \quad (8)$$

which is establishes independence of the protein partition coefficient (at its limiting value σ_2^0) and hence elution volume upon cosolute concentration. In that regard it is noted that the exp $(-B_{2X}C_X^\beta)$ term in Eq. (8) is the alternative means for accommodating the standard-state offset $(\mu_2^\beta)_{T,\mu_1,\mu_3}$.

In the event that the protein undergoes reversible self-association to a single oligomeric state (nA ⇆ C) the partition coefficient σ_2 in Eq. (8) needs to be replaced by its weight-average counterpart, σ_{av}, which is given by

$$\sigma_{av} = (\sigma_A c_A^\alpha + \sigma_C c_C^\alpha)/(c_A^\alpha + c_C^\alpha) = f_A\sigma_A + (1 - f_A)\sigma_C) \quad (9)$$

where f_A is the weight-fraction of monomer in the mobile phase. From Eq. (5) it is evident that γ_A^α and γ_C^α both increase with increasing cosolute concentration, a consequence of which is a systematic decrease in f_A with increase in C_3^α. Provided that the cosolute is sufficiently small to partition between the mobile and stationary phases of the SEC column, the observation of a cosolute-induced decrease in protein elution volume is the predicted outcome of enhanced protein self-association.

1.3. Chromatography in the presence of an excluded cosolute

Unfortunately, the above criterion for the existence of protein self-association is compromised by the use of a relatively large cosolute such as dextran 500, which is excluded from the stationary phase of many SEC matrices. Its presence in the mobile phase increases the thermodynamic activity z_2^α and hence necessitates the transfer of protein into

the stationary phase to achieve the situation $z_2^{\beta} = z_2^{\alpha}$ at partition equilibrium.

The system must therefore be considered in terms of two solute components (protein and cosolute), whereupon the protein chemical potential in the two phases needs to be written as

$$[\mu_2^{\alpha}]_{T,\mu_1} = \left[(\mu_2^0)^{\alpha}\right]_{T,\mu_1} + RT \ln z_2^{\alpha} \tag{10a}$$

$$[\mu_2^{\beta}]_{T,\mu_1} = \left[(\mu_2^0)^{\beta}\right]_{T,\mu_1} + RT \ln z_2^{\beta} \tag{10b}$$

to accommodate the non-identity of cosolute chemical potentials in the two phases. Although the expression for the protein partition coefficient [Eq. (4)] retains validity, the expression for the protein activity coefficient in the mobile phase [Eq. (6a)] becomes

$$\gamma_2^{\alpha} = \exp(2B_{22}C_2^{\alpha} + B_{23}C_3^{\alpha}) \tag{11}$$

to reflect the presence of cosolute in the mobile phase. Attainment of equal chemical potentials for protein in the two phases thus requires the transfer of protein to the stationary phase, whereupon its partition coefficient is increased. The counterpart of Eq. (8) is then

$$(\sigma_2)_3 = (z_2^{\beta}/z_2^{\alpha})\exp[2B_{22}(C_2^{\alpha} - C_2^{\beta}) - B_{2X}C_X^{\beta} + B_{23}C_3^{\alpha}] \tag{12}$$

which, after setting all terms in C_2 to zero because of the use of low protein concentrations in zonal SEC, becomes

$$(\sigma_2)_3 = (z_2^{\beta}/z_2^{\alpha})\exp(B_{22}C_3^{\alpha} - B_{2X}C_X^{\beta}) = \sigma_2^0 \exp(B_{23}C_3^{\alpha}) \tag{13}$$

An increase in protein partition coefficient from its value in the absence of cosolute and hence V_e with cosolute concentration are thus the predicted outcome for a nonassociating protein in the presence of a cosolute confined to the mobile phase [35]. The consequent increase in partition coefficient and hence elution volume as the result of protein–cosolute interaction clearly has the potential to over-ride any decrease in $(\sigma_{av})_3$ as the result of cosolute-induced protein self-association in the mobile phase.

From the foregoing considerations it is evident that a relatively small cosolute is likely to be required for SEC to become a useful procedure for the detection of protein self-association by molecular crowding effect. Large polymers such as dextran 500 should therefore be discarded in favour of smaller inert cosolute species to ensure concordance with the requirement for conduct of the experiments under the constraint of constant cosolute chemical potential. To that end the saccharides glucose, sucrose and raffinose [20–23] as well as the osmolyte trimethylamine N-oxide [24,25] have been used in ultracentrifuge studies to induce changes in either the self-association [22–24] or the isomerisation [20,21,25] state of enzymes.

2. Materials and methods

Salt-free lyophilized preparations of bovine serum albumin and α-chymotrypsin were obtained from Bio Basic (Canada) and used without further purification. Dextran 500 (M ≈ 500 kDa) was a product provided by Spectrum Chemicals (USA). Solutions were prepared by dissolving the albumin and α-chymotrypsin directly in acetate–chloride buffer, pH 3.9, I 0.2 M (0.18 M sodium chloride–0.02 M sodium acetate, pH adjusted with acetic acid). Samples (0.5 mL, 10 g/L) were applied to a Superdex 200 Increase column (1 × 30 cm) preequilibrated at 4 °C and 0.65 mL/min with the acetate–chloride buffer or the same buffer supplemented with various concentrations of sucrose. A lower flow rate (0.30 mL/min) was used in the series of experiments with dextran 500 as cosolute. The pre-equilibration medium was then used to generate a zonal elution profile that was recorded spectrophotometrically at 280 nm.

3. Results and discussion

In order to obtain an idea of the magnitudes of cosolute effects that are likely to be encountered in the detection of protein self-association by zonal SEC on Superdex 200, the theoretical dependence of partition coefficient upon cosolute concentration has first been calculated for α-chymotrypsin in acetate–chloride buffer (pH 3.9, I 0.20), conditions under which the protein undergoes reversible dimerization [21–23,37].

3.1. Predicted consequences of cosolute supplementation of protein solutions in SEC

To illustrate the consequences of supplementing protein solutions with high concentrations of an insert cosolute we present the predicted partition behaviour of bovine serum albumin (a nonassociating protein) and α-chymotrypsin (a reversibly dimerizing protein) on Superdex 200 equilibrated with acetate–chloride buffer, pH 3.9, I 0.2, conditions under which the dimerization of α-chymotrypsin is governed by an association constant (K_2) of 5.5 L/g [23]. A partition coefficient σ_2 of 0.353 has been assigned to serum albumin (M_2 = 65 kDa) on the basis of a calibration plot, $\sigma_2 = 1.72 - 0.284 \log M_2$, for a Superdex 200 column [38,39]. Respective partition coefficients of 0.471 (σ_A) and 0.385 (σ_C) for α-chymotrypsin monomer (M_A = 25 kDa) and dimer are obtained by the same procedure.

Evaluation of the average partition coefficient σ_{av} for the latter system requires specification of the magnitude of f_A (the weight fraction of monomer) by taking advantage of the following expression for the apparent dimerization constant (K_c^{app}) in the mobile phase [22,23],

$$K_2^{app} \approx K_2 \exp[(2B_{A3} - B_{C3})c_3/M_3] \tag{14}$$

where B_{i3}, the osmotic second virial coefficient for excluded volume interaction between solute and cosolute species, may be calculated from assigned species radii R_i via the relationship [19].

$$B_{i3} = 4\pi N_A (R_i + R_3)^3/3 \tag{15}$$

and the following species radii: 3.51 nm for bovine serum albumin [40]; 2.44 and 3.07 nm for α-chymotrypsin monomer and dimer respectively [23]; 0.34 nm for sucrose [19]; 4.5 nm for PEG 20000 on the basis of the relationship $R_3 = 0.0126\ M_3^{0.593}$ [41]; 18.7 nm for dextran 500 on the basis of the relationship $R_3 = 0.0271\ M_3^{0.498}$ [42]. Knowledge of K_2^{app} then allows (determination of c_A^{α}, the monomer concentration (g/L) in the mobile phase, by solution of the quadratic

$$K_2^{app}(c_A^{\alpha})^2 + c_A^{\alpha} - c_{tot}^{\alpha} = 0 \tag{16}$$

where the total protein concentration in the mobile phase (c_{tot}^{α}) has been assigned a relatively small value (0.10 g/L) in keeping with the situation pertaining in zonal size-exclusion chromatography [43].

The simulated dependence of partition coefficient σ_{av} for α-chymotrypsin upon concentration of sucrose (c_3) is summarized by the blue line in Fig. 1A, which illustrates the predicted inverse dependence of σ_{av} upon c_3 [Eq. (9)] in situations where the cosolute also partitions between the mobile and stationary phases. That the extent of the inverse dependence for a self-associating protein can be increased slightly by selecting a larger cosolute is illustrated by the green line in Fig. 1A – the calculated dependence of partition coefficient for α-chymotrypsin upon concentration of PEG-20000, a cosolute that also partitions between the two phases. In practice there would be little advantage to be gained from using the larger partitioning solute because the extra decrease in elution volume from a standard 1 × 30 cm Superdex 200 column (V^{σ} = 10.3 mL, V^{β} = 13.2 mL [38,39]) would be undetectable experimentally inasmuch as the predicted elution volumes [Eq. (1)] for α-chymotrypsin effected by 40 g/L sucrose and PEG-20000 are 16.01 and 15.97 mL respectively. The systematic decrease in elution volume for α-chymotrypsin effected by either cosolute is shown in Fig. 1B, which also illustrates the concentration-independent elution behaviour that is predicted for bovine serum albumin in the presence of either cosolute.

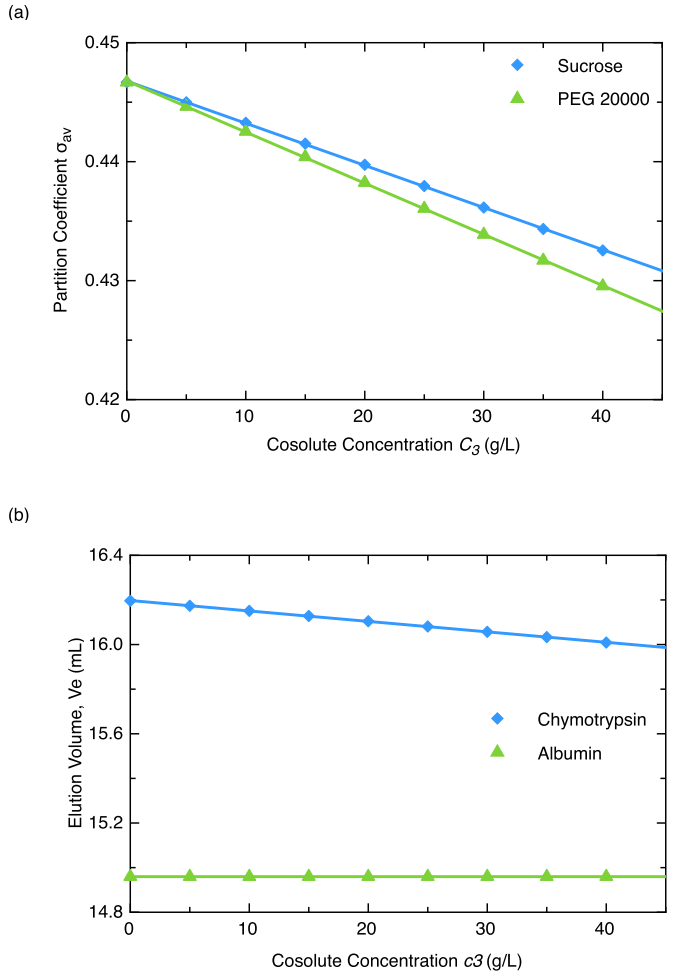

Fig. 1. Use of molecular crowding by an added cosolute for the detection of protein self-association by SEC. (A) Predicted effects of added sucrose (blue line) and PEG 20000 (green line) on the partition coefficient for α-chymotrypsin, a reversibly dimerizing system with K_2 = 5.5 L/g. (B) Corresponding predictions for the effects of cosolute concentration on the elution volumes of α-chymotrypsin and bovine serum albumin (a nonassociating protein) in SEC on a Superdex 200 column (1 × 30 cm) equilibrated with cosolute-supplemented acetate–chloride buffer (pH 3.9, *I* 0.2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

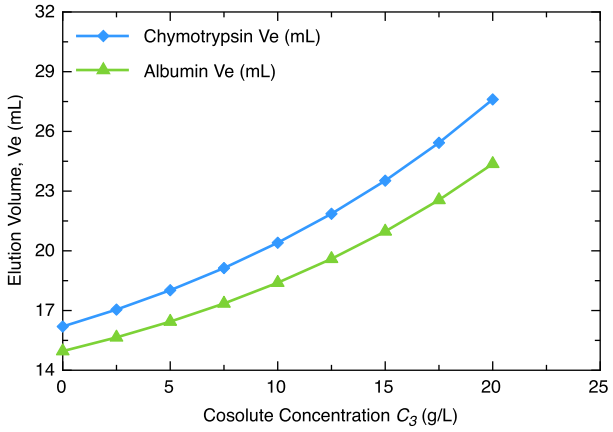

Fig. 2. Predicted concentration dependencies of elution volumes for the same α-chymotrypsin and bovine serum albumin systems (Fig. 1) in SEC experiments with dextran 500 as molecular crowding agent – a cosolute that is excluded from the stationary phase of the Superdex column.

The importance of selecting a partitioning cosolute for the detection of protein self-association is emphasized in Fig. 2, where positive concentration dependencies of the partition coefficient upon c_3 are predicted [Eq. (13)] for both proteins from simulations with an excluded species, dextran 500, as cosolute. In that regard allowance for the changing monomer – dimer distribution for α-chymotrypsin with dextran 500 concentration in the mobile (α) phase has entailed replacement of σ_2^0 in Eq. (13) by the value of σ_{av} deduced from the apparent association constant K_2^{app} [Eq. (14)] for each c_3^{α}. Clearly, the ability to detect the minor decreases in σ_{av} with increasing c_3^{α} is over-ridden by the need for transfer of protein (both oligomeric states of α-chymotrypsin) from the mobile to the stationary phase in order to meet the thermodynamic requirement for a fixed ratio of protein thermodynamic activities in the two phases [Eq. (3)]. Indeed, the form of the concentration dependence becomes strikingly similar to that predicted for the nonassociating protein, bovine serum albumin. Consequently, the use of molecular crowding for the detection of protein self-association is predicted to fail for a cosolute that is sufficiently large to be excluded from the stationary phase.

From the foregoing considerations is evident that the use of small partitioning cosolutes is required for size-exclusion chromatography to become a useful procedure for the detection of cosolute-induce protein self-association as the result of molecular crowding.

3.2. *Experimental consequences of cosolute supplementation of protein solutions in SEC*

Having illustrated the predicted consequences of cosolute supplementation on the SEC behaviour of nonassociating proteins and those undergoing reversible self-association, we now present qualitative experimental support for those theoretical predictions. Elution profiles obtained for bovine serum albumin from a 1 × 30 cm Superdex 200 column equilibrated with sucrose-supplemented acetate – chloride buffer (pH 3.9, *I* 0.2) are presented in Fig. 3A. Those from the corresponding experiments on α-chymotrypsin are shown in Fig. 3B. As predicted by Eq. (8), the elution volumes of albumin and its stable aggregate forms are independent of the sucrose concentration incorporated into the eluting buffer medium. On the other hand, the corresponding effect of sucrose supplementation on the elution profiles for α-chymotrypsin is a slight but progressive decrease in elution volume with increasing c_3, which is the prediction [Eq. (9)] for this reversibly dimerizing enzyme.

Although the results presented in Fig. 3b have demonstrated the potential use of macromolecular crowding for detecting reversible protein self-association by SEC, that observation only refers to experiments in which the cosolute also partitions between the mobile (α) and stationary (β) phases of the SEC column. As noted in the previous section, the use of polymeric cosolutes that are sufficiently large to be excluded from the stationary phase should lead to chromatographic profiles exhibiting a systematic increase in elution volume with increasing cosolute concentration for stable and reversibly-associating protein systems (Fig. 2). Experimental confirmation of that prediction is provided by elution profiles for bovine serum albumin (Fig. 4A) and α-chymotrypsin (Fig. 4B) in SEC experiments with dextran 500 as the molecular crowding agent. The progressive increases in elution volume with dextran 500 concentration are certainly smaller than those predicted in Fig. 2 on the basis of total cosolute exclusion from the stationary phase – a disparity that can be attributed, at least in part, to polydispersity of the dextran 500 preparation and hence partition into the stationary phase by molecules at the lower end of the molecular weight distribution. Irrespective of the consequent uncertainty about the concentration of excluded cosolute effecting the changes in elution volume, the important point to emerge from the results presented in Fig. 4A and B is the potential for those concentration-dependent increases in elution volume to be misconstrued as evidence of cosolute-induced protein dissociation rather than a consequence of using a

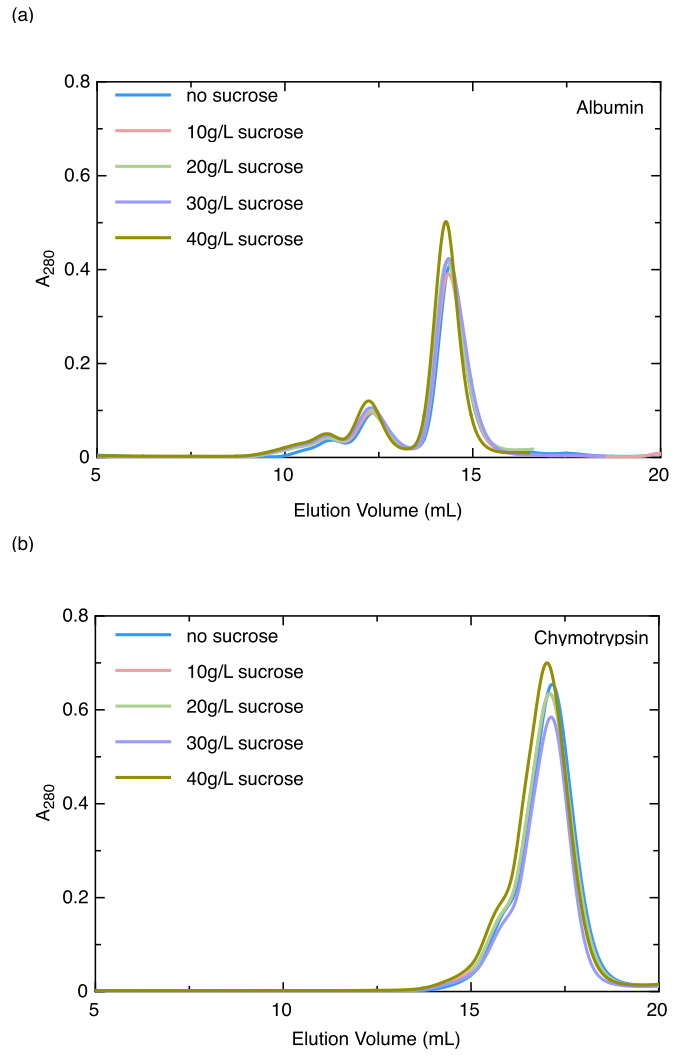

Fig. 3. Experimental verification of the predicted consequences of molecular crowding by sucrose on the SEC behaviour of proteins on a 1 × 30 cm column of Superdex 200 (pH 3.9, *I* 0.20). (A) Concentration independence of the elution volumes for bovine serum albumin and its stable aggregate states. (B) A systematic (albeit slight) inverse cosolute-concentration dependence for the dimerizing α-chymotrypsin system.

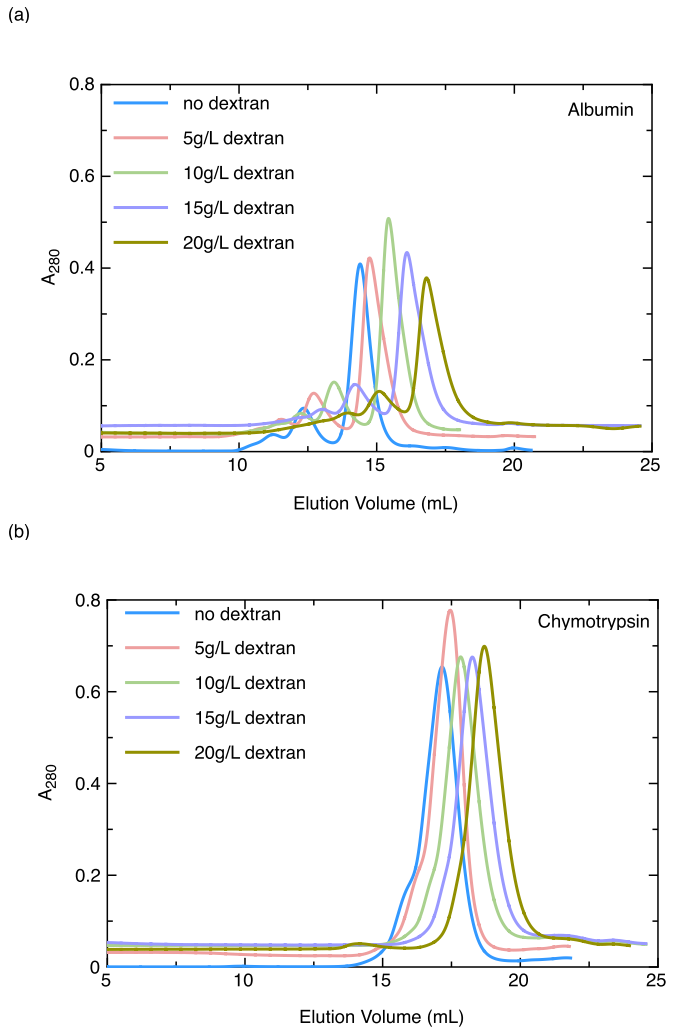

Fig. 4. Unsuitability of an excluded cosolute as molecular crowding agent for the detection of protein self-association by SEC. (A) The systematic increase in elution volume observed for bovine serum albumin and its aggregate states in SEC experiments on a 1 × 30 cm column of Superdex 200 equilibrated with acetate – chloride buffer (pH 3.9, I 0.20) containing the indicated concentrations of dextran 500. (B) The qualitatively similar systematic dependence of elution volume upon dextran 500 concentration for an enzyme (α-chymotrypsin) undergoing rapid reversible dimerization under the same conditions.

crowding agent that is largely confined to the mobile phase of the SEC column.

4. Concluding remarks

This investigation has served to illustrate the need for caution in the use of molecular crowding agents for the detection of reversible protein self-association by zonal size exclusion chromatography. Although cosolute-induced displacement of the self-association equilibrium position towards the oligomeric state always occurs in the mobile phase of the SEC column, that displacement is only manifested as a decreased elution volume for cosolutes that are sufficiently small to partition between the mobile and stationary phases of the SEC column. Use of a cosolute that is sufficiently large to be confined to the mobile phase gives rise to an increased elution volume that could well be misinterpreted as evidence of cosolute-induced dissociation. Fortunately, there is no particular advantage in choosing a large polymer as molecular crowding agent because the thermodynamic nonideality responsible for the equilibrium shift is governed by the product of the protein – cosolute second virial coefficient (B_{i3}) and the molar cosolute concentration [Eq. (14)]. The advantage of an enhanced magnitude of B_{i3} for a larger cosolute is thus offset by its correspondingly smaller molar concentration. The use of small cosolutes such as sucrose [20–23], PEG [35,44,45] and osmolytes such as trim ethylene N-oxide [24,25] as cosolute is therefore recommended for the detection of protein self-association by molecular crowding effects in size exclusion chromatography.

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