Resolution of Glycoproteins by Affinity-Based Reversed Micellar Extraction and Separation

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Affinity-based reversed micellar extraction and separation (ARMES) has proven effective in separating glycoproteins from nonglycosylated proteins from natural sources. The ability of ARMES to resolve closely related glycoproteins is of paramount importance if ARMES is to be used in glycoform resolution. It is demonstrated that ARMES can resolve the structurally similar soybean peroxidase (SBP; MW 37 kDa, pI 4.1) and α_1-acid glycoprotein (AGP; MW 43 kDa, pI 3.7), both of which have affinity for Concanavalin A (Con A) (the affinity ligand). SBP was almost exclusively extracted at pH 8 and above, with a separation factor greater than 50 (resolution $\sim$ 20), far better than was possible using Con A affinity chromatography ($R \sim 0.25$, separation factor $\sim$ 2). Model calculations suggest that differences in affinity measured by an equilibrium-building assay cannot account for the favorable extraction of SBP over AGP at higher pH. Hydrophobic interactions and/or charge shielding appear to affect partitioning of the lectin–glycoprotein complexes and add greatly to the selectivity of extraction in ARMES, especially at higher pH values.

Introduction

Many of the products of biotechnology, including recombinant genetic engineering, cell tissue culture, and monoclonal technologies, are glycoproteins. The position of glycosylation and the carbohydrate sequence determine to a large extent the physicochemical properties of glycoproteins. Despite the importance of glycoproteins in biotechnology and glycobiology, elucidating the precise role of glycosylated proteins in biological processes has been slowed, in part, due to difficulties associated with separating proteins that differ only in the glycosylation pattern. Lectin affinity chromatography has been effective for purifying glycoproteins from nonglycosylated proteins (Dakour et al., 1987; Leiner et al., 1986), and affinity matrices are available from a number of commercial sources. Nonetheless, such methods are difficult to scale and are unable to resolve closely related glycoproteins (Ohlson et al., 1989). We have developed a technique known as affinity-based reversed micellar extraction and separation (ARMES), which combines the affinity of lectin–glycoprotein interactions with the selective, liquid–liquid extraction of the affinity complex into a reversed micellar organic phase (RMOP). This technique has been shown to work with model systems (Paradkar and Dordick, 1991) and protein mixtures from natural sources (Paradkar and Dordick, 1993). ARMES appears to have advantages over other affinity techniques due to the inherent ease and scalability of liquid–liquid extractions. Moreover, ARMES affords selectivity at both the affinity step (because only those species that interact with the ligand will be extracted) and at the extraction step (where electrostatic and hydrophobic interactions of the ligand–ligate complex are important). Thus, high binding ligands are unnecessary, and dissociation of the ligate from the affinity ligand can be performed under relatively mild conditions that will not damage the protein of interest (Paradkar and Dordick, 1993).

In the present work, we examine the resolution of two structurally similar glycoproteins, both with affinity to the

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lectin, Concanavalin A (Con A). In particular, soybean hull peroxidase (SBP, 37 kDa, pI 4.1) and bovine α_1-acid glycoprotein (AGP, 43 kDa, pI 3.7) have similar molecular weights and isoelectric points. Hence, their separation, even in such a synthetic mixture, is difficult using gel permeation and ion-exchange chromatographies. The influence of various extraction parameters on the separation and resolution of SBP and AGP is evaluated using an equilibrium-binding model that reflects affinity constants, aqueous-phase pH, and the stoichiometry of lectin–glycoprotein association.

Materials and Methods

Materials

Concanavalin A from *Canavalia ensiformis* (jack bean, type IV); peroxidase from soybean (SBP); α_1-acid glycoprotein from bovine (AGP); bovine serum albumin; lectin from *Arachis hypogaea*; isooctane, 1,3-bis[tris(hydroxymethyl) methyl-amino] propane (bisTris propane); Aerosol OT (AOT; sodium di-(2-ethylhexyl) sulfosuccinate); 2,2-azino-di-[3-ethyl-benzothiazolin-6-sulfonic acid] (ABTS); 4-chloro-1-naphthol; Con A-Sepharose; bovine serum albumin (BSA); and α-D-methyl-mannopyranoside were obtained from Sigma (St. Louis, MO) and used without additional purification. TMB turbo (one step 3,3′5,5′-tetramethylbenzidine substrate for peroxidase) and bicinchoninic acid protein assay (BCA) were obtained from Pierce (Rockford, IL). Phastgel reagents and system were obtained from Pharmacia (Upsalla, Sweden). High protein-binding Maxisorp plates were from Nunc (Denmark).

General procedure for extractions

Forward Extraction at Different pH Values. Glycoproteins (10.8 nmol) were dissolved in 2.0-mL volumes of 10-mM bisTris propane, 100 mM NaCl, containing 2.92 mg (54 nmol) of Con A. After incubation at room temperature (5 min), the aqueous solutions were contacted with an equal volume of 40-mM aerosol OT (AOT) in isooctane (the reversed micellar organic phase (RMOP)) and shaken at 300 rpm and 25°C for 15 min. The phases were separated using a 1-min microcentrifugation step.

Back Extraction. The organic phase containing glycoproteins (and Con A) was contacted with an aqueous receiving phase consisting of 25-mM sodium pyrophosphate (pH 8.5), 200-mM NaCl, and 200-mM methyl α-D-mannopyranoside for 30 min at 300 rpm.

Con A Reextraction. To assess the total amount of glycoprotein extracted it was necessary to selectively remove Con A from the aqueous recovery phase. To that end, the aqueous phase pH was reduced to 5.8 with 30-mM HCl, followed by contacting the solution with an equal volume of 40 mM AOT in isooctane (20 min at 300 rpm), thereby resulting in selective extraction of Con A into the RMOP. SDS-PAGE verified the quantitative removal of Con A from the receiving phase. Total protein in the receiving phase was measured using BCA protein assay (Pierce), and the concentrations of SBP and AGP in the final extracted mixture were determined after measurement of SBP activity using ABTS as the chromogen (Pütter and Reinhild, 1983).

Microtiter Plate Equilibrium-Binding Assays

Con A (100 μL of 0.1 mg/mL in 10 mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS)) was directly adsorbed to high protein binding Maxisorp plates at 4°C for 48 h. The plates were blocked with BSA (300 μL of 0.5% w/v solution in PBS) for 2 h at room temperature and then rinsed well (6 well volumes) with PBS containing 0.05% (v/v) Tween-20. Plates were incubated for 2 h at room temperature with various concentrations of SBP (100 μL in BTP containing 0.05% (v/v) Tween-20). Color was developed using a 100 μL TMB turbo substrate to each well and incubating at room temperature for 4 min prior to the addition of 100 μL 0.5 M H_2SO_4. The absorbance was measured at 450 nm. Sigmoidal binding curves were assessed by nonlinear fitting algorithms. Competitive binding assays were performed in a similar manner with a fixed concentration of SBP (0.02 mg/mL or 5.4×10^{-7} M) and in the presence of different concentrations of competing AGP.

Preparation of Apo-Concanavalin A

Con A was treated to remove bound manganese and calcium, according to methods found in the literature (Toselli et al., 1981). Briefly, 100 mg of Con A was dissolved in 2 mL of 0.1 M HCl and dialyzed (MWCO 3500) against 1 L of 0.1 M ethylenediamine tetraacetic acid (EDTA) for 4 h at 4°C, pH 7.0. This was followed by extensive dialysis against 18 MΩ water. The apo-Con A was then lyophilized.

The Ca^{2+} and Mn^{2+} content in the apo-, native and metal-saturated Con A was determined by flame atomic absorption spectrometry using a Varian model AA-1475 atomic absorption spectrometer (Mulgrave, Australia) with an air/acetylene flame (Mn) or a nitrous oxide/acetylene flame (Ca). Standards and samples were prepared in 1.0% (v/v) HCl containing 2,000-μg/mL potassium (to minimize metal ionization).

Microtitration Calorimetry

Titration calorimetry was carried out using a Microcal microcalorimeter (Microcal, Northhampton, MA). Con A (5.0 mg/mL in 10-mM dimethylglutarate, 100-mM NaCl, pH 5.0) was thermally equilibrated in the cell prior to titration with glycoprotein. Injections of 6 μL of glycoprotein solution, dissolved in the same buffer as the Con A, were made using the computer-driven syringe at 8-min intervals. Calculations of thermodynamic properties were performed using the associated Microcal Origin software.

Affinity Chromatography

A Con A–Sepharose column (0.7 cm×20 cm, 10-mg Con A/mL packed resin) was equilibrated with 10-mM bistris propane–HCl, pH 7.5, containing 1-mL $CaCl_2$, 1-mM $MnCl_2$, and 100-mM NaCl. A mixture of SBP and AGP (0.8 mg and 0.92 mg, respectively) was loaded onto the column. Elution (0.1 mL/min) was made isocratically with either 20-mM α-D-methylmannopyranoside or in a linear gradient mode from 10 to 500 mM. Fractions were monitored at 280 nm (total protein) and 403 nm (Soret absorbance for the heme group of SBP), as well as for enzyme activity.

Results and Discussion

The ARMES process is shown in Figure 1. The separation of glycoproteins and their eventual resolution into positional and compositional glycoforms is a daunting task in glycobiology and biotechnology due to the complexity of the glycan chains and the often similar sizes and ionic properties of the proteins. For protein glycoforms, this is complicated further because the *only* difference in structure is governed by the glycans. This study demonstrates that ARMES can be used to separate glycoproteins that are very similar in their protein structure and that both show affinity for Con A.

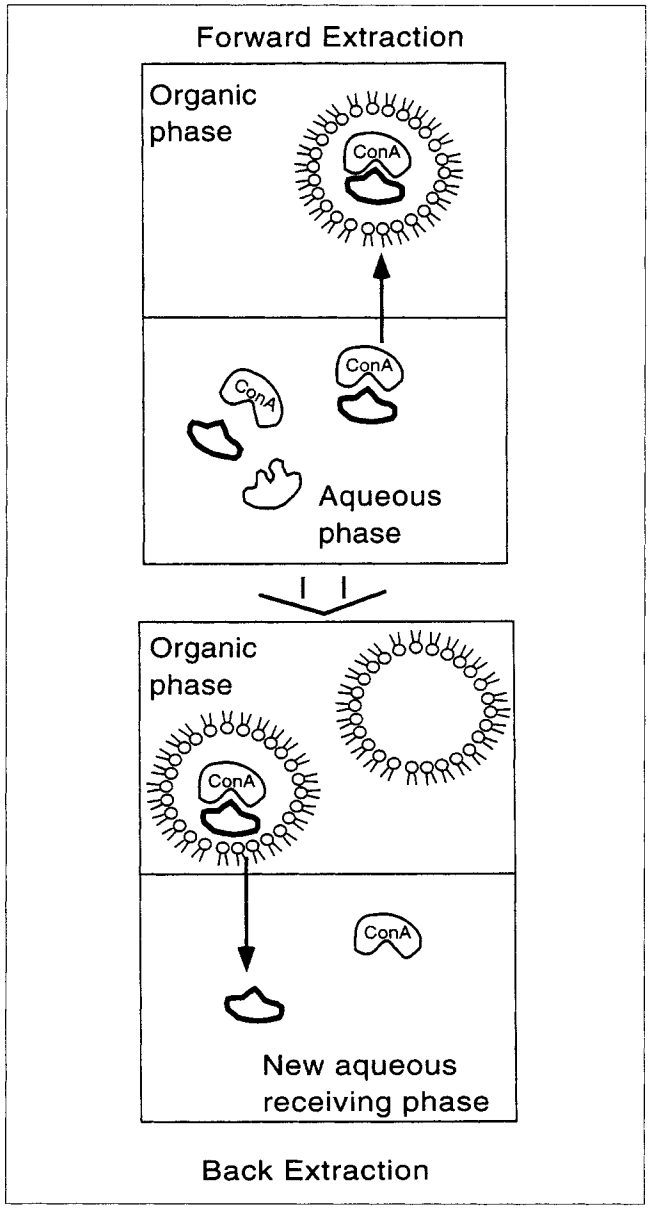

Figure 1. The ARMES process.

Forward extraction involves affinity interaction between a lectin (such as Con A) and a glycoprotein to separate from a mixture of glycoproteins. The backward extraction results in the recovery of the glycoprotein into an aqueous recovery phase.

Resolution of SBP and AGP by ARMES

Reversed micellar extraction of the acidic glycoproteins SBP and AGP occurs at pH values below their respective pIs where the proteins are positively charged and can interact with the negative charges of the AOT surfactant. At pHs above the respective pIs, this electrostatic interaction weakens and the extraction efficiency falls. Indeed, in the pH range used in this work (pH $\geq$ 6), the extraction efficiency is zero (data not shown). In the presence of Con A, however, both SBP and AGP are extracted at pH $\geq$ 6 using ARMES. Con A, a basic protein (Bhattacharyya and Brewer, 1990), can still interact with the AOT head anionic groups at pH $\geq$ 6, and is extracted. Any glycoproteins complexed to Con A are also extracted via ARMES at pHs well above their pI values (Choe et al., 1997).

The extraction efficiency of ARMES as a function of the aqueous-phase pH is shown in Figure 2. No extraction of either glycoprotein is seen in the pH range from 6.0 to 8.5 in the absence of Con A (data not shown). In the presence of excess Con A, however, both SBP and AGP are extracted using ARMES. Excess lectin is required due to the relatively weak affinity of Con A to glycoproteins (K_d about 10^{-6} M) as well as the tendency of Con A to extract on its own, thereby lowering the concentration of Con A available to bind to SBP or AGP in the aqueous phase.

In these single-protein experiments, SBP was extracted to a greater degree than AGP, and this effect was pH-dependent. Indeed, at pH 6.0, the fraction of SBP extracted is approximately 1.7-fold higher than that for AGP, and this value increases to nearly 3.2 at pH 8.5. The results obtained for each protein in a binary mixture of SBP and AGP differ from those for each glycoprotein individually (Figure 2). The efficiency of AGP extraction in a mixture with SBP is reduced

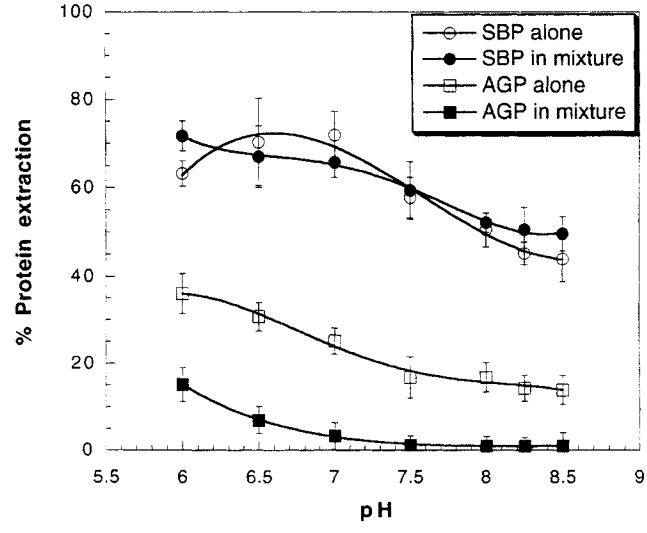

Figure 2. Amount of protein extracted via ARMES as a function of pH for (○) SBP in single protein extraction; (□) AGP in single protein extraction; (●) SBP from a binary mixture with AGP; (■) AGP from a binary mixture with SBP.

Conditions for extraction are as described in the "Materials and Methods" section. Mass balance closure is observed for all cases.

over the entire pH range, approaching zero at pH 7.5. The extraction efficiency of the SBP, however, is not appreciably altered by the addition of a competing glycoprotein into the starting mixture, possibly suggesting that the affinity interaction of SBP with Con A is significantly stronger than that for AGP. These results indicate that ARMES is able to resolve SBP and AGP from a synthetic mixture.

Determination of lectin–glycoprotein affinity constants

As depicted in Figure 1, ARMES consists of two discrete steps: a lectin–glycoprotein affinity interaction to form an extractable complex, followed by extraction of this complex into the RMOP. Both steps can be expected to influence the resolution of glycoproteins from a mixture. Fortunately, we can distinguish between affinity interaction and protein partitioning between the two phases. Enzyme-based microplate assays represent a convenient means of determining dissociation constants (Larvor et al., 1994). Moreover, it was convenient to use a simple colorimetric peroxidase assay using 3,3′,5,5′-tetramethylbenzidine as the chromogenic substrate to quantify the concentration of SBP. The activity of SBP was identical in the presence (1 mg/mL) and absence of Con A (data not shown).

The binding of SBP to Con A immobilized in microtiter plate wells was specific and could be prevented in the presence of α-D-methylmannopyrannoside and in wells coated either with BSA or a control lectin, *Arachis hypogaea*, which does not bind to SBP (data not shown). The binding of SBP to Con-A-coated plates displayed a concentration-dependent binding curve, and numerical analysis yielded a K_d of 2.4×10^{-6} M at pH 6.5 (Figure 3a). Table 1 shows the measured K_d values over the pH range of interest. The dissociation constant of Con A–SBP was relatively insensitive to pH in the range from pH 6.5 to 8, but decreased above pH 8.

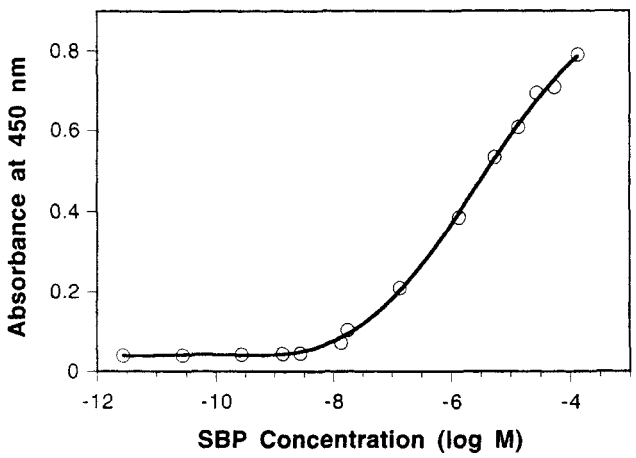

Figure 3a. Microtiter plate equilibrium binding assay between Con A (immobilized) and SBP at pH 6.5.

Conditions: plates were incubated for 2 h at room temperature with various concentrations of SBP (100 μL in BTP containing 0.05% (v/v) Tween-20). Color was developed using 100 μL TMB substrate to each well and incubating at room temperature for 4 min prior to the addition of 100 μL 0.5 M H_2SO_4. The absorbance was measured at 450 nm.

Table 1. Affinity Constants Measured in the Microtiter Plate Binding Assay

Aqueous Phase pH	6.5	7.0	7.5	8.0	8.5
SBP	2.4 μM	2.2 μM	1.7 μM	2.1 μM	5.7 μM
AGP	44 μM	41 μM	26 μM	13 μM	67 μM

It is important to note that each of the glycoproteins studied comprises a large number of glycoforms that may display different binding affinities with Con A. For example, while SBP is expected to have on average only one high mannose-type glycan chain, these glycans may have as many as six mannose residues attached to their trimannose core structure ($Man_3GlcNAc_2$) (Gray et al., 1996). Con A shows higher affinities for oligomannose glycopeptides with a greater number of mannose residues present. For example, Con A displays greater affinity for $Man_xGlcNAc_2$ when x is 7–9 than when x is 5 or 6 (Mandal and Brewer, 1993). Additional complex-type glycans can also be present for which Con A will have varying affinities. Thus, any affinity technique will generate an estimate of overall affinity for the population rather than the affinity with which Con A interacts with a particular glycan chain or glycoform. (Scatchard plots prepared based on the binding experiments were curved, reflecting a population of glycoforms having different interaction affinities (data not shown). This was expected as SBP (as well as AGP) consist of multiple glycoforms, each likely to have different affinity for Con A.) The binding constants reported herein, however, are useful for evaluating the overall behavior of each glycoprotein and also for providing information on the effect of pH on affinity.

Competitive binding assays in which AGP competes with SBP for binding to immobilized Con A show a concentration-dependent response (Figure 3b). The interaction between Con A and bovine AGP yielded a K_d of 4.4×10^{-5} M at pH 6.5. (It should be noted that a K_d of $0.99–1.73 \times 10^{-5}$ M was previously reported for the interaction of Con A and

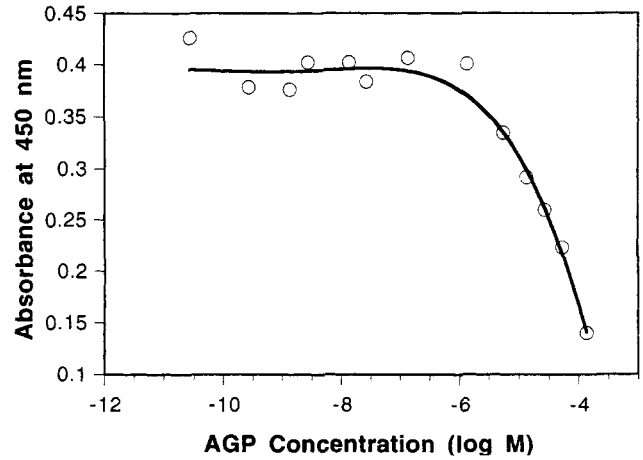

Figure 3b. Competitive binding assay with AGP and SBP for binding sites of Con A at pH 6.5.

Competition binding assays were performed in a similar manner with a fixed concentration of SBP (0.02 mg/mL or 5.4×10^{-7} M) and in the presence of different concentrations of competing AGP.

the closely related human AGP as determined by affinity electrophoresis (Bøg-Hansen and Takeo, 1980).) The interaction of Con A with AGP was about an order of magnitude weaker than that observed for SBP binding to Con A. The estimated K_d values for bovine AGP over the pH range of interest in this study are presented in Table 1. As was the case for SBP, no major differences in K_d were observed over a pH range from 6.5 and 8.5.

Lectin–glycoprotein stoichiometry and metal content

Con A is a multivalent lectin consisting of two glycan binding sites per dimer. Con A is either a dimer or a tetramer (with four binding sites) over the pH range of the current study (Mandal and Brewer, 1992), and may be expected to form cross-linked structures with glycoproteins (Bhattacharryya et al., 1990). Cross-linking can result in the formation of precipitates that cannot undergo extraction into the RMOP. However, these precipitates do not form under the conditions used for ARMES. This suggests that the actual stoichiometry of Con A–glycoprotein complexes is not multivalent. To test this hypothesis, microtitration calorimetry was performed at pH 5.0, using buffer conditions that matched as closely as possible those used in the ARMES extraction (see Figure 4). Because of the tendency for Con A to self-aggregate and precipitate at higher pH values (McKenzie et al., 1972), a pH of 5 was used. Nonlinear numerical analysis yielded an n coefficient of 0.12, based on a monomeric molecular weight of 27 kDa for Con A. This n is consistent with a single SBP molecule per four Con A dimers, much lower than the theoretical value of two carbohydrate-binding sites per Con A dimer. The stoichiometry measured for this interaction suggests that a significant portion of the Con A binding sites present are noninteracting.

Con A is a metalloprotein and requires both Ca^{2+} and Mn^{2+} ions for binding. Carbohydrate binding sites that lack both Ca^{2+} and Mn^{2+} ions are nonbinding. Therefore, it is possible that the observed stoichiometry of one SBP per four Con A dimers results from a portion of the sites being metal-deficient. In the present study, commercial Con A is used in the ARMES extractions without additional metal. Atomic absorption spectrometry indicated that this Con A contained 0.42 mol of Ca^{2+} and 0.10 mol of Mn^{2+} per mole of Con A dimer. Previous literature estimates indicated 1.20 mol of Ca^{2+} and 1.50 mol of Mn^{2+} per mole of Con A dimer purified from jack beans (Doyle et al., 1975). The amount of bound Ca^{2+} has a significant effect on the carbohydrate binding and precipitation activity of Con A, although the Mn^{2+} content has been shown to be less important (Obata et al., 1981). When 1 mM Ca^{2+} and Mn^{2+} were added to the buffer used in microtitration calorimetry experiments, the calculated stoichiometry increased ($n \sim 0.36$). Unfortunately, turbidity developed throughout the titration resulting from increased cross-linking, making it impossible to accurately calculate the stoichiometry and strength of the interactions (data not shown).

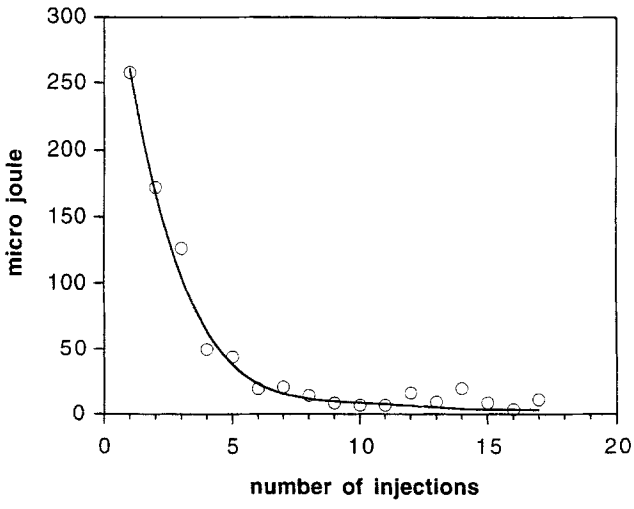

Figure 4. Integrated curve for the calorimetric titration of Con A with SBP.

Experimental conditions: Con A (5.0 mg/mL in 10-mM dimethylglutarate, 100-mM NaCl, pH 5.0) titrated with 5-μL injections of protein solution (SBP, 85.0 mg/mL in 10-mM dimethylglutarate, 100-mM NaCl, pH 5.0). Calculated value for $n = 0.12$.

Based on the aforementioned results, the influence of metal content, and thus stoichiometry, on ARMES extraction, was of interest for further study. As expected, apo-Con A, which contained less than 9% of the total possible metal content, was not effective in the ARMES extraction of SBP, extracting only 7% of the total SBP present. Commercially available Con A, used without added metal, extracted 55% of the SBP. Con A, fully saturated with metal (i.e., with 1 mM each of Ca^{2+} and Mn^{2+} added to the buffer), failed to extract any SBP into the RMOP. Large quantities of precipitate were observed in the aqueous feed phase, indicating the formation of cross-linked structures with SBP. These results suggest that large complexes, such as those formed between metal-saturated Con A and glycoprotein, are not well extracted into the RMOP. The reduced stoichiometry of the metal-deficient, commercially available Con A facilitates the formation of small and extractable complexes. Thus, the importance of valence as controlled by metal content on the efficiency of glycoprotein extraction by ARMES is apparent.

Modeling the ARMES extraction

To understand the observed resolution of glycoprotein extraction in the Con A-SBP-AGP system, we developed a simplified equilibrium extraction model as depicted in Figure 5. Con A interacts with both SBP and AGP with the respective

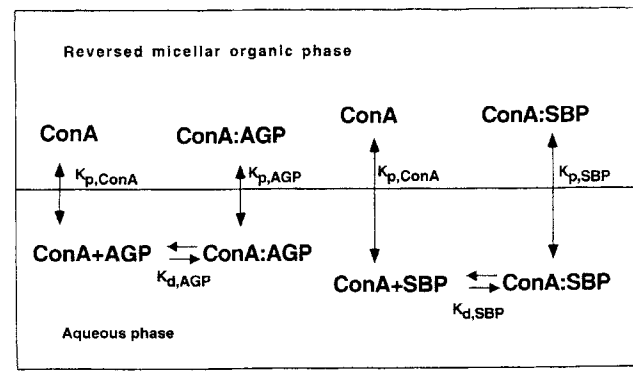

Figure 5. Equilibrium extraction model.

dissociation constants, and Con A partitions into the RMOP, either alone or in complexes with SBP and AGP. The separation of SBP from AGP in ARMES can be given by the separation factor (α), as depicted in Eq. 1, and this value can be determined over the entire pH range employed:

$$\alpha = \frac{[\text{SBP}]_{\text{org}}}{[\text{AGP}]_{\text{org}}} = \frac{K_{p_{\text{SBP}}}}{K_{p_{\text{AGP}}}} \times \frac{K_{d_{\text{AGP}}}}{Kd_{\text{SBP}}} \times \frac{[\text{SBP}]_{\text{aq}}}{[\text{AGP}]_{\text{aq}}}. \quad (1)$$

Ratios of partition coefficients and the concentrations of the free glycoproteins in the aqueous phase can be determined by simultaneous solution of eight equilibrium mass-balance expressions (Eqs. 2–9). The ratios of lectin–glycoprotein dissociation constants are obtained from Table 1:

$$K_{d,\text{SBP}} = \frac{[\text{SBP}_{\text{aq}}][\text{ConA}_{\text{aq}}]}{[\text{ConA:SBP}_{\text{aq}}]} \quad (2)$$

$$K_{d,\text{AGP}} = \frac{[\text{AGP}_{\text{aq}}][\text{ConA}_{\text{aq}}]}{[\text{ConA:AGP}_{\text{aq}}]} \quad (3)$$

$$K_{p,\text{SBP}} = \frac{[\text{ConA:SBP}_{\text{org}}]}{[\text{ConA:SBP}_{\text{aq}}]} \quad (4)$$

$$K_{p,\text{AGP}} = \frac{[\text{ConA:AGP}_{\text{org}}]}{[\text{ConA:AGP}_{\text{aq}}]} \quad (5)$$

$$K_{p,\text{ConA}} = \frac{[\text{ConA}_{\text{org}}]}{[\text{ConA}_{\text{aq}}]} \quad (6)$$

$$\text{SBP}_{\text{total}} = [\text{SBP}_{\text{aq}}] + [\text{ConA:SBP}_{\text{aq}}] + [\text{ConA:SBP}_{\text{org}}] \quad (7)$$

$$\text{AGP}_{\text{total}} = [\text{AGP}_{\text{aq}}] + [\text{ConA:AGP}_{\text{aq}}] + [\text{ConA:AGP}_{\text{org}}] \quad (8)$$

$$\text{ConA}_{\text{total}} = [\text{ConA}_{\text{aq}}] + [\text{ConA}_{\text{org}}] + [\text{ConA:SBP}_{\text{aq}}] + [\text{ConA:SBP}_{\text{org}}] + [\text{ConA:AGP}_{\text{aq}}] + [\text{ConA:AGP}_{\text{org}}]. \quad (9)$$

Assuming a 1:1 Con A–glycoprotein stoichiometry, the values of α as a function of aqueous-phase pH are shown in Figure 6a. Clearly, at pH $\geq$ 7.5, the value of α reaches ca. 50, indicating an extremely efficient separation between SBP and AGP. The ratios $K_{p_{\text{SBP}}}/K_{p_{\text{AGP}}}$ and $K_{d_{\text{AGP}}}/K_{d_{\text{SBP}}}$ are plotted vs. pH in Figure 6b. Two distinct regions are evident. For pH $\leq$ 7.0, the ratio of $K_{p_{\text{SBP}}}/K_{p_{\text{AGP}}}$ is small relative to $K_{d_{\text{AGP}}}/K_{d_{\text{SBP}}}$ and contributes less to a large separation factor. For pH $\geq$ 7.5, $K_{p_{\text{SBP}}}/K_{p_{\text{AGP}}}$ is relatively large compared to $K_{d_{\text{SBP}}}/K_{d_{\text{AGP}}}$ and contributes more to a large separation.

The values of $K_{d_{\text{AGP}}}/K_{d_{\text{SBP}}}$ do not differ substantially over the pH range employed (Table 1). Thus, significant differences in the relative *extraction* of the two different lectin–glycoprotein complexes must exist. One possible cause for this partitioning behavior is the shielding of charges on both the Con A and the glycoproteins upon formation of the two glycoprotein complexes. The relevance of charge shielding to ARMES has been discussed at length in our previous work (Choe et al., 1997). Nonetheless, inspection of Figure 2 provides a glimpse into the mechanism of ARMES. Substantial extraction of SBP and AGP occurs when the proteins are complexed with Con A even at pH 8.5, well above the pI

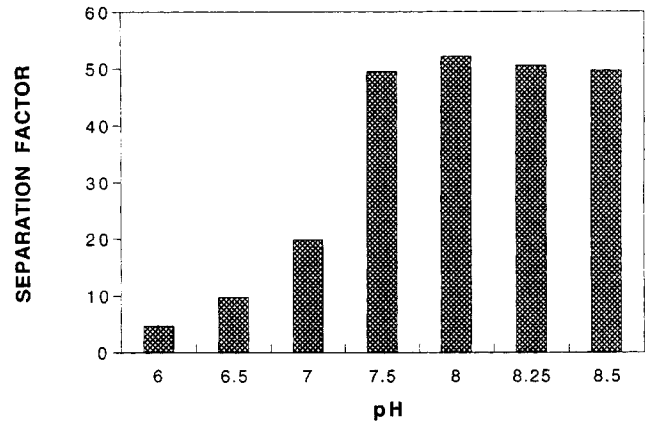

Figure 6a. Observed separation factor (α) as a function of pH for the ARMES resolution of SBP and AGP (Eq. 1).

values of either SBP or AGP and above the pI value of Con A (pI 8.0; Bhattacharyya and Brewer, 1990). Because the structures (and charge distribution) of SBP and AGP surely differ, the interaction of each Con A–glycoprotein complex with the AOT-based reversed micelles, which is dominated by electrostatics, is expected to vary. Thus, the values of $K_{p_{\text{SBP}}}/K_{p_{\text{AGP}}}$ differ, leading to high separation factors. Simply by changing the pH of the aqueous phase, significant differences in the observed separation factor (and therefore resolution) can be achieved.

This property of ARMES is not obtained using conventional affinity chromatography. Using a column prepared from commercially available Con A–Separose, SBP and AGP were poorly resolved using either standard isocratic or gradient elution with α-D-methylmannopyranoside. These separations afforded a resolution of only 0.25, corresponding to a separation factor of $\sim$ 2 (Figure 7), consistent with the similar K_d

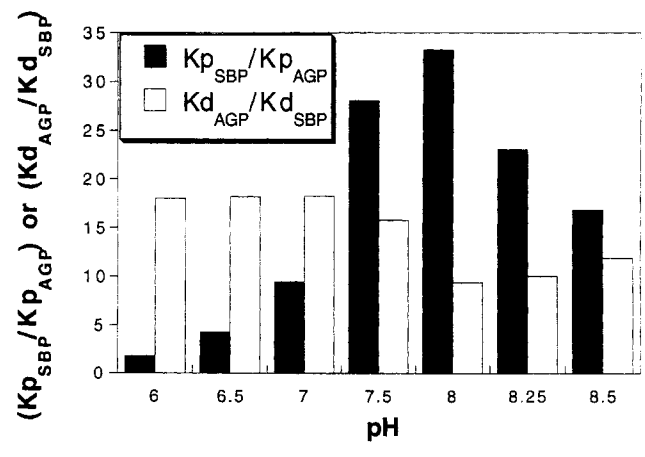

Figure 6b. Relative partition coefficient (K_p) and relative affinity (K_d) as a function of pH for SBP and AGP derived from experimental data and simultaneous solution of Eqs. 2–9.

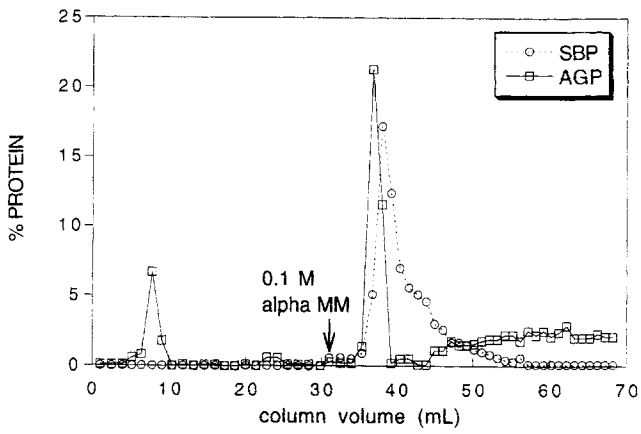

Figure 7. Affinity chromatographic separation of SBP and AGP.

Conditions: Con A Sepharose column (0.7 cm × 20 cm, 10-mg Con A/mL packed resin) equilibrated with 10-mM bistris propane-HCl, pH 7.5, containing 1-mM $CaCl_2$, 1-mM $MnCl_2$, and 100-mM NaCl. Sample: Mixture of SBP and AGP (0.8 mg and 0.92 mg, respectively). Elution: Isocratically with 20-mM α-D-methyl-mannopyranoside at 0.1 mL/min. Fractions were monitored at 280 nm and 405 nm as well as for enzyme activity. A similar elution profile resulted when a linear gradient elution was performed with 10–500-mM α-D-methylmannopyranoside.

values for Con A with SBP and AGP. This compares very poorly with the separation factor of 50 (at pH 7.0) achieved in ARMES. While it may be possible to improve somewhat the resolution of the affinity chromatography with further optimization, we do not believe it will be possible for affinity chromatography to approach the extremely high resolution obtained with ARMES.

Conclusions

Interactions between individual lectin binding sites and carbohydrates are often relatively weak, but nature has compensated for this by engineering in multimeric interactions. By contrast ARMES depends on weak monomeric interactions. Unlike other commonly used affinity techniques (e.g., antibody–antigen or avidin–biotin), high affinity interactions are not required in ARMES. Based on low-affinity monovalent interactions, ARMES is capable of resolving glycoproteins with nearly identical molecular weights and isoelectric points. Furthermore, the excellent resolution ($\alpha \sim 50$) achieved is substantially better than that afforded by the more traditional approach of Con A–Sepharose affinity chromatography ($\alpha \sim 2$), and reflects the added resolution achieved by combining affinity interactions with selective liquid–liquid extractions. ARMES is a relatively simple method that only requires the adjustment of the pH of the aqueous phase. Indeed, the best resolution of the two glycoproteins was pH $\geq$ 7.5, a region in which the partition coefficients have the greatest contribution to the separation factor. Further development of ARMES for the resolution of glycoforms will require a better understanding of the mechanism of extraction and the role of other parameters on separation, such as charge-shielding and hydrophobic interactions. Such studies are underway.

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