Parameters Affecting the Efficiency of Affinity-Based Reversed Micellar Extraction and Separation (ARMES) in Glycoprotein Purification

Jaehoon Choe,[†] Victoria A. VanderNoot,[†] Robert J. Linhardt,[†,‡] and Jonathan S. Dordick*[,†,‡]

Department of Chemical and Biochemical Engineering and Medicinal and Natural Product Chemistry, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242

Affinity-based reversed micellar extraction and separation (ARMES) is an effective method for purifying both low and high molecular weight glycoproteins via liquid-liquid extraction. A range of extraction conditions were examined to gain insight into the mechanism of ARMES. Concanavalin A (Con A) was used as the model affinity ligand to bind soybean peroxidase (SBP) and β-galactosidase as model glycoproteins. Factorial design was used to investigate the effect of various system variables on the extraction of SBP via ARMES. A quadratic model described the system well, resulting in a standard deviation of 7% between calculated and experimental extraction efficiencies. Sensitivity analysis suggested that the key criteria in ARMES were the NaCl concentration and pH of the aqueous feed phase. Extraction of both glycoproteins decreased above pH 7 but fell to zero only at pH values significantly above the pI of the model glycoproteins and the Con A affinity ligand. It is proposed that the complex of the affinity lectin with the glycoprotein results in a sufficiently hydrophobic species that can be extracted into a reversed micellar organic phase even at pH's far above the pI's of the individual proteins that comprise the complex. This finding has practical considerations for the use of ARMES in the resolution and purification of protein glycoforms.

Introduction

Glycoproteins are ubiquitous in eukaryotes and thus represent an important class of proteins. Indeed, the majority of secreted or membrane proteins in cells are glycosylated and glycoproteins function in such diverse roles as cell−cell recognition and protein folding (Montreuil, 1995). While affinity chromatographic methods employing carbohydrate-binding lectins have been used effectively to purify glycoproteins (Dakour, 1987; Leiner, 1986), such methods are generally discontinuous and are not always scaleable (Ohlson et al., 1989).

In previous work, we developed a new method for purifying glycoproteins via affinity-based reversed micellar extraction and separation (ARMES) (Paradkar and Dordick, 1991). ARMES combines an affinity step of a lectin−glycoprotein interaction with the selective extraction of the affinity complex into a reversed micellar organic phase (RMOP). This technique is convenient for separating both model (Paradkar and Dordick, 1991) and complex protein mixtures (Paradkar and Dordick, 1993). ARMES has advantages over other affinity techniques due to the inherent ease and scalability of liquid−liquid extractions. Moreover, ARMES affords selectivity at both the affinity interaction step and at the extraction step. Thus, high binding ligands are unnecessary, and dissociation of the ligate from the affinity ligand can be performed under relatively mild conditions (Paradkar and Dordick, 1993).

ARMES has already been described in detail in the literature (Paradkar and Dordick, 1993); consequently, only a brief description will be given here. The extraction is carried out under pH and ionic strength conditions in which the glycoprotein to be purified is not directly extracted into the RMOP. This may be accomplished by using an anionic surfactant (aerosol OT or AOT) and a pH that is greater than the pI of the glycoprotein (but not greater than the pI of the affinity ligand, the lectin). Under these conditions, the protein will have a net negative charge and will not interact with the negatively charged head groups of the AOT surfactant. The lectin will be positively charged and able to interact with the surfactant and will be extracted into reversed micelles. When the affinity ligand interacts with the target glycoprotein, the lectin then acts as a facilitative carrier of the glycoprotein into the RMOP. In complex mixture separations, a key step in the purification is a pre-extraction carried out at the conditions to be used for the purification but in the absence of the lectin affinity ligand. In this manner, any proteins that might directly partition into the RMOP are removed.

The presence of an affinity ligand makes the situation more complex in ARMES than conventional protein extraction into a RMOP (Goklen and Hatton, 1985; Wolbert et al., 1989). The extraction efficiency of ARMES depends on both the interaction of the lectin with the glycoprotein as well as the extractability of the complex. Various factors may affect the extractability of the glycoprotein complex including the physicochemical properties of the organic solvent, aqueous phase pH and ionic strength, and the chemistry and concentration of the surfactant. In the present study, the effect of extraction variables on ARMES is investigated using two model glycoproteins, soybean peroxidase (SBP) and β-galactosidase (GAL from *Aspergillus oryzae*). A factorial analysis

[†] Department of Chemical and Biochemical Engineering.
[‡] College of Pharmacy.
* Author to whom correspondence should be addressed at the Department of Chemical and Biochemical Engineering.

S8756-7938(97)00049-0 CCC: $14.00

was used to model the extractive behavior of SBP in ARMES, and sensitivity analysis of the model was used to identify the most important extraction variables for further study.

Materials and Methods

Materials. Concanavalin A from *Canavalin ensiformis* (jack bean, type IV), β-galactosidase (Grade XI) from *A. oryzae* (GAL), aerosol OT (AOT; sodium bis(2-ethylhexyl)sulfosuccinate), 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), *o*-nitrophenyl β-D-galactopyranoside (ONPG), isooctane, 1,3-bis[tris(hydroxymethyl)methylamino]propane (bisTris propane), 2,4,6-trinitrobenzenesulfonic acid (TNBS), ninhydrin reagent, and 4-chloro-1-naphthol were obtained from Sigma (St. Louis, MO) and used without additional purification. Peroxidase from soybean hulls was obtained from Enzymol International (Columbus, OH). Low-volume (0.5 mL) ultracentrifugation filters (10 kDa MWCO) were from Amicon (Beverly, MA). Phastgel Dry isoelectric focusing gels, pH 2.5−5 ampholytes, as well as the Phast System on which the gels were run were all obtained from Pharmacia (Upsalla, Sweden).

General Procedure for Extractions. Samples of individual glycoproteins (2.7 nmol) were dissolved in 1.0 mL volumes of the extraction buffer (typically 10 mM bisTris propane, 100 mM NaCl, pH 7), containing 1.5 mg (27 nmol) of Con A. For the sake of consistency, all of the lectin:glycoprotein mixtures were given a fixed amount of time (40 min) for the complexes to form prior to extraction, although the length of interaction did not noticeably affect the overall extraction efficiency (data not shown). After interaction, the aqueous solutions were contacted with an equal volume of AOT-containing isooctane solution (typically 40 mM AOT). The two phases were shaken in 4 mL vials at 300 rpm at room temperature for 15 min, following the procedure of Paradkar and Dordick (1991). After 2 min of microcentrifugation to obtain a clear phase boundary, the protein composition in each phase was determined via enzymatic assays. Extraction conditions were varied individually as indicated, while keeping all other parameters identical.

Enzyme Assays. SBP activity was measured colorimetrically using ABTS as the chromogen (Pütter, 1983). A 20 μL aliquot was added to 980 μL of assay buffer (0.1 M sodium phosphate buffer, pH 6.0). To this were added 0.1 mL of ABTS solution (0.11 g of ABTS in 10 mL of assay buffer) and 0.1 mL of 0.01 M H_2O_2 solution, and the absorbance was immediately monitored at 405 nm for 10 s to obtain initial reaction rates. The concentration of GAL was similarly determined using ONPG (Sambrook et al., 1989). A 30 μL aliquot was added to 66 μL of 4 mg mL^{-1} ONPG in 0.1 M sodium acetate, pH 4.5 (assay buffer), 3 μL of 0.1 M $MgCl_2$ (containing 4.5 M β-mercaptoethanol), and 201 μL of assay buffer. The reaction was incubated at 37 °C for 30 min, following which the reaction was terminated by the addition of 1.5 mL of 1 M Na_2CO_3. The reaction vials were centrifuged for 5 min prior to measuring the absorbance at 420 nm.

Organic phase assays required the initial removal of the organic solvent by vacuum drying at room temperature, followed by enzyme reconstitution in a like volume of the appropriate assay buffer prior to analysis. Control experiments were performed in which enzyme (either SBP or GAL) of known concentration was directly extracted into reversed micelle-containing isooctane at low pH and in the absence of the affinity ligand Con A. Mass balance calculations of the total protein concentration and enzyme activity of each phase verified that enzyme activity was not appreciably affected by this treatment, losing less than 3% of its total enzyme activity and thus remaining a good indicator of protein concentration.

Table 1. Range of Parameters Used in Factorial Analysis Modeling

		coded values		
parameter	range	(−1)	(0)	(+1)
pH	6.5−9.0	6.5	7.75	9.0
[NaCl]	0.1−0.2 M	0.1	0.15	0.2
[AOT]	0.01−0.04 M	0.01	0.025	0.04
log *P* (organic solvent)	3.5−5.1	3.5	4.3	5.1

Modification of Soybean Peroxidase by 2,4,6-Trinitrobenzenesulfonic Acid (TNBS). Lysine residues of SBP were modified using a variation of the procedure developed by Ugarova et al. (1979) for horseradish peroxidase. SBP (15 mg) was dissolved in 2.1 mL of 2.8% (w/v) TNBS in 2% (w/v) $NaHCO_3$, pH 8.3. The reaction mixture was incubated at 40 °C for 2 h. The modified SBP was separated from excess reaction material via three sequential ultracentrifugation steps using a 10 kDa MWCO filter, and the modified enzyme was reconstituted with extraction buffer.

The degree of modification was determined colorimetrically using the commercially available ninhydrin reagent as follows. SBP (20 μL) was added to a 10 mL vial containing 1.0 mL of 0.05% acetic acid and 1.0 mL of ninhydrin reagent. The reagents were mixed gently and incubated at 100 °C for 10 min. A 5 mL aliquot of 95% ethanol was added and the absorbance of the solution was read at 570 nm. The pI of the modified soybean peroxidase was established via gel isoelectric focusing in a pH 2.5−5 gradient. Bands were visualized by *in situ* peroxidase staining using 4-chloro-1-naphthol (Lagrimini and Rothstein, 1987).

Computer Simulation of Con A−Subtilisin Neoglycoprotein Docking. Con A−subtilisin BPN′ neoglycoprotein complexes were generated by computer simulation and energy minimization using Sybyl (Tripos Assoc.). The three-dimensional structure of subtilisin BPN′ was modified to include a glycosyl chain attached to the molecule via Lys_{256}. Computer-simulated docking of a Con A dimer (assumes a pH below 5.8 above which tetramers dominate; McKenzie, 1972) and the BPN′ molecule was followed by energy minimization. Distance calculations were made between charged amino acid residues on both molecules and their nearest neighbors within the Con A neoglycoprotein complex.

Results and Discussion

Factorial Design and Sensitivity Analysis. Factorial analysis was used to model the behavior of ARMES extraction of SBP. The parameters used in this model included the pH and NaCl concentration of the aqueous feed phase and the AOT concentration and the hydrophobicity of the organic phase. Though the Con A and SBP concentrations are both variables that are known to affect extraction in this system, they are interdependent and, therefore, have been excluded from this factorial design. Table 1 shows the ranges over which the parameters were investigated. A first-order factorial design using only two coded values to represent the parameter range (Box et al., 1978) was found to be inadequate to describe the system. This was most probably due to the interdependence of some variables such as pH and ionic strength. A second-order response surface design, therefore, was employed, and the three coded values of −1, 0, and 1 were used to describe each parameter's range (Lorenzen and Anderson, 1993; Box

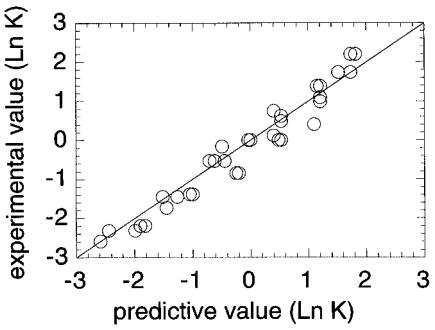

Figure 1. Comparison of experimental and predicted extraction efficiencies.

Table 2. Sensitivity Analysis of the SBP Extraction System[a]

parameter	effect value
pH	−1.660
[NaCl]	−1.293
$[AOT]^2$	−1.254
$(pH)^2$	−0.421
$[NaCl]^2$	−0.211
[NaCl] [AOT]	−0.128
$(\log P)^2$	−0.127
(pH) [AOT]	−0.122
(pH) (log *P*)	−0.117
[NaCl] (log *P*)	−0.078
[AOT] (log *P*)	0.047
(pH) [NaCl]	0.420
log *P*	0.471
[AOT]	1.522

[a] The coded effect values are derived from the coefficients of eq 1 and are presented for each variable. Parameters with extremely small effect values are excluded for simplicity.

and Draper, 1987). Data for the model were obtained by performing SBP extractions using a Box−Behnken design for four factors with a total of 25 experiments. The extraction of SBP via ARMES is described by eq 1, where

$$\ln K = -18.56 + 2.503(\text{pH}) - 36.89[\text{NaCl}] + 491.8[\text{AOT}] + 3.923(\log P) - 0.2691(\text{pH})^2 - 84.45[\text{NaCl}]^2 - 5576[\text{AOT}]^2 - 0.198(\log P)^2 + 6.712(\text{pH})[\text{NaCl}] - 6.48(\text{pH})[\text{AOT}] - 0.1173(\text{pH})(\log P) - 103.3[\text{NaCl}][\text{AOT}] - 3.04[\text{NaCl}](\log P) - 10.67[\text{AOT}](\log P) \quad (1)$$

K represents the extraction equilibrium constant, pH and [NaCl] refer to the pH and the salt concentration of the aqueous feed phase, respectively, [AOT] and log *P* are the surfactant concentration in, and the hydrophobicity of, the RMOP, respectively.

Equation 1, derived from only 25 extractions, was found to describe the system adequately and displayed a standard deviation of 7% between calculated and experimental extraction efficiency values for 65 test extractions (Figure 1). Using a prediction sum of squares (Myers and Montgomery, 1995), the coefficient of multiple determinations (R^2), used for prediction, was calculated to be 0.95.

Sensitivity analysis (Box and Draper, 1987) was performed on eq 1 to identify the factors that were most critical to the overall extractive behavior. The coefficient for each term in eq 1 was converted to a coded effect value, and a summary is presented in Table 2. Those variables with the greatest deflection from zero, in either the positive or the negative direction, are those expected to have the greatest effect on the extraction efficiency. For example, the pH and salt content of the aqueous phase were predicted to have the greatest influence on SBP extraction yields.

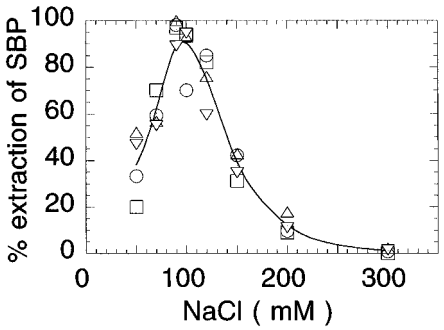

Figure 2. Extraction efficiency as a function NaCl concentration in the aqueous phase. Extraction was carried out at pH 6.5, other conditions as already specified.: (○) 20 mM AOT, (□) 40 mM, (△) 60 mM, (▽) 80 mM.

Table 3. Mass Balance Calculations of SBP Remaining in the Interface Region and Aqueous and Organic Phases Under a Variety of Conditions[a]

[NaCl], mM	%SBP in organic phase	%SBP in interface region	%SBP in aqueous phase
50	4	46	50
70	28	30	42
90	58	3	39
100	72	1	37
120	69	2	29

[a] Other extraction conditions are 10 mM bisTris propane (pH 7.5) and 40 mM AOT in isooctane.

Solution Phase Ionic Strength and pH Strongly Impact ARMES. Aqueous pH and NaCl concentration exhibited large and negative effect values for the single terms and smaller negative effect values for the squared terms (Table 2). The extraction behavior with respect to NaCl concentration is shown in Figure 2. At high ionic strength, the interaction between the proteins and the surfactant head group should decrease and this electrostatic shielding should also reduce the repulsion between surfactant head groups, resulting in smaller micelles (Hatton, 1989) and lower efficiencies of protein extraction. Indeed, this is observed at NaCl concentrations above ~100 mM. However, in low ionic strength feed phases, there is an increase in extraction efficiency from 50 to 100 mM NaCl (below 50 mM, the two phases form stable emulsions and do not separate cleanly). The increase in apparent extraction efficiency may reflect differences in partitioning of the protein complexes from the aqueous−organic interface and into the organic phase itself. Furthermore, at low NaCl concentrations, SBP becomes associated with the aqueous−organic interface, thereby lowering the effective fraction of enzyme being extracted into the RMOP (Table 3).

The impact of NaCl concentration on SBP extraction was studied with the aid of mass balance calculations (Table 3). At very low ionic strengths (<50 mM NaCl), SBP resides almost exclusively in either the aqueous phase or the interface (presumably complexed with Con A). This is consistent with previous observations of AOT-based protein extractions, where at low ionic strengths the bulk of the AOT will reside in the aqueous phase rather than the organic phase (Aveyard et al., 1986), thus disfavoring extractions as the majority of hydrophobic Con A−SBP−AOT complexes would reside at the interface rather than partition into the RMOP. As the concentration of NaCl is increased, the interfacial SBP is transformed into organic solvent-soluble Con A−SBP complexes. The net result is an optimum in extraction at about 100 mM NaCl. The effects of pH and ionic

Table 4. Extraction of SBP Employing Different Organic Solvents as Feed Phases[a]

solvent	hydrophobicity, log *P*	% extraction of SBP
ethyl acetate	0.68	0
amyl alcohol	1.3	0
toluene	2.5	0
hexane	3.5	42.2
octane	4.5	75.8
nonane	5.1	88.1

[a] Extraction conditions were 10 mM bisTris propane−HCl buffer (pH 7.0) and 40 mM AOT in each organic phase.

strength are interdependent in reversed micellar extraction systems (Nishiki et al., 1993). In our own experiments, this optimum with respect to NaCl is constant at least in the narrow pH range between pH 6.5 and 7.5 (data not shown).

The impact of solution pH on the extraction efficiency of SBP and GAL via ARMES is shown in Figure 2. Below pH 5.0, SBP (pI of 4.1; McEldoon et al., 1995) is extracted even in the absence of Con A, presumably as a result of favorable electrostatic interactions between the increasingly positively charged SBP and the anionic head group of the surfactant. Above pH 5.0, less than 1% of either SBP or GAL (pI 4.2; Tanaka et al., 1975) is extracted into the organic phase in the absence of Con A. Optimum facilitated extraction of both SBP and GAL was seen in the pH range 6−7. This result is consistent with the high affinity of Con A to glycoproteins near neutral pH (McKenzie, 1972).

Impact of Organic Phase Hydrophobicity and AOT Concentration on the Efficiency of ARMES. The hydrophobicity of the organic phase was determined to be relatively unimportant to the overall behavior in the range 3.0−5.1 (Table 4). This was suggested by the relatively small absolute value of the effect value for both the log *P* and the $(\log P)^2$ terms. Indeed, no extraction was observed in hydrophilic solvents ($\log P < 2$) or in hydrophobic protic solvents (e.g., octanol and nonanol), whereas alkanes with log *P* values of >3.5 (e.g., hexane or larger) supported increasing degrees of extraction. The lack of SBP facilitated extraction in octanol and nonanol, which have log *P* values comparable to that of hexane, may be explained by the tendency for alkanols to act as cosurfactants (Laane et al., 1987), thereby altering the geometry of the micelles and possibly reducing the overall interaction of the affinity complex with the polar head groups of the surfactant.

The situation was different in the case of the AOT concentration of the organic phase, in which the [AOT] term was moderately large and positive but the $[AOT]^2$ term was large and negative. At high concentrations, such as when the coded range values are 0 or +1, the two terms tend to counteract each others effect on the extraction coefficient, ln *K* (i.e., $1.522(+1) - 1.254(+1)^2 = 0.268$). At low concentrations, when the coded value is −1, the two terms act additively and result in a large and negative ln *K* value or very poor extraction efficiencies (e.g., $1.522(-1) - 1.254(-1)^2 = -2.776$). Thus, it was determined that extraction was only dependent on low concentrations of AOT. This was investigated further with additional experiments and was found to be consistent with the formation of a plateau above 30 mM AOT (Figure 3a). The position of the plateau is independent of the pH of the aqueous phase over the full pH range for facilitated extraction (i.e., pH 6.5 to >9). The results are similar in the case of *β*-galactosidase (GAL) (Figure 3b), although the plateau occurs at lower AOT concentrations (~10 mM) and may suggest that the mechanism of extraction of the Con A−GAL complex involves hydrophobic ion pairing. This was the mechanism proposed for the extraction of α-chymotrypsin into AOT-containing reversed micelles (Paradkar and Dordick, 1994) and may provide important clues as to the mechanism of ARMES and the effect of system variables on extraction efficiency (see below).

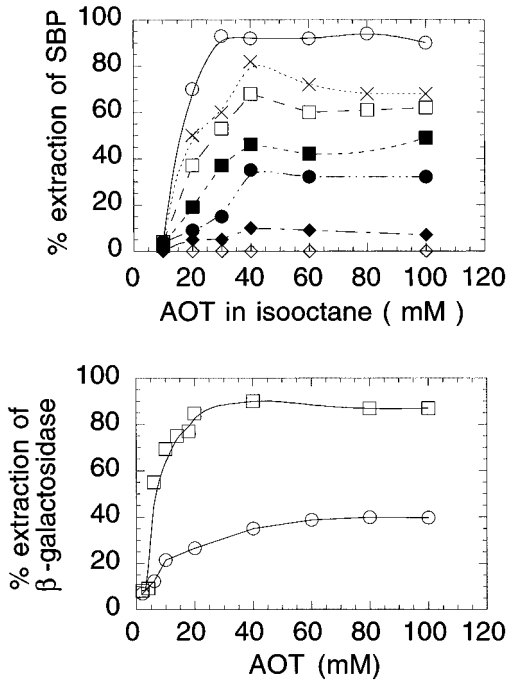

Figure 3. (a) Extraction efficiency of SBP as a function of aerosol OT (AOT) concentration in the organic phase: (○) pH 6.5, (×) pH 7.0, (□) pH 7.5, (■) pH 8.0, (●) pH 8.5, (◆) pH 8.8, (◇) pH 9.1. (b) Extraction efficiency of GAL as a function of aerosol OT (AOT) concentration in the organic phase: (□) pH 7.5, (○) pH 9.0. Other conditions are aqueous phase: 0.1 mg/mL (2.7 nmol) SBP or 0.28 mg/mL (2.7 nmol) *β*-galactosidase (GAL); 1.5 mg/mL concanavalin A (Con A); 10 mM bisTris propane buffer, 100 mM NaCl.

ARMES Is Capable of Extracting Protein Complexes Even in the Presence of Electrostatic Repulsion. The Con A−glycoprotein complex is expected to undergo extraction into the RMOP as long as the pH is below the effective pI of the complex. Interestingly, we observe significant extraction at pH values that are notably *higher* than the pI of Con A itself (pI ca. 8; Bhattacharyya and Brewer, 1990). Indeed, 20% of GAL was extracted at pH 9.5, over 5 pH units above GAL's pI, and 1.5 pH units above the pI of Con A (Figure 4). Similarly, SBP was extracted up to pH 9.3, also significantly higher than the pI's of either SBP or Con A, alone.

Assuming that any complex formed between Con A and SBP or GAL will have an effective pI intermediate between the individual values of 4.1 and ca. 8, then the complex will be negatively charged at pH 9 and should not be extracted into the negatively charged reversed micelle formed by AOT. This was observed for the extraction of the individual glycoproteins as well as Con A into reversed micelles (Figure 4). In fact, the pH at which the degree of direct extraction of the individual proteins into the RMOP fell to zero correlated well with the pI values of the individual proteins. Thus, it appears that there is a unique component associated with lectin complexation that extends the pH range for the extraction to such high pH values.

Charge Shielding and Effective Hydrophobicities. The results depicted in Figure 4 suggest that the effective pI values of the lectin−glycoprotein complexes may be nearer to pH 9 than between pH 4 and 8. While

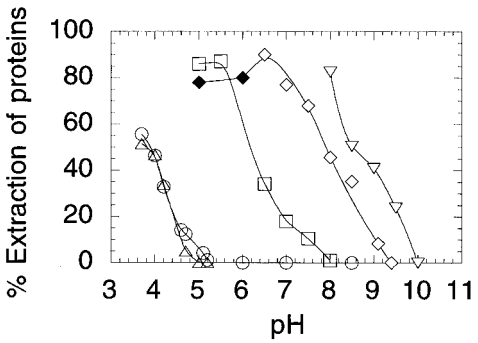

Figure 4. Extraction efficiency as a function of pH of the aqueous phase. Reversed micellar extraction of individual proteins (i.e., no affinity interaction): SBP (○), GAL (△) and Con A (□). ARMES extraction of SBP (◇) and GAL (▽). Extraction conditions are 10 mM buffer (bisTris propane, sodium phosphate, or acetic acid buffers as dictated by the desired pH), 40 mM AOT, and 100 mM NaCl except for ◆, which used 80 mM NaCl and 1 mM each of $CaCl_2$ and $MnCl_2$.

charges shielded from solution or from ampholytes used to obtain pI estimates may give rise to higher effective pI measurements than expected, the extractability of the protein complex is more complex than simply electrostatic effects. Paradkar and Dordick (1994) have suggested that the hydrophobicity of the complex is the physicochemical property that most influences protein extraction into a reversed micellar organic phase. Unlike the individual proteins, Con A−glycoprotein complexes are expected to have a substantial number of total charges (on both the lectin and the glycoprotein) shielded from the solution due to close association of the protein molecules. Thus, the number of charges per unit surface area of protein exposed to the aqueous solution is decreased.

Charge shielding, and its effect on exposed total charges can be evaluated by molecular simulation. Because crystal structures for SBP and GAL are unavailable, we simulated Con A interacting with a hypothetical neoglycoprotein constructed by molecular modeling. Specifically, we simulated the attachment of a biantennary, high-mannose carbohydrate chain to the surface Lys_{256} position of subtilisin BPN′, a structurally well-characterized protein (Alden et al., 1971). The carbohydrate chain ($Man_5GlcNAc_2$) is representative of the high-mannose portion of SBP's glycosyl moieties (Gray et al., 1996) and contains the trimannose core that is specifically recognized by Con A (Naismith and Field, 1996). The Con A was bound to the subtilisin neoglycoprotein by affinity for the glycosyl chain attached to the subtilisin and energy minimized using the Sybyl molecular graphics package (Figure 5). Despite the large size of the sugar chain, Con A is closely associated with the subtilisin and numerous charges on both the Con A and subtilisin are shielded (as defined as charges on one protein within 3.5 Å of the other protein). For example, nine positive charges are shielded from the bulk aqueous solution (Con A: Arg_{33}, Lys_{39}, Tyr_{22}, all three on both Con A monomers, and Tyr_{77} on one Con A monomer. BPN′: Arg_{247} and Lys_{237}); hence, a net change in charges *exposed* to the bulk solution is −9. Thus, the total charge of the Con A−subtilisin neoglycoprotein is lower than that of the two proteins taken individually.

An additional driving force is that, even at high pH values (but below the pK_a of ϵ-amino groups of Lys residues), there will be a suitable number of positively charged amino groups oriented outward toward the solution (and not subjected to charge shielding) that can ion pair with AOT molecules to increase the effective hydrophobicity of the Con A−glycoprotein complex. This

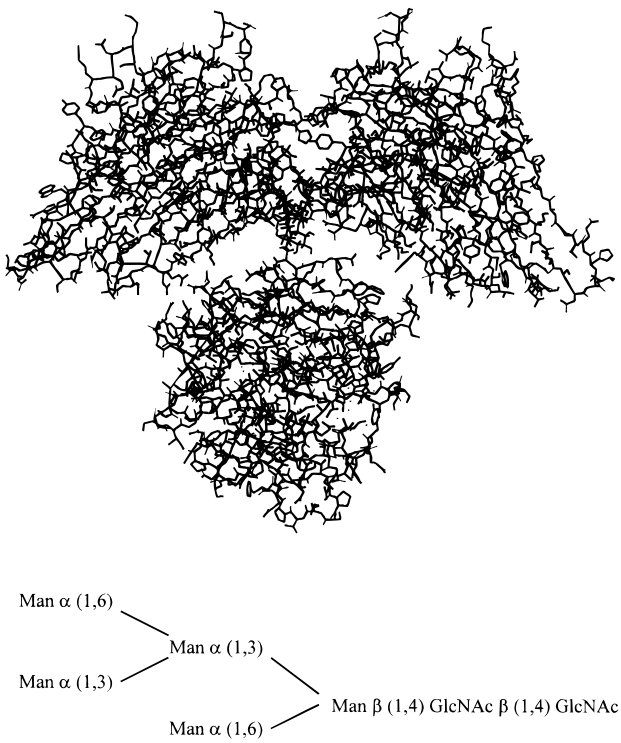

Figure 5. Simulated binding of Con A and a hypothetical neoglycoprotein. A complex of subtilisin BPN′, modified to include a carbohydrate chain at Lys 256, (bottom portion of figure) and a Con A dimer (top portion of figure) were generated by computer simulation and energy minimization using Sybyl (Tripos, Inc.). A total of nine charged groups were shielded by the proposed interaction.

complex, already with a lower total charge than each protein separately (see above), becomes sufficiently hydrophobic at relatively high pH values ($pH > pI_{effective}$) to undergo extraction into the RMOP. Such an effect is somewhat general; it is observed with the unrelated (and distinctly different with respect to size and structure) SBP and GAL. Con A, however, may be unique in providing the driving force for high effective hydrophobicity in a complex with a glycoprotein.

The mechanism involving effective hydrophobicity would suggest that extraction would be affected by changes to the number of amino groups available for ion pairing with the AOT. To that end, we chemically modified SBP with trinitrobenzenesulfonic acid (TNBS). This treatment blocked the ϵ-amino acid groups of surface exposed lysine residues, resulting in an overall reduction in pI as well as a reduction in the number of sites available for AOT interaction. Gel isoelectric focusing verified the drop in pI from the original value of 4.2 to 3.8 (Figure 6, inset). Ninhydrin assay of modified and native SBP indicated that 90% of the available lysines were modified, although the catalytic activity of the enzyme was found to be unaffected by the modification (data not shown), suggesting that the bulk structure of the SBP was unchanged. Extractions performed using TNBS−SBP yielded a curve of extraction *versus* pH that was shifted from the corresponding curve for native SBP toward lower pH values by approximately 0.5 pH units (Figure 6). This is consistent with the proposed model of extraction depicted in Figure 5 wherein a less hydrophobic Con A−SBP−AOT complex is less favorably extracted into the RMOP at high solution pH.

Conclusions

A consequence of ARMES extraction is the enhancement of the partition coefficient of a glycoprotein over a

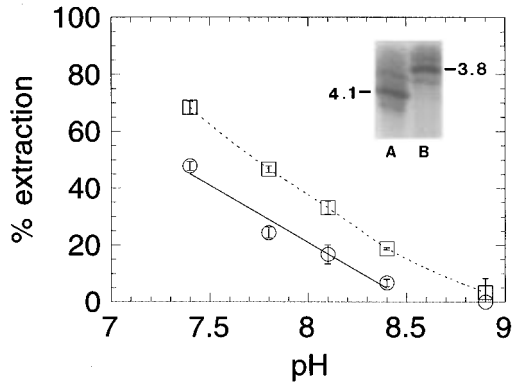

Figure 6. Effect of lysine modification of SBP on the extraction behavior. Extractions performed with native SBP (□) and with TNBS-modified SBP (○). Extraction conditions are 10 mM bisTris propane, 40 mM AOT, and 100 mM NaCl. Figure inset: Isoelectric focusing gel (pH 2.5−5) of native (A) and modified (B) SBP.

wide pH range. As demonstrated in Figure 4 for SBP, electrostatic interactions of the protein itself at pH values below the pI serve to carry it into the RMOP directly, although the calculated partition coefficient (K) falls from 1.3 at pH 3.75 to less than 0.1 at pH 5. Conversely, the binding of Con A leads to a K for facilitated extraction at pH 7 of 7.4. This represents a significant enhancement of the extractability of SBP into the organic phase. Hydrophobic interactions of the Con A−glycoprotein complex with the RMOP serve to extend the upper bound of pH for effective extraction. This represents a significant advantage of ARMES over other purification methods as it offers greater flexibility in the pH range over which useful purifications may be performed. This has clear implications for both batch and continuous operations.

Acknowledgment

We acknowledge the financial support of the National Institutes of Health (GM49056). We thank Dr. Joseph Rich for providing computer simulations of Con A−subtilisin neoglycoprotein.

Literature Cited

Alden, R. A.; Birktoft, J. J.; Kraut, J.; Robertus, J. D.; Wright, C. S. Atomic Coordinates for Subtilisin BPN′. *Biochem. Biophys. Res. Commun.* **1971**, *45*, 337.

Aveyard, R.; Binks, B. P.; Clark, S.; Mead, J. Interfacial Tension Minima in Oil-Water-Surfactant Systems. *J. Chem. Soc.. Faraday Trans.* **1986**, *82*, 125−142.

Bhattacharyya, L.; Brewer, C. F. Isoelectric Focusing Studies of Concanavalin A and the Lentil Lectin. *J. Chromatogr.* **1990**, *502*, 131−142.

Box, G. E. P.; Hunter, W. G.; Hunter, J. S. *Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building*; John Wiley and Sons: New York, 1978.

Box, G. E. P.; Draper, N. R. *Empirical Model-Building and Response Surfaces*; John Wiley and Sons: New York, 1987.

Dakour, J.; Lundblad, A.; Zopf, D. Separation of Blood Groups A-active Oligosaccharides by High-pressure Liquid Affinity Chromatography using a Monoclonal Antibody Bound to Concanavalin A Silica. *Anal. Biochem.* **1987**, *161*, 140−143.

Goklen, K. E.; Hatton, T. A. Protein Extraction Using Reverse Micelles. *Biotechnol. Prog.* **1985**, *1*, 69−74.

Gray, J. S. S.; Yang, B. Y.; Hull, S. R.; Venske, D. P.; Montgomery R. The Glycans of Soybean Peroxidase. *Glycobiology* **1996**, *6*, 23−32.

Hatton, T. A. In *Surfactant-Based Separation Processes*; Scamehorn, J. F., Harwell, J. H., Eds.; Marcell Dekker: New York, 1989; pp 55−89.

Laane, C.; Boeren, S.; Vos, K.; Veeger, C. Rules for Optimization of Biocatalysis in Organic Solvents. *Biotechnol. Bioeng.* **1987**, *30*, 81−87.

Lagrimini, L. M.; Rothstein, S. Tissue Specificity of Tobacco Peroxidase Isoenzymes and Their Induction by Wounding and Tobacco Mosaic Virus Infection. *Plant Physiol.* **1987**, *84*, 438−442.

Liener, I. E., Sharon, N., Goldstein, I. J., Eds. *Lectins: Properties, Functions and Applications in Biology and Medicine*; Academic Press: New York, 1986.

Lorenzen, T. J.; Anderson, V. L. *Design of Experiments: A No-Name Approach*; Marcel Dekker, Inc.: New York, 1993.

McEldoon, J. P.; Dordick, J. S. Soybean Peroxidase has Lignin Peroxidase-like Thermal Stability. *Enzyme Microb. Technol.* **1995**, *17*, 359−265.

McKenzie, G. H.; Sawyer, W. H.; Nichol, L. W. The Molecular Weight and Stability of Concanavalin A. *Biochim. Biophys. Acta* **1972**, *263*, 283−293.

Montreuil, J. In *Glycoproteins*; Montreuil, J., Vliegenthart, J. F. G., Schacter, H., Eds.; Elsevier: Amsterdam, 1995; pp 1−10.

Myers, R. H.; Montgomery, D. C. *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*; John Wiley and Sons, Inc.: New York, 1995; pp 49.

Naismith, J. H.; Field, R. A. Structural Basis of Trimannoside Recognition by Concanavalin A. *J. Biol. Chem.* **1996**, *271*, 972−976.

Nishiki, T.; Sato, I.; Kataoka, T.; Kato, D. Partitioning Behavior and Enrichment of Proteins with Reversed Micellar Extraction: I. Forward Extraction from Aqueous to Reversed Micellar Phase. *Biotechnol. Bioeng.* **1993**, *42*, 596−600.

Ohlson, S.; Hansson, L.; Glad, M.; Mosbach, K.; Larsson, P.-O. High Performance Liquid Chromatography: a New Tool in Biotechnology. *Trends Biotechnol.* **1989**, *7*, 179−186.

Paradkar, V. M.; Dordick, J. S. Purufication of Glycoproteins by Selective Transport Using Concanavalin-Mediated Reverse Micellar Extraction and Separation. *Biotechnol. Prog.* **1991**, *7*, 330−334.

Paradkar, V. M.; Dordick, J. S. Affinity-Based Reverse Micellar Extraction and Separation (ARMES): A Facile Technique for the Purification of Peroxidase from Soybean Hulls. *Biotechnol. Prog.* **1993**, *9*, 199−203.

Paradkar, V. M.; Dordick, J. S. Mechanism of Extraction of Chymotrypsin at very Low Concentrations of Aerosol OT in the Absence of Reverse Micelles. *Biotechnol. Bioeng.* **1994**, *43*, 529−540.

Pütter, J.; Reinhild, B. In *Methods and Enzymatic Analysis*, 3rd ed.; Bergemeyer, H. N., Ed.; Verlag Chemie: Deerfield Beach, FL, 1983; pp 286−293.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: New York, 1989; Chapter 16, pp 64−65.

Tanaka, Y.; Kagashiimi, A.; Kiuchi, A.; Horiuchi, T. Purification and Properties of β-Galactosidase from Aspergillus Oryzae. *J. Biochem.* **1975**, *77*, 241−247.

Ugarova, N. N.; Rozhkova, G. D.; Berezin, I. V. Chemical Modification of Lysine Residues in Horseradish Peroxidase and its Effect on the Catalytic Properties and Thermostability of the Enzyme. *Biochim. Biophys. Acta* **1979**, *570*, 31−42.

Wolbert, R. B. G.; Hilhorst, R.; Voskuilen, G.; Nachtegaal, H.; Dekker, M.; Van't Reit, K.; Bijsterbosch, B. H. Protein Transfer from an Aqueous Phase into Reversed Micelles: The Effect of Protein Size and Charge Distribution. *Eur. J. Biochem.* **1989**, *184*, 627−633.

Accepted May 20, 1997.[⊗]

BP970049H

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1997.