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Aqueous two-phase extraction for protein recovery from corn extracts

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Abstract

Corn has been used as an expression host for several recombinant proteins with potential for large-scale production. Cost-effective downstream initial recovery, separation and concentration remain a challenge. Aqueous two-phase (ATP) partitioning has been used to recover and concentrate proteins from fermentation broths and offers advantages for integration of those steps with biomass removal. To examine the applicability of ATP partitioning to recombinant protein purification from corn endosperm and germ, ATP system parameters including poly(ethylene glycol) (PEG) molecular weight (MW), phase-forming salt, tie line length (TLL), and pH were manipulated to control partitioning of extracted native proteins from each fraction. Moderate PEG MW, reduction of phase ratio, and added NaCl effected complete recovery of the hydrophobic model protein lysozyme in the top phase with ca. 5× enrichment and illustrates a favorable match of recombinant protein characteristics, expression host, and separation method. Furthermore, integration of protein extraction with the partitioning reduced the load of contaminating host proteins relative to the more traditional separate steps of extraction followed by partitioning. Performance of the integrated partitioning was hindered by endosperm solids loading, whereas for germ, which has ca. 35× higher aqueous soluble protein, the limit was protein solubility. For more hydrophilic model proteins (the model being cytochrome c), effective separation required further reduction of PEG MW to effect more partitioning of host proteins to the top phase and enrichment of the model protein in the lower phase. The combination of PEG MW of 1450 with 8.5 wt.% NaCl addition (Na_2SO_4 as the phase-forming salt) provided for complete recovery of cytochrome c in the lower phase with enrichment of 9× (germ) and 5× (endosperm). As a result of lower-phase recovery, the advantage of simultaneous removal of solids is lost. The lower solubility of native endosperm proteins results in higher purity for the same enrichment.
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1. Introduction

Utilization of transgenic crops as expression hosts offers several distinct advantages over more widely-used bacterial hosts including ability to carry out post-translational modifications to produce active proteins, easier scale-up, and established production methods [1–3]. Furthermore, the ability to target recombinant proteins to specific storage organs or tissues (e.g. seeds and tubers) allows for prolonged stable storage and may reduce the number of contaminants in extracts [1,4–6].

Corn is preferred over other plant hosts due to its lower water-soluble protein content. Furthermore, specific target tissue expression will benefit the downstream purification process via the potential of initial milling and dry fractionation to reduce contaminants and enrich the target protein concentration. Proper choice of the targeted tissue will increase these benefits. For instance, if a protein is targeted to endosperm, protein extraction with a neutral salt buffer will leave nearly 80% of the native proteins in the residue. For germ, only 30% of the native proteins would remain in the residue [6]. Furthermore, an endosperm extract would have much less oil than a germ-rich or whole kernel extract, which would also simplify purification [6]. With targeted expression of β-glucuronidase to germ, dry fractionation of the grain to eliminate the hull and much larger endosperm fractions produce a germ-rich fraction with nearly 10-fold higher β-glucuronidase/solids ratio than would have resulted from whole corn [1] and easier purification for transgenic aprotinin and β-glucuronidase [7,8].

Protein recovery from the starting material generally includes extraction, clarification, protein capture, purification, and polishing. The overall production cost is mainly determined by the

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efficiency of the initial capture and purification steps where feed volumes are large until biomass solids and oils are removed and the protein is concentrated [1,6]. Therefore, more efficient initial concentration and separation procedures need to be developed for recombinant protein recovery from transgenic corn.

An aqueous two-phase (ATP) system is formed when two water-soluble polymers, such as poly(ethylene glycol) (PEG) and dextran, or a polymer and a salt are dissolved in water beyond a critical concentration at which two immiscible phases form. Both phases can enhance protein stability [9]. In addition, ATP partitioning can replace a number of steps involved in conventional downstream processing such as extraction, clarification, concentration, and intermediate purification. Furthermore, the ease of scale-up and suitability for continuous operation make PEG–salt ATP systems very suitable for large-scale application [10,11].

Of the multiple factors determining selectivity of ATP partitioning, protein surface hydrophobicity and hydrophobic differences between phases can be dominant, with two such examples being in PEG-containing systems [12,13]. Furthermore, ATP systems with NaCl addition are more sensitive to protein surface hydrophobicity [13,14]. In such systems, the surface hydrophobicity difference between target protein and host contaminants is crucial for selective partitioning [14,15]. This factor has been exploited by attaching hydrophobic fusions to improve the selectivity of partitioning [16].

ATP partitioning has been applied to plants to fractionate gluten from wheat [17], purify peroxidase from soy [18], and separate tobacco leaf proteins [19]. To develop a suitable ATP separation method for recombinant protein from corn, the partitioning behavior of native corn proteins from different tissues and the relation between protein partitioning and ATP system parameters need to be known.

The effect of factors such as phase-forming salt, pH, tie-line length, phase ratio and sample loading for aqueous phase systems chosen to illustrate selectivity based on hydrophobicity differences will be discussed in this paper. Successful purification of a recombinant protein depends on behavior relative to the native host proteins. Lysozyme, ribonuclease A, and cytochrome c were chosen as a series of model proteins of decreasing hydrophobicity [20–22] to test performance over a range of potential recombinant protein characteristics. In addition, integration of extraction and partitioning was modeled by adding model proteins and corn solids directly to the ATP system.

1.1. Definition of parameters in ATP systems

Tie line length (TLL) characterizes the compositional differences between the two phases:

$$\mathrm{TLL} = ((\Delta C_{\mathrm{PEG}})^2 + (\Delta C_{\mathrm{salt}})^2)^{1/2} \tag{1}$$

$$\Delta C_{\mathrm{PEG}} = C_{\mathrm{PEG}}^{\mathrm{top}} - C_{\mathrm{PEG}}^{\mathrm{bottom}}, \quad \Delta C_{\mathrm{salt}} = C_{\mathrm{salt}}^{\mathrm{top}} - C_{\mathrm{salt}}^{\mathrm{bottom}} \tag{2}$$

where C_i^j is the concentration of component i in phase j.

The partition coefficient (K) is the ratio of concentrations in each phase:

$$K_i = \frac{C_i^{\mathrm{top}}}{C_i^{\mathrm{bottom}}} \tag{3}$$

The extent to which an ATP system can separate target protein from host protein in a single stage is the selectivity (α):

$$\alpha = \frac{K_{\mathrm{target}}}{K_{\mathrm{host}}} \tag{4}$$

The purification factor (PF) for recovery in the product phase is defined as the ratio of product-phase specific activity (SA, as units/mass for enzymatic assays and mass/mass for the absorbance assay) to initial extract SA

$$\mathrm{PF} = \frac{\mathrm{SA}^{\mathrm{product}}}{\mathrm{SA}^{\mathrm{initial}}} \tag{5}$$

In the case of extractive partitioning there was not an initial extract so we used the value of $\mathrm{SA}^{\mathrm{initial}}$ obtained by extraction with the buffer in which the phase-forming solutes were dissolved. As a result, the PF will be the combined result of selective partitioning (determined by α) and selective extraction (determined by the ratio of host proteins extracted by buffer relative to APS). Defined in this manner, it provides a ready comparison of the overall benefits of extractive partitioning, but does assume no difference in the ability to extract the target protein.

Phase ratio (Φ) is the relative volume of the two phases:

$$\Phi = \frac{V_{\mathrm{top}}}{V_{\mathrm{bottom}}} \tag{6}$$

where V_i is the volume of phase i. Total recovery (R_{T}) of protein is defined as the ratio of protein i dissolved in top and bottom phases to the mass of corn solids added, M_i:

$$R_{\mathrm{T}} = \frac{C_i^{\mathrm{top}} \times V_{\mathrm{top}} + C_i^{\mathrm{bottom}} \times V_{\mathrm{bottom}}}{M_i} \tag{7}$$

Yield (Y) of protein is defined as recovery in the product phase:

$$Y_i = \frac{C_i^{\mathrm{product}} \times V_{\mathrm{product}}}{M_i} \tag{8}$$

2. Materials and methods

2.1. Materials

Yellow dent corn endosperm commercially degermed and milled to a particle size range from 0.33 to 0.85 mm was supplied by Lauhoff Grain Co. (Danville, IL), defatted corn germ milled to a particle size range from 0.03 to 0.15 mm was supplied by ProdiGene Inc. (College Station, TX). PEG (molecular weight (MW) 8000, 3350, 1450, and 600 Da), hen egg white lysozyme (L7001), porcine pancreatic ribonuclease A (R5500), equine heart cytochrome c (C7752), torula yeast ribonucleic acid VI (R6750), and *Micrococcus lysodeikticus* (M3770) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Coomassie Plus® protein assay reagent kit was purchased from Pierce Biotechnology (Rockford, IL, USA). ACS

Table 1
Compositions of ATP systems for partitioning of host proteins

ATP system[a]	PEG[b] (wt.%)	Salt[b] (wt.%)	TLL[c]	Experiments[d]
PEG 8000–phosphate, short TLL	13.2	10.3	25	EP + UE + NaCl
PEG 8000–phosphate, long TLL	22.5	12	48	
PEG 3350–phosphate, short TLL	10.6	10.7	18	EP + UE + NaCl
PEG 8000–Na_2SO_4, short TLL	13.1	8.1	26	
PEG 8000–Na_2SO_4, long TLL	21.4	11.7	44	
PEG 3350–Na_2SO_4, medium TLL	15.7	8.9	32	
PEG 8000–Na_2CO_3, short TLL[e]	13.2	5.3	26	
PEG 8000–Na_2CO_3, long TLL[e]	24.1	9.1	51	
PEG3350–Na_2CO_3, medium TLL[e]	19.1	9.4	38	
PEG 8000–$(NH_4)_2SO_4$, short TLL	18.2	11.1	39	EP + NaCl
PEG 8000–$(NH_4)_2SO_4$, long TLL	27.6	17	63	
PEG 3350–$(NH_4)_2SO_4$, short TLL	18.2	12.3	38	EP + NaCl
PEG 8000–$MgSO_4$, short TLL	14.5	8.1	23	
PEG 8000–$MgSO_4$, long TLL	19.7	9.2	40	
PEG 3350–$MgSO_4$, short TLL	15.1	7.6	22	
PEG 8000–sodium citrate, short TLL	10	12.6	22	
PEG 8000–sodium citrate, long TLL	14	14.1	32	
PEG 3350–sodium citrate, short TLL	10	10.6	18	

[a] All ATP systems were prepared with 50 mM sodium phosphate buffer at pH 7.
[b] Φ is 1 for all systems without NaCl addition.
[c] TLL is the calculated from the equilibrium composition without NaCl addition.
[d] Clarified extract partitioning was tested in all ATP systems. Other cases were done only for systems noted. EP: extractive partitioning, UE: unclarified extract, NaCl: NaCl addition.
[e] pH 9; all others at pH 7.

Certified Grade salts, acids, and bases were from Fischer Scientific (Pittsburgh, PA, USA).

2.2. Methods

Phase diagrams for ATP systems used in this paper (Tables 1 and 2) have been reported [9,23–27]. Compositions were chosen to give phase ratios of 1 (for no added NaCl) and approximately matching tie line lengths of selected values. TLL, a measure of phase difference has been correlated with partition coefficient [9,23]. Three procedures were used to prepare the ATP systems: (1) clarified extract partitioning: mixing clarified extract, PEG stock solution, salt stock solution or solid salt; (2) unclarified extract partitioning: mixing unclarified extract, PEG stock solution, salt stock solution or solid salt; (3) integrated procedure: corn solids added directly to preformed ATP system for integration of extraction and partitioning.

2.2.1. Preparation of extracts and stock solutions

Corn endosperm or germ solids were added by weight to 50 mM sodium phosphate at the desired pHs 4, 7, or 9 at a level of 10 g solids/50 ml buffer. The slurry was stirred for 1 h by magnetic stir bar and pH was controlled by adding 1 M NaOH or 1 M HCl. To prepare clarified extracts, the slurry was centrifuged ($3000 \times g$, 22 °C, 30 min), decanted and the supernatant filtered through a 0.45 μm μStar cellulose acetate syringe filter (Costar Corp., Corning NY). Unclarified extracts were prepared only at pH 7.

Phosphate stock solution (40 wt.%, pH 7) for the ATP system was prepared by combining sodium mono- and potassium

Table 2
Composition and phase ratio of ATP systems used for partitioning of model proteins by extractive partitioning

ATP components[a]	Composition[a] wt.%	NaCl (wt.%)	Phase ratio (Φ)	Model proteins
PEG 3350	15.7	0	1	Lysozyme
Na_2SO_4	8.9	4.5	0.71	Ribonuclease A
	TLL[b] = 32%	8.5	0.71	Cytochrome c
PEG 1450	16.3	0	1	Cytochrome c
Na_2SO_4	13.5	4.5	0.67	
	TLL = 31%	8.5	0.67	
PEG 600	17.7	0	1	
Na_2SO_4	15.5	4.5	0.71	
	TLL = 33%	8.5	0.71	

[a] All ATP systems were prepared with 50 mM sodium phosphate buffer at pH 7.
[b] TLL are for systems without added NaCl.

Table 3
Salt content and phase ratio of equilibrium PEG-$K_nH_{3-n}PO_4$ systems of constant [PO_4]

ATP system	pH	[Salt]/wt.%	Phase ratio[a]
PEG 8000 (13 wt.%)–potassium phosphate (PO_4 8 wt.%)	4	9.7	1
	6	10.9	0.86
	8	11.5	0.55
	9	12.6	0.47
PEG 3350 (12 wt.%)–potassium phosphate (PO_4 9 wt.%)	4	11	1
	6	12.3	0.89
	8	12.9	0.47
	9	14.2	0.47

[a] Addition of corn extracts caused no significant differences in phase ratios.

dibasic phosphate (1:1.17 weight ratio) in deionized water. The mixed cations were required because of solubility limitations of the respective phosphate salts. Where pH was varied, the phosphate stock solution was prepared by titrating 30 wt.% phosphoric acid with 10 M potassium hydroxide solution to the appropriate pH. On dilution into the final ATP system, the wt.% PO_4 was held constant rather than wt.% salt (see Table 3). Other salt stock solutions (ca. 25 wt.% depending on solubility) and PEG stock solutions (50 wt.%) were prepared by dissolving the required amount of solute in 50 mM sodium phosphate, pH 7 buffer.

Lysozyme was dissolved in 66 mM potassium phosphate, pH 6.24 while ribonuclease A and cytochrome c were dissolved in water and buffer (40 mM tris-cacodylate with 10 μM EDTA, pH 6.0), respectively, as recommended by the supplier, to prepare 5 mg protein/ml stock solutions. Literature values of the surface hydrophobicities of the two model proteins and lysozyme are shown in Table 4 along with values obtained in the current study. Level of addition of the model proteins to the germ and endosperm extracts was chosen such that the concentration could be accurately assayed and at the same time to correspond to the range of expression levels that have been reported for transgenic corn and are felt to be economically viable. The latter levels are 1–10% of soluble proteins [28,29]. The levels used here were 1.5 mg/g germ (2.5% of soluble proteins) and 0.15 mg/g endosperm (9% of soluble proteins). With the 10× higher levels of endosperm added to the ATP system, this resulted in the same model protein levels/g ATP system, providing for matching assay accuracy.

2.2.2. Partitioning

Clarified endosperm extracts were partitioned in each of the ATP systems of Table 1, while clarified germ extracts were partitioned in all PEG–salt systems listed by Table 1. In addition, at pH 7 only, unclarified extract was partitioned in the PEG 3350– and short TLL PEG 8000–phosphate system and the integrated extraction/partitioning was carried out in the PEG 3350– and short TLL PEG 8000–phosphate and PEG 8000–$(NH_4)_2SO_4$ systems. The latter experiments were done with several levels of NaCl addition. The sample loadings were 0.1 g endosperm or 0.01 g germ solids for the integrated procedure, while the clarified and unclarified extracts (prepared at 1:5 w/v solid:buffer ratios) were added in volumes corresponding to initial solids equivalent to those added in the integrated procedure. The integrated procedure with added lysozyme was carried out in PEG 3350–Na_2SO_4 systems, examining the effects of NaCl addition, phase ratio and sample loading, while further reductions in PEG MW (Table 2) were used for cytochrome c. Na_2SO_4 was the final choice as sulfate salt because it has been reported that lysozyme recovery is poor with $(NH_4)_2SO_4$ [19].

ATP aliquots (1.5 g) were prepared by combining appropriate stocks of phase components, extracts or corn solids (germ 0.01 g/g ATP aliquots or endosperm 0.1 g/g ATP aliquots), any added NaCl and diluted to final mass with extraction buffer. For the integrated procedure and unclarified extracts, the mass of corn solids was not counted toward the 1.5 g total mass.

The mixtures were thoroughly mixed by vortexing for 30 s, equilibrated at room temperature (22 °C) for 1 h with mixing by tumbling, and centrifuged (3000 × *g*, 22 °C, 20 min) to expedite the phase separation. After recording the volume of each phase (with added NaCl the phase ratio changed from the value of one without NaCl), the top phase was carefully removed by transfer pipette and the bottom phase by piercing the centrifuge tube with a syringe; the region near the interface and the solids layer in tube bottom was left in the tube to avoid contamination of the phase samples. The total protein and model protein concentrations in samples from each phase were measured as described below.

For the model protein-spiked samples, 50 ml of ATP systems were prepared in bulk and the two phases were separated for later recombination in the partitioning experiments. Except for a series of varying phase ratio experiments, the recombination was in the ratio resulting from the bulk preparation. The individual phases were combined with the corn solids (germ 0.01 g/g ATP aliquots or endosperm 0.1 g/g ATP aliquots) and model protein

Table 4
Surface hydrophobicities of model proteins and their partitioning in different ATP systems

Model protein	MW (Da)	p*I*	HSF[a]	Log (*K*)[b]	Log (*K*)[c] PEG 3350–Na_2SO_4	
					4.5% NaCl	8.5% NaCl
Cytochrome c	12400	9.6	−100	−0.658	−1.33	−1.84
Ribonuclease A	13700	9.6	−60	−0.108	−0.72	−0.13
Lysozyme	14300	10.3	−20	0.218	2.11	2.25

[a] Surface hydrophobicity scale based on the logarithm of the partition coefficient of protein in the PEG–dextran systems [20].
[b] Partitioning in EOPO–dextran system [21].
[c] Partitioning in this work.

stock (providing 22.5 μg lysozyme) to prepare 1.5 g aliquots for partitioning.

2.2.3. Assay methods

Protein concentration was determined by Coomassie Plus® protein assay reagent kit (Pierce Rockford, IL), using bovine serum albumin (BSA) as standard. The appropriate blank aqueous system phase was employed to correct for minor interference from salt and PEG. The activity assays for lysozyme followed the standard protocol for enzymatic assay of lysozyme from Sigma-Aldrich [30]; activity assays for ribonuclease A followed the standard protocol for enzymatic assay of ribonuclease A from Sigma-Aldrich [31]; cytochrome c concentration was determined by absorbance at 410 nm using an extinction coefficient of 8.0 ml/mg/cm [32]. Assay blanks for all model protein assays were phases from integrated partitioning of corn solids with no added model protein. All partitioning experiments were replicated three times and each replicate was assayed twice.

3. Results and discussion

3.1. Native corn protein partitioning

3.1.1. Protein extraction from corn endosperm and corn germ

Fig. 1 shows that the extraction of total proteins (mg protein/g solids) from both germ and endosperm increases as pH increases from 4 to 9. Initially this is the result of moving away from the overall isoelectric point [33] and eventually to greater release of the glutelins fraction [6]. Furthermore, the amount of protein extracted from germ was much higher than from endosperm. Hence, when corn will be used for recombinant protein production, a recombinant protein stable at pH 4 could be more easily purified because the level of contaminating protein would be relatively low. Further, expression in endosperm avoids most soluble host proteins—a clear advantage assuming similar expression levels can be achieved with this targeting.

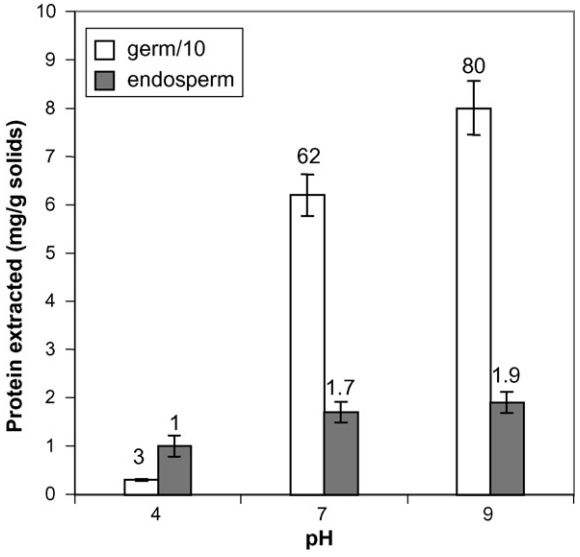

Fig. 1. pH dependence of protein extraction. For germ, the *y*-axis is 1/10 of the actual values. Numbers above the bars were the actual values. Conditions: 1 g solids: 5 ml buffer (50 mM sodium phosphate). The error bars represent the 95% confidence intervals.

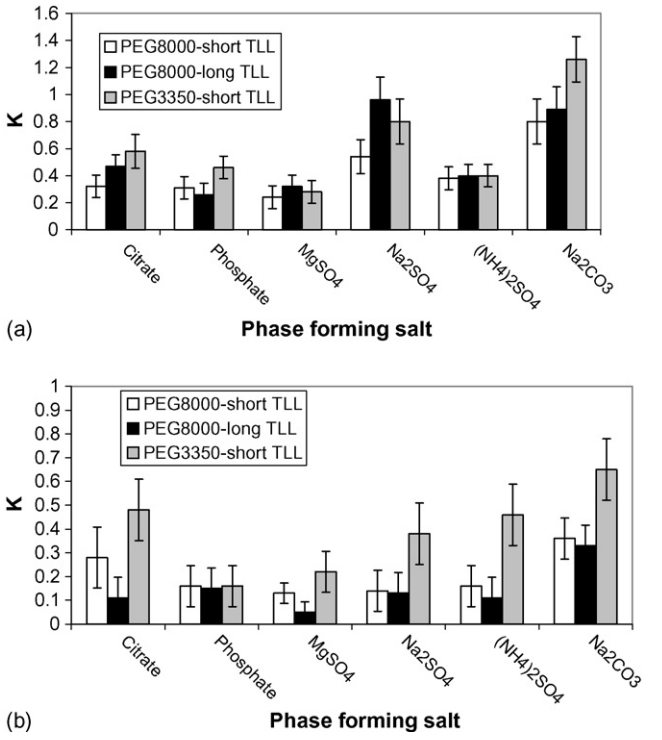

Fig. 2. Partition coefficient of total corn proteins. ATP systems were those listed in Table 1. (a) Endosperm protein extracted at pH 7, 50 mM phosphate buffer; loading: 125 μg protein/g ATP aliquots; (b) germ protein extracted at pH 7, 50 mM phosphate buffer; loading: 125 μg protein/g ATP aliquots. The error bars represent the 95% confidence intervals.

3.1.2. Partitioning of native corn proteins in ATP systems

The partitioning of extracted native protein from endosperm and germ was carried out for each of the ATP systems in Table 1. The results (Fig. 2a and b) show that (1) for similar TLL, *K* increases as PEG MW decreases, as is generally observed in

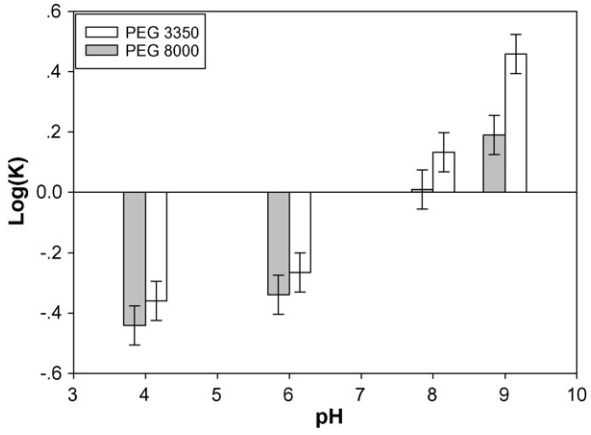

Fig. 3. pH dependence of endosperm protein partitioning. PEG, potassium phosphate systems listed in Table 4; endosperm protein extracted at pH 7; loading: 106 μg protein/g ATP aliquots. The error bars represent the 95% confidence intervals.

Table 5
Host protein partitioning in PEG-phosphate[a]

PEG MW	Corn fraction	Procedure	K	R_T[b] (μg/g aliquots)	Soluble protein loading[c] (μg/g aliquots)	Comments	
						Precipitation at interface	Location of corn solids
8000	Endosperm	Clarified	0.35 ± 0.07[d]	165 ± 6[d]	170	Yes[e]	–
		Unclarified	0.2 ± 0.05	163 ± 5	170	Yes[e]	Bottom
		Integrated	0.15 ± 0.05	162 ± 7	–	Yes[e]	Bottom
	Germ	Clarified	0.11 ± 0.03	620 ± 44	620	Yes[e]	–
		Unclarified	0.08 ± 0.03	650 ± 98	620	Light brown[f]	Bottom
		Integrated	0.07 ± 0.03	550 ± 48	–	Light brown[f]	Bottom
3350	Endosperm	Clarified	0.44 ± 0.1	155 ± 15	170	Yes[e]	–
		Unclarified	0.32 ± 0.07	155 ± 11	170	Yes[e]	Bottom
		Integrated	0.15 ± 0.03	157 ± 36	–	Yes[e]	Bottom
	Germ	Clarified	0.16 ± 0.02	595 ± 46	620	Yes[e]	–
		Unclarified	0.15 ± 0.03	580 ± 58	620	Light brown[f]	Bottom
		Integrated	0.13 ± 0.02	557 ± 45	–	Light brown[f]	Bottom

[a] ATP systems were PEG–phosphate ATP systems at pH 7 (Table 1, short TLL). Sample loading were 0.1 g endosperm or 0.01 g germ/g ATP system for the two solids-containing procedures and clarified extract from the same amount of solids for the clarified procedure.
[b] The total protein recovered in two phases was calculated from measured concentrations and volumes of the two phases. For the unclarified and integrated procedures, the accuracy was limited because particles at the interface and tube bottom make the accurate volume determination difficult. For all systems, protein that may have precipitated at the interface was not accounted for.
[c] For the two-step procedures, protein loading was calculated from the protein concentration of clarified extract and the volume of that extract (with or without solids) added to the system.
[d] ± ranges represent the 95% confidence intervals.
[e] White precipitate of low protein content.
[f] Mix of precipitate and fine germ particles.

ATP systems [9,23], (2) K depends on the choice of salt, (3) K of corn proteins was generally below 1 for endosperm and still lower for germ fractions, and (4) TLL was not a significant factor for K except for endosperm protein in the Na_2SO_4 system.

It is generally desirable to recover the target protein in the top phase, which is particle-free and better for protein stability; recovery in the bottom salt phase also requires desalting if followed by an ion exchange step. Therefore, systems with low K for the native proteins were explored further on the assumption that these would be well-matched to a class of recombinant proteins partitioning more strongly to the upper phase in these systems. This consideration favors the PEG 8000–salt systems, though, as will be seen, target protein behavior may still favor lower MW. Of these, the PEG–Na_2CO_3 was not favored because its higher pH 9 may destabilize some proteins. Of the remaining systems, PEG–$(NH_4)_2SO$ and PEG–phosphate were chosen to investigate the integrated procedure and manipulation of selectivity via NaCl addition. Furthermore, because TLL was not significant factor in these two systems, only ATP systems with the short TLL were used.

Table 6
Host protein partitioning in PEG-sulfate[a]

Corn fraction	Procedure	K	R_T[b] (μg/g aliquots)	Soluble protein loading[c] (μg/g aliquots)	Comments	
					Precipitation at interface	Location of corn solids
Endosperm	Clarified	0.39 ± 0.07[d]	153 ± 11[d]	170	Yes[e]	–
	Integrated	0.11 ± 0.05	100 ± 6	–	Yes[e]	Bottom
Germ	Clarified	0.16 ± 0.02	610 ± 54	620	Yes[e]	–
	Integrated	0.12 ± 0.01	306 ± 70	–	Light brown[f]	Bottom

[a] ATP systems were PEG 8000–$(NH_4)_2SO_4$ system at pH 7 (Table 1, short TLL). Sample loading were 0.1 g endosperm or 0.01 g germ/g ATP system for the solids-containing integrated procedure and clarified extract from the same amount of solids for the clarified procedure.
[b] The total protein recovered in two phases was calculated from measured concentrations and volumes of the two phases. For the integrated procedures, the accuracy was limited because particles at the interface and tube bottom make the accurate volume determination difficult. For all systems, protein that may have precipitated at the interface was not accounted for.
[c] For the two-step procedures, protein loading was calculated from the protein concentration of clarified extract and the volume of that extract (with or without solids) added to the system.
[d] ± ranges represent the 95% confidence intervals.
[e] White precipitate of low protein content.
[f] Mix of precipitate and fine germ particles.

One other potential tool to manipulate partitioning, pH [34,35], was examined for the PEG–phosphate system. The results (Fig. 3) indicated that the native endosperm proteins partition more to the top phase at basic pH. The MW of PEG also affected the partitioning of endosperm protein more significantly at basic pH, though the apparent MW dependence was confounded with the other changes occurring in parallel, namely the need for higher salt content (Table 3) resulting from titration of the phosphate to higher pH. Earlier studies of such pH effects [34,35] held wt.% salt constant, adjusted pH with addition of phosphoric acid, or were not specific as what wt.% referred to and as a result reported different effects of pH on phase ratios. Here, wt.% PO_4 was held constant (by adding the PO_4 as phosphoric acid and then titrating with base to the desired pH) and salt wt.% and phase ratio both changed with pH (Table 3). Hence, the change in partitioning may have resulted either from change in protein charge with pH or from change in the phase system/TLL (evidenced by phase ratio and salt concentration changing along with pH) or from a combination of both.

3.1.3. Extractive partitioning

For endosperm, *K* was lower for the integrated procedure than for using clarified extracts (Tables 5 and 6). The pattern was similar for germ but the difference was less. There were two likely reasons for the difference: (1) the mix (rather than the total as Tables 5 and 6 show that the amount of soluble protein was roughly the same for all procedures with a given solids fraction) of extracted native proteins was different for the two procedures; (2) the presence of corn solids influences partitioning as reported for other biosolids [36,37]. To examine the isolated influence of solids on partitioning, unclarified extracts were also partitioned (Table 5). *K* of endosperm protein with solids was significantly less than for clarified extracts and marginally higher than for the integrated procedure; however, the presence of germ solids had insignificant effect. Since the solids loading with germ was 10-fold lower, any solids influence would be lower; hence, the endosperm result indicates that the presence of solids was probably a factor affecting protein partitioning that would add to any effect of what proteins were extracted. Tables 5 and 6 also indicate the location of solids. With endosperm and its extracts, no precipitation of protein was observed and the solids settled to the bottom. With germ extract, protein concentration was higher and a white (protein) precipitate appeared at the interface. With extractive partitioning of germ solids, yellow solids settled to the tube bottom and the fine material that appeared at the interface was light yellow indicating a mix of precipitate and finer germ solids.

In the PEG–sulfate system (Table 6), for endosperm the amount of extracted protein was ca. 30% less in the integrated procedure, while for germ it was ca. 50% less. Therefore, the lower *K*'s in the integrated procedure could have resulted from either lower protein concentration (although these were still in the range that independence could be expected [38]) or different protein composition. The lower level of extracted proteins in the integrated procedure would result in less contamination in extracts of recombinant proteins whose solubility was not limited by the phase components.

3.1.4. NaCl addition in extractive partitioning

To realize the advantages of keeping solids and native proteins in the lower phase, it is necessary to direct the target protein to the upper phase. NaCl addition has been used to enhance selectivity for PEG–phosphate, PEG–sulfate and PEG–citrate systems [9,19,24]. But NaCl addition often changes target and contaminant protein partitioning in the same direction [19]. Therefore, to evaluate NaCl addition's effect on selectivity, its effect on native protein partitioning was characterized. For the integrated procedure, the *K* of endosperm protein increased from 0.1 at no NaCl addition to around 9 in PEG 8000–$(NH_4)_2SO_4$ with 10 wt.% NaCl addition (Fig. 4) while the increase was lower in PEG–phosphate systems. *K* of germ protein also increased but was still less than *K* of endosperm protein and below or around one in all ATP systems studied (Fig. 4). The extracted corn native

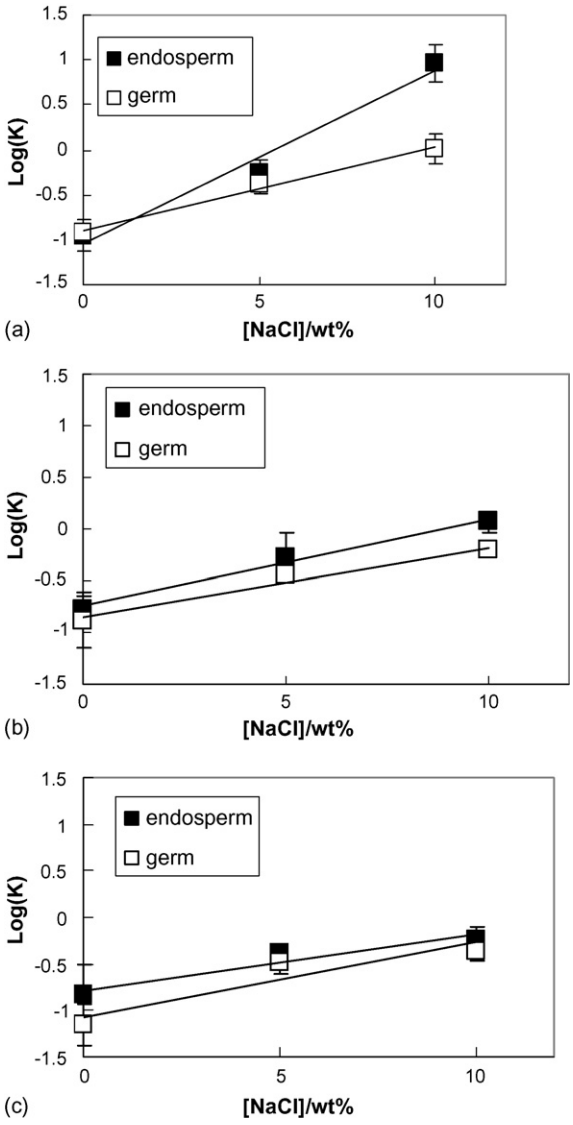

Fig. 4. Salt dependence of partition coefficients of total corn fraction proteins in extractive partitioning. Filled symbols: 0.10 g endosperm/g ATP aliquots; open symbols: 0.01 g germ/g ATP aliquots. (a) PEG 8000 (18.2%)–$(NH_4)_2SO_4$ (11.1%) at pH 7 (b) PEG 3350 (10.6%)–phosphate (10.7%) at pH 7; (c) PEG 8000 (13.2%)–phosphate (10.3%) at pH 7. The error bars represent the 95% confidence intervals.

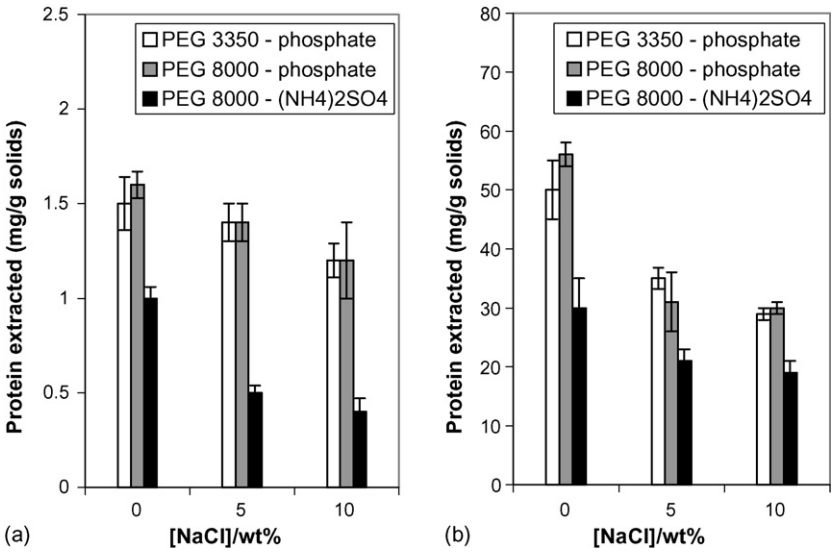

Fig. 5. Salt dependence of total corn fraction protein extraction in extractive partitioning. ATP systems were PEG 8000 (18.2%)–$(NH_4)_2SO_4$ (11.1%), PEG 3350 (10.6%)–phosphate (10.7%) and PEG 8000 (13.2%)–phosphate (10.3%) at pH 7. (a) 0.10 g endosperm/g ATP system; (b) 0.01 g germ/g ATP system. The error bars represent the 95% confidence intervals.

protein decreased with NaCl addition for all systems (Fig. 5), an advantage only if the targeted protein can still be extracted at high salt.

3.2. Model protein purification from corn extracts

Based on the above results and preliminary results (not shown) that favored PEG MW of 3350 for lysozyme partitioning, the PEG 3350–Na_2SO_4 was chosen to apply the ATP principles to purification of the series of three model proteins in integrated partitioning. The three model proteins were then reduced to two cases—purification of lysozyme in the upper phase and purification of cytochrome c in the lower phase.

Preliminary results (not shown) showed little effect of pH on selectivity for lysozyme in extractive partitioning of lysozyme-spiked germ as changes in K for native proteins and lysozyme were all small. However, NaCl addition influenced the partitioning of the model proteins in proportion to their relative hydrophobicity (Fig. 6). Data in Fig. 6 for partitioning of the three model proteins are averages of the K values obtained from germ and endosperm extracts, as the values were not significantly different (individual results are available in Supplementary Material). Fig. 6 offers the best perspective for selecting conditions under which proteins can be recovered in a desired phase from corn or germ extracts. Ribonuclease A behavior likely was too similar to that of host proteins to allow good separation. Lysozyme and cytochrome c each have the potential for selective recovery, though to different phases. The results for lysozyme and cytochrome c will be discussed separately at greater length because of the disparate requirements of targeting partitioning to opposite phases.

3.2.1. Lysozyme

3.2.1.1. NaCl addition. The K of lysozyme increased from 0.37 to above 100 (i.e. below the detectable limit in the lower phase after which calculated PF were based on using the detectable limit as the lower-phase concentration) with 4.5 wt.% NaCl addition, whereas K of corn germ and endosperm proteins only increased about two-fold (Fig. 6). Because lysozyme partitioned totally to the top phase at 4.5 wt.% NaCl, more NaCl addition only pushed more corn protein to the top phase and decreased the purification factor (Table 7). Where complete lysozyme activity was not in the top phase, the remainder was accounted for in the lower phase. In addition, the purification factor of lysozyme from germ was higher than from endosperm due to the lower partitioning of germ proteins to the top phase.

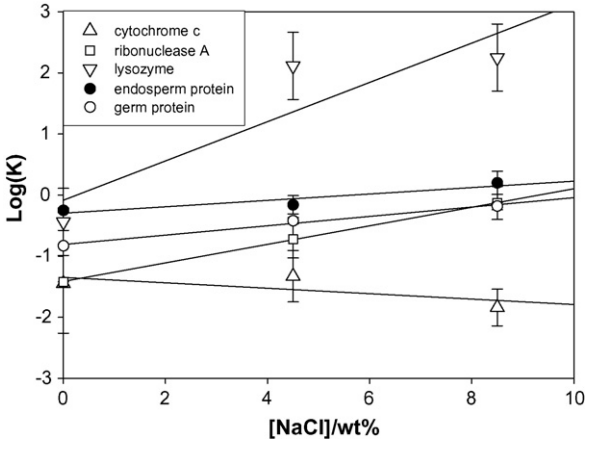

Fig. 6. Effect of NaCl addition on partition coefficients of proteins in PEG 3350 (15.7%)–Na_2SO_4 (8.9%) systems in extractive partitioning at pH 7. 0.10 g endosperm or 0.01 g germ combined with 15 μg model protein/g ATP system. K of model proteins were averages of the separate values from endosperm and germ as there was no significant difference in the values (see Supplementary Material information for individual values). K of corn proteins were the values from experiments without model proteins added. The error bars represent the 95% confidence intervals.

Table 7
Effect of NaCl addition on lysozyme purification from corn proteins by extractive partitioning[a]

Corn fraction	[NaCl] (wt.%)	Φ	PF	Y_{TP}^{top} %[b]	α	$Y_{lysozyme}^{top}$(%)	$Y_{lysozyme}^{bottom}$(%)
Endosperm	0.00	1	2.52 ± 0.23[c]	11 ± 2[c]	1.61 ± 0.2[c]	28 ± 2[c]	69 ± 3[c]
Endosperm	4.50	0.71	4.80 ± 0.96	23 ± 5	151 ± 33	108 ± 5	<DL[d]
Endosperm	8.50	0.71	3.48 ± 0.50	30 ± 5	140 ± 14	105 ± 5	<DL[d]
Germ	0.00	1	3.37 ± 0.55	8 ± 2	3.04 ± 0.49	27 ± 6	76 ± 8
Germ	4.50	0.71	8.43 ± 0.60	13 ± 1	627 ± 45	109 ± 6	<DL[d]
Germ	8.50	0.71	4.63 ± 0.73	23 ± 3	221 ± 37	103 ± 5	<DL[d]

[a] PEG 3350 (15.7%)–Na_2SO_4 (8.9%) phase system with added NaCl. Solids loading of 0.10 g endosperm or 0.01 g germ combined with 15 μg lysozyme/g ATP system.
[b] Corn protein yield based on assuming that the protein available from the solids was the protein that would be extracted by 50 mM phosphate buffer at pH 7 with solid:buffer ratio of 1 g:5 ml and that the amount recovered was that resulting from complete recovery of the top phase.
[c] ± ranges represent the 95% confidence intervals.
[d] Lysozyme activity below the detectable limit (DL); PF based on the DL of 10 U/ml.

3.2.1.2. Phase ratio. Having fixed the nature of top and bottom phases to provide favorable selectivity, product recovery and overall purification factor can still be manipulated by controlling the relative volumes of top and bottom phases (i.e. phase ratio). In principle, selectivity relative to individual proteins should not change; however, here we were looking at selectivity relative to a mixture of native proteins where changes in phase ratio can lead to nonuniform losses from precipitation at the interface so that the apparent *K* of native proteins and selectivity would also change. The integrated partitioning results of lysozyme with corn solids in PEG 3350–Na_2SO_4 with 4.5 wt.% NaCl at different phase ratios (0.71, 0.44 and 0.15) are shown in Fig. 7. The *K* (Fig. 7a) of native corn proteins decreased with increase in phase ratio, but, in terms of top phase impurities, host proteins *decreased* at the lower phase ratio because of the combination of less protein being extracted and the smaller volume of the top phase. Since lysozyme essentially partitioned completely to the top phase for all phase ratios, the net result was that the purification factor (Fig. 7b) and the concentration (Fig. 7c) of lysozyme in the top phase increased linearly when phase ratio decreased from 0.71 to 0.15, while maintaining complete recovery.

3.2.1.3. Sample loading. To consider processing capacity for lysozyme purification by ATPS, sample loading (maintaining 15 μg lysozyme to 0.1 g added endosperm (or 0.01 g added germ)) was also examined (Table 8). The endosperm loading experiments were only carried out at 1× and 2× loading due to the volume capacity of ATP systems for corn solids. The germ loading experiments were carried from 1× to 15×. *K* of endosperm protein increased slightly from 1× to 2×; *K* of germ protein only increased slightly at 5×. Lysozyme partitioned totally to the top phase for all sample loadings (except for 15× germ where it remained absent from the bottom phase but was not completely accounted for in the top phase; the same occurred for a pure lysozyme control). The extracted corn protein decreased slightly with sample loading. Purification factor only changed marginally with sample loading, increasing for endosperm and for germ samples until 10× loading and then decreasing because of the lysozyme loss.

3.2.2. Cytochrome c

Fig. 6 shows that *K* for lysozyme, germ proteins, endosperm proteins, and ribonuclease A increased with NaCl addition. In contrast, *K* for cytochrome c decreased (though to a statistically insignificant degree) with NaCl addition. Furthermore, *K* for endosperm proteins was higher than germ proteins, and the difference among *K*'s for different proteins increased with NaCl addition. The hydrophobic resolution (which is the ability of the system to discriminate between proteins with different hydrophobicities) of PEG 3350–Na_2SO_4 ATP system used here can be compared (Table 4) with previously reported systems, showing that this system was similar to PEG –dextran ATP sys-

Table 8
Effect of sample loading on lysozyme purification[a]

Loading[b]	$K_{corn\ protein}$	Y_{TP}^{top} (%)[c]	PF	$Y_{lysozyme}^{top}$ (%)[d]
1× endosperm	0.47 ± 0.15[e]	23 ± 5[e]	4.8 ± 0.96[e]	108 ± 5[e]
2× endosperm	0.62 ± 0.05	22 ± 1	4.48 ± 0.35	103 ± 7
1× germ	0.29 ± 0.04	13 ± 1	8.43 ± 0.60	109 ± 6
5× germ	0.36 ± 0.01	12 ± 1	7.80 ± 0.59	99 ± 5
10× germ	0.25 ± 0.01	9 ± 1	10.22 ± 0.73	98 ± 7
15× germ	0.24 ± 0.03	9 ± 1	7.06 ± 0.55	64 ± 5

[a] Extractive partitioning of lysozyme and corn fraction proteins in the PEG 3350 (15.7%)–Na_2SO_4 (8.9%) phase system with 4.5 wt.% NaCl.
[b] 1× sample loading: 0.10 g endosperm or 0.01 g germ solids combined with 15 μg lysozyme/g ATP system.
[c] Corn protein yield based on assuming that the protein available from the solids was the protein that would be extracted by 50 mM phosphate buffer at pH 7 with solid:buffer ratio of 1 g:5 ml and that the amount recovered was that resulting from complete recovery of the top phase.
[d] As there was no detectable lysozyme activity in the bottom phase, values <100% indicate inactivation or precipitation.
[e] ± ranges represent the 95% confidence intervals.

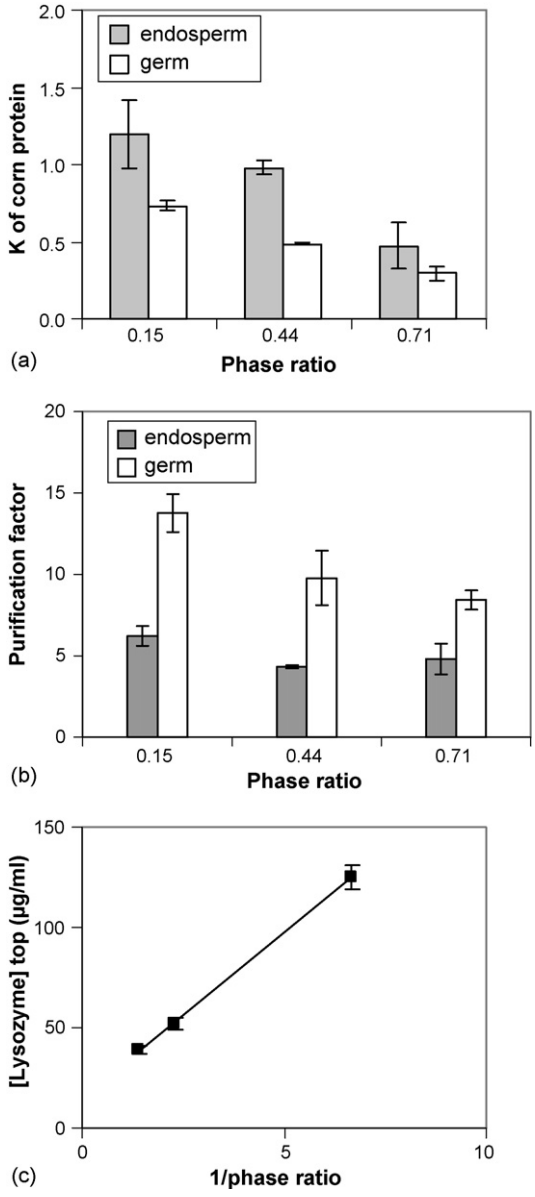

Fig. 7. Phase ratio influence on lysozyme purification and partitioning of corn fraction proteins in extractive partitioning. (a) Corn protein partitioning; (b) purification factor; (c) lysozyme concentration in top phase. PEG 3350–Na_2SO_4 phase system with 4.5 wt.% NaCl as in Table 2. 0.10 g endosperm or 0.01 g germ solids combined with 15 μg lysozyme/g ATP system. The corn solids did not change the phase ratio of the ATP systems. The error bars represent the 95% confidence intervals.

tem [20] and more discriminating (wider range of *K*-values) than EOPO–dextran ATP system [21]. The extent of discrimination corresponds to the spread of the published rankings of hydrophobicity of phase-forming components [9,23]. With the hydrophilicity of cytochrome c offering the opportunity for lower phase recovery, PEG MW effects were examined further for the potential to reduce native protein contamination in the lower phase.

3.2.2.1. PEG MW. Fig. 8 shows the extent to which host proteins can be moved to the upper phase by reduction of PEG MW. The salt effects in this same figure indicate that there

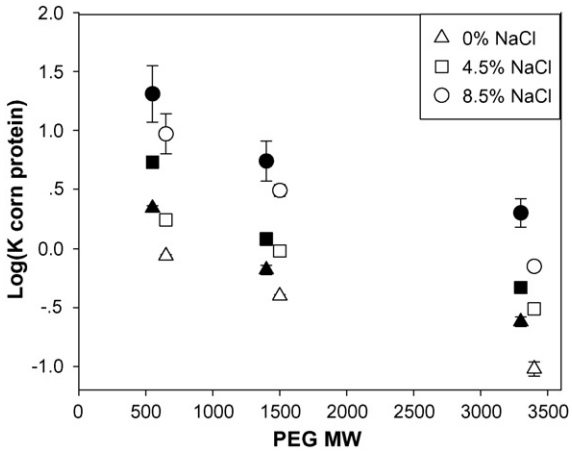

Fig. 8. PEG MW and NaCl addition influences on partition coefficients of native proteins in extractive partitioning. Filled symbols: 0.10 g endosperm combined with 15 μg cytochrome/g ATP system; open symbols: 0.01 g germ combined with 15 μg cytochrome/g ATP system. The compositions of ATP systems are listed in Table 2. Plots are displaced slightly on MW axis for clarity. The error bars represent the 95% confidence intervals.

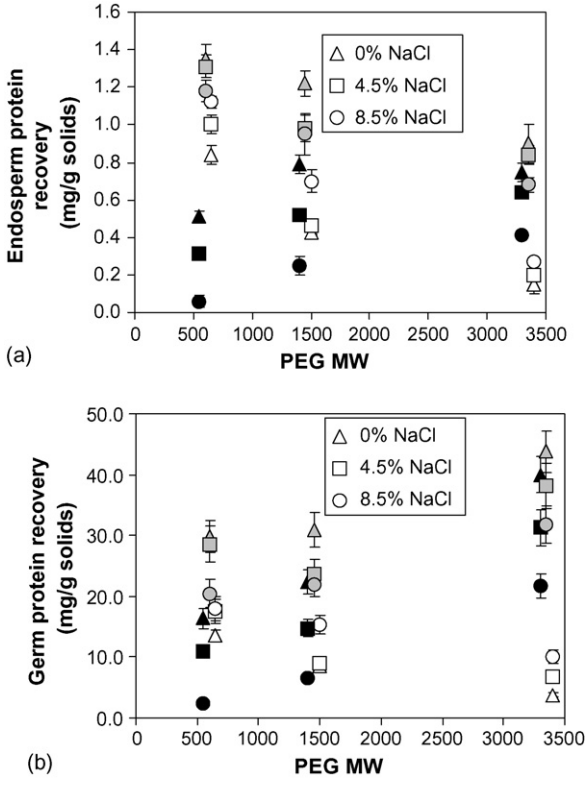

Fig. 9. PEG MW and NaCl addition influence on corn protein extraction in extractive partitioning. For comparison, recovery values for extraction with pH 7 buffer (Fig. 1) are 1.7 mg/g for endosperm (a) and 62 mg/g for germ (b). The compositions of ATP system are listed in Table 2. Filled (gray) symbols: two phases, filled (black) symbols: bottom phase only, open symbols: top phase only (a) 0.10 g endosperm combined with 15 μg cytochrome c/g ATP system; (b) 0.01 g germ combined with 15 μg cytochrome c/g ATP system. Plots are displaced slightly on MW axis for clarity. The error bars represent the 95% confidence intervals.

was little further difference in corn fraction behavior resulting from the hydrophobic resolution increase of added salt. For both corn-solid fractions, reduction of PEG MW and increase in NaCl had the desired effect of directing a higher fraction of the native proteins to the upper phase. From the perspective of total amount of native proteins extracted, lowering PEG MW decreased extraction of germ proteins, but it did increase the amount of endosperm protein (Fig. 9). This opposite trend was likely the result of the offsetting effect of lower salt content (Table 2) of the phase systems with higher PEG MW with the balance of the two effects differing for endosperm and germ extract proteins. This was evident in looking at the amount of protein extracted into the individual phases (Fig. 9) where germ protein concentration in the salt phase was noticeably higher for the higher MW while endosperm proteins were relatively unaffected. The somewhat higher levels of extracted endosperm proteins at lower MW remained low relative to the ca. 30-fold higher levels of extracted germ protein.

The simultaneous effects of these ATP system influences on ribonuclease A and cytochrome c purification in the bottom phase is seen Fig. 10 for PEG 3350. While modest purification occurs even at low salt for ribonuclease A, the response of cytochrome c to NaCl addition makes the high salt addition system effective, particularly for endosperm. For cytochrome c the additional benefits of PEG MW reduction on yield and purification are shown in Table 9 and Fig. 11. With the exception of the PEG 600 with 8.5 wt.% NaCl, there was consistently good recovery and better enrichment in the lower phase in the higher salt, lower PEG MW systems. While this was equally true for germ and endosperm, both as regards trends and average values, purity would be higher for endosperm for comparable expression levels because of the lower levels of extractable proteins from endosperm. The anomalous result for PEG 600

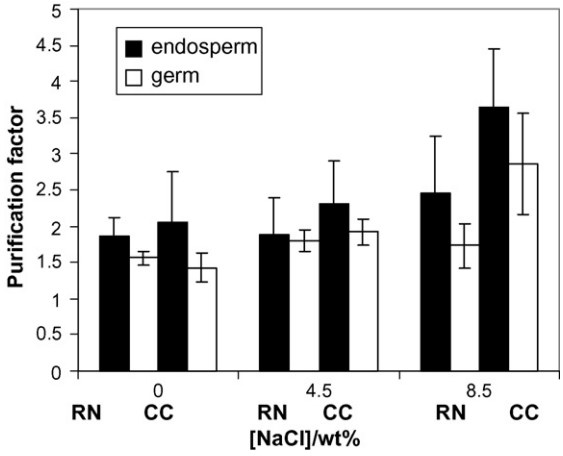

Fig. 10. NaCl addition influences on purification factors in the bottom phase of cytochrome c and ribonuclease A after extractive partitioning. ATP systems listed in Table 2. Filled bars: 0.10 g endosperm/g ATP system; open bars: 0.01 g germ/g ATP system. RN: ribonuclease A; CC: cytochrome c. The error bars represent the 95% confidence intervals.

with 8.5 wt.% NaCl addition can be attributed to the overall cytochrome c losses of 76% and 37% for endosperm and germ, respectively (Table 9). These losses were attributed to precipitation at the high NaCl/Na_2SO_4 levels in the PEG 600 system. Because of this loss, the optimal conditions were PEG 1450 with 8.5 wt.% NaCl addition, where PF = 4.7 with Y = 93% and PF = 9.1 with Y = 100% for recovery from endosperm and germ extracts, respectively. Cytochrome c loss and the high variability of that loss at 8.5 wt.% NaCl/PEG 600 also leads to the higher variability in purification factors seen in Fig. 11.

The drawback to such lower phase recovery with the integration of extraction and partitioning is that solids removal requires

Table 9
Effect of PEG MW, NaCl addition and corn fraction on partition coefficient and yield of cytochrome c in extractive partitioning[a]

Corn fraction	PEG MW	[NaCl] wt.%	K_{cyt}	Y_{cyt}^{bottom}(%)	Mass balance[b] of cytochrome[c] (%)
Endosperm	600	0	0.17 ± 0.1[c]	93 ± 2[c]	110 ± 13[c]
		4.5	0.32 ± 0.27	96 ± 5	117 ± 23
		8.5	7.51 ± 6.7	4 ± 1	24 ± 18
	1450	0	0.04 ± 0.04	99 ± 4	103 ± 6
		4.5	0.04 ± 0.01	102 ± 6	105 ± 6
		8.5	0.04 ± 0.01	93 ± 2	96 ± 2
	3350	0	0.03 ± 0.01	102 ± 8	106 ± 8
		4.5	0.04 ± 0.01	99 ± 5	102 ± 5
		8.5	0.02 ± 0.01	100 ± 7	101 ± 8
Germ	600	0	0.23 ± 0.03	87 ± 8	108 ± 9
		4.5	0.36 ± 0.3	87 ± 13	109 ± 7
		8.5	0.7 ± 0.66	42 ± 26	63 ± 29
	1450	0	0.27 ± 0.05	83 ± 14	106 ± 15
		4.5	0.06 ± 0.01	114 ± 9	119 ± 16
		8.5	0.03 ± 0.01	116 ± 37	119 ± 37
	3350	0	0.04 ± 0.01	88 ± 2	91 ± 2
		4.5	0.06 ± 0.03	83 ± 8	86 ± 7
		8.5	0.01 ± 0.01	103 ± 34	104 ± 34

[a] ATP systems were PEG–Na_2SO_4 systems listed in Table 2. Solids loading of 0.10 g endosperm or 0.01 germ combined with15 μg cytochrome c/g ATP system.
[b] % of cytochrome c accounted for in two phases.
[c] ± ranges represent the 95% confidence intervals.

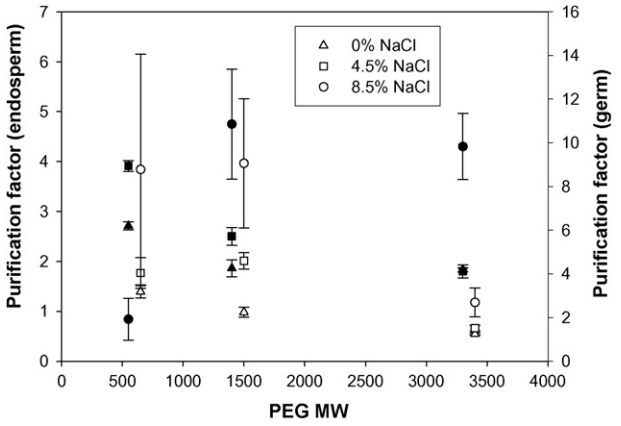

Fig. 11. PEG MW and NaCl addition influences on purification factor in the bottom phase of cytochrome c after extractive partitioning. ATP systems listed in Table 2. Filled symbols: 0.10 g endosperm/g ATP system; open symbols: 0.01 g germ/g ATP system. Plots displaced are slightly on MW axis for clarity. The error bars represent the 95% confidence intervals.

a separate step; however, there is an advantage in reduced need for PEG removal [39].

4. Conclusion

For purification of a recombinant (target) protein from corn, the purity achieved will depend on the properties of that protein, but also on the amount of native proteins in the extract and the native protein partitioning behavior. The amount of protein extracted from endosperm was much less than that from germ; in both cases the amount of extracted protein increased with pH. Assuming similar levels of expression of recombinant protein, this provides an initial advantage to targeting to endosperm or having a protein with high solubility at low pH if targeted to germ. Partitioning behavior is the result of interaction of the proteins with phase system components, in this case chosen to separate chiefly on the basis of hydrophobicity differences. Extracted corn protein favored the bottom phase in most PEG–salt ATP systems. K of endosperm protein was higher than that of germ protein in all ATP systems studied. K of corn protein increased with pH and decreased with PEG MW. TLL was not a significant factor for K in most PEG 8000–salt systems.

APS offers the possibility of integration of the extraction and partitioning steps. K of corn proteins decreased in the integrated extractive partitioning because of the presence of corn solids and the amount of host proteins extracted was also lower. NaCl addition increased K but decreased extraction of corn proteins in the integrated procedure. These effects were more evident in PEG 8000–$(NH_4)_2SO_4$ systems than in PEG–phosphate systems.

NaCl addition, aimed at increasing the hydrophobic resolution of the APS, enhanced the selective recovery of lysozyme (a relatively hydrophobic protein) in PEG 3350 (15.7 wt.%)–Na_2SO_4 (8.9 wt.%). At 4.5 wt.% NaCl, selectivity for lysozyme in the top phase relative to corn fraction proteins was 134 and 616 for endosperm and germ, respectively, with essentially complete recovery in the top phase. For a phase ratio of 0.15, the purification factor for lysozyme from germ protein was 14. Increasing corn solids and lysozyme loading did not change the purification factor significantly until 10× loading for germ.

A different set of conditions was required for selective recovery of the more hydrophilic protein, cytochrome c, in the lower phase. Lowering PEG MW and adding NaCl resulted in PF = 4.7 and Y = 93% and PF = 9.1 and Y = 100% for recovery from endosperm and germ extracts, respectively, in the PEG 1450–8.5% NaCl–Na_2SO_4 system.

Thus, it has been demonstrated that an APS can be chosen and optimized for recovery of either hydrophobic or hydrophilic proteins from corn extracts and that extraction can be advantageously integrated with the partitioning step.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2006.07.025.

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