Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Recovery of Recombinant Dog Gastric Lipase from Corn Endosperm Extract

Zhengrong Gu[a]; Charles E. Glatz[a]
[a] Department of Chemical and Biological Engineering, Iowa State University, Ames, IA

To cite this Article Gu, Zhengrong and Glatz, Charles E.(2007) 'Recovery of Recombinant Dog Gastric Lipase from Corn Endosperm Extract', Separation Science and Technology, 42: 6, 1195 — 1213
To link to this Article: DOI: 10.1080/01496390701241980
URL: http://dx.doi.org/10.1080/01496390701241980

Separation Science and Technology, 42: 1195–1213, 2007

ISSN 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390701241980

Recovery of Recombinant Dog Gastric Lipase from Corn Endosperm Extract

Zhengrong Gu and Charles E. Glatz
Department of Chemical and Biological Engineering, Iowa State University, Ames, IA

Abstract: Two separation methods, aqueous two-phase (ATP) partitioning and cation-exchange chromatography, were compared as alternative methods for the recovery of recombinant dog gastric lipase (r-DGL) from extracts of transgenic corn endosperm. r-DGL is a hydrophobic, acid-stable protein targeted for stable expression in endosperm. Polyethylene glycol (PEG) - salt ATP system parameters of PEG molecular weight, phase-forming salt, NaCl addition, Triton X-100 concentration and phase ratio were adjusted to achieve favorable partitioning. The purification factor and yield of r-DGL in the bottom phase of a PEG 3350 (14.2%)-Na_2SO_4 (8.5%)-NaCl (0.5%)-Triton X-100 (2 mM) system at pH 4 were 1.5 and 80%, respectively. A higher purification factor of 2.3 and nearly 100% yield of r-DGL was obtained in the top phase of a PEG 3350 (9.4%)-phosphate (14.3%)-NaCl(1.5%)-Triton X-100 (2 mM) system at pH 4.0. The yield, purification factor, and concentration factor were 90%, 7.7, and 3.6, respectively, for the alternative of cation-exchange on CM-Sepharose. Countercurrent ATP partitioning with 3–7 stages was calculated to achieve a purification factor equivalent to that from cation exchange but with a lower concentration factor. While the cation exchange was favored on this basis, the two approaches were close enough that further optimization and economic analysis would be needed to be definitive.

Keywords: Dog gastric lipase, extraction, Triton X-100, corn, endosperm, aqueous two-phase, PEG, cation exchange, transgenic

Received 19 June 2006, Accepted 7 January 2007
Address correspondence to Professor Charles E. Glatz, Department of Chemical and Biological Engineering, Iowa State University, 2114 Sweeney Hall, Ames, IA 50011. Tel.: (515) 294-8472; Fax: (515) 294-2689; E-mail: cglatz@iastate.edu

INTRODUCTION

Gastric lipases have been proposed for orally-administered treatment of exocrine pancreatic insufficiency such as occurs in cystic fibrosis (1). Their stability and activity in acidic gastric medium enables oral administration. Among several gastric lipases, dog gastric lipase (DGL), a 49 kDa glycoprotein of isoelectric point (pI) 6.3, has the highest activity on long-chain triacylglycerols (the main components of human dietary fats) and was judged to have the most potential for treatment of cystic fibrosis (1).

Recombinant gastric lipases have been expressed using insect cells (2–4) and yeast (5). However, plant hosts may be favored because they do not contain human or animal pathogens and can provide for glycosylation and folding (6). In addition, transgenic plants offer large and flexible production capacity and lower production cost than transgenic animals or mammalian cell cultures (7, 8).

The first transgenic plant hosts providing active r-DGL expression were tobacco (9) and maize (corn) (1). Maize has the advantages of longer storage stability in the seed, lower levels of phenolics, and the absence of toxic nicotine. Recovery can be simplified by targeted expression to endosperm, which can be separated from germ and hulls by dry-milling (10). Removal of the latter fractions greatly reduces the levels of oil and soluble native proteins (10). Further reduction of the separation burden can be achieved by selection of extraction conditions(11). These optimized strategies for upstream processing and extracting of r-DGL from corn seeds have been reported (12).

As a relatively low-cost recovery method, aqueous two-phase (ATP) partitioning offers the initial advantage of less stringent requirements for removal of residual solids from the extract slurry and the potential for continuous, multi-stage operation (13). PEG-phosphate systems have been widely studied for separating microbial lipases (14–19), especially hydrophobic microbial lipases (16), from fermentation broths. However, there are few applications of ATP partitioning to plant hosts (20–24). PEG-Na_2SO_4 systems have been successfully used for recovering lysozyme, a relatively hydrophobic protein, from tobacco (20) and extracts of corn endosperm (21).

The selectivity of ATP partitioning in PEG-salt systems can be controlled by the hydrophobic difference between the two phases and the hydrophobicities of target protein and host proteins (25). Based on amino acid composition, r-DGL is more hydrophobic than lysozyme (Grand Average of Hydropathicity (GRAVY) values of -0.069 and -0.15, respectively) (26), which has been purified from corn endosperm extracts by ATP partitioning (21).

The PEG-salt ATP parameters of PEG MW, the phase-forming salt, and NaCl addition are seen to be the most important factors for selective recovery of proteins in the PEG phase (27). NaCl addition causes relatively hydrophobic proteins to partition more to the PEG phase by increasing the hydrophobic difference between the two phases (28), promoting stronger hydrophobic interaction

between the proteins and PEG (29). Addition of surfactants can provide an additional means of enhancing partitioning. Triton X-100 was used as an affinity ligand to drive partitioning of relatively hydrophobic proteins to the hydrophobic top phase of thermo-separating ATP systems(15).

Once partitioning has been fixed by choice of system composition, the volume ratio of the top phase to the bottom phase (phase ratio) can also be manipulated as has been done for the purification of lysozyme from corn endosperm extracts (21).

In this work, the PEG-Na_2SO_4 and PEG-phosphate systems were chosen to provide a hydrophobic basis for separating r-DGL from endosperm extract. PEG MW, the phase-forming salt, and NaCl addition were manipulated to provide an initial favorable partitioning. Then Triton X-100 concentration and the phase ratio of the selected ATP system were manipulated to optimize the purity and recovery of r-DGL. The performance was compared to that obtained using cation-exchange chromatography, which has provided for selective capture of r-DGL (pI = 6.3) when run at pH 4–5.2 (1, 2). Such conditions should be applicable to separation from corn proteins, which have an average pI of 4.7 (30).

Definition of Parameters in ATP Systems

The tie line length (*TLL*) characterizes the compositional differences between the two phases:

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

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

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

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

where C_i is the concentration of component i (total protein (TP), native endosperm protein (NEP) or r-DGL). The total protein is expressed as mass, while r-DGL is measured as activity units (U). The concentration of the native endosperm protein is calculated by difference

$$C_{NEP} = C_{TP} - \frac{C_{rDGL}}{65\,\mathrm{U/mg}} \tag{4}$$

where 65 U/mg is the specific activity of pure r-DGL

The selectivity of a single stage (α) is:

$$\alpha = \frac{K_{r-DGL}}{K_{NEP}} \tag{5}$$

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

$$\phi = \frac{V_{top}}{V_{bottom}} \tag{6}$$

The purification factor (*PF*) for recovery in the product-phase is the ratio of product-phase specific activity (*SA*) to initial *SA* in the extract.

$$PF = \frac{SA^{product}}{SA^{initial}} \tag{7}$$

Yield (*Y*) of protein is the fractional recovery in the product-phase.

$$Y_i^j = \frac{C_i^j \times V_j}{M_i} \tag{8}$$

where M_i is the mass (or activity units) of r-DGL, native endosperm protein, or total protein added.

The mass balance (*MB*) on a protein is the fraction of protein accounted for in the two phases

$$MB_i = \sum_{j=1}^{2} \frac{C_i^j \times V_j}{M_i} \tag{9}$$

The concentration factor (*CF*) in the product phase is defined as the ratio of concentration of product ($C_{r-DGL}^{product}$) to initial $C_{r\text{-}DGL}$ in the extract.

According to the parameters defined above, the purification factor of the r-DGL in the top phase can be expressed as a function of partition coefficients, phase ratio, and mass balances.

$$PF_{TOP} = \frac{K_{r-DGL} \times MB_{r-DGL} \times (K_{TP} \times \phi + 1)}{K_{TP} \times MB_{TP} \times (K_{r-DGL} \times \phi + 1)} \tag{10}$$

MATERIALS AND METHODS

Materials

The 4-nitrophenyl butyrate (NPB), CM-Sepharose resin (CL-B6) and L-histidine were from Sigma-Aldrich (St. Louis, MO). Other chemicals were ACS certified grade from Fisher Scientific (Pittsburgh, PA). The water was deionized. The endosperm-enriched fraction of corn was prepared by dry milling of r-DGL (targeted expression to endosperm) corn (10) provided by Meristem Therapeutics (Clermont-Farrand, France).

r-DGL Extraction

Extracts were prepared (12) by stirring 1 g endosperm meal in 10 ml of extraction buffer (20 mM of sodium phosphate, 200 mM NaCl, 2 mM Triton X-100 at pH 3.0) for 6 h before clarification by centrifuging (10,000 × *g* for 30 min) and decanting through two layers of Whatman #4 filter paper (Fisher Scientific, Pittsburgh, PA). This "coarse-clarified" extract was assayed immediately for lipase activity and protein concentration. Before cation exchange, the coarse-clarified extract was adjusted to pH 4.5 and filtered (0.45 μm nitrocellulose membrane; Millipore, Billerica, MA) without loss of r-DGL or protein. To investigate the influence of Triton X-100 on the protein partition coefficient, an extract of r-DGL without Triton X-100 was also prepared.

ATP Partitioning

The PEG (50%) and sulfate (23%) stock solutions were prepared in 20 mM phosphate, 2 mM Triton X-100 and pH 4.0 buffer. The pH 4.0 phosphate (40%) stock solution containing 2 mM Triton X-100 was prepared by combining appropriate amounts of NaH_2PO_4 and K_2HPO_4 (10:1 by weight). The mixed cations were required because of solubility limitations of the respective phosphate salts. The same stocks were also prepared without Triton X-100. Final phase system compositions, after a combination of stocks and endosperm extracts are shown in Table 1. Tie line lengths in this table are calculated from published phase diagrams for these systems (27, 31).

Five ml of coarse-clarified extract was mixed with 6 ml total of phase stock solutions to form an 11 ml ATP system in a 16 ml graduated centrifuge tube. After 30 min of gentle mixing on an end-to-end shaker (Lab Industries Inc., Berkeley, CA) at room temperature (22°C), phases were separated by centrifugation at 1000 × g for 10 min. Final phase volumes were recorded, the top phase was removed by transfer pipette, and the bottom phase was collected by piercing the centrifuge tube with a syringe; the region near the interface was left in the tube to avoid contamination of the phase samples. The total protein and r-DGL concentrations in samples from each phase were analyzed. Each partitioning was replicated three times and each assay twice.

Cation Exchange Chromatography

Clarified extract (60 ml) was loaded onto 5 ml of CM-Sepharose (preliminary results gave low lipase recovery with SP-Sepharose) resin (Sigma-Aldrich, St. Louis, MO) in a glass Econo-Column with flow adaptor (200 × 10 mm; Bio-Rad, Hercules, CA) that had been equilibrated with 15 ml Buffer A (20 mM sodium acetate buffer at pH 4.5 with 200 mM NaCl). After sample loading, the column was washed with 20 ml of Buffer A. r-DGL was eluted

Table 1. Compositions of ATP systems[a]

ATP system	Concentration of components (wt%) PEG	Phase-forming salt	NaCl	Φ	*TLL*[b] (wt%)
PEG 1450 – Na_2SO_4 (pH 4)	12.7	9.6	0.5	1	23.2 (31)
			6.0	0.57	
PEG 3350 – Na_2SO_4 (pH 4)	12.4	8.5	0.5	1	23.4 (31)
			6.0	0.83	
PEG 8000 – Na_2SO_4 (pH 4)	13.0	8.0	0.5	0.57	26.4 (31)
			6.0	0.47	
PEG 3350 – NaH_2PO_4/ K_2HPO_4 (pH 4)[c]	14.0	12.0	0.5	1	
			1.5[d]	1	
			2	1	
			4	1	
			6	0.83	33.2 (27)
			8	0.83	
	3.1	18.5	1.5[d]	0.13	
	5.4	17.0		0.29	
	9.4	14.3		0.5	

[a]All systems contained 2 mM Triton X-100 except where noted in Table 3.
[b]In the absence of NaCl.
[c]NaH_2PO_4: K_2HPO_4 = 10:1 (w:w).
[d]Influence of phase ratio was investigated in these systems; overall PEG and salt wt% change only because of the phase ratios used.

in a linear gradient from 100% Buffer A to 100% Buffer B (20 mM sodium acetate buffer at pH 4.5 with 1 M NaCl) in 25 ml. An alternative of step elution by 20 mM sodium acetate buffer, pH 4.5, with 500 mM NaCl was also evaluated. After elution, the column was washed with 15 ml of 0.1 M NaOH to remove strongly absorbed components. The flow rate was 1 ml/ min throughout, output was monitored by A_{280}, and fraction volumes were 5 ml. The total protein and r-DGL concentrations in each fraction were analyzed.

Lipase Assay

The lipase activity assay followed the analytical method reported by Zhong and Glatz (32). This assay was chosen for its tolerance to the presence of nonionic detergents in the sample. Assays involved the emulsification of 6 μl of 0.15 M NPB dissolved in n-heptane in 1 ml of 50 mM L-histidine

buffer at pH 5.5 with 0.01% (w/v) Tween 80 by vortexing for 10 sec. The 6 μl lipase samples were added, the emulsion was transferred into a quartz cuvette, and the absorbance was monitored at 346 nm for 5 mins at 25°C. The initial slope was used to calculate the lipase activity. One unit of lipase activity corresponded to the production of 1 μmol of nitrophenol in1 min. Absorbance was converted to nitrophenol concentration based on a standard curve of pure nitrophenol at pH 5.5.

Protein Assay

The bicinchoninic acid (BCA) method (33) was chosen because Triton X-100 did not interfere. Reagent A, containing BCA, was purchased from Pierce (Rockford, IL), while reagent B, 4% cupric sulfate, was prepared in the lab. Assays followed the procedures of the Pierce manual, with incubation at 37°C for 30 min. Bovine serum albumin was used as the reference standard.

RESULTS AND DISCUSSION

ATP Partitioning

PEG-Na_2SO_4 systems

Effects of NaCl Addition and PEG MW

$K_{r\text{-}DGL}$ increased with NaCl addition (Table 2) as was anticipated based on the added hydrophobicity difference between phases after salt addition (28). K_{TP} did not increase with NaCl addition to quite the same extent as $K_{r\text{-}DGL}$ indicating that r-DGL is more hydrophobic than most of the extracted endosperm proteins. But the purification of r-DGL in the top phase was not enhanced greatly because K_{TP} also increased to above one for all PEG-Na_2SO_4 systems. In interpreting the magnitudes of PF in these $\Phi = 1$ systems, it should be noted that the maximum possible value (complete partitioning of r-DGL to the top phase) is 2 when there is even partitioning of total host proteins (see Eqn (11) below). Attaining high purification requires not only strong partitioning of lipase to the upper phase, but also favorable partitioning of the host proteins to the lower phase.

Furthermore, K_{TP} and $K_{r\text{-}DGL}$ generally decreased with higher PEG MW, most clearly at high NaCl. Because concentrations of both phase-forming components change along with PEG MW, the cause is not clearly pinpointed. However, this decrease is consistent with the stronger volume exclusion effect for higher MW PEG (34), which would exclude more proteins from the top phase.

Table 2 also shows that r-DGL activity was not completely recovered in all PEG-Na_2SO_4 systems. The losses of r-DGL activity were especially high in

Table 2. Partitioning of r-DGL containing corn endosperm extract in PEG-Na_2SO_4 ATP systems at pH 4

PEG MW	NaCl (%)	K_{TP}	$K_{r\text{-}DGl}$	$Y_{r\text{-}DGL}$ (%)			Y_{TP} (%)			PF		
				Bottom	Top	Third	Bottom	Top	Third	Bottom	Top	Third
1450	0.5	1.56 ± 0.08[a]	0.19 ± 0.02	79 ± 5	12 ± 1	ND[b]	45 ± 4	61 ± 6	—	1.66 ± 0.08	0.2 ± 0.02	—
	6	2.05 ± 0.09	11.9 ± 2.3	11 ± 2	74 ± 2	ND	48 ± 5	55 ± 5	—	0.23 ± 0.05	1.31 ± 0.03	—
3350	0.5	0.9 ± 0.03	0.04 ± 0.01	81 ± 6	3.5 ± 0.5	ND	54 ± 2	49 ± 3	—	1.51 ± 0.06	0.07 ± 0.01	—
	6	1.74 ± 0.11	3.69 ± 0.24	21 ± 1	63 ± 4	16 ± 5[c]	35 ± 2	62 ± 3	3 ± 3[c]	0.48 ± 0.03	1.02 ± 0.06	5 ± 1[c]
8000	0.5	0.91 ± 0.04	0.16 ± 0.02	36 ± 1	3.4 ± 0.3	ND	66 ± 7	34 ± 3	—	0.55 ± 0.01	0.1 ± 0.01	—
	6	1.41 ± 0.04	1.27 ± 0.17	24 ± 2	14 ± 1	ND	56 ± 6	36 ± 4	—	0.42 ± 0.06	0.38 ± 0.02	—

[a]$\pm$ Ranges are 95% confidence intervals.

[b]ND: not detected.

[c]The yields of r-DGL and total protein (TP) and purification factor were calculated based on amount added with the extract; third-phase volume was by difference (assuming a total of 100% recovery), because the third-phase volume was not accurately measured.

the PEG 8000-Na_2SO_4 system, where precipitation was observed at the interface and more than 60% r-DGL was lost. This high r-DGL loss is likely the result of reduced solubility of r-DGL in the presence of higher MW PEG (34).

6% NaCl addition resulted in the formation of a small volume of a viscous, yellow phase above the PEG-rich top phase for all PEG MW, but only for PEG 3350 was there detectable protein in this third phase. The third phase likely consisted of oil and oil-soluble species because the endosperm rich fraction still contains 1.63% oil (10). The activity assay of the third phase samples from the PEG 3350-Na_2SO_4-6% NaCl system showed high r-DGL activity, 55 ± 10 unit/ml compared to r-DGL concentration in the top and bottom phases of 10.2 ± 0.6 and 2.8 ± 0.2 unit/ml. Therefore, if the third phase of PEG 3350-Na_2SO_4-6% NaCl system could be collected separately, the r-DGL would be recovered in a more concentrated form though with lower yield (Table 2). The r-DGL activity was not detected in the third phases of PEG 1450 and PEG 8000 systems. Again, Na_2SO_4 and PEG concentration also varied among these three systems (Table 1). Thus, one can not conclude the difference is solely due to PEG MW.

Purification of r-DGL in Bottom Phase

Top phase PF for r-DGL was not high in any PEG-Na_2SO_4-6% NaCl systems because of high K_{TP} (above one) and r-DGL loss (Table 2). On the other hand, in PEG 3350 or 1450-Na_2SO_4 -0.5% NaCl ATP systems, the r-DGL was partially enriched and purified in the bottom phase because most r-DGL was retained in the bottom phase ($K_{r\text{-}DGL}$ was around 0.1), while the total protein was distributed almost evenly (K_{TP} was around 1) in the two phases (Table 2).

PEG 3350-Phosphate Systems

Effects of NaCl Addition

In contrast to the PEG-Na_2SO_4 systems, NaCl addition to a PEG 3350-phosphate system did lead to effective top phase recovery of r-DGL. NaCl addition gave much higher $K_{r\text{-}DGL}$ and yield of r-DGL in the top phase (Fig. 1a). The r-DGL partitioned almost completely into the top phase of PEG 3350-phosphate systems with 1.5% or higher levels of added NaCl. K_{TP} also increased with NaCl addition but not as much as $K_{r\text{-}DGL}$. The net result was improved r-DGL purification in the top phase with the best result between 1.5% ($PF = 1.62 \pm 0.03$, $Y = 89 \pm 3\%$) to 2% NaCl addition ($PF = 1.66 \pm 0.09$, $Y = 96 \pm 7\%$) (Fig. 1c).

Though top-phase selectivity for r-DGL relative to NEP was extremely high ($\alpha = 45$ from Eqn (5) and Fig. 1a), this was not as apparent in the purification factor because K_{NEP} was still higher than one. This can be conveniently seen from the limiting case of Equation (10) for phase ratio equal to one (Table 1), mass balances close to 100% (Fig. 1b) and

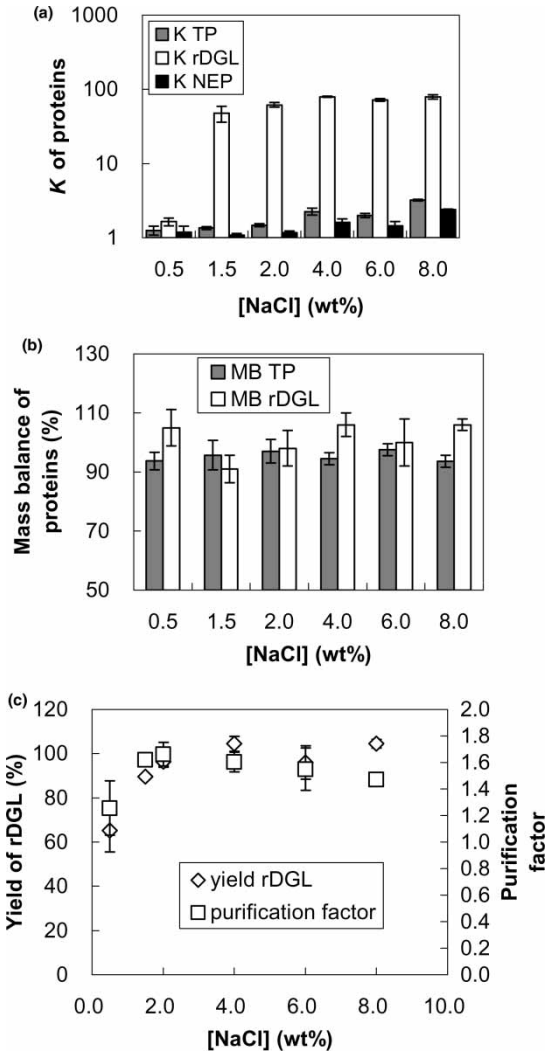

Figure 1. Influence of NaCl addition on partitioning of r-DGL-containing endosperm extract in PEG 3350 (14%)-phosphate (12%) systems at pH 4. (a) Partition coefficient. (b) Mass balance of r-DGL and total protein. (c) Purification factor and yield of r-DGL in the top phase. Error bars are 95% confidence intervals.

$K_{r\text{-}DGL} >> K_{TP} > K_{NEP} > 1$ (Fig. 1a), which gives

$$PF_{TOP} = \frac{K_{TP} + 1}{K_{TP}} < 2 \tag{11}$$

That K_{NEP} was more than one (Fig 1a) contrasts with an expectation of $K < 1$, based on our result for NEP under similar conditions (partitioning in

a similar pH4, PEG3350-phosphate system (21) but of pH 7 extract rather than pH 3 here). There are two possible explanations:

1. affinity of some of these proteins causes them to partition to the Triton rich top phase (15, 35) or
2. the Triton X-100 in the extract buffer dissolves a greater proportion of the hydrophobic proteins with inherently higher K.

This Triton X-100 influence is explored further in Table 3 where Triton levels in the extract and ATP system are varied. In the complete absence of Triton, $K_{NEP} < 1$ as expected (see above). Addition of Triton to either extract or ATP system increases K_{NEP}. Since the result is nearly the same for NEP as long as Triton is present for partitioning, extraction of a different set of host proteins is not likely responsible. The presence of Triton X-100 is chiefly desired for maintaining high yield of r-DGL in the top phase.

Both K_{TP} and $K_{r\text{-}DGL}$ decreased slightly with decreasing Triton X-100 concentration, while r-DGL losses were significant at lower Triton X-100 levels (Table 3). Therefore, Triton X-100 concentration was kept at 2 mM to avoid the significant yield loss.

The most important advantage of PEG 3350-phosphate over PEG 3350-Na_2SO_4 was that the top-phase yield and purification of r-DGL were higher (Fig. 1b and Table 2). In addition, in the PEG 3350-Na_2SO_4 -NaCl (6%) system, r-DGL also partitioned into the third phase (Table 2), while no third phase was observed in PEG 3350-phosphate systems.

There are two possible reasons for the sulfate/phosphate differences.

1. Triton X-100 is expected to be constrained to the PEG phase in the sulfate system with $K > 10$ (36). Therefore, the lack of Triton X-100 in the bottom phase causes oil and hydrophobic components including a portion of the r-DGL to become insoluble and form the third phase.
2. The stronger salting-out capability of SO_4 relative to phosphate (27) along with its ionic strength ($I = 1.5$) in the PEG 3350-sulfate system results in lower solubility of r-DGL in this system than in the PEG 3350-phosphate systems ($I = 1.3$). Only the more effective PEG 3350-phosphate system was studied further.

Effect of Phase Ratio

Having fixed the nature of the top and bottom phases to provide favorable selectivity, product recovery and overall purification factor can still be manipulated by controlling the phase ratio (21), though not always effectively (37). The r-DGL was partitioned completely to the top phase at phase ratios of 0.29 and higher (Fig. 2a). The apparent $K_{r\text{-}DGL}$ increase may be misleading as it results from assuming a bottom phase composition of the detectable limit when, in fact, none was detected. At the still lower phase ratio of 0.13,

Table 3. Triton X-100 influence on partitioning of r-DGL and endosperm protein in PEG 3350 (14%)-phosphate (12%)-NaCl (1.5%) system at pH 4 ($\Phi = 1$).

Triton X-100 concentration (mM)								MB (%)	
In extract	In final ATPS	$K_{r\text{-}DGL}$	K_{TP}	K_{NEP}	PF_{Top}	Purity[a] (%)	$Y^{Top}_{r\text{-}DGL}$ (%)	r-DGL	TP
2	1	39 ± 9[b]	1.34 ± 0.05	1.12 ± 0.05	1.34 ± 0.05	17 ± 1	72 ± 3	74 ± 4	94 ± 1
	2	48 ± 12	1.35 ± 0.08	1.09 ± 0.05	1.61 ± 0.08	21 ± 1	89 ± 3	90 ± 4	94 ± 1
0	0	26 ± 2	0.76 ± 0.07	0.71 ± 0.02	1.34 ± 0.12	17 ± 1	57 ± 5	59 ± 4	99 ± 2
	1	32 ± 2	1.17 ± 0.07	1.10 ± 0.07	1.31 ± 0.07	17 ± 1	70 ± 4	72 ± 4	100 ± 1
	2	44 ± 1	1.35 ± 0.14	1.21 ± 0.22	1.63 ± 0.14	21 ± 1	98 ± 3	100 ± 3	100 ± 1

[a]r-DGL as % of total proteins.
[b]$\pm$ ranges are 95% confidence intervals.

precipitation was observed on the interface and approximately 42% r-DGL and 13% total protein were not accounted for in the two phases (Fig. 2b).

At the same time, K_{TP} increased with decreasing phase ratio. In principle, the K of individual proteins would be independent of the phase ratio; however, here we are combining a mixture of native proteins, where changes in phase ratio can lead to changes in K_{TP} even if Ks of individual proteins remain constant. This can be seen by combining the definition of K_{TP} as the sum of

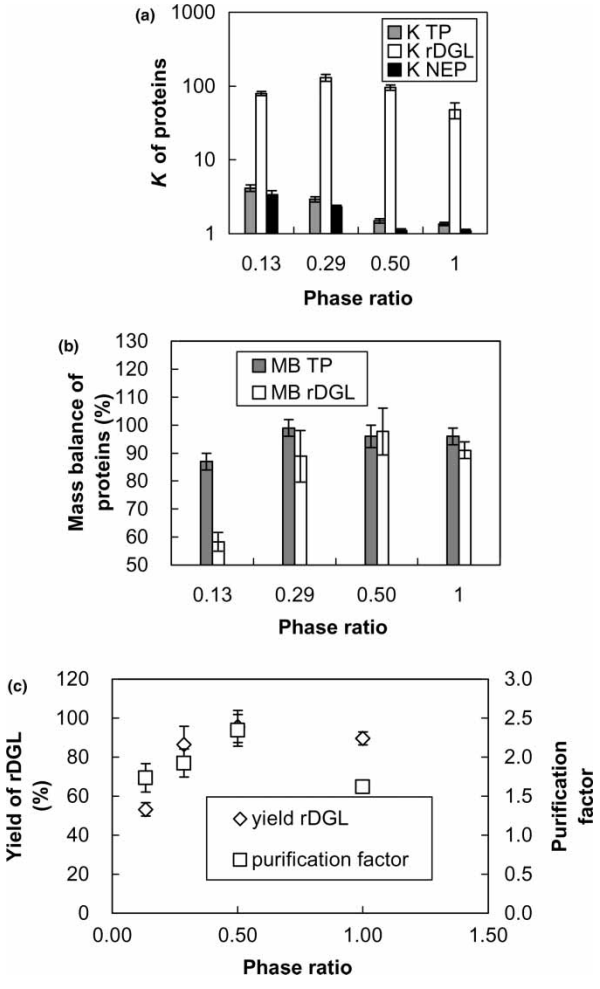

Figure 2. Influence of phase ratio on partitioning of r-DGL-containing endosperm extract in PEG 3350-phosphate-NaCl (1.5%) systems at pH 4 (compositions are shown in Table 1). (a) Partition coefficient. (b) Mass balance of r-DGL and total protein. (c) Purification factor and yield of r-DGL in the top phase. Error bars are 95% confidence intervals.

contributions from individual proteins and mass balances results in the following equation showing the dependence of K_{TP} on phase ratio, ø.

$$K_{TP} = \frac{\sum \frac{K_i}{1 + K_i \phi}}{\sum \frac{1}{1 + K_i \phi}} \tag{12}$$

Although K_{TP} increased at a lower phase ratio, less corn protein was recovered in the top phase because of the smaller volume of the top phase. As a result, the optimal purification with a purification factor 2.3, concentration factor 1.5, and 96% yield was achieved at phase ratio 0.5 (Fig. 2c), where the r-DGL was 30% of the total protein.

The concentration factor was limited because the extract loading used in ATP partitioning was 5 ml extract/11 ml final phase volume. Attempts to prepare more concentrated stocks or add the phase-forming components as solids led to significant losses (ca. 50% at 9 ml/11 ml final phase volume) of r-DGL (in some but not all cases with the appearance of a visible precipitate). In addition, the method, which enhances the purification of target proteins in the top phase by decreasing phase ratio, is limited by the solubility of the r-DGL (Fig. 2b).

Estimation of Countercurrent ATP Partitioning

Countercurrent staging of extraction improves the purification obtained and reduces material requirements. Based on the parameters obtained from the optimal ATP one-stage partitioning ($K_{r\text{-}DGL} = 96$, $K_{NEP} = 1.1$, phase ratio = 0.5, $MB_{NEP} = 96\%$, $MB_{r\text{-}DGL} = 98\%$), three stages of countercurrent ATP partitioning with introduction of extract into the fresh top phase would result in a calculated (38) purification factor, concentration factor, and yield of r-DGL in the top phase of 7.9, 1.4, and 92%, respectively. Because K_{NEP} is the apparent K of a protein mixture; it is likely (based on the phase ratio results of Fig. 2a) that K_{NEP} will increase with the number of stages. Recalculation based on a $K_{NEP} = 2.2$ results in purification factor, concentration factor and yield of r-DGL of 7.6, 1.3, and 85%, respectively, after seven stages (Table 4).

Cation Exchange Chromatography

Cation-exchange chromatography run at pH 4.5 was compared to ATP partitioning. Figure 3 shows that 5 ml of resin captured nearly all of the ca. 400 units of r-DGL loaded to the column in 60 ml of extract (6.7 U/ml). The r-DGL was mainly collected in Fraction 19 for gradient elution or in Fraction 17 for step elution. For higher recovery of r-DGL, Fractions 18 through 20 for gradient elution or Fractions 17 through 19 for step elution were pooled. The resulting

Table 4. Purification of r-DGL by cation exchange chromatography and countercurrent ATP partitioning normalized for the product capacity of a 1 L ATP-system stage volume

Separation method[a]		*V* of product (ml)	V_{buffer}[c] *(ml)*	Purity (%)	*CF*	*PF*	$Y_{r\text{-}DGL}$ (%)
Cation exchange (38 ml packed bed)	Gradient elution	114	562	95 ± 9	3.6 ± 0.1	7.7 ± 1	92 ± 3
	Step elution	114	562	95 ± 9	3.3 ± 0.1	7.9 ± 1	83 ± 2
Countercurrent	3 stages $K_{NEP} = 1.1$	367	545	95	1.25	7.9	92
ATP partitioning[b] (1 L/stage)	3 stages $K_{NEP} = 2.2$	367	545	50	1.25	3.9	92
	7 stages $K_{NEP} = 2.2$	367	545	95	1.16	7.6	85

[a]The processing capacity of r-DGL extract was 454 ml (5/11 L).
[b]Estimated by calculation.
[c]Volume of buffer consumed in chromatographic or partitioning steps.
$\pm$Ranges are 95% confidence intervals.

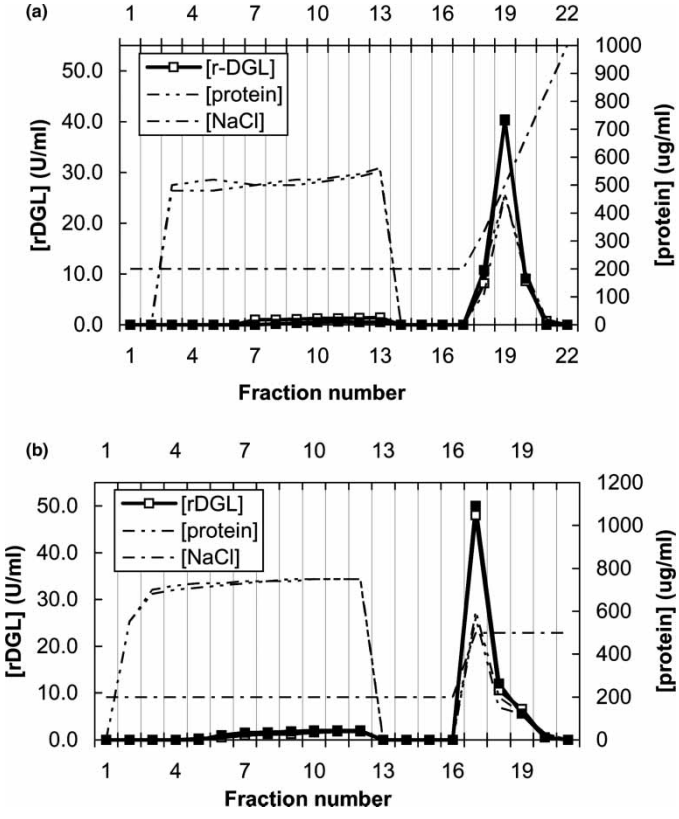

Figure 3. Cation-exchange chromatography of r-DGL. (a) Gradient elution; (b) Step elution. Details of the chromatography are presented in the text. Two replicate experiments using repeated extractions on the same column are plotted with the rDGL values from the two runs distinguished by the filled and open symbols.

purification factors of these pooled eluates were 7.7 and 7.9 for gradient elution and step elution, respectively. These results are shown in Table 4, where the volumes have been adjusted to show a scale corresponding to producing the same quantity of lipase as would be recovered in 1 L of ATP system stage volume. With these cuts, the gradient elution produced slightly higher yield–approximately 92% compared to 83% in step elution (Table 4).

The comparison of ATP and cation exchange processing of Table 4 is based on the result that 5 ml of extract can be partitioned in 11 ml of stage volume (or 455 ml in 1 L of stage volume) resulting in a top phase product volume of 3.7 ml (or 333 ml at 1 L scale). Since 5 ml of resin could process 60 ml of extract, 38 ml of resin would be used for 455 ml of extract. With staging, similar buffer consumption, yield and purification can be obtained, but the ATP process results in a more dilute product stream.

CONCLUSIONS

Proper selection of phase ratio, Triton X-100 concentration, and NaCl addition enables selective recovery of r-DGL in the top phase of a PEG 3350-phosphate ATP system that makes it a feasible alternative to cation exchange. Evaluation of cation exchange was limited to evaluating two resin chemistries and two modes of elution. ATP partitioning (1 stage) achieved a lower purification factor ($PF = 2.3$) than cation-exchange chromatography ($PF = 7.7$); however, it was calculated that countercurrent ATP partitioning could achieve a similar purification factor as cation exchange chromatography by employing three countercurrent stages (assuming $K_{NEP} = 1.1$) or seven stages (assuming $K_{NEP} = 2.2$) stages. Purity and concentration factors for the partitioning and ion exchange alternatives were 95% and 1.4 and 95% and 3.6 respectively, and buffer usage was similar. Thus the only clear advantage of the cation exchange was that the product fraction is more concentrated. Economic analysis combined with further optimization of each alternative would be needed to make a definitive discrimination.

ACKNOWLEDGEMENTS

This work was sponsored by the USDA CREES Grant Nos. 2003-34496-14026 and 2003-34515-14027. The authors thank Dian Octaviani, Steve Fox and Nathalie Vignaux at the Center for Crops Utilization Research of Iowa State University for supplying r-DGL corn endosperm fraction and Qixin Zhong for suggesting cation-exchange conditions.

REFERENCES

1. Roussel, A., Miled, N., and Berti-Dupuis, L. (2002) Crystal structure of the open form of dog gastric lipase in complex with a phosphonate inhibitor. *J. Biol. Chem.*, 227 (3): 2266–2274.
2. Joliff, G., Vaganay, S., Legay, C., and Benicourt, C. (1998) Secretion of an active recombinant dog gastric lipase from baculovirus-infected insect cells. *Biotechnol. Lett.*, 20 (7): 697–702.
3. Canaan, S., Dupuis, L., Riviere, M., Faessel, K., Romette, J.L., and Verger, R. (1998) Purification and interfacial behavior of recombinant human gastric lipase produced from insect cells in a bioreactor. *Protein Expres. Purif.*, 14 (1): 23–30.
4. Wicker-Planquart, C., Cannan, S., Rivière, M., Dupuis, L., and Verger, R. (1996) Expression in insect cells and purification of a catalytically active recombinant human gastric lipase. *Protein Eng.*, 9 (12): 1225–1232.
5. Crabbe, T., Weir, A.N., Walton, E.F., Brown, M.E., Sutton, C.W., Tretout, N., Bonnerjea, J., Lowe, P.A., and Yarranton, G.T. (1996) The secretion of active recombinant human gastric lipase by *Saccharomyces cerevisiae. Protein Expres. Purif.*, 7 (3): 229–236.

6. Kusnadi, A.R., Nikolov, Z.L., and Howard, J.A. (1997) Production of recombinant proteins in transgenic plants: practical considerations. *Biotechnol. Bioeng.*, 56 (5): 473–484.
7. Evangelista, R.L., Kusnadi, A.R., Howard, J.A., and Nikolov, Z.L. (1998) Process and economic evaluation of the extraction and purification of recombinant β–glucuronidase from transgenic corn. *Biotechnol. Prog.*, 14 (4): 607–614.
8. Mison, D. and Curling, J. (2000) The industrial production cost of recombinant therapeutic proteins expressed in transgenic corn. *BioPharm. International*, 13 (5): 48–54.
9. Gruber, V., Berna, P., Arnaud, T., and Bournat, P. (2001) Large-scale production of a therapeutic protein in transgenic tobacco plants: effect of subcellular targeting on quality of a recombinant dog gastric lipase. *Mol. Breeding*, 7 (4): 329–340.
10. Vignaux, N., Octaviani, D., and Johnson, L.A. (2005) Efficiencies of different types of dry mills in recovering a fraction rich in recombinant protein expressed in endosperm. Annual Meeting of American Association of Cereal Chemists and the Tortilla Industry Association, San Diego, CA., Sept. 19–22. AACC/TIA Annual Meeting Program Book, Abstract 2005, 305, pp. 141.
11. Azzoni, A.R., Kusnadi, A.R., Everson, A.M., and Nikolov, Z.L. (2002) Recombinant aprotinin produced in transgenic corn seed: extraction and purification studies. *Biotechnol Bioeng.*, 80 (3): 268–276.
12. Zhong, Q., Gu, Z., and Glatz, C.E. (2006) Extraction of recombinant dog gastric lipase from transgenic corn seed. *J. Agri. Food Chem.*, 54 (21): 8086–8092.
13. Albertsson, P.A. (1986) *Partition of Cell Particles and Macromolecules*, 3rd ed.; Wiley: New York, NY.
14. Bompensieri, S., Gonzalez, R., and Kok, R. (1996) Purification of a lipase from *Acinetobacter calcoaceticus* AAC323-I by hydrophobic-interaction methods. *Biotech. Appl. Biochem.*, 23 (1): 77–81.
15. Nilsson, A., Mannesse, M., Egmond, M.R., and Tjerneld, F. (2002) Cutinase–peptide fusions in thermo-separating aqueous two-phase systems: prediction of partitioning and enhanced tag efficiency by detergent addition. *J. Chromatogr. A.*, 946 (1–2): 141–155.
16. Gupta, R., Gupta, N., and Rathi, P. (2004) Bacterial lipases: an overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol.*, 64 (6): 763–781.
17. Sebastiao, M.J., Cabral, J.M.S., and Aires-Barros, M.R. (1996) Improved purification protocol of a *Fusarium solani pisi* recombinant cutinase by phase partitioning in aqueous two-phase systems of polyethylene glycol and phosphate. *Enzyme Micro. Tech.*, 18 (4): 251–260.
18. Gulati, R., Saxena, R.K., and Gupta, R. (2000) Fermentation and downstream processing of lipase from *Aspergillus terreus. Process Biochem.*, 36 (1): 149–155.
19. Bradoo, S., Saxena, R.K., and Gupta, R. (1999) Partitioning and resolution of mixture of two lipases from *Bacillus stearothermophilus SB-1* in aqueous two-phase system. *Process Biochem.*, 35 (1): 57–62.
20. Balasubramaniam, D., Wilkinson, C., Cott, K.V., and Zhang, C.M. (2003) Tobacco protein separation by aqueous two-phase extraction. *J. Chromatogr. A.*, 989 (1): 119–129.
21. Gu, Z. and Glatz, C.E. (2007) Aqueous two-phase extraction for protein recovery from corn extracts. *J. Chromatogr. B.*, 845 (1): 38–50.
22. Maria, E.S. and Telma, T.F. (2000) Purification of soybean peroxidase (*Glycine max*) by metal affinity partitioning in aqueous two-phase systems. *J. Chromatogr. B.*, 743 (1–2): 287–294.

23. Platis, D. and Labrou, N.E. (2006) Development of an aqueous two-phase partitioning system for fractionating therapeutic proteins from tobacco extract. *Bioresource Technol.*, 98 (3): 696–699.
24. Vaidya, B.K., Suthar, H.K., Kasture, S., and Nene, S. (2006) Purification of potato polyphenol oxidase (PPO) by partitioning in aqueous two-phase system. *Biochem. Engr. J.*, 28 (2): 161–166.
25. Harris, D.P., Andrews, B.A., Wright, G., Pyle, D.L., and Asenjo, J.A. (1997) The application of aqueous two-phase systems to the purification of pharmaceutical proteins from transgenic sheep milk. *Bioseparation*, 7 (1): 31–37.
26. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., and Bairoch, A. (2005) Protein identification and analysis tools on the ExPASy Server; In *The Proteomics Protocols Handbook*. Walker, J.M. (ed.), Humana Press: Totowa, N.J., 571–607. http://www.expasy.org/tools/protparam.html.
27. Zaslavsky, B.Y. (1995) *Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications*. Marcel Dekker Inc.: New York.
28. Andrews, B.A., Schmidt, A.S., and Asenjo, J.A. (2005) Correlation for the partition behavior of proteins in aqueous two-phase systems: effect of surface hydrophobicity and charge. *Biotechnol. Bioeng.*, 90 (3): 380–390.
29. Li, Y. and Beitle, R.R. (2002) Protein purification via aqueous two-phase extraction and immobilized metal affinity chromatography: Effectiveness of salt addition to enhance selectivity and yield of GFPuv. *Biotechnol. Prog.*, 18 (5): 1054–1059.
30. Nielsen, H.C., Inglett, G.E., and Wall, J.S. (1973) Corn germ protein isolate preliminary studies on preparation and properties. *Cereal Chem.*, 50 (4): 435–443.
31. Snyder, S.M., Cole, K.D., and Szlag, D.C. (1992) Phase compositions, viscosities, densities for aqueous two-phase systems composed of polyethylene glycol and various salts at 25°C. *J. Chem. Eng. Data*, 37 (3): 268–274.
32. Zhong, Q. and Glatz, C.E. (2006) An enzymatic assay method for evaluating the lipase activity in complex extracts from transgenic corn seed. *J. Agric. Food Chem.*, 54 (9): 3181–3185.
33. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, G.B., and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, 150 (1): 76–85.
34. Juckes, I.R.M. (1971) Fractionation of proteins and virus with polyethylene glycol. *Biochim. Biophys. Acta.*, 229 (3): 535–546.
35. Yano, K., Hasegawa, T., and Kuboi, R. (1994) Characterization of surface properties of heat shock proteins for the separation using aqueous two-phase systems. *J Chem Eng JP*, 27 (10): 808–814.
36. Save, S.V. and Pangarkar, V.G. (1995) Behaviour of surfactants in aqueous two phase systems. *Bioseparations*, 5 (1): 27–33.
37. Marcos, J.C., Fonseca, L.P., Ramalho, M.T., and Cabral, J.M.S. (1998) Variation of penicillin acylase partition coefficient with phase volume ratio in poly (ethylene glycol)–sodium citrate aqueous two phase systems. *J Chromatogr. B.*, 711 (1–2): 295–299.
38. Harrison, R.G. (2003) *Bioseparations Science and Engineering*. Oxford University Press: New York, U.S.A.