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Coupled Application of Aqueous Two-Phase Partitioning and 2D-Electrophoresis for Characterization of Soybean Proteins

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A novel combination of 2-D electrophoresis with hydrophobic partitioning in aqueous two-phase systems (ATPS) was extended to an alternative ATPS and both systems used for the three-dimensional characterization of the proteins extracted from soybeans. The 3-D plots of molecular weight, isoelectric point, and surface hydrophobicity were obtained using two different phase-forming salts: Na_2SO_4 and potassium phosphate. Six proteins with known hydrophobicities were used to validate the ATPS-based method. Molecular properties obtained using the PEG-sulfate system resulted in a wider range of proteins characterized. The wide range of concentration and strongly hydrophilic character of the soy extracts limited the coverage obtained; reduction of the storage protein content aided detection. The number of proteins simultaneously and accurately characterized by this method as currently implemented is limited by the dynamic range of staining, the ability to quantify strongly partitioning proteins in both phases, and loss of proteins of limited solubility in the ATPS or during the removal of phase-forming components.

Keywords 2-D electrophoresis; aqueous two-phase systems; protein characterization; soybean

INTRODUCTION

During the last decades, plants have emerged as alternative hosts for large-scale production of recombinant proteins (1). Despite the numerous economical and technical advantages of the use of crops as bioreactors, the main factor for the establishment of a production system is the ease of purification, which directly influences the selection of the expression system (2). As much as 95% of the costs of the production of a recombinant protein by molecular farming come from extracting the protein from the plant and purifying it. Although the production yields are important, the development of efficient extraction and purification methods is of primary commercial interest (2,3). Several attempts have been reported on the use of alternative extraction and purification methods for recombinant proteins (e.g., ATPS, expanded bed adsorption) from particular hosts (4,5); however, the optimal choice of the separation method will depend on properties differences between the recombinant protein and the co-extracted host proteins.

Aqueous two-phase systems have emerged as a practical technique that allows the recovery and purification of biological products. They are formed when two water-soluble polymers or a polymer and a salt are mixed in aqueous solutions beyond the critical concentration at which two immiscible phases are formed (6). ATP partitioning has been used both for primary recovery and purification of proteins from plants (7–12) and also for the measurement of protein hydrophobicity (13,14).

The establishment of adequate strategies demands the characterization of the contaminant proteins from plant extracts. It is clear that a better understanding of the molecular characteristics (MW distribution, hydrophobicity, pI, etc.) of the potential contaminants will benefit the process of selection, optimization, and design of the downstream strategies (15). In this context, proteomics can provide tools to measure protein properties that can be exploited to facilitate the extraction and/or purification. 2-D electrophoresis (2-DE) enables the separation of complex mixtures of proteins with simultaneous determination of p*I, MW*, and relative abundance. It can be used for quantitative protein profiling of multicomponent protein mixtures, like soybean protein extracts (16,17).

The hydrophobic properties of a protein play a fundamental role defining its behavior in solution and how the protein relates to other biomolecules. It is in fact, the ruling principle in hydrophobic interaction chromatography (HIC), a separation technique being used in most industrial processes for protein purification as well as in laboratory scale (18). Several methods have been widely used to

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measure the hydrophobicity of proteins, namely HIC, reverse phase chromatography (RPC), and $(NH_4)_2SO_4$ precipitation. The use of aqueous two-phase systems (ATPS) for measuring the functional hydrophobicity of proteins, previously reported by several authors (13,19,20) measures the surface hydrophobicity of a protein as the result of the interaction between the residues on the surface of the protein and the solvent.

Gu and Glatz (14) first reported the integration of 2-DE and ATPS for the characterization of protein mixtures, establishing the protocol for a three-dimensional analysis of corn proteins based on their molecular properties. Recently an extension of this experimental approach was reported for alfalfa green-tissue proteins (21). This method of 3D mapping consisted of the use ATP partitioning on the basis of hydrophobicity, followed by 2D electrophoresis of the proteins in each ATP phase to estimate MW, pI, and concentration with the concentration measure providing for the calculation of the partition coefficient. Representation of the host proteins by three-dimensional plotting of their properties allowed visualization of potentially useful property differences to guide the selection of a separation strategy or choice of host for a particular recombinant protein.

The aim of this research was to extend this three-dimensional approach previously reported for corn protein extracts to a soybean protein extract, selected for being a prolific source of protein that has been previously reported as host for recombinant protein production (22,23). Additionally an alternative ATPS with different phase-forming salt (24) was evaluated for the hydrophobicity determination. The characterization of soybean proteins in 2-DE (MW and pI), together with ATPS composed by PEG 3350 (14.8%)-potassium phosphates (10.3%)-NaCl (3%) and the previously reported system used for corn proteins PEG 3350 (15.7%)-Na_2SO_4 (8.9%)-NaCl (3%) were used to simultaneously measure the partition coefficient of soybean proteins and obtain three-dimensional scatter plots. The application of this 3D technique to soybean proteins extends this method to another potential plant host and serves as a test of its broader applicability.

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MATERIALS AND METHODS

Materials

Defatted soybean flour was kindly provided by Mark Reuber from the Center for Crops Utilization Research at Iowa State University. Poly(ethylene glycol) MW 3350, DL-dithiothreitol (DTT) and the selected model proteins: chymotrypsin A (CHY), cytochrome C (CYC), ribonuclease A (RNA), α-lactalbumin (LAC), bovine serum albumin (BSA) and lysozyme (LYS) were purchased from Sigma-Aldrich Chemicals (St Louis, MO). The Ready-Prep® rehydration buffer, 11 cm ReadyStrip® IPG strips (pH 3–10), iodoacetamide, Precision Plus® protein standards and Criterion® precast polyacrylamide gels (4–20%) were from Bio-Rad (Hercules, CA). Coomassie Plus protein assay kit from Pierce Biotechnology (Rockford, IL). Coomassie blue R250 from Acros Organics (Geel, Belgium). Salts and other reagents were from Fischer Scientific (Pittsburgh, PA).

Protein Extraction

Defatted soybean flour was suspended in 20 mM phosphate buffer, pH 7 at a proportion of 1.0 g solids/10 mL buffer. The slurry was stirred for 1 h with constant pH monitoring, and then centrifuged ($3000 \times g$, 30 min, room temperature; Centrifuge 5424, Eppendorf, Hamburg, Germany) and decanted to eliminate waste solids. The supernatant was filtered using 0.22 mm syringe filter (mStar CA filter, Costar Corp., Corning, NY). An additional sample of soluble soybean proteins was obtained by isoelectric precipitation of the original extract following the protocol reported by Thanh and Shibasaki (25) for depletion of the two main storage proteins.

Protein Assays

Total protein determination for the soybean extract, phase samples, and TCA precipitates was made using microplate Bradford reaction with BSA as standard (EL340, Bio-Tek Instruments, Winooski, VT) (26). The concentration of model proteins was measured spectrophotometrically at 280 nm (Pharmacia Biotech, Uppsala Sweden). All protein determinations included calibration curves using proper solvents and blank ATPS for correction of any interference from phase-forming components.

Aqueous Two-Phase Partitioning

PEG 3350–sodium sulfate and PEG 3350–potassium phosphate systems were formulated based upon previous reports on the 3D characterization of plant proteins using hydrophobic partitioning (14,21) and the corresponding binodal curves reported by Zaslavsky (27) to give a fixed weight of 5.0 g in the case of soybean protein extract and 2.0 g for the model proteins previously listed. The following total compositions were evaluated: 15.7% PEG 3350, 8.9% Na_2SO_4, 3% NaCl for sulfate-ATPS and 14.8% PEG 3350, 10.3% potassium phosphate, 3% NaCl for phosphate-ATPS. Protein load was 1 mg/g ATPS for soybean extract and the model protein mixture and 0.35 mg/g ATPS for individual proteins. Sodium chloride's effect on protein partitioning was also evaluated using 0%, 1.5%, and 3% levels of NaCl. All partitioning experiments with soybean and model proteins were run in triplicate at pH 7. When partitioning was combined with 2-DE, TCA precipitates of each phase's protein were analyzed by 2-DE with replicate gels of each. The TCA precipitate from a

non-partitioned total extract sample (5.3 mg total protein/mL) was run simultaneously with the replicate gels.

Sample Preparation

To eliminate interference from phase-forming compounds and increase protein concentration, trichloroacetic acid (TCA) precipitation was performed to the top and bottom samples before isoelectric focusing according to the protocol reported by Gu and Glatz (14). The final protein pellet was completely re-dissolved using the lowest possible volume of 8 M urea depending on the amount of precipitate present; 0.12 mL for the top phase and the model protein mixture samples and 0.24 mL and 0.48 mL for the bottom phase and the total protein samples. Protein concentration in all samples was measured at this step for a mass balance check and correction factors were obtained to account for protein losses for each sample.

Isoelectric Focusing

Protein precipitates redissolved in urea were diluted with rehydration buffer (8M urea, 2% CHAPS, 50 mM DTT, 0.2% BioLyte) to a volume according to its protein concentration to reach 200 μg of protein per strip (maximum load). The immobilized pH gradient strips were rehydrated with 185 μL of this protein solution for 16 h at room temperature. Isoelectric focusing (IEF) was carried out in an Amersham Ettan IPGphor II® IEF cell for a total of 132,000 Vh.

Second Dimension Electrophoresis

For the second dimension, the focused IPG strips were equilibrated with 6 M urea, 0.375 M Tris, 2% SDS, 20% glycerol, pH 8.8 and 2% w/v DTT for 15 minutes, and then acetylated using 2.5% w/v iodoacetamide instead of DTT for another 15 min. Strips were placed onto 4–20% w/v gradient polyacrylamide gels (Criterion® precast gels, Bio-Rad) and the electrophoresis was performed using a Criterion Dodeca Cell (Bio-Rad) unit. The gels were visualized by staining with Coomassie Blue G-250, and scanned using a flatbed ImageScanner (GE Healthcare) at 600 dpi in transmissive mode and analyzed with PDQuest (Bio-Rad). The mass of protein for individual spots was calculated from the spot volumes (area multiplied by the pixel intensity) relative to the total amount of protein and total spot volume for each gel. A non-partitioned sample of the extract was run in parallel with the samples from ATPS (top-bottom from sulfate and phosphate systems) and used as the reference to match spots from partitioned samples and calculate the mass balances from 2D gels. Protein concentrations from spot volumes were used to calculate K_p of individual proteins. A correction factor specific to each type of sample (top phase, bottom phase or initial extract) was applied to compensate for the average losses at the TCA precipitation step, as calculated from the mass balances of protein before and after this step.

RESULTS AND DISCUSSION

Protein Extraction

The soy protein extracts had an average protein concentration of 30.7 mg/mL. This represents 0.31 ± 0.01 g protein extracted/g soy flour. Protein content of soy is reported as up to 48% w/w with higher soluble yields at more alkaline pH than used here (24,25,28).

Protein Partition Experiments

The criteria for suitability of an ATPS as the hydrophobicity determinant in this method are

1. providing partition coefficient (K_p) centered about 1.0, in order to obtain the highest number of proteins in both phases;
2. high recovery of the proteins in the two phases rather than precipitated at the interface; and
3. partitioning dominated by protein surface hydrophobicity, rather than other protein properties (21).

Earlier work showed that the last criterion could be met without sacrificing the first two by moderate addition of NaCl.

In order to test the capacity of ATPS formulated with phosphate and sulfate salts to provide an accurate hydrophobicity value, six model proteins (CHY, CYC, RNA, LAC, BSA, and LYS) with known hydrophobicities were partitioned. The log K_p values measured in ATPS were correlated with their corresponding hydrophobicities calculated from the precipitation curves (m^* values) reported by Hachem et al. (13) and Gu and Glatz (14). Figure 1(a,b) shows the same ranking for the six different model proteins for both systems. The correlation factor ($R^2 = 0.86$) obtained between $1/m^*$ and Log K_p measured in PEG/phosphate system demonstrated that it also provided a reliable method to measure the functional hydrophobicity of model proteins as well as the previously reported PEG/sulfate system ($R^2 = 0.83$). The specific linear relationship between Log K_p and Log $(1/m^*)$ was dependent on the selection of the phase-forming salt to formulate the systems. However, both measures are seen to be very strongly correlated (Fig. 1c). Differences in absolute values of Log K_p are the expected result of the different intrinsic hydrophobicities of the two systems. The PEG/sulfate system provided a wider Log K_p range between BSA and LYS than did the PEG/phosphate system, suggesting that the former would have a better resolving capacity. Both systems met the third criterion for a hydrophobicity determinant: partitioning dominated by protein surface hydrophobicity.

Hydrophobic Partitioning of Soybean Proteins

The two-phase systems were evaluated using a soybean protein extract for hydrophobic partitioning in order to evaluate whether PEG/phosphate or PEG/sulfate could

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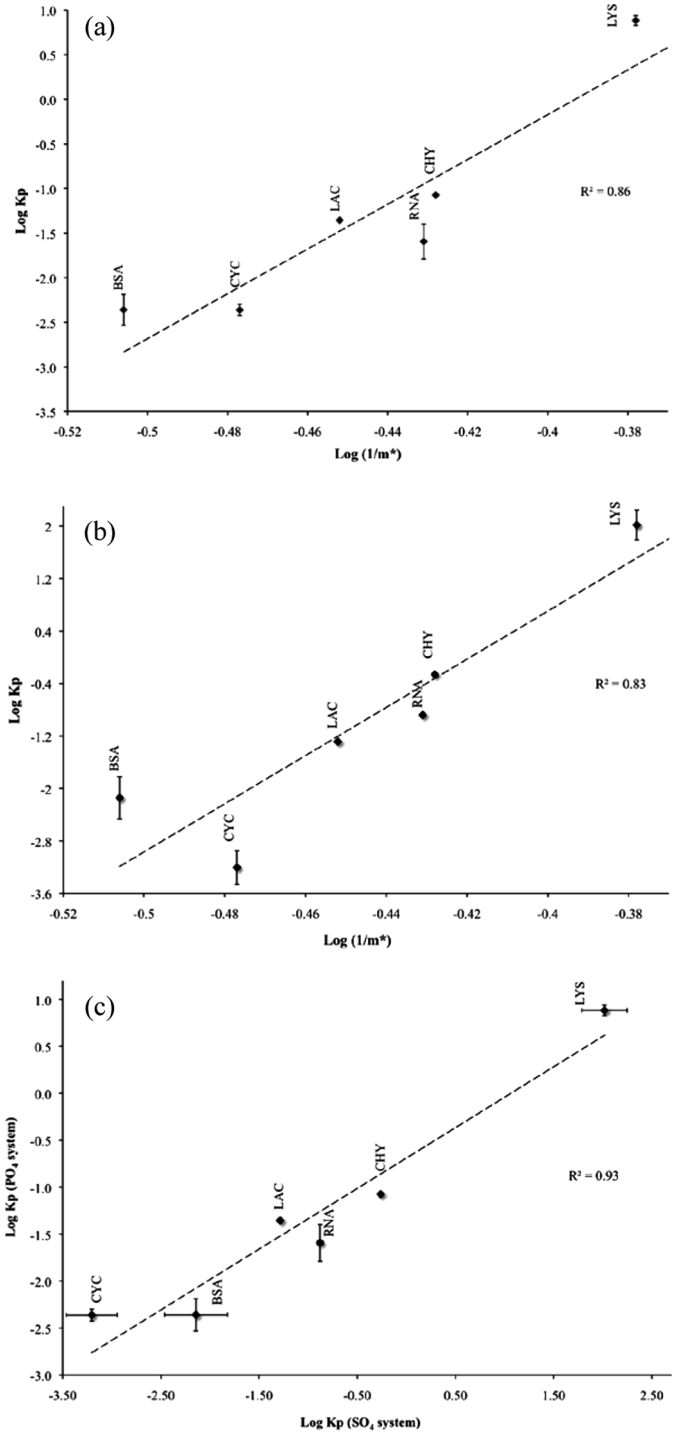

FIG. 1. Correlation between surface hydrophobicity in ATPS (log K_p) with $1/m^*$. K_p is the partitioning coefficient of protein in PEG-salt system at pH 7 and protein loading of 0.4 mg/g ATPS each, and m^* is the concentration of salt at the discontinuity point in $(NH_4)_2SO_4$ precipitation curve, reported by Hachem et al. [13] for selected proteins using an initial concentration of 2 mg/mL. (a) ATPS: PEG 3350 (14.8%), potassium phosphate (10.3%), NaCl (3%). (b) ATPS: PEG 3350 (15.7%), Na_2SO_4 (8.9%), NaCl (3%). (c) Cross-correlation between two ATPS for hydrophobicity measurement (a vs b).

provide partitioning and recovery meeting the previously stated criteria for simultaneous hydrophobicity determination of this protein mixture. Table 1 shows the total recovery percentages obtained from the ATPS for soybean proteins as well as for a model protein mixture (LAC-BSA-LYS). The recovery percentages after ATP partitioning showed that PEG/phosphate system was better for protein recovery than PEG/sulfate, for both mixtures. Mass balance after TCA precipitation was also considered here, since the removal of the phase-forming components is a crucial step in the two-dimension analysis and its effects will be discussed later. Loss at the interface does not affect K_p for proteins detected in subsequent gels of both phases but could lead to a component being below detection limits in the gel analysis.

Table 2 shows the effect of NaCl on the hydrophobic partitioning of the soybean native proteins. In the case of sulfate system, there is a small increase in K_p of soybean protein when the sodium chloride increases from 0% to 3%. This behavior may be explained by the increase in the hydrophobic difference between the two phases (13,20). In the case of the phosphate system, NaCl addition is not effective for K_p modification. This characterization was originally developed using corn extracts which gave the overall partition coefficients nearly an order of magnitude higher than observed here. Two conclusions stem from this:

1. soy proteins are more hydrophilic; and
2. fewer proteins are likely to be measurable in the top phase, limiting the number of proteins for which K_p values can be obtained

Given the slightly higher K_p and previous experiences reported for corn protein (8), the system with 3% NaCl content was again selected for hydrophobic partitioning of soybean proteins.

Application of 3D Method to a Model Proteins Mixture

In order to assess the feasibility of obtaining a 3D map of a complex mixture of proteins, the three-dimensional technique was first tested using a mixture of three model proteins (BSA, LAC, and LYS). Figure 2 shows the result of the application of this technique to the artificial mixture of proteins fractionated in ATPS. K_p for each model protein was calculated by the ratio of the spot volumes obtained from the corresponding gel (top, or bottom). The different hydrophobicity values obtained from the use of two different ATPS (sulfate and phosphate systems) are expected from Fig. 1. The differences in Log K_p values obtained by the 3D method (Fig. 2) and during validation of the systems using individual proteins (Fig. 1) are greater than reported by Gu and Glatz (14) and likely reflect the more variable protein losses at the TCA precipitation step in our hands (Table 1). These losses (60 to 70% in some cases) affected the mass balances for individual proteins

TABLE 1
Percentage of recovered protein after sequential ATP partitioning and TCA precipitation of different protein sample[a]

	From ATPS[b]		From TCA precipitation[c]	
Type of sample partitioned	Phosphate system[d]	Sulfate system[e]	Phosphate system	Sulfate system
Soybean Protein				
Top phase	4.9 ± 1	5.4 ± 1	46.5 ± 3	32 ± 9
Bottom phase	81.7 ± 1	51.2 ± 1	89.2 ± 13	104 ± 5
Interface	13.4 ± 1	43.4 ± 1	–	–
Model proteins mixture[f]				
Top phase	15.1 ± 0.2	14.6 ± 0.2	31.9 ± 9	74.3 ± 14
Bottom phase	84.6 ± 0.6	78.8 ± 1.5	41.3 ± 12	39.9 ± 4
Interface	0.3 ± 0.4	6.6 ± 0.8	–	–

[a]Standard errors reported are the result of three independent measurements for TCA precipitation and duplicates for ATP partitioning.
[b]Expressed as the percentage of the initial amount of protein added to the system (1.0 mg/g ATPS). The amount of protein at the interface was estimated as the necessary to complete 100% recovery.
[c]Percentage relative to the amount of protein measured on top/bottom phase previous to precipitation step.
[d]ATPS is PEG 3350 (14.8%)-potassium phosphates (10.3%)-NaCl (3%) at pH 7.
[e]ATPS is PEG 3350 (15.7%)-Na_2SO_4 (8.9%)-NaCl (3%) at pH 7.
[f]Mixture of LYS, BSA and LAC as model proteins.

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after partitioning and thus the K_p estimation by spot densitometry of 2D gels. In some cases the differences could be due to errors in the single protein determinations as these were based on total proteins including evident impurities (esp. lysozyme aggregates), whereas the impurities could be excluded on the 2D gels.

With both ATPS formulations the MW and pI of the spots detected were similar to that reported by Gasteiger et al. (29) for these proteins. With both ATPS this 3D technique is feasible for the simultaneous determination of the molecular characteristics of a protein mixture, as has been demonstrated for a larger set of proteins (30). However, special attention should be paid to the TCA concentration step, in order to avoid or minimize protein losses that may affect further K_p estimation.

Molecular Characterization of Proteins from Soybean

Removal of the phase-forming components before performing IEF represents one of the main drawbacks encountered from the combination of ATP partitioning with 2-DE. In the case of the top phase, the polymer from this phase was removed as it could potentially interact with proteins and interfere with protein mobility on polyacrylamide gels. The high conductivity of the bottom phase prevents direct use for IEF runs. In this context, TCA precipitation was used to isolate proteins from phase-forming components. In Table 1, a quantification of protein loss was evaluated for the different samples treated (i.e., soybean protein sample, three model protein mixture). Protein losses up to 68% were observed after the TCA precipitation from the top-phase samples, and 60% for

TABLE 2
Effect of NaCl addition on the partition coefficient of soybean proteins in ATPS

	NaCl content (w/w)[a]		
	0%	1.5%	3.0%
TLL: 27% w/w			
PEG 3350 (14.8% w/w)	0.08 ± 0.0001	0.08 ± 0.001	0.08 ± 0.003
Phosphate (10.3% w/w)			
TLL: 32% w/w			
PEG 3350 (15.7% w/w)	0.06 ± 0.01	0.06 ± 0.003	0.09 ± 0.01
Na_2SO_4 (8.9% w/w)			

[a]Systems formulated at pH 7 and 25°C. Sample load was 1.0 mg protein/g ATPS.

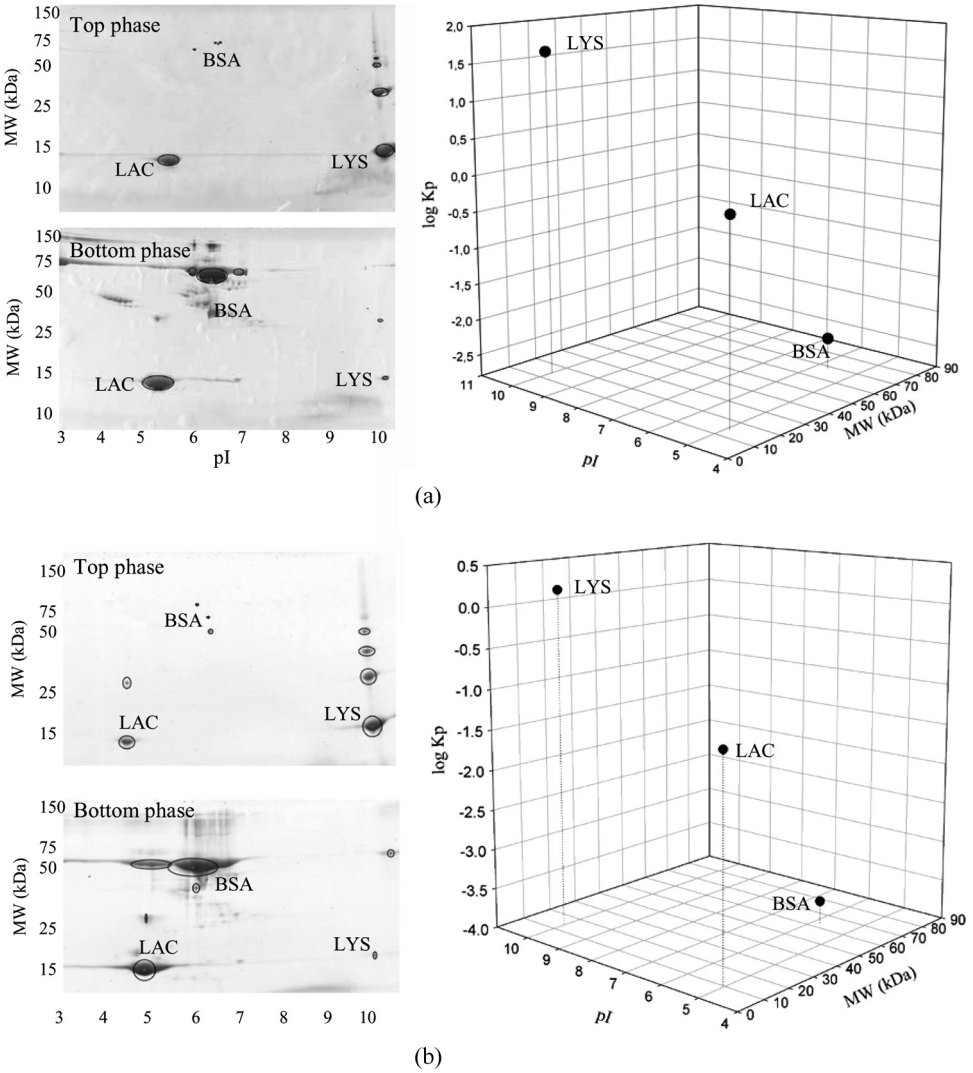

FIG. 2. 2D gels and 3D map of artificial mixture of three model proteins fractionated in ATPS. PEG-salt system at pH 7 and protein loading of 0.35 mg/g ATPS for each protein. (a) ATPS: PEG 3350 (14.8%), potassium phosphate (10.3%), NaCl (3%). (b) ATPS: PEG 3350 (15.7%), Na_2SO_4 (8.9%), NaCl (3%).

the bottom-phase samples. As with the interfacial losses in the partitioning, losses at the TCA step could also lead to detection limitations in the gel analysis. Additionally, if the differences in losses for the two phases are not corrected for, the TCA step losses would also affect K_p. The correction used was to assume that the losses were uniform for all proteins in a given phase and correct for this loss in calculating the phase concentrations from spot volume determinations. The validity of this assumption can then be assessed by mass balances on individual proteins (discussed later).

In most of the cases, the mass balances performed after every ATPS step (Table 1), resulted in recovery percentages higher than 90%. These findings differ from those reported for soybean proteins partitioned in similar hydrophobic systems (24). Such behavior or differences can be attributed to the increased TLL used in the previously reported study (42–56% w/w) compared to that used here (i.e., 27%). It has been reported that an increase in TLL results in biomass accumulation at the interface (31).

Table 3 lists soy extract proteins that could be quantified in both top and bottom phase gels for the two different ATPS. The full list of protein spots detected in 2-DE gels is provided as supplementary material. From the total list of spots detected, only those in Table 3 were found to be present in both phases (11 spots in the PEG-phosphate system and 15 spots in the PEG 3350-sulfate system) and matched as the same protein. This is a likely consequence of the relatively low partition coefficient for soy proteins (<0.1) for both systems resulting in top phase

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TABLE 3
Three-dimensional properties and mass balance of selected soybean proteins partitioned in ATPS

ATPS[a]	Id. #	MW (kDa)	pI	log K_p	Amount of Protein (mg)[b]	% Recovery[c]
PEG 3350-PO_4	1A	13.5	4.9	−0.361	0.186 ± 0.01	65.9
14.8% PEG 3350	2B	25.0	5.4	−2.409	0.531 ± 0.20	90.4
10.3% Phosphate	3C	22.5	5.7	−1.469	0.147 ± 0.02	104.0
3.0% NaCl	4	23.4	5.8	0.164	0.03 ± 0.01	95.0
	5D	24.1	6.0	0.353	0.051 ± 0.001	50.4
TLL 30%	6E	27.3	6.2	−0.386	0.017 ± 0.001	62.0
Vr ~ 1.25	7F	29.7	7.1	1.295	0.056 ± 0.02	157.9
	8G	12.9	7.1	−0.144	0.003 ± 0.001	6.0
	9	16.2	7.9	−2.168	0.171 ± 0.05	363.1
	10	30.2	7.8	1.064	0.088 ± 0.001	2155.9
	11	31.0	9.5	0.386	0.008 ± 0.001	41.2
PEG 3350-SO_4	12	24.7	4.7	−0.761	0.004 ± 0.002	28.6
15.7% PEG 3350	13	26.0	4.7	−0.688	0.047 ± 0.004	85.0
8.9% Na_2SO_4	14A	17.5	4.8	−1.017	0.115 ± 0.03	45.4
3.0% NaCl	15	28.0	5.0	−1.998	0.202 ± 0.005	75.7
	16	59.4	5.2	−2.746	0.198 ± 0.07	57.1
TLL 32%	17B	27.5	5.4	−2.268	0.335 ± 0.04	94.6
Vr ~ 1.0	18	33.7	5.4	−2.874	0.134 ± 0.001	73.1
	19	14.5	5.6	−1.722	0.039 ± 0.02	37.5
	20C	27.9	5.6	−2.687	0.49 ± 0.13	98.3
	21E	29.6	6.2	−1.995	0.006 ± 0.006	11.3
	22D	25.2	6.3	−1.234	0.066 ± 0.05	278.4
	23	25.0	6.3	−0.699	0.004 ± 0.002	41.8
	24F	31.0	6.7	0.160	0.02 ± 0.006	56.6
	25G	16.6	6.7	−1.164	0.005 ± 0.004	24.5
	26	69.8	7.5	−1.400	0.008 ± 0.001	239.2

[a]All data are the average of duplicate experiments run at pH 7 and 25°C. Load of protein was 1.0 mg/g ATPS.
[b]Amount of each protein present per 5 g ATPS, obtained as the sum of the amount of each protein quantified in top and bottom phases and considering losses during ATP partitioning and TCA precipitation steps for each sample.
[c]Calculated using the sum of top + bottom matched protein divided by the total amount of the same protein spot matched in the gel where no partition experiment was performed (total extract) and considering TCA loss for each case.
[d]Capital letters show presumed storage subunits commonly identified in both systems (see text).

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concentrations close to the detectable limits (0.04 ppm). In the case of the PEG 3350-sulfate system a total of 72 protein spots were detected and quantified. Only 15 of these were in both top and bottom gels such that their K_p could be determined (listed in Table 3) but they do comprise 44% of the total protein detected in both phases (from 72 spots). In a similar way, a total of 68 spots were detected and quantified from PEG-phosphate system, but only 11 spots were found to be present in both top and bottom phases resulting in a lower amount of protein detected and quantified when compared with the total extract. These spots comprised 25% of total protein detected in both phases (68 spots). PEG/sulfate system, thus, characterized a higher % of the total soybean protein. Supplementary tables augment the number of proteins of Table 3 by providing a limiting hydrophobicity value for the proteins not appearing in the top phase, assuming that they are present at the level of the detection limit by using the density of the faintest spot detected in top phase as the lower detection limit.

The presence of the dominant storage proteins was evident from the gel images (Figs. 3 and 4), and marked as corresponding capital letters in Table 3. The main spots detected in both phases correspond to the subunits of glycinins and β-conglycinin. β-conglycinin is a trimer of three subunits (α, α', and β) with α and α' MW around 57 kDa and the β subunit around 42 kDa. The reported properties of glycinin subunits (40 kDa; pI 4.7–5.4 for the acidic subunits and 20 kDa; pI 8–8.5 for the basic subunits), were reasonable matches for the most prominent spots detected in Fig. 3 for the phosphate system (spots 2B and 3C for acid subunits and 9 and 10 for basic subunits) with the

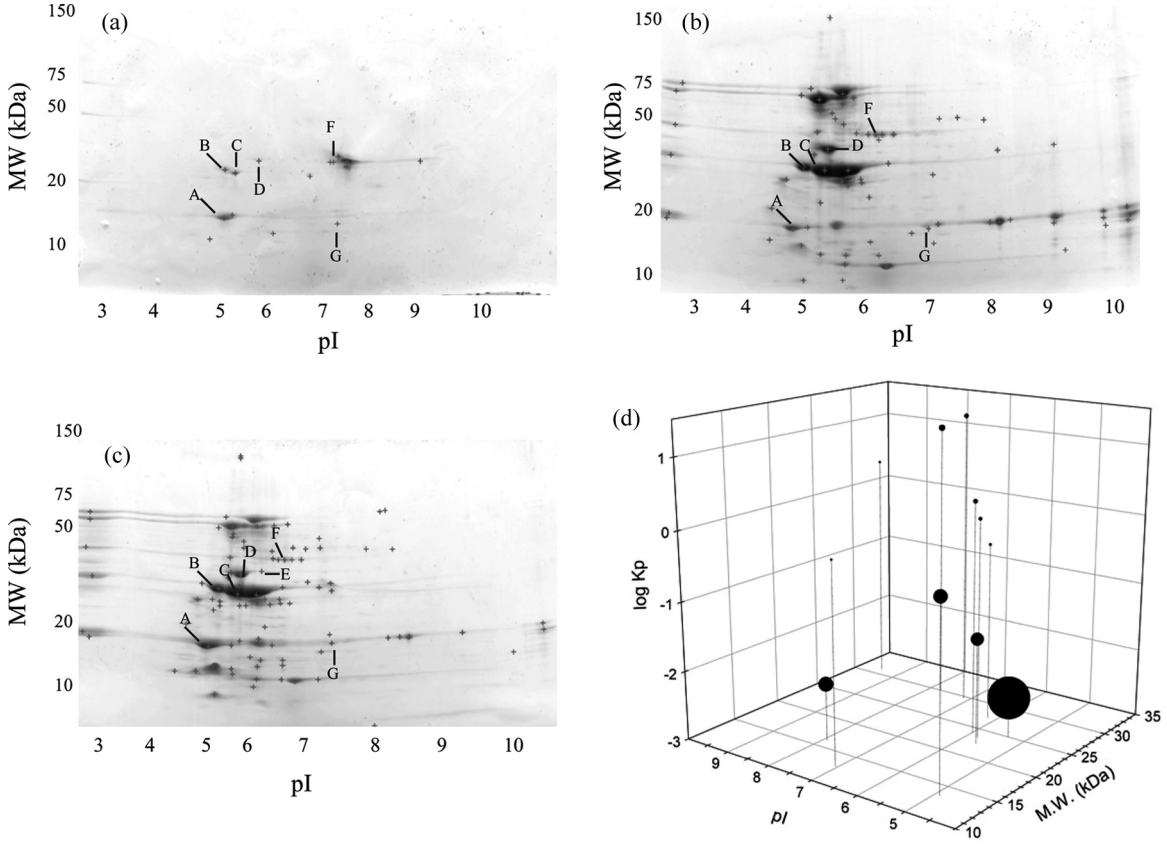

FIG. 3. 2D gels and 3D scatter plot of proteins from soybean using PEG 3350-phosphate system. ATPS: PEG 3350 (14.8%), potassium phosphate (10.3%), NaCl (3%) at pH 7 and 1 mg protein/g ATPS. (a) top phase gel, (b) bottom phase gel, (c) initial soybean extract gel, (d) 3D scatter plot of soybean proteins detected in both phases (see data in Table 3). Spot volumes are proportional to the relative protein content. Letter labels on gels correspond to those in the ID# of Table 3. Molecular weight and pI scales shown are only approximate and were not used to assign the values in Table 3.

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greatest deviation occurring in the MW measure (28). The PEG-sulfate system also allowed detection of the acidic subunits of glycinin (spots 17B and 20C), but additionally showed faint upper phase spots corresponding to the α and α' subunits of β-conglycinin (#16, MW 59.4 kDa; pI 5.2). PEG/sulfate system allowed the characterization of a few more proteins over a wider range of MW. An additional verification for the main storage proteins identification is the Log K_p values obtained for both systems. In the PEG-sulfate system, K_p values are more consistent for the glycinin subunits which should partition together as the multimer (spots 17 and 20).

The overall partitioning result was influenced by the partition behavior of these hydrophilic storage proteins. Similar results were reported for green tissue samples from alfalfa extracts (21) where partitioning of Rubisco dominates. An increase in the amount of proteins added to the gel will not necessarily improve the detection of low abundance proteins due to the limiting capacity of isoelectric focusing strips. Isoelectric precipitation was explored as a potential strategy to remove the dominant storage proteins (24,25) and allow characterization of lower abundance proteins. A sample of soybean proteins where a large proportion of abundant proteins was depleted by isoelectric precipitation (25) was run in 2D gel (Fig. 5). The gel resulted in a higher number of spots corresponding to low abundance proteins in the pI range of 6 to 8, not detected previously on Figs. 3c and 4c. However, after partitioning of this depleted sample in ATPS, the additional proteins did not partition to the top phase in detectable amounts; perhaps not surprising, as these soy whey proteins are likely quite hydrophilic. Some protein spots originally detected in relatively low quantities (Figs. 3c and 4c) were now dominant in the 2D gel of the depleted sample.

Compared with the previous experiments with corn proteins (14) the extension to soybean analysis yielded a lower number of protein spots. This low number (11 for PEG 3350-phosphate and 15 for PEG 3350-sulfate) is a combined result of the loss of proteins during the partitioning stage and TCA precipitation step (Table 1), but also explained by the hydrophilic nature of soybean proteins. Use of fluorescent stains would increase the sensitivity of

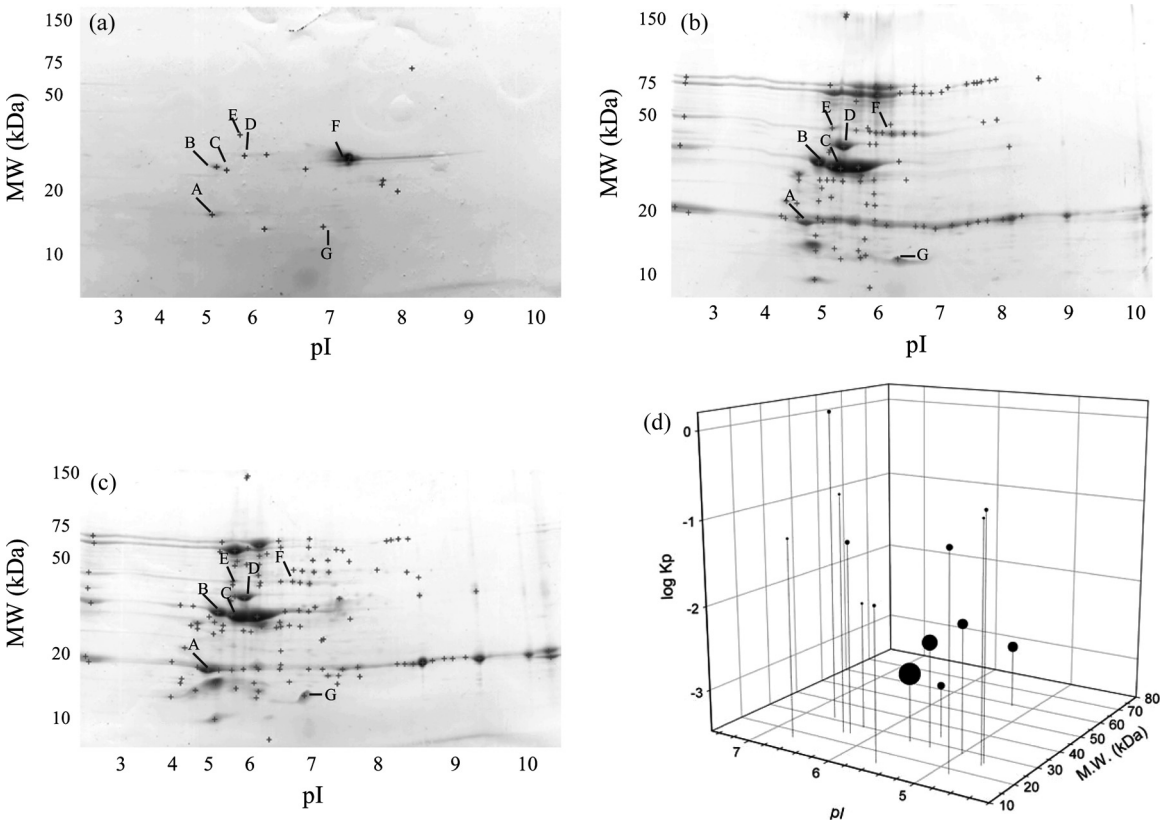

FIG. 4. 2D gels and 3D scatter plot of proteins from soybean using PEG 3350-Na_2SO_4 system. ATPS: PEG 3350 (15.7%), Na_2SO_4 (8.9%), NaCl (3%) at pH 7 and 1 mg protein/g ATPS. (a) top phase gel, (b) bottom phase gel, (c) initial soybean extract gel, (d) 3D scatter plot of soybean proteins detected in both phases (see data in Table 3). Spot volumes are proportional to the relative protein content. Letter labels on gels correspond to those in the ID# of Table 3. Molecular weight and pI scales shown are only approximate and were not used to assign the values in Table 3.

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detection and permit identification of such low abundance proteins in the upper phase.

The recovery percentages for specific proteins (Table 3) were based on the total amount of the same protein matched in the extract gel. For this calculation, normalized spot volumes from the gels were corrected for losses during TCA precipitation by assuming the total protein loss for each phase sample during this step applied to individual proteins as well. Errors in that assumption resulting from protein-specific differences in precipitation account for some of the deviations from 100%. Major deviations likely result from spot mismatches involving proteins of very different abundance. The less plentiful proteins tend to have less satisfactory mass balance. The cross correlation shown in Fig. 6 includes those proteins commonly identified by both systems according to the molecular properties and corresponding to the most abundant ones (see Table 3). Using Fig. 6 outliers check for consistency between the two ATPS shows the 2–17 pair as the most likely not corresponding. The correlation coefficient among the final set of matches shifts from 0.75 to 0.86 with the elimination of this pair. However, a reasonable correspondence

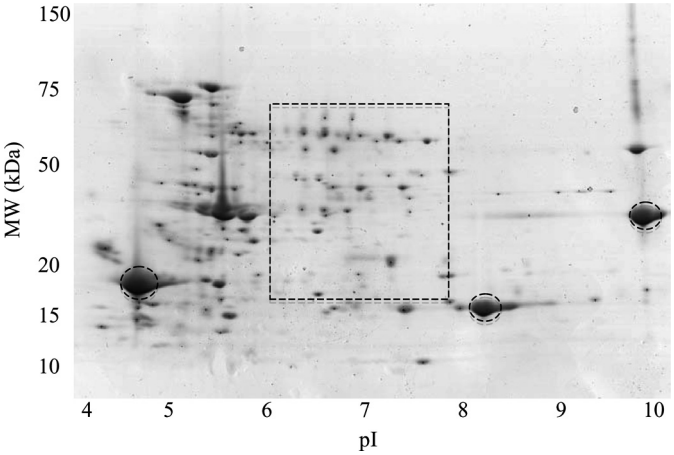

FIG. 5. 2D gel of a soybean protein extract after depletion of main storage proteins by isoelectric precipitation. Squared area is showing low abundant proteins appearing after partial depletion of main storage proteins. Circled spots indicate proteins with higher relative abundance (see Figs. 3c and 4c). Molecular weight and pI scales shown are only approximate.

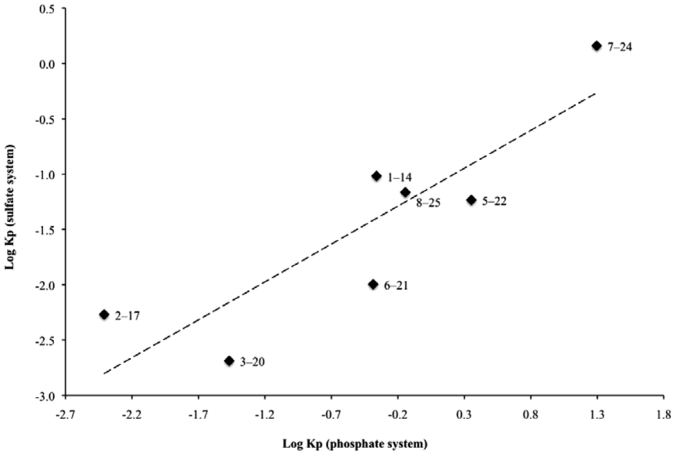

FIG. 6. Cross-correlation between surface hydrophobicity values (Log K_p) obtained for selected soybean proteins using two ATPS formulations. Numbers indicate corresponding proteins as listed in Table 3.

between the two hydrophobicity scales, confirms what was previously seen with the model proteins (Fig. 1c).

A final comparison between the K_p values obtained by a common chemical method of measuring protein concentration (Bradford reagent) and spot densitometry is presented in Table 4. For the PEG 3350-sulfate system there is no significant difference between the K_p values obtained using the two methods. In the case of PEG 3350-phosphate system the K_p value measured by Bradford reaction was somewhat higher than that measured by 2-DE method, likely the result of this system having fewer proteins detected in the top-phase gels.

This work demonstrates that the phosphate system is a potential alternative ATPS to characterize protein hydrophobicity; however, this alternative does not solve the problem of extending the application of the method to protein mixtures composed of very hydrophilic proteins. In fact, it does not achieve characterization of as many proteins as the sulfate system. For characterization of proteins over wide ranges of hydrophobicity using a single system, detection using a more sensitive stain would be helpful. For prediction of ease of separation in particular cases it may be sufficient to know that log K_p is beyond some limiting value.

CONCLUSIONS

The application of 3D characterization approach resulted in valuable information about the molecular nature of those host cell proteins that would be the likely impurities to be removed when a target protein is expressed in soybean. The application of this experimental approach to soybean protein presented new challenges and limitations, since the majority of the proteins detected in 2D gels were derived from two main storage proteins and the limited number of spots detected in top phase gels restricted the number of 3D spots characterized. A more detailed and complete protein profile could be obtained if some of the drawbacks of the hydrophobic ATP partitioning can be overcome. The composition of the PEG-sulfate system (PEG 3350 15.7%, Na_2SO_4 8.9%, NaCl 3%) resulted in a higher number of spots detected from both phases in 2DE gels, compared with the PEG-phosphate system (PEG 3350 14.8%, potassium phosphate 10.3%, NaCl 3%). PEG 3350-sulfate system, allowed the characterization of a higher number of proteins from soybean, including some subunits of the main storage proteins. It was also demonstrated that the phase-forming salt is a significant factor that influences the intrinsic hydrophobicity of the phase system used for K_p determination, modifying consequently the resolving capacity of ATPS for complex protein mixtures.

TABLE 4
Comparison between the K_p obtained simultaneously by 2DE spot densitometry and total protein assay of the two phases.[a]

Method	Sample[b]	ATPS[c]	
		PEG 3350-potassium phosphate	PEG 3350-sodium sulfate
K_p-Bradford assay	Soybean Protein	0.08 ± 0.003	0.09 ± 0.01
	Model mixture[d]	0.13 ± 0.003	0.13 ± 0.001
K_p-2DE[e]	Soybean Protein	0.04 ± 0.01	0.11 ± 0.03
	Model mixture	0.11 ± 0.001	0.13 ± 0.004

[a]All data are the average of duplicate experiment in the case of 2DE and triplicate for protein assay.
[b]Protein load was 1 mg/g ATPS for soybean samples and model proteins mixture.
[c]ATPS: PEG 3350 (14.8%)-potassium phosphate (10.3%)-NaCl (3%) and PEG 3350 (15.7%)-Na_2SO_4 (8.9%)-NaCl (3%) at pH 7.
[d]Mixture: lysozyme, bovine serum albumin and α-lactoalbumin.
[e]Calculated as the sum of proteins detected by spot densitometry in top divided by the sum of the proteins detected in bottom phase for each sample.

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SUPPLEMENTARY MATERIAL

TABLE 1
Total protein spots detected and quantified from 2D gels after partitioning in PEG 3350-Na_2SO_4 system

MW (kDa)	pI	Protein Concentration in ATPS, ppm		Total soybean Extract (ppm)	% Recovery	Log K_p
		Top phase	Bottom phase			
19.0	3.0		5.26	18.71	75.0	<−1.960
19.9	3.0		21.59	107.30	53.7	<−2.620
37.5	3.0			8.75	0.0	*
32.2	3.1		26.70	45.89	155.2	<−2.713
67.9	3.1		9.34	63.99	38.9	<−2.257
76.7	3.1		3.00	34.63	23.1	<−1.279
13.3	4.3			20.92	0.0	*
15.5	4.4			11.14	0.0	*
16.2	4.4		1.80	8.31	57.8	<−1.523
21.2	4.5		5.00	34.16	39.0	<−1.965
13.3	4.6			118.64	0.0	*
25.4	4.6		1.59	11.23	37.8	<−1.469
18.1	4.7		15.67	38.05	109.8	<−2.463
24.7	4.7	0.30	1.70	15.54	35.6	−0.761
26.0	4.7	2.68	13.03	77.31	56.5	−0.688
17.5	4.8	2.82	36.40	356.98	29.8	−1.017
10.2	4.9		18.00	129.06	37.2	<−2.548
13.5	5.0		48.74	224.13	58.0	<−2.966
28.0	5.0	0.70	69.53	361.16	52.0	−1.998
17.4	5.1		7.54	48.54	41.4	<−2.145
24.8	5.1		1.97	36.16	14.5	<−1.557
71.5	5.1		7.77	35.01	59.2	<−2.158
12.6	5.2		2.80	19.04	39.2	<−1.716
14.0	5.2			48.20	0.0	*
32.2	5.2		5.19	33.07	41.9	<−1.987
40.3	5.2		7.87	46.87	44.8	<−2.188
59.4	5.2	0.11	68.32	447.53	40.8	−2.746
21.4	5.3		2.32	8.66	71.4	<−1.612
34.4	5.3		12.42	82.05	40.4	<−2.362
267.3	5.3		1.98	5.18	101.9	<−1.565
304.2	5.3		1.24	2.04	162.1	<−1.363
17.9	5.4		27.17	55.24	131.2	<−2.480
27.5	5.4	0.65	115.29	650.58	47.6	−2.268
33.7	5.4	0.06	46.46	245.55	50.5	−2.874
61.2	5.4		13.07	117.89	29.6	<−2.384
14.5	5.6	0.18	13.44	163.12	22.3	−1.722
27.9	5.6	0.35	169.31	739.39	61.2	−2.687
42.7	5.6			24.33	0.0	*
54.5	5.6		14.41	56.64	67.8	<−2.301
65.1	5.6		51.45	262.78	52.2	<−2.874
9.0	5.7		4.18	23.12	48.2	<−1.526
25.2	5.7		11.86	117.28	27.0	<−2.327
18.3	5.9		39.09	202.85	51.4	<−2.879

(*Continued*)

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TABLE 1
Continued

MW (kDa)	pI	Protein Concentration in ATPS, ppm		Total soybean Extract (ppm)	% Recovery	Log K_p
		Top phase	Bottom phase			
24.8	5.9		6.60	30.63	57.5	<−2.102
26.2	5.9			51.30	0.0	*
69.5	5.9		39.10	24.46	426.3	<−2.838
20.7	6.0		1.88	7.08	70.8	<−1.566
40.0	6.0		3.75	7.06	141.6	<−1.842
40.1	6.0		6.81	33.12	54.8	<−2.118
63.2	6.0		44.38	34.72	340.9	<−2.810
15.7	6.1			18.31	0.0	*
29.6	6.2	0.04	4.11	35.47	31.3	−1.995
39.9	6.2		14.75	71.69	54.9	<−2.428
63.8	6.2		5.06	6.36	212.2	<−1.989
25.0	6.3	0.22	1.05	13.08	27.0	−0.699
25.2	6.3	0.46	22.28	82.60	73.8	−1.234
44.8	6.3			11.49	0.0	*
39.2	6.4		6.27	45.17	37.0	<−2.087
72.6	6.4		2.28	2.13	285.4	<−1.650
30.7	6.5		3.00	15.85	50.5	<−1.745
44.4	6.5			17.08	0.0	*
17.7	6.6		3.31	17.14	51.5	<−1.691
16.6	6.7	0.20	2.93	12.51	67.8	−1.465
31.0	6.7	7.26	5.03	23.99	156.8	−0.141
44.2	6.7			17.83	0.0	*
17.2	6.8		17.04	67.54	67.3	<−2.524
31.3	6.8	79.29		40.52	652.3	–
30.2	6.9	30.16		19.67	511.1	–
59.8	6.9		2.24	5.31	112.5	<−1.573
18.1	7.2		19.17	47.80	106.9	<−2.501
26.1	7.3	3.65		57.35	21.2	–
45.5	7.4		0.89	6.71	35.4	<−1.220
69.8	7.5	0.12	2.68	6.99	108.0	−1.400
18.5	7.7		4.52	11.18	107.8	<−1.948
18.6	8.0		21.00	63.49	88.2	<−2.514
34.5	8.0		1.77	8.16	57.8	<−1.532
18.8	8.1		16.33	159.66	27.3	<−2.503
19.2	8.2		5.87	16.81	93.1	<−2.027
19.7	8.7		4.49	22.70	52.7	<−1.920
19.5	8.9		20.22	134.91	40.0	<−2.590
20.1	9.7		9.33	65.17	38.2	<−2.238
19.8	10.0		7.78	81.30	25.5	<−1.969
20.7	10.0		6.48	119.43	14.5	<−2.038

Total protein concentration (ppm): Top phase, 129.2; Bottom phase, 1215; Total soybean extract, 6671.

Total spots count: 83*11 spots not found in either phase.

Partition coefficient: 0.11.

All data are the average of two experiments using two-phase system: 15.7% PEG 3350, 8.9% Na_2SO_4, 3% NaCl, pH 7, $V_r = 1.25$ at 25°C. Load of protein was 1.0 mg/g ATPS from a total soybean extract of 5.3 mg protein/mL. Protein concentration is expressed in parts per million in the ATPS. % Recovery calculated using the sum of top + bottom protein divided by the total amount of the same protein matched in the gel where no partition experiment was performed (total extract). Log K_p values for proteins not detected in top phase were estimated as the highest K_p possible taking the concentration of the faintest spot quantified as the limit of detection.

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TABLE 2
Total protein spots detected and quantified from 2D gels after partitioning in PEG 3350-phosphate system

MW (kDa)	pI	Protein concentration in ATPS, ppm		Total soybean Extract (ppm)	% Recovery	Log K_p
		Top phase	Bottom phase			
16.0	3.0		17.60	167.06	26.1	<−1.884
42.3	3.0		9.34	58.60	39.5	<−1.869
15.1	3.1		2.36	28.67	20.4	<−1.528
30.7	3.1		11.67	55.48	52.2	<−1.923
62.8	3.1		7.07	64.01	27.4	<−0.975
71.4	3.1		9.80	51.77	46.9	<−1.889
10.0	4.4		1.74	8.68	49.7	<−1.395
17.6	4.6		5.89	25.32	57.7	<−1.925
10.0	4.7			87.77	0.0	*
23.4	4.8		19.47	74.84	64.5	<−2.172
28.2	4.8			2.80	0.0	*
13.5	4.9	23.77	55.25	387.64	56.4	−0.361
7.5	5.0		6.63	129.29	12.7	<−1.537
10.6	5.0		15.07	200.00	18.7	<−1.982
20.8	5.0			6.24	0.0	*
23.6	5.0			20.98	0.0	*
13.1	5.1		5.34	12.57	105.4	<−1.882
21.7	5.1			11.18	0.0	*
22.9	5.1			17.17	0.0	*
26.6	5.1		104.29	435.35	59.4	<−2.849
54.8	5.1		14.99	0.58	6409.5	<−1.866
30.1	5.2		9.67	32.77	73.2	<−1.821
37.6	5.2		15.84	40.06	98.1	<−2.054
65.0	5.2		7.44	27.67	66.7	<−1.771
9.9	5.3		2.99	60.81	12.2	<−1.631
13.7	5.3			50.42	0.0	*
31.8	5.3			43.07	0.0	*
48.7	5.3		36.45	86.59	104.4	<−2.478
55.7	5.3		272.54	495.08	136.5	<−3.288
25.0	5.4	1.19	249.96	767.51	81.3	−2.409
14.3	5.4			5.37	0.0	*
21.7	5.4			6.43	0.0	*
27.1	5.4			133.48	0.0	*
30.9	5.4		128.00	269.01	118.0	<−3.000
65.3	5.4			19.36	0.0	*
250.0	5.4		2.82	5.25	133.2	<−1.208
10.9	5.5		0.75	9.82	18.9	<−1.033
21.8	5.5	3.65		3.34	374.5	–
31.5	5.5			21.29	0.0	*
43.5	5.5		10.11	14.68	170.8	<−2.160
8.3	5.6		0.38	19.44	4.8	<−0.739
9.0	5.6		18.95	36.51	128.7	<−2.144
14.0	5.6			15.87	0.0	*
37.3	5.6		4.59	10.55	107.9	<−1.816
40.7	5.6		10.89	20.91	129.2	<−1.942
61.9	5.6		149.21	292.77	126.4	<−2.940

(*Continued*)

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TABLE 2
Continued

MW (kDa)	pI	Protein concentration in ATPS, ppm		Total soybean Extract (ppm)	% Recovery	Log K_p
		Top phase	Bottom phase			
10.5	5.7		4.56	34.07	33.2	<−1.813
11.4	5.7			29.70	0.0	*
14.4	5.7		51.43	114.84	111.1	<−2.566
22.5	5.7	1.14	68.17	194.76	88.8	−1.469
24.8	5.7		375.38	467.16	199.3	<−3.311
32.1	5.7			5.13	0.0	*
55.5	5.7		51.05	137.53	92.1	<−2.623
17.0	5.8			3.99	0.0	*
22.0	5.8		12.02	32.28	92.3	<−1.868
23.4	5.8	3.17	9.69	20.81	167.7	0.164
36.8	5.9		3.55	14.69	59.9	<−1.704
40.4	5.9			2.48	0.0	*
56.5	5.9		10.93	32.71	82.9	<−2.193
11.0	6.0		3.04	19.67	38.3	<−1.337
24.1	6.0	8.85	11.72	157.32	37.8	0.353
36.7	6.0		5.19	8.71	147.8	<−1.870
36.7	6.1		7.19	35.37	50.4	<−1.778
57.9	6.1			5.00	0.0	*
9.2	6.2		62.59	74.99	207.0	<−2.583
22.4	6.2			6.41	0.0	*
27.3	6.2	2.14	5.21	18.53	109.3	−0.386
36.7	6.2		21.12	26.98	194.1	<−2.164
42.0	6.2			7.85	0.0	*
36.7	6.3		29.28	51.74	140.3	<−2.133
30.1	6.4		5.66	10.96	128.1	<−1.908
41.7	6.4		6.55	14.30	113.6	<−1.971
12.5	6.6		4.62	22.38	51.2	<−1.317
41.8	6.6		0.31	15.40	5.0	<−0.651
47.0	6.6		6.79	6.83	246.5	<−1.986
13.7	6.8		21.06	43.48	120.1	<−2.121
55.2	6.8			2.01	0.0	*
25.9	6.9	0.14		40.59	1.2	–
12.9	7.1	0.69	0.48	33.24	10.7	−0.144
29.7	7.1	18.78	0.48	50.64	129.4	1.295
29.8	7.2			2.08	0.0	*
44.8	7.3		1.94	2.44	197.2	−1.163
65.9	7.3			3.36	0.0	*
68.3	7.4			6.24	0.0	*
71.4	7.5			2.54	0.0	*
93.6	7.5			0.81	0.0	*
14.9	7.6		16.90	2.33	1798.8	<−2.383
72.7	7.6			3.06	0.0	*
41.3	7.7		1.32	4.13	79.3	<−0.959
30.2	7.8	29.23	1.26	2.72	3797.3	1.064
16.2	7.9	0.48	80.62	64.06	314.7	−2.168
15.0	8.0		5.61	172.11	8.1	<−1.904

(*Continued*)

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TABLE 2
Continued

MW (kDa)	pI	Protein concentration in ATPS, ppm		Total soybean Extract (ppm)	% Recovery	Log K_p
		Top phase	Bottom phase			
15.8	8.8		71.08	136.69	129.0	<-2.598
31.0	9.5	2.30	0.47	12.44	72.7	0.386
12.5	9.6			1.03	0.0	*
16.5	9.6		19.42	64.58	74.6	<-2.209
17.7	10.0		34.53	113.54	75.4	<-2.461

Total protein concentration (ppm): Top phase, 95.52; Bottom phase, 2218; Total soybean extract, 6671.

Total spots count: 97 *29 spots not found in either phase.

Partition coefficient: 0.04.

All data are the average of two experiments using two-phase system: 14.8% PEG 3350, 10.3% potassium phosphate, 3% NaCl, pH 7, $V_r = 1.25$ at 25°C. Load of protein was 1.0 mg/g ATPS from a total soybean extract of 5.3 mg protein/mL. Protein concentrations are expressed in parts per million in the ATPS. % Recovery calculated using the sum of top +bottom protein divided by the total amount of the same protein matched in the gel where no partition experiment was performed (total extract). Log K_p values for proteins not detected in top phase were estimated as the highest K_p possible taking the concentration of the faintest spot quantified as the limit of detection.

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