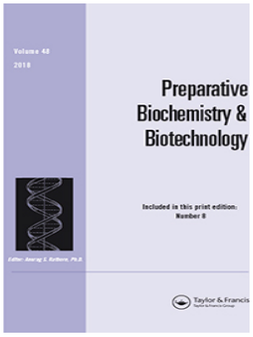

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Aqueous two phase partitioning of *Pisum sativum* lectin in PEG/citrate salt system

Rashmi Bommenahalli Shashidhara and Regupathi Iyyaswami

Department of Chemical Engineering, National Institute of Technology Karnataka, Surathkal, Mangalore, India

ABSTRACT

Pisum sativum lectin (Psl) is a metalloprotein which is in the center of research interest because of its HIV-1 reverse transcriptase inhibitory activity and mitogenic activity. The application of this lectin in various fields demands the economically feasible and scalable purification strategy other than affinity chromatography. The suitability of aqueous two phase system (ATPS) composed of poly ethylene glycol (PEG) with different salts (sodium citrate, potassium citrate, and ammonium citrate) was evaluated for better partitioning of Psl. The significant factors such as molar mass and concentration of PEG, type and concentration of salts, the effect of tie line length (TLL), ionic strength, and pH were studied to select a suitable system for better partitioning of Psl. ATPS comprising of 18% PEG 6000, 16% sodium citrate, 1% NaCl at the operating condition of pH 8, 40.23% of TLL, and the volume ratio of 1.32 was found to be the best system which gave a maximum partition coefficient and yield of 14.5% and 98.66%, respectively.

KEYWORDS

Lectin; partitioning coefficient; phase diagram; tie line length

Introduction

Lectin is a protein of non-immune origin which is different from other plant proteins due to its higher degree of specificity and reversible binding capacity to carbohydrates. A wide variety of applications are exploited based on the carbohydrate specificity of lectin. Even though lectin is widely distributed among plants, animals, and microorganisms, the legume seeds are considered as the richest source of lectins.[1] The demand for different types of lectins from different sources is drastically increasing due to their potential application in various fields such as lectins in diagnostics—agricultural and food industry, environmental detection,[2] medical sample analysis,[3] lectin in separation and characterization of glycoproteins and glycopeptides—lectin-based affinity tag for one-step protein purification,[4] glycan profiling,[5] and therapeutic applications of lectins—diagnosis of malignant tumors,[6] drug delivery,[7] antiviral, immunomodulatory activity, and antifungal activity.[8]

Pisum sativum known as "green peas" is believed to be originated from the countries of Mediterranean and slowly spread to other countries. Canada, United States, Europe, China, India, Russia, and Australia are the leading countries in the production of peas. Peas belong to the Leguminosae family and share few characteristics with *Concavalin, Canavilla*, and *Lentil* lectins.[9] Lectin from *P. sativum* is a metalloprotein which is made up of two identical subunits α and β (heterotetramer ($\alpha_2\beta_2$)) which are non-covalently linked together making up to a molecular weight of 50 kDa. Pea lectin has specificity to mannose and glucose carbohydrates.[10] Mitogenic and anti-HIV reverse transcriptase activity[11] of pea lectin holds the interest of researchers toward its purification and characterization. It was stated in the literature that "pea lectin can be developed as a drug as it shows significant mitogenic activity from mouse splenocytes at a low dose of 4.7μg/mL, which suggests the possibility of the drug development using Psl."[1] Because of the potential application of *P. sativum* lectin (Psl), there is a demand for extraction and purification of this lectin from pea plant seed. Few research groups have tried the extraction and purification of this lectin using chromatographic techniques.[1,11] Almost all the commercially available lectins were purified using affinity chromatography.[12] The other chromatographic techniques like gel filtration chromatography,[13] ion exchange chromatography,[14] and hydrophobic interaction chromatography[15] are also employed to purify lectin. Some of the drawbacks of these techniques such as higher cost with longer time consumption, low handling capacity, and difficulty in scaling up limit their application at the industrial level. To overcome the practical limitations of chromatographic methods, few authors employed the methods like magnetic separation,[16] reverse micellar extraction,[17] membrane separation,[18] and affinity precipitation[19] for the purification of Psl. The respective drawbacks of above listed techniques like complexity associated in the design of magnetic particles, denaturation of proteins due to the anionic or cationic surfactants, fouling of membranes and protein-protein interaction, higher cost

CONTACT Regupathi Iyyaswami regupathi@nitk.ac.in Department of Chemical Engineering, National Institute of Technology Karnataka, Surathkal, Mangalore 575025, India.

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of affinity ligands and matrix have limited the implementation of these methods in the industrial scale.

Aqueous two phase extraction (ATPE) emerged as a versatile extraction and purification technique which incapacitates the limitations with many advantages, such as a system comprising more than 80% of water which reduces the denaturation of protein thereby making it suitable for sensitive proteins, scope for continuous process, can be integrated with other techniques, cost-effective, time-saving, and some of the phase components like polyethylene glycol (PEG) stabilize the protein and retain its activity. Enrichment of desired biomolecules in one of the phases necessitate the proper understanding of the major forces involved in partitioning and the biomolecule characteristics such as surface hydrophobicity, charge, the isoelectric point of protein, and molecular weight.

Aqueous two phase system (ATPS) consisting of PEG/citrate salts is exploited for the lectin purification from few legume seeds belongs to beans family, such as *Canavalia brasiliensis* and *ensiformis*[20,21] and *Cratylia mollis*.[22] It was well documented that the source of the lectin is very important because the lectin properties and their applications vary with the sources. The intrinsic properties of Psl, such as isoelectric point (6.8–7.2),[23] molecular weight (50 kDa),[11] subunit association manner, and amino acids distribution, are distinctly different from the lectins of other legume seeds. The aqueous two phase partition of commercially available pure Psl from its aqueous solution was studied in the present work. Partitioning studies of pure protein reveal the information about the suitability of a specific ATPS through the interactive forces with the specific biomolecule due to the effect of process variables like phase forming components and their concentrations, pH, TLL, volume ratio etc. on partition coefficient. The pure protein studies may reveal the extent of specific interactive forces exist between the phase system and biomolecule in the absence of interferences caused by other molecules and impurities which are actually present in the real-time crude. The variation in the magnitude of the major non-covalent forces like Van der Waals force of attraction and hydrophobic interaction involved in the partitioning, which was varying for different ATPS, may be realized. Based on the information obtained from pure protein studies, the process variables can be further tuned to recover the desired proteins with higher yield and purity from the complex sources like crude extracts. Hence, the different PEG/Salt ATPSs are verified for the partition of commercial Psl by considering their properties and the effect of significant variables, such as PEG molar mass and concentration, type of salt and concentration, ionic strength, pH, TLL, and volume ratio on the partitioning characteristics of Psl.

Material and methods

Chemicals

The list of chemicals with their chemical formula, molar mass, purity, and manufacturer details are shown in Table 1. All the chemicals were of analytical grade and used as received without any further purification.

Aqueous two phase partitioning of Psl

Development of binodal curve

The binodal curve for PEG 6000, 8000/sodium citrate was constructed at 25 °C using cloud point method which was explained in detail by Sindhu and coworkers.[24] The binodal data for PEG 4000-trisodium/tripotassium/triammonium citrate system and PEG 2000/sodium citrate was referred from the literary work of Kalaivani and Regupathi[25] and Murugesan and Perumalsamy,[26] respectively. The developed binodal data was included in the supplementary material Table S1.

Tie line determination

The tie lines were generated for the PEG 6000-sodium citrate system by adding the appropriate weight of PEG 6000 and sodium citrate stock solution and 1 mL of Psl solution having the concentration of 0.5 mg Psl/mL. The total system was prepared on 5 g basis by adding the required amount of deionized water to the solution. The prepared systems were subjected to vortex for uniform mixing for 1 min and centrifuged at 2000 RCF for 5 min and incubated at 25 °C for 6 hr. After the appearance of fine separation between the phases, phase volume was recorded and separated by using micropipette. Each phase was analyzed for both salt and PEG concentration. PEG concentration was measured using the refractive index (RI) method (Digital refractometer RX-500, ATAGO Co. Ltd, Japan). The refractive index values for the homogeneous solutions of PEG and sodium citrate salt, which was formed by considering the compositions in the single-phase region, was obtained. The relation between the refractive index (*n*D) and the mass fraction of polymer (*w*p) and salt (*w*s) is developed (Equation (1)) through regression analysis and used for the determination of

Table 1. Details of the list of chemicals used in the present study.

S. No	Chemicals	Chemical formula	Molar mass	Purity	Manufacturer
1	Polyethylene glycol (PEG)	$[CH_2CH_2O)_n$-H]	2000, 4000, 6000, and 8000 g/mol	99%	Sigma-Aldrich, USA
2	*Pisum sativum* lectin (L5380)	—	50 kDa	99%	Sigma-Aldrich, USA
3	Bicinchoninic acid reagent (BCA)	$(HO_2CC_9H_5N)_2$	344.33 g/mol	98%	Sigma-Aldrich, USA
4	Trisodium citrate	$Na_3C_6H_5O_7.2H_2O$	294.10 g/mol	99%	Merck, India
5	Tripotassium citrate	$K_3C_6H_5O_7$	342.42 g/mol	99%	Merck, India
6	Triammonium citrate	$C_6H_{17}N_3O_7$	243.22 g/mol	98.5%	Merck, India
7	Sodium chloride	NaCl	58.44 g/mol	99%	Spectrum chemicals, India
8	Citric acid	$C_6H_8O_7.H_2O$	210.14 g/mol	99.5%	Spectrum chemicals, India
9	Copper sulfate	$CuSO_4.5H_2O$	159.609 g/mol	99%	Spectrum chemicals, India

PEG6000 concentration in the top and bottom phases of the ATPS by knowing the sodium citrate concentration.

$$n_D = a_0 + a_1 w_P + a_2 w_S, \quad (1)$$

where $a_0 = 1.332$, $a_1 = 0.1312$, and $a_2 = 0.1921$ were obtained for the regression analysis of the refractive index obtained for the homogeneous mixture of PEG 6000-sodium citrate system.

Salt concentration in the top and bottom phase was measured using flame photometer (Elico Ltd., CL-378, India) by diluting the samples appropriately. The calibration curve was prepared with the known concentration of salt. It was used to determine the salt concentration in the samples. Tie line length was determined using Equation (2).

$$\text{TLL } (\%) = \sqrt{\left[W^T_{Salt} - W^B_{Salt}\right]^2 + \left[W^T_{PEG} - W^B_{PEG}\right]^2}, \quad (2)$$

where W^T_{Salt} and W^B_{Salt} refer to the concentration of salt in weight percentage in top and bottom phases and W^T_{PEG} and W^B_{PEG} refer to the concentrations of PEG in weight percentage in top and bottom phases.

Psl partitioning in the ATPS

Psl partitioning behavior in different ATPS was analyzed initially to identify a suitable citrate salt by preparing the ATPS with PEG 4000 and three different citrate salts, namely trisodium/tripotassium/triammonium citrate at different concentrations. Furthermore, the effect of PEG molar mass on the Psl partitioning was studied by preparing the ATPS with trisodium citrate salt and PEG molar mass of 2000, 4000, 6000, and 8000. The ATPSs are formed by adding the appropriate amount of PEG and salt solution from stock solutions (PEG 50% and salt 30%) with 1 mL of Psl solution having the concentration of 0.5 mg Psl/mL on 5 g basis by adding deionized water in a 10 mL graduated conical tubes. The prepared systems were subjected to vortex for uniform mixing for 1 min and centrifuged at 2000 RCF for 5 min. The samples were incubated for 6 hr at 25 °C for complete phase separation. After the appearance of fine separation between the phases, phase volume was recorded, and phases were separated using micropipette and used for further analysis.

The effect of pH on the partitioning coefficient was studied by preparing the ATPS at different pH (5, 6, 7, 8, and 9) by mixing the sodium citrate stock solution, whose pH was adjusted by the addition of either citric acid (adjust to pH of 5, 6, 7, 8) or sodium hydroxide (adjust to pH of 9), and the required quantity of PEG 6000 solution, deionized water, and the protein was added in appropriate quantity to the total weight of 5 g. The pH values of the solutions were measured precisely with a digital pH meter. The crystal NaCl of known weight was directly added to the ATPS to study the effect of additives (NaCl) on the partitioning coefficient.

Protein determination

The concentrations of Psl in the top and bottom phases were estimated using Bicinchoninic acid assay (BCA) in UV/Visible spectrophotometer at 562 nm.[27] The absorbance of protein in the phases was measured by considering the similar phase (only with PEG and salt solution) without protein as a blank to nullify the interference of phase forming components on the absorbance measurement. The quantification of protein in both the phases was performed, and the mass balance of protein was analyzed by considering the initial amount added to the system.

The Psl partition coefficient (K_L) (Equation (3)) and % yield (Equation (4)) were calculated.

$$K_L = \frac{C_{LT}}{C_{LB}}. \quad (3)$$

The yield of the Psl (Y_L) was defined as the ratio of total concentration of lectin present in the top phase (C_{LT}) and the total concentration of lectin present in the initial feed (C_{LF}), expressed as percentage as shown in Equation (4).

$$Y_L\ (\%) = \frac{C_{LT} \times V_T}{C_{LF} \times V_F} \times 100, \quad (4)$$

where V_T and V_F represent the volume of the top phase and the volume of feed protein solution, respectively.

All the experiments were conducted in duplicates ($n = 2$), and the error bars representing the standard deviation are presented. Origin version 9.1 was used for graphical analysis of the data.

HPLC

Reverse phase HPLC (Shimadzu, LC-20AD, Japan) analysis was done to confirm the stability of Psl during the ATPS extraction using C18 (Capcell Pak C18 MG II, Shiseido, Japan) column. Mobile phase A (0.1% trifluoroacetic acid in water) and B (100% Acetonitrile) was used. Column was equilibrated with mobile phase for 15 min before injecting the sample. Column temperature and the flow rate were maintained at 25 °C and 0.5 mL/min, respectively. Binary gradient was used in order to monitor the elution rate. The binary gradient mode was adopted and maintained as 5% solvent B from 0.01 to 2 min, and 80% solvent B was linearly increased till 21–23 min, kept constant for 2 min and reequilibrated from 27 to 35 min by 5% of solvent B. The total run time was 35 min. Absorbance was recorded using a UV detector at 214 nm. The top and bottom phase samples were diluted appropriately to prevent the blocking of chromatographic column due to their higher viscosity and concentration.

Result and discussion

Effect of different salts on Psl partitioning

The partitioning characteristics, partition coefficient (K_L) and yield (Y_L) of Psl, in the PEG-salt ATPSs formed with three different citrate salts, namely sodium citrate, potassium citrate, and ammonium citrate were studied (Fig. 1), because the citrate salts possess good salting out, biodegradable, and nontoxic characteristics. As the properties of the citrate salts vary with associated cations and their concentration, two

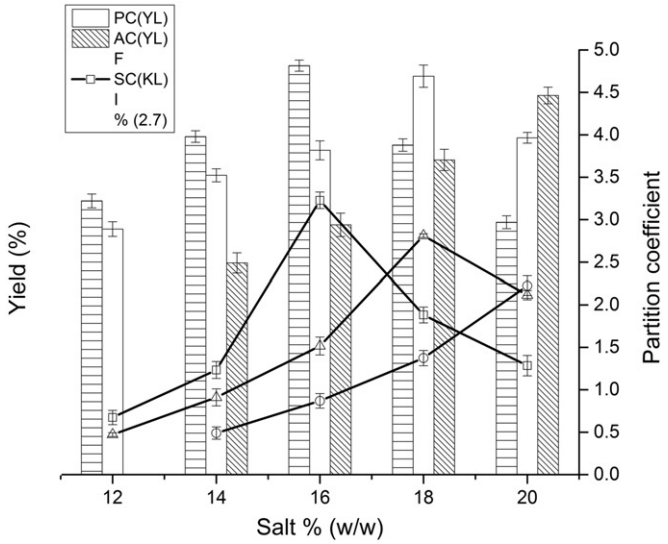

Figure 1. Effect of type of salts at different concentration (SC: sodium citrate; PC: potassium citrate; AC: ammonium citrate) with 20% (w/w) PEG 4000 (g/mol) on partition coefficient (K_L) and yield % (Y_L) of the lectin.

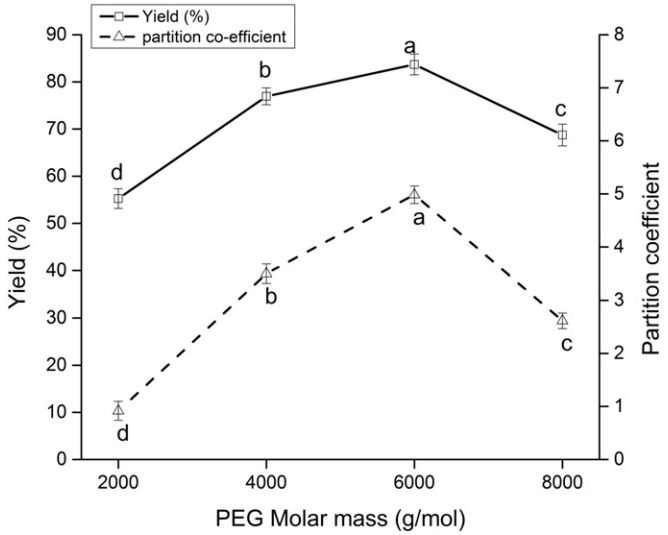

Figure 2. Effect of molar mass of PEG (g/mol) on partition coefficient (K_L) and yield % (Y_L) of the lectin in the ATPS of PEG 20% (w/w) and sodium citrate 16% (w/w).

different ionic environments formed in the top and bottom phases of ATPS and subsequently their specific interaction with the protein differs during the partitioning. The charged protein experiences a chemical potential difference between the phases and resulted in a net partitioning. The effect of citrate salts in the ATPS was retrieved by conducting the experiments at a constant concentration of 20 wt% PEG 4000 with three different salts concentration between 12% and 20% (w/w). It was observed that maximum K_L varied in the order of $3.23 \pm 0.05 > 2.81 \pm 0.06 > 2.23 \pm 0.10$ and maximum Y_L varied in the order of $77.04 \pm 1.05\% > 75.07 \pm 1.29\% > 71.42 \pm 1.58\%$, respectively for $Na^+ > K^+ > NH_4^+$ (Fig. 1). The difference in partitioning coefficient and the salting out ability of the ATPS varies with the properties of the associated cations of the salts having similar anion, citrate. Salting out ability of different salts were analyzed based on the Gibbs free energy of hydration ($\Delta G_{hydration}$), size, and effective excluded volume (EEV) of the cation. It was reported that the salt which contains more negative Gibbs free energy of hydration ($\Delta G_{hydration}$) has higher salting out ability. The highest K_L and Y_L of lectin was observed for sodium citrate salt because the sodium citrate (Na^+: −375 kJ/mol) has more negative $\Delta G_{hydration}$ than potassium citrate (K^+: −304 kJ/mol) and ammonium citrate (NH_4^+: −285 kJ/mol).[28] Furthermore, it is reported that the salting out capacity of the cation also differ with their ionic radius and cations with lesser ionic radius has more salting out capacity.[29] Na^+ (0.102 Å) has lesser ionic radius compared to K^+ (0.138 Å) and NH_4^+ (0.143 Å). The higher salting out characteristic was observed for sodium citrate due to the favorable $\Delta G_{hydration}$, and smaller ionic radius, which limits the solubility of the lectin in salt rich phase and eventually, expelled it to PEG rich top phase through salting out process. The hydrophobic domains of the Psl are exposed and interact with the PEG molecules. However, the EEV of the system, which depend on the molar mass of the PEG and type of the phase forming salt, had a significant effect on the protein partitioning. Because the EEV of PEG 4000 and sodium citrate salts (49.57 g/mol) were higher than the potassium (48.11 g/mol) and ammonium (37.56 g/mol) citrate salts,[25] 20% PEG 4000 and 16% sodium citrate provide a maximum Psl partitioning. The decreasing salting out ability and EEV of potassium citrate and ammonium citrate indicate the requirement of the larger concentration of salt to achieve a favorable partitioning, and hence the ammonium citrate was unable to form two phase at 12% concentration (Fig. 1). However, the maximum partitioning for potassium citrate and ammonium citrate was achieved at a concentration of 18% and 20%, respectively (Fig. 1). The PEG-sodium citrate system was successfully employed not only for the partitioning of *C. brasiliensis* (ConBr) lectin[20] and also for different other proteins like α-La from whey[30] and glycomacropeptide.[31] The sodium citrate salt was selected as a phase forming salt for further studies as it shows better performance on Psl partition coefficient and yield when compare to other two citrate salts.

Effect of PEG molar mass on partition of Psl

The multiple binding interactions of polymer and protein are a prime factor along with the other factors for the proteins like Psl, which get partition in the PEG phase. However, it is difficult to form multiple binding sites with protein for too shorter or longer chain length of PEG[32]; hence, the experiments were designed to screen the appropriate PEG molar mass which consists of optimum chain length to interact with the Psl and enhance its partition into the top phase. The partitioning experiments were carried out with constant total phase composition of PEG 20% (w/w) and sodium citrate 16% (w/w) with different molar mass of PEG from 2000 to 8000 (Fig. 2). The Psl partition into the top phase was found to increase with increasing molar mass up to 6000 and further increase in molar mass to 8000 resulted in lesser Psl partitioning. As the molar mass increases, the hydrophobicity of the phase also increases due

to the increase in the length of the PEG chain with increasing repeating nonpolar CH_2 group in polymer chain. The partitioning of protein to either of the phases depends on proteins intrinsic factors such as protein size, hydrophilic/hydrophobic characteristics, and conformational structure and extrinsic factors such as temperature, pH, ionic strength, and interaction between protein molecules. The isoelectric point of Psl is in the range of 6.8–7.2.[23] The maximum partitioning of Psl by retaining its functionality was observed at PEG 6000 when compared to other molar mass of the PEG (Fig. 2) due to the optimum chain length and the hydrophobicity which enhances the interaction between the PEG and Psl. The lower partitioning of Psl was observed at PEG 2000 molar mass may be due to the lower hydrophobicity of the top phase. The protein molecular weight also influences the accommodation of proteins in top phase. The molecular weight of Psl is 50 kDa, which is intermediate when compared to most of the other proteins in general. Accordingly, its steric exclusion rate is also low when compared to very high molecular weight glycoproteins. Interface precipitation of Psl in higher molar mass (PEG 8000) decreases the partition coefficient due to volume exclusion effect. However, lectin from *Canavalia ensiformis*[21] (molecular weight, MW = 50 kDa) and *C. mollis*[22] (MW = 60 kDa) were partitioned to the bottom phase of ATPS formed with PEG 8000-sodium citrate by utilizing the volume exclusion effect of the PEG 8000. In contrary, *C. brasiliensis* (MW = 60 kDa) lectin partitioned to top phase with a recovery yield of 70% in the ATPS formed by very low molar mass of PEG 600 with citrate salt.[20] The maximum partitioning characteristic was reported for ATPs formed by PEG 6000 for other proteins like serine protease (MW = 65 kDa) from mango waste[33] and lysozyme (MW = 14.4 KDa) partition from chicken egg where in the partitioning is governed by hydrophobicity.[34] The higher yield and partition coefficient of Psl were observed in the PEG 6000-sodium citrate system, hence PEG 6000 was selected for further studies.

Effect of concentration of PEG on Psl partitioning

The experiments were conducted to acquire the effect of PEG 6000 concentration with constant sodium citrate concentration of 16% (w/w) on the partition behavior of Psl. The K_L and Y_L were found to increase with increasing PEG 6000 concentration, and maximum K_L and Y_L was observed at 18% PEG (Fig. 3). The increased hydrophobicity with increasing PEG concentration promotes the PEG-Lectin interaction and resulted in higher partitioning and yield. The presence of hydrophobic binding site in legume lectins was identified using hydrophobic ligands[35] and reported that the aminoacids like valine, isoleucine, and phenylalanine were the major aminoacids present in the hydrophobic site[36] which has binding affinity toward hydrophobic molecule, such as PEG. The hydrophobic interaction was further confirmed by the work of Rathnasamy and his coworkers[37] with the partitioning studies of lysozyme from quail egg in 25% PEG and 18% ammonium sulfate and reported that the

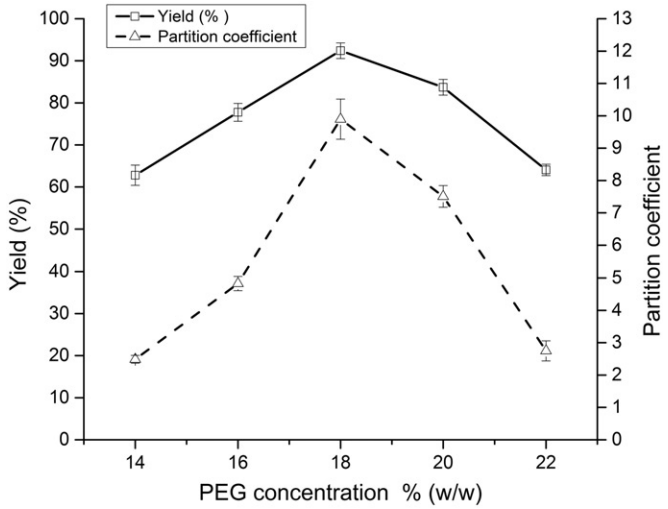

Figure 3. Effect of PEG 6000 (g/mol) concentration on partition coefficient (K_L) and yield % (Y_L) of the lectin at constant sodium citrate concentration of 16% (w/w).

increase in the partitioning of enzyme to top phase was mainly because of hydrophobic interaction. The similar effect was further reported for the partitioning studies of *Canavalia grandiflra* lectin by Porto and his coworkers.[38] The lesser K_L and Y_L was observed at 14% and 16% PEG (Fig. 3) due to the insufficient interactive force between the PEG and Psl, including the lower hydrophobicity of the PEG phase at lower PEG concentration. The K_L and Y_L found to be less even at higher concentration of PEG (20%) (Fig. 3) due to intermolecular hydrophobic bonding takes place between the PEG molecules and resulted in the compact structure of PEG which occupies more space. Hence, the free volume available for the Psl was reduced and consequently excluded from the PEG phase and may be forced to the interface or bottom phase. The ATPS with PEG 6000 concentration of 18% was selected for further studies.

Effect of concentration of sodium citrate on partition of Psl

The influence of salt concentration on Psl partitioning was studied with different salt concentration by keeping constant PEG 6000 concentration of 18%. Salts affect the protein partitioning in PEG/salt systems by changing the distribution of charged amino acids by imparting electrostatic effect. A gradual improvement in the partition of Psl was observed for increasing salt concentration from 12% to 16%, and the partition of Psl was found to decrease for the salt concentrations of 18% and 20% (Fig. 4). As the citrate concentration increases, the solubility of the Psl decreases in the bottom phase due to the salting out process. Furthermore, the hydration effect of ion increases with increasing salt concentration and resulted in the dehydration of proteins with the exposure of hydrophobic sites which in turn favors the interaction of Psl with PEG and facilitating the migration of Psl toward top phase. The increase in sodium citrate concentration also increases the ionic strength in the system which facilitates the movement of negatively charged

proteins to top phase by electrostatic repulsive effect.[38] However, the higher salt concentration (18% and 20%) fails to partition the Psl to top phase due to the reduction in volume ratio at higher salt concentration (Fig. 4). The desired protein gets concentrated in the PEG phase and reaches the maximum possible concentration in the reduced volume. The combined effect due to the non-availability of the free solvent in the PEG phase and the salting out characteristics of the bottom phase promoted the interphase precipitation. The similar trend was observed in Immunoglobulin-G (IgG) partition in PEG/phosphate system, and a significant loss of IgG at interface was reported due to increased salting out process.[39] Porto et al. also reported that the *Canavalia grandiflora* lectins partition coefficient increasing to 8.67 in the system composed of 20% PEG 600 and 20% sodium citrate and concluded that the salting out capacity of salt was the main reason for increasing yield of lectin in the top phase.[38] Based on the observations, 16% sodium citrate was selected as an optimum concentration for further experiments. It was noticed from the study that the salting out dependent hydrophobic interaction was playing prominent role in partitioning of lectin toward top phase. 16% of sodium citrate was considered as an optimum concentration for further studies as it gives maximum K_L and Y_L.

Effect of tie line length (TLL) on Psl partitioning

Tie line is the line connecting the equilibrium compositions of the components present in the two phases of ATPS formed by mixing the defined quantity of phase forming components. As the partition characteristics of the solute are governed by the equilibrium composition of the phase forming components (PFC), it is very important to obtain the effect of TLL in order to obtain better partition and yield of target molecule. Partition of protein in the system can be well understood with the equilibrium composition of phases rather than feed composition.[30] The effect of TLL was evaluated by considering different composition of phase forming components which result the TLL in the range of 30–48% (Table 2). The phase diagram of the system which indicates the binodal and tie lines with total and equilibrium concentrations of the present system was represented as Fig. 5. The hydrophobicity and salting out ability of the PEG/salt system can be varied with TLL. The TLL of 40.23% provided maximum K_L and Y_L of Psl (Fig. 6), in which the combination of hydrophobic interaction and salting out effect played a major role for the efficient partitioning of Psl. The optimum equilibrium concentration of PEG in top phase (34.1%) enhances the number of hydrophobic interaction with the exposed hydrophobic sites of Psl and favors its movement to top phase; consequently, optimum concentration of salt (27.56%) creates high ionic strength which favors the partition of Psl to top phase due to electrostatic repulsion. However, the increase in TLL to 47.34% increases the concentrations of PEG in top phase and salt in bottom phase to 39.43% and 32.14%, respectively; consequently, K_L and Y_L were found to reduce to 3.11% and 56.91%, respectively due to the reduction in the water content and free volume of phases to accommodate the Psl. The denaturation and interface precipitation of Psl was observed due to the higher PFC, particularly the salt concentration. Furthermore, the increased density and viscosity of the phases with TLL also makes the system more tedious for the process operation.[40] However, the lower TLL also fails to provide good resolution in the distribution of Psl due to the lesser salting out and hydrophobic properties, which were not enough to push the Psl to a particular phase.

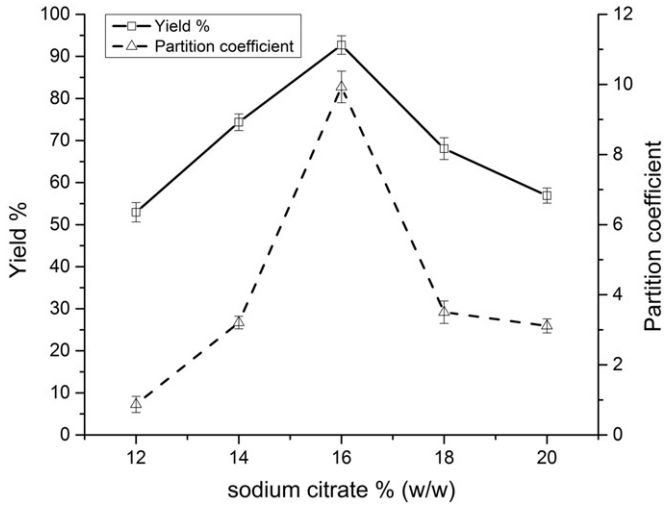

Figure 4. Effect of sodium citrate salt concentration on partition coefficient (K_L) and yield % (Y_L) of the lectin at constant PEG 6000 (g/mol) concentration of 18% (w/w).

Effect of NaCl on partition of Psl

The addition of electrolyte salts as additives in the ATPS system increases the hydrophobic resolution between the PEG and salt phases due to the change of surface properties of the proteins like exposing the hydrophobic interactive sites.[41] The larger hydrophobic difference between the phases improves the selective partitioning of desired proteins to any one of the phases. The improvement of partitioning

Table 2. Equilibrium concentration of the phases with their TLL, partition coefficient, and top phase yield of *Pisum sativum* lectin.

Feed (%,w/w)		Top phase (%, w/w)		Bottom phase (%,w/w)		Tie line length (%)	Partition coefficient of lectin, KL	Top phase yield of lectin, YL (%)
PEG 6000	Tri sodium citrate	PEG 6000	Tri sodium citrate	PEG 6000	Tri sodium citrate			
18	12	27.12	1.2	5.2	24.23	32.16	0.87 ± 0.08	52.95 ± 1.34
16	16	29.96	1.16	4.86	26.19	35.59	4.82 ± 0.14	77.75 ± 1.45
18	16	34.1	1.26	4.32	27.56	40.23	9.89 ± 0.12	92.75 ± 0.94
20	16	36.94	1.2	3.98	29.23	43.76	7.50 ± 0.16	83.70 ± 1.20
22	16	38.94	1.35	4.32	30.24	45.66	2.75 ± 0.09	64.05 ± 1.06
18	20	39.43	1.17	4.26	32.14	47.34	3.11 ± 0.09	56.91 ± 0.16

characteristics and subsequent increase in the yield and purity by the addition of electrolyte salt was reported for proteins like antibodies,[42] amylase,[43] and bovine serum albumin.[44] Hence, the electrolyte salt, NaCl was considered as an additive because of its ability to enhance the partitioning of protein to hydrophobic phase. Varied range of NaCl from 0% to 5% (Fig. 6) was considered to study its influence on the partitioning of Psl in the system consists of 18% PEG 6000 and 16% sodium citrate with a TLL of 40.23%. Maximum K_L and Y_L was achieved at 1% NaCl and above which there was a slight decrease in yield up to 3% of NaCl. However, a gradual decrease in both yield and partition coefficient was observed beyond the addition of 3% NaCl.

The accumulation of electrochemical species among the two phases creates difference in electrostatic potential which acts as a driving force for the movement of protein to a phase along with the increase in hydrophobic resolution among the phases. Similar increase in partitioning coefficient to the NaCl concentration of 5% was observed in the partitioning of *C. brasiliensis* lectin to top phase in PEG 600/sodium citrate system.[20]The addition of Nacl has improved the partitioning of *Zihua snap-bean* lectin toward PEG phase evidencing the role of NaCl in partitioning of proteins to top phase.[45] It was confirmed by the behavior of K_L and Y_L that Nacl of 1% drive the lectin toward top phase and 1% of NaCl was used for further studies.

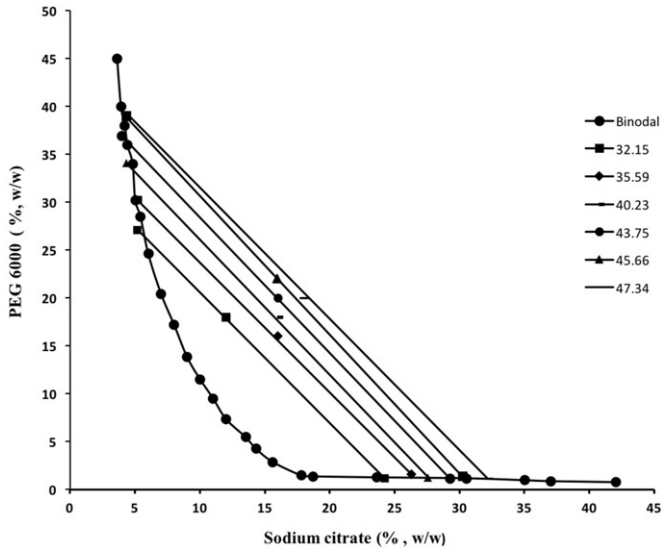

Figure 5. Binodal curve and tie lines for PEG 6000-sodium citrate system with Psl.

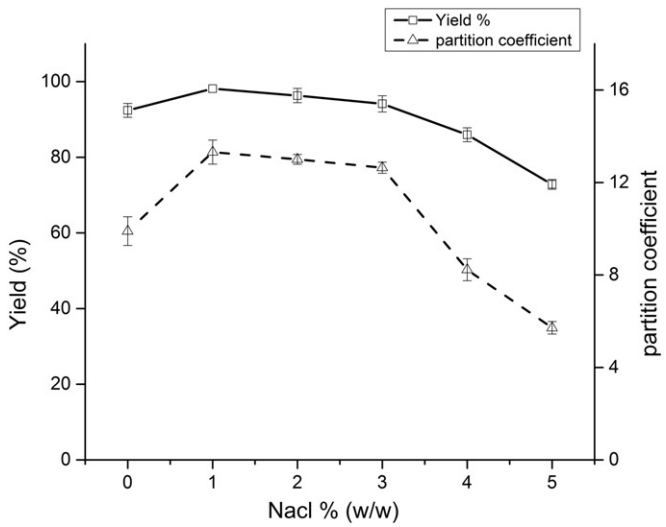

Figure 6. Effect of NaCl as an additive on yield and partition coefficient of Psl in the ATPS system made-up of 18% PEG 6000 and 16% sodium citrate at 40.23% TLL.

Effect of pH on Psl partitioning

The change in the pH controls the ionization state of the amino acids present on the surface of a protein. The partition of the desired protein can be fine-tuned by manipulating the pH. In the present study, the effect of pH was studied at different range of pH from 5 to 9 on the partition coefficient and yield of Psl in a system composed of 18% PEG 6000 and 16% sodium citrate with a TLL of 40.23% and the addition of 1% NaCl. The surface property of the protein and also the composition of ions in the phases vary with pH. The amine groups present on the surface of the proteins get protonated, and the proteins acquire positive charge at acidic pH. Conversely, protein becomes negatively charged in the alkaline pH by deprotonating the carboxyl group. The net surface charge of the protein depends on its pI. When the pH changes, generally the protein acquire positive charge below its pI and partitioned in the salt-rich bottom phase. But, the proteins gain negative charge above its pI and moves to the PEG-rich top phase.[46] Accordingly, the maximum partition of Psl in the PEG-rich phase was noticed at a system pH of 8, which is slightly higher than the pI of the Psl (6.1–7.2)[23] because the Psl acquires negative charge and thus partitions to positively charged PEG rich phase with maximum K_L (Fig. 7). In addition to that, the increasing pH also increases the trivalent citrate concentration in the bottom phase and increases the salting out process thereby Psl expelled to top phase. Similar observation was reported for many proteins like lipase (pI of 6.3) gave maximum partition in pH 7–8[47] and xylanase (pI of about 4.5) gave more partition coefficient with increasing pH to 6.[48] However, lower partition was observed in acidic pH because of the disassociation of α and β subunits of the Psl and destabilization of its structure. Leaching out of divalent metal ions also takes place at acidic pH where metal ions are essential for the stability of the lectin. pH of less than 6 and more than 8 gave lesser partition coefficient and yield which shows that lectin prefers a slightly basic pH than acidic or too basic. Hence, pH 8 was selected as an optimum pH for further studies.

Effect of volume ratio on Psl partition

The accommodation of target biomolecule in particular phase depends on the free volume of solvent available in that particular phase. It is possible to manipulate the extraction performance of the system by altering the volume ratios of individual phase by keeping the total volume constant

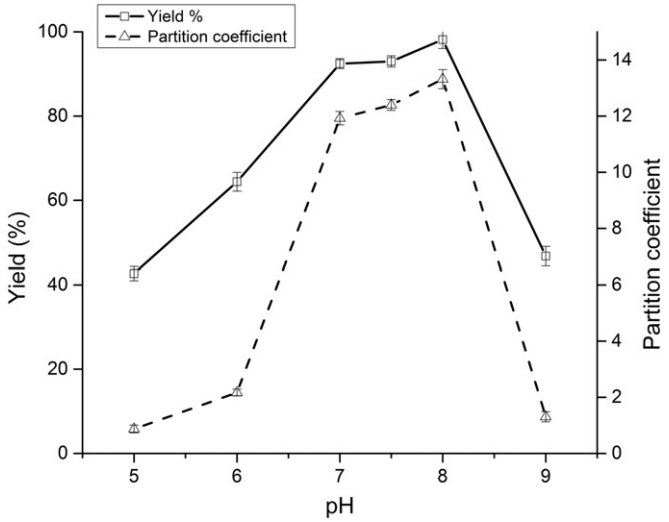

Figure 7. Effect of pH on the yield and partition coefficient of Psl in the ATPS system made-up of 18% PEG 6000, 16% sodium citrate, and 1% NaCl at 40.23% TLL.

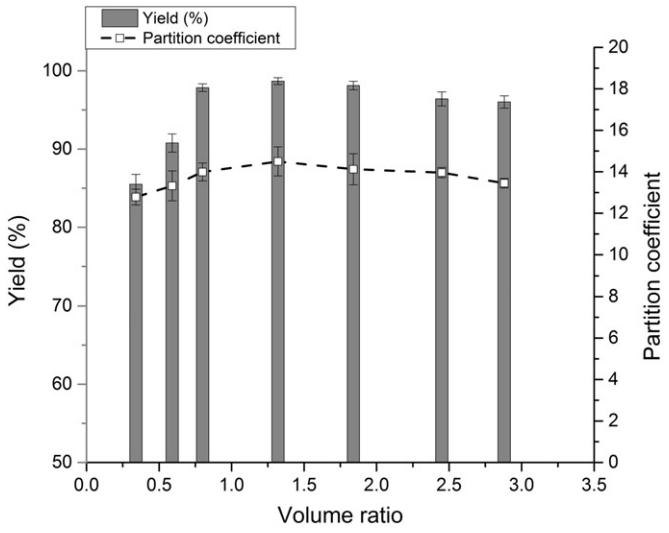

Figure 8. Effect of volume ratio on the yield and partition coefficient of Psl along the same tie line of (40.23) in 18% PEG 6000, 16% sodium citrate, and 1% NaCl at pH 8.

where the equilibrium composition of both the phases remains constant with respect to their tie line. The extractive performance of the system based on phase volume ratio was evaluated by considering different compositions of phase components on tie line (40.23%), and the volume ratios varied from 0.34 to 2.88. The effect of Vr on K_L and Y_L were shown in Fig. 8. Partition coefficient of a Psl remains almost constant for different volume ratios along the tie line and there was a slight decrease in yield at the volume ratio of 0.34 and 0.59 because of decrease in volume of top phase which may lead to precipitation of lectin.[47] K_L and Y_L tend to increase as volume ratio increases, and the maximum K_L of 14.5 ± 0.65 and Y_L of $98.66 \pm 0.82\%$ was observed at the volume ratio of 1.32 after this there was a very less decrease in the Y_L and K_L and at 2.88 Vr there was a decrease in both the parameters, this may be due to the dilution of the protein by increased top phase.

Qualitative analysis of extracted lectin

The Psl subjected to ATPS was analyzed in the RP-HPLC to infer the effect of phase forming component and operating condition on the stability of Psl by comparing it with the commercially available Psl. Peak elution was observed at 27.41 (Psl1) and 27.72 (Psl2) min at appropriate chromatographic conditions for pure pea lectin (Fig. S1). At the same chromatographic conditions, peak elution for extracted lectin was (Fig. S2) found at 27.44 and 27.76 min for Psl1 and Psl2, respectively. A slight variation in the peak of extracted Psl was observed due to presence of few ions in the sample, but the peak structure is similar when compared to pure Psl. Maximum intensity was observed at 214 nm.

Conclusion

The PEG 6000-sodium citrate salt was identified as an efficient ATPS for the partition of pure Psl. The analysis of all the studied parameters such as molar mass and concentration of PEG, salt type and concentration, ionic strength, and pH has significanteffect on the partitioning of Psl except the volume ratio. 18% PEG 6000 and 16% sodium citrate in 1% NaCl at 40.23% TLL in pH 8 and volume ratio of 1.32 were found to be the optimized process condition to obtain maximum Psl yield of 98.66% from the synthetic solution of Psl. The superior partitioning and yield characteristics of the present system suggest the implementation of the identified system for the extraction and purification of Psl from the crude extract of *P. sativum* after studying the effect of variables on the purity of Psl.

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