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Aqueous two phase based selective extraction of mannose/glucose specific lectin from Indian cultivar of *Pisum sativum* seed

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ABSTRACT

Pisum sativum lectin (Psl) being a high-value protein has marked its application in the biomedical and therapeutic field. Aqueous two phase extraction (ATPE) was implemented as a selective partitioning technique for the partial purification of Psl from its seeds. PEG/citrate based biodegradable aqueous two phase system (ATPS) was screened and the factors such as the type and concentration of citrate salts, molar mass and concentration of polyethylene glycol (PEG), tie line length (TLL) and additive (NaCl) concentration, pH, crude load and volume ratio were studied for the selective partition of Psl. The Psl was successfully extracted to the top phase in the ATPS formed with 18% PEG 6000/16% sodium citrate at 41.01% TLL, 2% NaCl and pH of 7.5. A volume ratio of 0.76 and a crude load of 20% showed maximum activity yield of 122.12% with the purification factor of 16.26. The subunits of Psl namely α and β were identified with a molecular weight of 6 and 18 kDa respectively during the purity analysis using SDS PAGE and HPLC.

1. Introduction

Lectins, the glycoproteins, can bind carbohydrates reversibly and specifically without any chemical modification. Lectins are divalent or polyvalent comprising two or more carbohydrate binding sites. Distribution of the lectins is ubiquitous; they can be found in different sources ranging from plants, animals, viruses, and even in microorganisms [1]. Lectins can be majorly classified based on their structure, carbohydrate specificity and evolutionary relationship [2]. The seeds of the legume plants contain a high amount of lectin when compared to its other parts. Natural sources of a protein are always given higher priority due to their lesser toxicity, relative safety when used as a drug. The beneficial properties of lectin, like ribosomal inactivation property [3], antimicrobial: anti-fungal, anti-bacterial [4], anti-viral [5], anti-cancer [6], diagnostic tools [7], and their role in drug delivery [8] made the application of lectins possible in different areas such as biomedical field, biotechnological industry, food industry, and agricultural industry. The source and properties of lectins have a prominent influence on their specific applications. As lectin properties vary tremendously depending upon the source, there is much scope to explore and extract lectin from various sources.

Pisum sativum, the pea is a rich source of protein, which contains mannose/glucose specific lectin which has anti-HIV-1 reverse transcriptase, mitogenic activity [9], anticancer [10], anti-microbial [11] properties and finds its application majorly in the biomedical and therapeutic field. *Pisum sativum* lectin (Psl) is a metalloprotein which requires metal ions such as Ca^{2+} and Mn^{2+} for its functional activity. Basically, Psl is a heterotetramer which consists of two identical α and β subunits, which account for the molecular weight of 50 kDa [9]. Psl subunits associate into a dimer in their three-dimensional structure. Pea is considered to have a significant amount of storage proteins such as albumin and globulins; they make up a large proportion of pea proteins, whereas lectins are about 10–15% of total proteins. Lectins are usually present along with storage protein in the seeds [12]. The selective extraction of lectins from abundance presence of other storage proteins is quite challenging.

The demand for the lectin is increasing due to diversified applications and hence, a process which can deliver the lectin in large-scale with low cost is in high demand. Current production of the lectin relies mainly on chromatographic approaches like affinity chromatography [13], ion exchange [14] and hydrophobic interaction chromatography [15]. The extraction and purification process becomes intricate since the crude extract contains a variety of biomolecules (as impurities). Combinations of one or more chromatographic procedures were implemented for the extraction and purification of lectins [16]. The pretreatment of the crude samples and the use of carbohydrate media as a stationary phase make the procedures complex and expensive which directly influences the cost of the purified lectin. Even though,

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chromatographic separation became a versatile process for the purification of lectins; high operation cost, complexity, and scale-up issues limit its application. Some of the other techniques studied and reported for the bulk extraction and purification of lectins are reverse micellar extraction [17], magnetic separation [18], membrane technology [19] and affinity precipitation [20]. The harmful solvents, chemicals, the resulted resistance of the processes and high operation cost were the significant drawbacks, which restricted the industries from implementing the processes in large-scale. Hence, continuous research is focused on exploring a suitable technique to overcome the demerits.

Aqueous two-phase extraction is a simple and cost-effective selective separation process which has the advantage of process integration and high water content. High water content avoids the denaturation of labile proteins; hence, it is the primary criterion which made researchers to show interest in ATPE technique. The application of ATPS for extraction of the lectin from tobacco hairy root culture medium was first reported by Zang and his coworkers [21]. However the partitioning of lectin from the seed extract of *Canavalia* species was studied in 2010 and PEG/phosphate system was used to selectively partition the lectin from seeds of *Canavalia brasiliensis* with a yield of 100% and purity of 73.04% [22], *Canavalia ensiformis* was also studied in PEG/sodium citrate and the lectin was purified with the purification factor of 11.5 and obtained maximum activity yield of 100% [23]. Later this method was successfully extended to *Cratylia mollis* seeds. The partitioning of the lectin from *Cratylia mollis* seed was explored in PEG/sodium citrate ATPS and 125% of activity yield and a purification factor of 13.28 was achieved [24]. The partitioning in the ATPS relies mainly on the properties of the protein, like the isoelectric point, molecular weight, hydrophobicity, amino acid distribution, number of isoforms, and quaternary structure [25].

Even though the aqueous two phase partitioning of the lectin from few beans family legume seeds were studied, the ATPE of Psl was not attempted. Recently the authors studied the partitioning characteristic of the Psl in the PEG 6000-sodium citrate system from the aqueous solution of commercial Psl and reported a maximum partition coefficient and yield of 14.5 and 98.66%, respectively in the PEG phase [26]. However, the partition behavior of protein may be altered with the influence of other proteins and nonproteinaceous substances in the crude extract. Hence, the present study was organized to analyze the effect of process parameters like, molar mass of PEG, type of salt, the concentration of PEG and salt, the effect of tie line length, and NaCl, pH, crude load and volume ratio on the selective partition of Psl, activity yield and purification factor.

2. Material and methods

2.1. Materials

Analytical grade Polyethylene glycol (PEG) [$(CH_2CH_2O)_n$-H] of different average molar mass ranging from 2000, 4000, 6000, 8000 $g{\cdot}mol^{-1}$ of purity 99%, analytical grade sodium chloride (NaCl-58.44 g/mol) of purity 99.5% and bicinchoninic acid reagent (BCA) was purchased from Sigma-Aldrich, India. Sodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$-294.10 g/mol), potassium citrate ($C_6H_5K_3O_7{\cdot}H_2O$-324.41 g/mol), ammonium citrate ($C_6H_{17}N_3O_7$-243.22 g/mol) all of analytical grade and of purity 99%, 99% and 98.5% respectively and bovine serum albumin (BSA-66 KDA), analytical grade of purity 98% was procured from HiMedia, India. Acetonitrile (C_2H_3N-41.05 g/mol) and trifluoroacetic acid ($C_2HF_3O_2$-114.02 g/mol) of HPLC grade with a purity of 99.9% were procured from Merck, India. Citric acid monohydrate ($C_6H_8O_7{\cdot}H_2O$-210.14 g/mol), copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$-249.68 g/mol) of ACS grade with a purity of 99.7% and 99% respectively were purchased from Merck, India. Potassium chloride (KCl-74.5513 g/mol), di-sodium hydrogen phosphate (Na_2HPO_4-141.96 g/mol), potassium dihydrogen phosphate (KH_2PO_4-172.114 g/mol) of analytical grade with a purity of 99.7% was purchased from SRL, India. Deionized water (Siemens, Labostar) was used throughout the experiment. All the chemicals were used as it is without any further purification.

2.2. Methods

2.2.1. Crude extract preparation

Pisum sativum seeds were purchased from local market of Mangalore city, Karnataka state, India. Collected seeds were freeze-dried and powdered in an ice-cold mixer. Powdered seed sample (10% w/v) was mixed with phosphate buffer saline (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) of pH 7.4 and stirred overnight at 4 °C. The extract obtained was filtered using a muslin cloth and centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant was collected and used as a crude sample for all the experiments.

2.2.2. Aqueous two-phase system

The details of the binodal curve of all the systems considered in the present work were provided elsewhere [26–29]. Phase systems of sodium citrate (SC), potassium citrate (PC), and ammonium citrate (AC) with PEG 4000 were prepared at different concentrations to form the ATPS based on the binodal curves for the determination suitable ATPS to selectively partition the Psl from its seed source. The influence of PEG molar mass was assessed by preparing the systems with different PEG molar mass (2000, 4000, 6000, 8000 g/mol) with trisodium citrate. Total weight of the system was maintained at 10 g by adding the required weight of phase forming components (PEG and salt), 20% w/w of the crude sample as a feedstock for all the experiments except crude loading studies and deionized water. The systems were incubated at 25 °C (5 h) to reach equilibrium after vortexed for 1 min and centrifuged for 2000 rpm. Phase volume was recorded after the fine separation of the phases and the phases were separated by using micropipette for further analysis. All partition studies were repeated thrice for reproducibility. The effect of pH on the extraction parameters was studied at the pH of 5, 6, 7, 8 and 9 by adjusting the pH of the stock solution of trisodium citrate using citric acid monohydrate. The effect of electrolyte additives on extraction parameters was studied by adding the NaCl crystals directly while preparing the system. The effect of crude concentration and loading capacity of the system was analyzed by adding a different amount of weighed crude to the system.

The sodium citrate concentration in the equilibrated top and bottom phases were estimated using the flame photometer (Elico Ltd., CL-378, India). The calibration curve developed using various known concentrations of sodium citrate was used to determine the concentration of salt in the samples. PEG concentration in the equilibrated phases was measured using the refractive index value, which was obtained using refractometer (Automatic digital refractometer, Atago Co. Ltd., RX-5000R) as mentioned elsewhere [26]. The equilibrium concentration of salt and PEG in both the top and bottom phases were used to estimate the TLL (tie-line length) (Eq. (1)).

$$\mathrm{TLL}\ (\%) = \sqrt{[W^{T}_{Salt} - W^{B}_{Salt}]^2 + [W^{T}_{PEG} - W^{B}_{PEG}]^2} \quad (1)$$

where $W_{Salt}{}^{T}$ and $W_{Salt}{}^{B}$ are the weight fraction of sodium citrate salt at top and bottom phase, respectively. $W_{PEG}{}^{T}$ and $W_{PEG}{}^{B}$ weight fraction of PEG 6000 at top and bottom phase, respectively.

2.2.3. Calculations

The concentration of total protein in the top and bottom phase were determined using Bicinchoninic acid assay (BCA) in UV/Visible spectrophotometer (UV3000+, Labindia) at 562 nm [30]. Interference of phase components was nullified by measuring all the systems against blank which contains only phase components without the crude sample. The quantification of protein in both the phases was done by using the calibration curve developed using the standard BSA. The total protein partition coefficient (Kp) was calculated as;

$$Kp = \frac{C_{TP}}{C_{BP}} \quad (2)$$

where, C_{TP} and C_{BP} are the total protein content in the top phase and bottom phase, respectively.

Hemagglutination assay (HA) was carried out for the samples of top and bottom phase according to the procedure described by Correia and Coelho [31]. Phase samples (50 µL) were 2-fold serially diluted in U shaped bottom microtiter plate with PBS of pH 7.2. 2% w/w of O^{+ve} human erythrocytes (Informed consent was obtained from the donors) was added to this diluted mixture and the plate was slightly tilted and allowed for 1-hour incubation. Wells without samples and only with PBS and erythrocytes which were diluted in 2 fold serial dilution fashion was maintained as 'control'. The appearance of a diffused mat of erythrocytes indicates positive results and the appearance of a red dot inside the well indicates negative results. Hemagglutination unit (HU) was defined as 'the reciprocal of highest dilution which shows visible agglutination' and specific hemagglutination activity (SHA) was defined as 'hemagglutination unit per mg of protein (HU/mg)' and SHA of the top phase (SHA_T) and the bottom phase (SHA_B) were calculated (Eq. (3)). Further, the specific hemagglutination activity partition coefficient (K_{SHA}) was calculated using Eq. (4). The hemagglutination activity yield (Y_{HA}) was calculated and expressed as a percentage (Eq. (5)).

$$SHA_T = \frac{HA_T}{C_{TP}} \text{ (or) } SHA_B = \frac{HA_B}{C_{BP}} \quad (3)$$

$$K_{SHA} = \frac{SHA_T}{SHA_B} \quad (4)$$

$$Y_{HA} = \frac{HA_T \times V_T}{HA_C \times V_C} \quad (5)$$

where, HA_T and HA_B are hemagglutination activity in the top and bottom phase respectively. V_T and V_C represent the volumes of the bottom phase and the crude extract, respectively. The purification factor (PF) was calculated as 'the ratio of specific activity in the top phase (SHA_T) to the initial specific activity in the crude extract prior to partition (HA_C/C_{CP})', expressed as follows:

$$PF = \frac{SHA_T}{SHA_C} \quad (6)$$

where HA_C (HU/mL) and C_{CP} (mg/mL) represent the hemagglutination activity and total protein concentration in the crude extract, respectively.

2.2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of the extracted Psl was analyzed by the SDS-PAGE. Discontinuous polyacrylamide gel comprising of 12% resolving gel and 5% stacking gel was prepared [32]. All the samples were dissolved in sample buffer of pH 6.8, containing 1% SDS and incubated at 90 °C for 5 min. Phase samples were dialyzed overnight at 4 °C against deionized water to minimize the interference of phase components. The interface was collected from the system and mixed with PBS and subjected to centrifugation; settled cell debris was discarded and the supernatant was used as a sample. Marker, standard Psl, *Pisum sativum* seed crude extract, samples from top phase, interphase and bottom phase of optimized ATPS were loaded to respective wells and electrophoresis was carried out at a constant current of 50 V for 3 h. The gel was stained with Coomassie brilliant blue R-250 and destained with a destaining solution.

2.2.5. High performance liquid chromatography (HPLC)

Reverse phase HPLC (Shimadzu, LC-20AD, Japan) analysis was done to confirm the purity of Psl during the ATP extraction of the lectin from *Pisum sativum* crude extract using C18 (Capcell Pak C18 MG II, Shiseido, Japan) column. Mobile phase A (0.1% trifluoroacetic acid in water) and B (100% Acetonitrile) were used. The 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. 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 to 23 min, kept constant for 2 min and re-equilibrated 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 the chromatographic column due to their higher viscosity and concentration.

2.2.6. Statistical analysis

All the experiments were performed in triplicate and average values were reported. Nonlinear regression analysis was performed using 'Microsoft Excel' for the quantitative variables considered in the experimental design to analyze the significance of the variables on the responses. The statistical parameters were estimated and discussed in detail.

3. Result and discussion

3.1. Selection of suitable citrate salt

Phase separation in PEG/salt varies with the type of salt which affects the water structure and hydrophobic interaction differently [33]. To determine the most applicable type of ATPS for selective extraction of the lectin from the crude extract of *P. sativum* seed, three citrate salts (sodium citrate (SC), potassium citrate (PC), and ammonium citrate (AC)) were considered and studied. The effect of salt type was examined in PEG 4000 with varying concentrations of salt by considering the respective binodals and constant PEG concentration of 20%. The performance of the various salts was assessed by hemagglutination activity yield (Y_{HA}), purification factor (PF) (Fig. 1a) and specific hemagglutination activity partition coefficient (K_{SHA}) (Fig. 1b). The Y_{HA} and PF varied respectively in the range of 14.19 ± 0.13% to 56.87 ± 0.62% and 0.48 ± 0.02 to 1.55 ± 0.08 for SC and 6.8 ± 0.09% to 52.50 ± 1.25% and 0.27 ± 0.02 to 1.37 ± 0.07 for PC. The SC and PC systems exhibited relatively good performance in terms of Y_{HA} and PF. However, more salt concentration (18%) of PC was required to give nearly the same Y_{HA} and PF than SC (16%). In contrast to these results, AC yields lesser Y_{HA} (46.23 ± 1.91%) and PF (1.12 ± 0.07) at a very high concentration of salt (20%). The concentration of salt required to form the two phases depends on its salting out ability, and thus SC and PC require less concentration while AC requires more concentration (Fig. 1a & b). The salting out effect of citrate salts used in the study can be evaluated by the concept of EEV (effective excluded volume), Gibbs free energy of hydration $\Delta G_{hydration}$, and the ionic radius of the cation [34]. Na^+ being highly kosmotropic has more negative $\Delta G_{hydration}$, and has more EEV and less cationic radius in comparison with potassium and ammonium ions. Thus sodium citrate salt exhibited more salting-out ability (also maximum Y_{HA}, PF, and K_{SHA}) when compared to the other two salts. The concept was elaborated in the previous paper of the author [26]. The specific activity of the lectin was maximum in the top phase for SC, PC, and AC which was evidenced by K_{SHA} of 10.37 ± 0.42, 9.22 ± 0.32 and 8.42 ± 0.40 respectively. The differential partitioning of Psl and other proteins was assessed by K_{SHA} and Kp. The high K_{SHA} with low Kp indicates that the target protein was successfully partitioned to top phase [35] and maximum K_{SHA}, PF, and yield were given by sodium citrate. The native solution pH of the system was around 8.1–8.2 for the ATPS formed by all the three salts, which was favorable for Psl to preferentially partition to the top phase as it has an isoelectric point (pI) of 6.8–7.2 [36]. 'Salting out' property of salts excluded protein from the bottom phase to the top phase by limiting its solubility. The distribution of ions in the system alters with

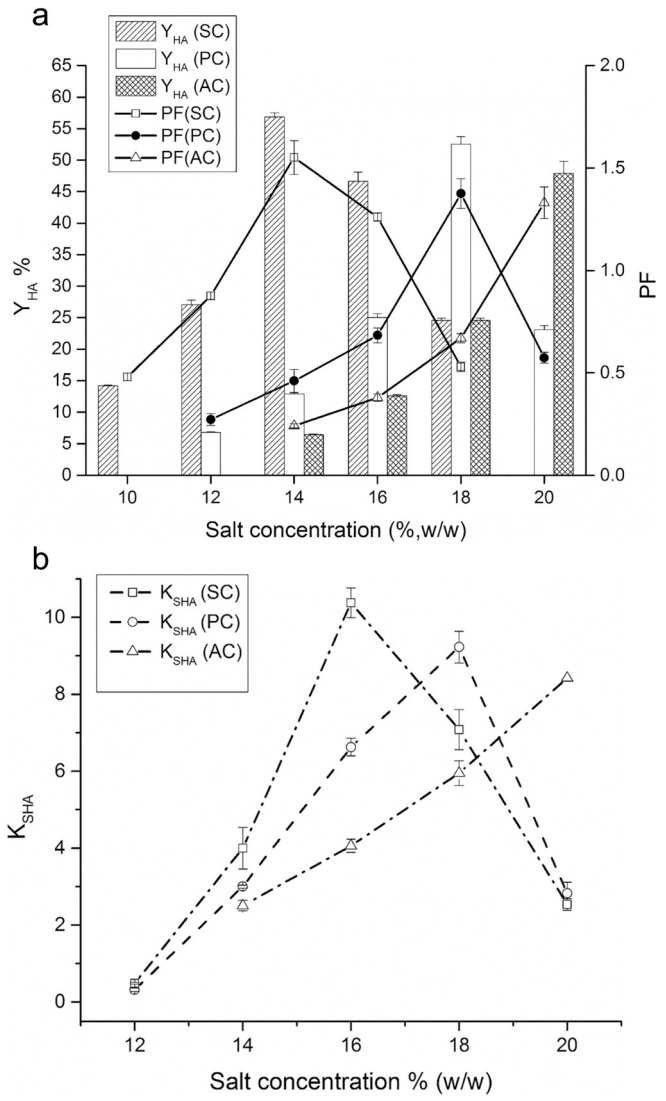

Fig. 1. a: Effect of type of salts at different concentration (SC: sodium citrate; PC: potassium citrate; AC: ammonium citrate) with 20% (w/w) PEG 4000 (g/mol) on hemagglutination activity yield (Y_{HA}) and Purification factor (PF) of Psl.
b: Effect of type of salts at different concentration (SC: sodium citrate; PC: potassium citrate; AC: ammonium citrate) with 20% (w/w) PEG 4000 (g/mol) on hemagglutination specific activity partition coefficient (K_{SHA}) of Psl.

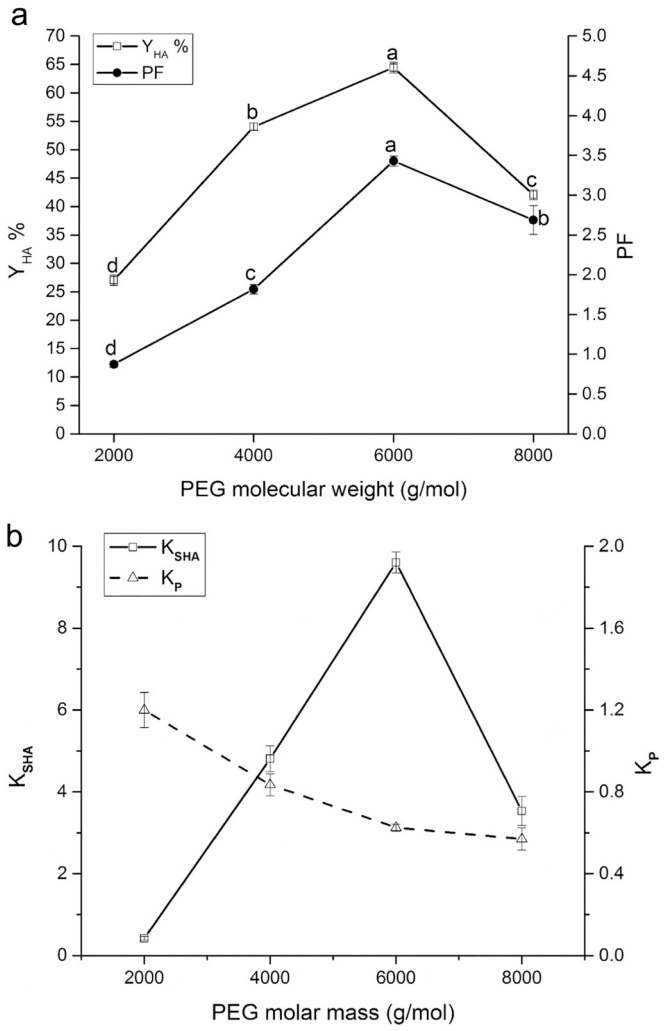

Fig. 2. a: Effect of molar mass of PEG (g/mol) on hemagglutination activity yield % (Y_{HA}) and purification factor (PF) in the ATPS of PEG 20% (w/w) and sodium citrate 16% (w/w).
b: Effect of PEG molar mass on Hemagglutination specific activity partition coefficient (K_{SHA}) and total protein partition coefficient (Kp) in the ATPS of PEG 20% (w/w) and sodium citrate 16% (w/w).

the different type of salts which affects the partition of lectin in the system [37]. Many complex sources such as bovine pancreatic homogenate [38], fermentation broth of *Aspergillus tamarii* URM4634 [39] and *B. cereus* [40] were explored to extract individual biomolecules like trypsin, protease, cyclodextrin glycosyltransferase respectively by employing PEG/sodium citrate combination as a phase forming component. In the current study, sodium citrate salt outperforms than the potassium and ammonium salts with better purification factor and hemagglutination activity recovery. Hence the PEG/sodium citrate ATPS was selected for further studies.

3.2. Selection of molar mass of PEG

Hydrophobicity is one among the main parameter which affects the extent of partitioning of proteins in PEG/Salt systems. The hydrophobicity of the system varies mainly with the molar mass of PEG which affects the PEG-protein interaction. The effect of PEG molar mass on the selective partitioning of Psl from its seed crude extract was studied in the ATPS formed by 20% PEG of different molar mass (2000, 4000, 6000 and 8000) and 16% SC system. As the molar mass of PEG varies, there will be a variation in the count of the ethylene oxide group per PEG, which is the main determinant of hydrophobicity [41]. Multiple fold increase in the specific activity and purification factor were observed for increased PEG molar mass from 2000 to 6000 g/mol (Fig. 2a and b). The significant increase in hydrophobicity with increasing PEG molar mass helped to selectively partition the lectin towards the PEG phase. ATPS aims to achieve not only a good partitioning coefficient for a protein but also enrich the desired protein towards a particular phase by discriminating the contaminant proteins to another phase.

The specific hemagglutination activity partition coefficient (K_{SHA}) increases with increasing molar mass of PEG up to 6000. The specific activity of the crude was 6.44 ± 0.03 U/mg, and the maximum specific activity achieved in the top phase at PEG 6000 was 22.53 ± 0.65 U/mg. The K_{SHA} of 0.42 ± 0.05, 4.80 ± 0.32, 9.60 ± 0.26, 3.53 ± 0.35 obtained in the current study for PEG 2000, 4000, 6000 and 8000, respectively and corresponding decrease in the Kp for PEG 2000 to 8000 proves that the Psl was selectively partitioned towards the top phase with the increase in molar mass. However, Y_{HA}, PF, and K_{SHA}

increased till PEG 6000 and decreased at PEG 8000 due to the unavailability of free water solvent. Maximum specific activity (22.53 ± 0.65 U/mg) was achieved with low total protein content in top phase (0.62 mg/mL), which was the desirable condition for the selective extraction, for PEG 6000-SC ATPS.

The simultaneous increase in enzyme activity with molar mass was also reported by Mohammadi and his coworkers where recombinant phenylalanine dehydrogenase was produced using E. coli and the enzyme was concentrated towards the PEG phase. It was observed that the enzyme partition coefficient and enzyme recovery increased with increase in molar mass from 2000 to 6000 and then gradually decreases above 6000 [42]. The results observed by Ooi et al. was also in agreement with the current trend where the selectivity of Burkholderia pseudomallei lipase increased with increasing PEG molar mass from 3000 to 6000 [43]. The enzyme recombinant neutral protease [44] has also been successfully partitioned to the PEG-rich phase at PEG 6000.

The hydrophobic interaction of Psl and PEG play the main role and thus the specific activity increases with the increase in molar mass. However, the K_{SHA} decreases at PEG 8000 with the loss of protein at the interphase; this behavior may be due to the volume exclusion effect exerted by the PEG 8000. Polymers with high molar mass have a greater difference in density and interfacial tension because of which they tend to induce phase separation with a lower amount of phase components. Accordingly, in the present study, PEG 2000 and 4000 require more concentration for effective phase separation when compared to PEG 6000. There was a poor resolution in the partitioning of lectin and contaminating proteins towards opposite phases in low molar mass PEG such as 2000 which leads to lowest Y_{HA} and PF of 27.02 ± 0.92% and 0.87 ± 0.03 respectively. Hence, PEG 6000 was selected as an optimal molar mass for further studies as it was able to give good selectivity with maximum Y_{HA} and PF of 64.43 ± 0.94% and 3.42 ± 0.06 respectively.

3.3. Effect of PEG concentration

The effect of PEG concentration on selective partitioning of the target protein was studied by varying PEG 6000 concentration from 14% to 22% with a constant SC concentration of 16% (Fig. 3). The partition coefficient for total protein (Kp) was < 1 at all the PEG concentration and the Y_{HA} and PF were maximum in the top phase, which indicates that most of the contaminant proteins accumulated in the bottom phase and the Psl were preferentially concentrated in the top phase. However, Nascimento et al. observed the partitioning of *Cratylia mollis* lectin into the bottom phase with high activity partition

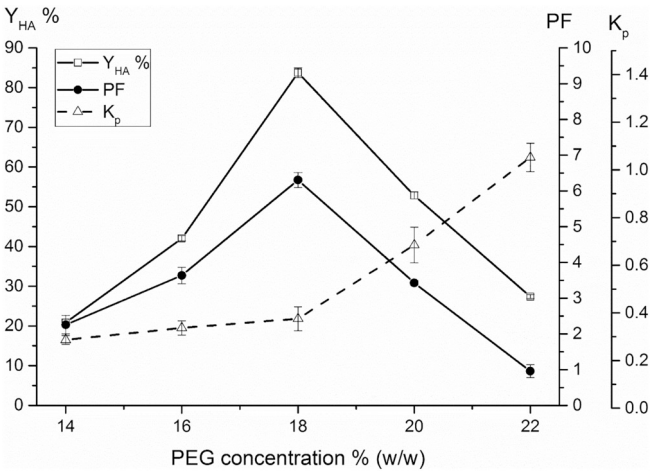

Fig. 3. Effect of PEG 6000 (g/mol) concentration on Hemagglutination activity yield (Y_{HA}), purification factor (PF) and total protein partition coefficient (Kp) at constant sodium citrate concentration of 16% (w/w).

coefficient and low total protein partition coefficient in the PEG/citrate system [23]. Increase in the PEG concentration from 14% to 18% showed a significant effect ($p < 0.05$) as it leads to a maximum Y_{HA} and PF of 83.75 ± 1.25% and 6.3 ± 0.20 respectively. The total protein content increased linearly in the top phase with the PEG concentration. However, maximum PF was achieved at 18% and gradually decreased there onwards (Fig. 3). The behavior observed may be due to the migration of contaminant proteins along with the Psl. This indicates that there was no efficient separation of the lectin from other proteins at higher concentrations of PEG, which was confirmed by the increasing profile of Kp (Kp > 1). It was reported in the literature that all legume lectins are characterized by a hydrophobic cavity other than the carbohydrate binding domain [45]. 18% PEG at the molar mass of 6000 creates a favorable hydrophobic environment in the top phase which facilitates the interaction of PEG with the hydrophobic cavity of Psl. This hydrophobic interaction of PEG - Psl was mainly responsible for the enhanced migration of Psl towards the PEG phase. However, the increased hydrophobicity due to the increase of PEG concentration over and above 18% attracts the other proteins and the selectivity of the separation was lost. A similar trend was observed by Bim and Franco [46], where alkaline xylanase extracted in PEG 6000/dipotassium phosphate system from *Bacillus pumilus* showed increased activity partition coefficient (of 46.9) with decreased Kp (0.1) when the PEG concentration is increased from 16 to 22% and decreases thereafter.

The decrease in purification factor and Y_{HA} above 18% PEG 6000 was mainly due to the attraction of other contaminant proteins towards the top phase as evidenced by an increased total protein partition coefficient. At very high concentration (22%), the volume exclusion effect of PEG molecule pushes the Psl to the bottom phase and leads to low Y_{HA} and PF. A decrease in the bromelin partition coefficient was reported with increasing PEG concentration due to volume exclusion effect of PEG [47]. The high extraction rate with better purification factor was achieved at 18% PEG and this concentration of PEG was used as an optimal concentration for further studies.

3.4. Effect of sodium citrate concentration

The influence of SC concentration on the partitioning of Psl from its crude extract was studied in the ATPS formed with 18% PEG 6000 at different concentration of SC. The solubility of the protein diminishes in the salt phase with an increase in the salt concentration thereby expelling the proteins to the top phase, which was observed by the increasing Kp. However, a reasonable degree of separation between Psl and other proteins was observed with a gradual increase in activity recovery and PF, leading to higher purification factor till 16% of SC due to the selectivity of the phases (Fig. 4). This was further confirmed by Kp < 1 and K_{SHA} > 1. Thus 16% (w/w) of sodium citrate had a significant effect ($p < 0.05$) on Y_{HA}, PF, and K_{SHA}.

The movement of protein towards the top phase was mainly due to the interaction of the salt ions with the hydration layer of the protein which forms a double electric layer; the layer makes protein to dehydrate with the exposure of hydrophobic sites and allows its interaction with other hydrophobic molecules [48]. Increased sodium citrate concentration in the bottom phase strengthens the electrostatic repulsion between the anion and the negatively charged Psl which facilitates the movement of Psl to the PEG phase [49]. A similar pattern was observed in the extraction of luciferase from fireflies where the enzyme activity coefficient increased several times more than the total protein concentration with increase in salt concentration from 12 to 16% [50]. The trend of increasing activity and PF with increasing salt concentration was also observed in aqueous two-phase extraction of bromelain from *Ananas comosus* pulp [51]. It was observed in the present study that an increase in salt concentration above 16% increases the partitioning of undesired protein towards top phase leading to a lesser degree of separation between contaminating proteins and the target protein where Kp > 1 and PF reduced from 6.3 ± 0.2 to 2.68 ± 0.18. High lectin

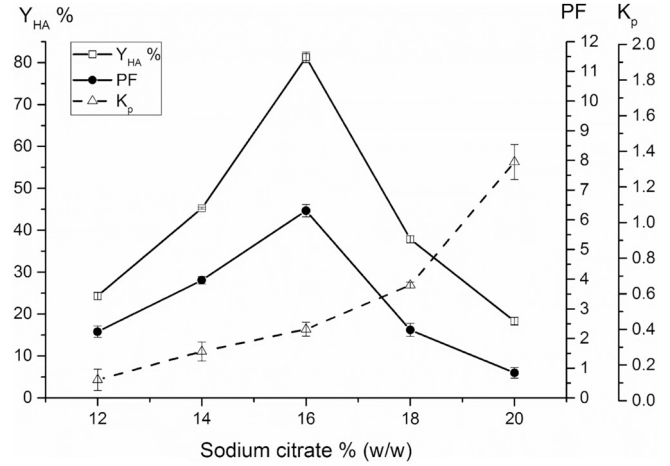

Fig. 4. Effect of sodium citrate salt on Y_{HA} (Hemagglutination activity yield), PF (purification factor) and Kp (total protein partition coefficient) at constant PEG 6000 concentration of 18% (w/w).

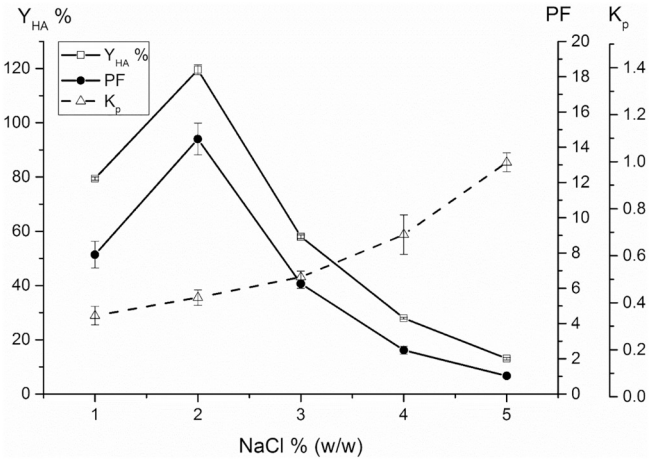

Fig. 5. Effect of NaCl as an additive on Y_{HA} (Hemagglutination activity yield), PF (purification factor) and Kp (total protein partition coefficient) in the ATPS system made-up of 18% PEG 6000, 16% sodium citrate at TLL of 41.01%.

activity with less Kp was achieved at 16% SC which was used as an optimum salt concentration for further studies.

3.5. Effect of tie line length

Experiments were conducted at different TLL (30 to 50%) to study the net effect of the equilibrium concentration of phase forming components on selective extraction of Psl in PEG 6000 – sodium citrate system. The equilibrium phase compositions at six different tie line and the corresponding extraction performance parameters like Y_{HA}, PF, K_{SHA}, and Kp are presented in Table 1. Manipulating the TLL is one among the way to alter the magnitude of phase hydrophobicity.

The favorable magnitude of hydrophobic force exerted by PEG and salting out ability exerted by sodium citrate for the selective partitioning was achieved at the TLL of 41.01%. It was noticed that both Kp and K_{SHA} were gradually increasing till the TLL of 41.01%. The equilibrium composition of 34.1% PEG in the top phase and 28.5% of SC in the bottom phase gave maximum K_{SHA} of 19.96 at low Kp of 0.40 mg/mL. The specific activity was raised to 41 ± 1.10 U/mg when compared to crude extract (6.44 ± 0.03 U/mg) at this TLL. The balanced environment at TLL of 41.01% facilitates an enhanced interaction of PEG–lectin and leads to a maximum Y_{HA} and PF. At the lower TLL (33%), the lesser equilibrium concentration of phase forming components (PFC) in either of the phases (27.12% of PEG in the top phase and 24.23% of SC in the bottom phase) were not enough to facilitate the movement of more Psl towards top phase, which was witnessed with low Kp of 0.25 ± 0.02 mg/mL along with low Y_{HA} and PF. There was a decrease in both the extraction parameters (Y_{HA} and PF) as the TLL exceeded 40.01%, where Kp increased and K_{SHA} decreased. Higher equilibrium composition of 40.03% PEG in top phase and 32.86% of SC in bottom phase limits the Psl solubility in both the phases and slight precipitation was observed at the interface at extreme TLL of 48.59%. Further, increased concentration of salt in the bottom phase increases the concentration of Psl in the top phase due to the volume reduction of top phase and more of the contaminant proteins partitioned into the comparably larger volume of bottom phase. TLL of 41.01% was considered as optimum as it gave better performance in terms of Y_{HA}, PF, and K_{SHA}. A similar phenomenon was observed by Bolar and his coworkers [52], where enzyme partition coefficient and activity yield of glutaminase increases with increasing TLL, but reduced at very high and low TLL.

3.6. Effect of NaCl

The addition of neutral salts as an additive into the ATPS influences the preferential responses of individual proteins present in the extract. The effect of NaCl concentration on the partitioning characteristic of Psl was studied by adding 1 to 5% NaCl in the ATPS consisting of 18% PEG 6000/16% sodium citrate at 41.01% TLL (Fig. 5). It was observed that the addition of NaCl enhances the partition of Psl towards the PEG-rich phase. The prominent effect on Y_{HA} and PF was observed at 1 to 2% of NaCl addition, where both the factors increased rapidly from 79.41 ± 0.84% to 119.5 ± 1.8% and 7.4 ± 0.7 to 14.5 ± 0.8, respectively due to the increased relative hydrophobicity and electrostatic property differences between the phases [53]. The same trend was observed by Ng et al., where the addition of NaCl at 4% to PEG/sodium citrate ATPS increased the CGTase (Cyclodextrin Glycosyltransferase) partition towards PEG rich phase and exhibited highest purification factor of 16.3 at 4% NaCl [40]. Similarly, the increasing lipase activity partition coefficient was observed with the addition of 1% NaCl in the

Table 1
Equilibrium and extraction characteristics of the ATPS PEG 6000 (18%)-sodium citrate (16%).

Feed (%, w/w)		Top phase (%, w/w)		Bottom phase (%, w/w)		TLL %	Y_{HA}	PF	Kp	K_{SHA}
PEG	TSC	PEG	TSC	PEG	TSC					
18	12	27.12	1.20	5.20	24.23	33.27	20.8 ± 0.16	2.26 ± 0.26	0.25 ± 0.03	4.00 ± 0.54
16	16	30.32	1.50	5.14	27.50	36.47	42.04 ± 0.19	3.63 ± 0.11	0.32 ± 0.01	12.29 ± 0.47
18	16	34.10	1.31	3.86	28.50	41.01	81.25 ± 1.25	6.30 ± 0.20	0.40 ± 0.02	19.96 ± 1.00
20	16	37.12	1.18	3.86	30.32	44.62	64.44 ± 0.60	3.43 ± 0.06	0.62 ± 0.02	9.60 ± 0.25
22	16	39.07	1.28	4.12	30.95	46.34	27.39 ± 0.48	0.95 ± 0.05	1.16 ± 0.06	1.74 ± 0.22
18	20	40.03	0.80	4.18	32.86	48.59	18.31 ± 0.16	0.84 ± 0.02	1.34 ± 0.05	0.74 ± 0.05

Note: PEG: polyethylene glycol, TSC: tri-sodium citrate, Y_{HA}: hemagglutination activity yield, PF: purification factor, K_{SHA}: specific hemagglutination activity partition coefficient, Kp: total protein partition coefficient.

PEG/potassium phosphate system [42].

The storage proteins present along with the Psl can bind to Psl through ionic interactions, however, they may be unbound at higher ionic strength [54]. Hence, the sweeping increase in Y_{HA} and PF was observed initially with increasing NaCl concentration. The Kp was found to increase with increasing concentration of NaCl which was prominent beyond the NaCl concentration of 2%. The selectivity for the Psl partitioning was lost at the higher NaCl concentration since other proteins are also attracted towards the top phase which was confirmed by Kp > 1. The addition of more NaCl increases the ionic strength of the phases and attracts low molecular weight proteins and small hydrophobic molecules to the PEG phase which in turn reduces the activity and purification of a specific protein [55]. Moreover, the hemagglutination activity was found to reduce drastically at higher NaCl concentration even with the increased Kp due to the dissociation of Psl subunits. High concentration of NaCl above 3% leads to precipitation of Psl along with the other proteins (visually observed). The significant effect ($p < 0.05$) of NaCl which leads to maximum Y_{HA} and PF with lower Kp was observed at 2% NaCl and the subsequent experiments were conducted with the addition of 2% NaCl.

3.7. Effect of pH

The pH of the system is responsible to preserve the native structure of a protein which in turn enhances the activity. The impact of pH on Y_{HA}, PF, and Kp in a system composed of 18% PEG 6000, 16% sodium citrate at 41.01% TLL with 2% NaCl was analyzed in the pH range of 6 to 9 (Fig. 6). The biomolecules acquire specific charge on their surface based on the isoelectric point (pI) and ionic composition of the system at different pH. In general, protein acquires a negative charge above its pI and positive charge below its pI [56]. The pI of Psl is around 6.8–7.2 and hence the Psl acquired a negative charge in the PEG/sodium citrate system, whose native pH was 8.1–8.2. The specific hemagglutination activity was found to increase until pH 7.5 and Y_{HA} reached a maximum of 121.33%; accordingly, the PF also found to increase which proves the significant effect ($p < 0.05$) of pH on studied responses (Fig. 6). The dimer form of lectin is essential to exhibit hemagglutination activity and Psl maintains its dimer form in a slightly basic pH of around 7–8. Very acidic or basic pH leads to dissociation of the alpha and beta subunits which in turn leads to the loss of hemagglutination activity [11]. Further, the major contaminant proteins in Pea seeds extract are legumin and vicilin and they associate to form hexamer and trimer [57] at slightly basic pH and thus transfer to bottom phase due to volume exclusion effect. Hence, higher purity was achieved at a pH of 7.5. However, the total protein content in the top phase gradually increased as the system pH increased. The increase in pH from 7.5 to 8 enhanced the Kp from 0.26 ± 0.006 to 0.38 ± 0.01 mg/mL besides, the gradual decrease of PF (Fig. 6). This behavior may be due to the migration of other contaminant proteins which have a similar isoelectric point as Psl. The pH influences not only the Psl's surface property but also the surface properties of other contaminating proteins in the extract which might attract them to the top phase at higher pH. When pH is reduced to 7.5 from 8 and above, these contaminants can be discriminated and maximum PF and Y_{HA} can be achieved. It's always good to maintain pH slightly more than pI rather than increasing too much. The salting out of proteins due to the dominance of citrate ions with an increase in pH also helps to partition all the proteins to the top phase and resulted in higher Kp and lower PF. These behaviors depict the distinct influence of pH on the solute partition due to electrostatic interactions. The system pH was maintained at 7.5 for the efficient partitioning where Psl partitioned to the top phase with maximum Y_{HA} of 121.33 ± 1.52% and PF of 16.10 ± 0.61 with a minimum total protein partition coefficient of 0.37 ± 0.02 (Fig. 6).

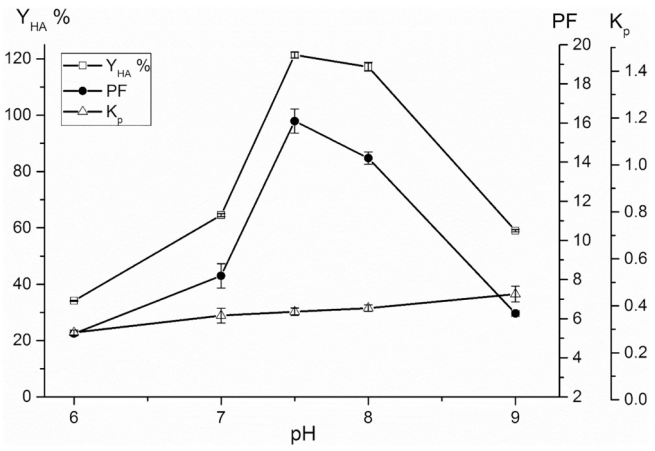

Fig. 6. Effect of pH on the on Y_{HA} (Hemagglutination activity yield), PF (purification factor) and Kp (total protein partition coefficient) in the ATPS system made-up of 18% PEG 6000 and 16% sodium citrate and 2% NaCl at 41.01% TLL.

3.8. Effect of crude load

The effect of crude load on 18% PEG 6000 and 16% sodium citrate at 2% NaCl was studied by varying crude load from 10 to 50% at the pH of 7.5 (Fig. 7). The maximum uptake capacity of the Psl in the top phase and the other impurities in the bottom phase of the ATPS was realized by increasing the crude concentration in the system. As the crude concentration varies, the partition behavior of the desired protein and the physical properties of the phases also altered. It was observed from Fig. 7 that the Y_{HA} and PF increase until the addition of 20% (volume) crude. The accumulation of precipitate at the interphase was observed as the crude load increased above 20% due to the saturation of both the phases, which lead to a loss of Psl along with the non-target proteins. The salting out effect of the bottom phase and the excluded volume effect of the top phase are the major reasons for the rejection of proteins from the respective phases. Further, the results indicate that the higher concentration of impurities associated with the crude had a direct effect on the partitioning of the Psl and the ATPS may provide higher Y_{HA} with higher PF if the major impurities are removed from the crude before subjecting to the ATPS partitioning. Crude load of 20% was used in further experiments as it had a significant effect on ($p < 0.05$) Y_{HA} and PF.

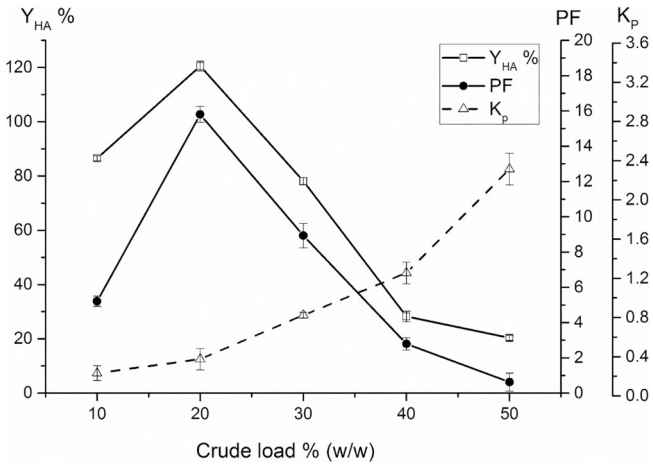

Fig. 7. Effect of crude load on hemagglutination activity yield (Y_{HA}), purification factor (PF) and total protein partition coefficient (Kp) in the ATPS of 18% PEG 6000 and 16% sodium citrate and 2% NaCl at pH of 7.5 and at 41.01% TLL.

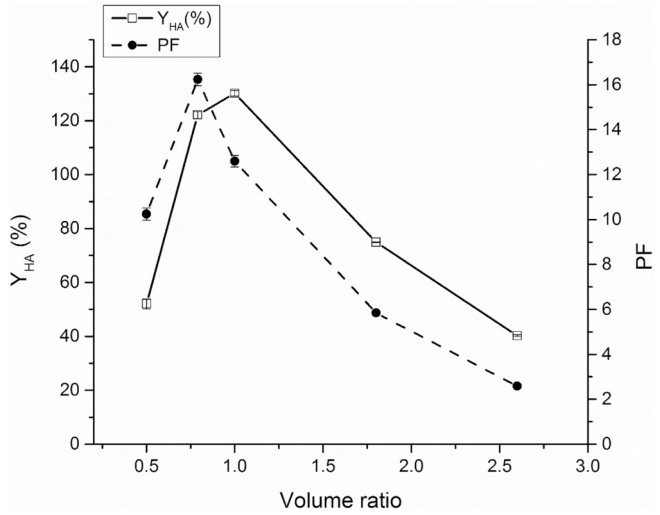

Fig. 8. Effect of volume ratio on hemagglutination activity yield (Y_{HA}) and purification factor (PF) in the ATPS composed of 20% crude load, 18% PEG 6000 and 16% sodium citrate and 2% NaCl at pH of 7.5 and at 41.01% TLL.

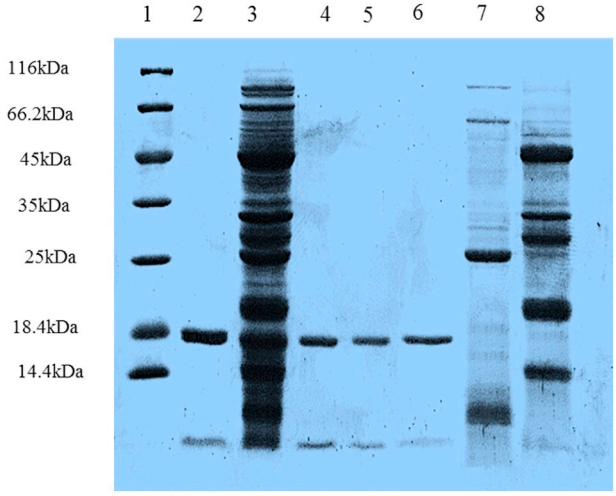

Fig. 9. SDS–PAGE analysis of different samples. Lane 1: molecular weight marker (116KD-14.4KD), Lane 2: pure Psl, Lane 3: crude extract of *P. sativum* seeds, Lanes 4, 5, 6: Extracted Psl to top phase, Lane 7: Interface, Lane 8: Bottom phase of ATPS.

3.9. Effect of volume ratio (Vr)

The volume ratio of the phases (top by bottom phase volume) along a specific tie line provides the constant equilibrium characteristics of the system. The maximum possible solute recovery with higher concentration may be achieved by varying the phase volume ratio of the system. Five different volume ratios were considered along the same tie line to study the influence of volume ratio on the partitioning of Psl with 20% crude load. The recovery and PF were observed to increase initially with increasing volume ratio from 0.4 to 0.76, however, most of the unwanted proteins were accommodated at the increased volume ratio (Kp > 1). The highest PF of 16.10 ± 0.63 with a recovery of 122.12 ± 1.36% was achieved at the volume ratio of 0.76 (Fig. 8). The obtained result indicated that the top phase was able to attract the maximum concentration of Psl at the volume ratio of 0.76. Higher volume ratio decreases the degree of purification as it promotes the partitioning of contaminants into the top phase, due to the solubility of impurities in the available free solvent of top phase and the rejection of proteins from the salt phase through salting out effect at the reduced salt phase volume. The decrease in volume ratio below 0.76 resulted in the lower recovery and PF with precipitation at the interface due to the solubility limit of the phases. The reduced top phase volume with maximum Psl concentration obtained at lower phase volume ratio also help to handle the subsequent purification process with reduced volume. By considering the maximum PF, the volume ratio of 0.76 was selected as optimum volume ratio even though the volume ratio of 1 gave high activity yield of 130.17 ± 1.95% (Fig. 8).

3.10. Regression analysis

The significance of all the independent variables considered in the present work were analyzed statistically by performing the non-linear regression analysis. The fitted non-linear models along with the coefficient of determination (R^2) and *p*-values for the coefficients were determined and presented in Table 2. The R^2 represents the fraction of the overall variance of the 'dependent' variable explained by the 'independent' variable. The *p*-values explain the significance of

Table 2
The fitted models and statistical parameters (the coefficient of determination (R^2) and *p*-value) of regression analysis for the quantitative variables considered.

Independent variable	Dependent variables	R^2 value	*p* value	Fitted models
PEG concentration (%, w/w)	Y_{HA} (%)	0.9834	0.0245	$Y = -2.9x^2 + 107.88x - 912.67$
	PF	0.9759	0.0154	$Y = 0.03x^2 - 1.02x + 8.30$
	Kp	0.9848	0.0165	$Y = -0.24x^2 + 8.37x - 68.90$
TSC concentration (%, w/w)	Y_{HA} (%)	0.9682	0.0206	$Y = -2.86x^2 + 90.67x - 653.32$
	PF	0.9879	0.0124	$Y = -0.23x^2 + 7.04x - 49.59$
	Kp	0.9923	0.0156	$Y = 0.02x^2 - 0.49x + 3.96$
Tie line length (%, w/w)	Y_{HA} (%)	0.9952	0.0124	$Y = -0.97x^2 + 79.14x - 1544$
	PF	0.9654	0.0267	$Y = -0.07x^2 + 5.39x - 102.66$
	Kp	0.9754	0.0145	$Y = 0.007x^2 - 0.51x + 9.43$
NaCl (%, w/w)	Y_{HA} (%)	0.9980	0.0147	$Y = -16.59x^2 + 79.70x + 21.47$
	PF	0.9979	0.0256	$Y = -1.96x^2 + 9.47x + 1.48$
	Kp	0.9997	0.0170	$Y = 0.039x^2 - 0.08x + 0.39$
pH	Y_{HA} (%)	0.9516	0.0253	$Y = -37.49x^2 + 560.21x - 1986.43$
	PF	0.9755	0.0145	$Y = -4.62x^2 + 68.45x - 239.94$
	Kp	0.9981	0.0236	$Y = 0.07x^2 - 0.86x + 2.95$
Crude load (%, w/w)	Y_{HA} (%)	0.9856	0.0145	$Y = -0.04x^2 + 0.018x + 104.10$
	PF	0.9534	0.0235	$Y = -0.024x^2 + 1.33x - 4.079$
	Kp	0.9653	0.0147	$Y = 0.001x^2 - 0.03x + 0.39$
Volume ratio	Y_{HA} (%)	0.9854	0.0186	$Y = -51.52x^2 + 137.19x + 22.76$
	PF	0.9755	0.0268	$Y = -1.90x^2 + 0.55x + 13.28$
	Kp	0.9786	0.0154	$Y = 0.31x^2 - 0.40x + 0.44$

Note: PEG: polyethylene glycol, TSC: tri-sodium citrate, Y_{HA}: hemagglutination activity yield, PF: purification factor, Kp: total protein partition coefficient.

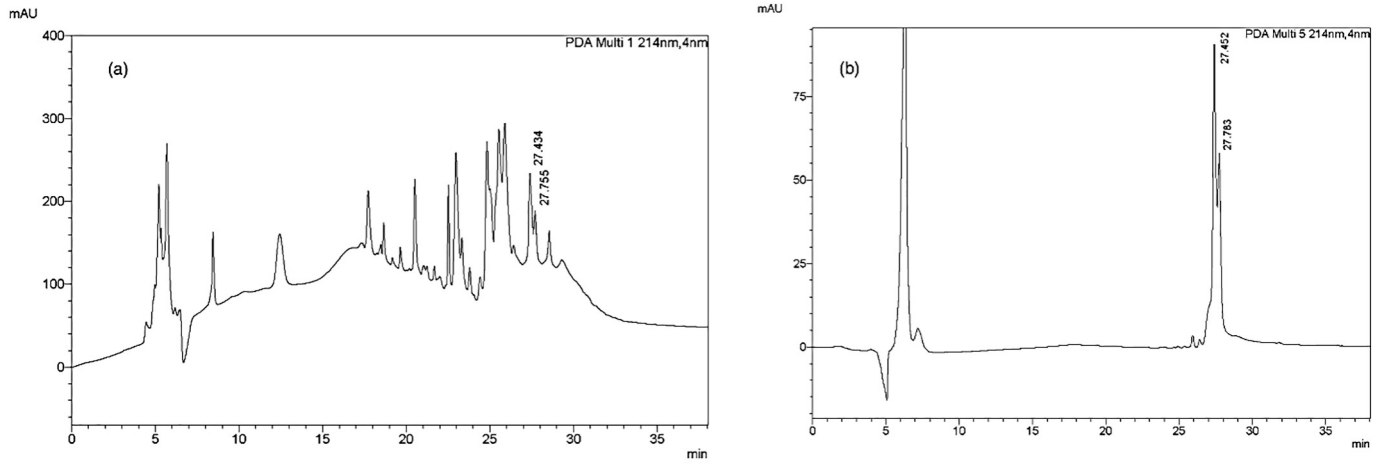

Fig. 10. Elution pattern of proteins in seed crude extract of *Pisum sativum* (panel a) and elution pattern of top phase extracted Psl (panel b) at the flow rate of 0.5 min/mL in binary gradient mobile phase (acetonitrile and tri-fluoroacetic acid) in column: RP-C18.

relationships developed between the dependent and independent variables. If the *p*-value for a variable is less than the significance level (0.05), the considered data provide enough evidence to reject the null hypothesis. The effect of an independent variable and their range of variation on the responses was found to be significant as the R^2 of all the variables are > 0.95 with the *p*-value of less than 0.05 ($p < 0.05$) (Table 2).

4. Purity analysis of extracted Psl

4.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

The purity of the partitioned pea lectin in PEG/sodium citrate ATPS was analyzed by SDS PAGE and shown in Fig. 9. Lane 1 represents the molecular markers used such as 116, 66.2, 45, 35, 25, 18.4 and 14.4 kDa. Lane 2 represents the standard Psl separated into two bands in the presence of β-mercaptoethanol. The two bands of 6 and 18 kDa correspond to smaller α and larger β chains respectively. Hence, the bands seen in the SDS–PAGE were most likely representing the subunits of a dimeric lectin. Lane 3 was loaded with the crude sample which shows the dense protein bands along with the Psl. Lanes 3, 4, 5 were loaded with top phase sample of optimized ATPS, where all of the three lanes represent similarity in electrophoretic pattern and molecular weight with standard Psl. The other contaminant proteins were concentrated in the bottom (lane 8) and interphase (lane 7). The obtained image clearly indicated that the presence of Psl with smaller α and larger β chains (lanes 4, 5 and 6) as similar to that of pure standard (lane 2). Further, it is evident that most of the contaminants are either accumulated in the bottom phase (lane 8) or interphase (Lane 7). The bands of Psl corresponding to 6 and 18 kDa were not visible in the Lane 7 and 8 confirm the selective partitioning of Psl in the top phase.

4.2. High performance liquid chromatography

High performance liquid chromatography (HPLC) was used to analyze the purity of protein qualitatively. Fig. 10a represents the chromatogram of a crude extract of Psl. The two isoforms of the Psl; larger β and smaller α were separated at a retention time of 27.434 and 27.755 min respectively along with the other protein peaks. Fig. 10b represents the chromatogram of partially purified top phase extracted Psl. The extracted Psl also shows the peaks of two isoforms (27.452 and 27.783 min), similar to crude Psl chromatogram without any peaks corresponding to contaminant protein. This proves that the ATPE was successful in the selective partition of Psl from other contaminant proteins with maintaining the stability of a protein. The HPLC chromatogram of standard Psl was available elsewhere [26].

5. Conclusion

The current research work proves the efficacy of PEG/sodium citrate ATPS for the successful selective partitioning of Psl directly from its seed crude extract without any pretreatment. It was noticed that the salt-dependent hydrophobic interaction played a significant role in the selective partition of Psl to PEG-rich phase by leaving other contaminants in the bottom phase and interphase. The additive NaCl and pH had a prominent effect on the selectivity of the protein and extraction parameters. Maximum hemagglutination activity yield 122.12% and purification factor 16.26 was achieved at 18% PEG 6000/16% sodium citrate representing the TLL of 41.01%, 2% NaCl and pH 7.5. Maximum Psl gets concentrated in the top phase at 20% loading of the crude in the system with the volume ratio of 0.76. The qualitative purity analysis by SDS-PAGE and HPLC confirms that the Psl has been selectively partitioned to top phase. The ATPE of Psl from the crude extract may be implemented at industrial scale by considering the appreciable performance of the PEG/sodium-citrate based ATPS at the optimized condition with the results obtained from the extended continuous pilot scale studies.

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Conflict of interest

All the authors express that there is no conflict of interest on the manuscript submitted.

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