Biotecnología

Revista Mexicana de Ingeniería Química

Vol. 14, No. 2 (2015) 383-392

PRODUCTION AND OPTIMIZATION OF A CHLOROPHYL-FREE LEAF PROTEIN CONCENTRATE FROM ALFALFA (*Medicago sativa*) THROUGH AQUEOUS TWO-PHASE SYSTEM

PRODUCCIÓN Y OPTIMIZACIÓN DE UN CONCENTRADO DE PROTEÍNA DE ALFALFA (*Medicago sativa*) LIBRE DE CLOROFILA MEDIANTE SISTEMAS DE DOS FASES ACUOSAS

P. Vázquez-Villegas, E. Acuña-González, L.A. Mejía-Manzano, M. Rito-Palomares y O. Aguilar*

Centro de Biotecnología-FEMSA, Tecnológico de Monterrey, Campus Monterrey, Ave. Eugenio Garza Sada 2501 Sur, Monterrey, NL 64849, México

Received October 23, 2014; Accepted April 8, 2015

Abstract

There is a growing interest in producing protein from green crops, being alfalfa (*Medicago sativa*) one of the more studied models. In this work we develop an aqueous two-phase systems (ATPS) process to obtain a chlorophyl-free leaf protein concentrate. A two-level factorial experimental design in order to study five factors that interferes with recovery of total protein in ATPS is performed, employing two model proteins (BSA and Lyz). The factors of polymer concentration and total protein concentration were statistically significative. From here, we applied steepest descent and central composite design (CCD) methods in order to optimize chlorophyl-free, total protein recovery from alfalfa (*Medicago sativa*) green tissue. An extraction of more than 80% of total protein was reached for bottom phase LPC and 51% of protein in powder from the lyophilized LPC.

Keywords: alfalfa, aqueous two-phase system (ATPS), central composite design, leaf protein concentrate.

Resumen

Existe un interés creciente en producir proteína a partir de tejido vegetal, siendo alfalfa (*Medicago sativa*) uno de los modelos más estudiados. En el presente trabajo, se desarrolla un proceso con sistemas de dos fases acuosas (ATPS, de sus siglas en inglés) para obtener un concentrado de proteína libre de clorofila, a partir de tejido verde. Se llevó a cabo un diseño experimental factorial de dos niveles para estudiar los factores que interfieren con la recuperación de proteína total, empleando dos proteínas modelo (albumina de suero bovino y lisozima). Los factores de concentración de polímero y concentración total de proteina fueron estadísticamente significativos. A partir de dichos resultados, se aplicó el método estadístico de búsqueda de descenso por gradiente (*steepest descent*) y un diseño central compuesto para optimizar la recuperación de proteína total libre de clorofila a partir de tejido verde de alfalfa (*Medicago sativa*). Un concentrado con más de 80% y 51% de proteína se obtuvo en fase inferior líquida y liofilizada, respectivamente.

Palabras clave: alfalfa, sistema de dos fases aquosas (ATPS), diseño central compuesto, concentrado de proteína.

1 Introduction

The use of green leafy crops has been identified as an economically attractive alternative for bulk, recombinant or functional protein production (Fiorentini and Galoppini, 1983; Ordiales *et al*., 2012; Schillberg *et al*., 2013; Melnik and Stroger, 2013). They offer numerous advantages including: the lack of animal pathogenic contaminants, low cost and flexibility in large-scale production and existing technology for harvesting and processing of plant material (Brodzik and Steplewski, 2009; Sharma and Sharma, 2009; Karg and Kallio, 2009). Specifically in the case of alfalfa, it is well known that *Medicago*

*Corresponding author. *E-mail*: : alex.aguilar@itesm.mx
Tel. 83-58-20-00, ext, 4821

sativa has high nutrients levels, high digestibility, and a unique proportion of structural to non-structural components (Yari *et al*., 2012). Soluble leaf protein concentrates (LPC) have better emulsifying stabilities than soy protein concentrate and an amino acid balance comparable to casein (Wang and Kinsella, 1976; Kerfai *et al*., 2011; Gachovska *et al*., 2013).

One of the main issues during green tissue proteins extraction is that chlorophyll can act as interference compound in many of the spectrophotometric and immunoaffinity techniques and for lipid and other pigments quantifications (Berges *et al*., 1993; Hua *et al*., 2012; Archanaa *et al*., 2012; Chen *et al*., 2012). Considerable losses of proteins have been observed during acetone washing of LPC for chlorophyll removal (Eze and Dumbroff, 1982). When producing whole LPC destined to human consumption, colour and taste play an important role, given so that green LPC are destined only for animal feed (Fiorentini and Galoppini, 1983; Kohler and Bickoff, 1975). Thus, new extraction techniques have to be developed to recover intact colourless protein fractions or chlorophyl-free LPC.

Some recent publications are related with the extraction of total protein from green tissues using ATPS (Aguilar *et al*., 2009; Ibarra-Herrera *et al*., 2011; Gu, 2014). When employing aqueous two-phase systems (ATPS), multiple aspects are involved in the partitioning behaviour of biomolecules. Typical factors that need to be optimized are: selection of the type of system components (polymers, phase-forming salts, ionic liquids, surfactants), molecular weight of the polymers, pH, ionic strength of the system (addition of neutral salts, such as NaCl), total composition, phase composition (tie line length - TLL), sample load, and phase volume ratio (Bernardo *et al*., 2014). Through years, an outstanding effort has been done in order to elucidate the partitioning mechanisms and how are they interacting with each other in order to favour product partitioning into a particular phase; mostly for polymer-polymer systems (González-Valdez *et al*., 2014; Wu *et al*., 1998; Haghtalab *et al*., 2003; Andrews *et al*., 2005; Perez *et al*., 2013; Ferreira *et al*., 2013).

Different strategies for rapid screening with a reduced amount of sample material have been proposed (Diederich *et al*., 2013; Benavides and Rito-Palomares, 2008). In this study, we develop a model to optimize the partitioning of total protein from crude extract of alfalfa (*Medicago sativa*), dealing with the partitioning of pigments, in order to obtain chlorophyll-free LPC from green tissue. Two different model proteins together with polymer molecular weight, phase composition, and protein load were considered as experimental variables, in order to obtain the maximum amount of alfalfa protein in the bottom phase. Only the significant factors were analysed to evaluate its effect in total protein partitioning. A central composite design (CCD) was employed to assess the interactive effects of such variables. With this approach, a complete downstream recovery strategy of total proteins from leafy green tissues using ATPS could be developed.

2 Materials and methods

2.1 Materials

Alfalfa plants (*Medicago sativa*) were harvested from a local producer (San Buenaventura Coahuila, Mex.) and frozen to -4ºC for further use. Polyethylene glycol (PEG) of two molecular weights (1000 Da and 8000 Da) and proteins bovine serum albumin (BSA, 66 kDa) and lysozyme (Lyz, 14.4 kDa) were purchased from Sigma Aldrich (St Louis, MO, USA). Sodium Chloride (NaCl), potassium and sodium phosphates (monobasic and dibasic) were purchased from Desarrollo de Especialidades Químicas (Monterrey, México). All dilutions were prepared with bi-distilled water.

2.2 Crude extract preparation

Aerial parts of alfalfa were harvested before flowering (leaves and first two stems) and immediately frozen at -20ºC for processing. 30 g of alfalfa were mixed with 300 ml of TBE buffer (0.45M Tris-HCl, 0.45 M H_3BO_3, 10 mM EDTA pH 8.0) (Aguilar and Rito-Palomares, 2014). The mixture was homogenized for 10 min and stirred in a tube rotator (VWR, Radnor, PA, USA) for 1 hr. After this, the slurry was centrifuged at 3100 x g for 20 min at 20ºC (IEC CL40R, Thermo Scientific). The supernatant was recovered and stored at 4ºC for further use.

2.3 Aqueous two-phase systems (ATPS)

Based upon previous experiences (Aguilar *et al*., 2009; Ibarra-Herrera *et al*., 2011), ATPS were initially formulated. Predetermined quantities of stock solutions of potassium phosphates and PEG of nominal molecular weights 1000 and 8000 Da were mixed with a dilution of 5 mg/mL of model proteins or alfalfa extract (adjusting the water content) to give

the desired PEG/salt composition (Table 1). Then, 5.0 or 2.0 g systems were formulated with 6 to 50% (w/w) of the total weight of model protein or alfalfa protein crude extract, respectively. The ATPS were immediately mixed with vortex. Phase separation was achieved by gravity and samples were carefully extracted from the phases (top and bottom phase) and analyzed. Visual estimates of the volumes of top and bottom phases were made in graduated tubes and used to estimate the experimental volume ratio (V_R= volume of the top phase/volume of the bottom phase). The top and bottom phase recoveries were estimated as the amount of protein present in the phase (volume of the phase x product concentration in the phase) and expressed relative to the original amount loaded into the system. All systems were adjusted to pH 7.0.

2.4 *Design of experiments*

An initial complete factorial design 2^5 blocked into 6 different days was carried out in order to obtain the significative factors and its interactions affecting the partitioning of proteins in ATPS. Using two model proteins of different molecular weight (Table 1), the effects of PEG and protein molecular weights, phase component concentration and protein load (in mg/g of total system) were studied. From here, a steepest ascent analysis was done in order to get the area were the partition coefficient of alfalfa protein had the lowest value and total recovery was the higher. Finally, a central composite face-centred design (CCD) was performed, along with confirmative trails in order to obtain the best response through a prediction of quadratic design. The Design-Expert Ver. 9 (Stat-Ease Inc., Minneapolis, USA) supported all designs and analysis (2k factorial design, surface response analysis, ANOVA, T-test and F-test). All experiments were carried out in triplicates and the average of protein concentration was used as response.

2.5 *Polishing of protein extract in bottom phase*

The selected system was scaled in order to have a 580 mL system. In order to remove phase components, bottom phase with a final volume of 390 ml was first passed through a 30 kDa cellulose membrane (10 psi; GE Healthcare, DF, Mexico), pumped at 20 ml/min (Watson Marlow, Wilmington, USA). Afterwards, 150 ml of retained volume were passed four times at 15 mL/min through a 1 kDa cellulose membrane (15 psi), adding 200 mL of water, every time. The retained volume (150 ml) was collected in four 50 mL tubes and lyophilized (Labconco, MO, USA) at -50ºC.

2.6 *Analytical methods*

Absorbance of samples was read in a 96-well plate reader (Biotek EPOCH; Tarrytown, NY, USA). Total protein content was analyzed by near UV absorbance at 280 nm and also through the Bradford colorimetric assay at 595 nm (Walker, 2002; Bradford, 1976), employing BSA for the calibration curve (0-05-1.4 mg/mL). Samples also were read at 645 nm and 663 nm wavelengths and total chlorophyll estimation (μg/ml) was carried out using the Arnon´s spectrophotometric calculation for total chlorophyll (Arnon, 1949).

3 Results and discussion

As previously reported, the most abundant proteins present in alfalfa (*Medicago sativa*) are the RuBisCO (ribulose-1,5-biphosphate) subunits, which are less than 60 kDa and account for c.a. 80% of total soluble proteins in green tissue (Aguilar *et al*., 2009). Thus, a previous experiment was performed with model proteins (BSA and Lyz) to observe if the molecular weight of the target proteins had a significative effect in the partioning of total protein and if an interaction with other factor could be observed. Moreover, the effect of molecular weight of PEG, phosphates and PEG composition and total protein added into the system were studied employing a 2^5 factorial design. The average of three replicates of each treatment is presented in Table 2.

Table 1. Coded factors and levels involved in the factorial design.

Code	Factor	Levels	
		Low (-1)	High (+1)
χ_1	PEG MW (Da)	1000	8000
χ_2	PEG % (w/w)[a]	17.9	21.95
χ_3	Salt % (w/w)[a]	10.65	12.3
χ_4	Protein (mg/g)	0.1	1.0
χ_5	Protein MW (Da)	14400 (Lyz)	66000 (BSA)

a. Selected based on previous experiences.

Table 2. Runs and experimental response for the partitioning of model proteins.

Run	χ_1	χ_2	χ_3	χ_4	χ_5	$\ln(K_p)^a$
1	-1	-1	-1	-1	-1	0.211
2	-1	-1	-1	-1	1	0.549
3	-1	-1	-1	1	-1	0.178
4	-1	-1	-1	1	1	-0.069
5	-1	-1	1	-1	-1	0.724
6	-1	-1	1	-1	1	1.028
7	-1	-1	1	1	-1	1.189
8	-1	-1	1	1	1	0.929
9	-1	1	-1	-1	-1	1.179
10	-1	1	-1	-1	1	1.269
11	-1	1	-1	1	-1	2.683
12	-1	1	-1	1	1	1.370
13	-1	1	1	-1	-1	1.888
14	-1	1	1	-1	1	1.920
15	-1	1	1	1	-1	3.284
16	-1	1	1	1	1	2.850
17	1	-1	-1	-1	-1	-1.494
18	1	-1	-1	-1	1	-2.240
19	1	-1	-1	1	-1	1.813
20	1	-1	-1	1	1	-0.967
21	1	-1	1	-1	-1	-2.653
22	1	-1	1	-1	1	-2.359
23	1	-1	1	1	-1	1.817
24	1	-1	1	1	1	-0.690
25	1	1	-1	-1	-1	-1.747
26	1	1	-1	-1	1	-2.379
27	1	1	-1	1	-1	-0.030
28	1	1	-1	1	1	-0.391
29	1	1	1	-1	-1	-1.118
30	1	1	1	-1	1	-1.713
31	1	1	1	1	-1	1.458
32	1	1	1	1	1	-0.090

a. Average of three replicates

As can be observed, the lowest K_P was obtained in the treatment # 21, where the levels employed are PEG of 8000 Da, 10.65% Phosphates, 21.95% PEG, 0.1 mg of protein per g of total system, and 14,400 Da molecular weight of protein. The highest partition coefficient was obtained in treatment # 15 with PEG of 1000 Da, 12.3% Phosphates, 21.95% PEG, a protein concentration of 1 mg/g of total system and 14,400 Da of MW.

In order to ensure that the selected effects are fare more likely to have an influence over the protein partitioning, those with a T-value above the Bonferroni limit were chosen.

Table 3. Analysis of variance (ANOVA) for the significant variables at the 5% confidence that represents the ATPS extraction of the two model proteins (BSA and Lyz) in the linear factorial model (R^2=0.660).

Source	Sum of Squares	df	Mean Square	F	p-value
Block	15.234	5	3.046		
Model	198.991	4	49.748	41.889	0.000
X_1	130.787	1	130.788	110.127	0.000
X_2	16.751	1	16.752	14.105	0.000
X_4	28.742	1	28.743	24.202	0.000
X_1X_4	22.709	1	22.710	19.122	0.000
Residual	102.134	86	1.187		
Total	316.360	95			

According to the ANOVA performed (Table 3) the significant factors obtained were PEG MW, PEG % and protein concentration. The obtained equation in terms of the coded factors was:

$$\ln(K_p) = -0.03 - 1.167\chi_1 + 0.42\chi_2 + 0.55\chi_4 + 0.49\chi_1\chi_4 \quad (1)$$

In the Eq. 1, the factor χ_1 refers to PEG MW (Da), χ_2 to PEG % (w/w), and χ_4 to protein concentration (mg/g). As the R^2 of the model was 0.660, it means that it is not a good option to predict values for total protein partitioning. Nonetheless, errors accomplishing normal distribution, constant variance and independence test support Eq. (1) as an adequate model to give us some hints to decide the following steps. First, while PEG MW is experimentally restricted to available commercial products, it was fixed at 8000 Da for high level, since a high molecular weight decreases the $\ln K_P$ due to limited excluded volume available for protein recovery on the top phase. Second, in order to maintain the lower concentration of phosphates, this parameter was fixed at the low level. Third, since MW of protein did not had a significative effect, it was not further considered. Apparently, excluded volume effects play an important role in this range of molecular weights of polymer and proteins (Ferreira *et al*., 2014); thus, this may work as a predictive model for partitioning behaviour of alfalfa total protein, even the differences in their isoelectric characteristics. Additionally, in previous work, the use of high MW polymers limits the partition of the hydrophilic proteins from alfalfa green tissue to the top phase, given the high hydrophobicity of the PEG-rich phase, besides the excluded volume effect (Aguilar, *et al*. 2009; Aguilar, *et al*. 2012).

Table 4. Results from a steepest descent experiment to optimize the extraction of total protein from alfalfa in ATPS

Treatment	PEG % (w/w)	Sample load in the system (% w/w)	ln (Kp)	Top phase protein recovery	Bottom phase protein recovery
A	32.22	53%	0.937	46%	8%
B	25.06	40%	0.056	43%	30%
C	17.9	30%	0.011	40%	32%
D	10.74	17%	-0.534	22%	55%
E	3.58	6%	-0.304	27%	54%

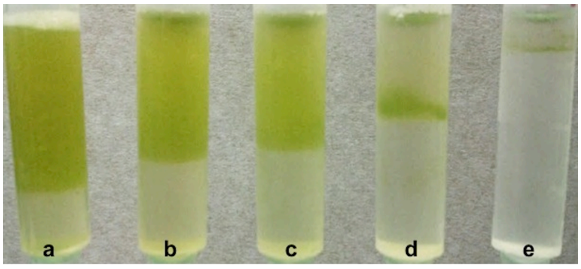

Fig. 1. Graphic demonstration of the steepest descent methodology in order to recover total protein from alfalfa crude extract. A to E are pictures that corresponds to each treatment in Table 4.

Eq. (1) shows that at a fixed partition coefficient, this later will decrease when decreasing both PEG composition and protein concentration. These two factors are easy to change within a wide range, consequently, a steepest descent was chosen and experiments carried out with alfalfa crude extract to reach a minimum of partition coefficient value. In this case, sample load to be added to the system was adjusted accordingly to the total protein in the alfalfa extract and the established protein concentration in the design. Usually, a 10% of load is preferred to add to the system to avoid saturation of the ATPS and to obtain better partition coefficients since higher sample loads (close to 40%) usually tends to accumulate proteins in the interphase (Benavides and Rito-Palomares, 2008).

In Table 4, the steepest descent direction can be observed, along with the respective $\ln(K_p)$. As observed, at the Treatment E, the partition coefficient started to increase, indicating that it had reached the lowest K_p value around the Treatment D. At this point, it was observed that chlorophyll was concentrating at the interphase (Fig. 1), and total protein could be recovered in top and bottom phases. Although, it is not desired that top phase have a lot of protein due its high viscosity (obstructing subsequent polishing steps) it was decided to optimize the protein recovery in the bottom phase, together with chlorophyll concentrated at the interphase.

A CCD with three central points was designed around the Treatment D in order to create a quadratic model to recover total protein from alfalfa. The conditions of each treatment are described in Table 5.

Table 5. Runs and experimental response of the partition coefficient of alfalfa total protein in ATPS employing a central composite face-centered design (CCD)

No.	X_2	X_4	PEG concentration (% w/w)	Sample load in the system (% w/w)	ln (Kp)
1	-1	-1	6.480	10%	0.318
2	1	-1	15.00	10%	0.385
3	-1	1	6.480	24%	-0.956
4	1	1	15.00	24%	0.950
5	-1	0	6.480	17%	-0.853
6	1	0	15.00	17%	0.901
7	0	-1	10.74	11%	0.639
8	0	1	10.74	24%	0.812
9	0	0	10.74	17%	1.206
10	0	0	10.74	17%	0.677
11	0	0	10.74	17%	0.866

Table 6. Analysis of variance (ANOVA) for the significant variables that represent the liquid-liquid extraction of protein from alfalfa (*Medicago sativa*) in the ATPS response surface quadratic model (R^2=0.901)

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Model	4.607	5	0.921	9.110	0.015[a]
X_2	2.316	1	2.316	22.901	0.005
X_4	0.048	1	0.048	0.472	0.523
X_2X_4	0.846	1	0.846	8.362	0.034
X_2^2	1.287	1	1.287	12.722	0.016
X_4^2	0.000	1	0.000	0.003	0.957
Residual	0.506	5	0.101		
Lack of Fit	0.362	3	0.121	1.680	0.394[b]
Pure Error	0.144	2	0.072		
Cor. Total	5.113	10			

a. Significant. b. Not significant.

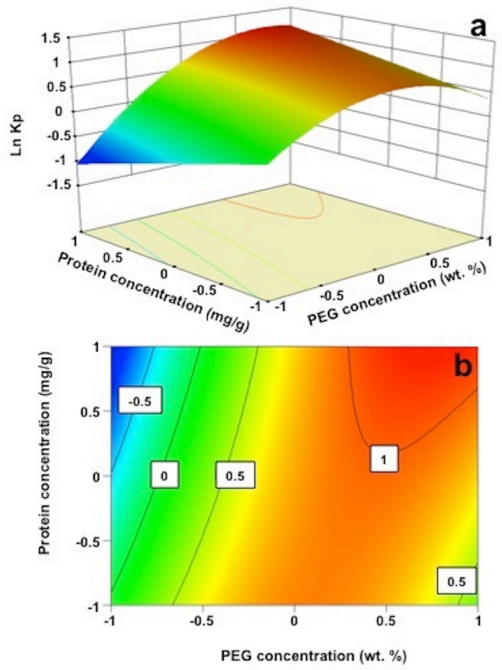

Figure 2. Quadratic surface response area of the optimization of total protein recovery (ln Kp) from alfalfa employing a central faced CCD.

Total protein into the system was calculated according to the total protein in the alfalfa crude extract. Total protein from fresh tissue of alfalfa is reported to be 30-94 mg/g, while moisture content can vary from 37.9 to 81% (Gachovska *et al*., 2013; Abranches *et al*., 2005; Colas *et al*., 2013). This may vary depending on the variety, stage of maturity and multiple thawing cycles (Yari *et al*., 2012; Levitt, 1980). Total protein in the crude extract was 57 mg/g approximately and was diluted to standardized protein content as needed. ANOVA for this experiment was performed (Table 6), giving the next quadratic equation for the partitioning behaviour:

$$\ln(K_p) = 0.844 + 0.621\chi_2 - 0.089\chi_4 + 0.460\chi_2\chi_4 - 0.713\chi_2^2 - 0.011\chi_4^2 \quad (2)$$

The surface response analysis of Eq. (2) can be observed in Fig. 2. This analysis predicted that the best response could be obtained with a high level of protein concentration and a low level of PEG concentration. As observed in Fig. 3, system C is the one that best collects the chlorophyll in the interphase with approximately a 5-fold concentration. A confirmatory analysis was done in order to prove those levels as the most adequate. Substituting the coded values in the resultant Eq. (2), a $\ln(K_p)$ of -1.05 is calculated. From the experiment, the total recovery was 14% for top phase and 80% for lower phase and Ln (K_p) of -0.937, which is very close to the predicted value. This experiment was scaled 300 times to get a 580 mL system. In this case we obtained a $\ln(K_p)$ of -0.920, and a recovery of 13% and 70% of total protein in top and bottom phases respectively with 4.38 fold concentration of chlorophyll in 35 mL of recovered interphase (estimated from 11.05 ± 0.13 to 49.83 ± 1.43 mM). The small difference in partition coefficient value may be attributed to the geometry of the container, since in the absence of centrifugation step, the time for phase coalescence and reach adequate separation is usually longer and

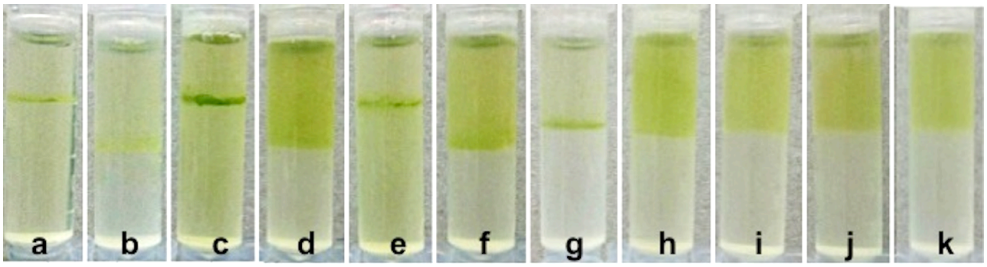

Fig. 3. Graphic demonstration of the CCD methodology in order to recover total protein from alfalfa crude extract. A to K are pictures that corresponds to the 1 to 11 treatments of Table 5.

dependent on the H/D rate of the container (Solano-Castillo and Rito-Palomares, 2000). A large interphase area (low H/D) is recommended to decrease phase separation times.

Afterwards, the bottom phase extract was filtered in order to get rid of the phase-forming components and contaminants. The concentrated protein, distributed in 50 mL tubes was then lyophilized obtaining 1.32 g of white yellowish powder with 33.23% w/w of total protein. The total recovery of protein was 51.41% from that added to the ATPS (150 mL of crude extract). This lyophilizate contain other bioactive compounds such as fibre and also flavonoids that are commonly reported to be present in aerial parts of the plant (Stochmal *et al*., 2001) responsible for the yellowish appearance giving potential antioxidant activity for the extracts.

The experimental optimization performed in ATPS allowed the production of an alfalfa protein powder with protein content comparable to extracts obtained through different methods. Commercial alfalfa green powder (spray dried) may have around 15% of proteins, while concentrated alfalfa extract 50.6% of protein (Luzerne and Extraluz products from Désialis, France) (Kerfai *et al*., 2011). These extracts also contain crude fiber and fat, and are high in mineral content (Gachovska *et al*., 2006). Knuckles and Kohler (1982) obtained an 88.6% crude protein concentrate from alfalfa leaf by carrying out grinding, pressing, heating, centrifugation, filtering, ultrafiltration, gel filtration, and spry drying/freeze drying procedure. In this respect, the lower number of operation units and low energy consumption of the phases are the main advantages of using ATPS.

The lowering in chlorophyll content of the final extract using ATPS could also be coupled with the simultaneous depletion of other relevant proteins from green tissues for industrial purposes (Ibarra-Herrera *et al*., 2011). The approach proposed here represent a starting point to establish a practical protocol that could also be extended to different protein sources from green tissues in general with the advantages of avoiding the use of centrifugal forces and the possibility of direct up-scaling (Espitia-Saloma *et al*. 2014).

Conclusions

In this work, a design the experiments was performed to evaluate the recovery of total protein from alfalfa (*Medicago sativa*). With the employed approach it was possible to establish a fast methodology using model proteins to predict the partition behaviour of a complex protein sample without the need of a large amount of sample. In this case, the size of the protein varying between 14,400 and 66,000 Da was not significative. Most of the proteins from the plant extract partitioned to the bottom phase, in accordance with the model, and chlorophyll pigments were retained at the interphase.

Employing a steepest descent and CCD methodology, it was possible to obtain a chlorophyl-free LPC from alfalfa green tissue with more than 80% of protein or a 33% chlorophyl-free protein powder. The results can be generalized for tissues of the same characteristics given that most of the proteins present in green tissues are quite similar and could be further employed for antioxidant capacity studies. However, variation of other factors such pH and ionic strength, are recommended in order to recover specific proteins in either phase, or pigments from interphase.

Acknowledgements

The authors wish to acknowledge the financial support of Tecnológico de Monterrey, Bioprocesses and Synthetic Biology Group and CONACyT for the fellowships of E. Acuña-González and L.M. Mejía-Manzano, No. 332683 and No. 252731, respectively.

References

Abranches, R., Marcel, S., Arcalis, E., Altmann, F., Fevereiro, P. and Stroger, E. (2005). Plants as bioreactors: a comparative study suggests that *Medicago truncatula* is a promising production system. *Journal of Biotechnology 120*, 121-134.

Aguilar, O., Glatz, C. and Rito-Palomares, M. (2009). Characterization of green tissue protein extract from alfalfa (*Medicago sativa*), exploiting a 3-D technique. *Journal of Separation Science 32*, 3223-3231.

Aguilar, O., Rito-Palomares, M. and Glatz, C.E. (2010) Coupled application of aqueous two-phase partitioning and 2D-electrophoresis for characterization of soybean proteins. *Separation Science and Technology 45*, 2210-2225.

Aguilar, O. and Rito-Palomares, M. (2014). Aqueous two-phase system strategies for the recovery of proteins from plants. In: *Methods in Molecular Biology*, (N.E. Labrou, ed.), Pp. 89-100. Humana Press, Totowa.

Andrews, B.A., Schmidt, A.S. and Asenjo, J.A. (2005). Correlation for the partition behavior of proteins in aqueous two-phase systems: effect of surface hydrophobicity and charge. *Biotechnology and Bioengineering 90*, 380-390.

Archanaa, S., Moise, S. and Suraishkumar, G.K. (2012). Chlorophyll interference in microalgal lipid quantification through the Bligh and Dyer method. *Biomass and Bioenergy 46*, 805-808.

Arnon, D. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. *Plant Physiology 24*, 1- 15.

Benavides, J. and Rito-Palomares, M. (2008). Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products. *Journal of Chemical Technology and Biotechnology 83*, 133-142.

Berges, J.A., Fisher, A.E. and Harrison, P.J. (1993). A comparison of Lowry, Bradford and Smith protein assays using different protein standards and protein isolated from the marine diatom *Thalassiosira pseudonana. Marine Biology 115*, 187-193.

Bernardo, S.C., Azevedo, A.M. and Aires-Barros M.R. (2014). Integrated platforms for the clarification and capture of monoclonal antibodies. *Revista Mexicana de Ingeniería Química 13*, 349-357.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry 72*, 248-254.

Brodzik, K.R., Steplewski, Z. (2009) Production of antibodies in plants: approaches and perspectives. *Current Topics in Microbioly and Immunology 332*, 55-78.

Chen, Z., Liu, J., Zeng, M., Wang, Z., Yu, D., Yin, C., Jin, L., Yang, S. and Song, B. (2012). Dot immunobinding assay method with chlorophyll removal for the detection of southern rice black-streaked dwarf virus. *Molecules 17*, 6886-6900.

Colas, D., Doumeng, C., Pontalier, P.Y. and Rigal, L. (2013). Twin-screw extrusion technology, an original solution for the extraction of proteins from alfalfa (*Medicago sativa*). *Food and Bioproducts Processing 91*, 175-182.

Diederich, P., Amrhein, S., Hämmerling, F. and Hubbuch, J. (2013). Evaluation of PEG/phosphate aqueous two-phase systems for the purification of the chicken egg white protein avidin by using high-throughput techniques. *Chemical Engineering Science 104*, 945-956.

Espitia-Saloma, E., Vázquez-Villegas, P., Aguilar, O., Rito-Palomares, M. (2014). Continuous aqueous two-phase systems devices for the recovery of biological products. *Food and Bioprodicts Processing 92*, 101-112.

Eze, J.M.O. and Dumbroff, E.B. (1982). A comparison of the Bradford and Lowry methods for the analysis of protein in chlorophyllous tissue. *Canadian Journal of Botany 60*, 1046-1049.

Ferreira, L., Fan, X., Mikheeva, L.M., Madeira, P.P., Kurgan, L., Uversky, V.N. and Zaslavsky, B.Y. (2014). Structural features important for differences in protein partitioning in aqueous dextran-polyethylene glycol two-phase systems of different ionic compositions. *BBA - Proteins and Proteomics 1844*, 694-704.

Ferreira, L., Madeira, P.P., Mikheeva, L., Uversky, V.N. and Zaslavsky, B. (2013). Effect of salt additives on protein partition in polyethylene glycol-sodium sulphate aqueous two-phase systems. *BBA - Proteins and Proteomics 1834*, 2859-2866.

Fiorentini, R., Galoppini, C. (1983). The proteins from leaves. *Plant Foods for Human Nutrition 32*, 335-350.

Gachovska, T.K., Ngadi, M., Oluka, S. and Raghavan, V. (2013). Electro-plasmolysis of alfalfa mash. *Pulsed Power Conference, 19th IEEE 1*, 16-21.

González-Valdez, J., Mayolo-Deloisa, K., González-González, M. and Rito-Palomares, M. (2014). Trends in Bioseparations. *Revista Mexicana de Ingeniería Química 13*, 19-27.

Gu, Z. (2014). Recovery of recombinant proteins from plants using aqueous two-phase partitioning systems: an outline. In: *Methods in Molecular Biology*, (N.E. Labrou, ed.), Pp. 77-87. Humana, Totowa.

Haghtalab, A., Mokhtarani, B. and Mauer, G. (2003). Experimental results and thermodynamic modeling of the partitioning of lysozyme, bovine serum albumin and α-amylase in aqueous two phase system of PEG and (KH_2PO_4 or Na_2SO_4). *Journal of Chemical Engineering Data 48*, 1170-1177.

Hua, X., Yang, J., Wang, L., Fang, O., Zhang, G. and Liu, F. (2012). Development of an enzyme linked immunosorbent assay and an immunochromatographic assay for detection of organophosphorus pesticides in different agricultural products. *PLoS ONE 7*, e53099.

Ibarra-Herrera, C.C., Aguilar, O. and Rito-Palomares, M. (2011). Application of an aqueous two-phase system strategy for the potential recovery of a recombinant protein from alfalfa (*Medicago sativa*). *Separation and Purification Technology 77*, 94-98.

Karg, S.R. and Kallio, P.T. (2009). The production of biopharmaceuticals in plant systems. *Biotechnology Advances 27*, 879-894.

Kerfai, S., Mathé, S., Alfenore, S. and Fernandez, A. (2011). Intensive ion exchange process for soluble proteins recovery from industrial alfalfa green juice. *Chemical Engineering Transactions 25*, 1061-1066.

Knuckles, B.E. and Kohler, G.O. (1982). Functional properties of edible protein concentrates from alfalfa. *Journal of Agricultural and Food Chemistry 30*, 748-752.

Kohler, G.O. and Bickoff, E.M. (1975). Industrial production of leaf protein in the USA. In: *Food protein sources* (N.W. Pirie, ed.), Pp. 141. Cambridge University Press, Cambridge.

Levitt, J. (1980). *Responses of Plants to Environmental Stresses*. Academic Press, New York.

Melnik, S. and Stoger, E. (2013). Green factories for biopharmaceuticals. *Current Medicinal Chemistry 20*, 1038-1046.

Ordiales, E., Martín, A., Benito, M.J., Hernández, A., Ruiz-Moyano, S. and Córdoba, M.G. (2012). Technological characterization by free zone capillary electrophoresis (FCZE) of the vegetable rennet (*Cynara cardunculus*) used in "Torta del Casar" cheese-making. *Food Chemistry 133*, 227-235.

Perez, B., Pellegrini-Malpiedi, L., Tubío, G., Nerli, B. and Pessoa-Filho, P.A. (2013). Experimental determination and thermodynamic modeling of phase equilibrium and protein partitioning in aqueous two-phase systems containing biodegradable salts. *Journal of Chemical Thermodynamics 56*, 136-143.

Schillberg, S., Raven, N., Fischer, R.M., Twyman, R. and Schiermeyer, A. (2013). Molecular farming of pharmaceutical proteins using plant suspension cell and tissue cultures. *Current Pharmaceutical Design 19*, 5531-5542.

Sharma, A.K. and Sharma, M.K. (2009). Plants as bioreactors: Recent developments and emerging opportunities. *Biotechnology Advances 27*, 811-832.

Solano-Castillo, C. and Rito-Palomares, M. (2000). Kinetics of phase separation under different process and design parameters in aqueous two-phase systems. *Journal of Chromatography B 743*, 195-201.

Stochmal, A., Piacente, S., Pizza, C., De Riccardis, F., Leitz, R. and Oleszek, W. (2001). Alfalfa (*Medicago sativa* L.) Flavonoids. 1. Apigenin and Luteolin Glycosides from Aerial Parts. *Journal of Agricultural and Food Chemistry 49*, 753-758.

Walker, J.M. (2002). *Protein Protocol handbook*. Humana Press, Totowa.

Wang, J.C. and Kinsella, J.E. (1976). Functional properties of novel proteins: Alfalfa leaf protein. *Journal of Food Science 41*, 286-292.

Wu, Y.T., Lin, D.Q., Zhu, Z.Q. (1998) Thermodynamics of aqueous two-phase systems-the effect of polymer molecular weight on liquid-liquid equilibrium phase diagrams by the modified NRTL model. *Fluid Phase Equilibria 147*, 25-43.

Yari, M., Valizadeh, R., Naserian, A.A., Ghorbani, G.R., Rezvani-Moghaddam, P., Jonker, A. and Yu, P. (2012). Protein molecular structures in alfalfa hay cut at three stages of maturity and in the afternoon and morning and relationship with nutrient availability in ruminants. *Animal Feed Science and Technology 172*, 162-170.