Partitioning Optimization of Proteins from *Zea mays* Malt in ATPS PEG 6000/CaCl2

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ABSTRACT

This work aimed to establish the relationship between the compositions and pH of ATPS PEG 6000/CaCl$_2$ and the proteins partition from maize malt and also to simplify the process optimization in ATPS for a statistical model, established by response surface methodology (RSM). Results showed that these were no influence of pH on the phase diagrams and on the composition of tie line length of PEG 6000/CaCl$_2$ ATPS. SRM analyses showed that elevated pH and larger tie line length were the best conditions for recovering of maize malt proteins. The maximum partition coefficient by PEG 6000/CaCl$_2$ ATPS was about 4.2 and was achieved in ATPS in a single purification step. The theoretical maximum partition coefficient was between 4.1-4.3. The process was very suitable for continuous aqueous two-phase purification due to the stability of proteins (e.g. α and β-amylases) and could increase their content into middle.

Key words: Partitioning, optimization, aqueous two-phase systems, maize malt, PEG 6000, CaCl$_2$

INTRODUCTION

Aqueous two-phase systems (ATPS) have been widely and successfully used in the extraction and purification of biological macromolecules, such as proteins, nucleic acids and antibiotics (Diamond and Hsu, 1992). Compared with other traditional purification techniques, ATPS has the advantages, such as high water content in two-phases (70-90%, w/w), high biocompatibility, low biomolecules degradation, high resolution, relatively high capacity and ease to scale-up (Albertsson, 1986; Mattiasson and Kaul, 1986). However, the exact mechanism governing the partition of

biomolecules is still not well understood. Therefore, many investigators have tried to elucidate the physical interaction and develop mathematical model for describing factors that influence the purification efficiency (Diamond and Hsu, 1992; Gunduz, 2000; Zaslasvsky, 1995). One of the challenges is due to protein partitioning in ATP system that depends on the characteristics of proteins, such as hydrophobicity, molecular size, electrochemical properties, molecular conformation and biospecificity as well as environmental conditions, such as phase-forming polymers or salts, pH, buffer, ion strength and temperature. Mathematical modeling that can

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predict the protein partition behavior and provide additional insights into the protein partitioning mechanisms is of critical importance.

The partition of a solute (e.g., a protein) between the phases is described a partition coefficient, K, defined as the ratio between the concentration of solute in the upper and lower phase.

$$K = \frac{C_{upper}}{C_{lower}} \qquad (1)$$

Formally, we can resolve the partition coefficient in a number of factors:

$$\ln K = \ln K^0 + \ln K_{el} + \ln K_{hfob} + \ln K_{biosp} + \ln K_{size} + \ln K_{conf} \qquad (2)$$

Where the indices el, hfob, biosp, size, and conf stand for electrochemical, hydrophobic, biospecific, size-dependent, and conformation contributions, respectively, to the partition coefficient. The K^0 part includes all other factors, such as general relative solvatation of the solute molecule in the phases. The logarithmic form of the relation above is especially useful when the various effects are studied (Albertsson, 1986; Silva and Franco, 2000).

Polyethylene glycol is one of the most useful polymers in ATPS. Its solubilization in water is attributed to the attachment of water molecules to many or all of the ether oxygen sites along the polyethylene oxide chain. This attachment occurs by a hydrogen-bonding mechanism. It was found that the addition of monovalent cations to polyethylene-oxide products decreases their solubility; this decrease in the cloud point happens when the competition of salt ions for water effectively reduces the amount of free water available to solubilize the polyethylene. Some inorganic salts are more able to promote this effect (e.g., $CaCl_2$, $MgCl_2$, $AlCl_3$) when the ions form association complexes with the ether groups (Silva and Franco, 2000). According to Cleland et al (1992), polyethylene glycol can significantly enhance the refolding of recombinant proteins when accumulated in the form of inclusion bodies that need to be solubilized and refolded to recover activity.

Response surface methodology (RSM) is an effective statistical tool and widely used in process optimization, which includes experimental design, model fitting, validation and condition optimization. An effective statistical design is the basis for response surface optimization and the reported designs include Plackett-Burman design, Box-Behnken design, Graeco-Latin square design and central composite design, which is the most popular among RSM designs and has the characteristics of orthogonality, uniform precision

and rotatability (Barros Neto et al., 2001; Zhi et al.,2005). Gunduz (2001) investigated the partition behavior of pure bovine serum albumin in ATPS PEG/ dextran. The concentration of NaCl and pH were considered as factors influencing K. Optimal empirical model had multiple correlation 0.966 and 99.5 of explain variance obtained by Box-Wilson experimental design.

Recently, Zhi et al (2005) reported a modeling approach based on a empirical model obtained by response surface methodology and investigated the influence of the PEG, citrate and sodium chloride concentrations, which directly affected the partition of α-amylase from *Bacillus subtilis* in a PEG/citrate ATPS. This work aimed to establish the relationship between the partition of maize malt proteins and the compositions and pH of ATPS PEG 6000/$CaCl_2$ and also to simplify the process optimization in ATPS for a statistical model, established by response surface methodology.

MATERIALS AND METHODS

Materials
Maize seed were obtained from EMBRAPA-SE, Brazil. PEG 6000 was provided by SIGMA from Germany and $CaCl_2$ and mono and bi basic phosphate were provided by VETEC from Brazil.

Maize malt obtaining
Maize seeds were cleaned and steeped for 24 h and germination under controlled conditions on moist cotton at 27 °C for 48 h. Germinated seeds were dried at 54 °C in an air oven for 5 h and vegetative growth portions were removed by gentle manual brushing. Devegetated seed (maize malt) were powdered and weighed 2 g and used for the extraction of amylases into 100 ml of phosphate buffer (0.015 mol.l⁻¹) at pH 5, 6 and 7, with agitation for 6 h (Biazus et al., 2005; Nirmala and

Muralikrishna, 2003; Malavasi, U. C. and Malavasi, 2004; Santana, 2003).

Determination of phase diagram
Solution of PEG 6000 and $CaCl_2$ of known concentrations were prepared into phosphate buffer ($0.015mol.l^{-1}$) at pH 5, 6 and 7. Binodal curves were determined according to Albertsson (1986). $CaCl_2$ solution of known concentration was added slowly to the concentrated solution of PEG, until turbidity appears at room temperature. While the system composition point was close to the bimodal curve, 1 ml distilled water was added into above solution. The above steps were repeated until enough points were obtained to form the bimodal curves.

Partition of malt protein
PEG 6000 was used in solid form. Aqueous two phase systems were prepared at room temperature by mixing required amounts of PEG, $CaCl_2$ solution and 400 μl maize malt solution, in 15-ml graduated tubes with conical tips. Distilled water was added to obtain 8 g of the final weight. After vortexing for 1 min, phase separation was accelerated by centrifugation at 800x*g* for 3 min. Total protein was determined in sample of bottom and top phases by Bradford method (Bradford, 1976). Partition coefficient was obtained by equation 1.

Experimental design
Orthogonal experimental design by star methods was used to partition optimization of maize protein by ATPS PEG/ $CaCl_2$. Several empirical models with **K** and **lnK** were tested. Factorial planning 2^3 with two factors: **pH** (x_1) and tie line length, **TL** (x_2), and one response, the coefficient partition (**K**) were made. Assays are shown in Table 1 (Barros Neto et al., 2001; Higuty et al., 2004).

$$x_1 = \frac{pH_i - pH_0}{\Delta pH}$$
(3.a)

$$\text{and } x_2 = \frac{TL_i - TL_0}{\Delta TL}$$
(3.b)

Model fit was evaluated by ANOVA and the optimization by RSM in software Statistic for Windows 5.0 (Barros Neto et al., 2001). The analysis of variance (ANOVA) was employed for the determination of significant variables. ANOVA consists of classifying and cross-classifying statistical results and was tested by the means of a specified classification difference, which was carried out by Fisher's statistical test (*F*-test). The *F*-value is defined as the ratio of the mean square of regression (MRR) to the error (MRe) (*F*=MRR/MRe), representing the significance of each controlled variable on the tested model. The regression equations were also submitted to the *F*-test to determine the coefficient R^2.

RESULTS AND DISCUSSION

Fig. 1 shows the pH effect on phase diagrams of PEG $6000/CaC_2$ ATPS. These was no influence of pH on binodal curves this ATPS. For reduced pH, there was a large need of salt concentration for two-phase formation, as the PEG was more soluble at lower pH (Silva and Franco, 2000). However, according Diamond and Hsu (1992), the pH effect on PEG-salt systems was not full elucidated.

Tables 1, 2 and 3 show the tie line compositions for PEG 6000/ $CaCl_2$ at pH 5, 6 and 7, respectively. There were significant differences among the composition systems. PEG/$CaCl_2$ rates of composition in tie line lengths were about: 16.5/1.6 (1st), 20.5/1.7 (2nd) and 25.5/1.8 (3rd) (w/w) for all studied pH.

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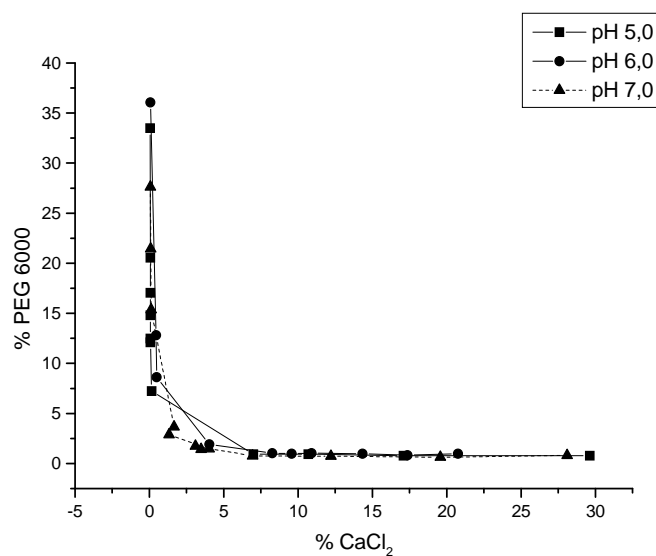
Figure 1 - pH effect on PEG 6000/ CaCl$_2$ ATPS behavior.

Table 1 - Tie line compositions of PEG 6000/CaCl$_2$ ATPS at pH 5.

Tie line	1ª			2ª			3ª		
Composition (% w/w)									
Phase	H$_2$O	CaCl$_2$	PEG	H$_2$O	CaCl$_2$	PEG	H$_2$O	CaCl$_2$	PEG
System	81.39	1.63	16.98	76.74	1.68	21.58	72.09	1.73	26.18
Upper	68.18	0.02	31.80	59.53	0.01	40.46	50.21	0.01	49.78
Lower	94.30	3.85	1.85	94.22	3.95	1.83	94.14	4.06	1.80

Table 2 - Tie line compositions of PEG 6000/CaCl$_2$ ATPS at pH 6.

Tie line	1ª			2ª			3ª		
Composition (% w/w)									
Phase	H$_2$O	CaCl$_2$	PEG	H$_2$O	CaCl$_2$	PEG	H$_2$O	CaCl$_2$	PEG
System	83.24	1.71	15.05	78.56	1.75	19.69	73.50	1.86	24.64
Upper	72.65	0.10	27.25	62.19	0.06	37.75	52.61	0.04	47.25
Lower	93.98	3.15	2.87	93.97	3.37	2.66	93.68	3.87	2.45

Table 3 - Tie line compositions of PEG 6000/CaCl$_2$ ATPS at pH 7.

Tie line	1ª			2ª			1ª		
Phase	H$_2$O	CaCl$_2$	PEG	H$_2$O	CaCl$_2$	PEG	H$_2$O	CaCl$_2$	PEG
System	82,11	1,72	16,17	77,63	1,77	20,60	73,02	1,83	25,15
Upper	70,04	0,06	29,90	60,21	0,04	39,75	49,97	0,03	50,00
Lower	94,97	3,00	2,03	94,78	3,33	1,89	94,66	3,52	1,82

Experiments according to the design in Table 4 were carried out and relevant results of partition coefficient (*K*) are shown in experimental (*lnK$_{exp}$*) and predict (*lnK$_{pred}$*) forms. Table 1 showed that in larger tie line and high pH (about pH 7 and *TL* 3, assays 4 and 11) the maximum partitioning coefficient (about 4.2) occurred in this PEG 6000/CaCl$_2$ ATPS in a single purification step. The theoretical maximum partitioning coefficient was between 4.1-4.3.

For further convenience, the relative model equation of coded variables fitted by regression analysis and its standard error are given by:

$$\ln K = 1.0519 + 0.1245\,x_1 + 0.1718\,x_2 - 0.0186\,x_1^2 + 0.0872\,x_2^2 + 0,0017\,x_1 x_2$$
$$(0.0049)\ \ (0.0030)\ \ \ \ (0.0030)\ \ \ \ \ (0.0036)\ \ \ \ (0.0036)\ \ \ \ (0.0043) \tag{4}$$

Table 4 - Planning matrix for partitioning optimization of malt maize protein extraction.

Assay	x_1	x_2	pH	TL	lnK$_{exp}$	lnK$_{pred}$
1	-1	-1	5	1	0.837	0.826
2	1	-1	7	1	1.082	1.072
3	-1	1	5	3	1.185	1.166
4	1	1	7	3	1.437	1.419
5	0	0	6	2	1.061	1.052
6	0	0	6	2	1.051	1.052
7	0	0	6	2	1.044	1.052
8	-1.41	0	4.6	2	0.824	0.839
9	0	-1.41	6	0.6	0.974	0.983
10	1.41	0	7.4	2	1.176	1.191
11	0	1.41	6	3.4	1.447	1.468

The analysis of variance is employed for the determination of significant variables. The regression equations were submitted to the *F*-test to determine the coefficient *R^2*. Table 5 lists the significant parameters and statistical test results of the models. The *F*-values and variance value of the model equation show that this model was significant and the model determination coefficient (*R^2*) indicated a good response between model prediction and experimental data (Barros Neto *et el*., 2001; Gunduz, 2000; Zhi et al., 2005).

Figs. 2 and 3 show the partition optimization of maize malt protein by response surface methodology according to equation 4. The partition coefficient increased when the pH and tie line length were increased. According to Silva an Franco (2000) for PEG-salts systems, salting-out effects appeared to operate with increasing tie line length, shifting proteins form the salt phase into the PEG-rich phase, and if protein solubility in the PEG phase is not sufficient, they tended to precipitate at the interface. Solubility and salting-out limits are dependent on the properties of individual proteins; therefore, a differential response is expected when a mixture of proteins is handled. Cabral and Aires-Barros (1993) showed that increasing the pH there was an increase in partition coefficient due the hydrophobic properties of PEG that must enhance to hydrophobic residuals of proteins. These figures showed that the best partitioning condition of maize malt protein by PEG 6000/CaCl$_2$ ATPS was in high PEG 6000 and high CaCl$_2$ concentration, at about pH 7 at environment temperature. It showed that maize malt proteins had affinity to PEG 6000-rich phase and this ATPS was suitable for the purification of this proteins.

Table 5 - Variance Analysis of the model equation.

Source	Square sum	Degree freedom	Square mean	F$_{Tab}$	F$_{ratio}$
Regression	0.414	5	0.0827	5.05	203.941
Residual	0.002	5	0.0004		
Fitting fault	0.002	3	0.00063	19.16	8.592
Error	0.000	2	0.00007		
Total	0.416	10			
% explaining variance				99.965	
Multiple correlation (R^2)				0.9951	
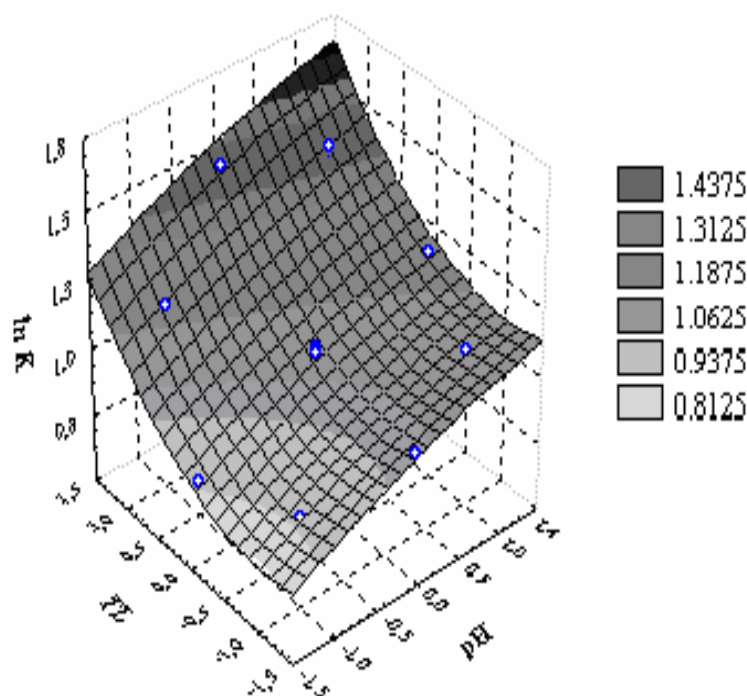
Figure 2 - Response surface for showing the *lnK* dependence of pH and tie line length (TL).
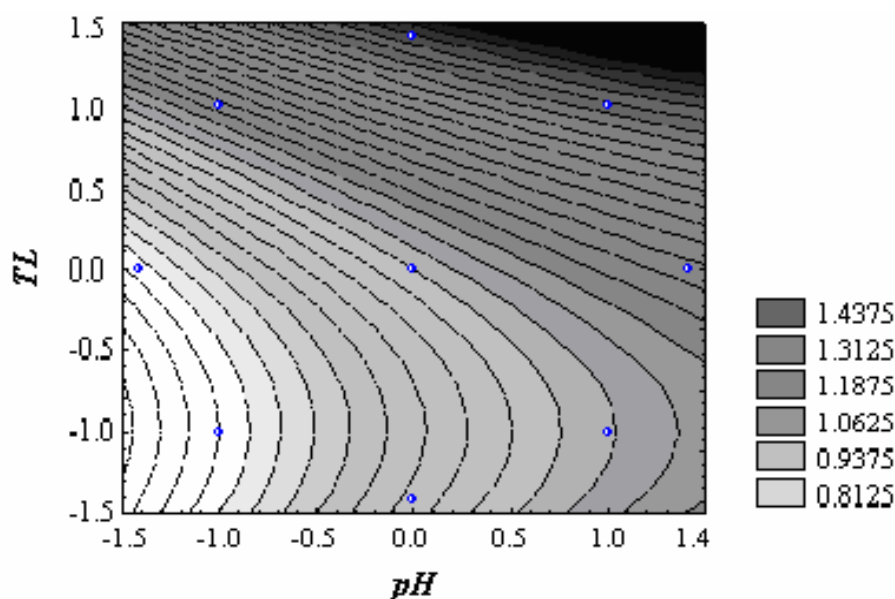
Figure 3 - Level curves for showing the ***lnK*** dependence of pH and tie line length (TL).

CONCLUSIONS

The results showed that this was no influences of pH on binodal curves and on the composition of tie line length of PEG 6000/CaCl$_2$ ATPS. RSM analyses showed that in high pH and larger tie line length was the best condition for recovering of maize malt proteins. The maximum partitioning coefficient by PEG 6000/CaCl$_2$ ATPS was about 4.2 and was achieved in ATPS in a single purification step. The theoretical maximum partition coefficient was between 4.1-4.3. The process was very suitable for continuous aqueous two-phase purification due to the stability of proteins (e.g. α and β-amylases) and it could increase their content into middle.

The model was proved to be useful in designing and conducting ATPS with proper viscosity and high selectivity as well. Further chromatography application, such as counter-current chromatography for protein purification is expected here to be integrated with ATPS to obtain high quality products.

ACKNOWLEDGEMENTS

RESUMO

Este trabalho objetivou encontrar uma relação entre a composição e o pH do sistema bifásico aquoso (SBA) PEG 6000/CaCl2 e a partição de proteínas do malte de milho, e assim simplificando a otimização do processo por um modelo estatístico, estabelecido por metodologia de superfície de resposta (RSM). Os resultados mostraram que não houve influência do pH sobre os diagramas de fases e sobre a composição das linhas de amarração do SBA PEG/CaCl$_2$. As analises RSM mostraram que em pH elevado e nas maiores linha de amarração encontra-se a melhor condição para a recuperação das proteínas do malte de milho. O coeficiente de partição máximo foi cerca de 4,2 para uma única etapa de purificação no SBA 6000/CaCl$_2$. O coeficiente de partição máximo encontrado teoricamente esteve entre 4,1-4,3. O processo é adequado para a purificação contínua via sistemas bifásicos aquosos, já que as proteínas do malte (ex: α e β-amilases) são estáveis e podendo elevar sua concentração no meio.

Palavras chave: partição, otimização, sistemas bifásicos aquosos, malte de milho, PEG 6000, CaCl$_2$.

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