African Journal of Food Science Vol. 6(4), pp. 85-90, 29 February, 2012
Available online at http://www.academicjournals.org/AJFS
DOI: 10.5897/AJFS11.209
ISSN 1996-0794 ©2012 Academic Journals

Full Length Research Paper

Effects of polyethylene glycol 6000 and tripotassium phosphate on protopectinase partition in the aqueous two-phase systems using response surface methodology

Chuanhui Fan[1,2], Zhanmin Liu[1,2]*, Jiaying Hu[1,2], Bin Niu[1,2], Junyi Huang[1,2] and LifengYao[1,2]

[1]School of Life Sciences, Shanghai University, 99 Shangda Road, Shanghai, 200444, P. R. China.
[2]Experimental Center of Life Sciences, Shanghai University, 99 Shangda Road, Shanghai, 200444, P. R. China.

Accepted 7 February, 2012

The quantitative effects of polyethylene glycol (PEG) 6000 and tripotassium phosphate on protopectinase partition in the aqueous two-phase systems were investigated using response surface methodology. With an increase in PEG 6000 less than about 3.6 g protopectinase partition increased and decreased later beyond about 4.75 g. Furthermore, protopectinase partition improved continually and reached the maximum with the increase of K_3PO_4 at the range of about 1.40 to 1.70 g, the statistic analysis showed that the effect of PEG ($P < 0.0089$) had significant effects on protopectinase partition, but the effects of the tripotassium phosphate ($P < 0.9475$) and the interaction of PEG 6000 and tripotassium phosphate ($P < 0.2712$) were not significant at the significance level of 0.05. The experimental values were shown to be in good agreement with predicted values since the correlation coefficient was 0.9410. At the optimal partition condition (4.14 g PEG 6000 and 1.71 g tripotassium phosphate in 10 ml two-phase systems), the maximum of protopectinase activity was 17.27 U/ml, which is about 2.27 folds than the other one.

Key words: Central composite design (CCD), two-phase systems, polyethylene glycol/tripotassium phosphate, protopectinase partition.

INTRODUCTION

Protopectinases (PPases) are used to a heterogeneous group of enzymes {Cavalitto, 1997 #34} (Cavalitto et al., 1997) {Cavalitto, 1997 #34} which produce the enzymatic solubilization of pectin from protopectin, the water-insoluble parental pectic substance present in plant tissues. Enzymatic extraction of pectin with protopectinases as biocatalysts has been investigated recently (Ptichkina et al., 2008; Contreras-Esquivel et al., 2006; Contreras-Esquivel et al., 1997). Several PPases from different microorganisms (bacteria, yeast or fungi) have been purified and characterized in traditional methods (Takao et al., 2000; Nagai et al., 2000).

However, the purification protocols on PPases involve ammonium sulphate precipitation, chromatography, dialysis and filtration, which increase the cost of the process and reduce the yield. Therefore, simpler and more efficient purification processes are needed in the biotechnology industry (Malpiedi et al., 2008).

An aqueous two-phase system (ATPS) is formed when combinations of hydrophilic solutes (polymers or polymer and salts) display incompatibility in aqueous solution above critical concentrations (Rito-Palomares, 2004). Extraction by ATPS has become an important emerging technique for separation, concentration and purification of proteins, enzymes and pharmaceutical products (Rangel-Yagui et al., 2004). ATPSs have been successfully used for separation and purification of several proteins and

*Corresponding author. E-mail: zhmliu119@yahoo.com.cn.Tel: +86-21-66135166. Fax: +86-21-66135166.

Table 1. Coded and actual values of central composite rotatable design (CCD) along with the experimental and predicted values of PPase activity.

Trial no.	x_1:PEG 6000	x_2:K_3PO_4	*Y*: PPase (U/ml)	
			Actual	Predicted
1	-1 (2.5)	-1 (1.4)	10.950	10.999
2	-1 (2.5)	1 (2.0)	9.213	9.325
3	1 (5.0)	-1 (1.4)	12.532	13.646
4	1 (5.0)	1 (2.0)	14.018	15.195
5	-1.41 (1.98)	0 (1.7)	8.470	8.6110
6	1.41 (5.52)	0 (1.7)	16.00	14.634
7	0 (3.75)	-1.41 (1.28)	13.572	13.004
8	0 (3.75)	1.41 (2.12)	13.572	12.915
9	0 (3.75)	0 (1.7)	17.733	18.476
10	0 (3.75)	0 (1.7)	19.219	18.476

have shown significant advantages over traditional methods (Albertsson, 1970), such as the high-water content of phases, signifying high biocompatibility, thereby minimizing bio-molecule degradation, low interfacial tension, low-cost and easily scaled-up (Coimbra et al., 2009; Malpiedi et al., 2008; Albertsson, 1970).

There are many reports in literatures concerning the partition of different enzymes and proteins in ATPS (Farruggia et al., 2011; 2007; Singh and Verma, 2010; Jiang et al., 2008; Pico et al., 2007), But reports on the use of such systems for partitioning and purification of protopectinase were not available. For industrial purposes, polymer/tripotassium phosphate systems are the most commonly used (Malpiedi et al., 2008); in the present work, we report the partitioning of protopectinase from microbial fermentation broth by PEG/tripotassium phosphate two-phase systems and effects of PEG content and the additions of tripotassium phosphate on protopectinase partition were investigated by response surface methodology.

EXPERIMENTAL PROCEDURES

Chemicals

PEG 6000 and other chemical reagents were purchased from Shanghai Branch of Sinopharm Chemical Reagent Co, Ltd. (Shanghai, China). All other reagents were of analytical grade.

Preparation of protopectins

Protopectins were prepared from persimmon peel according to the method of Cavalitto et al. (1999). Dry persimmon peel powder was repeatedly washed with sterilized water until the soluble substances that reacted with carbazole-H_2SO_4 were washed off, yielding water-washed protopectins. Then, it was washed with 2% sodium hexametaphosphate solution, pH 4.0, until a negative reaction with the carbazole-H_2SO_4, reagent was obtained. The remaining amounts of substance were finally carried out by vacuum freeze-drying to remove residual water, weighed and kept as protopectin.

Microbial fermentation and protopectinase production

Aspergillus terreus SHPP01 used in this study was provided by the Laboratory of Food Safety and Biotechnology, Shanghai University. The strain was cultured on the potato dextrose agar (PDA) slant at 28°C for one day, and then maintained at 4°C. Inoculum was prepared by transferring one loop full of culture from PDA slant to an Erlenmeyer flask (250 ml) containing 50 ml seed medium containing (g/L) glucose 20, yeast extracts 2, peptone 4, K_2HPO_4 3, KH_2PO_4 1, $MgSO_4$ 0.5, and the initial pH at 5.0. The seed cultures were grown at 28 ± 2°C on a rotary shaker incubator at 180 rpm for 18 h. After incubation, 3 ml of the seed culture was transferred into an Erlenmeyer flask (250 ml) containing 50 ml of the fermentation medium containing (g/L) glucose 20, yeast extracts 2, peptone 4, and the initial pH at 5.0. The fermentation cultures were then incubated at 28 ± 2°C on a rotary shaker at 180 rpm for 48 h.

Partition of protopectinase

10 ml of aqueous two-phase systems was constructed by weighing requisite amount (Table 1) of PEG 6000 and K_3PO_4 into a suitable vessel, equilibrated at 20°C and allowed to make two-phase systems. For analysis of protopectinase activity in the 4 ml of fermentation broth added into previous ATPSs, aliquots were removed at regular time intervals and centrifuged at 5000 r/min for 5 min, by which time, microbial cells formed a pellet at the bottom of the centrifuge tube and phase separation occurred. After equilibration and phase separation, the upper phase was carefully removed with a pipette and after removing the interface, the lower phase was collected. Because of little enzyme activity in the upper phase, protopectinase activities in the lower phase were analyzed for enzyme activity as responses in studies, indicating protopectinase partition in two-phase systems. Protopectinase activity is measured by sulphate-carbazole method (McComb and McCready, 1954).

Experimental design

The central composite design (CCD) is the most common experimental design used in response surface methodology (RSM), which has been equal predictability in all directions from the center (Reddy et al., 2000; Liu and Tzeng, 1998). In addition, CCDs are optimized designs for fitting quadratic models. The numbers of experimental points in the CCD are sufficient to test statistical

Table 2. Analysis of variance (ANOVA) for regression model.

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob>F
Model	101.88	5	20.38	12.76	0.0143
Lack-of-fit	5.28	3	1.76	1.59	0.5138
Pure error	1.10	1	1.1		
Corrected total	108.26	9			
R^2 = 0.9410, adj R^2 = 0.8673					

Table 3. Estimates of the parameter of regression for PPase partition.

Term	Esitmate	Standard error	t-ratio	p value > |t|
Interpret	18.476323	0.893376	20.68	<.0001
X_1	2.1294406	0.446688	4.77	0.0089
X_2	-0.031322	0.446688	-0.07	0.9475
X_1* X_2	0.8056593	0.631712	1.28	0.2712
X_1*X_1	-3.426904	0.590912	-5.80	0.0044
X_2*X_2	-2.758289	0.590912	-4.67	0.0095

validity of the fitted model and lack-of-fit of the model. The central point in CCD is replicated several times to estimate the error due to experimental or random variability.

A CCD was employed for the response surface methodology (RSM) study and the two factors chosen were PEG 6000 and K_3PO_4, which were designated as X_1 and X_2, respectfully. The low, middle and high levels, of each variable were designated as -1, 0, and +1, respectively (Table 1). The variables were coded according to the Equation (1):

$$x_i=(X_i-X_0)/\Delta X \quad (1)$$

Where x_i codes value of the variable X_i, X_0 is the value of X_i at the centre point and ΔX is the step change. Table 2 shows the actual design of experiments. The role of each variable, their interaction and statistical analysis to obtain the predicted yield were explained by applying the following quadratic equation:

$$Y=\beta_0+\beta_1 X_1+\beta_2 X_2+\beta_{11} X_1^2+\beta_{22} X_2^2+\beta_{12} X_1 X_2 \quad (2)$$

Where Y is the response; x_1 and x_2 are input variables; β_0 is a constant; β_1, and β_2, are linear coefficients; β_{12} is cross-product coefficients; β_{11} and β_{22} are quadratic coefficients. The statistical software package, JMP 7.02 (SAS Institute Inc., Cary, NC, USA) was used for the regression analysis of the experimental data. The statistical significance of the model equation and the model terms was evaluated via the Fisher's test. The coefficient of determination (R^2) and adjusted R^2 (Adj R^2) were used for the verification of the significance of the second-order polynomial model (Aziz et al., 2010). Based on the analysis of variance, parameters with a significance level (P) greater than 5% were eliminated to obtain the final reduced model. This final model can be displayed as three-dimensional response surface plots.

RESULTS AND DISCCUSION

Regression model of responses

Through, the 10 sets of experiments, experimental values of response were determined in Table 1. There was a considerable variation in the protopectinase activity depending upon the partition conditions. The replication at the centre point conditions resulted in higher protopectinase activities (17.733, 19.219 U/ml, respectively) than those at other levels. By applying multiple regression analysis methods, the data obtained were analyzed according to second-order polynomial model (Kim and Barrington, 2008). The predicted response Y for PPase activity can be obtained and given as:

$$Y=18.48+2.13X_1-0.031X_2+0.81X_1*X_2-3.43X_1^2-2.76X_2^2 \quad (3)$$

A summary of the analysis of variance (ANOVA) for the selected quadratic model was shown in Table 2.

The correlation measures for testing the goodness of fit of the regression equation are the multiple correlation coefficients R and the determination coefficient R^2. The value of R (0.9701) for Equation (3) being close to 1 indicates a high degree of correlation between the observed and predicted values. The value of the determination coefficient R^2 (0.9410) suggests that only about 6% of the total variation are not explained by the model. It is evident that the model is significant, as it is evident from the model F-value and a low-probability value (F = 12.76, P model = 0.0143). The value of lack-of-fit for regression Equation (3) is not significant (P = 0.5150), indicating that the model equation was adequate for predicting the protopectinase activity under any combination of values of the variables.

The coefficient values of Equation (3) were calculated and tested for their significance in Table 3. The P-values are used as a tool to check the significance of each of the

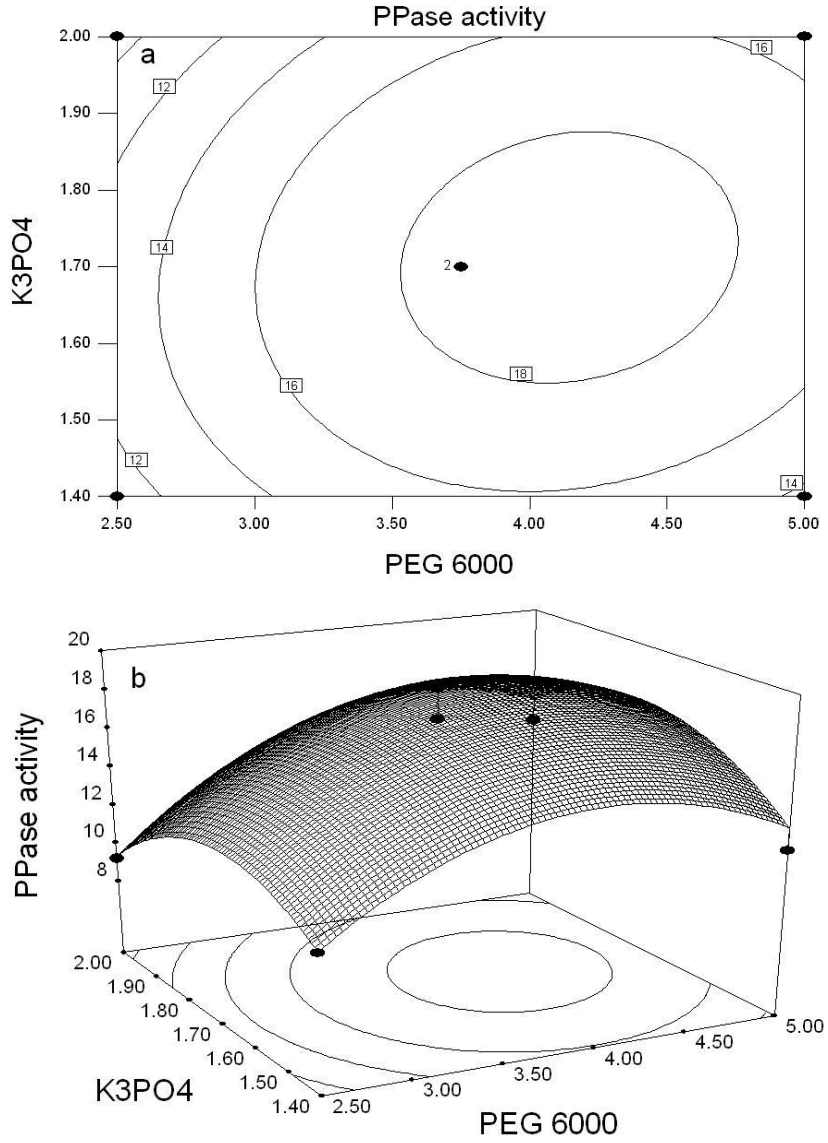

Figure 1. A contour plot (a) and three-dimensional response surface plot (b) showing effects of PEG 6000 and K_3PO_4 and their mutual interactions on protopectinase partition. The data on the contour map are protopectinase activity.

coefficients, which in turn may indicate the pattern of the interactions between the variables. The smaller the value of P, the more significant is the corresponding coefficient. It can be seen from Table 3 that the X_1 linear coefficient, two interaction terms (X_1*X_1 and X_2*X_2) were significant, the P-values being very small ($P < 0.05$), but the effect of X_2 ($P < 0.9475$) and X_1*X_2 ($P < 0.2712$) were not significant.

Response surface plot and its contuor plot showing effects of PEG 6000 and K_3PO_4 on protopectinase partition

The fitted response surface for protopectinase partition by the regression (Equation 3) was generated using the JMP software and is given in Figure 1. As can be seen from Figure 1 that an increase in PEG 6000 caused protopectinase partition to increase firstly at the range of less than about 3.6 g and decrease later beyond about 4.75 g; protopectinase partition was found to increase with an increase in PEG 6000 until the top point of response surface was reach; it increasingly reached the maximum at the range of 1.55 to 1.88 g K_3PO_4 and decreased at other ranges (Figure 1a). Furthermore, at the range of about 1.40 to 1.70 g K_3PO_4 protopectinase partition improved continually and reached the maximum with the increase of K_3PO_4. However, protopectinase partition decreased when K_3PO_4 was out of that range (Figure 1a).

It is evidence that in the area investigated, protopectinase partition is sensitive to minor alterations of the test variables, that is, PEG 6000 and K_3PO_4, and there exists a maximum value of protopectinase partition (Figure 1b). In addition, the interaction between K_3PO_4 and PEG 6000 was not significant. An elliptical nature of the contour plots indicates that the interaction between the corresponding variables is significant.

By analyzing the plots in Figure 1, the optimal values of protopectinase partition condition for obtaining approximately 18.4 U/ml of protopectinase activity lie in the following ranges of the tested variables: PEG 6000 = 4.0 g, K_3PO_4 = 1.7 g .The optimum value was obtained by deriving Equation (3) and by solving the inverse matrix, getting the optimization level of protopectinase partition at PEG 6000, 4.14 g and K_3PO_4, 1.71 g, respectively and the maximum value of protopectinase activity was 18.81 U/ml. Through verified experiment at the optimal condition, protopectinase activity reached 17.27 U/ml, which indicated that the error is only 8.2% between actual values and predicted one.

Although, bio-molecule partitioning in ATPS is a complex function of a variety of factors, the bio-molecular size, its surface properties, net charge, the system temperature and the polymer molecular weight (Baskir et al., 1989), there is no much information about such properties in the case of protopectinase. Many literatures report that the partition coefficient decreases as the PEG chain length increases. Furthermore, in the systems containing sodium chloride the enzyme partition coefficients can reduce in the systems without this salt (Wongmongkol and Prichanont, 2006). Therefore, a simple and efficient partition method was developed in our investigation without sodium chloride, and lower molecular weight PEG to partition of protopectinase from microbial fermentation broth.

For all ATPS studied, proteins were partitioned predominantly in the PEG-rich top phase, principally those with hydrophobic characteristics (Reh et al., 2002). In general, negatively charged proteins prefer the upper phase in PEG–salt systems, while positively charged proteins normally partition selectively to the lower phase (Del-Val and Otero, 2003). Hence, protopectinase partitioned in the lower phase might be positively charged.

PEG 6000/ K_3PO_4 system is one of the polymer/salt systems, which result in higher selectivity in partitioning, with high yields in the first extraction step. Utilizing the PEG/phosphate system, about 2.80 and 2.26 times higher alkaline protease production was reported than that of homogeneous fermentation (Hotha and Banik, 1997). Similar higher yields were reported for xylanase production (Kulkarni et al., 1999). In our study, protopectinase had the highest yield in the lower phase, which is similar to *Aspergillus oryzae* alpha-amylase partition in ATPS (Porfiri et al., 2011).

RSM involving an experimental design and regression analysis has been utilized in modeling and optimization condition of proteins purification or partition in ATPSs (Pericin et al., 2009; Kammoun et al., 2009; Zhi et al., 2005), which indicates that RSM provides a promising tool for experimental design of protein purification by an aqueous two-phase system. In this study, RSM was used to evaluate the effects of PEG 6000/K_3PO_4 on protopectinase partition and was effective in finding the optimum value of them. A predictive model for protopectinase partition was established as a function of PEG 6000/ K_3PO_4 in the aqueous two-phase systems. The optimal PEG 6000/ K_3PO_4 system in 10 ml consisted of 4.14 g PEG 6000 and 1.71 g K_3PO_4 to gain the maximum value of protopectinase partition about 2.27 fold than the fifth set of two-phase systems in our experiments.

ACKNOWLEDGEMENTS

This work was financially supported by Shanghai Science and Technology Committee (11DZ2272100) and Shanghai Key Laboratory of Bio-Energy Crops.

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