sciendo

Polish Journal of Chemical Technology, 22, 2, 42—49, 10.2478/pjct-2020-0015

Three phase partitioning as a rapid and efficient method for purification of plant-esterase from wheat flour

Liang Dong[1*], Linxin He[1], Danqun Huo[2]

[1]*Sichuan University of Science & Engineering, Zigong 643000, China*
[2]*Key Laboratory of Biorheology Science and Technology, Ministry of Education, College of Bioengineering, Chongqing University, Chongqing 400044, China*
[*]*Corresponding authors: e-mail: dongliang@suse.edu.cn*

Three-phase partitioning (TPP) was used to purify plant-esterase from wheat flour. Effect of various process parameters has been evaluated and plant-esterase was purified to 11.35-fold by optimized single step TPP system (50%, (w/v) $(NH_4)_2SO_4$ saturation, 1:1 (v/v) ratio of crude extract: *t*-butanol at pH 4).The enzyme was found to be exclusively partitioned in the aqueous phase. Using TPP system, plant-esterase quickly purified to homogeneity with very high purity and activity. On the basis of single factor research, purification process was optimized by using response surface method, established a new type of high efficient purification plant-esterase method. To the best of our knowledge, this is the first report for purification and characterization of plant-esterase by using three phase partitioning (TPP). The results indicated that, TPP is a simple, quick, economical and very attractive process for purification of plant-esterase compared to conventional chromatographic protocols.

Keywords: Purification, Plant esterase, Three phase partitioning

INTRODUCTION

Organophosphorus compounds (OPs) have become key components in current agricultural pesticides and herbicides. While these compounds have achieved enormous commercial success, they have also become major environmental pollutants. Due to their wide spectrum of insecticidal activity, organophosphorus pesticides account for 38% of the total pesticides applied in crop protection all over the world[1, 2]. In addition to agricultural applications, OPs have also found more sinister uses as nerve agents. In the event of a chemical weapon attack, it would be imperative to have a rapid on-site OP monitoring system in place.

As Acetyl cholinesterase(AchE) is a biorecognition element highly sensitive to the inhibition of organophosphates, carbamate pesticides, and nerve gases, AchE-based biosensors for OPs have been extensively investigated in past decades[3–7].Nevertheless, both the high cost and lesser accessibility (extracted from animal blood or tissue) of AChE greatly limit its wide application. Recently, as an alternative of AChE, plant-esterase (EC 3.1.1.X) has received much attention because plant esterase and AChE share a similar sensitivity and inhibition mechanism with OP pesticides. Moreover, plant-esterase can be extracted from a number of plants such as wheat, soybean, rice, and sorghum at low cost and with easy obtainment[8–10], thus holding great potential in OPs detection.

In our previous study, we developed a much faster and simpler optical detection protocol based on spectrophotometric changes of the H_2TPPS_1-plant-esterase complex in both liquid and solid state[11]. Recently, we developed a new cost-effective, simple, sensitive, reliable PLaE--CS/ AuNPs-GNs composite-based biosensor holds great potential in OPs detection for food and environmental safety[12]. However, the commercial-scale production of OPs sensors based on plant-esterase would be hampered by the requirement for large quantities of highly purified enzyme with appropriate properties.

Various researchers have developed several methods for the purification of plant-esterase[10, 13, 14]. However, almost all these methods involve a large number of steps. In our previous study, purification of plant-esterase from flour in an aqueous two-phase system (ATPS) was developed[8]. It is known that the higher the number of steps, the higher the loss of product yield. Furthermore, the scale-up of these methods is difficult and expensive. It may be noted that 50–90% of the production cost is due to the purification steps[15]. Hence, efficient and economical large-scale bioseparation methods are needed, to provide high purity, high yield plant-esterase that is biologically activity. One purification method that meets all these criteria is Three-phase partitioning (TPP).

TPP is a simple and efficient method for the separation and enrichment of protein compounds such as enzymes from complex mixtures. It consists in the sequential addition of a sufficient amount of salt (typically ammonium sulphate) and an organic solvent (mainly *t*-butanol) in the crude extract and after agitation and decantation, the mixture separates into three distinct phases: a *t*-butanol rich layer and an aqueous layer which are formed above and under the precipitated protein layer[16]. Pigments, lipids and inhibitors contained in crude extract are generally concentrated in the top phase, whereas polar compounds such as saccharides are enriched in the lower aqueous phase[17]. The third phase is formed by a protein-enriched intermediate layer and generally, an increase of purification fold in this intermediate layer can be attained[18]. During the TPP process, activation of enzyme is also possible. This may be due to the phenomenon that the increased flexibility of enzyme can lead to higher catalytic activity in TPP process[19, 20]. A remarkable increase in the catalytic activity and yield of ficin[19] and invertase[21] obtained from TPP has been reported. Sulfate ion and *t*-butanol are known to be excellent protein structure markers or kosmotropes[18]. Kosmotropes, inversely to chaotropic agents, cause water molecules to favorably interact, which also stabilizes intramolecular interactions in the proteins[22].

MATERIAL AND METHODS

Chemicals

Wheat flour was purchased from the local market (Chongqing, China). Bovine serum albumin (BSA) used for calibration of protein estimation was purchased from Sigma (St. Louis, MO, USA). 1-Naphthyl acetate (1-NA) used for calibration of Plant-esterase activity was purchased from YuanJu Biotechnology (Shanghai, China). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and N,N,N',N'-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA,USA). Fast Blue B salt were purchased from YuanJu Biotechnology (Shanghai, China). Other analytical grade chemicals were purchased from Beibei Chemical Reagent Factory (Chongqing, China). Water used for preparation of aqueous solutions was from a Millipore Direct-Q Water system (resistivity, 18.2 MΩ cm).

Preparation of crude extract

A 1:5 (w/v) solution of flour in distilled water was made. The mixture was incubated at 35°C for 30 min in a water bath, then centrifuged at 8000 rpm for 10 min at 4°C. The clear supernatant was collected and was kept frozen at –20°C for future use.

Purification of plant-esterase by three-phase partitioning (TPP)

TPP experiments were carried out as described by Dennison[18] with slight modifications. Briefly, the crude plant-esterase enzyme extract (5 ml containing 67.47 units and 33.83 mg protein) was saturated at room temperature with 20% ammonium sulfate, followed by addition of a half volume of *t*-butanol. The mixture was gently vortexed and then allowed to stand at 35°C for 40 min in a water bath. Afterwards, the mixture was centrifuged at 4500 rpm for 10 min at 4°C to facilitate the separation of the three phases. The upper *t*-butanol phase was removed by a Pasteur pipette. The lower aqueous phase and the interfacial phase were separated carefully and analyzed for enzyme activity and protein content. The interfacial precipitate was dissolved in 0.04 M, pH 8.0 sodium phosphate buffer.

The effect of salt concentrations (20,30, 40, 50, 60 and 70%)(w/v) on the crude enzyme extract for the TPP at the constant crude extract:t-butanol ratio (0:0.5) was investigated. After that, various *t*-butanol ratios (crude extract: *t*-butanol; 1:0.5, 1:0.75, 1:1, 1:1.25; 1:1.5, and 1:2) were employed with a constant ammonium sulfate saturation at 50% at 35°C.

The temperature of 4°C, 15°C, 25°C, 35°C, 45°C, 55°C were chosen to study its effects on partitioning of plant--esterase. The rest parameters were kept constant such as pH 7, ammonium sulfate 50% and crude extract to *t*-butanol ration of 1:1.

After that, effects with different pH values of medium study were tested. Crude extract was saturated with 50% ammonium sulfate and pH was adjusted to 3, 4, 5, 6, 7, 8 and 9, then 1:1 *t*-butanol was added and the incubation temperature was 35°C.

After optimization of the parameters, three repetitions were conducted to confirm the overall results using 50% ammonium sulfate, 1:1 ratio crude extract to *t*-butanol and a pH of 4.0 and the incubation temperature was 35°C. The interfacial phase always containing the higher plant-esterase activity was collected, dissolved in 0.04 M, pH 8.0 sodium phosphate buffer, dialyzed overnight against the same buffer and concentrated by ultrafiltration using Amicon Ultra device witha 10 kDa MWCO. The concentrated enzyme was stored at –20°C until use for further characterization studies in order to determine the general biochemical properties.

Enzyme activity assay

Plant-esterase activity was determined by the colorimetric method of VanAsperen[23]. The general buffer was 0.04 M sodium phosphate, pH 6.5. 1-Naphthylacetate (1-NA) (16 mM) was used as substrate. The mixture consisting of phosphatebuffer (1.95 ml), enzyme solution (0.5 ml) and 1-NA (50 µl) was incubated at 35°C for 15 min in a water bath. The hydrolysis of 1-NA was terminated by adding 0.5 ml of Fast Blue B salt–SDS solution. The absorbance at 595 nm was measured by spectrophotometer (PerkinElmer Instruments, USA) after 10 min.

Protein determination

The total amount of soluble proteins present in the sample was measured by the modified Bradford dye-binding assay[24]. Interference from phase-forming components was eliminated by using identical phase systems without sample as a blank in the spectrophotometric assay.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE of the protein samples with 10% gel was performed according to the method of Laemmli[25] with slight modification. For protein staining, the gel was stained with Coomassie Brilliant Blue R-250 for 1 h and then destained with 50% (v/v) methanol and 12% (v/v) acetic acid for 2–3 h.

Optimization of PlaE purification process by response surface method

TPP process was optimized using response surface methodology. For this purpose, four factors, namely ammonium sulphate saturation(w/v%), temperature, pH and crude extract/t-butanol(v/v) were selected, Purification fold as a response value. 29 groups of experiments were arranged, including 24 groups of factorial experiments, 5 groups of central experiments(used to estimate experimental errors). Ranges of variation of these factors were respectively: ammonium sulphate saturation(A): 40%–60%; crude extract/t-butanol(B):1:0.75 to 1:1.25; pH(C):3–5; temperature(D):25–45°C. Each of the 29 experiments was performed three times. Application of experimental design software Design-Expert 8 carries on the experiment design, the data analysis and the model establishment.

RESULTS AND DISCUSSION

Effect of ammonium sulfate saturation on Plant-esterase

Salting out can be used to separate proteins based on their solubility in the presence of high salt concentration. Ammonium sulfate is the traditional kosmotropic salt used for protein salting out because of its high solubi-

lity. The principle of SO_4^{2-} ion for salting out protein has been viewed in five different ways namely, (1) ionic strength effects, (2) cavity surface tension enhancement osmotic stressor (dehydration), (3) kosmotropy, (4) exclusion crowding agent and (5) the binding of SO_4^{2-} to cationic sites of protein[26].

Additionally, NH_4^+ and SO_4^{2-} are at the ends of their respective Hofmeister series and have been shown to stabilize protein structure. Ammonium sulfate concentration plays a major role in TPP as it is responsible for protein-protein interaction and precipitation. It causes protein precipitation by salting out mechanism. Protein solubility is influenced by the ionic strength of the solution which subsequently depends on salt concentration[27]. On addition of sufficient salt proteins precipitation occur, this is known as salting out effect. At higher salt concentration, water molecules are attracted by salt ions result in stronger protein–protein interactions and the protein molecules coagulate through hydrophobic interactions[28]. Generally, in TPP process, the researchers start minimum salt concentration of 20% (w/v) to optimize the partitioning conditions[18].

Herein, the experiment was carried out by keeping the experimental parameters as follows: ammonium sulfate loading 20% (w/v), crude extract: *t*-butanol ratio 1.0:0.5, temperature 35°C and pH 7. Ammonium sulfate concentration was varied from 20% to 70% w/v of the crude extract concentration. At lower concentration (20 and 40%) ammonium sulfate may not able to alter the hydrophobic surface of plant-esterase. Similar trend has been observed in the extraction of peroxidase[29]. Fig. 1. reveals that with an increase in $(NH_4)_2SO_4$ concentration, purity of plant-esterase is significantly increased up to 6.42-fold. Further increase in $(NH_4)_2SO_4$ concentration reduces fold purity. Higher salt concentrations leads to the reduction of purification fold, which may be due to the irreversible denaturation of protein as reported in earlier studies[18, 30]. As maximum purity was achieved at 50% $(NH_4)_2SO_4$ concentration, it was selected as optimum value or next set of experiments. This salt concentration was sufficient enough to concentrate the plant-esterase, a finding in agreement to its known property to precipitate at 50–60% $(NH_4)_2SO_4$ saturation[31, 32].

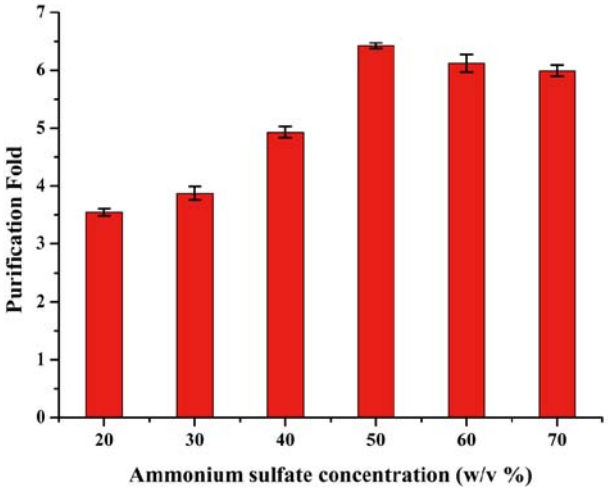

Figure 1. Effect of varying saturations of ammonium sulfate on the degree of purification of plant-esterase enzyme. The crude extract (5 ml containing 67.47 units and 33.83 mg protein) was brought to different levels of saturation and *t*-butanol was added in the ratio 1.0:0.5 (v/v) with respect to the volumes of the aqueous extract. The incubation temperature was 35°C. The pH value was 7

Effect of crude extract to *t*-butanol ratio on plant-esterase partitioning

After the selection of $(NH_4)_2SO_4$ saturation, the effect of crude extract to *t*-butanol ratio for plant-esterase partitioning in the TPP system was investigated. *t*-Butanol has been selected here due to its several advantages over other solvents and from the reference of earlier published reports on TPP[18, 19, 33, 34]. Due to its size and branched structure, *t*-butanol does not easily permeate inside the folded protein molecules and hence does not cause denaturation. It also shows significant kosmotropic and crowding effects that enhances the partitioning of enzyme[26]. Different crude extract: *t*-butanol ratio effect for the partitioning of plant-esterase at the constant saturation of $(NH_4)_2SO_4$ (50%, w/v), at 35°C is shown in Fig. 2. It can be seen from Fig. 2 that the highest purity was obtained at the ratio of 1:1 and purity fold decreased with subsequent increase in crude to *t*-butanol ratio. At 1:0.5 and 1:0.75 ratios, there is less purification fold which may be due to smaller amount of *t*-butanol does not adequately synergize with $(NH_4)_2SO_4$[18, 26]. Similar results have been reported by Ozer et al. who used the TPP for the purification of invertase from tomato[17]. If it is higher than 1:1, it is likely to cause protein denaturation as reported in many earlier studies[19, 35]. Otherwise, many previous studies and in agreement to our findings, a ratio of 1:1, crude extract to *t*-butanol was reported to give maximum partitioning[17, 26, 28] Thus, further experiments were carried out at 1:1 ratio of feed to *t*-butanol.

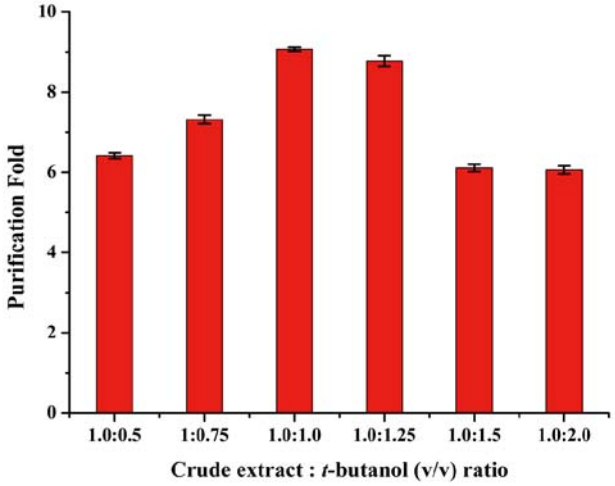

Figure 2. Effect of varying crude extract to *t*-butanol on the degree of purification of plant-esterase enzyme. Various amount of *t*-butanol was added to crude extract and saturated with 50% ammonium sulfate. The incubation temperature was 35°C. The pH value was 7

Effect of temperature on plant-esterase partitioning

Temperature is an important parameter that affects the enzyme configuration and overall stability. It is well known that the reaction rate of enzymes increases with increasing temperature, but after a certain temperature

activity, it decreases due to denaturation[17, 36, 37]. As TPP operates through multiple effects, which includes conformational tightening and changes in protein hydration, it was considered worthwhile to explore the effect of varying process temperature. Most concentration processes are usually carried out at low temperatures. The use of low temperatures in solvent or salt precipitation dissipates the heat generated, ensuring minimal protein denaturation. The requirement of low temperatures in TPP has not been clearly reported in the literature, however Dogan and Tari have observed the improved yield and purification of exo-polygalacturonase using TPP at 25°C rather than 37°C[38]. We investigated the effect of temperature from 4 to 55°C while keeping all other parameters at a constant value such as, 50% $(NH_4)_2SO_4$ saturation concentration, pH 7, broth to *t*-butanol ratio 1:1 (v/v). Fig. 3 illustrated the effect of temperature on partitioning behavior of plant-esterase in TPP. Purity of enzyme was increased with an increase in the temperature. At 35°C temperature, purity was found 9.07-fold. Decrease in purification fold at higher temperature (above 35°C) may be due to thermal deactivation of enzyme. Probable reason behind this may be , as already explained, at 4–35°C temperature, *t*-butanol imparts significant kosmotropic and crowding effects and enhances partitioning of plant--esterase. At higher temperature(above 35°C), there may not be significant kosmotropic and crowding effects which resulted in decreased purity[18]. By considering economic and operational feasibility further optimization study was performed at 35°C. Similar results have been reported by Romy et al. who used the TPP for the purification of nattokinase from fermentation[39].

Effect of pH on plant-esterase partitioning

One of the other important parameter effecting to the enzyme activity is pH. Protein concentration by salting out depends on the sulphate concentration and pH-dependent net charge of the proteins. Electrostatic forces and binding of sulphate anions to cationic protein molecules, which promote macromolecular contraction and conformational shrinkage, are the main causes of the strong sulphate pH dependency in salting out. Proteins tend to precipitate most readily at their pI (isoelectric point). Below the pI, proteins are positively charged and can be quantitatively precipitated out by TPP.

On the other hand, negatively charged proteins are more soluble and not easily precipitated. Reports of invertase[17], laccase[34] precipitating selectively at its pI and leaving most of the contaminant proteins in the aqueous phase, confirms the relevance of pH as an important parameter. Fig. 4. shows the effect of pH on the partition behaviour of plant-esterase during TPP. The purification fold of plant-esterase increases when the pH is increased from 3 to 4. With further increase in pH, the purification fold falls down again. The purification fold of 11.35 at pH 4 could be optimum value for TPP purification due to the better conformational stability of plant-esterase towards *t*-butanol at this pH.

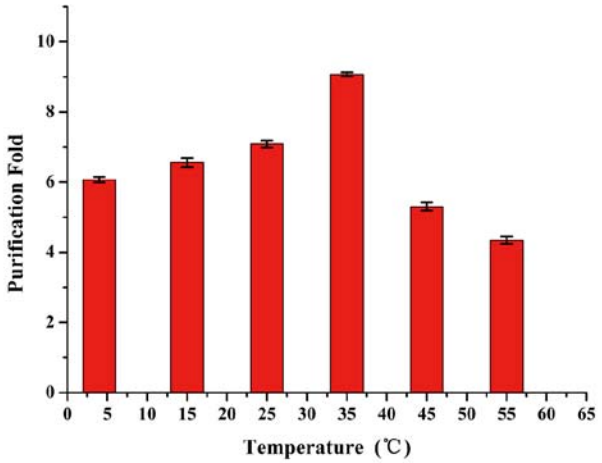

Figure 3. Effect of incubation temperature on the degree of purification of plant-esterase enzyme. Ammonium sulfate (50%, w/v) was added to the crude extract (5 ml containing 67.47 units and 33.83 mg protein). It was followed by addition of *t*-butanol in a ratio of 1.0: 1.0 (v/v).The pH value was 7

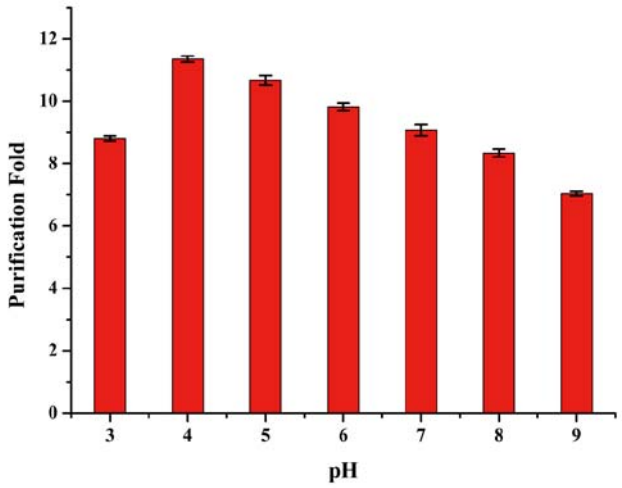

Figure 4. Influence of pH on the degree of purification of plant-esterase enzyme. Ammonium sulfate (50%, w/v) was added to the crude extract (5 ml containing 67.47 units and 33.83 mg protein). The pH of the medium was adjusted to different pH values. This was followed by addition of *t*-butanol in a ratio of 1.0: 1.0(v/v). The incubation temperature was 35°C.

Establishment of regression equation and significance checking for response surface design

Experimental design scheme and results such as Table 1. The results showed that the purification ratio of PlaE was between 4.95515 and 11.5491. Using Design--Expert 8 software to analyze the data in Table 1, study the relationship between various factors and response values, and establish a polynomial regression model of two times. Fitting equation:

Yield = 10.12555 + 1.19846 * A + 0.01189 * B – 0.35748 * C – 0.58404 * D + 1.21266 * A * B – 2.13838 * A * C – 0.73428 * A * D – 0.56449 * B * C + 1.13627 * B * D – 0.59023 * C * D – 0.86222 * A2 – 1.13784 * B2 – 1.76257 * C2 – 1.87381 * D2

ANOVA was used to evaluate the significance of the model, and the results of variance analysis were shown in Table 2. The model F = 51.53231, P < 0.0001 indicates that the model has reached a very significant level; The failure term of the equation is not significant, F = 5.957723, P = 0.0501, indicating that the failure term is not significant relative to the pure error, indicating

Table 1. BBD with the observed response for extraction yield

Run	A:Saturations [%]	B:ration	C:pH	D:temperature [°C]	Yield
1	50	1:1.25	5	35	6.28956
2	40	1:1	3	35	4.95515
3	60	1:1	5	35	6.01511
4	50	1:0.75	3	35	6.78451
5	50	1:1	4	35	9.99267
6	50	1:1	3	45	6.71219
7	60	1:0.75	4	35	7.85798
8	60	1:1	4	25	9.75493
9	50	1:1	4	35	10.04090
10	40	1:0.75	4	35	7.67867
11	50	1:0.75	5	35	7.38942
12	60	1:1	3	35	11.54910
13	50	1:1.25	3	35	7.94260
14	50	1:1.25	4	45	7.45403
15	50	1:1.25	4	25	6.34542
16	40	1:1	4	25	6.01740
17	40	1:1	5	35	7.97471
18	50	1:1	4	35	10.07020
19	50	1:1	4	35	10.42260
20	40	1:1	4	45	6.24542
21	50	1:0.75	4	25	9.29179
22	60	1:1.25	4	35	10.99940
23	50	1:1	3	25	6.63151
24	50	1:1	4	35	10.10130
25	50	1:1	5	45	5.16810
26	50	1:0.75	4	45	5.85533
27	40	1:1.25	4	35	5.96941
28	50	1:1	5	25	7.44833
29	60	1:1	4	45	7.04585

that the regression model can be fitted well with the measured value. Model $R^2 = 0.980964$, the correction of $R^2 = 0.961928$, indicating that the experimental values are highly correlated with the predicted values. The model R^2 is very close to the corrected R^2, mainly due to the large enough number of experimental groups. In one item, A, C, and D, all the interaction items and two terms had significant effect on the purification multiple of plant esterase ($P < 0.05$), and the other items were not significant ($P > 0.05$).

Response surface analysis and optimization

To intuitively illustrate the main factors that affect the response value and the interaction between factors, the software draws a three-dimensional response surface map and a two-dimensional contour map based on the experimental data, as shown in Fig. 5 and Fig. 6. The response surface diagram and contour map reflect the influence of other factors on the response value when two factors remain at 0 level. For example, the response surface diagram Fig. 5 AB and the contour map Fig. 6 AB reflect the interaction between the reaction of pH (C) and the temperature (D) in 0 water, and the reaction between the saturation of ammonium sulfate (A) and the ratio of material to liquid (B). Fig. 5AB data showed that the extraction rate increased significantly with the increase of ammonium sulfate saturation (A); With the increase of material liquid ratio (B), the extraction rate increased significantly. The curve slope of the former is obviously greater than that of the latter, indicating that the effect of ammonium sulfate saturation (A) on the purification multiple of PLaE is greater than that of the liquid ratio (B) on the PLaE purification multiplier, of which ammonium sulphate saturation (A) is at 0.8 level, the corresponding ammonium sulfate saturation is 58%, the ratio of material to liquid (B) is 0.5, and the corresponding ratio of liquid to liquid is 1:1.125, its PLaE purification ratio reached the maximum, reaching 10.7390. Similarly, the best conditions of the other 5 response surfaces can be obtained. The results are shown in Table 3.

The contour map can directly determine the influence of the interaction of factors on the response value. The circular indicates that the interaction is not significant, and the ellipse indicates significant interaction. From Fig. 7, we can see that the interaction between A and B and the interaction between A and C have a significant impact on response value.

Table 3. The optimal solution of different response surface operability and the optimum PLaE purification multiple

Cross effect	A	B	C	D	PF
AB	0.8	0.5	0	0	10.7390
AC	1	0	–0.5	0	11.2691
AD	0.8	0	0	–0.25	10.7082
BC	0	0	0	0	10.1255
BD	0	0	0	–0.25	10.1544
CD	0	0	0	–0.25	10.1544

Table 2. ANOVA for the response surface quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob>F
Model	95.59933	14	6.828524	51.53231	< 0.0001
A- saturations	17.23566	1	17.23566	130.071	< 0.0001
B-ratio	0.001696	1	0.001696	0.012802	0.9115
C- pH	1.533512	1	1.533512	11.57284	0.0043
D-temperature	4.093207	1	4.093207	30.8899	< 0.0001
AB	5.882159	1	5.882159	44.39045	< 0.0001
AC	18.2906	1	18.2906	138.0323	< 0.0001
AD	2.156646	1	2.156646	16.2754	0.0012
BC	1.274594	1	1.274594	9.618886	0.0078
BD	5.164418	1	5.164418	38.97392	< 0.0001
CD	1.393463	1	1.393463	10.51594	0.0059
A∧2	4.8222	1	4.8222	36.39134	< 0.0001
B∧2	8.397881	1	8.397881	63.37566	< 0.0001
C∧2	20.15124	1	20.15124	152.0739	< 0.0001
D∧2	22.77514	1	22.77514	171.8754	< 0.0001
Residual	1.855134	14	0.13251		
Lack of Fit	1.738417	10	0.173842	5.957723	0.0501
Pure Error	0.116717	4	0.029179		
Cor Total	97.45447	28			
R-Squared	0.980964				
Adj R-Squared	0.961928				

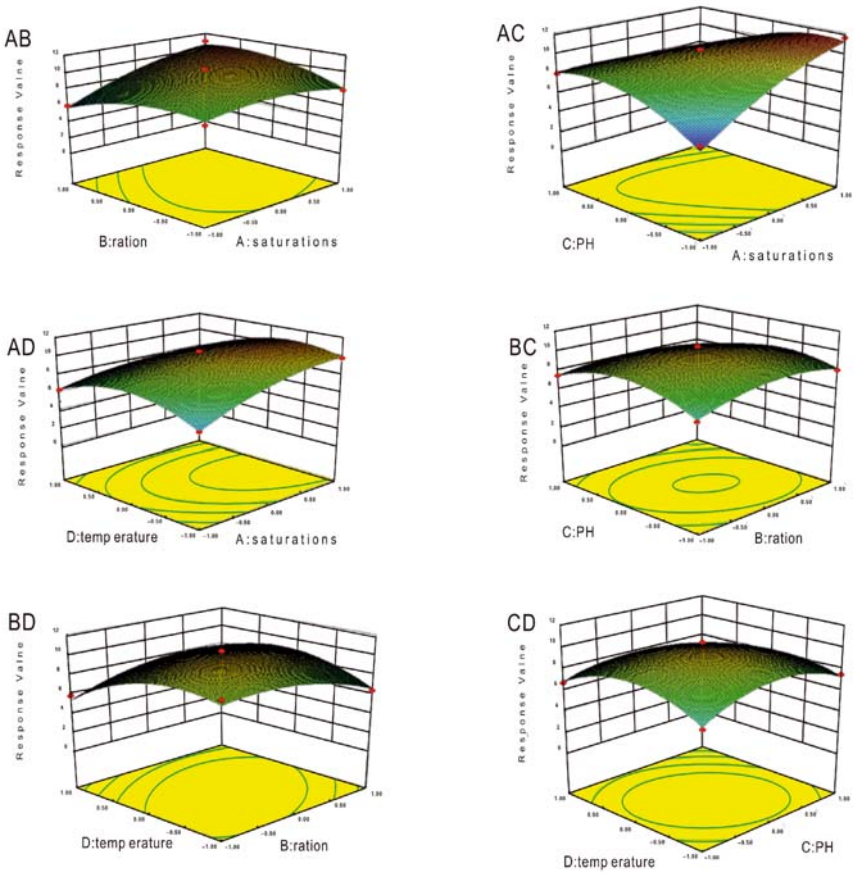

Figure 5. Response surface plots. (A: saturations, B: ration, C: pH, D: temperature)

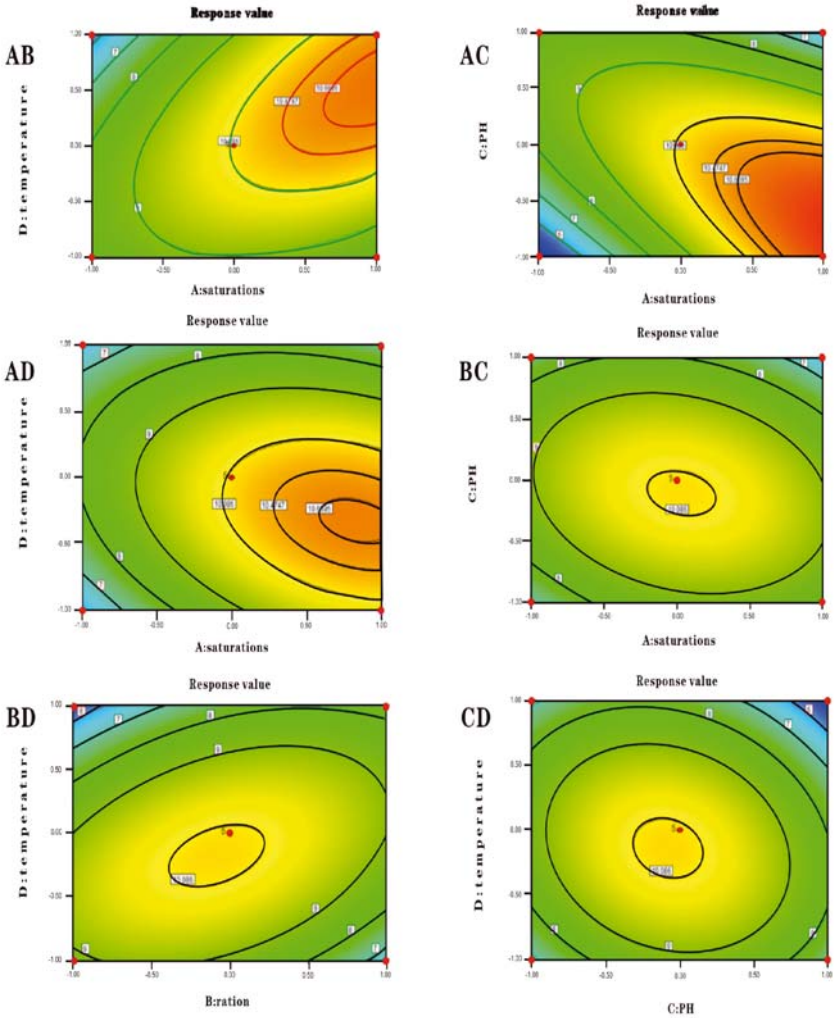

Figure 6. Contours plots. (A: saturations, B: ration, C: pH, D: temperature)

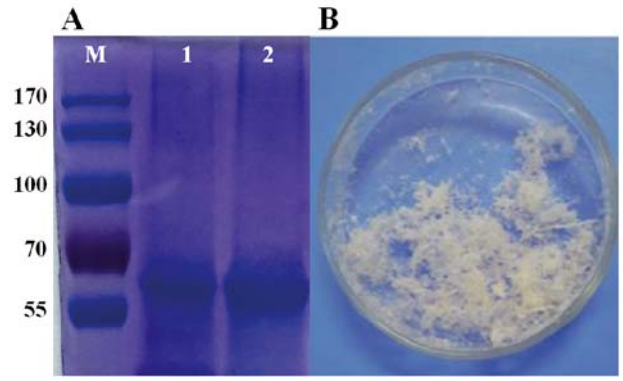

Figure 7. (A) SDS-PAGE of plant esterase (M: molecular mass standards; lane 1: purified plant esterase using TPP system; lane 2: crude extract of plant esterase.); (B) Purified plant esterase in powder form

Verification of the best process

By solving the equation by Design-Expert 8.0 software, the optimum purification conditions are as follows: the saturation level of ammonium sulfate is 1, the corresponding saturation is 60%, the ratio of material to liquid is 0.8, the corresponding ratio of material and liquid is 1:1.2, the level of pH is –0.75, the corresponding pH is 3.25, the corresponding temperature is 0, the corresponding value is 35, and the corresponding PLaE purification times The maximum value of the number theory is 11.9323. The experiment was repeated 3 times according to the optimum conditions, and the actual value was 12.0326. The results showed that the model was accurate and reliable, and could be used for the purification process of plant esterase.

SDS-PAGE ANALYSIS

As shown in the SDS-PAGE gel analysis (Fig. 7A), TPP system can significantly improve the purity of the as-prepared plant-esterase (lane 1 vs lane 2). The molecular weight of the purified plant esterase was measured to be about 68 kDa, which is in good agreement with the reported value[8, 12]. Fig. 7B shows the purified plant--esterase in white powder after freeze-drying.

CONCLUSIONS

To the best of our knowledge, this is the first report for purification and characterization of plant-esterase by using three phase partitioning (TPP). In comparison to chromatographic methods, TPP is very cheap, simple and efficient method. Effect of various process parameters has been evaluated and plant-esterase was purified to 11.35-fold by optimized single step TPP system (50%, (w/v) $(NH_4)_2SO_4$saturation, 1.0:1.0 (v/v) ratio of crude extract: *t*-butanol at pH 4).The enzyme was found to be exclusively partitioned in the aqueous phase. Using TPP system, plant-esterase quickly purified to homogeneity with very high purity and activity, showing an effective activity compared to the classical purification protocols. It can be concluded that, as fast elegant non-chromatographic separation technique, TPP could be attractive for the purification of plant-esterase.

Acknowledgment

The present study was supported by The Sichuan Provincial Education Office Project(17ZA0267),Talent introduction project of Sichuan University of Science and Engineering(2018RCL24) and Key Laboratory of fine chemicals and surfactant in Colleges and universities of Sichuan Province(2018JXY05), Sichuan Science and Technology Department Project (2019YJ0463), Innovation and entrepreneurship training program for College Students(S201910622037&cx2019050).

Conflict of Interest: The authors declare that they have no conflict of interest.

LITERATURE CITED

1. Liu, R.H., Yang, C,. Xu, Y.M., Xu, P., Jiang, H. & Qiao, C.L. (2013). Development of a Whole-Cell Biocatalyst/Biosensor by Display of Multiple Heterologous Proteins on the Escherichia coli Cell Surface for the Detoxification and Detection of Organophosphates. *J. Agric. Food Chem.* 61, 7810–7816. DOI: 10.1021/jf402999b.

2. Singh, B.K. (2009). Organophosphorus-degrading bacteria: ecology and industrial applications. *J. Nat. Rev. Microbiol*. 7, 156–164. DOI: 10.1038/nrmicro2050.

3. Wang, M., Gu, X., Zhang, G., Zhang, D. & Zhu, D. (2009). Continuous Colorimetric Assay for Acetylcholinesterase and Inhibitor Screening with Gold Nanoparticles. *J. Langmuir*. 25, 2504–2507. DOI: 10.1021/la803870v.

4. Zhang, L., Zhang, A., Du, D. & Lin, Y. (2012). Biosensor based on Prussian blue nanocubes/reduced graphene oxide nanocomposite for detection of organophosphorus pesticides. *J. Nanoscale.* 4, 4674–4679. DOI: 10.1039/c2nr30976a.

5. Wei, M. & Wang, J. (2015). A novel acetylcholinesterase biosensor based on ionic liquids-AuNPs-porous carbon composite matrix for detection of organophosphate pesticides. *J. Sens. Actuator B-Chem.* 211, 290–296. DOI: 10.1016/j.snb.2015.01.112.

6. Xia, N., Wang, Q. & Liu, L. (2015). Nanomaterials-Based Optical Techniques for the Detection of Acetylcholinesterase and Pesticides. *J. Sensors* 15, 499–514. DOI: 10.3390/s150100499.

7. Zhao, H., Ji, X., Wang, B., Wang, N., Li, X., Ni, R. & Ren, J. (2015). An ultra-sensitive acetylcholinesterase biosensor based on reduced graphene oxide-Au nanoparticles- beta-cyclodextrin/ Prussian blue-chitosan nanocomposites for organophosphorus pesticides detection. *Biosens. & Bioelectr.* 65, 23–30. DOI: 10.1016/j.bios.2014.10.007.

8. Yang, L., Huo, D., Hou, C., He, K., Lv, F., Fa, H. & Luo, X. (2010). Purification of plant-esterase in PEG1000/NaH2PO4 aqueous two-phase system by a two-step extraction. *J. Process Biochem.* 45, 1664–1671. DOI: 10.1016/j.procbio.2010.06.018.

9. Cummins, I., Burnet, N. & Edwards, R. (2001). Biochemical characterisation of esterases active in hydrolysing xenobiotics in wheat and competing weeds. *J. Physiol. Plantarum.* 113, 477–485. DOI: 10.1034/j.1399-3054.2001.1130406.x.

10. Cummins, I. & Edwards, R. (2004). Purification and cloning of an esterase from the weed black-grass (Alopecurus myosuroides), which bioactivates aryloxyphenoxypropionate herbicides. *Plant J.* 39, 894–904. DOI: 10.1111/j.1365--313X.2004.02174.x.

11. Huo, D., Yang, L. & Hou, C. (2009). Optical Detection of Dimethyl Methyl-Phosphonate with Monosulfonate Tetraphenyl Porphyrin-Plant-Esterase Complex. *J. Sensor Letters.* 7, 72–78. DOI: 10.1166/sl.2009.1012.

12. Bao, J., Hou, C., Chen, M., Li, J., Huo, D., Yang, M., Luo, X. & Lei, Y. (2015). Plant Esterase-Chitosan/Gold Nanoparticles-Graphene Nanosheet Composite-Based Biosensor for the Ultrasensitive Detection of Organophosphate Pesticides. *J. Agric. Food Chem*. 63, 10319–10326. DOI: 10.1021/acs.jafc.5b03971.

13. Li, J.K., Zhou, Y.L., Wen, Y.X., Wang, J.H. & Hu, Q.H. (2009). Studies on the Purification and Characterization of Soybean Esterase, and Its Sensitivity to Organophosphate and Carbamate Pesticides. *J. Agric. Sci. China.* 8, 455–463. DOI: 10.1016/S1671- 2927(08)60232-1.

14. Jiang, B., Feng, Z., Liu, C., Xu, Y., Li, D. & Ji, G. (2015). Extraction and purification of wheat-esterase using aqueous two-phase systems of ionic liquid and salt. *J. Food Sci. Technol.-Mysore.* 52, 2878–2885. DOI: 10.1007/s13197-014-1319-5.

15. Diamond, A.D. & Hsu, J.T. (1992). Aqueous two-phase systems for biomolecule separation. *Adv. Biochem. Engineer. Biotechnol*. 47, 89–135. DOI: 10.1007/BFb0046198.

16. Sagu, S.T., Nso, E.J., Homann, T., Kapseu, C. & Rawel, H.M. (2015). Extraction and purification of beta-amylase from stems of Abrus precatorius by three phase partitioning. *J. Food Chem.* 183, 144–153. DOI: 10.1016/j.foodchem.2015.03.028:

17. Ozer, B., Akardere, E., Celem, E.B. & Onal, S. (2010). Three-phase partitioning as a rapid and efficient method for purification of invertase from tomato. *Biochem. Eng. J.* 50, 110–115. DOI: 10.1016/j.bej.2010.04.002.

18. Dennison, C. & Lovrien, R. (1997). Three phase partitioning: Concentration and purification of proteins. *J. Protein Express. Purific.* 11, 149–161. DOI: 10.1006/prep.1997.0779.

19. Gagaoua, M., Boucherba, N., Bouanane-Darenfed, A., Ziane, F., Nait-Rabah, S., Hafid, K. & Boudechicha, HR. (2014). Three-phase partitioning as an efficient method for the purification and recovery of ficin from Mediterranean fig (Ficus carica L.) latex. *J. Separat. Purific. Technol.* 132, 461–467. DOI: 10.1016/j.seppur.2014.05.050.

20. Duman, Y. & Kaya, E. (2013). Purification, recovery, and characterization of chick pea (Cicer arietinum) beta-galactosidase in single step by three phase partitioning as a rapid and easy technique. *J. Protein Express. Purific.* 91, 155–160. DOI: 10.1016/j.pep.2013.08.003:

21. Akardere, E., Ozer, B., Celem, E.B. & Onal, S. (2010). Three-phase partitioning of invertase from Baker's yeast. *J. Separat. Purific. Technol.* 72, 335–339. DOI: 10.1016/j.seppur.2010.02.025.

22. Moelbert, S., Normand, B. & Rios, PD. (2004). Kosmotropes and chaotropes: modelling preferential exclusion, binding and aggregate stability. *J. Biophys. Chem.* 112, 45–57. DOI: 10.1016/j.bpc.2004.06.012.

23. Vanasperen, K. (1962). A study of housefly esterases by means of a sensitive colorimetric method. *J. Insect. Physiol*. 8, 401–416. DOI: 10.1016/0022-1910(62)90074-4.

24. Bradford, M.M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *J. Anal. Biochem.* 72, 248–254. DOI: 10.1016/0003-2697(76)90527-3.

25. Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of head of bacteriophage-t4. *J. Nature* 227, 680–685. DOI: 10.1038/227680a0.

26. Gagaoua, M., Hoggas, N. & Hafid, K. (2015). Three phase partitioning of zingibain, a milk-clotting enzyme from Zingiber officinale Roscoe rhizomes. *Internat. J. Biologic. Macromol.* 73, 245–252. DOI: 10.1016/j.ijbiomac.2014.10.069.

27. Vetal, M.D., Shirpurkar, N.D. & Rathod, V.K. (2014). Three phase partitioning coupled with ultrasound for the extraction of ursolic acid and oleanolic acid from Ocimum sanctum. *J. Food Bioprod. Process.* 92, 402–408. DOI: 10.1016/j.fbp.2013.09.002.

28. Narayan, A.V., Madhusudhan, M.C. & Raghavarao, K. (2008). Extraction and Purification of Ipomoea Peroxidase Employing Three-phase Partitioning. *J. Appl. Biochem. Biotech.* 151, 263–272. DOI: 10.1007/s12010-008-8185-4.

29. Vetal, M.D. & Rathod, V.K. (2015). Three phase partitioning a novel technique for purification of peroxidase from orange peels (Citrus sinenses). *J. Food Bioprod. Process.* 94, 284–289. DOI: 10.1016/j.fbp.2014.03.007.

30. Pike, R.N. & Dennison, C. (1989). Protein Fractionation by 3 Phase Partitioning (Tpp) In Aqueous Tert-Butanol Mixtures. *J. Biotech. Bioeng.* 33, 221–228. DOI: 10.1002/bit.260330213.

31. Qiao, Y.Y., Tong, J.F., Wei, S.Q., Du, X.Y. & Tang, X.Z. (2009). Economic methods of ginger protease's extraction and purification. p. 1619–1628. DOI: 10.1007/978-1-4419-0213-9_12.

32. Nafi', A., Ling, F.H., Bakar, J. & Ghazali, H.M. (2014). Partial Characterization of an Enzymatic Extract from Bentong Ginger (Zingiber officinale var. Bentong). *J. Molecules.* 19, 12336–12348. DOI: 10.3390/molecules190812336.

33. Wati, R.K., Theppakorn, T., Benjakul, S. & Rawdkuen, S. (2009). Three-phase partitioning of trypsin inhibitor from legume seeds. *J. Process. Biochem.* 44, 1307–1314. DOI: 10.1016/j.procbio.2009.07.002.

34. Rajeeva, S. & Lele, SS. (2011). Three-phase partitioning for concentration and purification of laccase produced by submerged cultures of Ganoderma sp. WR-1. *Biochem. Eng. J.* 54. DOI: 10.1016/j.bej.2011.02.006.

35. Duman, Y.A. & Kaya, E. (2013). Three-Phase Partitioning as a Rapid and Easy Method for the Purification and Recovery of Catalase from Sweet Potato Tubers (Solanum tuberosum). *J. Appl. Biochem. Biotechnol.* 170, 1119–1126. DOI: 10.1007/s12010-013-0260-9.

36. Kumar, V.V., Sathyaselvabala, V., Premkumar, M.P., Vidyadevi, T. & Sivanesan, S. (2012). Biochemical characterization of three phase partitioned laccase and its application in decolorization and degradation of synthetic dyes. *J. Molec. Catal. B-Enzymatic.* 74, 63–72. DOI: 10.1016/j.molcatb.2011.08.015.

37. Bayraktar, H. & Onal, S. (2013). Concentration and purification of alpha-galactosidase from watermelon (Citrullus vulgaris) by three phase partitioning. *J. Separat. Purific. Technol.* 118, 835–841. DOI: 10.1016/j.seppur.2013.08.040.

38. Dogan, N. & Tari, C. (2008). Characterization of three--phase partitioned exo-polygalacturonase from Aspergillus sojae with unique properties. *J. Biochem. Eng.* 39, 43–50. DOI: 10.1016/j.bej.2007.08.008.

39. Garg, R. & Thorat, B.N. (2014). Nattokinase purification by three phase partitioning and impact of t-butanol on freeze drying. *J. Separ. Purific. Technol.* 131, 19–26. DOI: 10.1016/j.seppur.2014.04.011.