Selective Extraction and Purification of Papain Using Polyethylene Glycol (PEG 4000)/Potassium Citrate Aqueous Two Phase

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Mathematical models concerning the purification of papain from the crude papain in an aqueous two-phase system which are composed of polyethylene glycol (PEG 4000)/potassium citrate are established with response surface methodology. The concentration of PEG 4000 and potassium citrate, the concentration of additional salt (KCl), the temperature and pH on the papain purification have been investigated. Results showed that papain tends to partitions to the more hydrophilic salt-rich phase. The steepest ascent method was used to locate the optimal domain. Papain was successfully extracted to the bottom phase of a system composed of 15 % (w/w) PEG 4000, 17 % (w/w) citrate and 5.0 % (w/w) KCl at pH 5.0 and 30 °C. Under those conditions, the specific activity, purification factor and activity yield for papain were 1703.02 U/mg, 1.89 and 94 %, respectively. The purity of extracted papain was confirmed by SDS-PAGE analysis.

Keywords: Aqueous two-phase system, Papain, Purification, Fractional factorial design, Response surface methodology.

INTRODUCTION

Papain is an enzyme which is found naturally in unripe papayas. This enzyme has a number of interesting applications in cell isolation, leather, cosmetic, textiles, detergents, food and pharmaceutical industries[1-3]. Papain is usually produced as a crude, dried material by collecting the latex from the fruit of the papaya tree. However, the purified enzyme using traditional methods still remains problematic or it is contaminated with other proteases and cannot be used in certain pharmaceutical applications[4-6]. A purification step is necessary to remove contamination. This purification consists of the solubilization and extraction of the active papain. Different methods, such as precipitation methods[7,8] and chromatography methods[9-12], can be applied to purify papain from papaya latex. However, the purified enzyme still remains contaminated by other proteases and the initial processing of the latex is essential before samples are applied on a chromatography column[4]. It is desirable to develop new techniques that either improve or totally or partially replace some stages of the current purification procedures.

Aqueous two-phase extraction has been proved to be a versatile technique for separating and purifying mixtures of biomolecules. Compared to other separation techniques, aqueous two phase extraction offers many advantages such as biocompatible environment for the biomolecules, ease of scale-up, lower interfacial tension and scope for continuous operation[13-17]. As a pioneering work, PEG-phosphate system has been reported for separation and purification of papain from papaya latex[18]. The papain was extracted to the PEG rich phase by modifying PEG with Procion Blue. In polyethylene glycol and ammonium sulfate system, Reactive Red 120 was added to the aqueous two-phase system as a free affinity dye ligand[6]. Papain was obtained in the PEG rich phase. However, the yield was low (50.6 %). In the present work,as an effective alternative, aqueous two phase extraction was attempted for the separation and purification of papain from the crude papain. As the citrate anion is both biodegradable and nontoxic[19], we employed potassium citrate as a substitute for phosphate and sulfate salts. Moreover, the structure-making anions, $C_6H_5O_7^{3-}$ which exhibit stronger interaction with water molecules, are beneficial to the aqueous two-phase system formation[20].

In present work, a detailed study was carried out for the purification of papain using polyethylene glycol (PEG 4000)/ potassium citrate aqueous two-phase system. The optimum conditions were achieved with less cost and shorter time using response surface methodology (RSM). The papain was obtained in the bottom phase and the majority of other protein contaminats were extracted in the other phase in a single step. PEG 4000/potassium citrate aqueous two-phase system is used to purify papain from the crude papain successfully with the specific activity 1703.02 U/mg and activity yield of 94 %.

EXPERIMENTAL

Crude papain was provided by Nanning Dongheng Huadao Biotechnology Limited Company. Potassium citrate, citric acid, *tris*(hydroxymethyl)aminomethane, trichloroacetic acid (TCA), coomassie Blue G250 and R250 were purchased from Sinopharm Chemical Reagent Co., Ltd.. papain, L-tyrosine and casein were purchased from Sigma. PEG (Kermel), L-cystesine (Jpana), EDTA disodium salts (Tieta, Shandong Laiyang) were used without further purification. All the reagents were of analytical grade. Pure water was used throughout.

Preparation of the papain solution and aqueous two-phase systems (ATPS): The potassium citrate buffers were prepared by adding a small amount of 50 % (w/w) citric acid solution to 50 % (w/w) potassium citrate solution to reach the desired pH. Aqueous two-phase systems were prepared by mixing appropriate amounts of 50 % (w/w) PEG 4000 stock solution, 50 % (w/w) potassium citrate buffer solution, papain solution and water in order to achieve the desired final system composition. All the systems were mixed using a vortex mixer and centrifuged for 10 min at 1500 rpm after placed at a certain temperature for 30 min. The volume of each phase was measured when the system turned clear. Top and bottom phases were separated with pipettes. The protein concentration and protease activity of those two phases were assayed separately.

The activity partition coefficient (K_a) was defined as the ratio of enzyme activity (U/mL) in the top phase to that in the bottom phase. The activity yield (Y_a) was determined as the ratio of the total activity in the top phase to that in the initial extract. The specific activity (A) was expressed in units per milligram of protein (U/mg) in the top phase. The purification factor (PF) was calculated as the ratio of the specific activity in the bottom phase to the specific activity in the crud papain.

Analytical methods: The protein concentration was determined by the Bradford method[21] using Coomassie Blue G250 with bovine serum albumin (BSA) as the standard. The optical density was measured at 595 nm. Proteolytic activity was assayed by method of Arnon[22] with slight modification. The reaction mixture (containing 200 μL of enzyme solution, 400 μL of 50 mM *Tris*-HCl buffer pH 7 and 50 mM cysteine-20 mM EDTA disodium salts pH 7) was incubated at 37 °C for 5 min. Then 2 mL of 1 % (w/v) casein was added to start the reaction. After 10 min, the reaction was stopped by adding 6 mL of 5 % (w/v) trichloro acetic acid (TCA) and then placed for 1 h at room temperature. The precipitate was separated by centrifugation. The absorbance of the supernatant fluid was measured at 275 nm. The blank sample was prepared with addition of trichloro acetic acid, followed by the enzyme. One unit (U) of proteolytic activity was expressed as the amount of enzyme which produced 1 μmol tyrosine min^{-1} mL^{-1}.

Electrophoresis: The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli[23], using a 12 % polyacrylamide separating gel and a 5 % stacking gel. Commercial pure papain (Sigma) was used to identify the papain bands. Electrophoresis was run at a constant voltage of 100 V. The protein samples migrated towards the cathode during electrophoresis. Proteins bands were stained with Coomassie Blue R250 according to the method of Meyer *et al.*[24].

RESULTS AND DISCUSSION

The five factors which were considered to affect the protein purification in the aqueous two-phase system systems were the concentration of PEG 4000 (X_1), the concentration of potassium citrate (X_2), the concentration of potassium chloride (X_3), pH (X_4) and temperature (X_5). The central composite face-centred design (CCF) was applied using SAS software (Version 9.2 in Windows). The details of the design and result are shown in Table-1. The linear regression equations for the

TABLE-1
CONDITIONS AND RESULTS OF THE FRACTIONAL FACTORIAL DESIGN SELECTED FOR PAPAIN EXTRACTION BY PEG4000/POTASSIUM CITRATE AQUEOUS TWO-PHASE SYSTEMS

Run	X_1^a (%)	X_2^b (%)	X_3^c (%)	X_4^d	X_5^e (K)	K_a^f	A^g (U/mg)	Y_a^h (%)	PF^i
1	15	15	0	5	323	0.46	1506.74	72	1.67
2	21	15	0	5	303	0.49	1317.63	37	1.46
3	15	19	0	5	303	0.45	1344.88	72	1.49
4	21	19	0	5	323	0.96	1211.64	58	1.34
5	15	15	8	5	303	1.91	671.27	34	0.75
6	21	15	8	5	323	1.58	763.87	41	0.85
7	15	19	8	5	323	1.90	577.29	37	0.64
8	21	19	8	5	303	11.54	103.97	5	0.12
9	15	15	0	8	303	0.63	1103.47	64	1.22
10	21	15	0	8	323	4.14	256.65	14	0.28
11	15	19	0	8	323	1.14	888.51	60	0.99
12	21	19	0	8	303	1.09	829.80	47	0.92
13	15	15	8	8	323	1.28	536.85	35	0.60
14	21	15	8	8	303	—	0.00	0	0.00
15	15	19	8	8	303	—	0.00	0	0.00
16	21	19	8	8	323	—	0.00	0	0.00
17	18	17	4	6.5	313	0.52	1130.12	67	1.25
18	18	17	4	6.5	313	0.46	994.60	59	1.10
21	18	17	4	6.5	313	0.59	1065.80	63	1.18

$^a X_1$: PEG 4000 concentration (w/w), $^b X_2$: potassium citrate concentration (w/w), $^c X_3$: potassium chloride concentration (w/w), $^d X_4$: pH, $^e X_5$: temperature, $^f K_a$: activity partition coefficient, $^g A$: protease activity in the bottom phase, $^h Y_a$: activity yield in the bottom phase; ^{i}PF: purification factor in the bottom phase

responses could be obtained from the regression results of fractional factorial experiment (in uncoded levels).

$$\text{Activity recovery } (Y_a) = 54.7 - 3.58\, X_1 - 0.56\, X_2 - 4.25\, X_3 - 5.67\, X_4 + 0.36\, X_5 \quad (1)$$

$$\text{Purification factor (PF)} = 3.20 - 0.050\, X_1 - 0.042\, X_2 - 0.10\, X_3 - 0.18\, X_4 + 0.0026\, X_5 \quad (2)$$

In general, the magnitude of the coefficients of the linear regression models could be used to evaluate the contribution of the corresponding independent variables to the dependent variables[25]. The linear regression equations reveal that the concentration of potassium citrate (X_2) and temperature (X_5) are the less significant factors for both activity recovery (Y_a) and purificatino factor (PF). In the following studies, both of the two factors are fixed at constant value. The factorial analysis of variance in Table-2 also indicates the concentration of PEG 4000 (X_1) and potassium chloride (X_3) are significant factors ($p < 0.05$) for Y_a and the concentration of potassium chloride (X_3), pH (X_4) are found to be significant factors ($p < 0.05$) for PF. In these cases the 'Pr > F' less than 0.05 indicates the particular term is statistically significant[26].

Path of steepest ascent: According to the regression equations and analysis results from fractional factorial experiment, the path of steepest ascent experiment is to maximize the response. Table-3 displays how the variables should be oriented. The direction of improvement starts from the center point of the fractional factorial experiment and moves away from it. It is obvious that the aqueous two-phase system 6 is up to the general vicinity of the optimum. The activity recovery (Y_a) and purification factor (PF) are to 88 % and 1.77, respectively.

Response surface method experiments: A central composite face-centred design (CCF) was employed to optimize the three most significant factors, identified by fractional factorial experiment [PEG 4000 concentration (X_1), KCl concentration (X_3) and pH (X_4)] for enhancing both the recovery and the purification factor. Table-4 shows the design and the results of this experiment. Statistical analysis of papain purification using regression model was checked by the Fisher's F-test for analysis of variance (ANOVA) and the results are shown in Table-5. Moreover, the p-value of the models is inferior to 0.05, while the p-value of the lack of fit is higher than 0.05, confirming that all the models are statistically significant with 95 % confidence[27]. The F-values and 'probability > F' values of all the regression equations show that these models are significant. The model determination coefficient R_2 indicates a good response between model prediction and experimental data[28]. The quadratic models for each response variable, including activity partition coefficient (K_a), activity yield (Y_a) and purification factor (PF), are given by (in uncoded levels):

$$K_a = 10.69 - 0.57\, X_1 - 1.66\, X_3 - 1.43\, X_4 + 0.0044\, X_1^2 + 0.042\, X_1 X_3 + 0.061\, X_1 X_4 + 0.057\, X_3^2 + 0.12 X_3 X_4 + 0.018\, X_4^2,\ R^2 = 92.79\,\% \quad (3)$$

$$Y_a = -1675.35 + 68.43\, X_1 + 109.59\, X_3 + 387.40\, X_4 - 2.15\, X_1^2 - 3.33\, X_1 X_3 + 1.88\, X_1 X_4 - 1.45\, X_3^2 - 8.1\, X_3 X_4 - 36.37\, X_4^2,\ R^2 = 88.18\,\% \quad (4)$$

$$PF = -23.44 + 1.17\, X_1 + 1.89\, X_3 + 4.96\, X_4 - 0.037\, X_{12} - 0.066\, X_1 X_3 + 0.046\, X_1 X_4 - 0.015\, X_3^2 - 0.13\, X_3 X_4 - 0.52\, X_4^2,\ R^2 = 83.5\,\% \quad (5)$$

Response surface plots were obtained for the measured responses based on the experimental model. These plots provide a method to visualize the relationship between responses and experimental levels of each variable as well as the type of interactions between two test variables[6,29]. With only three variables, it is possible to obtain numerous response surfaces.

TABLE-2
EFFECTS OF DIFFERENT FACTORS FOR Y_a AND PF

Variables	Test of significance (Y_a)[a]		Test of significance (PF)[b]	
Code	t	Pr > |t|	t	Pr > |t|
X_1[c]	-2.47117	0.0281	-2.0261	0.0638
X_2[d]	-0.25861	0.8000	-1.13361	0.2774
X_3[e]	-3.90789	0.0018	-5.48307	0.0001
X_4[f]	-1.95395	0.0726	-3.66604	0.0028
X_5[g]	0.833301	0.4197	0.349908	0.7320

[a]Y_a: activity yield in the bottom phase, [b]PF: purification factor in the bottom phase, [c]X_1: PEG 4000 concentration (w/w), [d]X_2: potassium citrate concentration (w/w), [e]X_3: potassium chloride concentration (w/w), [f]X_4: pH, [g]X_5: temperature

TABLE-3
EXPERIMENTAL DESIGN OF THE ASCENT AND CORRESPONDING RESULTS

Run	X_1[a] (%)	X_3[b] (%)	X_4[c]	K_a[d]	A[e] (U/mg)	Y_a[f] (%)	PF[g]
1	18.00	4.00	6.50	0.50	1331.60	83	1.48
2	17.80	3.70	6.30	0.53	1311.73	78	1.46
3	17.60	3.40	6.10	0.47	1313.40	78	1.46
4	17.40	3.10	5.90	0.43	1459.29	84	1.62
5	17.20	2.80	5.70	0.40	1556.97	88	1.73
6	17.00	2.50	5.50	0.52	1594.89	88	1.77
7	16.80	2.20	5.30	0.52	1583.01	85	1.76
8	16.60	1.90	5.10	0.50	1583.48	80	1.76
9	16.40	1.60	4.90	0.46	1385.09	64	1.54
10	16.20	1.30	4.70	0.69	1434.84	55	1.59
11	16.00	1.00	4.50	0.57	1217.91	17	1.35

[a] X_1: PEG 4000 concentration (w/w), [b] X_3: potassium chloride concentration (w/w), [c] X_4: pH; K_a: activity partition coefficient, [d] K_a: activity partition coefficient, [e] A: protease activity in the bottom phase, [f] Y_a: activity yield in the bottom phase, [g] PF: purification factor in the bottom phase

TABLE-4
UNCODED VALUES OF THE VARIABLES USED IN THE DIFFERENT EXPERIMENTAL ASSAYS OF THE
CENTRAL COMPOSITE DESIGN AND THE CORRESPONDING EXPERIMENTAL RESULTS

Run	X_1^a (%)	X_3^b (%)	X_4^c	K_a^d	A^e (U/mg)	Y_a^f (%)	PF^g
1	15	0	5	0.82	1048.40	41	1.16
2	19	0	5	0.48	1306.52	50	1.45
3	15	5	5	0.38	1765.01	97	1.96
4	19	5	5	0.58	1142.02	55	1.27
5	15	0	6	0.71	766.44	44	0.85
6	19	0	6	0.31	1498.98	76	1.66
7	15	5	6	0.59	1209.25	75	1.34
8	19	5	6	1.33	441.66	25	0.49
9	15	2.5	5.5	0.25	1350.33	80	1.50
10	19	2.5	5.5	0.31	1516.73	79	1.68
11	17	0	5.5	0.64	1650.02	84	1.83
12	17	5	5.5	0.60	1315.82	74	1.46
13	17	2.5	5	0.32	1379.25	70	1.53
14	17	2.5	6	0.22	1524.17	88	1.69
15	17	2.5	5.5	0.26	1389.18	76	1.54
16	17	2.5	5.5	0.25	1480.20	81	1.64
17	17	2.5	5.5	0.32	1360.98	75	1.51

[a]X_1: PEG 4000 concentration (w/w), [b]X_3: potassium chloride concentration (w/w), [c]X_4: pH, [d]K_a: activity partition coefficient, [e]A: protease activity in the bottom phase, [f]Y_a: activity yield in the bottom phase, [g]PF: purification factor in the bottom phase

TABLE-5
ANALYSIS OF VARIANCE (ANOVA) FOR THE QUADRATIC MODELS
PREDICTED FOR EACH RESPONSE VARIABLE

Response variable	soure	DF	SS	MS	F	Pr > F
K_a^a	Model	9	1.197314	0.133035	10.00664	0.0031
	Error	7	0.093063	0.013295		
	Lack of fit	5	0.090438	0.018088	13.78427	0.0690
	Pure error	2	0.002624	0.001312		
	Total	16	1.290376			
Y_a^b	Model	9	5424.732	602.748	5.80055	0.0151
	Error	7	727.3856	103.9122		
	Lack of fit	5	654.7189	130.9438	3.603957	0.2314
	Pure error	2	72.66667	36.3333		
	Total	16	6152.118			
PF^c	Model	9	1.694213	0.188246	3.938121	0.042
	Error	7	0.334607	0.047801		
	Lack of fit	5	0.325041	0.065008	13.5919	0.0699
	Pure error	2	0.009566	0.004783		
	Total	16	2.02882			

[a] K_a: activity partition coefficient, [b] Y_a: activity yield in the bottom phase, [c] PF: purification factor in the bottom phase

The plots that will be shown in this section are those that generated a significant change on the response variables under analysis[27]. The 3D plots are shown in Fig. 1. By analyzing the surface plots, the effects of KCl concentration and pH on the response were observed. Fig. 1(a) indicates that the lowest value of K_a occurs at a low pH within the range of experimentation. The similar phenomenon has been reported by Ling *et al.*[6]. Correspondingly, the highest activity recovery was obtained at pH 5.0 in the bottom phase (Fig. 1b). This trend can be interpreted by the net negative charge of the papain surface. According to the previous studies[30-32], the negatively charged proteins tended to be distributed to the upper phase and the positively charged proteins to the bottom phase in the PEG-salt systems. The response surface plot (Fig. 1c) for the purity of papain in the bottom phase shows that higher purification factors are generally achieved with high concentrations of KCl. This means that the contaminant proteins partition

preferentially to the top phase while the papain remains in the bottom phase at the high concentration of KCl. The optimal value of purification factor (1.95) for papain was obtained.

Using a fractional faction design followed by a RSM design, the final optimum values of the parameter are predicted to be at pH 5.0 and 30 °C containing 15 % (w/w) PEG4000, 17 % (w/w) potassium citrate, 5 % (w/w) KCl. The experiments (seven parallel experiments) were conducted to validate the predicted optimum. The average of activity partition coefficient, specific activity, purification factor and activity recovery for papain in the bottom phase are 0.47, 1703 U/mg, 1.89 and 94 %, respectively. Values of experimentally determined and statistically predicted responses reveal that the established models fitted well with the experimental data. It indicates that the applied statistical design and modeling is a reliable tool in the designing processes of the investigated aqueous two-phase system[33].
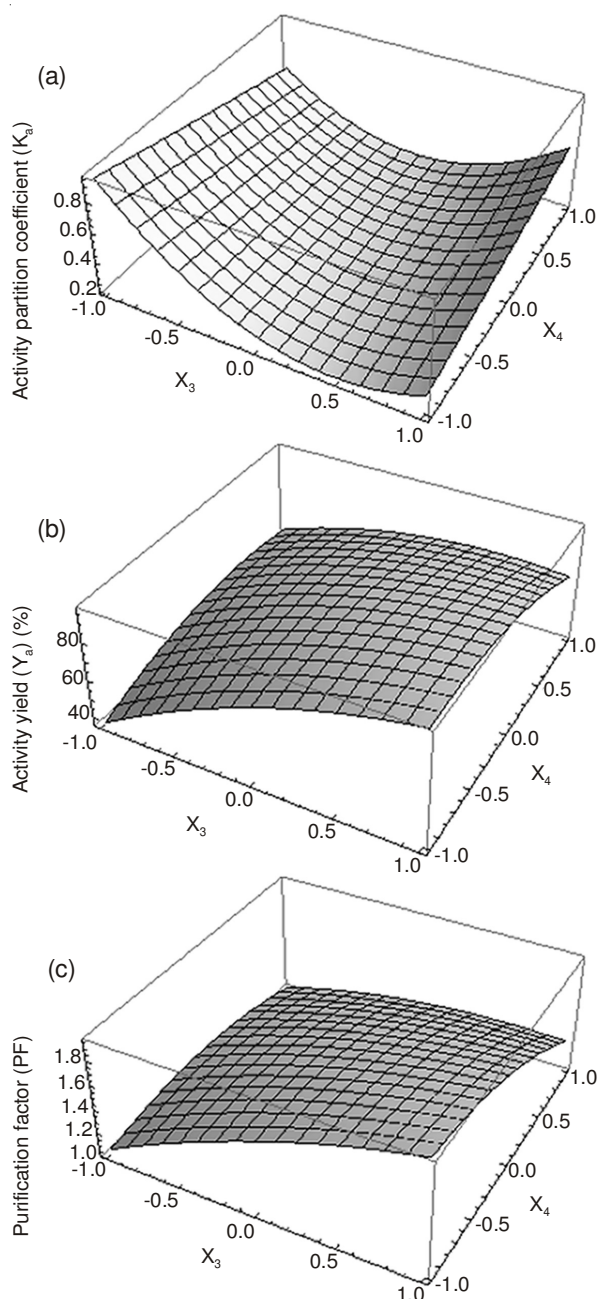
Fig. 1.　Response surface plot for a PEG 4000/potassium citrate aqueous two-phase system for the purification of papain. The actual levels corresponding to the coded levels of each variable-X_3: potassium chloride concentration (w/w) (-1: 0, 1: 5 %); X_4: pH (-1: 5, 1: 6). Other conditions used in the study: 15 % (w/w) PEG 4000; 17 % (w/w) potassium citrate; T = 30 °C
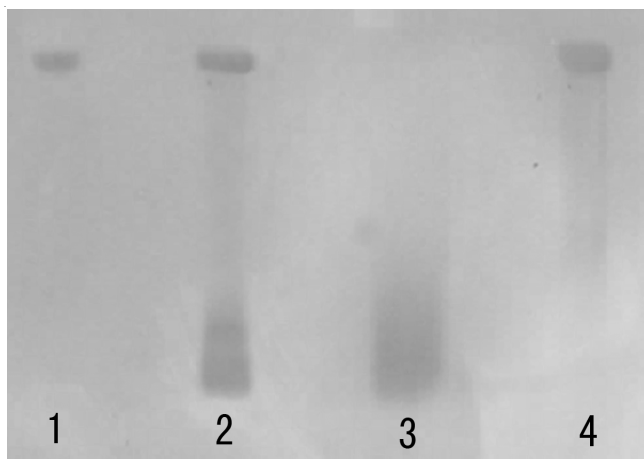

Fig. 2.　SDS-PAGE profile of papain during purification by PEG4000/potassium citrate aqueous two-phase system. Samples on the gel are arranged as followed: (1) papain standard (2) crude papain (3) the PEG-rich phase product after extraction (4) the bottom phase product after extraction.

Conclusion

PEG 4000/potassium citrate aqueous two-phase system was suitable for papain purification. Three successive experimental designs were employed in the present work to optimize the conditions of papain purification. The feasibility of quadratic models and the optimization of papain purification by the RSM were estimated. The papain was recovered in the bottom phase with an activity yield of 94 % and a purification factor of 1.89. Furthermore, the purity of the papain in the bottom phase was identified by SDS-PAGE. Overall results indicated the excellent separation and purification ability of aqueous two phase extraction for papain without the need of multiple steps.

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Evaluation of papain purity by SDS-PAGE: Under the optimal conditions, samples of crude papain, the top and the bottom solutions obtained from the extraction system were identified using SDS-PAGE (Fig. 2). The result (line 3) reveals that the contaminant proteins partitioned to the top PEG-rich phase. Line 4 demonstrates that the papain in the crude papain retained in the bottom phase. The reduction in the number of bands indicates the purity of papain. These results suggested that PEG 4000 / potassium citrate aqueous two-phase system extraction has the potential to be an efficient method and may partially replace some stages of the current purification procedures for purification of papain.

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