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Rhodopseudomonas palustris COLLAGEN-LIKE RECOMBINANT PROTEIN PURIFICATION USING AN AQUEOUS TWO-PHASE SYSTEM

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ABSTRACT: The potential use of recombinant collagen-like protein (recCLP) extracted from bacteria as disease-free collagen has been studied over the past decade. However, the complexity of the downstream processing generates high demand for an efficient and low-cost purification method. Aqueous two-phase system (ATPS) was adopted as a new approach to the recovery of biomolecules due to its simple, benign, and straightforward process. This study aimed to purify recombinant collagen-like protein from *Rhodopseudomonas palustris* using ATPS formed by a polymer/salt system. Recombinant collagen-like protein from *R. palustris* was partitioned in ATPS composed of polyethylene glycol (PEG) and potassium phosphate and several factors that influence the protein partitioning such as volume ratio, system pH, the concentration of polymer and salt were studied. Then, optimization of the selected ATPS conditions (PEG and salt concentration) were performed using response surface methodology (RSM). Results showed that the optimum conditions were found in ATPS with 24.80% (w/w) PEG 2000 and 29.23% (w/w) potassium phosphate with recCLP concentration of 3.23 ± 0.12 mg/mL with purification factor 7.48 ± 0.3. In comparison with the affinity chromatography method, ATPS was found to be low-cost, and time-saving with a higher protein recovery. Hence, this study demonstrated the potential application of ATPS in the recovery of recombinant CLPs for large-scale downstream processing.

ABSTRAK: Potensi penggunaan protein seperti kolagen rekombinan (recCLP) yang diekstrak daripada bakteria sebagai kolagen bebas penyakit telah dikaji sejak sedekad yang lalu. Walau bagaimanapun, kerumitan pemprosesan hiliran menjana permintaan yang tinggi untuk kaedah penulenan yang cekap dan berpatutan. Sistem akueus dua fasa (ATPS) telah diterima pakai sebagai pendekatan baharu dalam pemulihan biomolekul kerana prosesnya yang mudah. Tujuan utama kajian ini adalah untuk menyaring protein seperti kolagen rekombinan daripada *Rhodopseudomonas palustris* menggunakan ATPS yang dibentuk oleh sistem polimer/garam. Protein seperti kolagen rekombinan daripada *R. palustris* telah dibahagikan dalam ATPS yang terdiri daripada polietilena glikol (PEG) dan kalium fosfat dan beberapa faktor yang mempengaruhi pembahagian protein seperti nisbah isipadu, pH sistem, kepekatan polimer dan garam telah dikaji. Kemudian, keadaan ATPS terpilih (PEG dan kepekatan garam) telah dioptimumkan menggunakan metodologi permukaan tindak balas (RSM). Keputusan menunjukkan bahawa keadaan optimum dalam ATPS adalah 24.80% (b/b) PEG 2000 dan 29.23 % (b/w) kalium fosfat dengan kepekatan recCLP 3.23 ± 0.12 mg/mL dengan faktor penulenan 7.48 ± 0.3. Berbanding dengan kaedah kromatografi afiniti, ATPS didapati menjimatkan kos dan

masa dengan pemulihan protein yang lebih tinggi. Oleh itu, kajian ini menunjukkan potensi aplikasi ATPS dalam pemulihan CLP rekombinan untuk pemprosesan hiliran berskala besar.

KEYWORDS: *recombinant collagen-like protein; R. palustri; aqueous two-phase system; purification; chromatography*

1. INTRODUCTION

Collagen is the main structural protein in the extracellular matrix of the animal kingdom comprised of specific amino acid compositions such as glycine (Gly), proline (Pro), and hydroxyproline that twisted together in the form of a triple-helix structure [1]. It strengthens the skin and benefits its elasticity and firmness besides helping in tissue and organ development. Widespread applications of collagen in cosmetic, biomedical, and pharmaceutical industries make collagen a major industrial component [2]. Furthermore, owing to its high biodegradability and biocompatibility thus making it is applicable as a biomaterial in the biomedical field [3].

Apart from mammalian and marine collagen, scientists have discovered collagen-like proteins (CLPs) annotated in bacteria, viruses, and archaea that shared the similar (Gly-Xaa-Yaa)n repetitive amino acid sequences of mammalian collagen but different in terms of amino acid content and distribution [4]. CLPs in bacteria can form triple helix structures similar to mammalian collagens with high thermal stabilities (Tm = 36.5-40 °C) despite the lack of hydroxyproline (Hyp), which is important in the stabilization of protein structure and promote self-association [5]. Furthermore, the result of calorimetric studies showed that high Hyp content increases the enthalpic contribution thus providing stability to the proteins [6].

It has been reported that some of the CLPs work as a virulence factor to evade the immune system of higher animals and thus promote host cell invasion [7]. Hence the availability of CLPs with similar features with mammalian collagen could benefit humans in establishing well-defined and novel collagen-based biomaterials [8,9]. Furthermore, using recombinant technology, these CLPs can be produced in large quantities [10]. Recombinant DNA technology is one of the recent advances in biotechnology that involve the joining together of DNA segments from different organisms and introduced into *E. coli* to produce a new genetic organism that is useful to science and industry [11]. This technology enables the production of disease-free products, uniform quality and abundance in quantity thus have been successfully exploited in various fields [12].

In recent years, downstream processing remains the major challenge in the production of recombinant protein. Conventional methods like precipitation, filtration, and chromatography need multiple stages to produce highly purified protein. These multi-step purification processes lead to high process cost, time consumption, low yield, and difficulty in scale up is viewed as a significant disadvantaged [13,14]. According to Warner and co-workers [15], nickel columns in affinity chromatography can give higher yield compared to other types of columns.

These difficulties are avoided using an aqueous two-phase system (ATPS) method, a liquid-liquid extraction technique formed when two incompatible water-soluble phase components are mixed at appropriate conditions resulting in the formation of two phases at equilibrium [16]. ATPS is more preferable due to its simplicity, low cost, ease of scale-up, higher biocompatibility, and shorter processing time [17]. Its high water content and low interfacial tensions provide the mild environment for sensitive biomolecules [18]. It has

been documented to be an effective method for protein purification such as bromelain [19], protease [20], collagenase [21], lipase [22], papain [23], pepsinogen [24] and interferon [12]. However, there is still a lack of studies regarding the purification of recCLP using ATPS because of the complex process of protein partitioning that can be affected by many factors. The pH of the ATPS, concentration of salt, molecular weight, and concentration of polymers, are among the factors that can manipulate the distribution of protein molecules [12].

The potential factors that can affect the performance of ATPS such as volume ratio, pH, concentration of PEG, and salt were studied and optimized. In addition, affinity chromatography method was conducted to compare the purification effect of ATPS. The purpose of this study is to provide a simple yet effective method of purifying the recombinant collagen-like protein from *E. coli* crude extract. To date, recombinant collagen-like protein has been purified using the chromatography method [25,26] and no study has been attempted for purification of recombinant collagen-like protein from *E.coli* using the ATPS method, which could be promising in CLPs research.

2. MATERIALS AND METHOD

2.1 Materials

Polyethylene glycol with molecular weight of 2000 g/mol (PEG 2000), dipotassium hydrogen phosphate (K_2HPO_4), and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Merck-Schuchardt (Munich, Germany). Vegitone Luria Bertani agar and Vegitone Luria Bertani Broth were obtained from Sigma-Aldrich (St. Louis, MO, USA) while Enzyfluo Collagen assay kit was procured from BioAssay Systems (Hayward, USA). All chemicals used in this paper are analytical grade.

2.2 Experimental Setup

2.2.1 Microorganism

The recombinant plasmid pColdII-CLP-*R. palustris* was prepared by subcloning the sequence of CLP-*R. palustris* from the source plasmid (PUC57, Genescipt, Hong Kong) to the target plasmid (pColdII TKR-3322, Takara, Japan). The recombinant plasmid was then transformed into *E. coli* B strain (BL21DE3) (New England BioLabs, USA) competent cell using cold shock expression method [20]. Ampicillin selection and colony PCR were performed to verify the successfully transformed pColdII-CLP into BL21 after overnight incubation at 37 °C on LB agar (unpublished data).

2.2.2 Protein Expression

The positive clone of *E. coli* BL21DE harboring collagen-like protein from *R. palustris* was cultured in 100 mL of M9 minimal medium at 37 °C, 250 rpm until OD600 nm reached 0.8-1.0 in an incubator shaker (Infors HT Ecotron, Switzerland). The recCLP culture was then induced at 15 °C by adding 1 mM isopropyl ß-D-1 thiogalactopyranoside. After 24 hours, cultures were centrifuged in a refrigerated centrifuge (Heraeus Multifuge X1R refrigerated centrifuge, Thermo Scientific, USA), at 4696 x*g* for 10 minutes at 4 °C, to remove the debris and tissue. The crude extract was stored at -20°C until further processing.

2.2.3 Cell Lysis

The cell pellets were resuspended in the lysis buffer (20 mM Na_2HPO_4, 0.5 M NaCl, pH 7.4) and sonicated at 40% amplitude, 10 seconds with 20 seconds interval between

each sonication for 10 times using an ultrasonicator (LABSONIC®P, Sortarius, Germany) to break open the cell, therefore releasing the recCLP. Subsequently, the culture was then centrifuged using Eppendorf 580 4R refrigerated centrifuge (Eppendorf, Germany), at 12,000 rpm and 4 °C for 30 minutes to obtain a clear supernatant. The supernatant was considered as the soluble fraction whereas the pellet was known as the insoluble fraction.

2.2.4 Aqueous Two-phase System

ATPS was prepared on fixed mass basis in a 2.0 mL microcentrifuge tube by mixing predetermined quantities of PEG 2000, potassium phosphate buffer at pH 7.0 and 10% (w/w) of crude extract. Distilled water was added to the system to give a total weight of 2 g. The mixture was vortexed to mix for 1 minute and then centrifuged at 1000 x*g* (Heraeus Multifuge X1R, Thermo Scientific, USA), and 25 °C for 10 minutes to speed up phase separation. After that, top and bottom phases were carefully collected for further analysis. The volume of the phases was then used to estimate the volume ratio (VR). It was noted that the most potent ATPS could not be determined by monitoring the total protein in the phases. Thus, the total amount of recombinant collagen-like protein in both phases, partition coefficient (KE), as well as concentration of recCLP, were quantified using fluorometric collagen assay kit.

The binodal curves were referred elsewhere [28]. The strategy behind the selection of the experimental system is well described elsewhere [22]. According to Arshad and Amid [27], the tie-line length (TLL) was estimated graphically by using volume ratio and it can be calculated using the equation as follows,

$$TLL = \sqrt{[(\Delta x^2) + (\Delta y^2)]} \quad (1)$$

where ΔX and ΔY indicate the difference between salt and PEG concentration, respectively.

2.2.5 One-factor-at-a-time (OFAT)

OFAT (one-factor-at-a-time) method was designed and applied for ATPS separation. The tested parameters involved in this study were volume ratio, pH, and concentration of PEG and salt. The response was concentration of recombinant collagen-like protein and purification factor. Data from experiments were expressed as mean ± standard error. Analysis of variance (ANOVA) was performed on the data and statistical significance was defined at $p<0.05$.

2.2.6 Optimization

Response surface methodology (RSM) using Design of Expert 10.0.3 software (State-Ease Inc., Minneapolis MN, USA) was used for the optimization of ATPS conditions. A set of 11 experiments with two independent variables (concentration of polyethylene glycol and potassium phosphate) were coded using the face-centered central composite design with triplicate center point. The values of the center point were obtained based on previous ATPS separation by OFAT. The recCLP concentration and purification factor (PF) were chosen as the responses in each run.

2.2.7 Affinity Chromatography (Fast-Protein Liquid Chromatography)

Affinity chromatography was conducted using 1 mL HisTrap™ High Performance chromatography column (GE Healthcare, USA) prepacked with Ni Sepharose with an inner diameter of 7 mm connected to AKTA Prime Plus® (GE Healthcare, USA). The collected supernatant after cell lysis was filter-sterilized using 0.45 µm pore filter. Then, 2 ml of the solution was loaded into the packed column at a flow rate of 1 mL/min. The

unbound proteins were washed with binding buffer and the target protein was eluted using elution buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). Collected fractions were analyzed by SDS-PAGE and fluorometric collagen assay.

2.2.8 Quantification of Recombinant collagen-like Proteins (recCLP)

The concentration and total recCLP in ATPS samples were measured using a commercially available collagen assay kit (BioAssay Systems, USA). Briefly, collagen in the sample was enzymatically digested into peptides by mixing equal amounts of samples with digest enzyme and incubated at 37°C for 1 hour. Then, dye reagent was added to react with the N-terminal glycine peptides in the solution, incubated at 37°C for 10 minutes, thus forming a fluorescent complex [26]. The sample was transferred to a 384-black flat bottom microplate reader (Corning, USA) and ready for fluorescence reading at $\lambda_{ex/em}$ 375/465 nm using a Spark® multimode microplate reader (Tecan, Switzerland). In this study, bovine collagen type I was used as a standard for collagen assay. The fluorescent intensity, measured at $\lambda_{ex/em}$ 375/465 was directly proportional to the collagen concentration in the sample.

2.2.9 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein sample from crude extract, top phase in ATPS and FPLC fractions were analyzed using SDS-PAGE method. The samples were diluted to 1:1 ratio prior to loading into 12% resolving gel and 4% stacking gel. Electrophoresis is run at 120 V, 400 mA for 60 min, the gels are stained using ReadyBlue™ Protein Gel Stain (Sigma, USA) or silver staining method.

2.3 Calculation

The volume ratio is defined as,

$$V_R = \frac{Volume\ in\ top\ phase}{Volume\ in\ bottom\ phase} \quad (2)$$

The partition coefficient (KE) of the recCLP was calculated as the ratio of total recCLP in two phases,

$$KE = \frac{Total\ collagen\ in\ top\ phase}{Total\ collagen\ in\ bottom\ phase} \quad (3)$$

The purification factor (PF) of the recCLP was calculated according to the equation below,

$$PF = \frac{Collagen\ amount\ in\ sample}{Collagen\ amount\ in\ crude\ lysate} \quad (4)$$

3. RESULTS AND DISCUSSION

3.1 Selection of ATPS

The result of system tie-line length (TLL) and partition coefficient (KE) for ATPS samples composed of PEG 2000/potassium phosphate with VR value of 1.0 are shown in Table 1. The selection of binodal curves was decided according elsewhere [28]. To identify the best combination of ATPS, a quantitative fluorometric collagen assay was done to both top and bottom phases. The highest partition coefficient (KE) was achieved by the system with 26% (w/w) PEG 2000 and 26% (w/w) potassium phosphate with tie-line length (TLL) of 54.78% (w/w), therefore proving that most of the protein of interest were favored in one phase which is PEG-rich top phase. The concentration of salt in the

bottom phase increase as the TLL increase and this may drive recCLP towards the bottom phase as a consequence [12]. Therefore, this combination of ATPS was chosen for further analysis. The bottom phase of ATPS with 26% (w/w) PEG 2000 and 26 % (w/w) potassium phosphate showed a low concentration of recCLP in the quantitative assay (unpublished data). Therefore, in the further experiment, it was decided to conduct detection of recCLP in the top-phase only.

Table 1: The tie-line length (TLL) and partition coefficient (KE) of ATPS with VR of 1.0

% PEG 2000 (w/w)	% Potassium phosphate (w/w)	TLL	KE
25	25	50.51	1.33
26	26	54.78	4.03
27	27	60.26	1.55
28	28	64.42	1.28

3.2 Screening of ATPS Factors Using OFAT (one-factor-at-a-time)

3.2.1 Effect of VR on recCLP

The impact of different volume ratios along the same tie-line of a system consisting of 26% (w/w) potassium phosphate and 26% (w/w) of PEG 2000 at constant pH 7.0 was assessed and shown in Table 2. Five points indicate five different volume ratios (0.33, 0.8, 1.0, 1.57, 3.5) within the same tie-line. In defined ATPS, volume of top phase proportionally increases with the volume ratio resulting in more free volume available for the protein of interest to participate in the top phase. This situation caused a significant negative impact of free volume in the bottom phase [29]. On the other hand, an extremely low volume ratio may have limited free volume, thus limiting the partition of protein [12]. From the preceding result, either increasing or reducing the volume ratio did not cause any significant effect on recCLP concentration in the top phase. As a rule of thumb, researchers who are using ATPS method for the first time are advised to use volume ratio equal to 1, making it convenient to measure the volume of each phase during partition experiments [27].

Table 2: Influence of different volume ratios on recCLP in top-phase.
The data are presented as means ± standard error, $p<0.05$

Volume ratio	recCLP (mg/mL)	Purification factor (PF)
0.33	0.98 ± 0.004	2.77 ± 0.01
0.80	0.84 ± 0.003	5.78 ± 0.02
1.00	1.03 ± 0.006	8,84 ± 0.05

3.2.2 Effect of PEG 2000 % (w/w) on recCLP

The results of PF and concentration of recCLP in different PEG concentration were shown in Table 3. A higher concentration of recCLP with PF of 9.04 ± 0.25 was achieved in ATPS with 26% (w/w) PEG2000. Volume exclusion effect in top phase and salting-out effect in bottom phase are the crucial factors in partition behavior of biomolecules in the polymer-salt system [30]. Those effects are responsible for reducing the available space in the top phase and thus obstruct the partition of biomolecules in the top phase. This hypothesis was concurrent with the behavior of recCLP and PF when the PEG concentration was decreased from 26% (w/w) to 29% (w/w). Meanwhile, the trend with

PEG concentration from 20% (w/w) to 26% (w/w) and from 29% (w/w) to 32% (w/w) was parallel with the theory stated that an increase of polymer concentration caused the movement of biomolecules to PEG-rich phase due to the hydrophobic interactions between biomolecules and PEG [31]. Increasing PEG concentration leads to the increase of top phase volume, resulting in the rising of free volume available for the protein of interest to participate in the top phase [29,32]. Therefore, ATPS with 26% (w/w) of PEG 2000 was further employed for the next factor. However, the best concentration of PEG 2000 in the purification of recombinant recCLP cannot be corroborated with any studies due to a lack of references regarding the application of ATPS in the partition of recCLP.

Table 3: Influence on the concentration of PEG on recCLP concentration and purification factor. The data are presented as means ± standard error, $p<0.05$

%PEG 2000 (w/w)	recCLP (mg/mL)	Purification factor
20	0.944 ± 0.04	6.04 ± 0.27
23	1.08 ± 0.05	7.36 ± 0.33
26	1.18 ± 0.03	9.04 ± 0.25
29	0.95 ± 0.04	7.28 ± 0.33
32	1.10 ± 0.05	9.34 ± 0.39

3.2.3 Effect of Potassium Phosphate % (w/w) on recCLP

In this part, the effect of different potassium phosphate concentrations from 20 to 32% (w/w) at pH 7.0 on the concentration of recCLP was investigated and presented in Table 4. As shown in Table 4, the highest concentration of recCLP was shown in ATPS with 29% (w/w) potassium phosphate, with 0.97 ± 0.006 mg/mL recCLP and PF of 6.33 ± 0.04. The concentration of potassium phosphate % (w/w) is one of the factors which affect the partition behavior of the target protein. Theoretically, ionic strength will increase as the salt concentration in the system increases, thus pushing the proteins to the top phase. Besides, the ability of salt to capture water molecules will increase, hence moving biomolecules to the top phase [8,29]. However, a high concentration of salt also can lead to protein precipitation and denaturation thus reducing the amount of targeted protein in the phase [8,15]. This hypothesis supports the trend shown for concentration of recCLP when the concentration of salt decrease from 29% (w/w) to 32% (w/w). Meanwhile, due to the decreasing volume of top phase (20 to 32% (w/w)), PF decreased. When the free volume in the phase is preferred by protein of interest decreased, it will cause them to concentrate when the limits of protein solubility are exceeded [25]. Thus, the protein content in that phase will decline.

Table 4: Influence on the concentration of potassium phosphate on recCLP concentration and purification factor. The data are presented as means ± standard error, $p<0.05$

% Potassium phosphate (w/w)	recCLP (mg/mL)	Purification factor (PF)
20	0.78 ± 0.03	8.25 ± 0.28
23	0.93 ± 0.03	6.83 ± 0.22
26	0.92 ± 0.01	6.04 ± 0.06
29	0.97 ± 0.006	6.33 ± 0.04
32	0.95 ± 0.006	4.64 ± 0.03

3.2.4 Effect of pH on recCLP

The effect of different pH values on the partitioning of recCLP in ATPS was determined by varying the pH from 6.0-8.0 using different compositions of potassium phosphate salts (KH_2PO_4 and K_2HPO_4), ± 0.5 accuracy of pH. System pH affects the partitioning of proteins by altering the charge and surface properties of the solute. The partitioning behavior of recCLP is expected to be sensitive to the pH levels increased. This is due to the isoelectric point (pI) of the collagen (<7.0), which makes the protein become negatively charged and favors the PEG-rich top phase. According to [33], the zwitterion along collagen molecules are negatively charged, making the entire collagen is negative. Moreover, the pI glycine residues are 5.97 due to its acidic side chain, thus making the collagen-like protein carried a negative charge [34]. When the pH increases from 7.0 to 8.0, the protein of interest favored a partition in the bottom phase, which in turn decreased in protein concentration. Besides that, at higher pH, more contaminants become negatively charged and tend to partition to the top phase [17]. The presence of contaminants in the top phase lowered the concentration of collagen-like protein. Per the results shown in Table 5, the system of pH 7.0 exhibited the highest values for the responses, 1.37 ± 0.13 mg/ml of recCLP and PF of 6.44 ± 0.61. As a result, ATPS with pH 7.0 was chosen for further study.

Table 5: Influence of pH on recCLP concentration and purification factor.
The data are presented as means ± standard error, $p<0.05$

% Potassium phosphate (w/w)	recCLP (mg/mL)	Purification factor (PF)
6	0.76 ± 0.06	3.99 ± 0.32
7	1.37 ± 0.13	6.44 ± 0.61
8	1.22 ± 0.11	5.72 ± 0.51

3.3 Optimization of ATPS Experiment Using Design of Expert (DOE)

Response surface methodology with face-centered central composite design (FCCCD) was applied to determine the optimum condition of the ATPS purification method. The effect of two factors known as concentration of PEG 2000 and potassium phosphate on concentration of recCLP was studied. The temperature of phase separation and pH of the system was kept constant at 25 °C and 7.0 respectively. The design matrix and the result of optimization were summarized in Tables 6 and 7, respectively. For the successful development of ATPS purification method, the separation of recombinant collagen-like protein was optimized using a statistical experimental design involving the concentration of PEG 2000 (A) and concentration of potassium phosphate (B) at pH 7.0. Analysis of variance (ANOVA) of a quadratic model for the responses were employed to determine the significant variable and their interaction with the response variable (Tables 8-10). According to the analysis of variance, the F-value represents the accuracy of the model while the p-value indicates the interaction between the model terms. A significant model proved by the p-value that was less than 0.05 while a p-value greater than 0.10 is considered as an insignificant model. The coefficient of determination (R^2) and adjusted R^2 indicate the quality fit of the model equation [35].

It was found that the F-value of 7.65 and the p-value of 0.0217 (<0.05) indicate that the model is significant. Furthermore, a non-significant value of 0.2674 for lack of fit showed that the quadratic model was valid for this study. The model coefficient of determination (R^2) and adjusted R^2 value was 0.8843 and 0.7687, respectively. Both

values represent the correlation between actual and predicted data. R^2 value obtained in this present study is acceptable as it exceeded 0.8 and the small difference between R^2 and adjusted R^2 is preferable and proved the good correlation for recCLP partitioning. The model contains many insignificant terms proved by the huge difference between R^2 and adjusted R^2 [20,23]. In this experiment, R^2 of 0.8843 indicates that the predicted model could explain 88.43 % of the variability in the response. As well, the adjusted R^2, 0.7687 is also good to demonstrate a good correlation between actual and predicted data [20], [23]. From the three-dimensional plot in Figs. 1 to 3, an increasing pattern showed when the concentration of PEG and salt increase to the intermediate values and decline as the concentrations of PEG and salt decrease. In summary, the maximum values of the response appeared near the center of the graph, indicating that the center point selected for this experiment is appropriate. The result from the software implied that optimized conditions for recCLP partitioning occurred when the concentration of PEG 2000 and potassium phosphate was set at 24.8% (w/w) and 29.23% (w/w) respectively with 3.026 mg/ml recCLP.

Table 6: Factors for face-centered central composite design (FCCCD) study

Symbols	Factor	Range and levels		
		-1	0	1
A	Concentration of PEG 2000 % (w/w)	20	25	30
B	Concentration of potassium phosphate % (w/w)	25	28.5	32

Table 7: Design of experiment for optimisation of ATPS of recCLP using FCCCD

% PEG 2000 (w/w)	% Potassium phosphate (w/w)	recCLP concentration (mg/mL)
20	25	1.913
30	25	1.450
20	32	2.351
30	32	1.794
20	28.5	2.151
30	28.5	2.178
20	25	1.913
25	25	1.874
25	32	2.945
25	28.5	2.974
25	28.5	3.271
25	28.5	2.904

Table 8: Analysis of variance (ANOVA) for the quadratic model for concentration of recombinant collagen-like protein

Sources	Sum of Squares	P-value Prob>F
Model	3.10	0.0217
A-PEG 2000 concentration	0.16	0.2142
B-Salt concentration	0.57	0.0450
AB	2.209E-003	0.8754
A^2	1.24	0.0113
B^2	0.52	0.0519
Lack of fit	0.33	0.2674 (not significant)
R^2	0.8843	
Adjusted R^2	0.7687	

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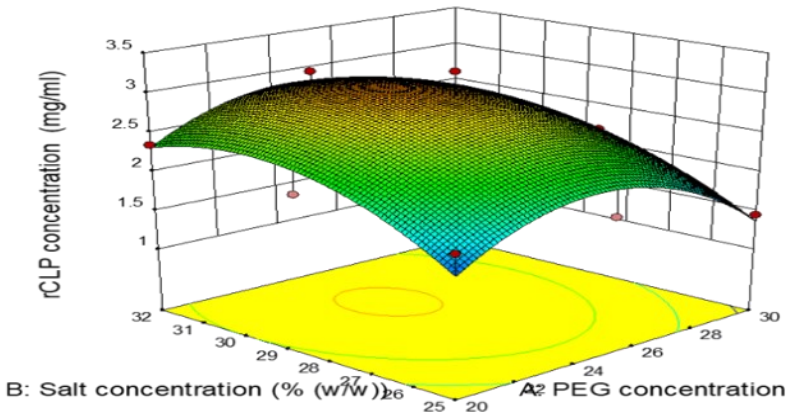

Fig. 1: Three-dimensional surface plot of response surface analysis showing the effect of the interaction of concentration of PEG 2000 and potassium phosphate on the recCLP.

Table 9: Analysis of variance (ANOVA) for the quadratic model for fluorescence intensity of recombinant collagen-like protein

Sources	Sum of Squares	P-value Prob>F
Model	1.610E+008	0.0238
A-PEG 2000 concentration	8.587E+006	0.221
B-Salt concentration	2.921E+007	0.050
AB	1.325E+005	0.869
A^2	6.482E+007	0.012
B^2	2.715E+007	0.056
Lack of fit	1.798E+007	0.024 (not significant)
R^2	0.8798	
Adjusted R^2	0.7596	

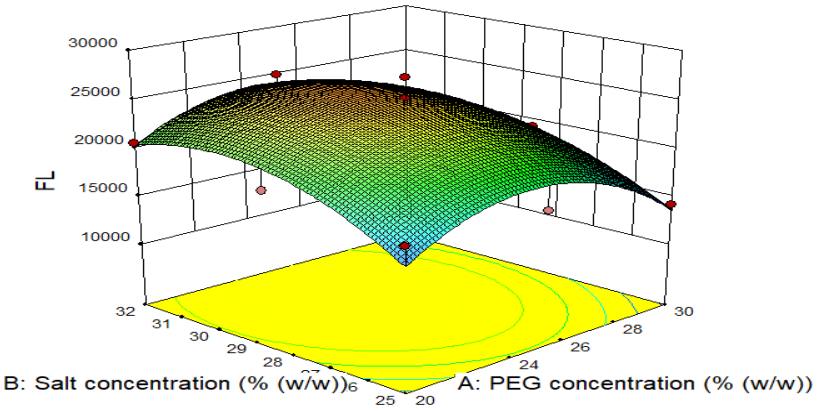

Fig. 2: Three-dimensional surface plot of response surface analysis showing the effect of the interaction of concentration of PEG 2000 and potassium phosphate on the fluorescence intensity.

Table 10: Analysis of variance (ANOVA) for the quadratic model for purification factor of recombinant collagen-like protein

Sources	Sum of Squares	P-value Prob>F
Model	17.96	0.0614
A-PEG 2000 concentration	0.62	0.417
B-Salt concentration	0.12	0.716
AB	0.033	0.847
A^2	6.65	0.034
B^2	5.96	0.041
Lack of fit	3.44	0.1905
R^2	0.8192	
Adjusted R^2	0.6383	

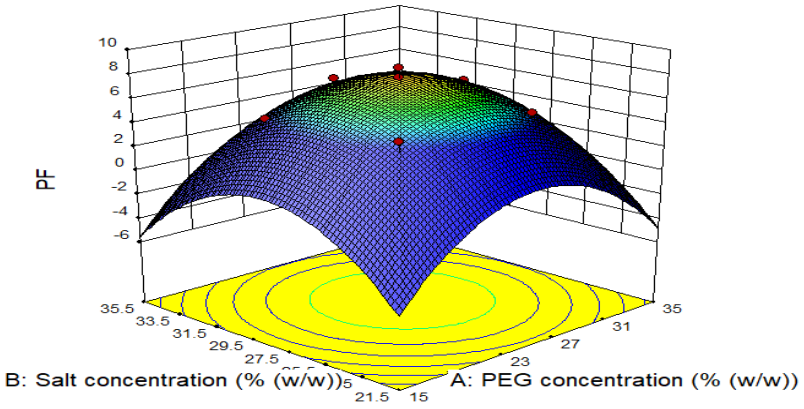

Fig. 3: Three-dimensional surface plot of response surface analysis showing the effect of the interaction of concentration of PEG 2000 and potassium phosphate on the purification factor.

The quadratic model for each response of fluorescence intensity, concentration of recCLP and purification factor coefficient were predicted from equations as follows:

$$\text{Fluorescence intensity (FL)} = 25175.95 - 1196.33C_{PEG2000} + 2206.50C_{PP} - 182.00C_{PEG2000}C_{PP} - 5058.37C_{PEG2000}^2 - 327.87C_{PP}^2 \quad (5)$$

$$\text{Concentration of recCLP} = 2.98 - 0.17C_{PEG2000} + 0.31C_{PP} - 0.023C_{PEG2000}.C_{PP} - 0.70_{PEG2000}^2 - 0.45C_{PP}^2 \quad (6)$$

$$\text{Purification factor (PF)} = 7.71 + 0.32C_{PEG2000} + 0.14C_{PP} + 0.091C_{PEG2000}.C_{PP} - 1.62C_{PEG2000}^2 - 1.53C_{PP}^2 \quad (7)$$

ATPS with only one polymer in the presence of salt (polymer/salt) is the better choice with cheaper cost, ease of handling, and low viscosity [13,36]. A similar finding, conducted by de Albuquerque Wanderley and co-workers [21], showed that the best condition for purification of collagenase by ATPS is 15% (w/w) PEG 3350 and 12.5% (w/w) phosphate salt.

3.4 Validation of Model

Validation of the model was conducted by carrying out three replicate experiments and the results were compared with the predicted results suggested by FCCCD of RSM as tabulated in Table 11. Under the optimized condition which composed of at 24.8% (w/w)

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PEG 2000 and 29.23% (w/w) potassium phosphate at pH 7.0, the predicted recCLP and PF were 3.026 mg/mL and 7.654 respectively. The experiments for validation of the predicted model resulting recCLP of 3.233 ± 0.12 mg/mL. The minimal difference between predicted and experimental values proved RSM is a suitable tool for optimization of ATPS conditions for purification of recCLP.

Table 11: Validation of model. Experiments were conducted in triplicate with data were presented as means ± standard error, $p<0.05$

	Model predicted	**Experimental**	**Percentage Error (%)**
Concentration of recCLP (mg/mL)	3.026	3.233 ± 0.12	6.9
Purification factor	7.654	7.476 ± 0.29	2.3
	Model predicted	Experimental	Percentage Error (%)

3.5 Comparison of Purification by ATPS and Affinity Chromatography

As suggested by Xu and co-workers [10], pH of 7.4 was used for binding buffer containing 20 mM of Na_2HPO_4 and 0.5 M NaCl. Ni-NTA resin with pH between 7.0 and 8.0 is preferable for most of the his-tagged proteins to prevent non-specific binding [37]. In this present study, the elution buffer containing 20 mM of Na_2HPO_4, 0.5 M NaCl and 0.5 M imidazole, pH 7.4 eluted the target protein from the column at fractions 17 to 19 (Fig. 4.). Affinity chromatography was performed under native conditions since the protein of interest is in soluble form [38].

Based on the results, the sample at the peak of the chromatogram (fraction 18) was analyzed by collagen assay and SDS-PAGE and compared with purified recombinant recCLP from ATPS purification method. The purification efficiency of recombinant collagen-like protein purified by ATPS and chromatography is summarized in Table 12. Both methods were performed directly after cell lysis stage. In the present study, Ni Sepharose chromatography (affinity) resulted in concentration of recCLP of 2.036 mg/mL and with purification factor of 0.524. Meanwhile, sample from ATPS with (24.8% w/w) of PEG2000 and (29.20 % w/w) of potassium phosphate buffer at pH 7 recCLP exhibited favorable result with 3.23 mg/ml and 7.48 purification factor. The yield is defined as the amount of purified total proteins divided by the initial amount of total protein (defined as 100%).

Table 12: Purification efficiency of recombinant collagen-like protein by different purification methods.

Purification methods	**ATPS**	**Affinity chromatography**
Fluorescence Intensity	27069	18355
Concentration of recCLP (mg/mL)	3.233	2.036
Purification Factor	7.476	0.524
Total protein (mg)	0.233	0.172
Yield (100%)	19.74	17.28
Purification methods	ATPS	Affinity chromatography

Based on economic analysis point of view as presented in Table 13, ATPS exhibited shorter processing time with lower cost of operation compared to the chromatography method. Due to expensive prepacked column with resin and chemicals used for 1h processing time, affinity chromatography is considered an expensive downstream purification method, thus making it unsustainable for bigger scale and long-term production. Despite the high cost of operation, affinity chromatography is advantageous in terms of reusability. The chromatography resin can be re-used up to 100 times since the hydrophobic stationary phase is washable [39]. However, additional cost needs to be considered for the stripping and recharging chromatography columns [19]. In the case of ATPS, polymer and salt cannot be recycled as they were part of the final purified protein [19]. Such economic analysis lays out a better understanding for future study, and ATPS method is proven to be cost-effective, time-saving with higher recovery that may be considered for the scale-up process.

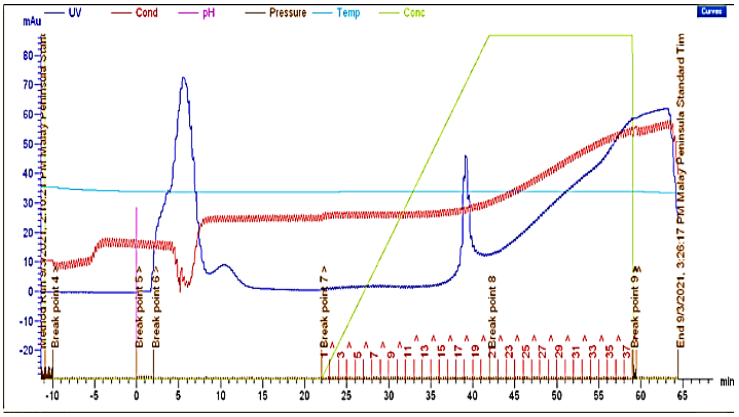

Fig. 4: Chromatogram of the purified samples from affinity chromatography of 2 mL loading volume of recombinant collagen-like protein lysates on Ni-Sepharose resin. Binding buffer contain 20 mM of Na_2HPO_4, 0. 5M NaCl at pH 7.4. Elution buffer contained 20 mM Na_2HPO_4, 0.5 M NaCl and 0.5 M imidazole, at pH 7.4. Fractions (1 mL/tube) were collected at flow rate of 1 mL/min.

Table 13: Direct comparison between chromatography and ATPS method for downstream processing of recombinant collagen-like protein

Purification methods	**ATPS**	**Affinity chromatography (FPLC)**
Processing time	30 minutes	1 hour
Cost prepacked column	-	228.36/5mL
Cost of chemicals (per kg)	K_2HPO_4: USD 235.4 KH_2PO_4: USD 163.98 PEG 2000: USD 91.6	Na_2HPO_4: USD 91.3 NaCl: USD 128.02 Imidazole: USD 543.88
Cost of operation per system	USD 490.86	USD 991.39

3.6 SDS-PAGE Analysis of Recombinant Collagen-like Protein

The presence of recombinant collagen-like protein purified by ATPS with optimized conditions of was analyzed by SDS-PAGE electrophoresis under reducing conditions and compared with purified samples from the chromatography method. Figure 5. shows the presence of a protein band with molecular weight of 36 kDa for ATPS sample, indicating the recCLP was successfully partitioned in the top phase. This was supported by the single band formed at the same size in samples from FPLC fractions. However, several bands were observed between 45 kDa and 60 kDa and above 75 kDa. This is explained by the fact that the bottom phase was unable to attract all of contaminants and unwanted proteins.

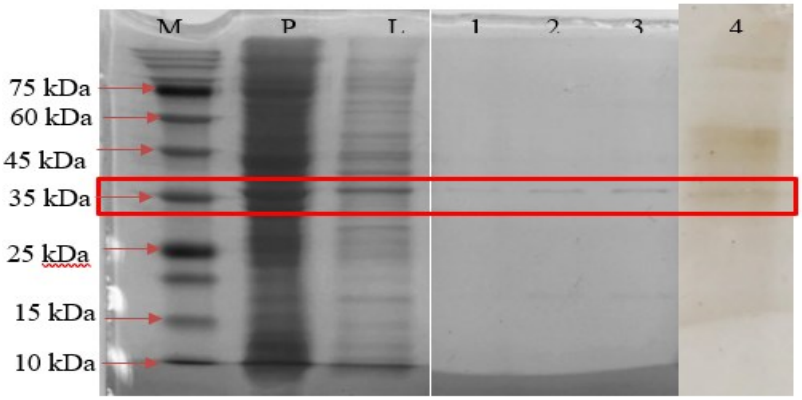

Fig. 5: SDS-PAGE analysis of purified sample of ATPS and affinity chromatography. Lane M: standard protein marker. Lane P: cell pellet. Lane L: crude cell lysate. Lane 1: FPLC fraction (17). Lane 2: FPLC fraction (18). Lane 3: FPLC fraction (19). Lane 4: ATPS top phase. Note: For SDS-PAGE analysis, 20 µl of sample with sample buffer was loaded into a 12% resolving and 4% stacking gel. Gel with FPLC fractions was stained with ReadyBlue™ Protein Gel Stain and the gel with ATPS sample was stained with silver staining after electrophoresis at 120 V, 400 mA for 60 minutes.

4. CONCLUSION

The present study had demonstrated the potential application of ATPS in purification of recCLP using PEG2000/potassium phosphate system. The optimum conditions of ATPS comprised of 24.8% (w/w) PEG 2000 and 29.20% (w/w) potassium phosphate at pH 7.0 which resulted in 27068 ± 900, 3.233 ± 0.12 mg/mL, 7.476 ± 0.29, for fluorescence intensity, concentration of recombinant collagen-like protein and purification factor, respectively. Moreover, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the molecular weight of the recCLP, which is 36 kDa. This study clearly demonstrated ATPS was proven to be time-saving, cost-effective with higher recovery that may be considered for the scale-up process. The obtained results are pivotal for the design of a purification process and understanding the complex mechanisms controlling the phase behavior. Besides, due to the lack of application of ATPS in purification of CLPs specifically from *R. palustris*, this present study can be used as a reference and deliver new information for future studies.

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