PURIFICATION OF RECOMBINANT COLLAGEN-LIKE PROTEIN FROM *Rhodopseudomonas palustris* EXPRESSED IN *Escherichia coli* USING AQUEOUS TWO-PHASE SYSTEM

BY

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ABSTRACT

Recombinant collagen-like protein (recCLP) is a collagen-like molecule extracted from microorganisms and expressed in *Escherichia coli* host. The surge in demand for high-quality collagen is due to the multitude of applications in the end-user industries such as pharmaceuticals, food, nutraceuticals and cosmetics. The awareness of the halal status of collagen among Muslim consumers encouraged the discovery of protein from non-mammalian sources, especially from microorganisms. The collagens from microorganisms have great industrial potential because they are free from any zoonotic diseases, contamination and side effect issues. Moreover, the difficulties of the present downstream processing, especially at the purification step, encourage the application of an aqueous two-phase system (ATPS). This study aimed to identify the optimum ATPS conditions for purification of recombinant collagen-like protein expressed in *E. coli* that initially extracted from *Rhodopseudomonas palustris*. Recombinant collagen-like protein from *R. palustris* was purified using the aqueous two-phase system consisting of polyethylene glycol (PEG)/ potassium phosphate. First, the five binodal curves representing five different molecular weights of PEG (1500, 2000, 4000, 6000 and 8000 g/mol) were constructed using the node determination method. Binodal curve that divides the region of two aqueous phases from one phase is important so that a systematic choice of system can be used for portioning experiments. Then, several factors involved in the partitioning behaviour of recCLP such as volume ratio, system pH, the concentration of polymer and salt were studied. The selected ATPS conditions (PEG and salt concentration) were optimised using the response surface methodology (RSM) method. Purification by affinity chromatography was carried out and further compared with ATPS in terms of efficiency and economic aspects to evaluate its potential application as a purification method for recCLP. The binodal curves obtained proved that a high molecular weight of PEG required a low concentration of potassium phosphate to form a two-phase system. As PEG molecular weight increased, the curved was distorted towards the origin. The highest partition coefficient (KE) was found in the system with 26 % (w/w) PEG 2000 and 26 % (w/w) potassium phosphate, making it the best ATPS combination for the OFAT analysis and optimisation process. The range of volume ratio, pH and concentration of PEG and potassium phosphate on the partitioning of recombinant collagen-like protein by ATPS were successfully obtained from OFAT method prior to the optimisation study. Optimisation of ATPS conditions using face-centered central composite design (FCCCD) in Response Surface Methodology (RSM) with 11 runs showed the optimum conditions of ATPS with 24.80 % (w/w) PEG 2000 and 29.20 % (w/w) potassium phosphate with recCLP concentration of 3.23 ± 0.12 mg/mL. Analysis of variance showed the coefficient of determination (R2) were 0.8823, 0.8823, and 0.8193 for fluorescence intensity, the concentration of collagen-like protein and purification factor, respectively. Lastly, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the molecular weight of the recCLP, which is 36 kDa. In addition, results showed that ATPS is a low cost, time-saving with a high recovery method that may raise the consideration for substitution of chromatography method. In conclusion, the purification method through ATPS to purify recCLP has high potential, cost-effective, can replace the tedious and expensive downstream processing. Furthermore, this study can serve as a reference and deliver new information for future work.

ملخص البحث

البروتين الشبيه بالكولاجين (recCLP) هو جزيء شبيه بالكولاجين يتم استخراجه من الكائنات الدقيقة ويعبّر عنه في مضيف *Escherichia coli*. وتعزى الزيادة في الطلب على الكولاجين عالي الجودة إلى تعدد التطبيقات في صناعات المستخدمين النهائيين مثل المستحضرات الصيدلانية والأغذية والمكملات الغذائية ومستحضرات التجميل. وشجّع الوعي بحالة الحلال للكولاجين لدى المستهلكين المسلمين على اكتشاف البروتين من مصادر غير ثديية ، ولا سيما من الكائنات الدقيقة. وللكولاجين من الكائنات الدقيقة إمكانات صناعية كبيرة لأنها خالية من أي أمراض حيوانية أو تلوث أو آثار جانبية. وبالإضافة إلى ذلك، فإن الصعوبات التي تواجه عملية المعالجة الحالية، وخاصة في خطوة التنقية، تشجّع على تطبيق نظام مائي من مرحلتين (ATPS). وتهدف هذه الدراسة إلى تحديد الظروف المثلى لنظام ATPS لتنقية البروتين الشبيه بالكولاجين المعاد تركيبه المعبّر عنه في *E. coli* والمستخرج بدايةً مِنْ *Rhodopseudomonas palustris*. تم تنقية البروتين الشبيه بالكولاجين المستخرج من *R.palustris* باستخدام النظام المائي ثنائي الطور المكوّن من البولي إيثيلين جليكول (PEG)/فوسفات البوتاسيوم. أولا ، تم بناء المنحنيات الثنائية الخمسة التي تمثل خمسة أوزان جزيئية مختلفة من PEG (1500 ، 2000 ، 4000 ، 6000 ، 8000 جم/مول) باستخدام طريقة تحديد نقطة تقاطع المدارين. المنحنى الثنائي الذي يقسم المنطقة من مرحلتين مائيتين عن مرحلة واحدة هو أمر مهم بحيث يمكن استخدام الاختيار المنهجي للنظام لتقسيم التجارب. وبعد ذلك، تمت دراسة عدة عوامل تؤثر على سلوك تقسيم recCLP مثل نسبة الحجم والأس الهيدروجيني للنظام وتركيز المبلمر والملح. تم تحسين الظروف المختارة لـ ATPS (PEG وتركيز الملح) باستخدام منهجية سطح الاستجابة (RSM). وأُجريت عمليات تنقية باستخدام كروماتوغرافيا التقارب ، كما أُجريت مقارنات أخرى مع ATPS من حيث الكفاءة والجوانب الاقتصادية لتقييم إمكانية تطبيقها كأسلوب لتنقية البروتين الشبيه بالكولاجين (recCLP). وأثبتت المنحنيات الثنائية التي تم الحصول عليها أن الوزن الجزيئي العالي من PEG يتطلب تركيزًا منخفضًا من فوسفات البوتاسيوم لتشكيل نظام ثنائي الطور. أدت زيادة الوزن الجزيئي PEG إلى انحراف المنحنى نحو نقطة الأصل. وقد وُجد أعلى معامل للتقسيم في النظام مع 26%(كتلة/كتلة) من PEG 2000 وَ 26% (كتلة/كتلة) من فوسفات البوتاسيوم، مما يجعله أفضل تركيبة ATPS لكلٍّ من تحليل OFAT والأمثليّة . ودُرس تأثير نسبة الحجم والأس الهيدروجيني وتركيز فوسفات البوتاسيوم على تقسيم البروتين الشبيه بالكولاجين المعاد تركيبه بواسطة الـ ATPS. وقد أظهر الاستخدام الأمثل لظروف الـ ATPS باستخدام التصميم المركزي المركب المركز على الوجه (FCCCD) في منهجية منهجية سطح الاستجابة (RSM) مع 11 دورة أظهرت الظروف المثلى لـ ATPS مع 24.80%(كتلة/كتلة) من PIG 2000 و 29.20% (كتلة/كتلة) من فوسفات البوتاسيوم مع تركيز recCLP بقيمة 3.23±0.12 ملجم/مل. وأظهر تحليل التباين أن قيم معامل التحديد (R^2) كانت 0.8823 و 0.8823 و 0.8193 بالنسبة لكثافة الفلورسنت وتركيز البروتين الشبيه بالكولاجين ومعامل التنقية، على التوالي. وأخيرا ، أكّد الفصل الهلامي الكهربائي (SDS-PAGE) الوزن الجزيئي لـ recCLP ، والتي تبلغ 36 كيلودالتون. وبالإضافة إلى ذلك ، أظهرت النتائج أن الـ ATPS هي طريقة منخفضة التكلفة ومقتصدة للوقت مع استرداد عالٍ قد تثير النظر في الاستعاضة عن طريقة الكروماتوغرافيا. وختامًا، فإن طريقة استخدام طريقة ATPS لتنقية recCLP تنطوي على إمكانات عالية وفعالة من حيث التكلفة، ويمكن أن تحل محل التجهيز الشاق والمكلف للمعالجة النهائية.وعلاوة على ذلك، يمكن لهذه الدراسة أن تكون مرجعًا وأن تقدم معلومات جديدة للعمل في المستقبل.

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APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Halal Industry Science).

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF SYMBOLS

g	gram
g	gravitational force
h	hour
l	litre
kda	kilo Dalton
mg	milligram
mL	millilitre
min	minute
rpm	revolutions per minute
%	percentage
w/w	weight over weight
w/v	weight over volume
v/v	volume over volume
°C	degree celcius
µl	micro litre

LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase	His	histidine
ANOVA	analysis of variance	IgG	immunoglobulin
ATPS	aqueous two-phase system	NCBI	National Center for Biotechnology Information
BSA	bovine serum albumin	IgG	immunoglobulin
BSE	bovine spongiform encephalopathy	OFAT	one factor at a time
CLP	collagen-like protein	PCR	Polymerase chain reaction
recCLP	Recombinant collagen-like protein	PEG	Polyethylene glycol
DEAD	diethyl aminoethyl cellulose	PF	Purification factor
FCCCD	face-centered central composite design	RSM	response surface methodology
FL	fluorescence intensity	Scl	Streptococcal collagen-like protein
FMD	foot-and-mouth disease	SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel
FPLC	fast protein liquid chromatography	TSE	transmissible spongiform encephalopathies
GRAS	Generally regarded as safe	Vr	volume ratio

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Collagen is the most abundant protein in mammals and the primary structural protein in all animals, comprising about 25-30 % of total animal proteins (Kiew & Don, 2013). It is the main structural material of the extracellular matrix of all connective tissues such as skin, bones, ligaments, tendons, and cartilage as well as interstitial tissues of all parenchymal organs (Felician et al., 2018). It is defined by a unique structure known as triple-helical conformation that consists of three polypeptide chains (α-chains) super-coiled together around a common axis to give a rope-like structure (Figure 1.1(a)) (Gould, 2016; Knupp & Squire, 2003; Yd et al., 2013). Figure 1.1(b) illustrates the repeating structure of amino acid of collagen Gly-Xaa-Yaa with the unusual abundance of three amino acids: glycine (Gly), proline, and hydroxyproline (Knupp & Squire, 2003; Lukomski et al., 2018). Due to its unique structure, collagen plays a big role in fibril formation, mechanical properties and responsible for the interaction with a wide range of molecules (Brodsky & Ramshaw, 1997).

Collagen can be derived from various sources such as land animals, marine animals, birds, and microorganisms. Mammalian collagen (mainly bovine and porcine) is the major industrial source of collagen owing to the multitude of applications in industries. Recently, instead of focusing on animal-derived collagen, most studies focused on non-mammalian sources such as bacteria and marine sources. The rise of potential disease transmission and religious issues triggered researchers to come up with an alternative to animal collagen. Therefore, for the past 10 years, more than 100 putative collagen-like proteins (CLPs) have been discovered from various bacterial genomes and eight of them have been recombinantly expressed in *Escherichia coli* (Xu et al., 2010). Collagen-like protein (CLP) in microorganism or bacterial collagen has been identified based on the repeating signature (Gly-Xaa-Yaa)n sequence

characteristic of triple-helix. Furthermore, their uniqueness is that bacterial collagen has a different amino acid structure from animal collagen but is able to function well as animal-derived collagen (Xu et al., 2014). Bacterial collagen has a big potential to be further developed and commercialised due to the high demand for collagen in various industries such as the pharmaceutical, biomedical, food, and cosmetic industry. This study is focusing on collagen from microorganisms as they are purer and disease-free compared to animal collagen.

Figure 1.1 (a) The Collagen Triple-Helix Structure (b) The Repeating Signature Triplets Gly-Xaa-Yaa in Collagen Amino Acid Sequences (Knupp & Squire, 2003)

In the recent era, recombinant DNA technology comes under the spotlight of researchers and scientists due to tremendous advancement and various applications in the medical, pharmaceuticals, and agriculture industry (Khan et al., 2016). Recombinant technology for the production of collagen from either plant or bacterial sources is currently under investigation and has been used in some animal studies (Peng et al., 2012; Shilo et al., 2013; Wang et al., 2013). This technology would allow the production of non-animal collagen in an animal-free system with the improvement of final products in terms of purity, yield, and safety (Gould, 2016). To keep up, there is a need to develop an efficient and cost-effective downstream process (Ratanapongleka, 2013; Rosa et al., 2010). Recently, a technique known as aqueous two-phase systems (ATPS) has become the alternative method for the purification of biomolecules which can reduce the number of stages as well as the overall cost (Raja et al., 2011).

The main purpose of this study is to purify the recombinant collagen-like protein from bacteria using an efficient and cost-effective method known as an aqueous-two-phase system (ATPS). Several potential factors can affect the performance of ATPS and they were investigated using the one factor at a time (OFAT) and optimised using Response Surface Methodology (RSM) method.

1.2 PROBLEM STATEMENT

The demand for high-quality collagen is due to the multitude of applications in industries such as pharmaceuticals, food, and cosmetic industry. Collagen is normally extracted from human sources or animal sources mainly from pigs and cows (Vázquez et al., 2016). However, Muslims and Jews are prohibited from consuming collagen from pigs whereas bovine sources are prohibited for Sikhs and Hindus (Eriksson et al., 2013). At present, the awareness of halal authentication among the consumers has been spread to other industries such as pharmaceuticals and cosmetics. The halal status of collagen is depending on the origin of the raw sources and the process conditions. Nonspecific collagen is highly suspected to contaminate with porcine elements and haram for used by the Muslim consumers.

Besides, another challenge in producing collagen from animal sources is the emergence of potential disease transmission, contamination, and side effect issues to the consumers. Production of animal-recombinant collagen is complex and requires additional processes results in the high cost of production. Therefore, recombinant technology has elevated the production of recombinant collagen from bacteria.

In recent years, recovery and purification of recombinant protein are the major challenges due to its complexity and high cost. The purification process is the most crucial step and makes up more than 70% of downstream processing costs (Diamond & Hsu, 1992; Goja et al., 2013). The conventional methods consist of several unit operations that cause the high cost of operation and maintenance (Raja et al., 2011). For

instance, precipitation needs to be combined with another process such as chromatography processes, which are complex, tedious, time-consuming, and often produced low yields (Cao & Xu, 2008). Furthermore, the high unit operation involved caused loss of target molecules that will result in a low yield of the product.

Therefore, in this study, the aqueous two-phase system is proposed as an alternative for the purification method. This method is very simple, mild, and free from protein denaturation due to the high water content and stabilising effect supplied by the polymers (Asenjo & Andrews, 2012). Thus, this method can maintain the native structure of proteins. Polyethylene glycol (PEG) is the commonly used polymer in ATPS as it is available at a low cost and able to form a two-phase system when reacts with other neutral polymers as well as salts (Raja et al., 2011).

1.3 RESEARCH QUESTIONS

The research questions in this study are as following:

i. What is the most suitable molecular weight and concentration of polymer that able to separate CLPs?
ii. What is the most suitable concentration of salt that able to separate CLPs?
iii. What are the optimum conditions for purification of recombinant collagen-like protein using aqueous two-phase system?

1.4 RESEARCH OBJECTIVES

The objectives of this project are as the following:

i. To identify suitable concentration and molecular weight of polymer for purification of recombinant collagen-like protein (CLP) by binodal curve determination.
ii. To identify suitable salt concentration for purification of collagen-like protein (CLP) using binodal curve determination.
iii. To optimize the purification method for collagen-like protein (CLP) by response surface methodology (RSM).

1.5 RESEARCH SCOPE

The present work focused on the application of aqueous two-phase system as the purification method for recombinant collagen-like protein. Firstly, five different binodal curves that represents the five molecular weight of polyethylene glycol (PEG) were developed using node determination method. After that, the most suitable molecular weight of PEG and concentration of PEG and salt required to purify recCLP were identified.

One Factor at A Time (OFAT) technique was adopted in this study as a tool for screening of optimum range of the selected parameters; molecular weight of polyethylene glycol (1500, 2000, 4000, 6000 and 8000), volume ratio (0.33-3.5), concentration of PEG (20- 32) % (w/w), concentration of salt (20-32) % (w/w) and pH (6.0-8.0). The responses involved were fluorescence intensity, concentration of collagen-like protein and purification factor.

Afterwards, the optimisation of ATPS conditions was done using Face Centered Central Composite Design (FCCCD) in Response Surface Methodology (RSM) where

the factors are concentration of PEG and salt. The responses involved were fluorescence intensity, concentration of recombinant collagen-like protein and purification factor. In addition, purification of recCLP using affinity chromatography was conducted and the result obtained were compared with ATPS purification method.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter starts with the general introduction of collagens including its structure, biosynthesis, function, stability, types of collagens that have been discovered followed by their applications in various industries. It also includes the details of the purification method involved in the purification of collagen such as the theory, mechanisms, advantages and disadvantages.

2.2 COLLAGEN

2.2.1 Structure of Collagen

Collagen is the main structural protein that is ubiquitously found in the extracellular matrix of animals, including all vertebrates and invertebrates that comprised of amino acids such as glycine (Gly), proline (Pro), and hydroxyproline (Figure 2.1) (Dutson, 1976; Yamazaki et al., 2010). The most common collagen, type 1, contains amino acid sequence of Gly-Xaa-Yaa triplets that are commonly occupied with proline (Pro) and hydroxyproline (Hyp) in Xaa and Yaa position, respectively (Gelse et al., 2003a; Krishnan & Perumal, 2013; Ricard-blum, 2011). The prevalence of Gly-Pro-Hyp sequence in collagen triplets is about 10.5% (Domene & Wajid, 2016; Gorres & Raines, 2010). These triplets are bound together to form polypeptide chains that twisted together in the form of a triple helix structure (Brodsky & Ramshaw, 1997).

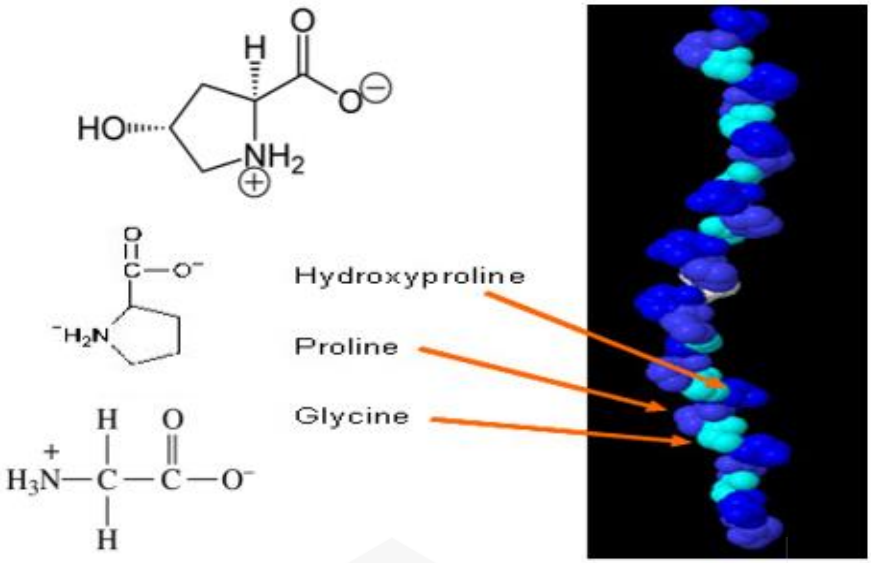

Figure 2. 1 Structure of collagen (Yamazaki et al., 2010)

2.2.2 Biosynthesis of Collagen

The process of collagen synthesis occurs intracellularly and extracellularly in the fibroblast cells which are well known in the synthesising of collagen and stroma (Fidler et al., 2018; Wu et al., 2020). The basic outline of collagen synthesis is illustrated in Figures 2.2 (a) and (b). During intracellular biosynthesis, two types of peptide chains known as pre-procollagen with a signal peptide and registration peptide (N-telopeptide and C-telopeptide) on each end are formed before it travels to the endoplasmic reticulum (ER) for post-translational modification and become procollagen (Mandal, 2019; Prockop et al., 1976; Ricard-blum, 2011). Prior to triple helix formation, collagen undergoes multiple steps of post-translational modification that involve a number of enzymes and molecular chaperones to assist their folding and trimerisation (Gelse et al., 2003a; Kadler et al., 2007). Once outside the cells, collagen peptidase act as an enzyme that cleaves the ends of procollagen and the molecules become tropocollagen. Lastly, a copper-dependent enzyme known as lysyl oxidase acts on lysine, hydroxylysine and covalent bonding to transform tropocollagen into collagen fibrils (Mandal, 2019). The smallest amino acid, glycine (Gly) located in the centre of the triple helix in every third position of the polypeptide chains acts as a structural prerequisite for the assembly of collagen (Brodsky & Ramshaw, 1997; Gelse et al., 2003a; Muyonga et al., 2004).

Meanwhile, more bulky amino acids fill out the outer position resulting in a close packaging along the central axis of the molecule (Gelse et al., 2003a). The biosynthesis of collagen is quite similar to other proteins, but collagen biosynthesis is distinguished based on two crucial features: (1) a precursor of collagen known as procollagen which fulfils important functions formed and (2), the involvement of several unusual post-translational modifications (Prockop et al., 1976).

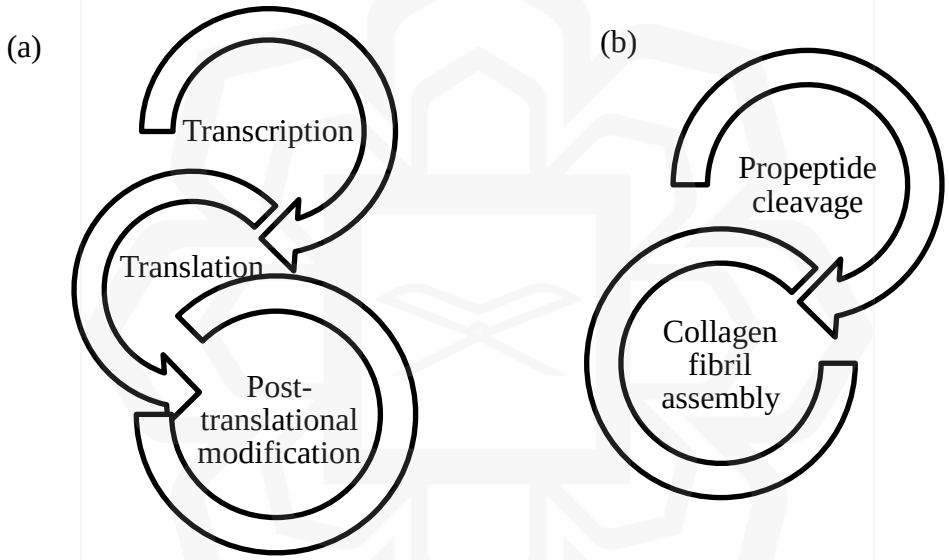

Figure 2.2 (a) Intracellular Synthesis of Collagen (b) Extracellular Synthesis of Collagen

2.2.3 Stability of Collagen

The high content of amino acids, interchain hydrogen bond and the presence of Gly at every tripeptide are the important stabilising factors in collagen triple helix structure (Brodsky & Ramshaw, 1997; Persikov et al., 2005). The most common and stabilising

tripeptide is the Gly-Pro-Hyp sequence in (Gly-Yaa-Xaa)n pattern (Persikov et al., 2005). However, the mechanism of triple helix stabilisation in mammalian collagen is controversial since the hydroxyl group of hyp points outward from the triple helix and cannot form any direct intramolecular hydrogen bonds to any other group within the molecule (Mohs et al., 2007; Xu et al., 2014). Despite the absence of direct hydrogen bonding, a similar and sufficient effect can be achieved through bridging water molecules between hyp hydroxyl group and Gly C=O within the same chain, and hyp C=O of the adjacent chain (Bella et al., 1995; Brodsky & Ramshaw, 1997). This has been proved by the crystal structure in Figure 2.3 (Bella et al., 1995)**.** Furthermore, the result of calorimetric studies carried out by Privalov (1982), showed the high hyp content increases the enthalpic contribution thus provide stability to the proteins.

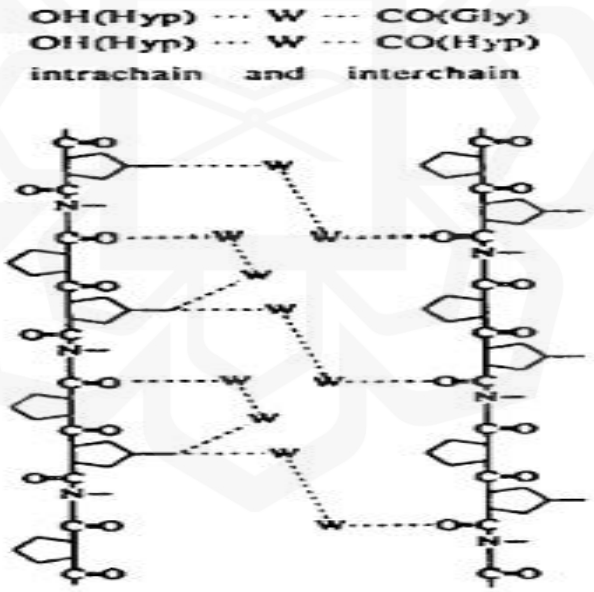

Figure 2.3 Water Mediated Hydrogen Bonding Linking Hydroxyproline OH Groups and Carbonyl Groups.

2.2.4 Types of Collagens

As of now, scientists have identified 29 types of collagen that consist of 46 different polypeptide chains (Marina et al., 2012; Silva et al., 2014; Sionkowska et al., 2020). All of them shared a similar triple helix structure but differs in the length of the helix. The five common types of fibril-forming collagens in the body and their sources were tabulated in Table 2.1. Type I collagen accounts for about 90 % of collagen in the body followed by type II and type III. The abundance of type I collagen is due to its high prevalence in connective tissues.

Table 2.1 Type of collagen (Silvipriya et al., 2015)

Types	Sources
Collagen I	Skin, bone, teeth, tendon, ligament, vascular ligature, organs
Collagen II	Eyes and cartilage
Collagen III	Reticulate, skin, muscle, blood vessels.
Collagen IV	Forms the epithelium-secreted layer of the basement membrane and the basal lamina.
Collagen V	Hair, cell surfaces and placenta.

2.2.5 Sources of Collagen

Although collagen is absent in plants, it can be abundantly found in many sources such as land animals, marine animals and microorganisms.

2.2.5.1 Land Animals: Bovine and Porcine

Collagen can be extracted from skin and bones of terrestrial mammals, mainly pigs and cattle that commonly addressed as porcine and bovine collagen, respectively (Hashim et al., 2015). The bovine and porcine collagens are the major industrial sources of collagen as they are available in plenty and largely applications in various industries such as pharmaceutical, biomedical, food, cosmetic and leather industries (Kittiphattanabawon et al., 2015; Lim et al., 2019; Raman & Gopakumar, 2018; Sadowska et al., 2003). Other than pigs and cattle, chicken neck, rat-tail tendon, duck feet, kangaroo tail, equine skin, bird's feet, alligator bones and skins, sheepskin and frog skin, are the examples sources of collagen (Avila Rodríguez et al., 2017).

However, the rising of transmissible diseases from the use of this source such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathies (TSE), foot-and-mouth disease (FMD), spongiform encephalopathies ('Mad cow disease') and avian influenza which leads to anxiety have posed a threat to human (Myllyharju, 2003; Nagai, 2010; Silvipriya et al., 2015). Besides, prolonged usage of mammalian collagen tends to be allergenic and misfolded and leads to a genetic disorder known as osteogenesis disorder (Avila Rodríguez et al., 2017; Lupi, 2002). Unlike bovine collagen, porcine collagen caused fewer allergic responses to humans as its structure is quite similar to human collagen. Due to religious constraints, porcine collagen is prohibited in Islam and Jewish cultures and bovine collagen is prohibited in Hinduism (Dayton, 2008; Hashim et al., 2015; Silva et al., 2014). At present, halal authenticity does not only exist in the food industry, but halal awareness has extended to other industries such as the cosmetics and pharmaceuticals industries. The halal status of collagen is mainly depends on the origin of the raw sources and collagen products with unclear sources are highly discouraged for use by the Muslims (Hashim et al., 2015).

2.2.5.2 Marine Collagen

The ocean which is inhabited by diverse living organisms covers more than 70% of the surface earth. This environment serves as a promising source of collagen that is utilised in different fields including food manufacturing, pharmaceutical, biomedical and cosmetic industries (Hashim et al., 2015; Raman & Gopakumar, 2018; Shin et al., 2016; Venkatesan et al., 2017). Considering the religious sentiments and potential drawbacks related to collagen originated from land animals, marine collagen can be seen as an appropriate alternative. Marine based collagen can be isolated from invertebrate animals such as squid (Raman & Mathew, 2014), cuttlefish, sea anemone, prawn, starfish (Felician et al., 2018), jellyfish (Krishnan & Perumal, 2013), sea urchin, octopus (Kimura et al., 1981), and marine sponges (Swatschek et al., 2002). Another type of marine collagen falls under marine vertebrates which include fish and marine mammals (Felician et al., 2018). In fishes, besides meat, collagen is usually extracted from their offal including processing waste such as skin, bones, fins and heads (Felician et al., 2018; Raman & Gopakumar, 2018; Sadowska et al., 2003). Several studies were reported on collagen isolation from fish waste (Nagai & Suzuki, 2000), skin of underutilised fishes (Nagai, 2010), and Pacific whiting Surimi processing waste (Kim & Park, 2004) and these under-utilised sources can be considered as sustainable and cost-effective (Raman & Gopakumar, 2018; Senaratne et al., 2006).

These types of collagen are found to have more advantages such as being easily extracted, water-soluble with better chemical and physical durability, and available in abundant quantities (Lim et al., 2019; Silvipriya et al., 2015). Moreover, it is safer compared to bovine and porcine collagen as it is free from the risk of transmitting diseases and pathogens and has been considered as GRAS (Generally recognized as safe) by Food and Drug Administration (FDA) (Avila Rodríguez et al., 2018; Felician et al., 2018; Lim et al., 2019; Silva et al., 2014; Silvipriya et al., 2015). The extraction processes of marine collagen normally involved acid extraction and enzyme hydrolysis techniques. The latter is preferable due to its improved functionality, high nutritional value and low processing time (Raman & Gopakumar, 2018; Schmidt et al., 2016).

Despite its advantages in availability, safety and extraction process, marine-based collagen still lacks in terms of its stability due to low denaturation temperature (Schmidt et al., 2016). The short amino acid (proline and hydroxyproline) in fish collagen is the main reason behind its lower thermal stability (Subhan et al., 2015). This limitation resulted in the need for stabilisation techniques using chemical or physical cross-linking methods to make marine-based collagen as a substitute for mammalian collagen in biomedical, food and cosmetic applications (Subhan et al., 2015; Yunoki et al., 2004).

2.2.5.3 Bacterial Collagen

There have been 18,874 collagen-like proteins (CLPs) annotated in bacteria, 695 in viruses, and 157 in archaea (search conducted on 9/24/16 in Uniprot database) (An et al., 2014; Rasmussen et al., 2003; Xu et al., 2014). They shared the similar Gaa-Xaa-Yaa repetitive amino acid sequences of mammalian collagen but significantly different in terms of amino acid content and distribution (Ilamaran et al., 2019; Peng et al., 2012; Ramshaw et al., 2014; Xu et al., 2014). Based on *in silico* studies performed by Rasmussen et al. (2003), CLPs from bacteria and viruses have low proline content and it was preferentially found in the Xaa position of Gly-Xaa-Yaa triplets. Besides, threonine is frequently found in bacterial collagens compared to mammalian collagens and the most dominating amino acids in Yaa position of 17 out of 53 CLPs (Rasmussen et al., 2003). Some of the CLPs come from pathogenic microorganisms may work as a virulence factor to avoid the immune system of higher animals and thus promote host cell invasion (An et al., 2014; Humtsoe et al., 2005; Ramshaw et al., 2014). For example, Streptococcal collagen-like protein (Scl-1) and Scl-2 from *Streptococcus pyogenes*, *Bacillus cereus*, *Bacillus anthracis* and Scl-C-Sc-lI from *Streptococcus equi* subspecies (Kananaviciute et al., 2020).

Unlike animal collagen with the presence of notable and high content post-translationally modified amino acid, hyroxyproline (Hyp), CLPs in bacteria are unique

as they are lack amino acid Hyp. Hyp amino acid is responsible for stabilising the triple helix structure, promote self-association, especially in cross-linking purposes in the human body, (Gorres & Raines, 2010; Kar et al., 2006; Myllyharju, 2003; Ramshaw et al., 2014; Timmins et al., 2017; Xu et al., 2014). This is due to the absence of prolyl 4-hydroxylase (P4H), a crucial enzyme that catalyses the post-translational modification of all Pro residues into Hyp (Gorres & Raines, 2010; Timmins et al., 2017).

However, collagen-like protein from bacteria can form a stable triple helix structure similar to mammalian collagens with high thermal stability (*Tm* = 36.5-40 °C) in defiance of hyp (Xu et al., 2014). According to Mohs et al. (2007), most of the studies in collagen structure concluded that Pro hydroxylation is responsible for the triple helix stabilisation for eukaryotic cells, whereas prokaryotes develop different molecular strategies to achieve stable protein. In CLPs, ion pairs, hydration mediated hydrogen-bonding network and Gly-Pro-Pro may contribute to stabilise the triple helix structure (Mohs et al., 2007). Hence, the availability of CLPs with the same stability as mammalian collagen creates a turning point from the interest of their role in pathogenesis to the application of recombinant bacterial collagens to establish well-defined and novel collagen-based biomaterials (An et al., 2014; Mohs et al., 2007)

2.3 RECOMBINANT PROTEIN

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 (Clark, 2005; Purandarey, 2018). This technology enables the production of disease-free products, uniform quality and abundance in quantity thus have been successfully exploited in various fields such as pharmaceuticals, gene therapy, agriculture, food processing and bioremediation (Khan et al., 2016; Palomares et al., 2004; Ramshaw et al., 2014). However, there still lack of examples of the production of recombinant protein with material properties such as silk, elastin and collagen.

2.3.1 Production of Recombinant Collagen-Like Protein (recCLP)

To date, significant effort has been made in producing human collagens and animal collagens in recombinant systems such as mammalian, plant, insect, yeast and bacteria (An et al., 2014; Ramshaw et al., 2014)**.** Nevertheless, as described in the problem statement (section 1.2), a variety of specific post-translational modifications and proteolytic cleavage are needed for the replication of mammalian collagen in a recombinant system like yeast, insects, bacteria and plants making it a tedious and complex process (An et al., 2014; Toman et al., 2000; Vaughan et al., 1998; Xu et al., 2014). Other obstacles include the risk of pathogen, diseases, allergy and inability of sequences modification to achieve different biological purposes (Xu et al., 2014).

On the other hand, mammalian expression system has the ability of post-translational modifications but, it has low yield, expensive and difficult to scale up. Thus, as a solution, *Escherichia coli*, a bacterial expression system, has been a promising host for recombinant protein production due to its simple procedure, cost-effective and easy to scale up (An et al., 2014). Besides, using a bacterial host provide an opportunity for genetic modification of the protein structure to produce high protein expression (Ramshaw et al., 2014).

2.3.2 Method of Producing recCLP

Initially, the sequences with high potential to form a stable triple-helix structure are searched from NCBI microbial genome database. Then, DNA fragments of the gene of interest with restriction sites are before being sub-cloned into pCold II vector and transformed for cold shock expression in *E. coli* BL21(DE3) as a host cell. The positive clone harbouring the CLPs gene is verified using ampicillin selection and colony polymerase chain reaction (PCR) prior to protein expression and purification (Xu et al.,

2010). In this method, the right expression system for any specific application is the key to success in the recombinant production (Yang & Shu, 2014).

There are a few examples of CLPs from bacteria that have been successfully expressed as recombinant proteins including Scl1 and Scl2 from bacterium group A *Streptococcus pyogenes*, *Methylobacterium*, *Solibacter usitatus*, *Rhodopsedomonas palustris*, *Clostridium perfringens* and *Corynebacterium diphtheria* (Sionkowska et al., 2017; Xu et al., 2010). Based on a study carried out by Xu et al. (2010), several collagen-like proteins were identified from genome databases using a criteria that the number of triplet repeats should be greater than 35 as presented in Table 2.2. Collagen proteins from *Methylobacterium* sp. *4-46*, *Rhodopseudomonas palustris*, and *Solibacter usitatus* were identified as soluble proteins whereas collagen from *Clostridium perfringens* was found as inclusion bodies (Xu et al., 2010).

Table 2.2 Predicted bacterial collagen-like proteins from genome databases (Xu et al., 2010)

Bacteria	**Protein**	**Molecular weight (kDa)**	**Isoelectric point (pI)**
Closridium perfringens, SM101	ABG86771.1	42.1	4.7
Methylobacterium, sp 4-46	ACA18713.1	33.5	8.6
Rhodopsedomonas palustris	YP_0019930	22.1	9.3
Solibacter usitatus	YP_822627.1	40.8	5.4
Corynebacterium diphtheria	CAE50366.1	25.8	8.85

2.3.3 Collagen-Like Proteins (CLPs) from *Rhodopsedomonas palustris*

Rhodopseudomonas palustris is a rod-shaped, gram-negative purple bacterium that belongs to class of alphaproteobacterial under the genus *Rhodopseudomonas.* It is a **nonpathogenic** bacteria that is widely found in nature inhabiting marine and soil ecosystems (Larimer et al., 2004; Xu et al., 2014). Typically, *R. palustris* are phototrophic. However, it is known for its metabolic versatility and ability to grow by any one of the four different modes of metabolism: phototrophic, photoheterotrophic, chemoheterotrophic and chemoautotrophic (Larimer et al., 2004). Non-toxic *R. palustris* is able to degrade various complex organic compounds into biomass and bioenergy (Bent, Gucker, Oda, & Forney, 2003; Zhang et al., 2015). Therefore, it makes them interesting and widely used in industry for bioremediation, sewage treatment, biofertiliser and removal of phytotoxic compounds (He et al., 2017; Liu, Ghosh, & Hallenbeck, 2015; Lo, Lee, & Liu, 2020; Wong et al., 2014; Zhang et al., 2015).

Current finding by Xu et al. (2010), **a phototrophic organism** and **non-pathogenic gram-negative** *R. palustris* was found to contain soluble collagen-like proteins (CLPs) and **capable of successful folding and *in vitro* refolding**. Figure 2.4 presented the evaluation of amino acid content at Xaa and Yaa positions conducted by Xu et al. (2010), using ProtParam tool. *R. palustris* has notably high amount of proline (pro), 37 % of all residues in the Xaa aand Yaa position. Besides, the collagen-like domains also contain 35% charged residues, 15% polar residues and 6% small residues.

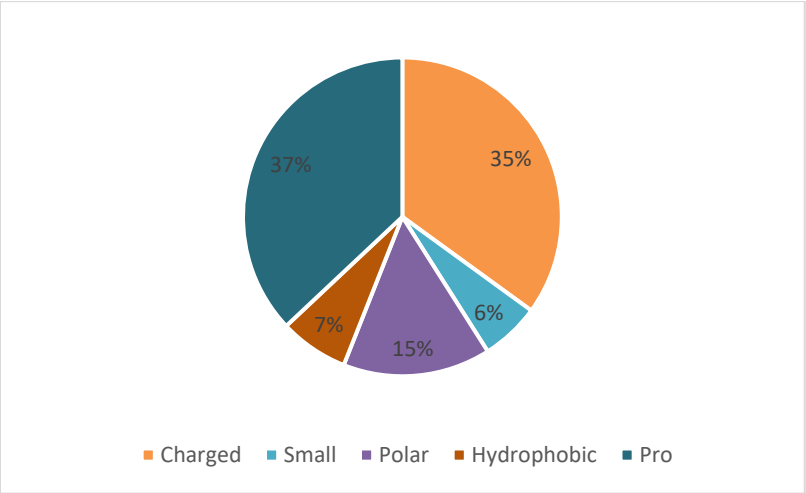

Figure 2.4 Pie Chart Representation of The Composition of The Non-Gly Residue of the Bacterial Collagen-like Domains. The following groups are shown: polar (Ser, Thr, Cys, Asn, Gln), charged (Asp, Glu, Lys, Arg, His), small (Gly and Ala), Pro, and hydrophobic (Val, Ile, Leu, Met, Phe, Trp, Cys) (Xu et al., 2010)

2.4 PURIFICATION OF COLLAGEN AND THE RESEARCH GAP

The recovery and purification of proteins remain as the major challenges in the production of protein and make up more than 70% of the downstream processing cost (Alves et al., 2000; Stitt & Weatherley, 1994). Precipitation, filtration and chromatography are examples of conventional purification method that involved several unit operations, tedious and time-consuming (Cao & Xu, 2008; Mazzola et al., 2008; Wang et al., 2017). Therefore, it is crucial to develop an efficient and cost-effective purification process for the development of high purity of recombinant collagen. Table 2.3 shows characteristics of methods used to purify collagen irrespective of their sources.

2.4.1 Chromatography

Chromatography is a method to separate mixture that involves two phases which are mobile phase and stationary phase. The compounds are subjected to flow by mobile phase through the stable surface of stationary phase (Li et al., 2013). Purification of proteins frequently consists of multi-step chromatography processes that involved several unit operations (Coskun, 2016). There are many types of chromatography that employ the same basic principles such as liquid chromatography, gas chromatography, ion-exchange chromatography and affinity chromatography. However, only affinity chromatography and ion-exchange chromatography will be further discussed as they are the most frequently used method in collagen purification.

2.4.1.1 Affinity Chromatography

Affinity chromatography has a functional group known as ligand that binds to the matrix to form a coordination complex. It is one type of chromatography technique based on the specific biological interactions between the separation material and desired component in the ligand (Magdeldin & Moser, 1996). During this process, the purified protein will bind to the ligand while non-bound components leave the column before elution process occurred by altering the buffer conditions (Coskun, 2016). Examples of columns available for this method are zinc, cobalt, nickel, and copper (Magdeldin & Moser, 1996).

This chromatography technique is well known as a powerful purification method with the use of peptide affinity tags commonly polyhistidine tag (His-tag), which are fused to the target protein (Bornhorst J.A. & Falke, 2000). A widely employed method to purify recombinant proteins with His-tag is known as immobilised metal-affinity chromatography (IMAC) (Riguero et al., 2020). Affinity chromatography

was performed under native conditions since the protein of interest is in soluble form (Bornhorst J.A. & Falke, 2000).

A research carried out by Warner et al. (2007), used nickel column in the affinity chromatography procedure to purify the recombinant collagen and it gives a higher yield compared to other types of columns. Meanwhile, only a 0.009% yield of recombinant human collagen is obtained using affinity chromatography principle through fast-protein liquid chromatography (FPLC) system (Rutschmann et al., 2013). Despite being the most preferable protein purification method for the past years due to its high selectivity, its complexity along with high cost of maintenance and low yield becomes the major drawback (Block et al., 2009; Pontis, 2017). Besides, another growing concern is the way affinity tags used may alter the protein activity (Adhikari et al., 2010; Bornhorst J.A. & Falke, 2000).

2.4.1.2 Ion-Exchange Chromatography

The principle behind this separation process is based on electrostatic interactions between charged protein groups, and solid support material (matrix). This method applies the idea of interaction between molecules and the stationary phase which are charged oppositely to each other. Negatively charged protein will be absorbed by positively charged ion-exchanged matrices or anion exchanged matrices whereas cation-exchange matrices will attach to the positively charged proteins (Adhikari et al., 2010). There are various types of ion exchanger matrix such as dextran, polystyrene, cellulose and acrylic (Coskun, 2016). Filter paper-based diethyl aminoethyl cellulose (DEAD) column chromatography is an example of ion-exchange chromatography used to purify type V collagen from porcine intestine (Sato et al., 2003). According to Sato et al. (2003), this method can prevent clogging problems and separates the proteins with high purity. However, the preparation of the experiment is tedious and there is high possibility of contamination to occur such as organic and bacterial contamination from resin (Block et al., 2009).

2.4.2 Precipitation

Precipitation is a conventional method of purification that is widely used in downstream processing of biomolecules and can be done in various techniques. Precipitation techniques that are commonly used in collagen purification are salt precipitation, acid precipitation and pepsin precipitation.

2.4.2.1 Salt Precipitation

This process refers to the formation of an insoluble salt when two solutions containing soluble salts are combined. The addition of salt will increase the solubility of globular proteins and this effect is termed salting-in. Meanwhile, salting out occur at the high salt concentration and protein solubility decreases that leads to precipitation (Wingfield, 2016). The increase in salt concentration will attract more water molecules. Hence, fewer water molecules interact with the charged part of the protein. The formation of contaminants alongside the salting-out process is a major challenge in salt precipitation. According to Cao & Xu, (2008), a combination of pepsin digestion, salt precipitation by NaCl and ion-exchange chromatography is used to purify type II collagen from chick sternal cartilage. Thus, an additional purification process such as chromatography is needed to obtain a purer protein.

2.4.2.2 Acid Precipitation

Acid precipitation is a scalable and efficient purification method for biomolecules. This technique is specifically used in the purification of recombinant protein to remove the contaminating bacterial host protein (Kiew & Don, 2013). The principle of this method is similar to salt precipitation which is the contaminants will tend to precipitate due to

the addition of acid. This is because the proteins are least soluble at a pH lower than 5. Although this method is favourable in purification of recombinant collagen, the acidic conditions may lead to adverse effects on the stability of the triple helix in these bacterial proteins (Werkmeister & Ramshaw, 2015).

2.4.3 Filtration

Another conventional method in the separation of biomolecules is filtration process. The basic understanding for this method is the removal of contaminants by passing the sample through a permeable medium but traps the contaminants or solid particles (Shon et al., 2011). This method is widely used in water treatment plants in environmental industry. Meanwhile, for protein purification, especially collagen, membrane ultrafiltration is commonly used method as a pre-concentration step (Machado et al., 2018; van Reis & Zydney, 2007). Glatz (2009), employed membrane ultrafiltration followed by chromatography to purify recombinant type I collagen. However, membrane fouling due to the pore blockage problem is a common issue that needs to be solved, thus increasing the cost of maintenance (Aimar & Bacchin, 2010). Additionally, filtration is also not suitable in the purification of low molecular weight species (Tang et al., 2011). Despite its disadvantages, membrane ultrafiltration is considered simple, eco-friendly and energy-saving, which is suitable in collagen extraction (Jiang et al., 2009).

Table 2.3 Characteristics of included publications regarding purification of collagen (Awang et al., 2020)

Reference	Sources of collagen	Method	Advantages	Disadvantages	Yield
Sato et al. (2003)	Porcine intestine (type V collagen)	Filter-based paper DEAD –cellulose column chromatography (ion-exchange chromatography)	• inexpensive	• column chromatography need to be prepared manually	Qualitative
Werkmeister & Ramshaw (2015)	Recombinant non-animals collagen	• Acid precipitation • Proteolysis (pepsin)	• Simple and effective • Inexpensive • Easy to scale up	• Acidic conditions can affect the stability of the collagen-triple helix structure • Further polishing steps needed for biomedical application	Qualitative
Rutschmann et al. (2013)	Human collagen	Affinity chromatography	• High selectivity • High purity	• Expensive • Low yield	Quantitative & Qualitative Yield: 0.009%
Warner, Blasick, Brown, & Oxford (2007)	Recombinant	Nickel affinity chromatography	• High selectivity • Reduce the risk of cross-contamination	• Low recovery • Expensive • Complex to scale up	Quantitative Yield: 15%

Table 2.3 continued

			(prepacked column)		
Glatz (2009)	Corn grain-derived recombinant collagen	• Membrane ultrafiltration • Chromatography (4 steps) (IEC)	• High yield and purity	• Pore blockage problem • Complex process	Quantitative Yield: 16 %
Singh & Tavana (2018)	Rat tail collagen: Type I	Aqueous two-phase system	• Simple and benign • Cost-effective • Rapid separation	• Not easy to scale up because PEG can cause corrosion of equipment	Quantitative Sys A: 62% Sys B: 34% Sys C & D :58%
Xu et al. (2010)	Recombinant Bacteria	Affinity chromatography	• High selectivity	• Expensive • Low recovery	Quantitative & Qualitative Yield: 11-12 %
Yd et al. (2013)	Sea cucumber (*Bohadschia bivitatta*)	Pepsin-solubilised collagen (PSC)	• High yield compared to acid precipitation	• Religious sentiments	Quantitative Yield: 65%
Jiang et al. (2009)	Jellyfish	Ultrafiltration	• Eco- friendly • Energy saving • Low maintenance	• Low yield/recovery	Quantitative Yield: 4.2%
Cao & Xu (2008)	Cartilage of chick	• pepsin digestion • NaCl precipitation	• High purity	• Time-consuming	Qualitative

Table 2.3 continued

		• Ion exchange chromatography			
Singh, Benjakul, Maqsood, & Kishimura (2011)	skin of striped catfish (*Pangasianodon hypophthalmus*)	• Acid Precipitation • Porcine pepsin	• No effect on triple helix structure	• Collagen not completely solubilized in acid	Qualitative and Quantitative ASC: 5.1% PSC: 7.7%

2.4.4 Aqueous Two-Phase System (ATPS)

Aqueous two-phase system (ATPS) or aqueous biphasic system (ABS) is a liquid-liquid fractionation technique and has been used throughout the years in biotechnological applications and purification of biomolecules such as proteins, enzymes, nucleic acids, virus, antibodies and cell (Iqbal et al., 2016; Raja et al., 2011). In 1896, ATPS was accidently discovered by Martinus Willem Beijerinck, a Dutch microbiologist and botanist while mixing an aqueous solution of starch and gelatine for culturing bacteria (Diamond & Hsu, 1992; Iqbal et al., 2016; Teixeira et al., 2017). However, the real application was started over 60 years ago by Swedish biochemist named P.-Å. Albertsson. Since then, this purification technique has been widely used for a range of purpose (Kaul, 2000; Iqbal et al., 2016).

ATPS are formed when two incompatible water-soluble phase components are mixed above their critical concentrations, resulting in the formation of two immiscible phases at equilibrium (Alberto, 2018; Grilo et al., 2014; Nawab et al., 2019; Ratanapongleka, 2013). Then, the biological substances are evenly distributed between the phases. For polymer-polymer system, ATPS formed from unfavourable energy interaction when polymer segments bonded strongly to each other. The driving force for the demixing properties is the entalphy associated with the interactions of the components, which is opposed by the loss in entropy associated with the segregation of the components during phase separation (Kaul, 2000). In polymer-salt system, partition behaviour is governed by volume exclusion-effect and salting-out effect(de Albuquerque Wanderley et al., 2017).

Figure 2.5 illustrated the aqueous two-phase system involving polymer and salt system. Up to date, there are a few types of ATPS that has been discovered such as polymer/polymer (Rito-palomares et al., 2014) , polymer/salt (Grilo et al., 2014) and alcohol/salt (Lin et al., 2013). A summary of studies that applied ATPS as their purification method is presented in Table 2.4.

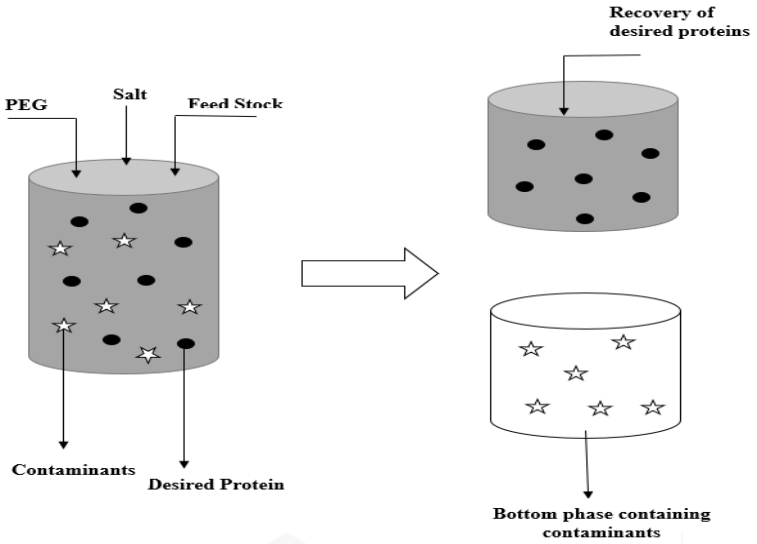

Figure 2.5 PEG/Salt Aqueous Two-Phase Extraction Principle (Stitt & Weatherley, 1994)

2.4.4.1 Polymer/Polymer System

The phase separation in two water-soluble polymers occur due to unfavourable energy interaction when the polymer segments bond strongly to each other (Ratanapongleka, 2013). The example of polymers used in ATPS are polyethylene glycol (PEG), polypropylene glycol (PPG), dextran, polyvinylpyrrolidone and hydroxypropyldextran (Ratanapongleka, 2013). Polyethylene glycol (PEG) becomes the most frequent polymer used in ATPS because it is cheaper and works well with other polymers and salts (Raja et al., 2011). The commonly used polymer/polymer system is combination of polyethylene glycol (PEG) and dextran and has been successfully applied in purification of type I rat tail collagen (refer table 2.4)**.** This combination is preferable because dextran is non-toxic polymer and can provide stabilising effect on microbial (Sinha et al., 2000)**.** However, due to high viscosity of the system and cost of dextran, other alternatives are found to substitute dextran such as cellulose, methylcellulose, agarose, polyvinyl alcohol and maltodextrin (Almeida, Venâncio, Teixeira, & Aires-Barros, 1998; Da Silva & Meirelles, 2000; Ratanapongleka, 2013; Rosa et al., 2010; Stitt & Weatherley, 1994). Other than that, ATPS with only one polymer in the presence

of salt (polymer/salt) is the better choice with cheaper cost, easy to handle and low viscosity (Ooi et al., 2009; Raja et al., 2011; Ratanapongleka, 2013).

2.4.4.2 Polymer/Salt System

One of the alternatives to the polymer/polymer system is polymer/salt system which formed when a water-soluble polymer and salt are mixed above critical concentrations, resulting a salt-rich bottom phase and a polymer-rich top phase (Pereira & Coutinho, 2020). Example of salt used to form two-phases especially with PEG are potassium hydrogen phosphate, potassium chloride, sodium dihydrogen phosphate, sodium carbonate, sodium citrate, magnesium sulfate and ammonium sulfate (Banik et al., 2003; Ratanapongleka, 2013). For polymer/salt systems, combination of PEG and phosphate salt is preferred since it is cheaper and stable in wide range of pH (6-9), and it is widely used by researchers in previous studies (refer Table 2.4).

The advantages of polymer/salt systems including their low costs, greater selectivity, easier manipulation and easy to scale up (Grilo et al., 2014). Additionally, the phases has low viscosity which can provide shorter times for the phase separation (Lladosa et al., 2012; Rosa et al., 2010). However, the high concentration of salt can denature the fragile biomolecules and in some cases, environmental problem can occur due to high salt concentration in the effluent (Da Silva & Meirelles, 2000). One solution for this drawback is using citrate salts due to their non-toxic and biodegradable characteristics (Goja et al., 2013). Despite of its wide applications in various studies, the mechanisms of how salts influence the phase separation is poorly understood (Iqbal et al., 2016). Due to the involvement of broad factors in the interaction of molecules during protein partitioning create a complex problem. A model allowing a *priori* calculation of protein partitioning for a wide range of polymer molecular weight and polymer and salt concentrations without the measurement of large parameters is not yet available (Kaul, 2000).

2.4.4.3 Alcohol/Salt System

The ATPS composed of alcohol and salt was first studied by Greve & Kula in 1991. In comparison to polymer/salt system that used unrecovered polymer, alcohol/salt system is an cost-effective extraction method that provide low-toxicity to environment (Zhi & Deng, 2006). Apart from that, the targeted biomolecules can be simply recovered using evaporation method (Lin et al., 2013). Nowadays, a lot of studies have been carried out using alcohol/salt system, for example, the recovery of human interferon alpha-2b from recombinant *E. coli*, achieved 74.64 % yield with 16.24 purification factor. However, compared to polymer/polymer and polymer/salt systems, the studies of the principle, theory, mechanisms and applications of polymer/alcohol systems in purification of biomolecules generally, and collagen specifically are still lacking.

2.4.4.4 Advantages of Aqueous Two-Phase System

The cost of chemicals becomes the dominant factor especially for large scale protein purification (Kaul, 2000). Therefore, the urge of using inexpensive yet effective chemicals is important in order to reduce the overall cost of purification process. This has favored the use of ATPS method generally and PEG/salt system specifically in protein purification. PEG/salt system is a good choice in replacing the expensive fractionated dextran (Ratanapongleka, 2013). Besides of the cheaper chemicals, ATPS also known for its extremely simple and powerful process without the complex and multi-step unit operations (Kaul, 2000). According to research by Rito-palomares (2006), on direct comparison between ion-exchange chromatography and aqueous two phase system for partial purification of penicillin acylase, ATPS approach resulted in the reduction number of unit operations involved and also cost of unit operation. Besides, in a study by Nitsawang et al. (2006), higher recovery (88%) and purity (100%) was achieved in purification of papain from wet *Carica papaya* latex. In purification of collagen, polymer/polymer ATPS system was used to localised collagen from rat tail into one phase and this simplified approach is very beneficial in biomedical applications such as cancer drug screening (Singh & Tavana, 2018)

Table 2.4 Summary of ATPS purification method comprises of type of ATPS, phase-forming components, biomolecules and their applications.

Type of system	Phase-forming components	Type of biomolecules	Application	References
Polymer/Polymer	PEG 1450 and Maltodextrin 4000	Bovine serum albumin (BSA)	Used in laboratory application as protein concentration standard and cell nutrient.	Alves et al. (2000)
Alcohol/Polymer	60% (v/v) ethanol and 15% (w/v) potassium phosphate	Glycyrrhizin	Used for anti-inflammatory compound, cosmetics and food additives.	Tianwei et al. (2002)
Polymer/Salt	29% (w/w) PEG 1000 and 9% (w/w) potassium phosphate	B-phycoerythrin	Act as colouring compound in cosmetics, food and pharmaceutical (fluorescent marker).	Benavides & Rito-Palomares (2006)
Polymer/Salt	8% (w/w) PEG 6000 and 15% (w/w) ammonium sulfate	Papain from *Carica papaya* latex	Widely used as meat tenderizer, defibrinating wounds, shrink proofing of wool, treatment of edemas.	Nitsawang et al. (2006)
Polymer/Salt	12% (w/w) PEG 6000, 10% potassium phosphate, and 15% (w/w) NaCl	Immunoglobulin (IgG)	Used as diagnostic tools, treat genetic, auto-immune diseases, and immune deficiencies.	Azevedo et al. (2007)
Polymer/Salt	12% (w/w) PEG 20000 and 7.33% (w/w) potassium phosphate	Alcohol dehydrogenase (ADH)	Used in metabolism of alcohol in biochemical and forensic studies.	Madhusudhan et al. (2008)
Polymer/Salt	14.5% (w/w) PEG 600 and 17.5% (w/w) phosphate	*β*-glucuronidase	Used as reporter gene to monitor gene expression in plant and mammalian cells.	Aguilar & Rito-palomares (2008)

Table 2.4 continued

Table 2.4 continued

Polymer/Salt	0.102 (g/mL) PEG 6000, 0.17 (g/mL) ammonium sulfate and 25 (mg/mL) NaCl	Papain	medical application, cell isolation, food, detergents, leather, textiles, cos- metic and pharmaceutical industries	Ling et al. (2010)
Polymer/Salt	18% (w/w) PEG 1500 and 15% ammonium sulfate	Pepsinogen	Collagen extraction, gelatine extraction, cheese making	Zhao et al. (2013)
Alcohol/Salt	18% (w/w) 2-propanol, 22% (w/w) ammonium sulfate and 1% (w/w) NaCl	Human interferon α-2b	Use in treatment of hairy cell leukemia, chronic myelogenous leukemia, condylar acuminate and chronic hepatitis B	Lin et al. (2013)
Polymer/Salt	20% (w/w) PEG 8000 and 11.6% (w/w) potassium phosphate	Fibrinolytic enzymes	Treatment for cardiovascular disease and stroke	Mohamed Ali et al. (2014)
Polymer/Salt	13% (w/w) PEG 6000 and 11% (w/w) potassium phosphate	Bromelain	food, beverage, tenderization, cosmetic, pharmaceutical and textile	Arshad et al. (2017)
Polymer/Salt	27% (w/w) PEG1500 and 34% (w/w) sodium citrate	Protease	used in the detergent industries.	Samad & Shukor (2017)
Polymer/Salt	15% (w/w) PEG 3350 and 12.5% (w/w) phosphate salt	Collagenase	Used for collagen hydrolysis, wound repair and production of bioactive peptides	de Albuquerque Wanderley et al. (2017)
Polymer/Polymer	5% (w/w) PEG 35000 and 7% (w/w) Dextran 500000	Type I rat tail collagen	Commonly used in cosmetics and food industries.	Singh & Tavana (2018)

Table 2.4 continued

Polymer/Salt	20% (w/w) PEG 4000 and 9.9% (w/w) Ammonium sulfate	Protease	Used indetergents, leather, food industry and bioremediation processes	Nawab et al. (2019)

2.4.4.5 Phase Diagram

Phase diagram is important in providing the concentration of phase-forming components which are required to form two-phase system that are in equilibrium. This diagram must be constructed at the early stage of aqueous two-phase system (Pereira & Coutinho, 2020). It consists of binodal curve (Figure 2.6) that divides the region of two aqueous phases (above the curve) formed by a suitable concentration of phase-forming components from one phase (below the curve). As described by Kaul (2000), there were three different methods used for construction of binodal curves, (i) turbidimetric titration, (ii) cloud-point titration, and (iii) node determination method. For purification of new discovering proteins using ATPS, researchers are advised to construct the binodal curves since the published equilibrium data may consist the unreported experimental error and addition of additives (Forciniti, 2000).

Apart of binodal curve, the phase diagram also consists of tie-line (TL) and critical point (C) as illustrated in Figure 2.6. The tie-line connect two nodes on the binodal curves that represents the final concentration of top and bottom phases (Pereira & Coutinho, 2020). All the nodes along tie-line (X, Y, Z) have different composition and volume ratios (Vr). Another crucial parameter is tie-line length (TLL) which is represented as the measurement for the relative difference between top and bottom phases (Liu et al., 2013). The tie-line length (TLL) can be calculated to express the effect of system composition on partitioning components (Kaul, 2000). Meanwhile, critical point has an equal volume of top and bottom phase or Vr of 1.00 and TLL of 0. According to Arshad & Amid (2018), the tie-line length (TLL) was estimated graphically by using volume ratio and can be calculated by the following equation:

$$TLL_{=}\sqrt{\Delta X^2 + \Delta Y^2} \qquad 2.1$$

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

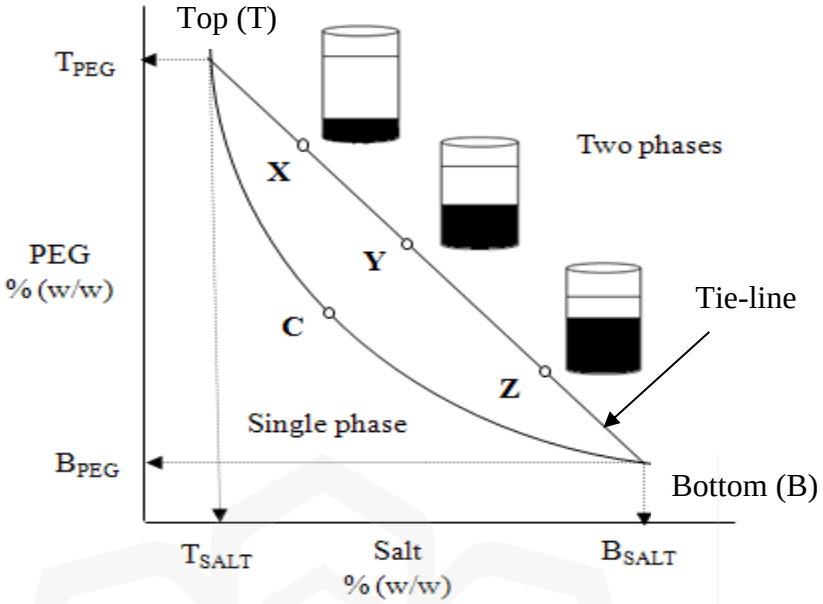

Figure 2.6 Binodal curve (Raja et al., 2011)

2.4.4.6 Factors Affecting Aqueous Two-Phase System (ATPS)

There are several variables that can affect the partitioning of biomolecules in ATPS such as molecular weight of polymer, concentration of polymer and salts, pH value, type of salt and temperature.

i. Polymer molecular weight

Polymer molecular weight can affect the partitioning process by altering the phase diagram and polymer-biomolecule interactions. Basically, increasing of molecular weight of polymer will push the biomolecules to another phase. This is because of the strong repulsive force between polymer and biomolecules. However, a very low molecular weight of polymer resulting in attraction of undesired biomolecules towards polymer phase due to low exclusion effect. Hence, it is not suitable to choose low molecular weight of polymer in ATPS. Therefore, selection of polymer molecular weight is considered as the key point in this technique.

ii. Polymer concentration and salt concentration

The effect of polymer concentration is closely related to density, refractive index and viscosity of the phase. For polymer-salt system, higher concentration of polymer is required for lower concentration of salt in ATPS. The concentration of polymer also depends on the molecular weight of polymer.

iii. pH

The pH value of ATPS may affects partitioning of biomolecules by altering the charge and surface properties of the solute. The net charge of the protein turns negative in case of higher pH than the isoelectric point (pI) and positive if lesser than pI. If the pH is equal to pI, net charge will be zero. It has been reported that the partitioning of negatively charged biomolecule in a higher pH system increases the partition coefficient and target biomolecule prefers the top phase. Higher pH values than pI of protein induce an affinity towards PEG-rich phase because of the positive dipole moment (Iqbal et al., 2016).

iv. Type of salt

Type of salt used in ATPS depends on their ability to promote hydrophobic interactions between molecules. The anion and cation of salt react with protein as counter ion depending on the net charge of the protein (Abd Samad, 2017). The binodal curve will move to water-rich corner with an increase of the anion hydrophobicity of the selected salt (H.-O. Johansson et al., 2011). Phosphate salt is widely used in partitioning of bioproducts using ATPS. Recently, the application of citrate and ammonium carbonate in ATPS are favoured due to their high selectivity, biodegradable and non-toxic (Goja et al., 2013).

v. Temperature

Different types of system have different effects of temperature. For example, high temperature needed in polymer/salt system to achieve two-phase system while low temperature required for polymer/polymer system to form two-phase system (G. Johansson & Walter, 1999). An increase in temperature will increase the concentration of PEG and salt thus reduce the water molecule for solute salvation in bottom phase. As result, the solubility of biomolecules in bottom phase will decrease. Moreover, the effect of temperature towards partitioning become significant when the system composition is close to critical point (Kaul, 2000).

In the present study, the effect of molecular weight of polymer, concentration of polymer and salts and pH value on the purification of recCLP using ATPS were chosen and studied. These factors are the most common factors studied by researchers involved in ATPS experiment and considered as the major factors. Compared to other factors, type of salt and temperature are the least common factors studied in purification of biomolecules using ATPS. In order to minimise the number of factors, type of salt and temperature were kept constant.

2.5 APPLICATIONS OF COLLAGEN IN INDUSTRIES

The role of collagen has been discovered and studied by scientist for over a century and widely used in both biomedical and industrial applications. Among the various applications of collagen, its involvement in biomedical, food and cosmetic industries are detailed in this section.

2.5.1 Biomedical Industry

Owing to its high biocompatibility, collagen type I is the commonly used collagen and is considered as the gold standard in this field (Lee et al., 2001; Silvipriya et al., 2015). Due to its immense characteristics such as weak antigenicity, cell attachment ability, biodegradability, biocompatibility, and triple-helix self-association, thus make it applicable as biomaterials in the biomedical field (Lim et al., 2019). In tissue engineering technology, collagen is used to replace ceramic scaffold in bone and cartilage regeneration in parallel to its function to provide structural and mechanical support to tissue and organ (Gelse et al., 2003b; Oliveira et al., 2010).

On top of that, collagen has been comprehensively and diversely utilised in drug delivery systems in various forms like film, sheet, disc, shield, sponge, gel, hydrogel and nanoparticle (Lee et al., 2001). The examples of applications of collagen in drug delivery systems are injectable microspheres, tablets, mini-tablets, nanoparticles and combination with liposomes for gel formulation (Vikash et al., 2013). Furthermore, collagen is used as shields in ophthalmology and sponges specifically for burn treatment (Cherim et al., 2016; Lee et al., 2001; Vikash et al., 2013).

2.5.2 Food

Alike pharmaceutical industry, collagen has a wide application in the food industry such as food additives, supplements, and drinks. Food additives are used to improve the texture, taste, colour, and appearance including antioxidants, emulsifiers, colorants, preservatives and thickeners. According to Neklyudov (2003), collagen acts as food additives to enhance the rheological and technological properties of sausages and frankfurters. Furthermore, Santana et al (2011) stated that the heated collagen fibre is a great emulsifier in acidic products as it allows the production of electrostatically stable emulsions at low pH. Other than that, due to high water-absorption capacity, its

derivative, gelatine is used as a thickener in pudding, ice cream and cakes (Coppola et al., 2020).

Collagen is greatly known for its health benefits to the consumers. It helps to uphold skin, nails, hairs and body tissues (King'ori, 2011). Thus, collagen is one of the crucial ingredients in most nutricosmetic products and usually can be found in the form of liquid, pills, or functional food. For example, the product of Munchy's Wheat Krunch Collagen has augmented with marine collagen to promote supplementation benefit (Hashim et al., 2015). The idea to combine vitamin C and collagen peptides in supplements helps the upregulation of collagen type I *in vivo* thus solving age-related skin thinning issues (Shibuya et al., 2014).

Moreover, collagen supplements are also used as sports nutrition to improve lean muscle gain and cardiovascular performances, shorten the recovery period and restructure the damaged joint. The hydrolysed collagen-containing arginine is used to promote muscle mass besides the natural creatine intake may help in the growth of new muscles (King'ori, 2011). Other than that, a controlled study by Wei et al. (2009) proved the ability of chicken type II collagen in reducing the pain and swollen joints of rheumatoid arthritis patients.

2.5.3 Cosmetic

As humans get older, the production of collagen is getting slow which results in the loss of elasticity in human skin. Besides, UV radiation is another factor that can reduce the amount of collagen in the skin. Thus, the most common ingredients in cosmetics especially in skin care products come from collagen. Nowadays, hydrolysed collagen is the alternative in cosmetic formulations due to the insolubility of collagen. Hydrolysed collagen contains small peptides and short polypeptides that are soluble in water (Sionkowska et al., 2017).

Collagen has great potential in cosmetic fields such as skin care, hair care and oral care since it is a natural humectant and moisturiser (Sionkowska et al., 2020). Due to its excellent ability to bind with water molecules, it helps to keep the skin moisturised, softened and hydrated during the day. The peptide occlusion responsible for making skin looks radiant and smooth besides avoiding skin and hair damage caused by mechanical impairments (Helfrich et al., 2008; Jin et al., 2001).

Furthermore, collagen is well known as a biodegradable injectable filler in aesthetic medicine to treat dermatological defects, skin texture and signs of aging before the introduction of hyaluronic acid in this field (Funt & Pavicic, 2013; Sionkowska et al., 2020). As the public acceptance of the dermal fillers grows, lots of collagen fillers have been commercialised such as Resoplast, Zyderm, Zyplast, Fibrel, Percamol and Evolence (De Maio & Rzany, 2014; Funt & Pavicic, 2013).

2.6 DESIGN OF EXPERIMENT

Design of Experiments (DOE) is a multipurpose statistical tool used for optimisation, variable screening, comparison of design and robust design. For the past 20 years, the application of DOE have been blowing up in manufacturing and non-manufacturing industries especially in scientific field such as engineering, biochemistry, medicine and computer science (Durakovic, 2017). The design of experiments is also used to study the effect of various factors on the responses variable (Arshad, 2016). Response Surface Methodology (RSM) is the most popular method used in DOE since it is able to reduce the period of experiments by fewer experiment sets. There are few types of statistical optimisation in RSM such as central composite design (CCD), Box–Behnken design, two-level factorial design, Placket– Burmann design and the Taguchi method (Cheng et al., 2013). As mentioned in subsection 2.4.4.6, partitioning of biomolecules in ATPS involving various factors. Therefore, DOE can be used as powerful strategy to identify the optimal purification process conditions as well as to evaluate the interactions

between them (Rosa et al., 2010). Furthermore, CCD was selected due to its high accuracy and optimised results.

One-factor-at-a-time (OFAT) is a method involving the testing of a factor, while the others are kept constant. Then, the other factors are changed until the best condition is found. Despite of demanding high number of experiments, OFAT design allows the researchers to find out whether a factor has any effect in a shorter time. Thus, they can monitor the data more rapidly (Qu & Wu, 2005). However, OFAT approach does not evaluate the interaction between the factors (Fukuda et al., 2018). Hence, DOE is required as a following method.

2.7 IMPORTANCE OF RESEARCH IN HALAL INDUSTRY

As commercial collagens are often extracted from animals, the source of the raw materials is a decisive factor in the formulation of halal end-products. According to the global market forecast by source reported by Visiongain Pharma in 2018, bovine and porcine collagen contributes a high percentage compared to other sources as illustrated in Figure 2.7. Porcine collagen is not permissible as it was extracted from pig meanwhile, bovine collagen can be doubtful if the sources were not slaughtered according to shariah. Hence, there is high possibility for Muslims to consume non-halal collagen. Furthermore, the potential disease transmission as explained in section 1.2 has become a serious issue regarding mammalian collagen.

Collagen from marine organism becomes a promising source of collagen due to their high content of collagen and free from any transmissible diseases (Lim et al., 2019; Subhan et al., 2015). However, pepsin is commonly used in the extraction step for marine collagen (Aukkanit & Garnjanagoonchorn, 2010; Hashim et al., 2015; Kiew & Don, 2013). Pepsin is an enzyme derived from pigs thus raising the issue of halal status of marine collagen (Ermis, 2017).

In the present study, the concept of halal and *toyyiban* is taken into consideration in choosing the source of collagen and the method of purification. Thus, this study involved a safe collagen source that comes from a non-pathogenic bacteria and a simple purification method with minimal chemicals without involving any non-halal and harmful ingredients.

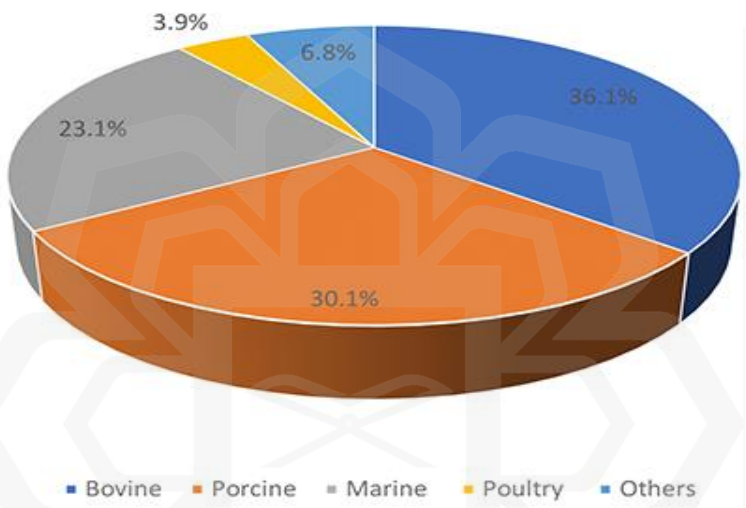

Figure 2.7 Global collagen market forecast, by source, (Visiongain pharma, 2018)

CHAPTER THREE

METHODOLOGY

3.1 INTRODUCTION

This chapter covers methodologies (Figure 3.1) used to achieve all objectives stated in Chapter 1. In this chapter, the materials and method are described for the ATPS process followed by its optimisation. Finally, the detailed experimental methods are elaborated comprehensively in this chapter.

3.2 MATERIALS

3.2.1 Chemicals

All chemicals and reagent were purchased from Merck Chemicals (Darmstadt, FR Germany), Sigma-Aldrich Chemicals (St. Louis, MO, USA), Bio-Rad (Hercules, California, USA) and Ncalai Tesque (Kyoto, Japan). All chemicals were of analytical grade. List of chemicals can be found in Appendix A.

3.2.2 Equipment and Instruments

Equipment used throughout this project were autoclave machine (Hirayama, Japan)**,** laminar flow (Esco, Singapore), incubator shaker (Sortorius, Germany), incubator shaker (HT Ecotron, Inforst, Switzerland), spectrophotometer (Thermo Fisher Scientific, USA), pH meter (Mettler Toledo, Switzerland), refrigerated centrifuged

machine (Eppendorf, Germany), weighing balance (Mettler Toledo, Switzerland), multimode reader (Tecan, Switzerland) and AKTA fast protein liquid chromatography (GE Healthcare, UK). The detailed list of equipment and instruments are shown in Appendix C.

3.2.3 Glassware Consumable Items

Erlenmeyer flasks, beaker and Schott bottles were the basic glassware used in this study. Consumable items such as autoclave tape, autoclave plastic bag, petri dishes, parafilm, microcentrifuges tubes, Falcon tubes, pipette tips, syringes, 96-well plate and the rest. were used in the present study. The list of consumables items can be referred in Appendix D.

3.3 METHODOLOGY

The overview of the overall experiments conducted in this present study is presented in Figure 3.1.

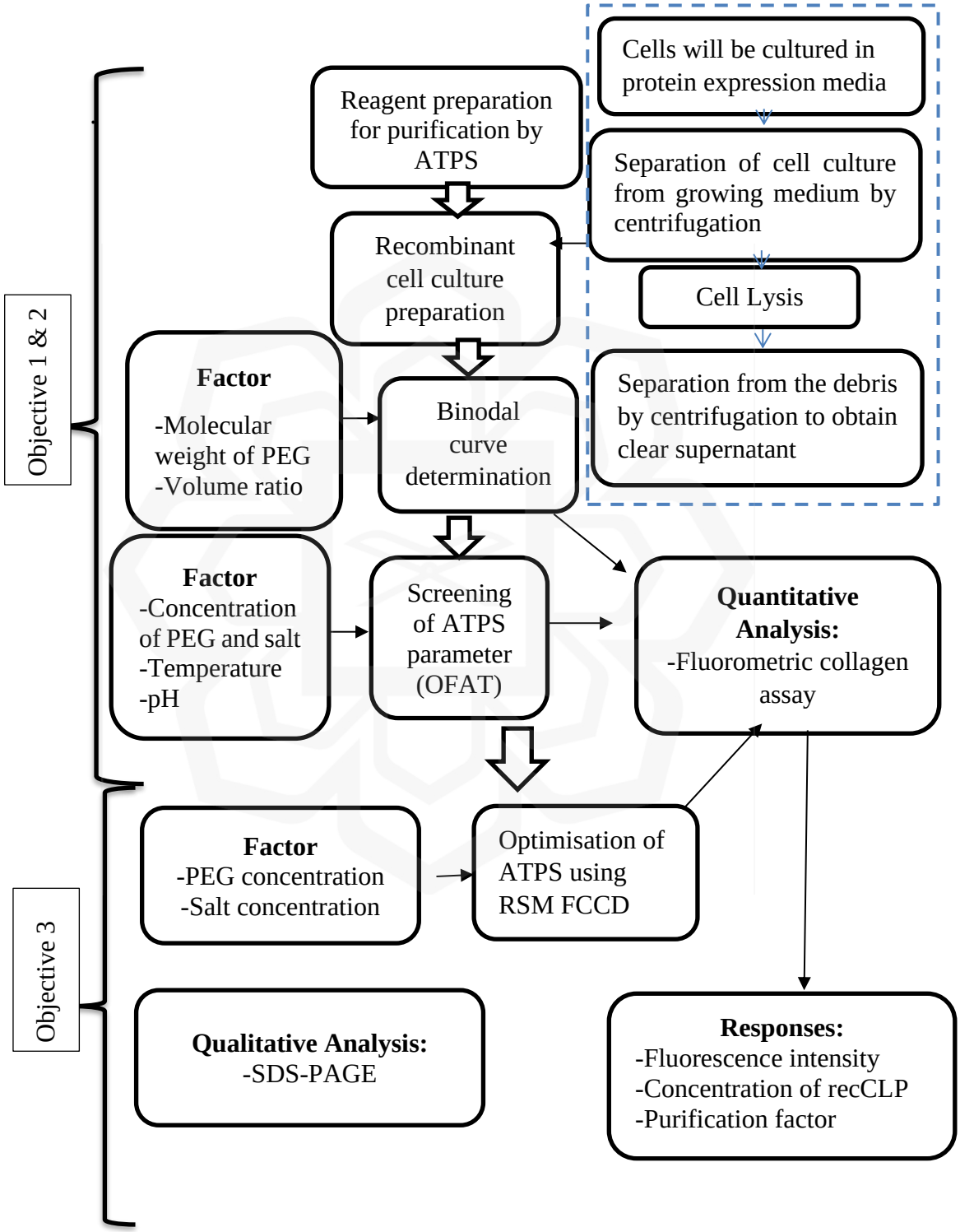

Figure 3.1 Flowchart showing general methodology of the study

3.3.1 Growth Condition for *Escherichia coli* BL21DE3 Harbouring Collagen-like Protein

The positive clone of *Escherichia coli* BL21DE harbouring collagen-like protein from *Rhodopseudomonas palustris* (cloned by another student from the same laboratory, Fatemeh Soroodi) was cultured in 50 mL M9 minimal medium supplemented with 50 ug/mL ampicillin and incubated in an incubator shaker (HT Ecotron, Inforst, Switzerland) at 250 rpm until the OD_{600nm} reached 0.8-1.0. Then, 1 mM IPTG was added and incubated for 24 hours at 15 °C (CERTOMAT® IS, Sartorius, Germany). After 24 hours, the cells were harvested by centrifugation (4 °C, 4696 x *g*, and 10 minutes) (5804R, Eppendorf, Germany), and the cell pellet was stored at -20 °C for further used in cell lysis step.

3.3.1.1 Cell lysis

The cell pellet of the harvested recombinant collagen-like protein was resuspended in the lysis buffer (20 mM Na_2HPO_4, 0.5 M NaCl, pH 7.4) before cell was disrupted by sonication (40% amplitude, 10 seconds on, 20 seconds off). The cell lysate was then centrifuged at 12 000 rpm for 30 minutes to obtain a clear supernatant for further analysis.

3.3.2 Purification of Recombinant Collagen-Like Protein using ATPS

Aqueous two-phase system (ATPS) technique had been applied in this study and affinity chromatography was conducted to compare the purification efficiency of ATPS. Figure 3.2 presented the flow chart process for the whole purification process. A detailed purification overview of ATPS is illustrated in Figure 3.3.

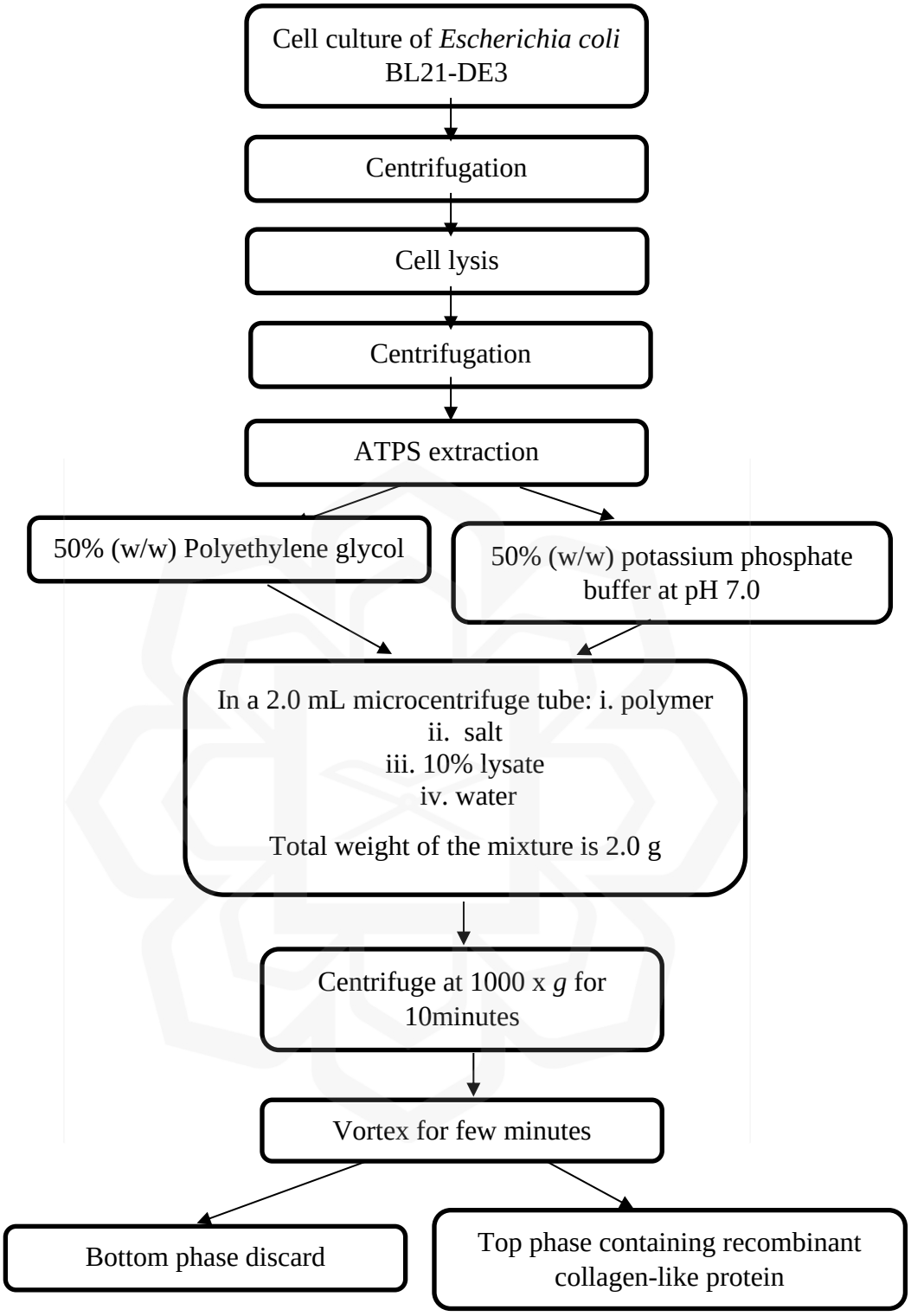

Figure 3.2 Flow chart of recombinant collagen-like protein purification by ATPS technique

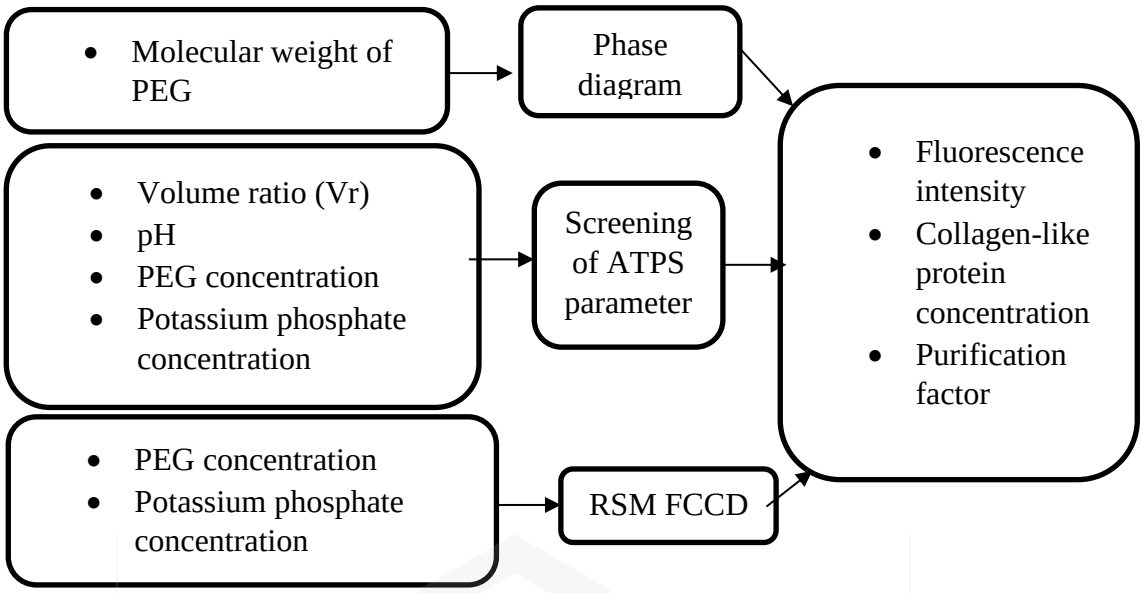

Figure 3.3 Detail of experiments for purification of recombinant collagen-like protein by ATPS technique

3.3.2.1 Development of Binodal Curve

The binodal curve was constructed using node determination method proposed by (Kaul, 2000). Prior to binodal curve determination, stock solution of polyethylene glycol (molecular weight: 1500, 2000, 4000, 6000, 8000) and potassium phosphate buffer at pH 7.0 with concentration of 50 % (w/w) were prepared. Stock solution of 50% (w/w) PEG was prepared by dissolving 50 g of PEG with 50 g of distilled water. For preparation of potassium phosphate buffer at pH 7.0, 15.4 g of dipotassium hydrogen phosphate was mixed to 9.6 g potassium dihydrogen phosphate and 25 g distilled water. The amount of each component in phosphate buffer was determined using Henderson-Hasselbalch equation and the details calculation was shown in Appendix F.

ATPS was set up in 2.0 mL microcentrifuge tube with total weight of 2 g, made up of different composition of each component. Out of 2 g, about 5 % (w/w) of

potassium phosphate buffer at pH 7.0 was mixed with 50 % (w/w) of PEG from stock solution, 10% of recombinant lysate and distilled water was added into the system to reached total weight of 2 g. The mixture was gently mixed by vortex for few minutes and centrifuged for 10 minutes at 1000 x *g* and 25 ˚C (Heraeus Multifuge X1R, Thermo Scientific, USA). The formation of two phases was observed and the process was repeated by increasing potassium phosphate concentration and stopped when two phases system was formed. This step was done in triplicate. Then, the steps were repeated again by changing the concentration of PEG on different concentration of potassium phosphate buffer. Concentration of phosphate that created two phases for each PEG concentration before the system turns into one phase were recorded and translated into graph of concentration of PEG versus concentration of salt (potassium phosphate buffer). After phase separation, top and bottom phases with volume ratio of 1 were collected for further analysis.

The volume ratio is defined as,

$$V_R = \frac{Volume\ in\ top\ phase\ (V_T)}{Volume\ in\ bottom\ phase\ (V_B)}. \quad (3.1)$$

Where V_T and V_B are the volume in top phase and bottom phase respectively. Protein concentration was measured using fluorometric collagen assay, which was further explained in section 3.3.5 and Appendix E.

Table 3.1 showed the concentration of PEG and potassium phosphate in each system that formed two phases and one phase and the end nodes represents the final concentration of potassium phosphate needed to create two phases with PEG before it turns into one phase. The binodal curve as shown in Figure 3.4 was constructed by connecting all the end nodes with the line. The binodal curve represent a line that separates one phase from two phase system. The example of data for PEG2000 was tabulated in Table 3.2. After experimental procedure of binodal curve development, several expressions has been used to corelate the binodal data using SigmaPlot® 14.5 software provided with non-linear regression and fitting tools (Capela et al., 2019; Xie et al., 2010).

Table 3.1 System composition of binodal curve representation for PEG2000 and potassium phosphate at pH7.0

PEG2000 % (w/w) / **Potassium Phosphate % (w/w)**	**50**	**45**	**40**	**35**	**30**	**25**	**20**	**15**	**10**	**5**	**3**
10	x	x	x	x	x	x	x	x	x	x	x
11	/	x	x	x	x	x	x	x	x	x	x
12	/	/	x	x	x	x	x	x	x	x	x
15	/	/	/	x	x	x	x	x	x	x	x
18	/	/	/	/	x	x	x	x	x	x	x
20	/	/	/	/	/	x	x	x	x	x	x
22	/	/	/	/	/	/	x	x	x	x	x
24	/	/	/	/	/	/	/	x	x	x	x
26	/	/	/	/	/	/	/	/	x	x	x
30	/	/	/	/	/	/	/	/	/	x	x
35	/	/	/	/	/	/	/	/	/	/	x
40	/	/	/	/	/	/	/	/	/	/	/

/ = system with two aqueous phases
x = system with one aqueous phase

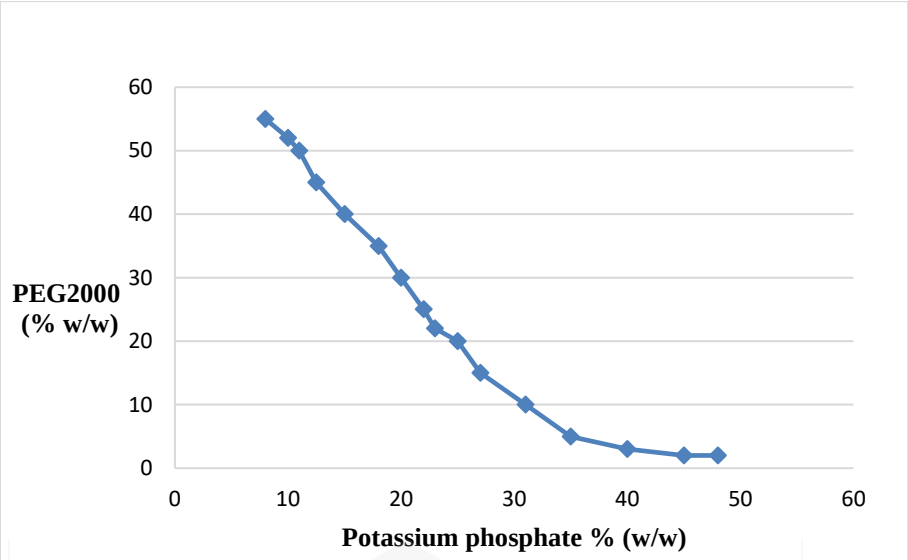

Figure 3.4 Phase diagram of a system containing PEG2000 and potassium phosphate at pH 7.0

Table 3.2 The end nodes for construction of binodal curve for PEG2000, and potassium phosphate at pH 7.0

Percentage of Potassium phosphate pH 7.0 (% in w/w)	**Percentage of PEG2000 (% in w/w)**
8	55
10	52
11	50
12.5	45
15	40
18	35
20	30
22	25
23	22
25	20
27	15
31	10
35	5

40	3
45	2
48	2

3.3.3 Total Protein (Bradford) Assay

The protein content in each phase of ATPS samples were quantified based on Bradford method using Bio-Rad Protein Assay (Bio Rad, Germany) at 595 nm with microplate reader (Multiskan^{TM} Go, USA). Bovine serum albumin was used as a standard for protein assay (Bradford, 1976). All samples were carried out in triplicate. 5 µL of sample was mixed with 250 µL of Bradford reagent in a clean 96-well microplate reader. Then, the mixture was gently mixed by pipetting up and down. After 10 minutes of incubation at room temperature, absorbance at 595 nm was measured. The advisable period of incubation by manufacturer is between 5 minutes and 1 hour. The standard curve of absorbance at 595 nm against concentration of BSA was used to determine the concentration of protein in sample.

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

SDS-PAGE was used to measure qualitatively the purity of purified collagen. The samples were diluted to 1:1 ratio before applying to 12% resolving gel (Appendix B5) and 4% stacking gel (Appendix B6). Electrophoresis was run at 120 V, 400 mA for 60 min, the gels are stained using ReadyBlue™ Protein Gel Stain (Sigma, USA) or silver staining method (Appendix B7).

3.3.5 Total Collagen Assay Using Fluorometric Collagen Assay

According to Yasmin et al. (2014), the amount of collagen purified in ATPS samples were measured based on quantitative fluorometric collagen determination technique using EnzyFluo Collagen Assay Kit (BioAssay Systems, USA) at $\lambda_{ex/em}$ 375/465 nm with black flat-bottom 96 well plate. Bovine collagen type I (Sigma, USA) was used as a standard for collagen assay.

20 µL of top phase ATPS sample and 30 µL of digest mix containing digest enzyme were mix in a clean microcentrifuge tube. The mixture was gently mixed by pipetting up and down the mixture followed by incubation at 37 °C for 1 hour. Then, 40 µL of dye reagent was added into the mixture. After 10 minutes of incubation at 37 °C, 8 µL of developer was added before another 10 minutes of incubation. Next, the sample was transferred to 384-black flat bottom microplate reader and ready for fluorescence reading at $\lambda_{ex/em}$ 375/465 nm using Multimode reader (Tecan, Switzerland). In this assay, sample blank with the absent of digest enzyme was used to correct the potential error in this assay. The standard curve of collagen concentration against $\Delta F_{375/465nm}$ was used to calculate the protein concentration in ATPS sample.

$$\Delta F_{375/465nm} = F_S - F_B \tag{3.4}$$

Where F_S and F_B is the fluorescence reading for sample and sample blank, respectively.

3.3.6 Screening of ATPS Separation Parameters Using One Factor at A Time (OFAT) Experimental Design

The experiment was set up based on OFAT designed for ATPS separation. The tested parameters were volume ratio, molecular weight of polyethylene glycol, pH,

temperature and concentration of PEG and salt. The responses were fluorescence intensity, concentration of collagen-like protein and purification factor.

3.3.6.1 Volume Ratio

The effect of different volume ratio on the purification using ATPS was studied by varying volume ratio on the tie-line from (0.33, 0.8, 1, 1.57, 3.5). Five points indicate five different volume ratios within the same tie-line. In define ATPS, volume of top-phase proportionally increases with the volume ratio. The top phase for each system were collected and fluorometric collagen assay was conducted on each sample to determine the fluorescence intensity, concentration of collagen-like protein and purification factor. The pH of system was kept constant.

3.3.6.2 Effect of PEG2000 Concentration

The concentration of PEG2000 is one of the factors that affect the purification of protein using ATPS method. Thus, the effect of five different concentration of PEG2000 (20%, 23%, 26%, 29%, 32%) on the fluorescence intensity, concentration of recCLP and purification factor was studied (Arshad, 2016). The other parameters such as pH and concentration of potassium phosphate were kept constant.

3.3.6.3 Effect of Potassium Phosphate Concentration

The effect of potassium phosphate concentration was varied from 20% (w/w) to 32% (w/w) with constant PEG concentration at 26% (w/w), pH 7 and 10% (w/w) of crude sample at 25 °C were studied (Arshad, 2016).

3.3.6.4 Effect of pH

The effect of different pH was studied by preparing ATPS system consisting of 10% crude lysate, 26% PEG2000 and 26% potassium phosphate buffer with pH was varied (6.0, 7.0, 8.0) (Goja et al., 2013; Kaul, 2000). The total volume was top-up to 100% after adjusting the pH with 0.1 M HCl and 1 M NaOH.

3.3.6.5 Determination of Partition Coefficient (KE) and Purification Factor (PF)

In order to evaluate the purification process, partition coefficient (KE), purification factor (PF) and concentration of collagen-like protein were calculated according to the equations below,

$$KE = \frac{Total\ collagen\ in\ top\ phase}{Total\ collagen\ in\ bottom\ phase} \quad 3.1$$

$$PF = \frac{Collagen\ Amount\ in\ sample\ (CAf)}{Collagen\ Amount\ in\ crude\ lysate\ (CAi)} \quad 3.2$$

The volume ratio was defined as,

$$V_R = \frac{Volume\ in\ top\ phase\ (V_T)}{Volume\ in\ bottom\ phase\ (V_B)} \quad 3.3$$

3.3.7 Optimisation of ATPS Procedure

Response Surface Methodology (RSM) was employed to optimize the purification of recombinant collagen-like protein using ATPS procedure. Face Centred Central Composite Design (FCCCD) with triplicate of centre points was applied for RSM to design the experiment using Design of Expert 10.0.3 software (State-Ease Inc., Minneapolis MN, USA). In this present study, a set of 11 experiments (Table 3.3) which consist of two independent variables (polyethylene glycol and potassium phosphate concentration) was performed for optimisation of ATPS procedure. The values of centre point were obtained based on previous ATPS separation by OFAT. The fluorescence intensity, recCLP concentration and purification factor were chosen as the responses in each run.

Table 3.3 Design of experiment for the optimisation of ATPS for purification of recombinant collagen-like protein.

STD	**Percentage of PEG concentration (% in w/w)**	**Percentage of Potassium Phosphate concentration (% in w/w)**
1	20	25
2	30	25
3	20	32
4	30	32
5	20	28.5
6	30	28.5
7	25	25
8	25	32
9	25	28.5
10	25	28.5
11	25	28.5

3.3.8 Affinity Chromatography (Fast-Protein Liquid Chromatography)

Affinity chromatography was applied to compare the purification effect of ATPS. The 1 mL HisTrap™ High Performance chromatography column (GE Healthcare, USA) was connected to AKTA Prime Plus® (GE Healthcare, USA) The column was prepacked with Ni Sepharose with an inner diameter of 7 mm. The collected supernatant after cell lysis was filter-sterilised 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 analysed by SDS-PAGE and fluorometric collagen assay.

3.4 SUMMARY

In this chapter, the detailed procedure for purification of recombinant collagen-like protein using aqueous-two phase system method and its optimisation was briefly explained. Firstly, fermentation of the recombinant *E. coli* harbouring collagen-like protein from *R. palustris* includes protein expression and followed by cell lysis. Then, the protein purification using ATPS was proceed. The first and second objective were conducted simultaneously by preparing the binodal curves using polyethylene glycol (PEG) with different molecular weight as polymer and potassium phosphate buffer as salt. Screening of significant parameters was done using OFAT. Optimisation of ATPS parameters was studied in third objective.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 INTRODUCTION

This chapter presents all experimental results and discussion for this study. The purification process of protein of interest using aqueous two-phase system were firstly discussed, followed by screening of parameters using one-factor at a time (OFAT) technique. Then, optimisation results were reported and discussed.

4.2 PURIFICATION PROCESS OF RECOMBINANT COLLAGEN-LIKE PROTEIN

Aqueous two-phase system (ATPS) technique was used in purification of recombinant collagen-like protein in this present study. The process flow for the whole purification method was presented in Figure 3.2 and a detailed experimental flow of ATPS purification was illustrated in Figure 3.3.

4.2.1 Development of Binodal Curve

The binodal curves for five different molecular weight of PEG (1500, 2000, 4000, 6000 and 8000 g/mol) with potassium phosphate as salt were presented in Figure 4.1. Each curve represents the composition of phase-forming and the range of concentration that cause in phase separation which depicted the borderline between one-phase and two-

phase region. Construction of binodal curves is necessary to determine the working concentrations of the components that give two-phase separation systems.

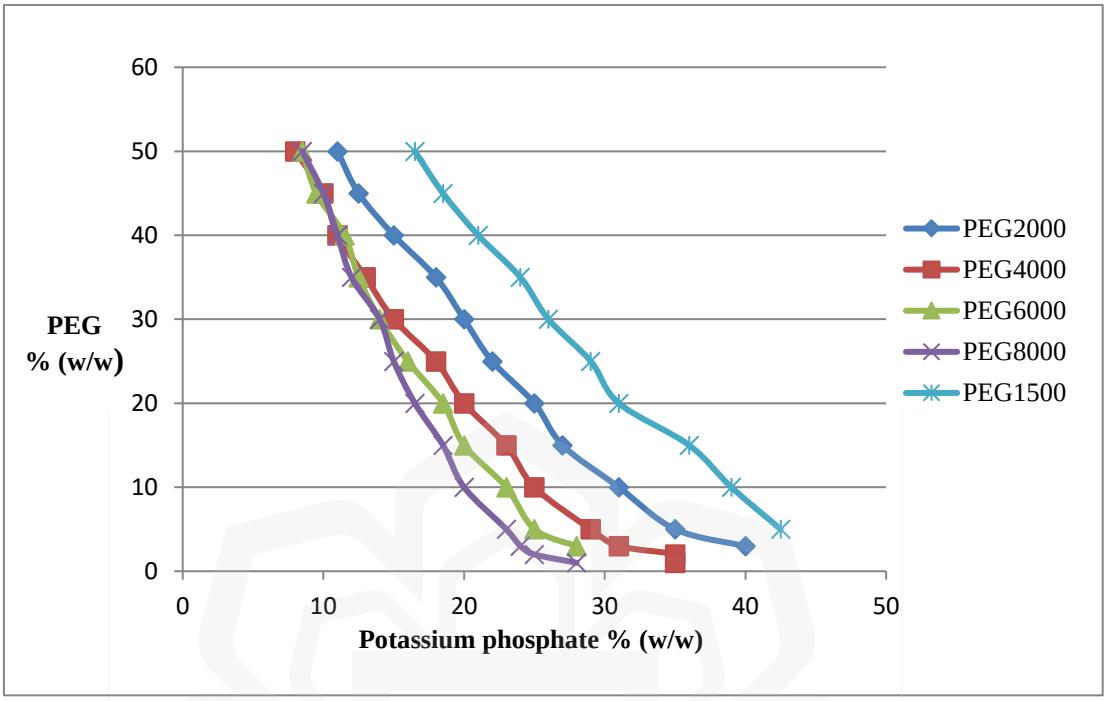

Figure 4.1 Phase diagram constructed using PEG/potassium phosphate system

Based on the analysis of binodal curves presented in Figure 4.1, the trend of the curves showed that as the molecular weight of PEG increases, the binodal curves were distorted towards the origin of the graph. This result in agreement with the hypothesis stating that higher molecular weight of PEG required low concentration of salt to form biphasic system (Raja et al., 2012). When the molecular weight of PEG decrease, high concentration of potassium phosphate is needed to achieve equilibrium as a result of higher hydrophobicity belongs to PEG of higher molecular weight (Capela et al., 2019). Besides that, this result was supported by previous studies conducted by Abd Samad (2017), Arshad (2016), Mohammadi et al. (2015) and Zhang et al. (2013). Furthermore, a similar trend can be observed from all the binodal curve that conclude the relationship between concentration of PEG and potassium phosphate which is high concentration of PEG requires low concentration of salt to reach equilibrium and form two aqueous phases.

Besides that, the relationship between concentration of PEG with different molecular weight of PEG were studied based on the concentration of PEG and salt required for the system to achieve volume ratio of 1 presented in Table 4.1. Volume ratio (Vr) is defined as the ratio of the volume at the top phase to the volume at the bottom phase in ATPS system. As molecular weight of polymer increase, low concentration of PEG and salt were needed for the ATPS system to achieve $V_R = 1$ as mentioned in Table 4.1.

Table 4.1 The composition of PEG and potassium phosphate when the volume of top phase and volume of bottom phase are equal.

Molecular weight of PEG (g/mol)	**$V_R = 1$**	
	PEG % (w/w)	**Salt % (w/w)**
1500	30	28
2000	23	22
4000	20	20
6000	20	19
8000	20	18

The correlation of binodal data was study on the basis of R^2 closer to 1.00. The better results were obtained using the following equations:

For PEG2000 + potassium phosphate buffer + water + recombinant collagen like protein:

$$f = Y_0 + a*\exp(-x/b) + c*\exp(-x/d) \quad (4.1)$$

For PEG (1500, 4000, 6000, 8000) + potassium phosphate buffer + water + recombinant collagen like protein:

$$f = Y_0 + a*x + b*x\text{^}2 + c*x\text{^}3 \quad (4.2)$$

where f and x are the concentration (% w/w) of polymer and salt, respectively. The coefficient *a*, *b*, *c*, *d* and Y_0 along with the R^2 for the investigated systems were obtained and result are tabulated in Table 4.2. Although it is merely empiric, the above equations provide a good description of the solubility curve and is particularly useful for "smoothing" experimental deviations or providing regions in the curve where poor or no experimental data exist.

Table 4.2 Coefficient of best-fitted empirical equations for the correlation of binodal curves of investigated ATPS at T= 25°C

ATPS	**Best-fit empirical system**	**Coefficients**					**R^2**
		a	**b**	**c**	**d**	**Y_0**	
PEG1500	Y_0+*a**x+*b**x^2+*c**x^3	-3.61	0.04	-0.0003	-	98.64	0.9976
PEG2000	Y_0+*a**exp(-x/*b*)+*c**exp(-x/*d*)	27.98	2.13	103.92	29.85	-24.59	0.9995
PEG4000	Y_0+*a**x+*b**x^2+*c**x^3	-3.24	0.01	0.0005	-	74.35	0.9969
PEG6000	Y_0+*a**x+*b**x^2+*c**x^3	-4.89	0.06	0.0001	-	86.32	0.9973
PEG8000	Y_0+*a**x+*b**x^2+*c**x^3	-3.88	-0.05	0.003	-	85.11	0.9986

In general, amount of protein interest or enzyme activity will be used as the criteria for the selection of binodal curve. However, in this study, the selection of the best binodal curve was decide based on the shape, smoothness of the curves and wide choices of salt concentration due to the problems associated to the detection of recCLP in the phases. Hence, the most suitable PEG in this purification process is PEG2000. Furthermore, a low molecular weight of PEG has a lower interfacial tension and cost

compared to high molecular weight of PEG. This decision was also made based on previous report that proteins in non-ionic ATPS attract to the aqueous phase with smaller polymer molecules if all the other conditions are kept constant (Albertsson et al., 1987). According to results from Table 4.2, highest R^2 of 0.9995 found for PEG 2000 supported the selection of PEG 2000 for the purification of recCLP.

4.2.1.1 Selection of Tie-Lines

After selection of the most appropriate phase-forming system for purification of recombinant collagen-like protein (PEG2000/potassium phosphate), this project was further continued by selection of a tie-line which involved different ATPS samples above binodal curve that showed Vr of 1.00. Different points represent different tie-lines (Figure 4.2). The tie-line length (TLL) with the same units as concentration was calculated and the results were tabulated in Table 4.3. TLL has the same units as the concentration, and it is used to express the influence of the system composition on the partition of solutes.

Table 4.3 The tie-line length (TLL) and KE for each tie-line

%PEG (w/w)	**%PP (w/w)**	**TLL**	**KE**
25	25	50.510	1.332
26	26	54.782	4.027
27	27	60.258	1.548
28	28	64.422	1.277

To identify the best tie-lines, a quantitative fluorometric collagen assay was done to both top and bottom phases. Figure 4.3 show the concentration of recombinant collagen-like protein in the PEG-rich top phase and salt-rich bottom phase of ATPS samples with Vr of 1.0. ATPS sample with 26% (w/w) PEG 2000 concentration and 26% (w/w) potassium phosphate concentration at pH 7.0 revealed the highest concentration of recCLP in the top phase and lowest reading in the bottom phase.

From Table 4.3, the highest partition coefficient (KE) belongs to the system with 26 % (w/w) PEG 2000 and 26% (w/w) potassium phosphate with tie-line length (TLL) of 54.78 which proved that most of protein of interest favoured in one phase which is PEG-rich top phase. Therefore, this combination of ATPS sample was chosen for further analysis. In the further experiment, due to the problem associated with the detection of recombinant protein in the bottom phase and limited collagen assay kit during the movement control order (MCO), thus it was decided to conduct detection of recCLP in the top-phase only and purification factor will be used as the response variable.

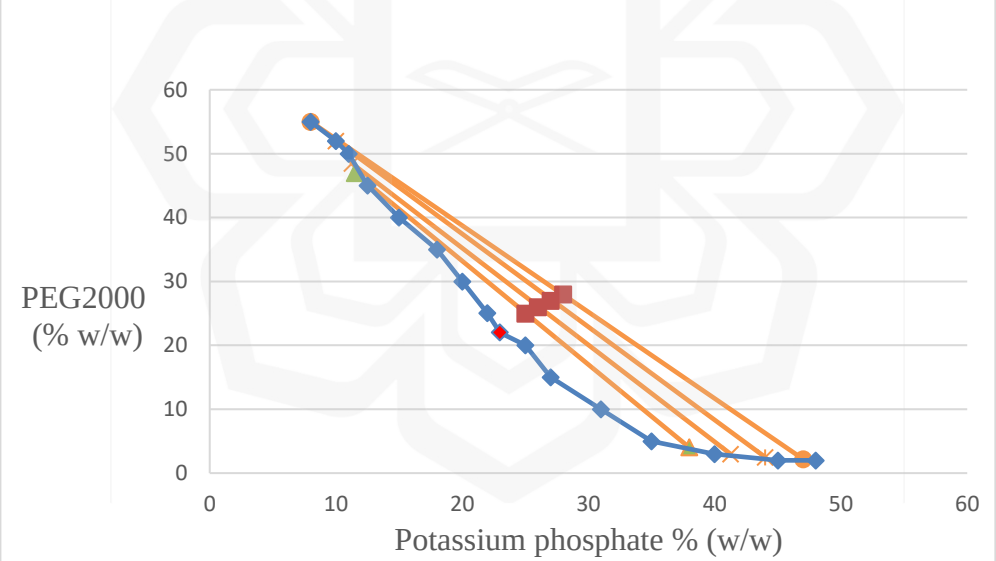

Figure 4.2 Binodal curve PEG 2000 with tie-lines

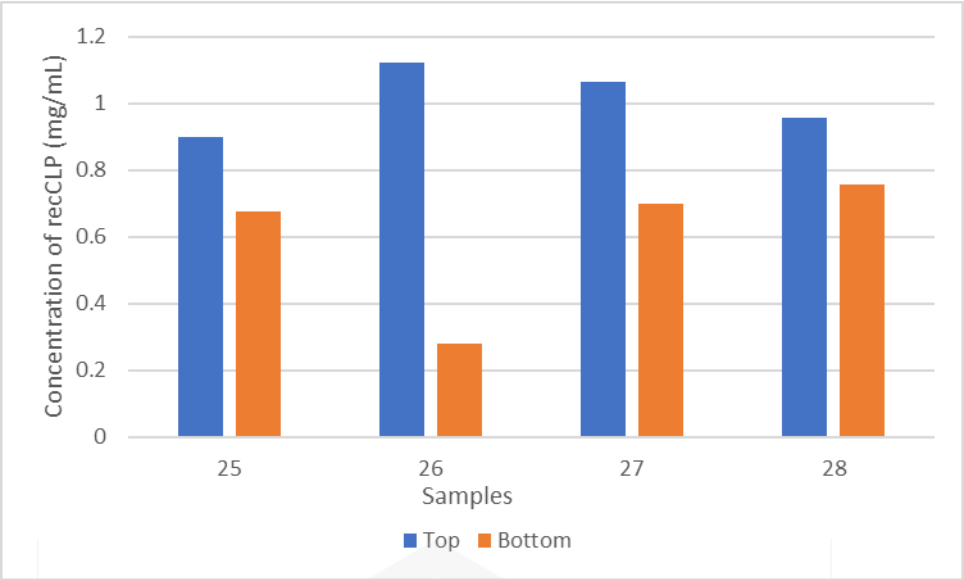

Figure 4.3 Concentration of recombinant collagen-like protein (recCLP) for selection of ATPS

4.2.2 Screening of ATPS Condition using OFAT (One Factor at A Time)

This section provides the complete interpretation of data collected from the experiment designed by OFAT method to identify the optimum range of certain parameters involved in ATPS separation such as volume ratio, concentration of PEG 2000 and potassium phosphate and pH.

4.2.2.1 Effect of Volume Ratio

Next, 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. Five points indicate five different volume ratio (0.33, 0.8, 1.0, 1.57, 3.5) that has been chosen to study the effect of volume ratio on the purification factor (Table 4.4).

Analysis of result in Figure 4.6 shows high purification factor (PF) was generally achieved in system with the highest Vr, whereas low PF was found at Vr of 0.33. Based on the rule of ATPS, an increase in volume ratio will cause the increment of volume in the top phase. As a result, more free volume available for the protein of interest to participate in the top phase, which also causes a significant negative impact of free volume in the bottom phase (Benavides & Rito-Palomares, 2006; Rito-palomares et al., 2014). However, in certain cases, high Vr can leads to the dilution of protein in concentrations (Mohamed Ali et al., 2014). Meanwhile, extremely low Vr in the phase may have limited free volume, thus limiting the partition of protein (Lin et al., 2013). Furthermore, when Vr decreases over limit of solubility of protein, the protein will precipitate at interphase that leads to loss of protein by precipitation (Mohamed Ali et al., 2014).

From the preceding results (Figure 4.4, 4.5, 4.6), either increasing or reducing the volume ratio did not cause a significant influence on fluorescence intensity (FL) and recCLP concentration in the top phase. As 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 (Kaul, 2000).

Table 4.4 Set of experiments for effect of volume ratio (Vr=1.0)

Top phase Volume(mL)	**% PEG (w/w)**	**% Potassium Phosphate (w/w)**	**Volume ratio (Vr)**
0.45	10	36.5	0.33
0.80	20	30.0	0.80
0.90	26	26.0	1.00
1.10	30	23.3	1.57
1.40	40	16.8	3.50

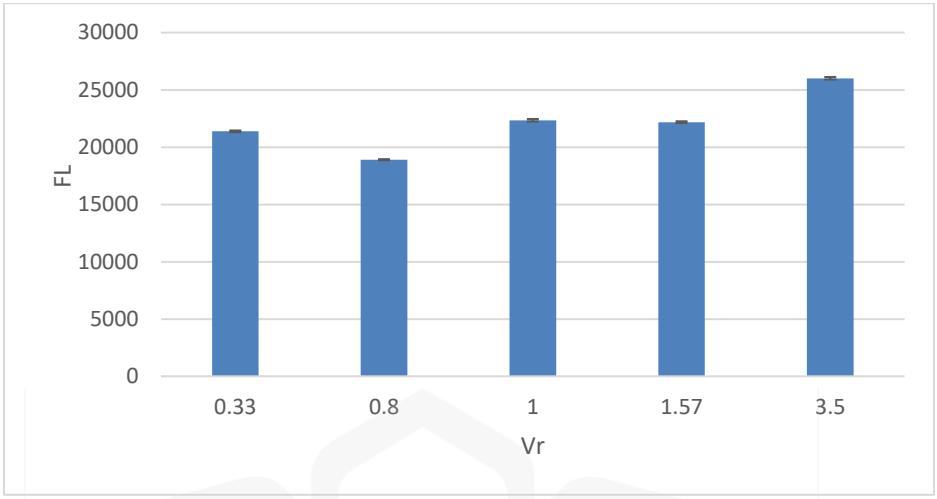

Figure 4.4 Influence of different volume ratio on fluorescence intensity (FL)

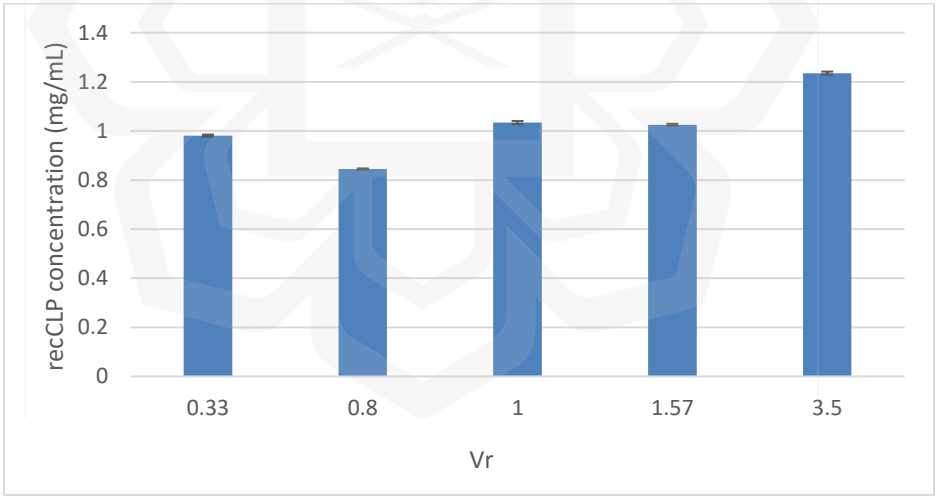

Figure 4.5 Influence of different volume ratio on recCLP concentration in the top phase

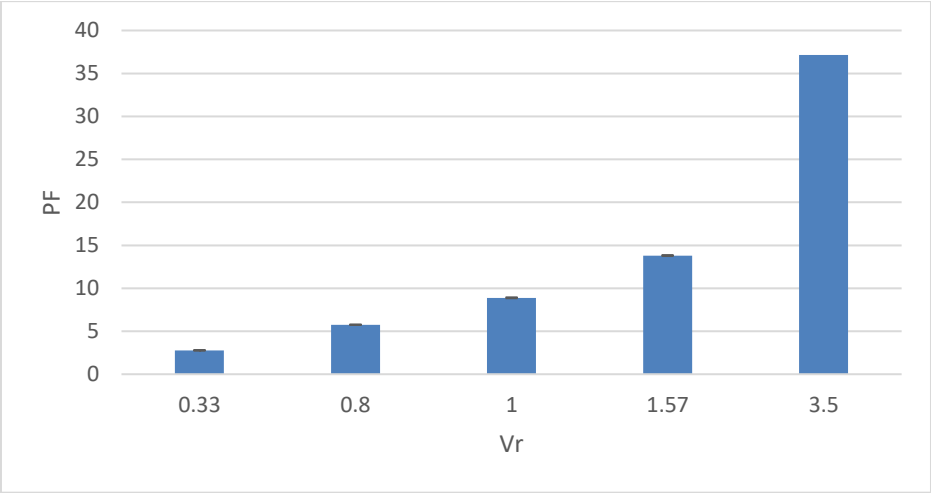

Figure 4.6 Influence of different volume ratio on purification factor

4.2.2.2 Effect of PEG Concentration %(w/w)

Next, the effect of different PEG 2000 concentration range between 20% (w/w) and 32% (w/w) with constant concentration of potassium phosphate (26%) at pH of 7.0 was assessed. As the concentration of PEG increased from 20% (w/w) to 26% (w/w), the fluorescence intensity, concentration of recCLP and purification factor (PF) in the top-phase were markedly increased as illustrated in Figures 4.7, 4.8, 4.9, respectively. This trend changed when PEG concentration dropped from 26% (w/w) to 29% (w/w) and increased again from 29% (w/w) to 32% (w/w). Highest FL, recCLP concentration and PF were generally achieved in ATPS system with 26% (w/w), whereas the lowest FL, recCLP concentration and PF were found at 29% (w/w) of PEG 2000.

Theoretically, for ATPS composed of polymer-salt system as applied in the present study, the partition behaviour of biomolecules depends on the volume exclusion effect in polymer-rich phase (top-phase) and salting-out effect in salt-rich phase (bottom-phase) (Babu et al., 2008). Those effects will cause the reduction of available space in the top phase and thus obstructed the partition of biomolecules in the top phase. This hypothesis concurrent with the behaviour of CLP when the PEG concentration was increased from 26% (w/w) to 29% (w/w). Furthermore, in general, increase in polymer

concentration will increase the difference in densities, refractive index and viscosity between the phases that leads to the large differences in properties between the phases (Ratanapongleka, 2013).

Meanwhile, the trend with PEG concentration from 20% (w/w) to 26% (w/w) and from 26% (w/w) to 29% (w/w) was supported by the theory explained by González-Tello et al. (1994), which is an increase of polymer concentration will increase the number of polymer units which resulted to the movement of biomolecules to PEG-rich phase due to the hydrophobic interactions between biomolecules and PEG. The increasing of PEG concentration may cause the increase of top phase volume, resulting in an increase in volume ratio due to the competition for water between salt phase (Zhao et al., 2013). As a result, more free volume available for the protein of interest to participate in the top phase, which also causes a significant negative impact of free volume in the bottom phase (Benavides & Rito-Palomares, 2006; Rito-palomares et al., 2014).

Therefore, ATPS with 26% (w/w) of PEG 2000 was further employed for the next factor which involving the effect of potassium phosphate buffer on the purification of recCLP using ATPS. However, the best concentration of PEG 2000 in purification of recCLP cannot be corroborated with any studies due to lack of references regarding the application of ATPS in partition of recombinant collagen-like proteins.

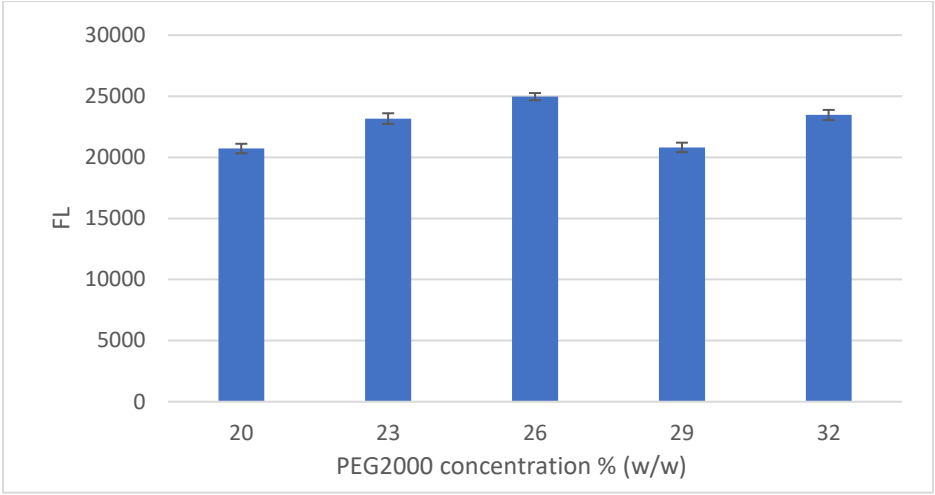

Figure 4.7 Influence of concentration of PEG 2000 on fluorescence intensity (FL)

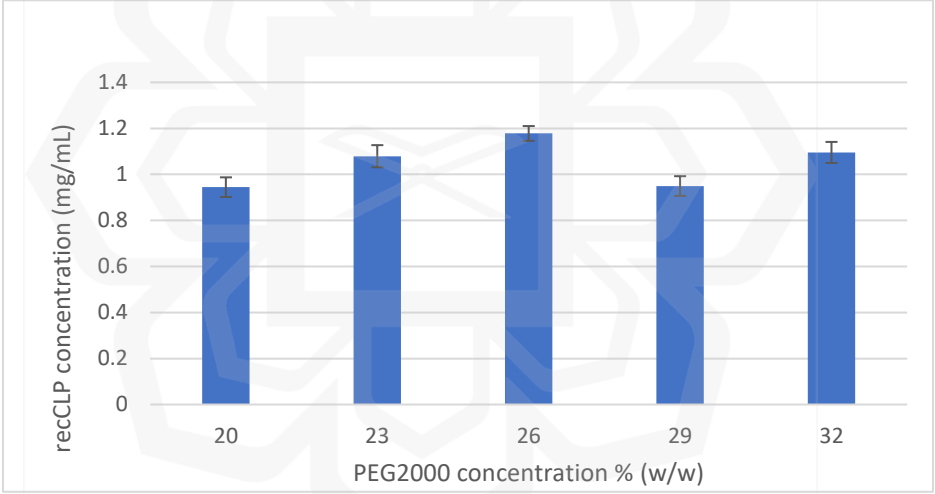

Figure 4.8 Influence on concentration of PEG on recCLP concentration

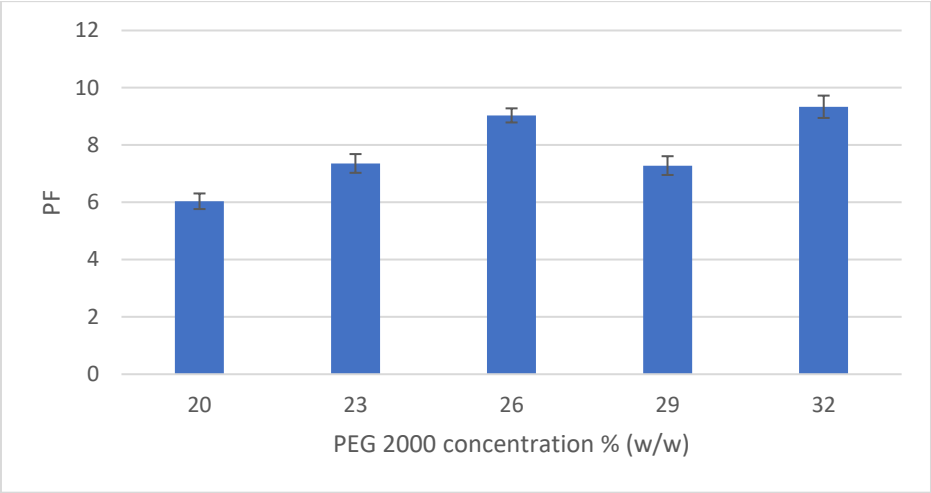

Figure 4.9 Influence of concentration of PEG 2000 on purification factor (PF)

4.2.2.3 Effect of Potassium Phosphate Concentration in (% w/w)

The concentration of potassium phosphate in (% in w/w) is one of the factors which affect the partition behaviour of target protein. In this experiment, potassium phosphate concentration was assessed in the range of 20 to 32% (w/w) at pH 7.0. As the concentration of salt increased from 20 and 23% (w/w), FL and recCLP concentration also increased and slightly declined at 26% (w/w) as illustrated in Figures 4.10, 4.11. This trend was repeated from 26% to 32% (w/w) of potassium phosphate. The highest FL and concentration of protein of interest was shown in ATPS with 29% (w/w) of salt. Meanwhile, purification factor in Figure 4.12 showed the decreasing trend from 20 to 32% (w/w) due to decreasing volume of top phase. 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 (Rito-palomares et al., 2014). Thus, the protein content in that phase will decline.

Theoretically, ionic strength will increase as the salt concentration in the system increase, thus pushing the proteins to the top phase. Besides that, the ability of salt to capture water molecules will increase, hence moving biomolecules to the top phase (Raja et al., 2011; Ratanapongleka, 2013). However, high concentration of salt also can

lead to protein precipitation and denaturation thus reduce the amount of targeted protein in the phase (Ratanapongleka, 2013; Samad & Shukor, 2017). This hypothesis supports the trend showed when the concentration of salt increase from 29% (w/w) to 32% (w/w). Thus, 29% (w/w) of potassium phosphate has been selected for further study.

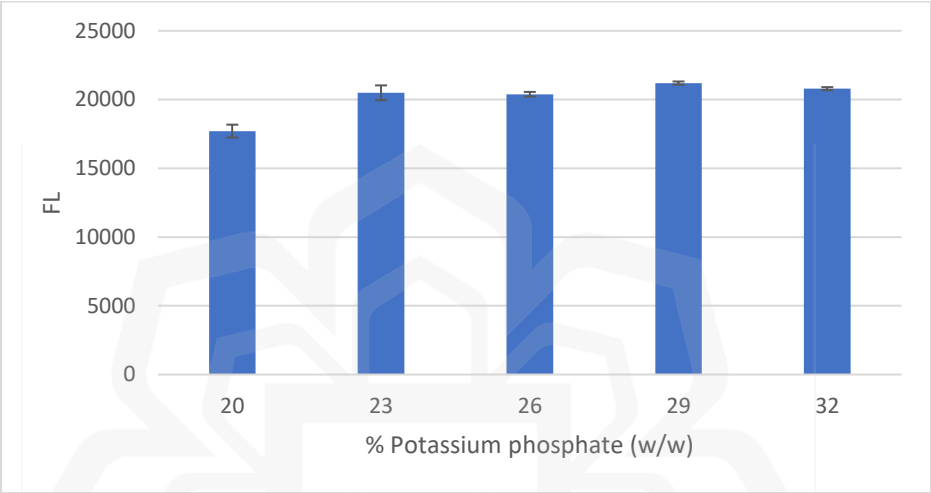

Figure 4.10 Influence of salt concentration on fluorescence intensity

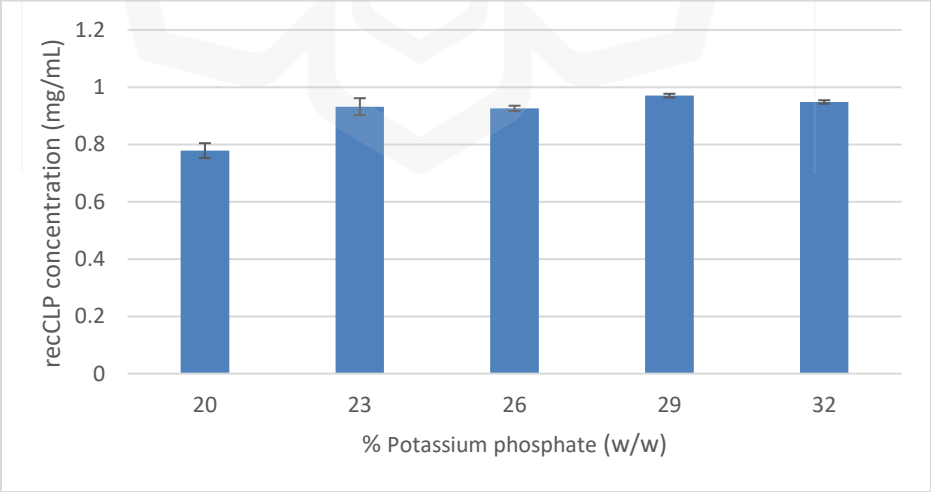

Figure 4.11 Influence of salt concentration on recCLP concentration

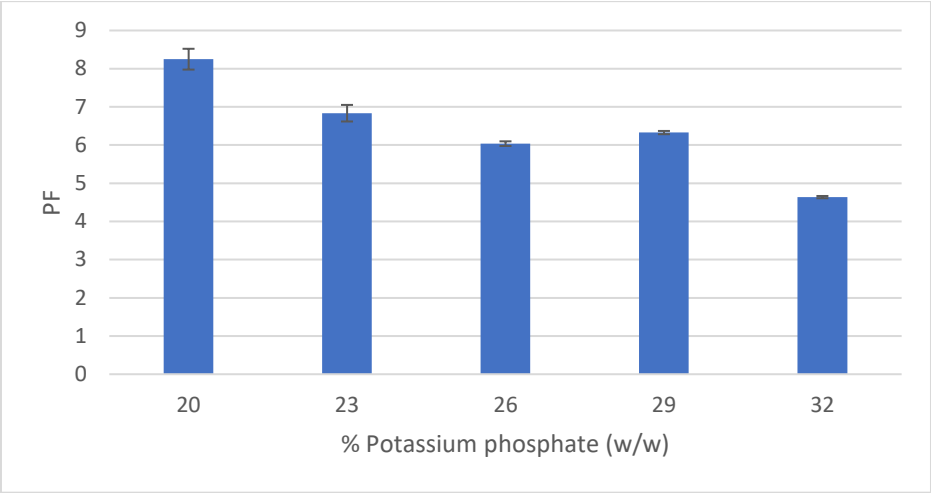

Figure 4.12 Influence of salt concentration on purification factor

4.2.2.4 Effect of pH

The effect of different pH values on partitioning of recCLP in ATPS was determined by varying the pH from 6.0-8.0 on the ATPS system containing 26% (w/w) of PEG 2000 and 29% (w/w) of potassium phosphate buffer. System pH affects the partitioning of desired biomolecules by altering the charge and surface properties of the solute. The partitioning behaviour of recCLP is expected to be sensitive to the pH levels since CLPs are charged molecules.

The influence of pH on the FL, recCLP concentration and PF is shown in Figures 4.13, 4.14, 4.15, respectively. When the pH increases from 6.0 to 7.0, the FL, recCLP concentration and PF increased. This is due to isoelectric point (pI) of the collagen (<7.0), which makes the protein become negatively charged and favours the PEG-rich top phase. According to Thomas & Kelly (1922**)**, the zwitterion along collagen molecules are negatively charged, making the entire collagen is negative. Moreover, the pI glycine residues is 5.97, thus making the collagen-like protein carried a negative charge (Borsook & MacFadyen, 1930). When the pH increases from 7.0 to 8.0, the protein of interest favoured a partition in the bottom phase, which in turn decreased in FL, protein concentration and PF. Besides that, at higher pH, more contaminants

become negatively charge and tend to partition to top phase (Lin et al., 2013). The presence of contaminants in the top phase lowered the PF of collagen-like protein. Per the results shown in Figure 4.13, 4.14, 4.15, system of pH 7.0 exhibited the highest values for the responses. As a result, ATPS with pH 7.0 was chosen for further study.

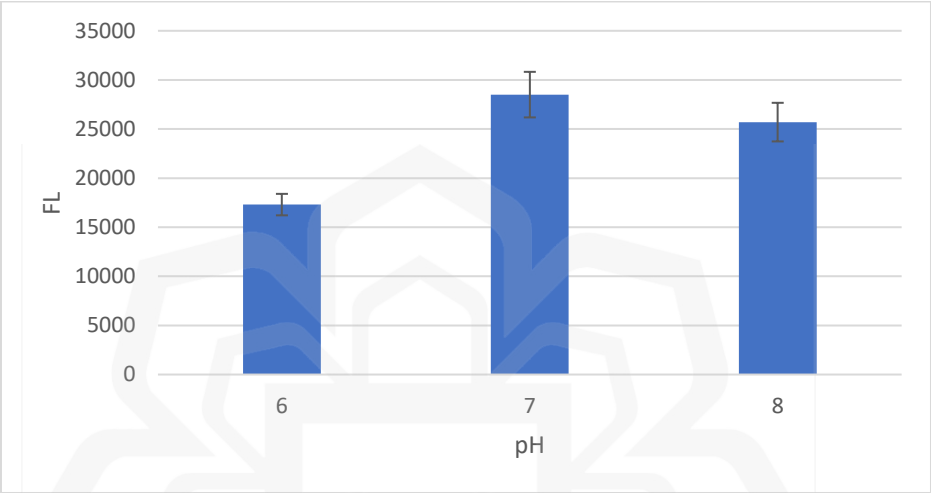

Figure 4.13 Influence of pH on the fluorescence intensity

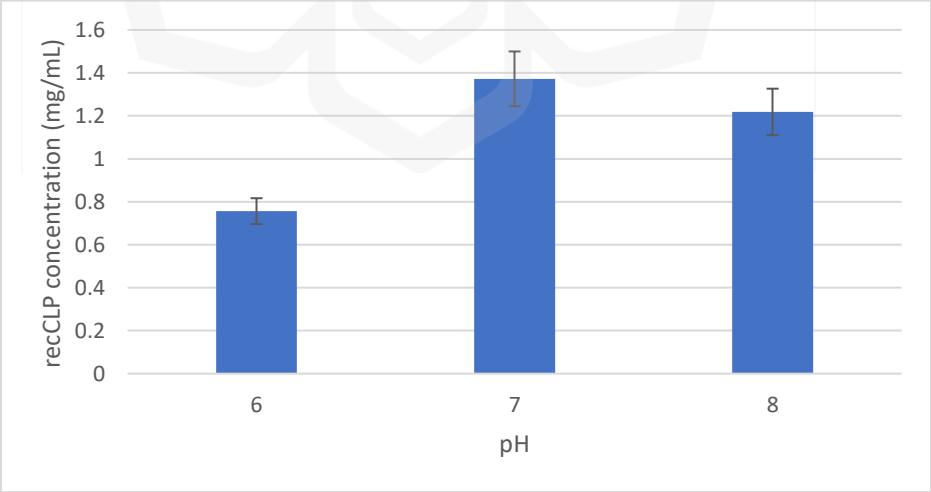

Figure 4.14 Influence of pH on the recCLP

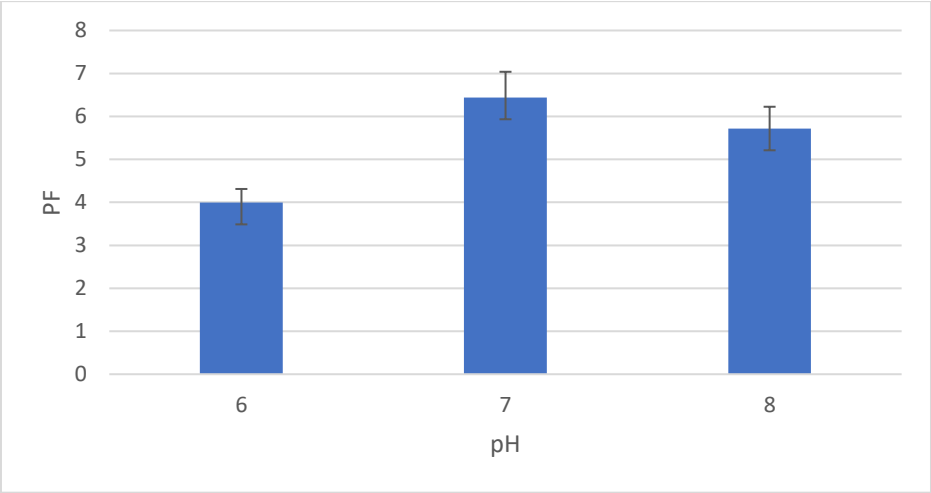

Figure 4.15 Influence of pH on the purification factor

4.2.3 Optimisation of ATPS Experiment Using Design of Expert

For the development of successful ATPS for purification of recombinant collagen-like protein, response surface methodology (RSM) was applied to determine the optimal levels of variables. The effect of two independent variables namely as concentration of PEG 2000 and concentration of potassium phosphate on the fluorescence intensity, concentration of recCLP and purification factor was investigated. In this experiment, a face-centered central composite design (FCCCD) was constructed with three replications in central point. Central point for concentration of PEG 2000 and potassium phosphate were chosen based on previous OFAT analysis. In this experiment, temperature of phase separation and pH of potassium phosphate were kept constant at 25 °C and 7.0, respectively. The high coded value, low coded value and center point were tabulated in Table 4.5 and the experimental design and results of ATPS purification are summarised in Table 4.6.

Table 4.5 Factors selected for central composite design (CCD) study to optimise the purification process by ATPS technique

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 4.6 A set of 11 runs using face-centered central composite design (FCCD) and the responses of fluorescence intensity (FL), recCLP concentration (mg/mL), purification factor (PF).

Run	**A: PEG 2000 concentration %(w/w)**	**B: Salt concentration % (w/w)**	**FL**	**recCLP concentration (mg/mL)**	**PF**
1	20	25	17560	1.913	5.008
2	30	25	14235	1.470	4.947
3	20	32	20686	2.351	4.397
4	30	32	16593	1.794	4.697
5	20	28.5	19188	2.151	4.826
6	30	28.5	19388	2.178	6.517
7	25	25	17175	1.874	4.906
8	25	32	24970	2.945	6.610
9	25	28.5	25178	2.974	7.787
10	25	28.5	27340	3.271	8.565
11	25	28.5	24669	2.904	7.604

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

$$\text{Fluorescence intensity (FL)} = 25175.95 - 1196.33C_{PEG2000} + 2206.50C_{PP} - 182.00C_{PEG2000}C_{PP} - 5058.37C_{PEG2000}^2 - 327.87C_{PP}^2 \quad (4.3)$$

$$\text{Concentration of recCLP} = 2.98 - 0.17C_{PEG2000} + 0.31C_{PP} - 0.023C_{PEG2000}.C_{PP} - 0.70_{PEG2000}^2 - 0.45C_{PP}^2 \quad (4.4)$$

$$\text{Purification factor (PF)} = 7.71 + 0.32C_{PEG2000} + 0.14C_{PP} + 0.091C_{PEG2000}.C_{PP} - 1.62C_{PEG2000}^2 - 1.53C_{PP}^2 \quad (4.5)$$

Analysis of variance (ANOVA) was conducted and described in Table 4.7, for the determination of significant variables and all their possible interactions on the response variables. According to analysis of variance, the F-value represents the accuracy of the model while p-value indicates the interaction between the model terms. A significant model proved by the p-value that was less than 0.05 while p-value greater than 0.10 is considered as an insignificant model. The coefficient of determination (R^2), adjusted R^2 and predicted R^2 indicates the quality fit of the model equation.

According to Table 4.7**,** it was found that F-value for FL, recCLP concentration was 7.32, 7.65 and the p-value of 0.0238 and 0.0217 (>0.05) respectively, indicating that both models are significant. In addition, non-significant values of 0.024 and 0.2674 for lack of fit for FL and recCLP respectively, showed that the quadratic model was valid for this study. Meanwhile, for purification factor, F-value of 4.53 with 0.0614 of p-value was calculated, which implies an insignificant model. However, this model showed a non-significant value of lack of fit (0.1905) which is good for the model.

The regression analysis of models for responses of FL and concentration of recCLP showed that the linear model B (concentration of salt) and quadratic model terms B^2 are significant (P>0.05). However, the linear model A (concentration of PEG), AB and quadratic model B^2 are insignificant. For responses of purification factor,

quadratic model of A2 and B2 were significant while the interaction term AB, A (concentration of PEG) and B (concentration of salt) were not significant.

The model coefficient of determination (R^2) was 0.8823, 0.8823, and 0.8193 for fluorescence intensity, recCLP concentration and PF respectively. The R^2 is always between 0 and 1, and value close to 1 shows better accuracy of the model. The R^2 value is acceptable as it exceeded 0.8 and the small difference between R^2 and adjusted R^2 is preferable. Moreover, the R^2 values were concordant with the adjusted R^2 of 0.7566, 0.7687, and 0.6383 for FL, recCLP concentration and PF respectively.

Table 4.7 (a) Analysis of variance of quadratic model for fluorescence intensity of recCLP

Fluorescence Intensity (FL)			
Source	**Sum of squares**	**F-value**	**p-value Prob > F**
Model	1.610E+008	7.32	0.0238 (significant)
A-PEG concentration	8.587E+006	1.95	0.221
B-Salt concentration	2.921E+007	6.64	0.050
AB	1.325E+005	0.030	0.869
A^2	6.482E+007	14.73	0.012
B^2	2.715E+007	6.17	0.056
Lack of Fit	1.798E+007	7.32	0.024 (not significant)
R^2: 0.8798 Adjusted R^2:0.7596 Predicted R^2:0.0102			Std. Dev.: 2097.67

Table 4.7 (b) Analysis of variance of quadratic model for concentration of recCLP

Concentration of recombinant collagen-like protein (recCLP)			
	Sum of squares	**F-value**	**p-value Prob > F**
Model	3.10	7.65	0.0217 (significant)
A-PEG concentration	0.16	2.02	0.214
B-Salt concentration	0.57	7.06	0.045
AB	2.209E-003	0.027	0.875
A^2	1.24	15.30	0.011
B^2	0.52	6.45	0.052
Lack of Fit	0.33	2.89	0.2674 (not significant)
R2: 0.8843 Adjusted R^2: 0.7687 Predicted R^2: 0.0505			Std. Dev.: 0.28

Table 4.7 (c) Analysis of variance of quadratic model for purification factor

Purification factor (PF)			
Source	**Sum of squares**	**F-value**	**p-value Prob > F**
Model	17.96	4.53	0.0614 (not significant)
A-PEG concentration	0.62	0.78	0.417
B-Salt concentration	0.12	0.15	0.716
AB	0.033	0.042	0.847
A^2	6.65	8.39	0.034
B^2	5.96	7.51	0.041
Lack of Fit	3.44	4.40	0.1905 (not significant)
R2: 0.8192 Adjusted R^2: 0.6383 Predicted R^2: -0.5153			Std. Dev.: 0.89

Accordingly, three-dimensional plots generated as pair combinations from two factors, in which the effect of both factors are shown were illustrated in Figures 4.16, 4.17 and 4.18, it can be seen that the intermediate values of PEG 2000 concentration and potassium phosphate concentration favoured the highest FL, recCLP concentration and PF. The plots exhibited an increasing pattern 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 centre of the graph, indicating that the center points selected for this experiment are appropriate.

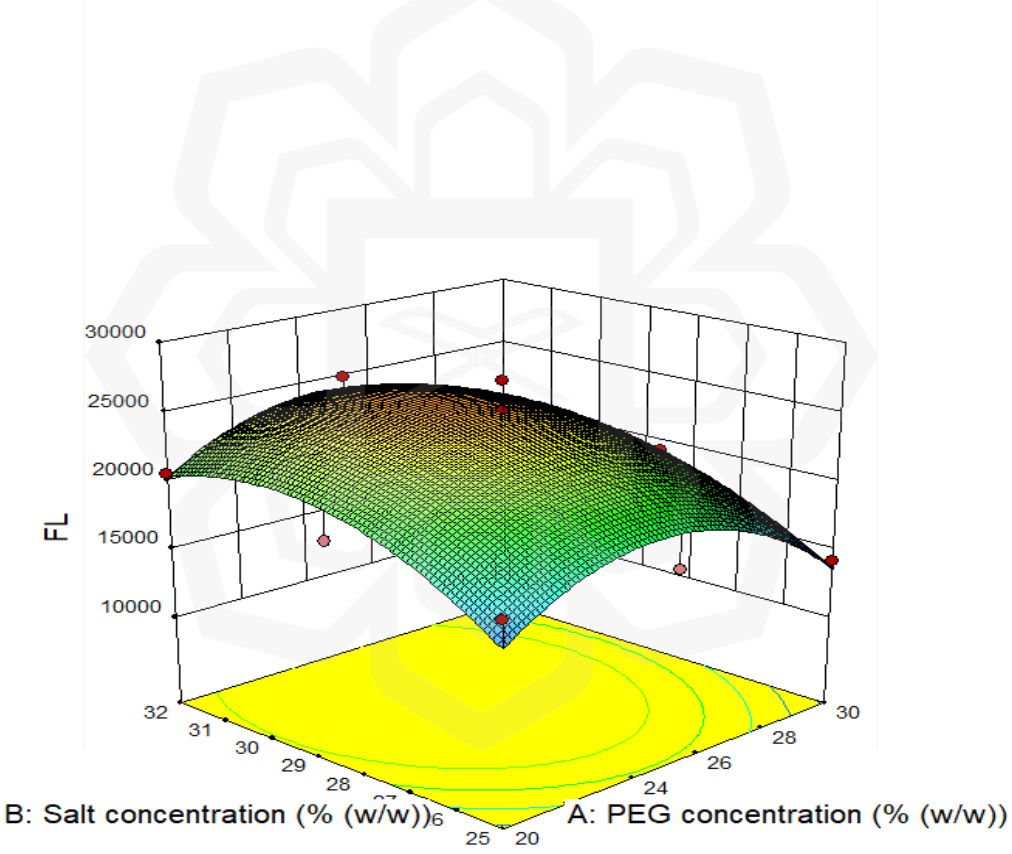

Figure 4.16 Three-dimensional surface plot of response surface analysis showing the effect of the interaction of concentration of PEG 2000 and potassium phosphate on the fluorescence intensity

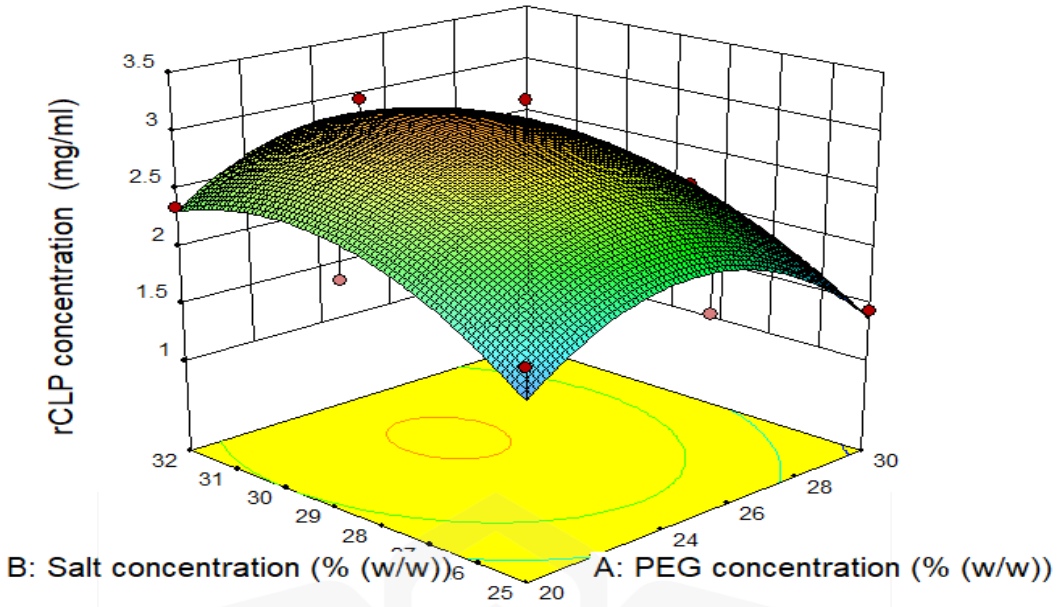

Figure 4.17 Three-dimensional surface plot of response surface analysis showing the effect of the interaction of concentration of PEG 2000 and potassium phosphate on the recCLP

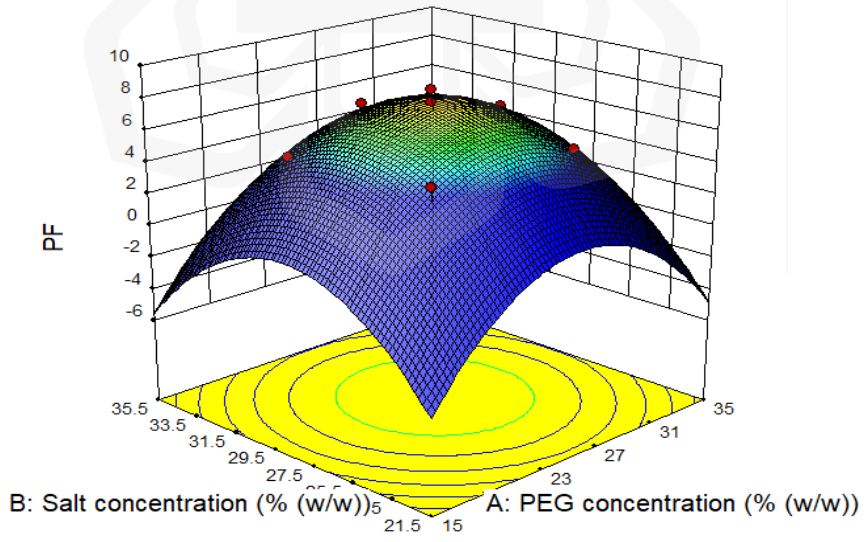

Figure 4.18 Three-dimensional surface plot of response surface analysis showing the effect of the interaction of concentration of PEG 2000 and potassium phosphate on the purification factor.

4.2.4 Validation of Model

To validate the model, the experiments were conducted in triplicate and the result was compared with the optimum results predicted by the software. The results were presented in Table 4.8. **Under the optimised condition** which composed of at 24.80 % (w/w) PEG 2000 and 29.20 % (w/w) potassium phosphate at pH 7.0, **the predicted recCLP was 3.026 mg/mL**. 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 optimisation of ATPS conditions for purification of recombinant collagen-like protein. Other responses such as fluorescence intensity and purification factor were calculated as 27068 ± 900 and 7.476 ± 0.2, respectively.

Table 4.8 Validation of model

PEG2000 concentration (% w/w)	Potassium phosphate concentration (% w/w)	**Model Predicted** Concentration of recCLP (mg/mL)	**Experimental** Concentration of recCLP (mg/mL)
24.80	**29.20**	**3.026**	**3.233 ± 0.12**

4.3 AFFINITY CHROMATOGRAPHY (HISTIDINE-TAG PURIFICATION)

As suggested by Xu et al. (2010), pH of 7.4 was used for binding buffer containing 20 mM of Na_2HPO_4 and 0.5 M NaCl. To avoid non-specific binding, Ni-NTA resin with pH between 7.0 and 8.0 is preferable for most of the his-tagged proteins (Zachariou, 2008)**.** In this present study, high concentration of imidazole was used to elute the target protein. 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 (Figure

4.19). Every fraction was subjected to SDS-PAGE analysis and the result in Figure 4.20 showed the expected size of 36 kDa.

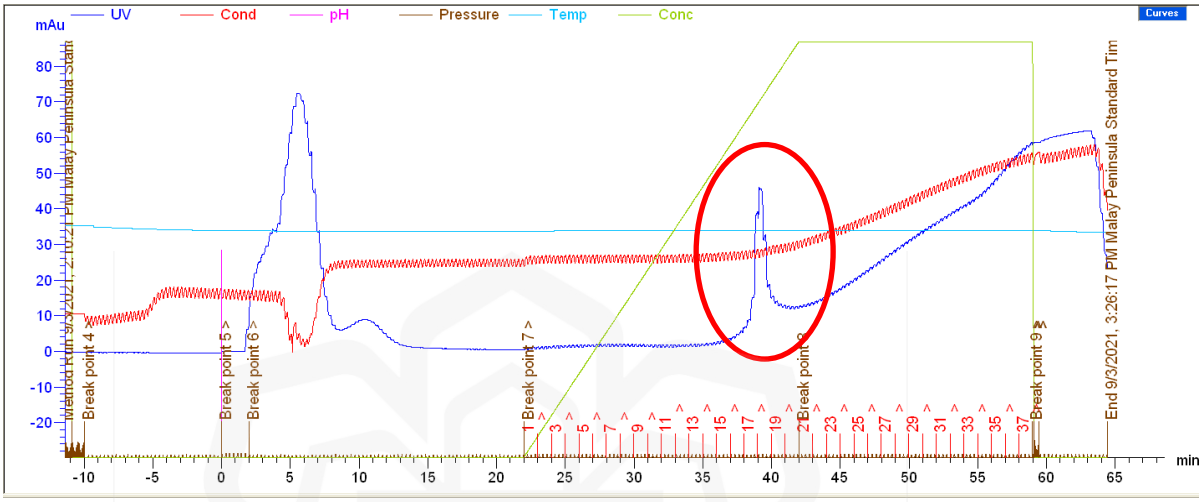

Figure 4.19 Chromatogram of the purified samples from affinity chromatography of 2mL loading volume of recombinant collagen-like protein lysates on Ni-Sepharose resin. Binding buffer contain 20 mM of Na_2HPO_4, 0.5 M NaCl at pH 7.4. Elution buffer contained 20 mM Na2HPO4, 0.5 M NaCl and 0.5 M imidazole, at pH 7.4. 1 mL fraction was collected at flow rate 1mL/min.

4.4 SDS-PAGE ANALYSIS

The presence of recombinant collagen-like protein purified by ATPS with optimised conditions of was analysed by SDS-PAGE electrophoresis under reducing conditions. The result was compared with purified samples from chromatography method. Figure 4.19 shows the presence of 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 in between 45 kDa and 60 kDa and above 75

kDa. This explained by the fact that the bottom phase was unable to attract all of contaminants and unwanted proteins.

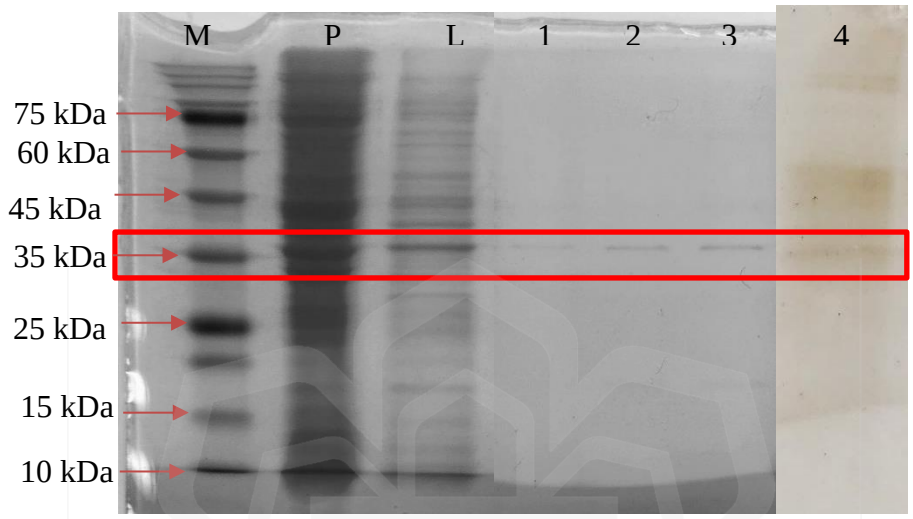

Figure 4.20 SDS-PAGE analysis of purified sample of ATPS and affinity chromatography. Lane M: standard protein marker. Lane P: cell pellet. Lane L: crude cell lysate. Lane 1: FPLC fraction (17). Lane 2: FPLC fraction (18). Lane 3: FPLC fraction (19). Lane 4: ATPS top phase. Note: For SDS-PAGE analysis, 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.

4.5 COMPARISON OF PURIFICATION BY ATPS AND AFFINITY CHROMATOGRAPHY

Based on the results from section 4.4, the sample at the peak of the chromatogram (fraction 18) was analysed by fluorometric collagen assay and compared with purified recombinant recCLP from ATPS purification method. The purification efficiency of recombinant collagen-like protein purified by ATPS and chromatography is summarised in Table 4.9**.** 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 favourable 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 4.9 Purification efficiency of recombinant collagen-like protein by different purification methods.

Purification Methods	**ATPS**	**Affinity Chromatography (FPLC)**
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

Based on economic analysis point of view as presented in Table 4.10, ATPS exhibited lower shorter processing time with lower cost of operation compared to chromatography method. Due to expensive prepacked column with resin and chemicals used for 1 h processing time, affinity chromatography is considered an expensive downstream purification method, thus making it not sustainable 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 (Oscar et al., 2006). However, additional cost needs to be considered for the stripping and recharging chromatography columns (Arshad et al., 2017). In the case of ATPS, polymer and salt cannot be recycled as they were part of the final purified protein (Arshad et al., 2017). Such economic analysis provides an understanding for future research, and ATPS method is proven to be time-saving, cost-effective with higher recovery that may be considered for the scale-up process.

Table 4.10 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**

4.6 SUMMARY

This chapter briefly discussed the purification of recombinant collagen-like protein using aqueous two-phase system (ATPS) as well as the effect of several factors such as volume ratio, pH of system, concentration of polymer and salt on the partitioning of recCLP. As a result, the validation of optimised results was based on concentration of PEG 2000 and potassium phosphate at pH 7.0; 24.8 % (w/w) and 29.20 % (w/w) with concentration of recCLP of 3.233 ± 0.12 mg/mL, respectively. Therefore, the concentration of recCLP produced in 50 mL fermentation volume was 1.292 ± 0.05 mg/mL. To evaluate the potential of ATPS as a purification method for recCLP, the comparison of ATPS with affinity chromatography was studied in terms of purification efficiency and economic aspect.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSION

This research demonstrated the potential application of ATPS in the purification of recombinant collagen-like protein. The aqueous two-phase system (ATPS) of PEG 2000/potassium phosphate was employed to purify the collagen-like protein. Based on the constructed binodal curves, the most suitable molecular weight and concentration of PEG for the purification of recombinant CLP from *R. palustris* are 2000 g/mol PEG2000 and 26% (w/w), respectively and the most suitable concentration of potassium phosphate buffer was identified at 26% (w/w).

The screening of factors involved in protein partitioning by ATPS such as volume ratio, pH and concentration of polymer and salt were conducted using OFAT and the fluorescence intensity, concentration of protein and purification factor were recorded as responses. Concentration of PEG 2000 (26%w/w) and concentration of potassium phosphate (29%w/w) showed the highest fluorescence intensity (21196 ± 118) and concentration of recCLP (0.97 ± 0.01 mg/mL). This was followed by pH at 7.00 (1.37 ± 0.13 mg/mL) as the best condition that provides the highest recCLP in the top phase. Then, optimisation using RSM based on FCCCD design model predicted ATPS with 24.8% (w/w) PEG 2000 and 29.20% (w/w) potassium phosphate at pH 7.0 as the optimum condition and validated as 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. ANOVA resulted in R^2 of fluorescence intensity, the concentration of recCLP and purification factor of 0.8798, 0.8843 and 0.8192, respectively. It was found that 1.292 ± 0.05 mg/mL recCLP was produced in 50 mL fermentation solution.

Herein, the comparison with affinity chromatography highlighted that ATPS was proved to be a low cost, time-saving with high recovery method that may raise the consideration for substitution of chromatography method.

The obtained results are crucial for the design of the purification process using ATPS and understanding the complex mechanisms controlling the partitioning behaviour of proteins. Besides, due to the lack of application of ATPS in the purification of collagen-like proteins specifically extracted from *R. palustris*, this present study can serve as a reference and deliver new information for future studies.

5.2 RECOMMENDATIONS

Based on the result obtained throughout this study, the following recommendations were proposed for future works:

i. This study only covers aqueous two-phase system made up of polymer and salt. Hence, it is suggested to do purification of recCLP by ATPS using polymer/polymer and alcohol/salt followed by a comparative study of ATPS. The purification of collagen has been conducted using polyethylene glycol (PEG) and dextran (polymer/polymer) (Singh & Tavana, 2018).
ii. The detection of recCLP in this study was conducted using a commercialised fluorometric collagen assay kit, which is quite expensive. It is suggested to develop a colorimetric detection method that quantitatively measures the amount of recombinant collagen-like protein from bacteria.
iii. Optimisation of the culture media followed by kinetic study of recCLP from *R. palustris* for the maximum production of recCLP. In this study, the qualitative assay (SDS-PAGE) was challenging due to the lower amount of proteins present in the sample.

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APPENDICES

APPENDIX A

List of chemicals and reagents

Process	Type	Chemicals		Formula	Brand
Fermentation	LB agar plate, LB media	Luria Bertani Agar, Vegitone		-	Sigma-Aldrich
		Luria Bertani Broth, Vegitone		-	Sigma-Aldrich
		Ampicilin			EMDC, United Kingdom
	M9 salt	Di-sodium hydrogen phosphate		Na_2HPO_4	Merck
		Potassium dihydrogen phosphate		KH_2PO_4	Merck
		Sodium Chloride		NaCl	Merck
		Ammonium chloride		NH_4Cl	Sigma-Aldrich
	M9 minimal media	Glucose		$C_6H_{12}O_6$	Merck
		Magnesium sulfate		$MgSO_4$	Merck
		Thiamine hydrochloride		$C_{12}H_{18}Cl_2N_4OS$	Sigma-Aldrich
		Biotin		$C_{10}H_{16}N_2O_3S$	Sigma-Aldrich
		Calcium chloride		$CaCl_2$	Merck
		Trace elements	Iron (III) chloride	$FeCl_3$	Merck
			Calcium chloride	$CaCl_2$	Merck
			Manganese (II) chloride	$MnCl_2$	Merck

			Zinc sulfate	$ZnSO_4$	Merck
			Cobalt (II) chloride	$CoCl_2$	Merck
			Copper (II) chloride	$CuCl_2$	Merck
			Nickel (II) chloride	$NiCl_2$	Merck
			Sodium molybdate	Na_2MoO_4	HmBG
			Sodium selenite	$NaSeO_3$	Merck
			Boric acid	H_3BO_3	Merck
Cell Lysis	Cell lysis buffer	Di-sodium hydrogen phosphate		NaH_2PO_4	Merck
		Sodium chloride		NaCl	Merck
Purification	ATPS Extraction	PEG1500		-	Ncalai Tesque
		PEG2000		-	Merck
		PEG4000		-	Merck
		PEG6000		-	Merck
		PEG8000		-	Merck
		Dipotassium hydrogen phosphate		KH_2PO_4	Merck
		Potassium di-hydrogen phosphate		KH_2PO	Merck
	Fast-protein Liquid Chromatography (FPLC)	Disodium hydrogen phosphate		Na_2HPO_4	Merck
		Sodium Chloride		NaCl	Merck
		Imidazole		$C_3H_4N_2$	Sigma Aldrich
		Ethanol		C_2H_5OH	Sigma Aldrich
Quantitative Assay	Protein assay	Bio-Rad Protein Assay		-	BioRad, Germany

	Collagen quantification assay	Collagen quantification assay kit	-	BioAssay System, USA
Qualitative Assay	Polymerase chain reaction (PCR)	Master mix	-	Axil Scientific, Singapore
		Primers	-	Axil Scientific, Singapore
	Gel electrophoresis	Agarose	-	Axil Scientific, Singapore
		TBE buffer		Axil Scientific, Singapore
		FloroSafe DNA stain	-	Axil Scientific, Singapore
		1Kb DNA ladder	-	Axil Scientific, Singapore
	SDS-PAGE	30% Acrylamide/Bis	-	BioRad, Germany
		0.5M Tris-HCl, pH 6.8	$NH_2(CH_2OH)_3HCl$	BioRad, Germany
		1.5 M Tris-HCl, pH8.8	$NH_2(CH_2OH)_3HCl$	BioRad, Germany
		10% SDS	$NaC_{12}H_{25}SO_4$	BioRad, Germany
		TEMED	$C_6H_{16}N_2$	BioRad, Germany
		10% APS	$(NH_4)_2S_2O_8$	BioRad, Germany
		ß-mercaptoethanol	C_2H_6OS	BioRad, Germany
		Bromo-phenol blue	$C_{19}H_{10}Br_4O_5S$	BioRad, Germany
		ReadyBlue™ Protein Gel Stain	-	Sigma, USA

APPENDIX B

Reagent preparation

B1. Preparation of 10X M9 salt.

Components	Molecular Weight (g/mol)	Final volume (250mL)
Na_2HPO_4	141.96	15 g
KH_2PO_4	136.09	7.5 g
NaCl	58.44	1.25 g
NH_4Cl	53.49	2.5 g
dH_2O	-	250 mL

- All components were weight and mixed until homogenous solution is achieved.
- The pH of the solution was adjusted to 7.2 before adding all water.
- The solution was autoclaved at 121°C for 20 minutes. After autoclaved, the solution was let to cool and stored at room temperature.

B2. Preparation of M9 minimal media (100 mL)

Components	Stock solution	Final Concentration	Amount
10x M9 minimal salts	10x	1 x	10 mL
20% glucose	20%	0.4 %	2 mL
1M $mgSO_4$	1M	2 mM	200 µL
Thiamine HCl (1mg/mL)	1 mg/mL	1 µg	100 µL
Biotin (1mg/mL)	1mg/mL	1 µg	100 µL
100x Trace Elements	100x	1 x	1 mL
1M $CaCl_2$	1M	0.1 mM	10 µL

Sterile dH_2O	-	-	86.34 mL
Ampicillin	100 mg/mL	100 µg/mL	250 µL

- All components (except $CaCl_2$) were mixed until homogenous solution is achieved. $CaCl_2$ was the last component added in the mixture.
- The remaining water was added and they were mixed until homogenous solution formed.

B3. Preparation of 1000X trace elements.

Components	Final concentration (mM)	Final volume (mL)
$FeCl_3$	20	100
$CaCl_2$	10	
$MnCl_2$	10	
$ZnSO_4$	2	
$CoCl_2$	2	
$CuCl_2$	2	
$NiCl_2$	2	
Na_2MoO_4	2	
$NaSeO_3$	2	
H_3BO_3	20	

B4. Preparation of cell lysis buffer

Components	Stock solution	Final concentration	Amount (Final volume: 100 mL)
Na_2HPO_4	1 M	20 mM	2 mL
NaCl	1 M	500 mM	50 mL

dH_2O	-	-	48 mL

- Prior to preparation of binding buffer, pH value for 1M Na_2HPO_4 was adjusted to 7.4.
- The mixture was filter-sterilized 0.45 µm pore filter and stored in chiller.

B5. Preparation of SDS-PAGE electrophoresis

Reagent	Amount	Preparation
10 % (w/v) SDS	10 g 90 mL distilled water	Dissolve SDS in distilled water
1.5 M Tris-HCl, pH 8.8	27.23 g Tris base 130 ml distilled water	Dissolve Tris base in 100 mL distilled water. Adjust the pH to 8.8 with 6 N HCl. Add the distilled water up to 100 ml of total volume.
0.5 M Tris-HCl, pH 6.8	6 g Tris base 90 mL distilled water	Dissolve Tris base in 60 mL distilled water. Adjust the pH to 6.8 with 6 N HCl. Add the distilled water up to 100 ml of total volume.
Sample buffer (SDS reducing buffer)	3.55 mL deionized water 1.25 mL 0.5 M Tris -HCl, pH 6.8 2.5 mL glycerol 2.0 mL 10 % (w/v) SDS 0.2 mL 0.5 % (w/v) Bromophenol blue 50 µL ß-mercaptoethanol	Mix all the components. The sample was mixed with the sample buffer with ratio 1:2 and heated at 95 °C for 5 min.
10X Running buffer, pH 8.3	30.3 Tris base 144 g glycine 10 g SDS	Dissolve all the components and add the volume of distilled water up to 1 L. For each electrophoresis run. 50 mL of 10X stock was diluted with 450 mL distilled water.
10 % (w/v) APS	0.1 g ammonium persulfate (APS) 1 mL distilled water	Dissolve ammonium persulfate with 1 mL of distilled water. It is

		advisable to prepare this solution fresh.

B6. Preparation of 12% resolving and 4% stacking gel

Materiasl	12 % resolving gel	4 % staking gel
Sterile dH_2O	1.7 mL	3.05 mL
30% Acrylamide	2 mL	0.65 mL
0.5 M Tris-HCl, pH 6.8	1.25 mL	-
1.5 M Tris-HCl, pH 8.8	-	1.25 mL
10 % SDS	50 µL	50 µL
10 % APS	50 µL	50 µL
TEMED	5 µL	10 µL
Total volume	5.055	5.06

- All the components were mixed (10% APS and TEMED were added when ready to pour).
- Distilled water was poured into the gel casting to check any leakage. Any changes in the water level were observed for 15 minutes.
- Resolving gel mixture was poured into the gel casting, leaving 2 cm below the bottom of the comb for stacking gel. Distilled water was added on top of it before leaving the gel to polymerized for 30 minutes.
- Distilled water was removed and stacking gel mixture was poured into the gel casting with the comb on top of it. Let the gel polymerized for 30 minutes.

B7. Preparation of silver staining reagents

Fixing solution: 50 % methanol, 12 % acetic acid, 0.05 % formalin	
Components	Volume
Methanol (99.8 %)	25 mL
Acetic acid (100%)	6 mL
Formaldehyde (35 %)	25 µL
Sterilised distilled water	19 mL
Washing solution: 35% ethanol	
Ethanol	73 mL
Sterilised distilled water	200 mL
Sensitizing solution: 0.02% $Na_2S_2O_3$	
Sodium thiosulfate ($Na_2S_2O_3$)	0.04 g
Sterilised distilled water	200 mL
Staining solution: 0.2 % $AgNO_3$, 0.0276 % formalin	
$AgNO_3$	0.2 g
Formalin (35 % formaldehyde)	76 µL
Sterilised distilled water	100 mL
Developing solution: 6 % Na_2CO_3, 0.05 % formalin, 0.0004 % $Na_2S_2O_3$	
Sodium carbonate	12 g
Formalin (35 % formaldehyde)	100 µL
Sodium thiosulfate	4 mL
Sterilised distilled water	196 mL
Stop solution: 50 % Methanol, 12 % acetic acid	
Methanol (99.8 %)	25 mL
Acetic acid (100 %)	6 mL
Sterilised distilled water	19 mL

- All buffers were freshly prepared.
- Formaldehyde was added just before used.

APPENDIX C

List of equipment

No	Equipment	Model	Company
1.	Autoclave machine	HiclaveTM HV-10	Hirayama
	Biophotometer	Biophotometer™ plus	Eppendorf, Germany
2.	Refrigerated Centrifuge	5804R	Eppendorf, Germany
		Heraeus Multifuge X1R	Thermo Scientific, USA
3.	Chiller	2D/DC-G	BERJAYA Steel Product Sdn. Bhd. Malaysia
4.	Digital balance	B204-S	METTLER TOLEDO, Switzerland
5.	FPLC	AKTA Prime Plus®	GE Healthcare, UK
6.	Freezer (-20°C)	Forma 900	Thermo Fisher Scientific, USA
7.	Freezer (-80°C)	Forma 7000	Thermo Fisher Scientific, USA
8.	Hot Plate	PC4200	Fisher Scientific, USA
9.	Incubator	INE400	Memmert GmbH & Co. KG, Germany
10.	Incubator shaker	CERTOMAT® IS	Sartorius, Germany
		HT Ecotron	Infors, Switzerland
11.	Laminar Flow	Airstream	Esco, SIngapore
12.	Magnetic hot plate stirrer	IKA C-Mag HS7	IKA, Germany
13.	Microcentrifuge	SIGMA 1-14	Sartorius, Germany

14.	Multimode reader	SPARK®	TECAN, Switzerland
15.	Micro Balance	Ohaus	Pioneer, China
16.	pH meter	Seven Compact	METTLER TOLEDO, Switzerland
17.	Spectrophotometer	Multiskan™ Go	Thermo Fisher Scientific, USA
18.	Thermo cycler (PCR)	5020 Arktik	Thermo Scientific, USA
19.	Rocker	Gyro-rocker SSL3	Stuart
20.	Ultrasonic homogeniser	Labsonic®	Sartorius
21.	Vortex Mixer	EVM-6000	ERLA, Korea
22.	Water bath	ISOTEMP 220	Fisher Scientific, USA

APPENDIX D

List of Glassware and Consumables

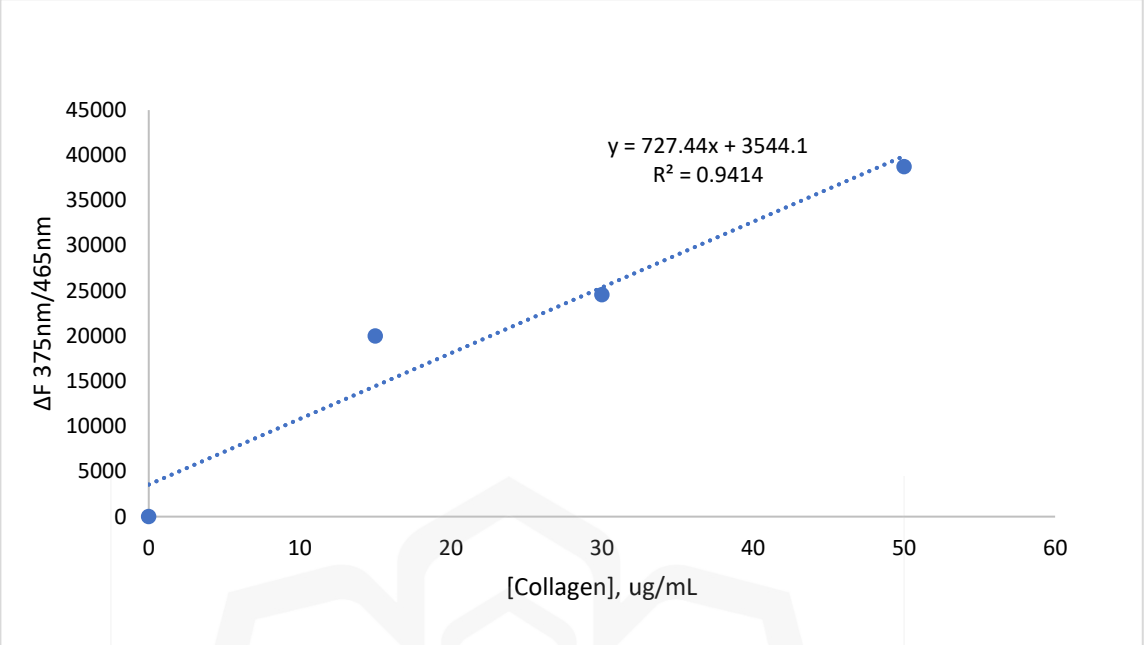
No	Items	Capacity
1.	Aluminium foil	
2.	Beakers	50, 100, 250, 500 mL
3.	Cuvettes	1 mL
4.	Erlenmeyer flasks	50, 100, 250 mL
5.	Sterile centrifuge tubes	15, 50 mL
6.	Filter and syringe	0.45 µm
7.	Graduated cylinder	100, 250, 500, 1000 mL
8.	Microcentrifuge tubes	0.2, 0.5, 1.5, 2.0 mL
9.	Micropipette tips	0.5-10 µL, 10-200 µL, 100-1000 µL, 1-5 mL
10.	Parafilm	-
11.	Petri dishes	-
12.	Schott bottles	100, 250, 500, 1000 mL
13.	Spatula	-
14.	Sterile hockey stick	-
15.	Sterile inoculum loop	-
16.	Weighing boat	-

APPENDIX E

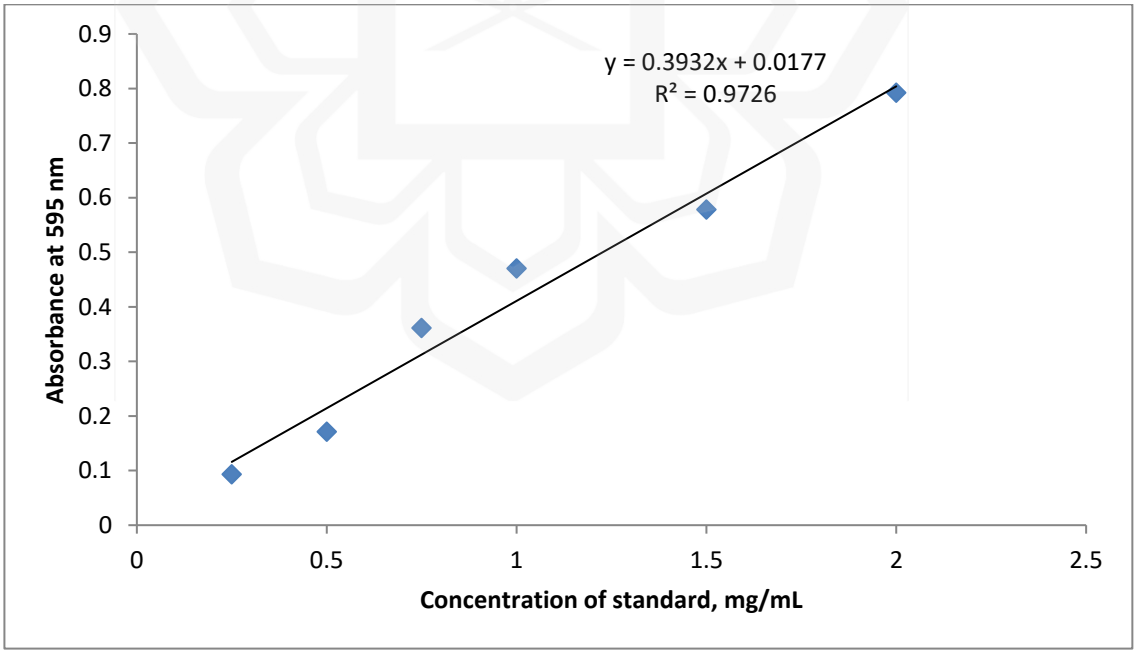
E1. Preparation of collagen assay.

i. Solution containing recombinant collagen-like protein were dilute with sterile distilled water (0.5µL cell lysate + 19.5µL dH_2O).
ii. 20 µL of samples were transferred in duplicate microcentrifuge tubes labelled as “sample” and “sample blank”.
iii. 30 µL of digest mix (30 µL buffer + 0.5 µL digest enzyme) was added in samples and 30 µL of buffer was added in each sample blank.
iv. The tubes were tapped to mix them thoroughly and incubated at 37 °C for 1 hour.
v. 40 µL of dye reagent was added into all tubes and they were incubated for 10 minutes at 37 °C.
vi. 8 µL of developer was added to all tubes.
vii. All samples were incubated at 37 °C for 10 minutes.
viii. Fluorescence at $\lambda_{ex/em}$=375/365 nm was read.
ix. The concentration of collagen was calculated based on the calibration curve (E2).

E2. Standard curve of collagen assay



E3. Standard curve for Bradford assay



APPENDIX F

Calculation for pH of phosphate buffer in ATPS using Henderson-Hasselbalch equation.

$$pH = pK_a + log_{10}\left(\frac{A^-}{HA}\right) \quad \textbf{(F1)}$$

i. Potassium phosphate buffer pH 6.0

$$6.0 = pKa + log\frac{A}{B}\ ;\ \ pKa = 7.21$$

$$\frac{A}{B} = 0.062$$

$$A = 0.062B$$

$$\frac{mol\ A}{174.18} = 0.062\ \frac{mol\ B}{136.086}$$

$$mass\ A = 0.079\ mass\ B$$

$$A + B = 10\ g$$

$$A = 0.73\ g \quad B = 9.27\ g$$

ii. Potassium phosphate buffer pH 7.0

$$7.0 = pKa + log\frac{A}{B}\ ; pKa = 7.21$$

$$\frac{A}{B} = 1.25$$

$$A = 1.25\ B$$

$$\frac{mol\ A}{174.18} = 1.25\ \frac{mol\ B}{136.086}$$

$$mass\ A = 1.6\ mass\ B$$

$$A + B = 10\ g$$

$$A = 6.16\ g \quad B = 3.84\ g$$

iii. Potassium phosphate buffer pH 8.0

$$8.0 = pKa + log\frac{A}{B}\ ; pKa = 7.21$$

$$\frac{A}{B} = 6.17$$

$$A = 6.17\ B$$

$$\frac{mol\ A}{174.18} = 6.17\ \frac{mol\ B}{136.086}$$

$$mass\ A = 7.9\ mass\ B$$

$$A + B = 10\ g$$

$$A = 8.8\ g \quad B = 1.12\ g$$