DIRECT RECOVERY OF PAPAIN FROM PAPAYA JUICE USING EXPANDED BED ADSORPTION CHROMATOGRAPHY

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DIRECT RECOVERY OF PAPAIN FROM PAPAYA FRUIT USING EXPANDED BED ADSORPTION CHROMATOGRAPHY

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Thesis submitted in fulfillment of the requirements
for the award of degree of
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MASTER OF ENGINEERING (BY RESEARCH)

Thesis submitted in fulfillment of the requirements for the award of the degree of Master of Engineering in Bioprocess Engineering.

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Dedicated to
My parents and all family members whose love, care and prayers enabled me to complete this task.

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In the name of The Great Almighty Allah SWT.

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ABSTRACT

Papain is an enzyme from *Carica papaya* which of great research interest in recent years due to its pharmaceutical and biological application. The adsorption of papain on Amberlite™ XAD7HP adsorbent with the mechanism of reverse phase liquid chromatography (RPLC) was investigated in this study. The equilibrium adsorption isotherm, binding buffer and elution buffer conditions were studied in a batch system. 15% ethanol was obtained as the optimum binding buffer with binding efficiency of 76.53% and 40% ethanol was confirmed as the optimum elution buffer with elution efficiency of 84.09%. At the optimum conditions, the adsorption capacity of Amberlite™ XAD7HP for papain was found to be 4.5 mg papain/g adsorbent. The equilibrium isotherm was fitted to Freundlich isotherm which has an n value of 1.3927 and the K of the adsorption of papain onto the Amberlite™ was 267.67 $(mg/g)(ml/mg)^n$, R^2 is 0.9746. Papain in the concentrated extract was purified 4.3-folds and 55% purity corresponding to around 15% of the total papain in the crude juice. More than 84% of the adsorbed papain was eluted using 40% ethanol as the elution buffer. The efficiency of purifying papain from papaya juice using the method developed above was around 65.5%. A purified papain with 65% yield and 50% purity was recovered.

A direct recovery of papain enzyme from unclarified *Carica papaya* juice was developed successfully using a reversed phase expanded bed adsorption chromatography (RP-EBAC). The dynamic binding capacity for the RP-EBAC at 10% breakthrough of 810.52 µg papain/g adsorbent was achieved at a linear flow velocity of 936 cm/h, bed expansion degree of 2 and feedstock viscosity of 2.68 mPa • s. Papain purity of 58.39% and purification factor of 4.96 was obtained by one-step elution in RP-EBAC. Two-step elution was employed to enhance the purity of papain in RP-EBAC which gives higher papain purity of 74.98% and higher purification factor of 7.04. Absolute ethanol (100%) was successfully used in the Clean-in-place (CIP) for cleaning of the Amberlite™ XAD7HP adsorbent. After the first CIP about 15% of total protein was still retained on the adsorbent and about 3% of total protein still retained on the adsorbent after second CIP. It shows that there is no strong cumulative effect of contaminants happens on the adsorbent. This work shows a great potential of using RP-EBAC to purify papain from unclarified *Carica papaya* juice.

ABSTRAK

Papain adalah enzim dari betik *Carica* yang mempunyai kepentingan penyelidikan dan aplikasi yang besar dalam farmasuetikal dan biologi kebelakangan ini. Dalam kajian ini, penjerapan papain pada Amberlite™ XAD7HP dengan mekanisme fasa terbalik cecair kromatografi (RPLC) telah disiasat. Keseimbangan isoterma penjerapan, penimbal pengikat dan penimbal elusi telah dikaji dalam sistem kelompok. Etanol 15% telah diperolehi sebagai penimbal pengikat optimum dengan kecekapan 76.53% dan etanol 40% sebagai penimbal elusi optimum dengan kecekapan elusi sebanyak 84.09%. Pada keadaan optimum, kapasiti penjerapan Amberlite™ XAD7HP untuk papain didapati adalah 4.5 mg papain/g adsorben. Isoterma keseimbangan telah di sesuaikan dengan isoterma Freundlich yang mempunyai nilai n ialah 1.3927 dan K penjerapan papain terhadap Amberlite™ XAD7HP, 267.67 $(mg/g)(ml/mg)^n$. Papain dalam ekstrak pekat telah ditulenkan 4.3 kali ganda ke tahap ketulenan 55%, iaitu kira-kira 15% daripada jumlah papain jus mentah. Lebih daripada 84% papain yang terjerap telah elut dengan menggunakan etanol 40% sebagai penimbal elusi. Dengan menggunakan kaedah yang dibangunkan di dapati kecekapan penulenan papain dari jus betik ialah 65.5%. Perolehan papain tulen adalah dengan 65% aktiviti dan 50% ketulenan.

Perolehan secara langsung enzim papain dari jus betik Carica yang tidak jernih telah berjaya dibangunkan dengan menggunakan fasa penjerapan lapisan terkembang kromatografi terbalik (RP-EBAC). Kapasiti dinamik pengikat untuk RP-EBAC pada 10% telah mencapai penjerapan sebanyak 810.52 μg papain/g pada halaju aliran linear 936 cm/h, lapisan kembang tahap dua dan kelikatan bahan suapan adalah 2.68 mPa•s. Papain tulen sebanyak 58.39% dan faktor purifikasi 4.96 telah diperolehi dari elusi satu langkah dalam RP-EBAC. Manakala, elusi dua langkah telah digunakan untuk meningkatkan ketulenan papain dalam RP-EBAC yang memberikan ketulenan yang tinggi, iaitu 74.98% dan juga faktor penulenan yang tinggi, iaitu 7.04. Etanol tulen (100%) telah digunakan dalam pembersihan secara terus (CIP) untuk membersihkan XAD7HP adsorben Amberlite™. Selepas CIP yang pertama kira-kira 15% protein masih dikekalkan pada adsorben dan selepas CIP yang kedua hanya 3% protein kekal pada adsorben. Ia menunjukkan bahawa tiada kesan pencemaran kumulatif yang berlaku pada adsorben. Ini menunjukkan bahawa RP-EBAC berpontensi besar untuk digunakan bagi penulenan papain dari jus betik Carica tidak jernih.

TABLE OF CONTENTS

SUPERVISOR'S DECLARATION **iii**

STUDENT'S DECLARATION **iv**

ACKNOWLEGEMENTS **vi**

ABSTRACT **vii**

ABSTRAK **viii**

TABLE OF CONTENTS **ix**

LIST OF TABLES **viii**

LIST OF FIGURES **xiv**

LIST OF SYMBOLS **xvi**

LIST OF ABBREVIATIONS **xvii**

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND INFORMATION 2
1.2 PROBLEM STATEMENTS 3
1.3 SCOPE OF THE RESEARCH 4
1.4 RESEARCH OBJECTIVE 5
1.5 SIGNIFICANCE OF THE RESEARCH 5
1.6 CONCLUSION 5

CHAPTER 2 LITERATURE REVIEW

2.1 PAPAIN 6

2.1.1 Papain characteristic and application 7
2.1.2 Purification of papain 11

2.2 REVERSED PHASE LIQIUD CHROMATOGRAPHY (RPLC) 13

2.2.1 Principles of RPLC 13
2.2.2 Stages in RPLC 16
2.2.3 Amberlite™ XAD7HP 19

2.3 EXPANDED BED ADSORPTION CHROMATOGRAPHY (EBAC) 21

2.3.1 Principles of EBAC 22
2.3.2 Important factors on EBAC performance 25
2.3.3 The purification of proteins by EBAC 26

CHAPTER 3 MATERIALS AND METHODS

3.1 ADSORBENT AND CHEMICALS 31

3.2 EQUIPMENTS 31

3.3 PREPARATION OF CRUDE SAMPLES 31

3.4 OPERATION OF BATCH ADSORPTION 32

3.4.1 Batch adsorption 32
3.4.2 Optimization of binding and elution conditions 33
3.4.3 Equilibrium of adsorption isotherm 33

3.5 OPERATION OF EBAC 34

3.5.1 Expansion characteristic of adsorbent 34
3.5.2 Dynamic binding capacity 34
3.5.3 Direct recovery of papain from feedstock suspension using EBAC 35
3.5.4 Enhancement of papain purity using stepwise elution on EBAC 36
3.5.5 Study of Clean-in-place (CIP) 36

3.6 ANALYTICAL PROCEDURE 36

3.6.1 Estimation of papain amount 38
3.6.2 Bradford assay 38
3.6.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 38

3.7 CALCULATIONS 39

CHAPTER 4 RESULTS AND DISCUSSION

4.1 EFFECT OF ETHANOL CONCENTRATION ON PAPAIN ACTIVITY 41

4.2 RECOVERY OF PAPAIN FROM CRUDE FEEDSTOCK OF PAPAYA BY BATCH ADSORPTION REVERSED PHASE LIQUID CHROMATOGRAPHY 42

4.2.1 Optimization of binding and elution conditions 42
4.2.2 Equilibrium and adsorption isotherm 44
4.2.3 Operation of batch adsorption 46

4.3 DIRECT RECOVERY OF PAPAIN FROM UNCLARIFIED PAPAYA JUICE USING REVERSED PHASE EXPANDED BED ADSORPTION CHROMATOGRAPHY (RP-EBAC) 50

4.3.1 Operation of RP-EBAC column 50
4.3.2 Operation of RP-EBAC 54

4.4 ENHANCEMENT OF PAPAIN PURIFICATION BY TWO-STEP ELUTION IN EBAC 56

4.5 CLEAN-IN-PLACE (CIP) 60

CHAPTER 5 OVERALL SUMMARY, CONCLUSION AND FUTURE PERSPECTIVE

5.1 OVERALL SUMMARY 62

5.2 CONCLUSION 65

5.3 FUTURE PRESPECTIVES 65

REFERENCE 67

APPENDICES 78

A Bio-imaging system (Alpha Innotech) 78

B EBAC system 78
C DV-III Viscometer 79
D BSA standard curve by Bradford assay 79
E Tyrosine standard curve 80

LIST OF TABLES

Table No.	Title	Page
2.1	The physical properties and suggested operation conditions of Amberlite™ XAD7HP	20
2.2	Bioproducts purified from the EBAC	29
4.1	Summary of the Freundlich models parameters for papain adsorption isotherms onto different adsorbents	46
4.2	Summary of the results, include of activity, amount, efficiency during the initial, binding, washing and elution stage, and purification factor of papain	48
4.3	Results of viscosity and the linear regression of the Richardson–Zaki plots	52
4.4	The performance of papain purification from feedstock suspension using RP-EBAC	55
4.5	The purification performance of papain from feedstock suspension using RP-EBAC with two-step elution	58
4.6	Protein amount in each stage of EBAC process	61

LIST OF FIGURES

Figure No.	Title	Page
2.1	Collecting the latex from green unripe papaya.	6
2.2	Amino acid sequence of papain	7
2.3	A space fill model of papain.	9
2.4	RPLC: Proteins with exposed hydrophobic region (red) will be able to bind to the immobilized hydrophobic stationary phase; the rest simply wash off.	14
2.5	Stages in an RPLC separation.	17
2.6	The chemical structure of Amberlite™ XAD7HP	19
2.7	A schematic diagram of an EBAC column.	23
2.8	The operational stages of EBAC system	24
3.1	Unripe green papaya fruits (*Carica papaya*)	32
3.2	Amount of papain corresponding to their enzyme proteolytic activity	37
3.3	Flowchart of the methodologies used in this research	40
4.1	Effect of ethanol concentration on the papain activity	42
4.2	Binding efficiency of papain on the Amberlite™ XAD7HP. The (A) profile with the different concentrations of ethanol and (B) the adsorption time with 15% ethanol	43
4.3	Optimization of ethanol concentration in the elution process	44
4.4	Adsorption isotherm of papain activity from papaya juice feedstock on Amberlite XAD7HP	45

4.5	Graphical illustration of SDS-PAGE of the protein fractions collected in optimized batch adsorption reversed phase liquid chromatography.	49
4.6	Bed expansion chearacteristics of Amberlite™ XAD7HP in equilibration 15% ethanol (■) and 15% ethanol mixed unclarified feedstock(◆)	51
4.7	Richardson–Zaki plot for Amberlite XAD7HP adsorbent. Binding buffer (■) and feedstock suspension (◆)	52
4.8	Breakthrough curve of the papain from unclarified feedstock onto Amberlite™ XAD7HP in RP-EBAC	54
4.9	Profile of separation of 14ml feedstock suspension on Amberlite™ XAD7HP in EBAC at a superficial velocity of 936cm/h during feeding.	55
4.10	Graphical illustration of the pooled protein fractions collected in RP-EBAC operation	56
4.11	Graphical illustration of the pooled protein fractions collected in RP-EBAC operation using two-step elution	60
5.1	Process scheme for the recovery of papain from papaya fruit juice using RPLC batch adsorption chromatography	63
5.2	Process scheme for the recovery of papain from papaya fruit juice using expanded bed adsorption chromatography	64

LIST OF SYMBOLS

A	Papain activity
Af	Papain concentration after binding
Ai	Initial concentration of papain
C	The equilibrium papain concentration in the sample solution
C_0	Initial concentration of papain in the feedstock suspension
H_0	Height of settled bed
H	Height of the expanded bed
K	Adsorption constant of Freundlich isotherm
m	Amount of the adsorbent
M	Tyrosine amount
n	Freundlich coefficient
Q	The amount of papain
Q_B	The dynamic binding capacity
u	Superficial velocity
u_t	Terminal settling velocity
V_b	The volume at 10% breakthrough
Vs	The volume of the samples was used for the binding
Vs	The settled volume of the adsorbent
ε	Expanded bed voidage

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
BV	Bed volume
CIP	Cleaning-in-place
DEAE	Diethylaminoethanol
EBAC	Expanded bed adsorption chromatography
EDTA	Ethylenediaminetetraacetic acid
HIC	Hydrophobic interaction chromatography
NCp-NiV	Nucleocapsid protein of Nipah virus
OFAT	One-factor-at-a-time
PBAC	Packed bed adsorption chromatography
PF	Purification factor
RPLC	Reversed phase liquid chromatography
RP-EBAC	Reversed phase expanded bed adsorption chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
THF	Tetrahydrofuran
UV-vis	Ultraviolet–visible

CHAPTER 1

INTRODUCTION

A large part of the plant source sample enrichment procedures currently available rely on the adsorption of the analytes of interest onto a suitable adsorbent. Adsorption enables the separation of selected compounds from dilute solutions. Compared to alternative technologies, adsorption is attractive for its relative simplicity of design, operation and scale up, high capacity and favorable rate, insensitivity to toxic substances, ease of regeneration and low cost. Additionally, it avoids using toxic solvents and minimizes degradation. Unfortunately, their applicability for the enrichment of polar and/or high molecular weight compounds, especially coupled to thermal desorption, is limited. Polar solutes can easily be adsorbed but can also be readily converted into different compounds on the surface catalyzed by active surface groups. High molecular weight compounds cannot be desorbed due to extremely strong interactions with the adsorbent and low volatility. As a solution, at least to a part of these problems, sample preparation techniques based on the reversed phase adsorption was described in Chapter 2. Non-aqueous enzymology has undergone significant progress in the past two decades, and the use of enzymes in organic solvents has extended the scale of their application and allowed the syntheses of biologically active enantiomer. Although the ability of enzymes to act as selective catalysts for a broad spectrum of organic reactions has been known for many years, their application as biocatalysts is rare because of the inappropriate stabilities. The stability of enzymes is one of the most difficult problems in protein chemistry, and accordingly there is a great interest in understanding the effects of organic solvents on the stability of enzymes, and in developing novel strategies to improve their productivity.

The work provides an overview on purification and fractionation of papain from

papaya by resins. Special attention has been devoted to recent developments on the recovery of biologically active papain. Additionally, some major challenges in batch and expanded bed adsoprtion are identified, and some selected major trends are discussed.

1.1 BACKGROUND OF THE STUDY

Papain (EC3.4.22.2), a minor constituent (5-8%) among the cysteine endopeptides is extracted from the latex of unripe *Carica papaya* (Kimmel and Smith, 1954). Papain digests most protein substrates more extensively than the pancreatic proteases (Arnon, 1970). The enzyme has been applied in many areas such as cell isolation (Kinoshita *et al.*, 2003), cosmetic, leather, textiles, detergents, food industry, peptides production (Xiang *et al.*, 2004), synthesis of molecules (Rajesh *et al.*, 2003) and pharmaceutical industries etc. (Huffman *et al.*, 1967; Walsh, 2002; Nie and Zhu, 2007; Li *et al.*, 2010). And same time, papain has been used in many medicinal areas like antibody production which with a great efficiency (Newkirk *et al.*, 1987). Thus, there is an increased requirement of papain in industry.

The purification of papain from papaya latex has been traditionally achieved by salt precipitation (Kimmel and Smith, 1954). Modern method for the purification of papain is the adsorption chromatography. The success of chromatography is associated with the selection of appropriate type of adsorption chromatography to be used when a protein sample is applied to the column. Adsorptive chromatography works by ionic interaction (electrostatic binding), hydrophobic interaction, or specific chemical interaction. Ion exchange chromatography, hydrophobic chromatography (Azarkan *et al.*, 2003), aqueous two-phase extraction (Nitsawang *et al.*, 2006; Li *et al.*, 2010) and dye affinity chromatography (Nie and Zhu, 2007) have been used for the purification of papain. Although purification in downstream process for papain by chromatography can reach high purity, all these studies show alternative small scale purification which only stands for laboratory scale. This is not adapting to the large quantity needs of highly pure papain in industry with low cost.

Fortunately, alternative large scale method such as expanded bed adsorption chromatography (EBAC) can be sought to improve the downstream process of papain.

EBAC has been used successfully with a number of different feedstock such as bacterial (Johansson *et al.*, 1996; Ling *et al.*, 2004), mammalian cell (Thömmes *et al.*, 1995), plant extract (Valdés *et al.*, 2003; Li and Chase, 2009b), yeast (Owen *et al.*, 1997), chicken egg white (Chang and Chang, 2006), fermentation broths and transgenic milk (Özyurt *et al.*, 2002; Tan *et al.*, 2006). Furthermore, EBAC combines three important steps of clarification, purification and concentration steps into one single operation. It is reported that when used in the recovery of proteins, EBAC can dramatically increase the process efficiency (González *et al.*, 2003), reduce operation cost (Bodo *et al.*, 2006), shorter process time (de Lamotte, 2005) and higher productivity (Cabanne *et al.*, 2005; Li and Chase, 2009a).

1.2 PROBLEM STATEMENTS

The major problem during the large scale production and purification of the papain from the *Carica papaya* is the low purity. Conventional method for the purification of papain is salt precipitation method (Kimmel and Smith, 1954). This method has been used for a long time, nevertheless, it only gives very low purity of papain of 39%, and it still contaminated with many kinds of proteins (Baines and Brocklehurst, 1979; Nitsawang *et al.*, 2006). In order to fulfill the willing of scale up production of papain with high purity, there must be an adsorptive method which able to treat large quantity of feedstock and also with high purification resolution.

EBAC is the successful hybrid of the conventional packed bed column chromatography and fluidized bed chromatography (Chase, 1994; Anspach *et al.*, 1999). The adsorbent beads in the expanded bed become fluidized and classified by an upward flow. When the bed becomes stable and the improved adsorption properties are similar to those encountered in a conventional packed bed. Unlike the conventional packed bed chromatography that requires particulate-free feedstock in the purpose of avoiding the particulates collage the adsorbent bed, EBAC combines three important steps of clarification, purification and concentration steps into one single operation which allows the feeding of particulate-containing feedstock. This will avoid the loss of the target protein which happens in the excessive procedures. And also it benefits the large scale production of biomolecules which with high requirements for the short processing time

and critical processing condition. Thus, EBAC would be an efficient method to purify papain in large scale from the unclarified papaya juice feedstock.

Although EBAC able to solve the requirement of large scale production, for the purpose of high purity would be another consideration on the adsorptive type and adsorptive mechanism. Papain was confirmed with high hydrophobic region (Jayaraj *et al.*, 2009; Rajasekaran and Vijayasarathy, 2011) and it enabled the use of mechanism of reversed phase liquid chromatography (RPLC) which adapt the high resolution purpose and result in high purity of target protein (Aguilar, 2003). RPLC and EBAC both have not been used for the purification of papain. This research would be the exploring work for the combination of these two methods in the purification of papain from unclarified papaya juice feedstock with high efficiency.

Amberlite™ XAD7HP is an industrial grade non-ionic aliphatic acrylic polymeric adsorbent can be applied in RPLC for the adsorption of enzyme in order to keep the enzyme structure and the enzyme activity. The direct capture of flavonoids from unclarified Ginkgo leaf extraction solution on Amberlite™ XAD7HP using EBAC has been evaluated and shown Amberlite™ XAD7HP is available to be used in EBAC (Li and Chase, 2009b). In order to combine the use of RPLC and EBAC, Amberlite™ XAD7HP was selected as the adsorbent. The hyrdrophobicity of papain properly worked and it enabled the binding of papain on the Amberlite™ XAD7HP with ethanol as the binding buffer. Elution of papain also been done by higher concentration of ethanol as the elution buffer. Enhancement of papain purity was successfully performed by stepwise elution method.

1.3 SCOPE OF THE RESEARCH

The selection, optimization of binding and elution buffer condition for the purification of papain on Amberlite™ XAD7HP from clarified papaya juice was performed in a batch adsorption system. The purification of papain using expanded bed adsorption chromatography (EBAC) using Fastline™ 10 EBAC column packed with Amberlite™ XAD7HP from unclearified papaya juice feedstock was performed in the optimal binding and elution conditions which obtained from the batch adsorption

system. Equilibrium adsorption isotherm of papain on the Amberlite™ XAD7HP was confirmed in a batch system. Bed expansion characteristic of Amberlite™ XAD7HP was obtained in EBAC. Enhancement of papain purity was investigated in EBAC by stepwise elution strategy.

1.4 RESEARCH OBJECTIVE

Therefore, the objectives of the current study were:

1. To establish a strategy to purify papain from unripe papaya juice using batch adsorption reverse phase liquid chromatography (RPLC).
2. To optimize the batch adsorption protocol of papain using Amberlite™ XAD7HP, a RPLC adsorbent.
3. To develop a direct purification method using EBAC packed with Amberlite™ XAD7HP to purify the papain from unclarified papaya juice.

All the objectives mentioned above were researched in laboratory scale.

1.5 SIGNIFICANCE OF THE RESEARCH

Papain is found naturally in *Carica papaya*, which is a versatile plant grows in a wide range of climate. It has been revealed to be an enzymatic protein having number of uses and enzymatic properties of significant biological and economic importance. This proteolytic enzyme works and makes itself valuable for a variety of purposes. Purification of papain becomes great important since its broad usage, especially in the pharmaceutical area which need highly purified papain. Present work on the purification of papain from papaya fruit would explore the efficient method of papain purification and make the large scale production of papain become possible.

1.6 CONCLUSION

This research made an exploring work on the frontal analysis for scaling up purification of papain from unclarified papaya juice feedstock using reversed phase expanded bed adsorption chromatography (RP-EBAC). It shows RP-EBAC is a potential method to purify papain in a large scale production.

CHAPTER 2

LITERATURE REVIEW

2.1 PAPAIN

In 1873, G.C. Roy first investigated the action of papain in an article published in the *Calcutta Medical Journal* entitled "The Solvent Action of Papaya Juice on Nitrogenous Articles of Food". In 1879, Wurtz and Bouchut first named partially purified and recognized proteolytically active constituent in the latex of tropical papaya fruit as papain, as shown in Figure 2.1 (Baines and Brocklehurst, 1979). Throughout the mid-1950s and 1960s, purification and separation techniques improved greatly and pure papain was isolated.

Figure 2.1: Collecting the latex from green unripe papaya

2.1.1 Papain characteristic and application

Papain was the first cysteine protease have its structure to be identified by X-ray methods (Drenth *et al.*, 1968). It is confirmed that papain is a single peptide chain of 211 residues folded into two parts that form a cleft and have one free sulfhydryl group (-SH) along the sequence of the cys-25 which is functional as shown in Figure 2.2 (Drenth *et al.*, 1971).

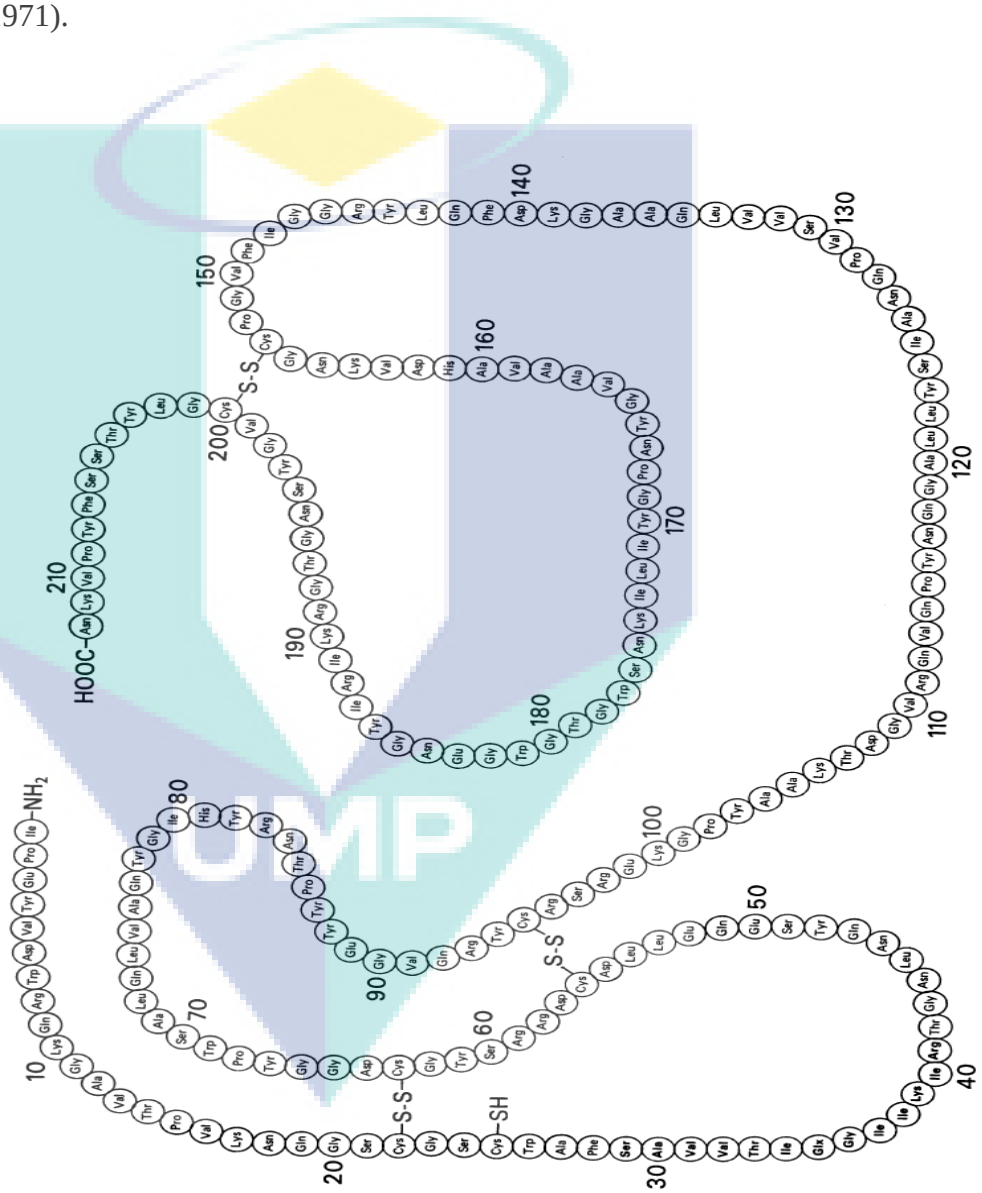
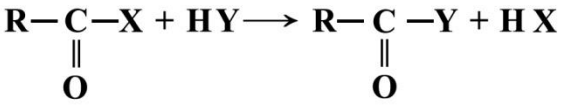


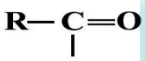
Figure 2.2: Amino acid sequence of papain

(Source: Drenth, 1971)

As a proteolytic enzyme, besides hydrolysis of peptide bonds, papain can also catalyze other type of reaction. Practically, all this reaction can be represented by formula. (2.1).



(2.1)

Thus papain can be considered as a catalyst for the transfer of acyl group  from a group X to group Y. The molecular weight of papain is 23.4 kDa (Drenth *et al.*, 1968).

Papain is highly stable even at elevated temperatures (Cohen *et al.*, 1986) and also active under a wide range of conditions. Papain is unusually defiant to high concentrations of denaturing agents, such as, 8 mol/L urea or organic solvent, nevertheless, it was observed that no decrease of activity even at up to 60% ethanol (Szabó *et al.*, 2006). Optimum pH for activity of papain is in the range of 3.0-9.0 which varies with different substrate (Edwin and Jagannadham, 2000; Ghosh, 2005). Papain is activated by cysteine, sulfide, sulfite, heavy metal chelating agents like EDTA and N-bromosuccinimide, isoelectric point is at 9.6 (Sluyterman and Wijdenes, 1972). It was inhibited by cystatin (El Moussaoui *et al.*, 2001), Hg^{2+} and other heavy metals, Sulfhydryl binding agents, PMSF etc. Papain's stabilizing agent is EDTA, cysteine and dimercaptopropanol (Bender *et al.*, 1966).

In general, native proteins have a hydrophobic core and a charged and/or polar group on the surface. The hydrophobic core helps to stabilize the tertiary structure of the protein by hydrophobic interaction while the outer polar surfaces preferentially interact with the exterior aqueous medium (Wang *et al.*, 2006). The carbon content distribution profile method shows that carbon content is maintained at 31.45% of carbon all along the sequence of papain (Amri and Mamboya, 2012). Some regions along the sequences have values above 31.45%, these are considered to be higher hydrophobic regions as it has previously been reported (Rajasekaran and Vijayasarathy, 2011). The overall hydrophobicity of papain is contribute to stability of protein (Jayaraj *et al.*, 2009).

Figure 2.3 is a space fill model of papain which shows the hydrophobic area on the surface of papain. High hydrophobic region enables the use of hydrophobic interaction chromatography or reversed phase liquid chromatography to purify the papain.

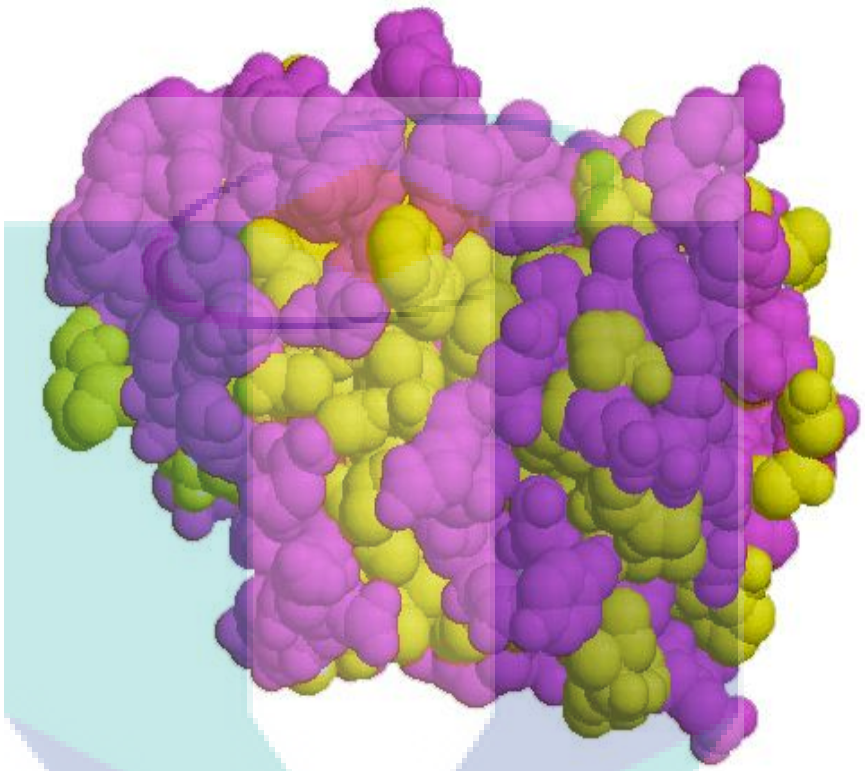


Figure 2.3: A space fill model of papain. The hydrophobic amino acids are colored purple. The backbone oxygen and nitrogen of the residues with the hydrophobic side chain are colored yellow.

(Source: http://www.oocities.org/bramsugar/intro4.html).

Papain present in the latex of *Carica papaya* has been extensively studied (Brocklehurst *et al.*, 1981; Mellor *et al.*, 1993; Thomas *et al.*, 1994) and it is an enzyme of a broad usage and of high research interest. Papain has been used in many areas through many years of research. Among the major applications of papain is in the food industry. The ability of papain as a protease to tenderize meat either from vitro or vivo is well confirmed. In vitro, papain is used to modify myofibrillar proteins and the connective tissue proteins which are the major meat proteins responsible for tenderness (Khanna and Panda, 2007). Several researchers have indicated that one of the

difficulties in vitro using of papain is the penetration of the enzyme through the tissue (Gottschall and Kies, 1942; Wang and Nehema, 1955; Tappel *et al.*, 1956). Huffman *et al*. (1961) reported a significant tenderizing effect through vivo effects by injection of crude papain to chickens. And injection of papain on cattle also induced the same result of tenderized beef (Huffman *et al.*, 1967). Papain has been reported to improve meltability and stretchability of Nabulsi cheese which more easy to melt when was heated and not easy to be pulled apart when it was stretched, resulting in outstanding fibrous structure enhancing superiority in the application in kunafa, pizza and pastries (Abu-Alruz *et al.*, 2009). It was also used for the beer chill-proofing, beer clarification (Caygill, 1979), brewing and baking (Li et al., 2010).

Papain is not only used in food industry, the most attractive application of papain is in the research and medicinal area. Papain has been applied in cell isolation (Kinoshita *et al.*, 2003) and it has been proven is more efficient and less destructive than other proteases on certain tissue such as neurons from postnatal rats (Huettner and Baughman, 1986) and smooth muscle cells (Driska *et al.*, 1999). Papain is reported to be useful in the understanding and design of the inhibitors of papain superfamily proteins such as cathepsin L and cathepsin K, which of the certain property can be used in preventing certain types of illnesses. (LaLonde *et al.*, 1998; Tsuge *et al.*, 1999; Gayosso-Garc á Sancho *et al.*, 2010). Arnon (1970) has indicated that papain will degrade most protein substrates more extensively than pancreas protease, and it has been proven to be a versatile protease for the production of a variety of peptides (Stehle *et al.*, 1990; Stevenson and Storer, 1991; Xiang *et al.*, 2004; Szab ó *et al.*, 2006), protein hyrolysates (Dupaigne, 1973), lipo amino acid-based surfactants, esters of amino acids and carbohydrate derivatives (Morcelle *et al.*, 2006), and it can help to synthesis of many other molecules (Rajesh *et al.*, 2003). Limited papain digestion has proven useful for structural studies of enzymes and other proteins (Margossian and Lowey, 1973; Shiozaki and Yanagida, 1991). And same time, papain was successfully used in antibody fragments production which shown a great efficiency (Newkirk *et al.*, 1987; Walsh, 2002). Briefly, papain cleaves antibodies into two Fab fragments, which recognize the antigen specifically with their variable region, and one Fc fragment. The fragments of antibody then can be purified by gel filtration, ion exchange, or affinity chromatography (Harlow and Lane, 1988), applying to the medicinal purpose.

As a protein digestant, papain is used as a digestion aid medicine (Deulgaonkar and Thorat, 2008), combating dyspepsia and other digestive disorders and disturbances of the gastrointestinal tract (Huet *et al.*, 2006). Papain bears with antifungal, antibacterial and anti-inflammatory properties (Nwinyi and Abikoye, 2010). For instance, papain has previously been reported to have significant analgesic and anti-inflammatory activity against symptoms of acute allergic sinusitis like headache and toothache pain without side effects (Mansfield *et al.*, 1985). Papain enzyme has a long history of being used to treat sports injuries, trauma, allergies (Deitrick, 1965), edemas and wounds (Li *et al.*, 2010). In some treatments, papain acts as a debris-removing agent with no harmful effect on sound tissues because of the enzyme's specificity, acting only on the tissues, which lack the α1-antitripsine plasmatic antiprotease that inhibits proteolysis in healthy tissues (Flindt, 1979). Besides all the applications were mentioned above, papain still has many usages in cosmetic, leather, textile, detergent and wool anti-shrinking etc.. Thus, there is a great increasing requirement of papain in various industries.

2.1.2 Purification of papain

The first purification of papain in the native crystalline state from fresh papaya latex was carried by Balls *et al.* (1937). In order to obtain the crystalline papain from dried latex in satisfactory yield, Kimmel and Smith modified the preparative procedure of Balls and Lineweaver using commercially available dry latex and it has been the classical method for papain preparation for many years (Kimmel and Smith, 1954) with some later modifications (Arnon, 1970; Baines and Brocklehurst, 1979). This method involves extraction of the latex, removal of insoluble material and precipitation by ammonium sulfate followed by three recrystallizations. The resulting protein contains three components, active papain, activatable papain, and nonactivatable papain. The differences between these components associated with the state of the active-site thiol group. The active papain in the thiol form, as judged from the proportionality between thiol content and enzyme activity (Kimmel and Smith, 1957; Sanner and Pihl, 1963). Activatable papain, which itself is inactive, can be converted to active papain by reaction with thiols (Klein and Kirsch, 1969; Blumberg *et al.*, 1970). Nonactivatable

papain cannot be activated to an enzymitally active material by the addition of thiols (Blumberg *et al.*, 1970; Burke *et al.*, 1974). Baines et al. (1979) spray dried latex of *Carica papaya* by a controlled and relatively mild process which was used to prepare papain. The paper reported a method modified from the method of Kimmel and Smith that optimizes the yield of papain from highly soluble papaya latex while avoiding contamination by other enzymes (Baines *et al.*, 1979). In 2000, Monti and Basilio reported a method for crystallizing papain from fresh papaya latex without the use of sulphydryl reagents by a precipitation method with the entire procedure carried out under bubbling with nitrogen for protection against atmospheric oxygen, which gave higher yields of 2.43 mg papain/g latex than previously reported. The papain thus obtained is practically pure and shows a single band when submitted to electrophoresis on polyacrylamide gel, and is identical to the papain obtained by other methods. Purification of papain from wet *Carica papaya* latex by extraction in aqueous two-phase system was studied and highly pure papain was obtained in a much shorter processing time directly from unclarified latex with the use of an aqueous two-phase system consisting of 8% (w/w) polyethylene glycol and 15% (w/w) ammonium sulfate (Nitsawang *et al.*, 2006).

Recently, chromatography draws a great attention to the researchers due to its characteristic of producing highly purified product. Many studies have performed alternative small scale papain isolation through chromatography with different mechanisms including ion exchange, covalent, or affinity chromatography. (Blumberg *et al.*, 1970; Brocklehurst *et al.*, 1973; D'Souza and Lali, 1999; Nitsawang *et al.*, 2006; Nie *et al.*, 2007; Nie and Zhu, 2007; Deulgaonkar and Thorat, 2008). For each mechanism here describes an instance for the application as following paragraph.

In 1970, Blumberg *et al*. successfully performed the papain purification using affinity chromatography with a covalently linking of a water insoluble derivative of a papain inhibitor, Gly-Gly-Tyr(Bz1)-Arg, on an agarose resin. The property of thiol group of papain with high reactivity towards 2, 2'-dipyridyl disulphide (2-Py-S-S-2-Py) at pH 4 was used in covalent chromatography by thiol-disulphide interchange on a A Sepharose-(glutathione-2-pyridyl disulphide) conjugate was performed by Brocklehurst *et al*. (1973) which shown papain containing 1 intact catalytic site per mol of protein

was readily prepared both from dried papaya latex and from commercial 2×crystallized partially active papain. Chromatography on cation-exchange supports has traditionally been used as the first purification step of papaya enzymes (Schack, 1967; Baines and Brocklehurst, 1982; Zucker *et al.*, 1985; Dubois *et al.*, 1988). Deulgaonkar and Thorat (2008) performed a ion exchange chromatography packed with cation exchange resins (SP-sepabeads) for the purification of papain which gives almost 4.5 to 5 folds more pure papain as compared to various precipitation techniques .

Nevertheless, up to now, there is no literature has described the purification of papain by reversed phase liquid chromatography (RPLC).

2.2 REVERSED PHASE LIQIUD CHROMATOGRAPHY (RPLC)

2.2.1 Principles of RPLC

Reversed phase liquid chromatography (RPLC) is a separation method based on the hydrophobicity of the protein. It is a kind of hydrophobic interaction chromatography (HIC). HIC is an important technique for protein purification, which exploits the separation of proteins based on hydrophobic interactions between the stationary phase ligands and hydrophobic regions on the protein surface (Lienqueo *et al.*, 2007). The term ‘reversed’ in RPLC was derived from its predecessor named ‘normal’ phase. In the 1970s, most HIC was performed based on "normal phase chromatography" using a solid support stationary phase (also called a "column") containing unmodified silica or alumina resins. In normal phase chromatography, the stationary phase is hydrophilic and therefore has a strong affinity for hydrophilic molecules in the mobile phase. Thus, the hydrophilic molecules in the mobile phase tend to bind (or "adsorb") to the column, while the hydrophobic molecules pass through the column and are eluted first. In normal phase chromatography, hydrophilic molecules can be eluted from the column by increasing the polarity of the solution in the mobile phase. During the development of affinity chromatographic support mediums, a number of “control” experiments were carried out. In most cases, the behavior of matrices containing spacer arms but no ligand behaved as expected, with no adsorption noted. However, in a few cases, proteins were found to bind strongly to hexametylene arms, even without charged

ends (amino or carboxylate). These observations were further developed the techniques of hydrophobic chromatography, for it was the interaction between aliphatic chains on the adsorbent and corresponding hydrophobic regions on the surface of the proteins that was causing binding, and this exactly RPLC comes from as illustrated in Figure 2.4.

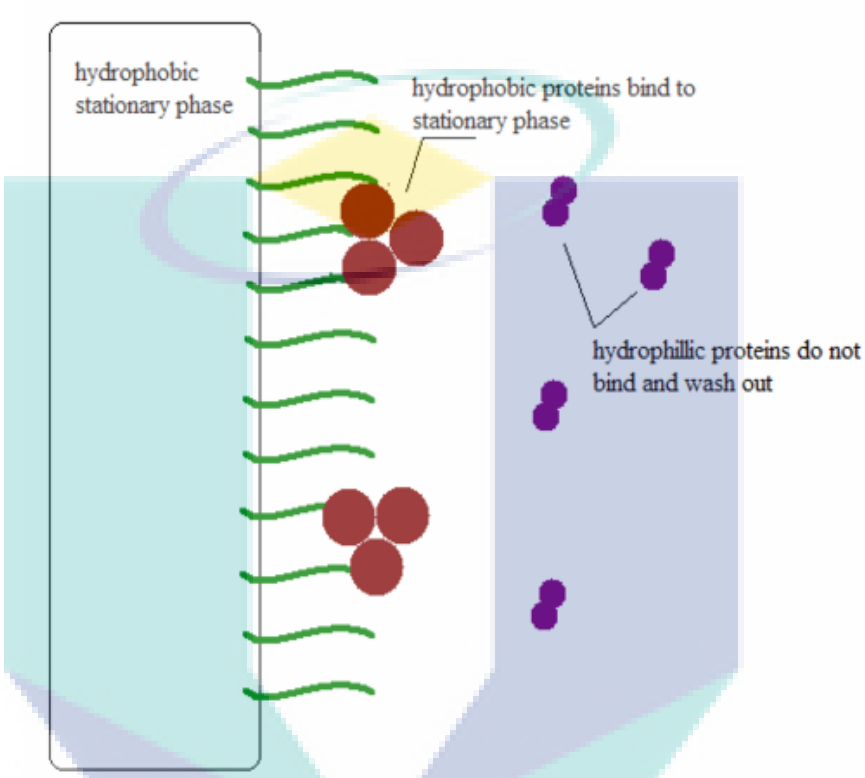


Figure 2.4: RPLC: Proteins with exposed hydrophobic region (red) will be able to bind to the immobilized hydrophobic stationary phase; the rest simply wash off.

(Source: Amersham, 1999; GE Healthcare, 2006)

RPLC involves the use of hydrophobic adsorbents, typically aliphatic chains between C8- and C18-, attached to silica beads. The samples are applied in an aqueous solvent, often a dilute acid, and are eluted by gradient of miscible organic solvent such as methanol or acetonitrile. In general, smaller proteins are resolved earlier and proteins above about 40 kDa may not be readily removed from the column. In many cases, the eluted protein is not fully native state; in fact, it has been proposed that RPLC of proteins takes place by denaturation on adsorption and renaturation on elution (Scopes, 1993).

RPLC has one major positive feature is of very high resolution. That is why it is so widely used, and in particular for peptides which are small, sometimes with no real secondary structure to preserve, thus cannot be denatured in the conventional sense. Consequently, RPLC became very important in the development of purification strategies for many of these proteins (Scopes, 1993). And it is also developed for many proteins with secondary structure.

Petrides (1980) has used an RPLC system in which normal α- and β-chains of human hemoglobin have been separated from several of their respective mutant chains which differ by single amino acid residues only suggests that RPLC is a powerful tool for the separation of medium-sized proteins with minimal structural difference. Three major types of human collagen (Types I, II, and III) (Fallon *et al.*, 1981) and human pro α1(I) and pro α2(I) procollagen chains (Van der Rest *et al.*, 1982) has also been completely separated by RPLC. A rapid and effective separation of the structural polypeptides of poliovirus of strains of all three serological types was developed in a RPLC system. All four virus polypeptides (VP1 to VP4) were obtained quantitatively in high purity by this one-step procedure of RPLC (Heukeshoven and Dernick, 1983). Moreover, 30S subunit of *Escherichia coli* ribosomes (Kerlavage *et al.*, 1982), the histones of *Physarum polycephalum* (Hallenbeck and Mueller, 1984) and individual neurofilament proteins (210,000, 160,000, and 70,000 Da) from the glial fibrillary acid protein (Hui *et al.*, 1986) has been purified by RPLC. These results showed that RPLC is a powerful technique for purification of proteins. Some enzymes also obtained an excellent separation by RPLC. Intracellular phospholipase A2 was purified to homogenity from rat spleen supernatant by RPLC. The recovery of the enzyme activity was greater than 70% with an about 23,000-fold purification. The solvent system did not affect the catalytic properties of the enzyme (Tojo *et al.*, 1984). The vasculitis autoantigen proteinase 3 was purified from neutrophil primary granules using RPLC. It was shown to be free of important contaminants, was enzymatically active and was antigenic to sera containing antineutrophil cytoplasmic antibodies with a cytoplasmic pattern (C-ANCA) by indirect immunofluorescence (Gaskin *et al.*, 1995). All this research indicates that RPLC is of great potential to be used in the separation of proteins and enzymes. The advantages of high resolution and time conserving of RPLC may lead

to a scalable use in the research or industry.

2.2.2 Stages in RPLC

2.2.2.1 Mobile phase conditions (solvent)

Mixtures of water or aqueous buffers and organic solvents are used in RPLC. The solvents must be miscible with water, and the most common organic solvents used are acetonitrile, methanol, tetrahydrofuran (THF), ethanol and 2-propanol. The polarity of the initial mobile phase must be low enough to dissolve the partially hydrophobic solute yet high enough to ensure binding of the solute to the stationary phase. The organic solvent is added to lower the polarity of the aqueous mobile phase. The lower the polarity of the mobile phase, the greater its eluting strength in RPLC. Elution can be performed isocratically (the water-solvent composition does not change during the separation process) or by using a gradient solution (the water-solvent composition changes during the separation process, usually by decreasing the polarity). (McCormick and Karger, 1980; Queiroz *et al.*, 2001) Briefly, A low-polar solvent (buffer B: ethanol) is then usually mixed with a high polar solvent or aqueous solution (buffer A: water) in slowly increasing proportion. The proportion of this mixture is usually represented as a percentage of the buffer mixture derived from buffer B (%B). When the mixture of buffer A and buffer B matches or exceeds the non-polarity of a molecule bound to the stationary phase, the molecule will elute into the buffer flowing over the column at that time. As the buffer flows over the entire column the same molecule will elute at the same point (in the same polarity of solvent). This will elute each molecule individually at its specific polarity and generate a peak of each molecule eluting from the column. Figure 2.5 is illustrating the stages in EBAC process, it including equilibration, sample application, elution, washing and regeneration (GE Healthcare, 2006).

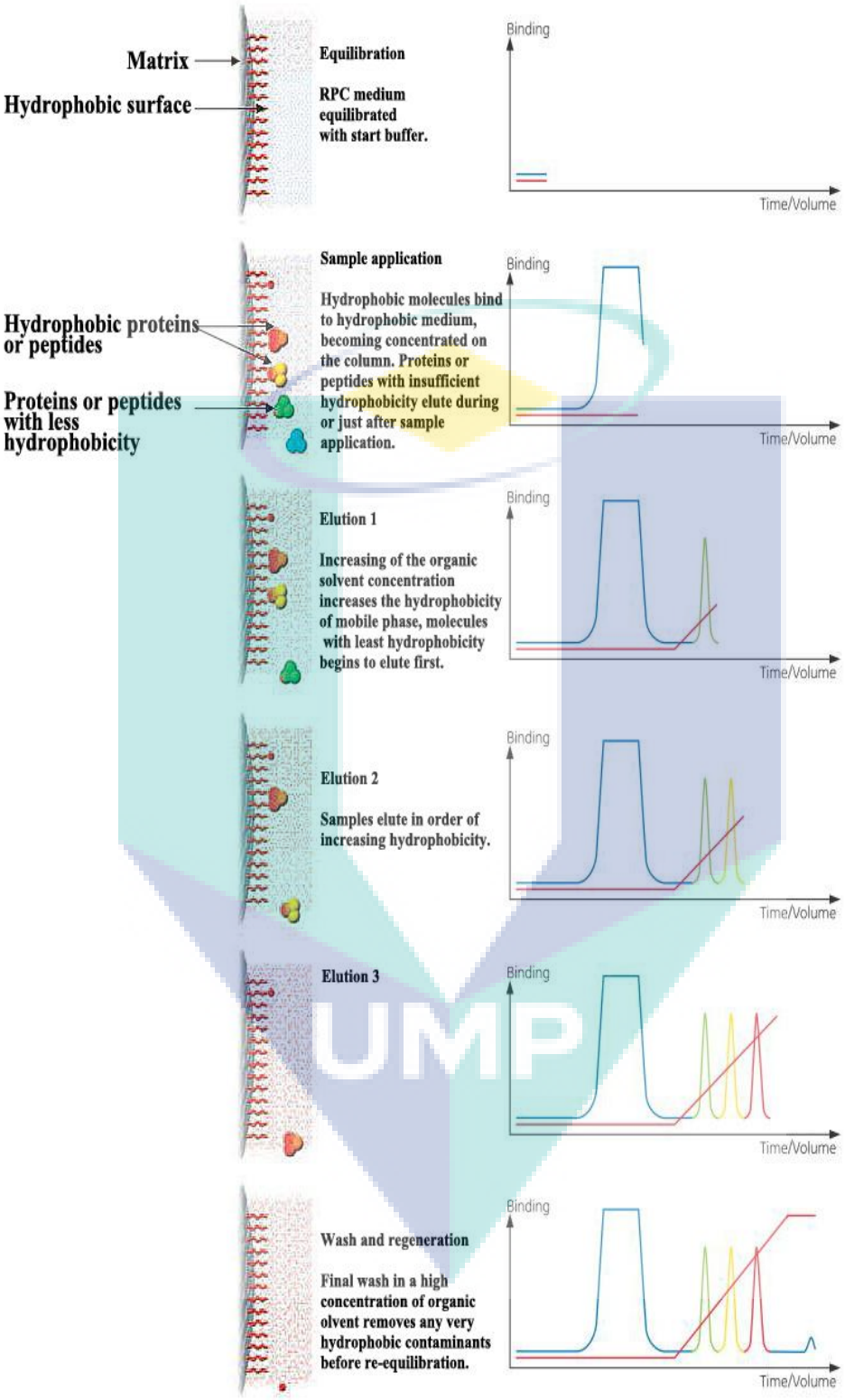


Figure 2.5: Stages in an RPLC separation

(Source: GE Healthcare, 2006)

2.2.2.2 Equilibration

In this step the hydrophobic stationary phase is primed by applying the specific sample buffer such as ethanol, acetonitrile etc. Because the stationary phase is hydrophobic, water molecules tend to be ordered at the junction between the stationary phase and the buffer.

2.2.2.3 Application of the Sample

The sample protein is loaded into the column. Proteins in the mixture that have a high percentage of exposed hydrophobic amino acid residues or with high hydrophobic region will be adsorbed to the stationary phase. Other proteins with insufficient hydrophobicity or with hydrophilicity would not bind to the stationary phase and then flow out.

2.2.2.4 Elution

Elution stage involves changing the buffer conditions to elute the bound hydrophobic protein. Elution begins by increasing the concentration of organic buffer to increase the hydrophobocity or lower the polarity of mobile phase. The higher the hydrophobicity or lower the polarity of the mobile phase, the greater its eluting strength in RPLC. Molecules with the lowest hydrophobicity or highest polarity will elute first. By controlling the increase in organic buffer, molecules are eluted differentially. The order in which proteins are desorbed is usually relative to the number of external hydrophobic residues each protein has. Proteins possess more hydrophobicity would move through the column slower. Those molecules with the highest degree of hydrophobicity will be most strongly retained and eluted last.(Queiroz *et al.*, 2001)

2.2.2.5 Washing and regeneration

This stage involves washing off any remaining protein from the stationary phase and returning the conditions back to the way they were at the start of the process. A wash step removes most of the tightly bound molecules at the end of elution. This

involves creating a more hydrophobic environment by greatly increase the concentration of buffer, such as 100%. The column is then re-equilibrated before the next run (Amersham, 1999).

2.2.3 Amberlite™ XAD7HP

Amberlite™ XAD7HP is an industrial grade non-ionic aliphatic acrylic polymeric adsorbent for removal, immobilization, and recovery of peptides, proteins and low molecular compounds from aqueous solutions. The chemical structure, physical properties and suggested operating condition of Amberlite™ XAD7HP from its manufacture are presented in Figure 2.6 and Table 2.1. The high surface area and aliphatic nature of Amberlite™ XAD7HP surface gives good adsorptive property from its macroreticular structure, containing both a continuous polymer phase and continuous pore phase. Due to the aliphatic characteristic, Amberlite™ XAD7HP can adsorb non-polar compounds from aqueous systems, and can also adsorb polar compounds from non-polar solvents. Excellent physical and thermal stability made the Amberlite™ XAD7HP a good choice for adsorption of peptides and proteins which would otherwise be damaged by the adsorption onto an aromatic adsorbent such as Amberlite™ XAD16 and Amberlite™ XAD1180. Refer to the aliphatic nature, and chemical structure of its adsorbent surface (Figure 2.6), Amberlite™ XAD7HP would be a potential adsorbent applied in RPLC for the adsorption of enzyme in order to keep the enzyme structure and the enzyme activity.

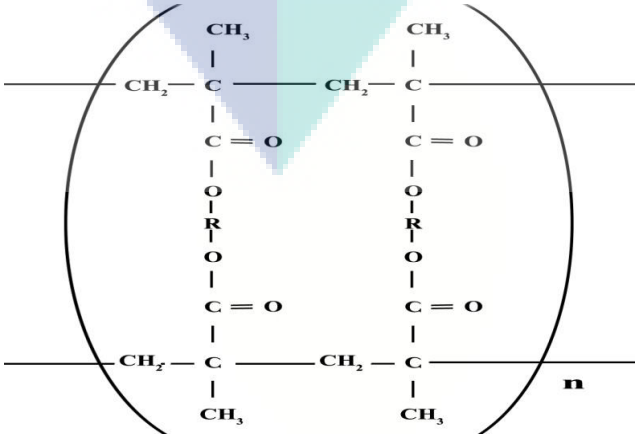


Figure 2.6: The chemical structure of Amberlite™ XAD7HP

Table 2.1: The physical properties and suggested operation conditions of Amberlite™ XAD7HP

Properties and suggested operation conditions of Amberlite™ XAD7HP	
Properties	
Matrix	Macroreticular aliphatic crosslinked polymer
Physical form	White translucent beads
Moisture holding capacity	61-69%
Shipping weight	655 g/L
Specific gravity	1.06 to 1.08
Particle size (harmonic mean size)	0.56-0.71 mm
Uniformity coefficient	≤2.0
Fines content	<0.300 mm:7.0% max
Coarse beads	>1.18 mm :8.0% max
Surface area	≥ 380 m^2/g
Porosity	≥0.5 ml/ml
Suggested operation conditions	
pH range	0-14
Maximum temperature limit	80-100 ℃
Minimum bed depth	75 cm
Flow rate of Loading	2-16 BV/h
Flow rate of displacement	1-4 BV/h
Flow rate of regeneration	1-4 BV/h
Flow rate of rinse	2-16 BV/h

Amberlite XAD7HP resin has been used for product purification in food processing, bioprocessing and pharmaceutical processing etc.. It has demonstrated an excellent ability to be used as a adsorbent for concentrating or removing perfluorochemical as high as 83% (Xiao *et al.*, 2012) and could be used as effective adsorbent for enrichment of phenolics and rosmarinic acid as purity of 58.81% from

initial purity of 16.66% and as purity of 63.16% from initial purity of 10.14%, respectively , from *Rabdosia serra*. (Lin *et al.*, 2012). The direct capture of flavonoids from unclarified Ginkgo leaf extraction solution using EBAC on Amberlite XAD7HP has been evaluated and shown to be an efficient operation in primary recovery (Li and Chase, 2009b). Aminoalkylation with 1, 2-diaminoethane followed by gluteraldehyde activation of Amberlite^{TM} XAD7HP allows binding of enzymes such as penicillin G acylase and glutaryl-7-ACA, and sodium hydroxide in 15% isopropanol followed by trichlorotriazine treated Amberlite^{TM} XAD7HP also provides a good support for immobilized pectinylase activity and stability (Fisher, 2008). Matsumoto *et al.* (2001) successfully isolated four components of anthocyanin of D3R, D3G, C3R, and C3G from black currant anthocyanin as fine crystals of flavylium chloride at >99.5% purity and the recovery yields were 47.8%, 153%, 50.7%, and 310% respectively, which combining the use of Amberlite XAD-7HP adsorption with acetate and phosphoric acid buffer and other treatments.

2.3 EXPANDED BED ADSORPTION CHROMATOGRAPHY (EBAC)

Many bioproducts from the biotechnological industry are proteins, which are of great interest because of their enzymatic activities, specific recognition interactions or other therapeutic actions. Such proteins may be synthesized in a variety of biological systems, however, proteins for most applications have to possess high degrees of purity. Purification schemes frequently contain an extensive sequence of separating steps that adds greatly to the overall production costs, and can result in significant loss of product (Chase, 1994). The running cost of this typical purification scheme is high, often contributes about 50–80% of the total production cost (Clonis, 2006). As there is a growing demand for therapeutic bioproducts in the market, such as recombinant proteins and enzymes, hence considerations on the total cost in downstream processing, purification and final polishing steps of the bioproducts are of great importance. In view of these, there has been a growing interest in new biotech among the manufacturers to develop an innovative separation and purification methods that are not only cost effective but also viable in preserving the biological activity of proteins, as well as fulfilling the stringent quality imposed on the therapeutic bioproducts. Hence, nowadays, development of an industry-desired procedure of purification of protein is very

important. Thus, a powerful method called expanded bed adsorption chromatography (EBAC) is extensively investigating to separate or purify biological products from different sources. It has been proved a very efficient purification process integrates the clarification, concentration and purification in one unit operation with many advantages such as generates robust, easy to scale up and biocompatible (Chase and Draeger, 1992; Chase, 1994).

2.3.1 Principles of EBAC

Traditionally, adsorption chromatography is often addressed as a conventional packed bed adsorption chromatography (PBAC). Although PBAC allows highly selective separation and high recovery of the target molecules, it requires critically removal of solid impurities from the feedstock before its feeding as these could result in severe operational problems as a consequence of the trapping of particulate matter in the voids of the bed (Chase and Draeger, 1992). An alternative solution to the conventional PBAC is the expanded bed adsorption chromatography (EBAC) (Ng *et al.*, 2007) which allows the direct extraction of target substance from large volume of crude feedstock and the particulates can pass through the voids of bed without being trapped. EBAC is the successful hybrid of the conventional PBAC and fluidized beds chromatography (Chase, 1994; Anspach *et al.*, 1999). There are two distinct features that separate EBAC from the PBAC, which are the liquid inlet and the top adapter in EBAC column. Figure 2.7 shows a schematic diagram of a typical EBAC column.

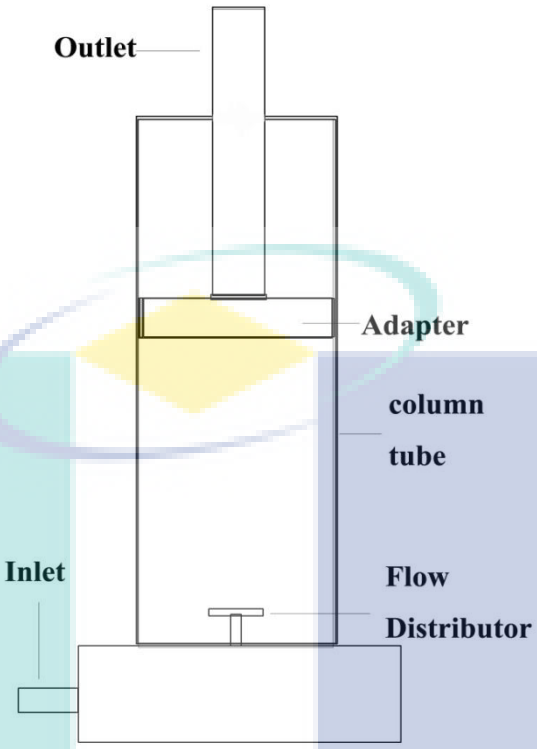


Figure 2.7: A schematic diagram of an EBAC column. Unclarified feedstock is pump into the column via the column inlet and leaves the column via the column outlet

Inlet of the feedstock is located at the base of the column, it allows the feedstock was pumped into the column in a upward direction. The column is equipped with suitable flow distributors designed to produce a stable plug flow. With these systems, higher linear flow rates and stable expansion characteristics can be obtained (Belter *et al.*, 1973). When an upward flow is applied to the column, the smaller and larger particles populate the upper and lower part of the column, respectively, forming a stable fluidized bed, mimicking the packed bed but with higher bed voidage (Ng *et al.*, 2007). Same time, the bed is not constrained by the presence of an upper flow adapter (Chase, 1994), in addition to that, the top adapter of the column is adjustable, thus allows the column to be operated at different bed heights, which is necessary during the different stages of the operation (Tong *et al.*, 2002).

The bed can be expanded and spaces open up between the adsorbent beads which allows the particulates can pass freely through the spaces in the bed. A minimum liquid velocity is needed before the bed starts to expand, thereafter, the gaps between the adsorbent beads widen as the liquid velocity increases, the voidage of the bed increases. When the bed has increased in height to approximately twice its settled height, or more, particulates pass freely through the bed, whilst the adsorbent is retained (Chase, 1994). The operational stages of EBAC system is illustrated in Figure 2.8.

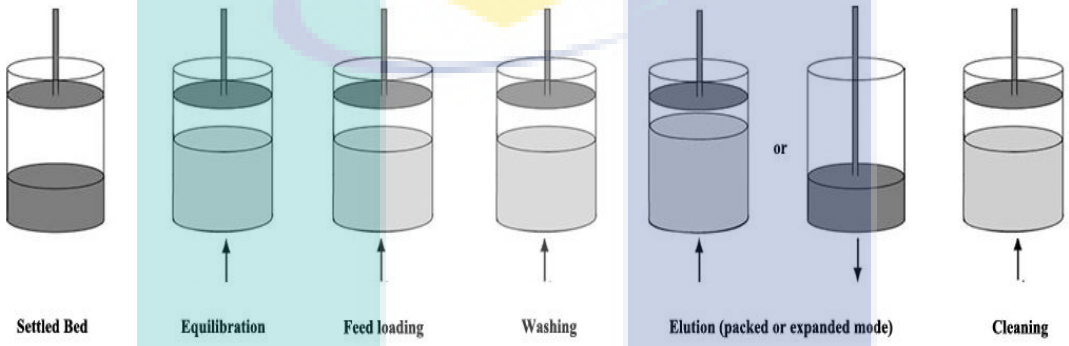


Figure 2.8: The operational stages of EBAC system

The ability to feed particulate-containing feedstock directly to the adsorbent bed in EBAC eliminates prior removal of cells and/or cell debris. Usually, application of feedstock is stopped when 10% breakthrough of the target products achieved. This is to prevent wastage of the target product, especially those with high value (Chase, 1994; Özyurt *et al.*, 2002). At the end of the adsorption phase, particulates remaining in the voids of the bed can be washed away, whilst maintaining the bed in an expanded mode (Fee and Liten, 2001). Adsorbed target products can then be eluted from the bed, which may be in either an expanded or packed mode. During the EBAC operation, matrix is exposed to various contaminants and foulants such as cell debris, proteins, nucleic acids etc., which will compromised the subsequent performance of column. Decontamination and regeneration of the adsorbents after each purification cycle are necessary to restore the original hydrodynamic and chromatographic properties of the adsorbents. Therefore cleaning-in-place protocols (CIP) are mandatory in EBAC after elution was carried out and usually this 'cleaning' stage is performed in expanded mode (Anspach *et al.*, 1999).

The use of expanded beds shows great potential for simplifying the downstream-processing for the recovery of a wide variety of proteins, with concomitant savings in equipment and operating costs.

2.3.2 Important factors on EBAC performance

The adsorption efficiency of the EBAC is critically dependent on the complex nature of the feedstock, as it could interfere with the hydrodynamic behavior of the adsorbent bed (Anspach *et al.*, 1999; Fernández-Lahore *et al.*, 2000; Ng *et al.*, 2008). And it also dependent on the superficial velocity used (Johansson *et al.*, 1996).

In EBAC, high flow velocity is usually employed, hence reduces the processing time and significantly increase productivity of purification cycle (Chang *et al.*, 1998). Furthermore, high flow velocity could reduce the mass transfer resistance of the compound of interest at the surface of the adsorbents, thus improving the system productivity (Güzeltunç and Ö. Ülgen, 2001). However, high flow velocity could simultaneously decrease protein residence time in the column, which could lead to early breakthrough and reduce the adsorption efficiency compared with that exhibited using lower flow velocity (Raymond *et al.*, 1998). It was shown that, a reduction in adsorption capacity at high flow rate is attributed to restrictions in pore diffusion (Chang and Chase, 1996).

Presence of the solids and other colloidal component such as DNA in the crude feedstock could influence the hydrodynamic behaviour of the bed (Theodossiou and Thomas, 2002; Lin *et al.*, 2003; Valdés *et al.*, 2003; Lin *et al.*, 2004; Vergnault *et al.*, 2004). Non-specific interaction between the cell debris with the solid stationary phase, will lead to aggregation of cells and adsorbents, which in turn deteriorates the bed stability and protein adsorption efficiency (Fernández-Lahore *et al.*, 1999; Feuser *et al.*, 1999; Fernández-Lahore *et al.*, 2000). In addition, adhesion of cells onto the surface of the solid stationary phase and into the pores will also decrease the static binding capacity of the stationary phase (Chase and Draeger, 1992; Zhang *et al.*, 2001).

Apart from that, high viscosity encountered in most of the feedstocks also plays a

major role in hydrodynamic performance of the EBAC (Lin *et al.*, 2003). Loading of various feedstocks could lead to uneven distribution of feedstock, muti-channeling as well as high axial mixing, and subsequently collapse of the bed (Fee, 2001; Bermejo *et al.*, 2003). In addition, viscous feedstock could also lower intraparticle protein diffusion, thus cause inefficient protein adsorption (Chang and Chase, 1996). Therefore, dilution of the feedstock is necessary in order to overcome the problem (Anspach *et al.*, 1999). It was shown that high flow rate used during application of less viscous feedstock, could reduce mass transfer resistance, hence increased protein adsorption (Bermejo *et al.*, 2003). Alternatively, addition of nuclease or spermin to remove DNA could also aid in reducing feedstock viscosity (Nayak *et al.*, 2001; Choe *et al.*, 2002).

2.3.3 The purification of proteins by EBAC

More recently, considerable interests being shown in the use of EBAC for the direct extraction of proteins from crude plant feedstock (Somers *et al.*, 1989; Gailliot *et al.*, 1990; Chase, 1994). Matrices and ligands are critical for the adsorption of protein on the adsorbent. The matrices used in EBAC are direct descendants of PBAC matrices. In contrast to the normal PBAC adsorbents, these adsorbents are incorporated with various composites such as quartz (Streamline, Amersham Pharmacia, Sweden), glass (Upfront Chromatography, Denmark) and ceramic (Biosepra, Marlborough, MA, USA). EBAC matrices are also available with various ligands and have been successfully used in many kinds of capture chromatography such as Ion-exchange (Kalil *et al.*, 2005), affinity (Zhou *et al.*, 2004; Tan *et al.*, 2006) and hydrophobic interaction (Smith *et al.*, 2002) chromatography, which has been extensively studied in direct recovery of desired compound from various sources of particulate-containing feedstocks. Ion exchangers are robust and relatively cheap adsorbent to use (Anspach *et al.*, 1999). In 2006, Bermejo *et al.* used EBAC to purify C-phycocyanin and allophycocyanin from microalgae *Spirulina platensis* with Streamline-DEAE. The yield of the overall purification process was 9.6% for C-phycocyanin and 9.5% for allophycocyanin which are higher than the value (around 1%) which without use of EBAC. Purification of C-phycocyanin from the cyanobacteria *Synechocystis aquatilis* has also been developed in EBAC with Streamline- DEAE, the yield of the EBAC step is in the range of 90–93% (Ramos *et al.*, 2011). B-phycoerythrin is a major light-harvesting phycobiliprotein in

some marine algae which was large-scale recovered by EBAC from the algae *Porphyridium cruentum* using Streamline-DEAE. The yield was in a high range of 71–78% (Bermejo *et al.*, 2007). Other ion exchangers also are used with a good performance. For instance, Amberlite 410 ion-exchange resin for the adsorption of amylases from maize malt was studied using a EBAC, which shows a good recovering of amylases from maize malt (Biazus *et al.*, 2006). β-1, 4-Xylanase, produced extracellularly by *Bacillus amyloliquefaciens* MIR 32, was isolated directly from the culture broth by adsorption on a cation exchanger, Amberlite IRC-50, in EBAC. The enzyme was eluted from the adsorbent by increase in pH, with a recovery of 82.3% and purification factor of 5.3. About 99.99% of the colony forming units, 82% of the contaminating neutral protease activity, and 100% of the reducing sugars present in the crude feedstock were removed at the end of the purification cycle.

Instances aforementioned shown that EBAC by ion-exchange chromatography maintains a high protein recovery while reducing both processing costs and times. However, the binding capacity of ion exchangers are greatly influenced by the presence of cells/cell debris and colloidal materials such as nucleic acids in the feedstock, particularly when ion exchangers are used (Theodossiou and Thomas, 2002; Vergnault *et al.*, 2004). Their lack of specificity and negative charges of many cell surfaces and other colloidal materials such nucleic acids could lead to aggregation of the adsorbents within the bed, which eventually cause higher back-mixing and unstable bed (Frej *et al.*, 1997; Theodossiou and Thomas, 2002). Nevertheless, besides ion exchangers, many other adsorbents also successfully employed in the EBAC, such as affinity adsorbent, hydrophobic adsorbent etc. Native potato proteins consisting mainly of protease inhibitors and patatin which possessing esterase activity were isolated from industrial potato fruit water and crude juice of potato tubers respectively by EBAC with a heterofunctional adsorbent ligand (mixed mode affinity resins, where the binding depends primarily on the pH, and is almost independent of the ionic strength) (Strætkvern *et al.*, 1998; Løkra *et al.*, 2008).

Using of hydropobic ligands in the EBAC for the purification of protein draws a great attention due to characteristic of protein which with the hydrophobic region on the surface. Hydrophobic adsorbent Streamline Phenyl in EBAC for fast and efficient

purification of Ca^{2+}-binding protein, α-lactalbumin, from milk was developed based on the unique property of removal of Ca^{2+} undergo a significant conformation change rendering them more hydrophobic, results in a reduction of the number of chromatographic purification steps (Noppe *et al.*, 1999). A direct recovery of recombinant nucleocapsid protein of Nipah virus (NCp-NiV) from crude *Escherichia coli* (*E. coli*) homogenate using a hydrophobic interaction with Streamline phenyl in EBAC was developed successfully. The adsorbed NCp-NiV was eluted with the buffer containing a step gradient of salt concentration. The purification has recovered 80% of NCp-NiV from unclarified *E. coli* homogenate with a purification factor of 12.5 (Chong *et al.*, 2010). Two lactic acid bacterium bacteriocins were isolated from fermentation medium through EBAC using a hydrophobic adsorbent, in which optimal adsorption was obtained in a medium of ammonium sulphate for amylovorin L471 of 94.9% and enterocin RZS C5 of 75.0%, and elution with 50% ethanol, buffered at pH 6.0, resulted in an optimal total recovery of the bacteriocin activity of 47.6 and 57.6%, respectively. The highest fold purification expressed as the increase in specific activity (AU/mg) corresponded to the highest recovery, being 140- and 1677-fold, respectively. Nevertheless, a total recovery of only 25.6% with an increase of the specific activity of 121 times was obtained after conventional isolation of ammonium sulphate precipitation (Moreno *et al.*, 2001). These processes demonstrated the feasibility and capability of EBAC with different kinds of adsorbents in the use of recovering potentially valuable proteins from plant biomasses.

EBAC has been demonstrated to be able to process larger volume of feedstock (Hjorth, 1997) from various sources such as *E.coli* homogenates, alga, yeast homogenates, mammalian cell culture, and plant extract. The performance of EBAC in purification of various feedstock sources are summarized in Table 2.2.

Table 2.2: Bioproducts purified from the EBAC

Adsorbent	Product	Source	Buffer system	Purification factor	Yield %	Reference
Streamline SP/ SP XL	Nattokinase	*Bacillus subtilis*	Sodium phosphate buffer	8.2	95	(Hu *et al*., 2000)
Streamline phenyl/Streamline Q(-sephatose)	Phycoerythrin	Red alga *Gracilaria verrucosa*	Ammonium sulfate	/	/	(Wang, 2002)
Streamline recombinant protein A	Recombinant antibody specific to hepatitis	Transgenic tabacco plant extract	Phosphate-buffered saline/ citric acid	24	60	(Valdés *et al*., 2003)
001×7 Styrene-DVB	Ephedrine hydrochloride	Ephedra herb	HCl/phosphate buffer	22	86	(Chen *et al*., 2004)
Streamline SP	Ilunilase	*K. marxianus* extracellular protein		10.4	74	(Kalil *et al*., 2005)
Streamline SP/Streamline Direct HST1	β-glucosidase	*Pichia pastoris*	Sodium acetate buffer	4.1	74	(Charoenrat *et al*., 2006)

Table 2.2: continued

Streamline DEAE	B-hycoerythrin	Microalga *P. cruentum*	Acetic acid/sodium acetate buffer	/	66	(Bermejo *et al*., 2006)
Amberlite IRA 410	Amylases	Maize malt	Phosphate buffer	564	/	(Biazus *et al*., 2006)
Amberlite IRA 410	α-amylases	*Aspergillus niger*	Phosphate buffer	8.45	4.7	(Toledo *et al*., 2007)
Streamline Phenyl-Sepharose/ Q-Sepharose	C-phycocyanin	*Spirulina platensis*	Ammonium sulfate	/	/	(Niu *et al*., 2007)
Amberlite XAD7HP	Flavonoids	*Ginkgo biloba.L*	Ethanol/deionized water	17	39.9	(Li and Chase, 2009b)
Amberlite IRA 410	Alkaline lipase	*Pseudomona cepacia*	Phosphate buffer	77.7	/	(da Silva Padilha *et al*., 2009)
Streamline SP	Ilunilase	*K. marxianus* extracellular protein	sodium chloride/sodium acetate buffer	10.4	74	(Kalil *et al*., 2005)

CHAPTER 3

MATERIALS AND METHODS

3.1 ADSORBENT AND CHEMICALS

Amberlite™ XAD7HP (Amberlite, Japan), Folin & Ciocalteus Phenol reagent, casein, L-cystein, L-tyrosine, papain standard (catalog no.: P4762), protein assay agents and chemicals for electrophoresis were purchased from Sigma-Aldrich (USA). Trichloroacetic acid (TCA) was supplied by Merck (Germany). Protein marker used was Precision Plus Protein™ All Blue Standards (#161-0373) from Bio-Rad (USA). All the chemicals used were of analytical grade.

3.2 EQUIPMENTS

Fastline™ 10 EBAC column (Inner diameter 1cm, height 40 cm, maximum expanded height 35 cm) employing localised stirring for fluid distribution was purchased from UpFront Chromatography (Copenhagen, Denmark). A unit of peristaltic pump (520S, Watson Marlow, UK) was used for the feeding of feedstock. Centrifuge (Eppendorf 5810R, Germany) with 6 x 85 ml High-speed fixed-angle rotor: F-34-6-38 was used to do the centrifugation. Viscosity measurement was performed by using the Spindle SC14-18 (rotating at a speed of 200 rpm) of the DV-III Viscometer (Brookfield Eng. Lab., USA). Briefly, sample was loaded into the viscometer cone and the viscosity of the sample was taken as the mean of three measurements.

3.3 PREPARATION OF CRUDE SAMPLES

Unripe green papaya fruits (*Carica papaya*) as shown in Figure 3.1 were collected from a selected papaya tree at a local farm, Gambang, Malaysia. For each

batch of the experiment, papaya was used is collected at the same time and the size is similar. After washed the freshly harvested unripe papaya fruit and the seeds was removed (if any), the pulp was blended using the juice extractor MJ-68M (Panasonic, Japan,http://www.panasonic.co.in/wps/portal/home/products/homeappliances/kitchenappliances/juicermixergrinder/MJ68M). In the batch adsorption process, the juice was collected and applied to centrifugation with 18514.08 kg*m/s^2, 5 ℃， spin for 5 min, and then the supernatant was collected as the feedstock for adsorption. The feedstock was added with L-cystein to make the final concentration of 1 mM, this was done to enhance and stable the papain activity (Homaei *et al.*, 2010). The preparation of the crude sample for the expanded bed adsorption is similar with the batch adsorption only the juice was collected without centrifugation.

Figure 3.1: Unripe green papaya fruits (*Carica papaya*)

3.4 OPERATION OF BATCH ADSORPTION

3.4.1 Batch adsorption

In this study, all the steps in batch adsorption were performed in a 50 ml conical flask and shaked on an orbital shaker at 120 rpm, 25 ℃. The feedstock was prepared and mixed with 5 ml of the binding buffer added with ethanol to obtain the final concentration of 15% (v/v). 1 g adsorbent of Amberlite^{TM} XAD7HP was equilibrated

with the binding buffer for 60 min before adding in 25 ml feedstock. The mixture was then allowed to mix well for binding stage for 15 min. The adsorbents were then washed with 5 ml of the binding buffer to remove unbound proteins and elution was carried out using 5 ml of the elution buffer of 40% ethanol solution.

3.4.2 Optimization of binding and elution conditions

In order to optimize the binding and elution behavior of papain on the Amberlite™ XAD7HP, the screening of ethanol concentration of the binding buffer, binding time and elution buffer were investigated using one-factor-at-a-time (OFAT) method (Montgomery, 1997). The papain was bounded onto the Amberlite adsorbent was performed by subjecting to the binding buffer added with ethanol to obtain the final concentration of 5%, 10%, 15% and 20% (v/v). Subsequently, the time for papain binding was studied in a range of 5-40 min with the optimum 15% (v/v) ethanol binding buffer. Fractions of unbound papain sample were collected and analyzed for the amount of total protein and papain. The amount of total protein was determined by the Bradford assay and the amount of papain was measured by the proteolytic papain activity assay.

Before the elution stage, the loosely bound papain was washed with 5 ml of binding buffer. For optimization of elution conditions, 5 ml of elution buffer with different concentration of ethanol of 0% to 50% were used to elute the bound papain. Eluted papain fractions were collected and analyzed for the amount of total protein and papain. The purity and yield of the papain amount obtained in the purified fractions were calculated as described by Ng *et al*. (2007). The equation is listed in Section 3.7.

3.4.3 Equilibrium of adsorption isotherm

A series of batch adsorption experiments of papain onto Amberlite™ XAD7HP were carried out to determine the equilibrium adsorption isotherm of papain, as modified from Bayramoğlu *et al*. (2002). A range of feedstock prepared in binding buffer which contained papain from 0.045 mg/ml to 0.9 mg/ml were mixed with 1 g of pre-equilibrated adsorbent respectively. The mixture was then allowed to mix well on a shaker for 15 min at 25 ℃. The amount of adsorbed papain was calculated by using the

following equation, which modified from Su *et al.* (2009) and Bayramoğlu *et al*. (2002).

$$Q = \frac{(Ai - Af) * Vs}{m} \quad (3.1)$$

Where Q (mg/g) is the amount of papain was bounded onto the unit amount of the adsorbent; Ai and Af (mg/ml) are the concentration of the papain in the initial and filtrates after binding respectively; Vs (ml) is the volume of the samples was used for the binding; m (g) is the amount of the adsorbent used.

3.5 OPERATION OF EBAC

3.5.1 Expansion characteristic of adsorbent

The expansion characteristics of Amberlite™ XAD7HP adsorbent were investigated in a Fastline™ 10 EBAC column at 25℃. Initial sedimented bed height is 5 cm. The adsorbent bed was expanded using binding buffer (15% ethanol) and the unclarified feedstock suspension (243.15 μg/ml papain with viscosity of 2.68 mPa s) with increasing superficial velocity. The degree of bed expansion corresponding to each velocity was recorded and expressed as a ratio of the height of the expanded bed, H, to the height of settled bed, H_0, thus, the degree of bed expansion is H/H_0. The data was then fitted with the Richardson–Zaki correlation (Asif, 1998):

$$u = u_t \varepsilon^n \quad (3.2)$$

Where u is the superficial velocity (cm/h), u_t is the terminal settling velocity (cm/h), ε is the expanded bed voidage, and, n is the bed expansion index.

3.5.2 Dynamic binding capacity

Dynamic binding capacity was studied in Fastline™ 10 EBAC column loaded with Amberlite™ XAD7HP to a settled bed height of 5 cm. The adsorbent bed was expanded to a degree of 2 with binding buffer at a superficial velocity of 1066.5 cm/h. Subsequently, the feedstock suspension was loaded at a flow rate of 936 cm/h and

protein fractions were collected and assayed for the papain concentration. The dynamic binding capacity, Q_B (mg of protein adsorbed per ml of settled adsorbent), was calculated as:

$$Q_B = \frac{C_0 V_b}{V_s} \tag{3.3}$$

Where C_0 is the initial concentration of papain in the feedstock suspension (mg/ml), V_b is the volume at 10% breakthrough (ml) and Vs is the settled volume of the adsorbent (ml).

3.5.3 Direct recovery of papain from feedstock suspension using EBAC

The Fastline™ 10 EBAC column was filled with 4 ml of Amberlite™ XAD7HP adsorbent corresponding to a settled bed height of 5 cm. Bed expansion and equilibration was done by 10ml of binding buffer through the mixing chamber and onto the column at a flow rate of 19.35 ml/min (520S peristaltic pump, http://www.watson-marlow.com/en-GB/representatives/search/?query=520s, Watson Marlow, UK, linear flow velocity of 1066.5 cm/h) until a constant bed height was obtained. The bed was allowed to expand to a bed height of 10 cm (corresponding to a two-fold expansion) and the adaptor was positioned at 12 cm prior to the loading phase. 14 ml of the unclarified feedstock suspension of 243.15 µg/ml papain was loaded into the column with flow velocity of 936 cm/h to maintain the bed expansion degree of 2 and then followed by washing with 25 ml 15% ethanol to remove weakly or unbound proteins from the voids of bed. Then 25 ml of 40% ethanol was used as the elution buffer. The elution of papain was also performed in the upwards flow expanded bed mode due to the design of Fastline™ 10 EBAC column without filter and net equipped at the bottom of column. Elution in the expanded mode was also reported with the advantage of avoiding potential problems that is the bed may becomes clogged due to the presence of residual solids in the voids between adsorbent particles if elution is operated in a packed bed mode (Thömmes *et al.*, 1996; Hjorth, 1999; Li and Chase, 2009b). Elution fractions were collected and subjected to protein analysis and quantification. The operational stages of RP-EBAC are referring to Figure 2.8.

3.5.4 Enhancement of papain purity using stepwise elution on EBAC

The adsorption of papain was performed as described in Section 3.5.3 described. Elution was carried out with stepwise elution method. Two-step elution was employed in this study. Screening of optimal elution conditions for bound papain was performed as the first- step elution with 15 ml different concentration of ethanol of 20%, 25%, 30% and 35% to elute respectively, And then followed second-step elution using 25 ml 40% ethanol. After these procedures, best combination of the elution conditions was selected.

3.5.5 Study of Clean-in-place (CIP)

Adsorption of papain was done as described in Section 3.5.3, and stepwise eluted by 25% ethanol and 40% according to the result of optimum elution conditions referred to Section 3.5.4. The CIP process is performed using 100% ethanol as cleaning solvent. This is base on the lowest polarity (highest hydrophibicity) of mobile phase will elute more hydrophobic proteins down from RPLC column. There are two CIP tests were performed. First CIP is performed on the new adsorbent. In the second CIP, the adsorbent which was used in the first CIP was used to perform the adsorption again as the aforementioned procedure. The second CIP was taken in place also with 100% ethanol as the cleaning solvent. Ethanol was used in this process is 40 ml. CIP process is performed by upward flow of velocity 975 cm/h (11rpm), each 5 ml was collected as a fraction. Totally eight fractions were collected and subjected to the protein amount analysis and papain amount analysis

3.6 ANALYTICAL PROCEDURE

3.6.1 Estimation of papain amount

The amount of papain from various samples was estimated by comparing their proteolytic activity value from the colorimetric determination with the help of an internal standard of corresponding papain amount (Figure 3.2). The papain concentration of the internal standards was developed from 0 to 1 mg/ml. The papain activity value was measured by the colorimetric determination using casein as a

substrate by the method as described by Cupp-Enyard (2008). The amount of released tyrosine from casein during the reaction was measured as an absorbance value on the UV-vis spectrophotometer (U-1800, Hitachi, Japan) using a wavelength of 660 nm.

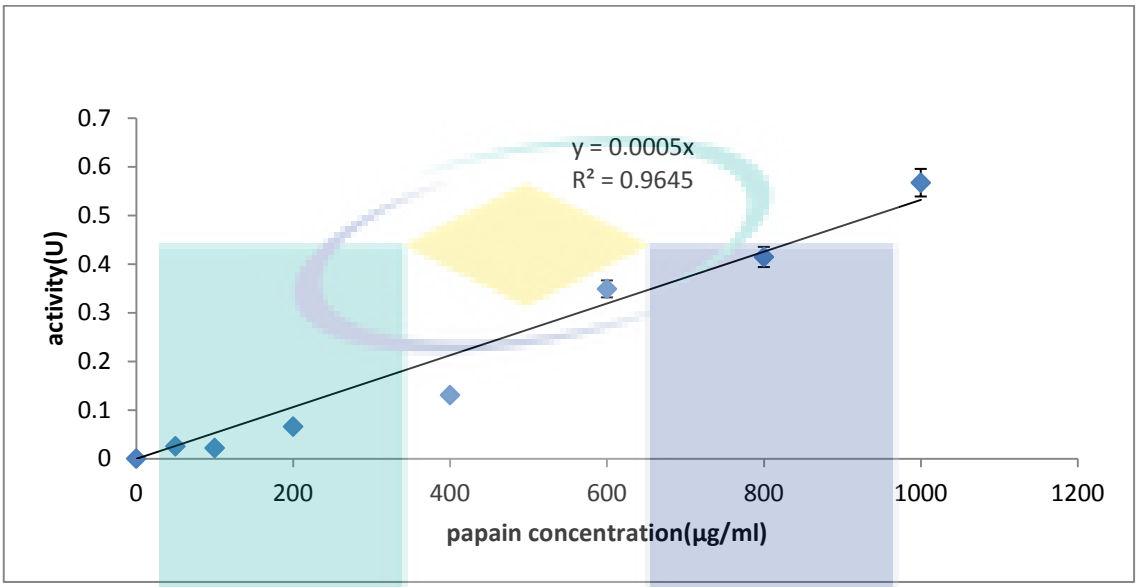


Figure 3.2: Amount of papain corresponding to their enzyme proteolytic activity. The data were obtained as duplicate analysis.

Briefly, 1 ml papain-containing sample was mixed with pre-equilibrated 5 ml 0.65% casein substrate and incubated for exact 10 min; 5 ml of 0.11 M trichloroacetic acid (TCA) solution was added to the mixture to stop the reaction and the mixture was incubated for another 30 min at 37 ℃ for completely stop the reaction. The mixture was filtrated with 0.45 µm polyethersulfone syringe filter and 2 ml filtrates was mixed with 5 ml of sodium carbonate to regulate the pH which may caused by the adding of Folin & Ciocalteus Phenol reagent. Afterwards, 1 ml of 2 N Folin & Ciocalteus Phenol reagent was added to the mixture and incubated for 30 min. Then, 2 ml filtrate of this mixture was transferred to 1 cm path glass cuvettes. The blank of the sample for the spectrophotometer measurement was prepared in the almost same way as aforementioned except the sequence of adding the TCA first and papain-containing sample afterwards to ensure the papain was denatured and no function to hydrolysis the casein. The amount of tyrosine that produced in the papain catalysis reaction was obtained from the absorbance value was compared with tyrosine standard curve. From

the standard curve, the activity of papain can be determined in terms of Units (U). The unit activity was defined as the amount in micromoles of tyrosine equivalents released from casein per min. The papain activity in U/ml sample was obtained from the following equation:

$$A = \frac{M \times 11}{1 \times 10 \times 2} \quad (3.4)$$

Where A is papain activity (U/ml). M is the tyrosine amount which was released in the reaction with the unit of μmol; 11 is total volume (in ml) of assay; 10 is duration of assay (in min) as per the Unit definition; 1 is volume of Enzyme (in ml) of enzyme used; 2 is volume (in ml) used in colorimetric determination.

3.6.2 Bradford assay

The amount of total protein in each sample fraction was quantified using the Bradford Assay. Briefly, 60 μl of protein sample was mixed and incubated with 3 ml Coomassie Brilliant Blue reagent (100 mg Commassie Brilliant Blue , 53 ml absolute ethanol and 100 ml phosphoric acid, top up to 1 L by water) for 5 min at room temperature. The absorbance of the mixture at 595 nm was determined by UV-vis spectrophotometer. The total protein amount was calculated based on the absorbance –concentration standard curve developed using bovine serum albumin (BSA) as a model protein (It was shown as figure D in Appendix in page 78).

3.6.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The collected fractions of papain were subjected to electrophoresis at 200 V on a CSL OmniPAGE Mini Electrophoresis System (Cleaver Scientific Ltd., UK). Acrylamide of 15% (W/V) and 5% (W/V) for resolving gel and stacking gel, respectively, were used in this study. After electrophoresis, the gel were then stained with Coomassie Brilliant Blue R-250 and detained with destaining solution containing 10% (v/v) methanol and 10% (v/v) acetic acid until a clear background was obtained.

3.7 CALCULATIONS

Binding efficiency is defined as the amount of papain was bounded on the adsorbent divided the amount of papain which existed in the initial sample:

$$\text{Binding efficiency (\%)} = \frac{\text{bound amount of papain}}{\text{initial amount of papain}} \times 100\% \quad (3.5)$$

Elution efficiency is regarded as the eluted amount of papain divided the amount of papain which was bounded on the adsorbent:

$$\text{Elution efficiency (\%)} = \frac{\text{eluted amount of papain}}{\text{bound amount of papain}} \times 100\% \quad (3.6)$$

Yield is defined as the amount or activity of papain obtained in elution divided by the initial amount of papain and it is usually expressed in percentage:

$$\text{Yield (\%)} = \frac{\text{eluted amount of papain}}{\text{initial amount of papain}} \times 100\% \quad (3.7)$$

Purification factor (PF) is regarded as the purity of the papain in elution divided by the purity of the papain in the initial sample:

$$\text{Purification factor (PF)} = \frac{\text{purity of papain in elution}}{\text{purity of papain in the initial sample}} \quad (3.8)$$

Purity of papain in the sample is calculated as the following equation:

$$\text{purity} = \frac{\text{papain amount}}{\text{total protein amount}} \times 100\% \quad (3.9)$$

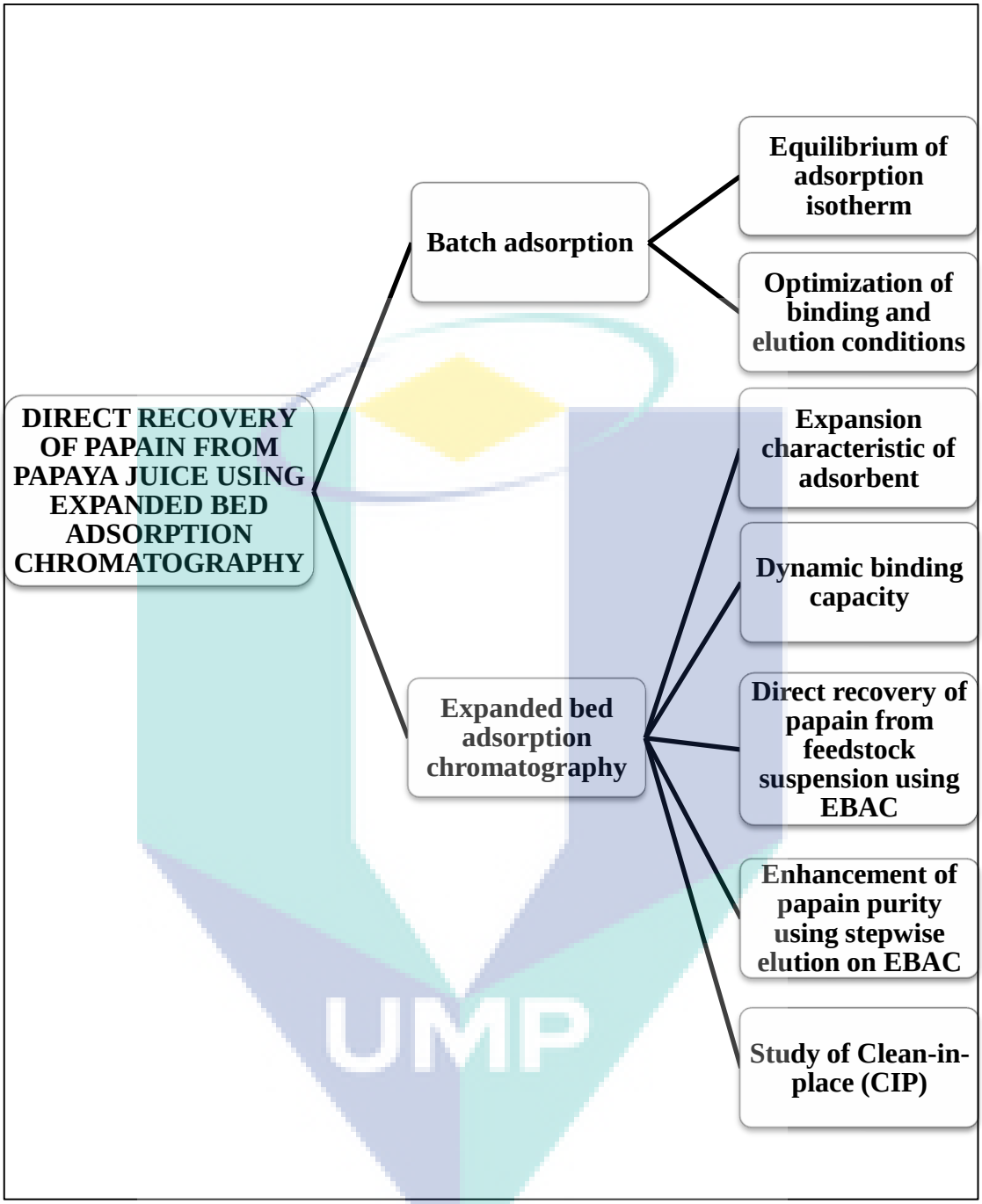


Figure 3.3: Flowchart of the methodologies used in this research

CHAPTER 4

RESULTS AND DISCUSSION

4.1 EFFECT OF ETHANOL CONCENTRATION ON PAPAIN ACTIVITY

Isolation, purification and quantification of biomolecules using RPLC is a common method to separate low molecular weight and highly charged dinucleoside polyphosphates and also for middle and high-molecular weight ampholytic biomolecules like peptides and proteins (Tsao and Otter, 1999; Perera *et al.*, 2012). Water miscible organic solvents (ethanol, 1, 4-dioxane and acetonitrile) in aqueous solution as the eluents in RPLC can modulate on the activity and conformational stability of papain (Simon *et al.*, 1998; Simon *et al.*, 2001). Ethanol was studied as the binding buffer and the eluent in the purification of papain using RPLC because 1, 4-dioxane and acetonotrile can cause toxic effects and high in cost.

Figure 4.1 shows papain exhibited high activity in aqueous ethanol. However, at the ethanol concentration above 50%, the papain activity was decreased. This effect of different concentrations of water miscible organic solvent ethanol was observed to be corresponding with Szabó *et al*. (2006). Replacement of the water molecules associated with the protein structure by organic solvent molecules could well be responsible for reduction in structural stability of papain (Kamphuis *et al.*, 1985). Szabelski *et al*. (2001) observed that the hydrolytic activity and the number of active sites of papain were decreased in aqueous organic solvents (aliphatic alcohols and dimethylformamide). Through the fluorescence and CD spectroscopic measurements performed by Szabó *et al*. (2006), the changes in the catalytic activity of papain in aqueous organic solvents were accompanied by alterations in the secondary and tertiary structures of the enzyme.

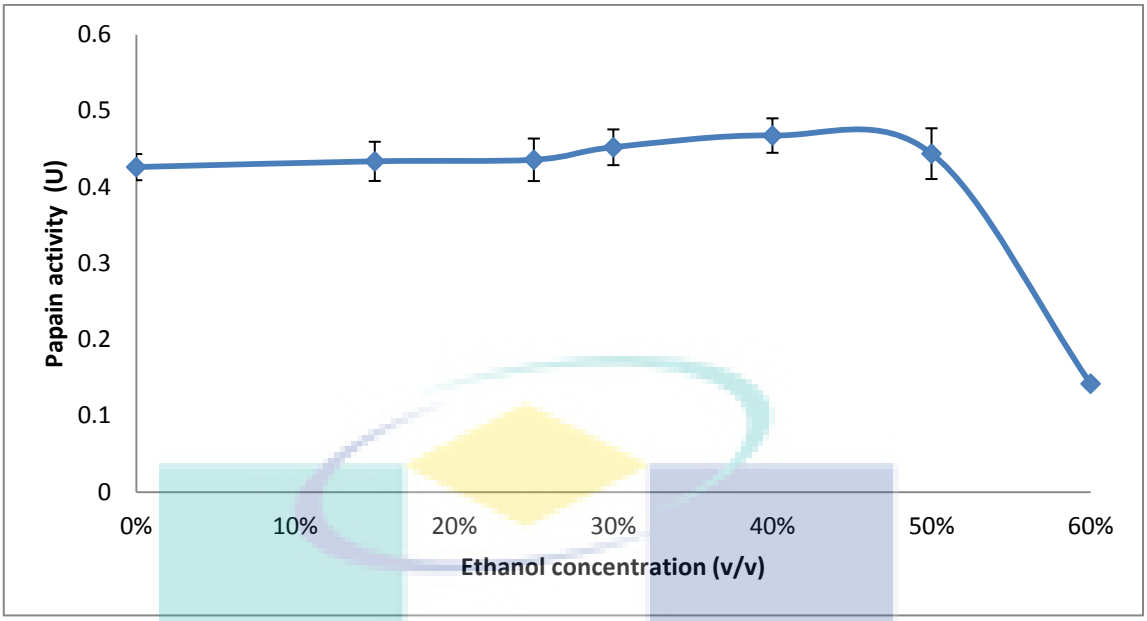


Figure 4.1: Effect of ethanol concentration on the papain activity. Initial activity of papain is 0.4 U at 25 ℃. The data were obtained as duplicate analysis.

4.2 RECOVERY OF PAPAIN FROM CRUDE FEEDSTOCK OF PAPAYA BY BATCH ADSORPTION REVERSED PHASE LIQUID CHROMATOGRAPHY

4.2.1 Optimization of binding and elution conditions

Optimization of binding conditions was carried out to determine the best binding buffer and duration to capture the papain with Amberlite™ XAD7HP from the crude juice feedstock. As shown in Figure 4.2, the highest binding efficiency of papain onto the Amberlite™ XAD7HP (76.53±1.39)% was obtained by using a 15% ethanol solution with adsorption time of 15 min. After the adsorption process, washing with 5 ml of 15% ethanol was carried out to remove the unbound protein. The elution process shown in Figure 4.3 was performed with 5 ml of aqueous ethanol solutions varying from 0% to 50% for 5 min. It is apparent from Figure 4.3 that a 40% aqueous ethanol is suitable for the elution of the majority of the adsorbed papain. In these experiments, the papain, being intermediate polar compound, can be absorbed from aqueous organic solvent onto the Amberlite™ XAD7HP, a moderately polar pacing material and subsequently desorbed by aqueous solutions of ethanol. The high concentration of

ethanol that is needed for elution confirms that there is a relatively strong interaction between Amberlite XAD7HP adsorbent and papain.

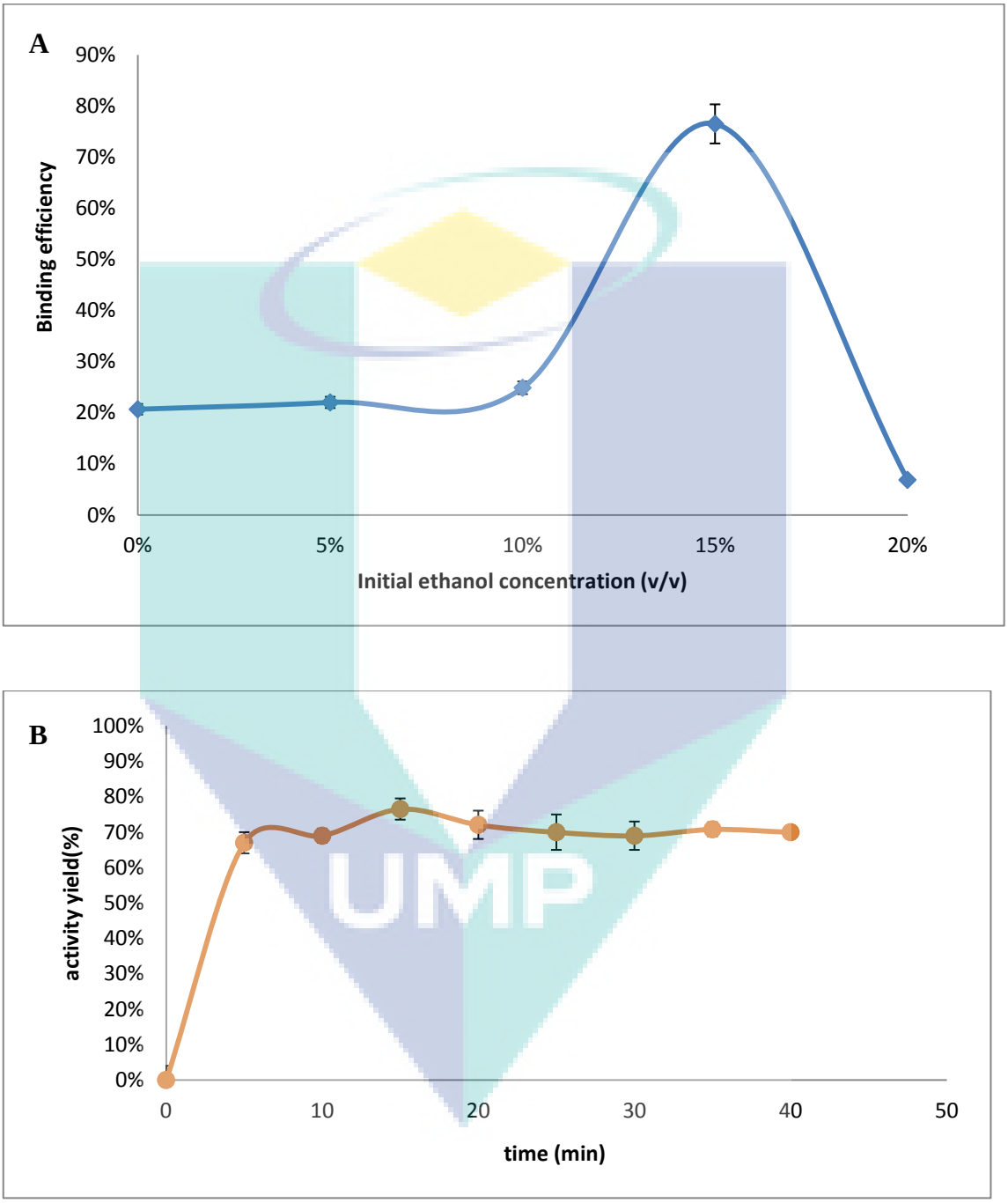


Fig 4.2: Binding efficiency of papain on the Amberlite^{TM} XAD7HP. The (A) profile with the different concentrations of ethanol and (B) the adsorption time with 15% ethanol.

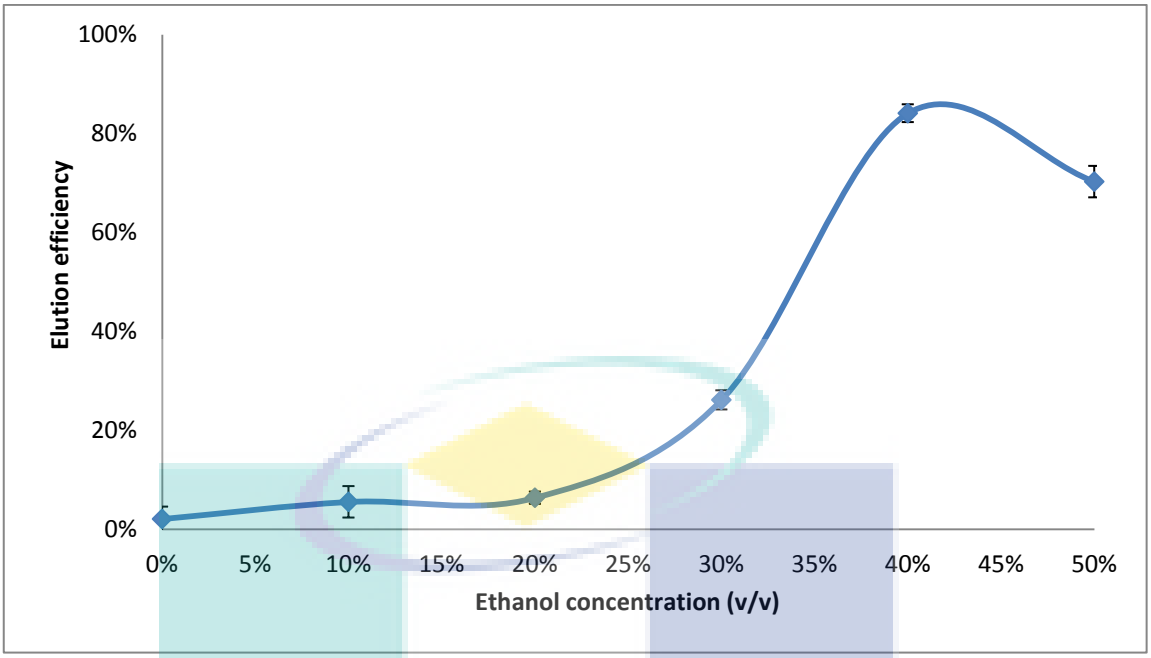


Fig 4.3: Optimization of ethanol concentration in the elution process

4.2.2 Equilibrium and adsorption isotherm

The equilibrium adsorption isotherm of papain activity from papaya juice feedstock onto the Amberlite™ XAD7HP was observed and fitted to the Freundlich isotherm (Bayramoğlu *et al.*, 2002) and Langmuir isotherm as shown in Figure 4.4. The isotherm equations are as following:

Freundlich equation: $$Q = K(C)^n \quad (4.1)$$

Linear form of Freundlich equation: $$\log Q = \log K + n \log C \quad (4.2)$$

Langmuir equation: $$Q = \frac{Q_m C}{k_d + C} \quad (4.3)$$

Linear form of Langmuir equation: $$\frac{1}{Q} = \frac{K_d}{Q_m} * \frac{1}{C} + \frac{1}{Q_m} \quad (4.4)$$

Where C is the equilibrium papain concentration in the sample solution (mg/ml) and Q is the equilibrium adsorption capacity (mg/g); K is the adsorption constant of Freundelich isotherm and n is the Freundlich coefficient. The K and n are the indicators of the adsorption capacity and adsorption intensity, respectively (Chen *et al.*, 2009). Q_m is the maximum adsorption capacity. K_d is the effective dissociation constant. The

ability of the Freundlich model was examined by the plot of log Q versus log C to generate the intercept value of K and the slope of n (Figure 4.4 B). The ability of the Langmuir model was examined by plot of 1/Q versus 1/C to generate the intercept value of $1/Q_m$ and slope of K_d/Q_m, then calculated Q_m and K_d (Figure 4.4 C).

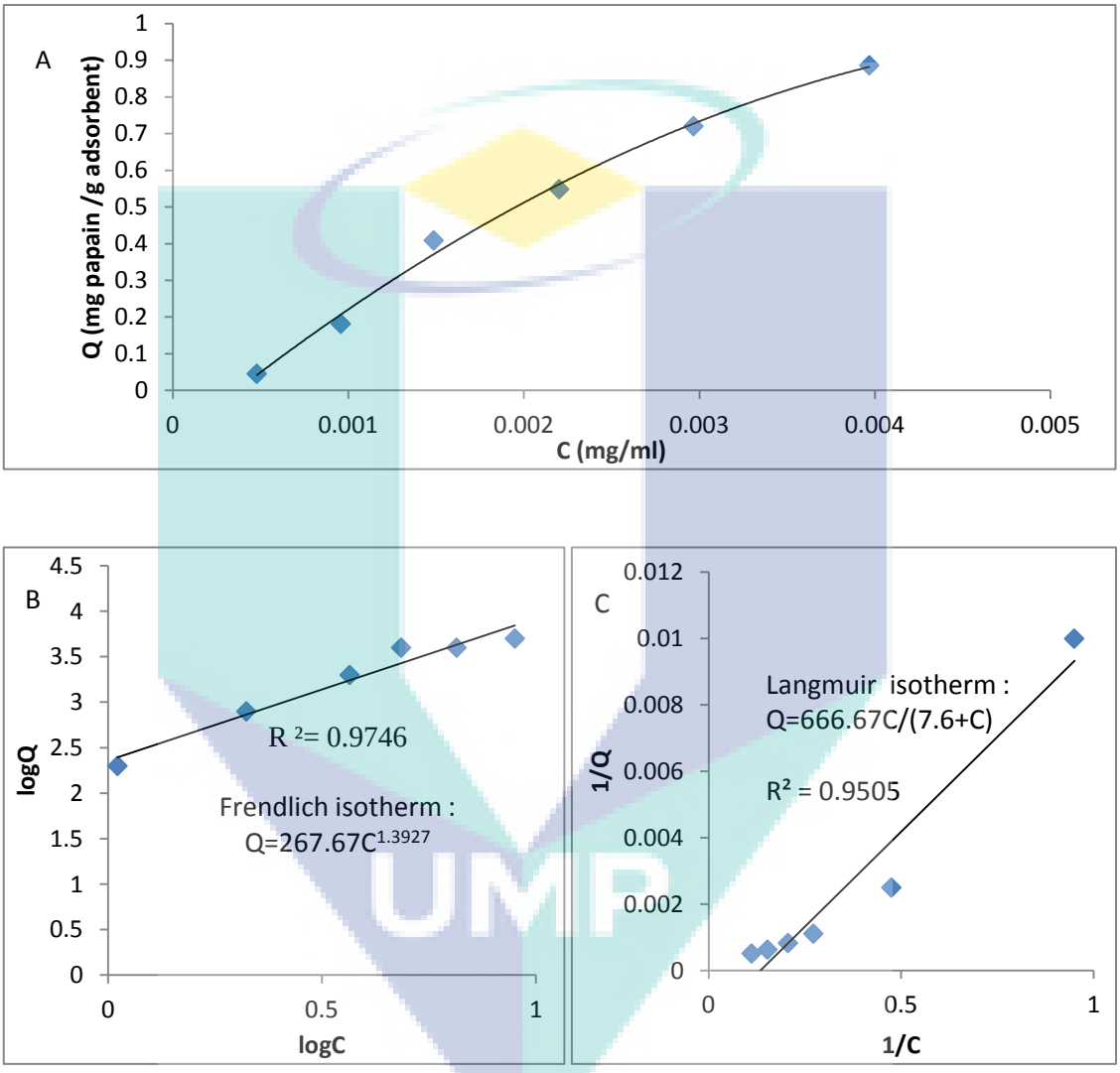


Figure 4.4: Adsorption isotherm of papain activity from papaya juice feedstock on Amberlite XAD7HP. A: Adsorption of papain on Amberlite™ XAD7HP. B: Freundlich isotherm plot of log Q versus log C. C: Langmuir isotherm plot of 1/Q versus 1/C.

The Freundlich isotherm has an n value of 1.3927 and the K of the adsorption of papain activity onto the Amberlite™ XAD7HP was 267.67 (mg/g)(ml/mg)^n, the R^2 is 0.9746. Langmuir isotherm has a Q_m value of 666.67 mg papain/g adsorbent and the K_d

is 7.6, the R^2 for Langmuir equation is 0.9505. Freundlich isotherm has a better linearity that Langmuir isotherm. The adsorption data fits the Freundlich isotherm well. Thus, the adsorption of papain on Amberlite™ XAD7HP can be modeled using Freundlich isotherm. This result of Freundlich isotherm suggests that favorable heterogeneous adsorption of papain from aqueous organic solvent with a high adsorption capacity of Amberlite™ XAD7HP. As seen in Table 4.1, the present study with the higher magnitude of K and n value, indicates an excellent adsorption capacity compared with the other researchers' works (Nie *et al.*, 2007; Nie and Zhu, 2007; Chen *et al.*, 2009).

Table 4.1: Summary of the Freundlich models parameters for papain adsorption isotherms onto different adsorbents

Type of purification method	**Freundlich constants**			**Reference**
	K	n	R^2	
CB F3GA-Chitosan-Nylon affinity membrane, 277 Kelvin	32.02	1.53	0.999	Nie *et al.*, 2007
CB F3GA- Chitosan-Nylon affinity membrane, 298 Kelvin	96.45	1.22	0.995	Nie *et al.*, 2007
CB F3GA- Chitosan-Nylon affinity membrane, 310 Kelvin	137.69	1.13	0.998	Nie *et al.*, 2007
CB F3GA- Chitosan-Nylon affinity membrane	75.4	1.2	0.996	Nie and Zhu, 2007
Red 120- Chitosan-Nylon affinity membrane	74.34	1.07	0.997	Chen *et al.*, 2009
Brown 10- Chitosan-Nylon affinity membrane	55.87	1.06	0.999	Chen *et al.*, 2009
RPLC, Amberlite™ XAD7HP	267.67	1.39	0.974	Present study

4.2.3 Operation of batch adsorption

A performance summary of the optimized batch adsorption reverse phase liquid chromatography, including activity, amount, efficiency during the initial, binding, washing and elution stage, and purification factor of papain in the elution experiments is presented in Table 4.2. It can be seen from Table 4.2 that the binding efficiency of

papain amount on the Amberlite™ XAD7HP is 78% and elution efficiency is 84%, hence, the efficiency of papain purification from papaya juice using the method was developed in this study is around 65.5%. The papain purity in the initial sample was 13.9%, after purification the purity was increased to 60.6%, almost 4.3 times of initial purity. In the elution stage 84% papain amount which adsorbed on the adsorbent was eluted. A papain yield of 65.6% and a purification factor of 4.3 was recovered from the optimized elution condition.

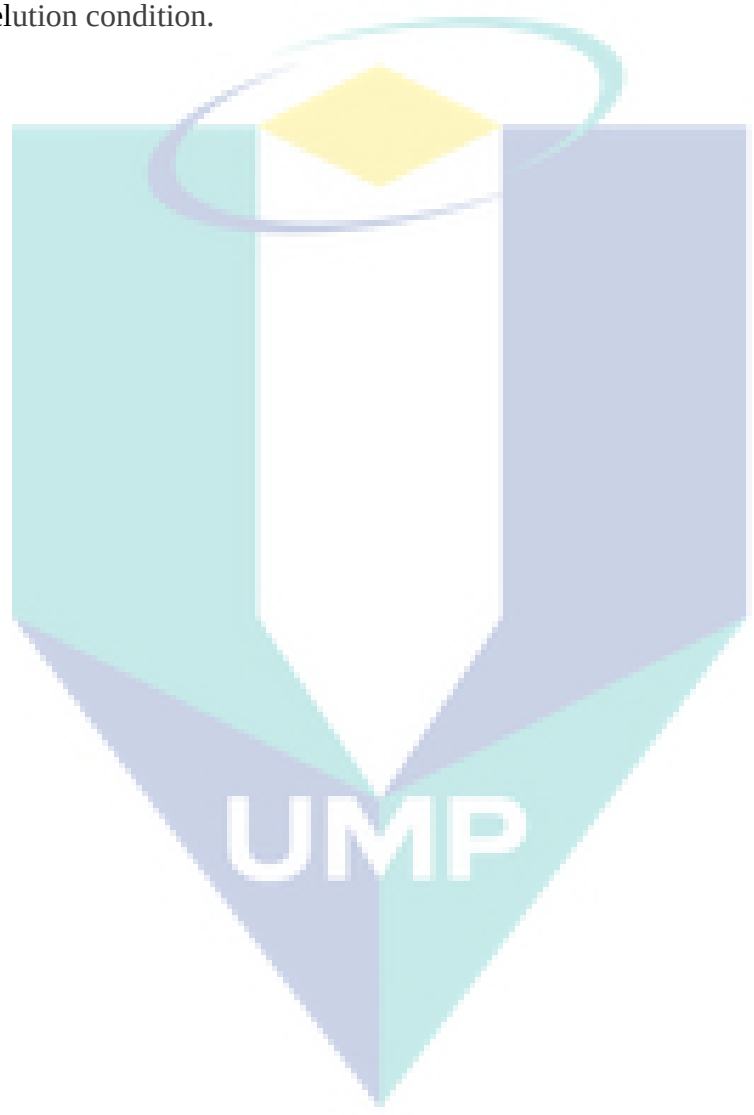


Table 4.2: Summary of the results, include of activity, amount, efficiency during the initial, binding, washing and elution stage, and purification factor of papain. The data were obtained as duplicate analysis.

Stage	Initial	Binding	Washing 1	Washing 2	Elution
Sample volume(ml)	25		5	5	5
Total papain activity (U)	3.466 ±0.168	2.705 ±0.125	0.048 ±0.002	0.023 ±0.002	2.273 ±0.019
Activity concentra tion (U/ml)	0.139 ±0.007		0.01 ±0.0005	0.005 ±0.0004	0.455 ±0.004
Papain amount(µg)	6931.818 ±336.32	5409.091 ±49.362	95.455 ±4.885	45.455 ±3.6	4545.455 ±37.541
Papain concentration (µg /ml)	277.273 ±13.453		19.091 ±0.977	9.091 ±0.720	909.091 ±7.508
Total protein amount(µg)	49704.545 ±136.536				7497.658 ±166.205
Purity（%）	13.9 ±0.6				60.6 ±1.8
Papain yield（%）					65.6 ±3.7
Purification factor					4.3 ±0.332
Binding efficiency（%）		78 ±4.5			
Elution efficiency（%）					84 ±0.1

The SDS-PAGE analysis (Figure 4.5) was carried on a 15% polyacrylamide gel. The lane was loaded with the papain fractions collected from the crude sample, binding, washing and elution stage of Amberlite^{TM} XAD7HP and the standard. The papain amount of the crude feedstock was 11.6%, and the papain amount in the elution fraction reached 50.5%. The purity factor of the papain was as good as 4.3. As shown in Figure 4.5, the band of the purified papain (Lane 2 and 3) matched well with standard (Lane 7), which was found to be around 23 kDa, the same molecular weight as papain (Drenth *et al.*, 1971). In this work, a direct capture of papain from papaya juice extraction solution using batch adsorption on Amberlite XAD7HP has been evaluated and shown to be an efficient scalable operation for primary recovery. Moreover, the enzyme activity of the purified papain from the batch adsorption operation was still preserved as demonstrated by the proteolytic activity value from the colorimetric determination using casein.

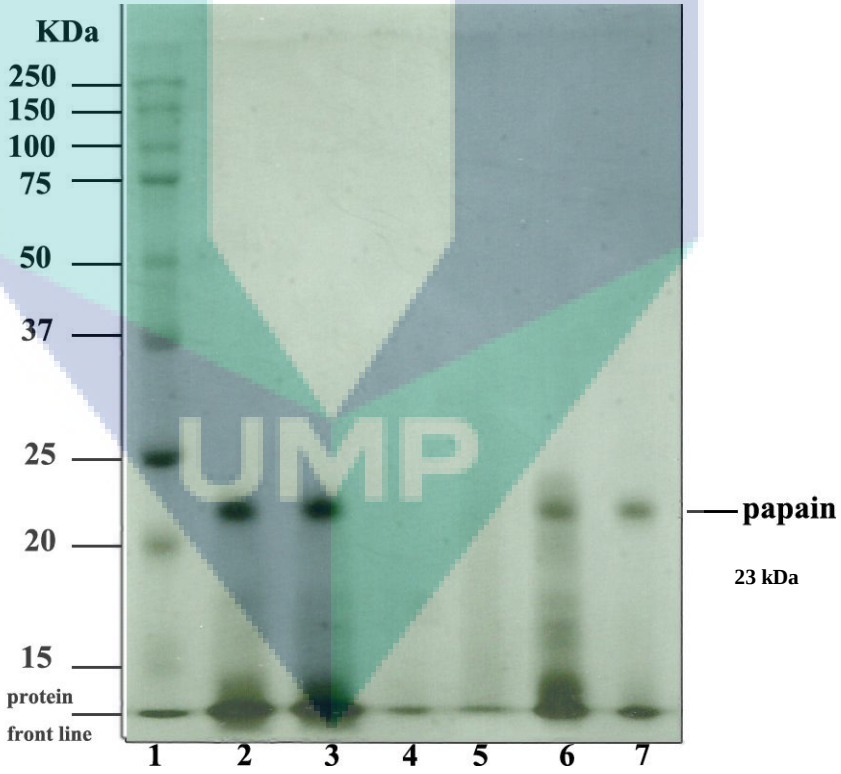


Figure 4.5: Graphical illustration of SDS-PAGE of the protein fractions collected in optimized batch adsorption reverse phase liquid chromatography. Lane 1； molecular weight maker; lane 2, lane 3: elution with 40% ethanol; lane 4, lane 5: washing with 15% ethanol for twice, lane 5 is the first washing, lane 4 is the second washing; lane 6: clarified papaya juice after centrifugation ; lane 7: papain standard.

4.3 DIRECT RECOVERY OF PAPAIN FROM UNCLARIFIED PAPAYA JUICE USING REVERSED PHASE EXPANDED BED ADSORPTION CHROMATOGRAPHY (RP-EBAC)

4.3.1 Operation of RP-EBAC column

4.3.1.1 Expansion characteristic of adsorbent

The degree of bed expansion is influenced by liquid superficial velocity, density of adsorbent particles and viscosity of feedstock, as reviewed by Anspach *et al.* (1999). The voidage of the expanded bed depends on the degree of bed expansion (H/H_0). The bed voidage ε of the expanded bed was calculated from Eq. 4.5.

$$\frac{H}{H_0} = \frac{1-\varepsilon_0}{1-\varepsilon} \qquad (4.5)$$

Where ε_0 is the settled bed voidage. A value of 0.4 was assumed for ε_0 (Nayak *et al.*, 2001; Theodossiou *et al.*, 2002; Tan *et al.*, 2006; Ng *et al.*, 2007; Chong *et al.*, 2010), thus, ε can be calculated from Eq. 4.5. The bed expansion characteristics of Amberlite™ XAD7HP were evaluated in various mobile phases, taken as the binding buffer (15% ethanol) and feedstock suspension (15% ethanol mixed unclarified crude papaya juice), as shown in Figure 4.6. The result shows that the bed expansion increased with the superficial velocity. At a constant superficial velocity of 1066.5 cm/h, the adsorbent was expanded evenly at a degree of 2 by using the binding buffer. When the feedstock suspension was loaded at 1066.5 cm/h, the bed expanded was higher by a degree around 2.5. The viscosity was measured using a viscometer (Table 4.3). The feedstock suspension had a 42% higher viscosity compared to binding buffer. A linear regression of the Richardson–Zaki equation ($u = u_t\varepsilon^n$) was used to assess the hydrodynamic stability of the fluidization of the adsorbent bed (Eq. 3.2). The Richadson–Zaki plot of ln u against ln ε is very successfully and show very good linearity: the R^2 value for the Richardson–Zaki plots of binding buffer is 0.9918 and that of feedstock suspension is 0.9989 (Figure 4.7). The values of n and u_t for different mobile phases were obtained and are summarized in Table 4.3. Bed expansion characteristics are dependent on the viscosity and density of process liquid and certain physical properties of adsorbent (Tan

et al., 2006). An increase in the viscosity in the mobile phase had resulted in a decrease in the value of terminal settling velocity. This is in line with the findings as by other researchers (Ng *et al.*, 2007; Chong *et al.*, 2010). Apart from that, the n value for each feedstock used is greater with the increase of the viscosity and these trends are similar to those previously reported (Dasari *et al.*, 1993; Chang and Chase, 1996; Ng *et al.*, 2007). The result of Richardson-Zaki coefficient of feedstock suspension, n, is 2.968. It is not very close to a laminar flow regime of 4.8. The flow inside of the column maybe a turbulence. Nevertheless, as we observed in the experiment, the bed is still stable. It demonstrated that the bed expansion by the feedstock suspension is available to be used in the EBAC.

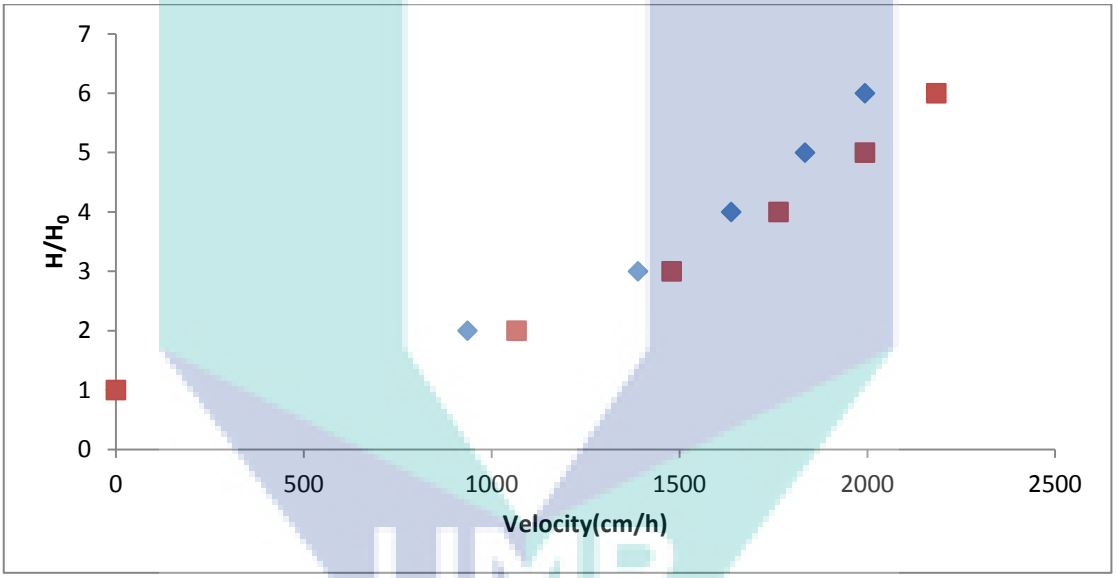


Figure 4.6: Bed expansion chearacteristics of Amberlite™ XAD7HP in equilibration 15% ethanol (■) and 15% ethanol mixed unclarified feedstock(◆)

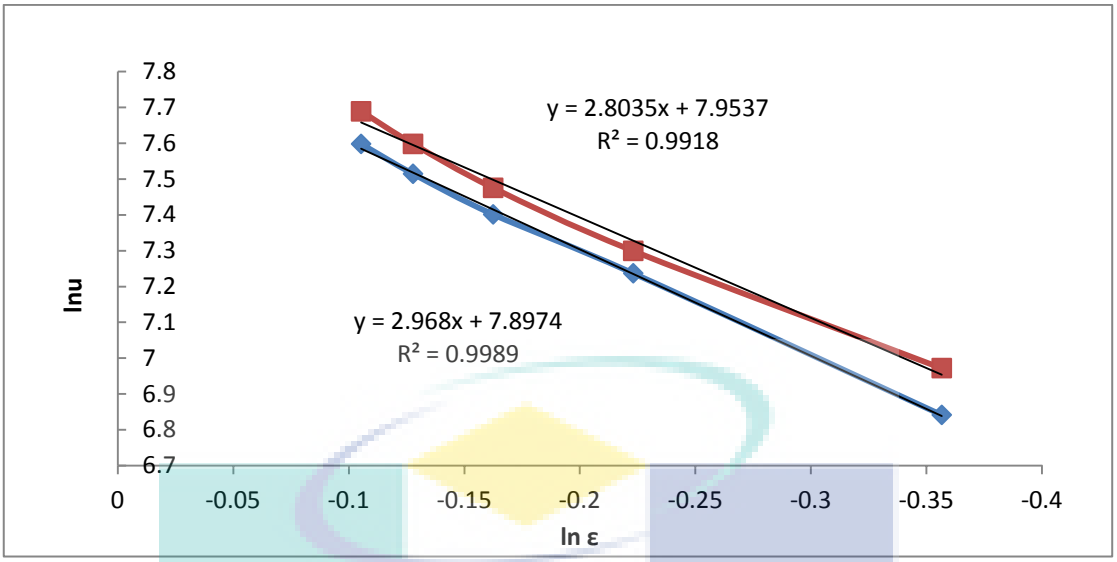


Figure 4.7: Richardson–Zaki plot for Amberlite XAD7HP adsorbent. Binding buffer (■) and feedstock suspension (◆)

Table 4.3: Results of viscosity and the linear regression of the Richardson–Zaki plots

	Binding buffer (15% ethanol)	**Feedstock suspension (15% ethanol mixed feedstock suspension)**
Viscosity(mPa s)	1.55	2.68
Richardson–Zaki coefficient, n	2.8035	2.968
Terminal settling velocity, u_t (cm/h)	2846.086	2690.279

4.3.1.2 Dynamic binding capacity

Frontal analysis observed from the breakthrough curve is important to estimate the maximum loading amount of target protein passed through adsorbent bed. Loading stage will be terminated at C/C_0=0.1 in most of the industrial practice in order to reduce the loss of target product in flowthrough (Lan *et al.*, 1999; Özyurt *et al.*, 2002; Ng *et al.*, 2007; Ramos *et al.*, 2011). The breakthrough curve of the papain adsorbed onto the Amberlite™ XAD7HP described above is shown in Figure 4.8. The dynamic binding capacity, Q_B, at the target permissible loss of 10% of papain in the effluent with the breakthrough volume of 14ml calculated from Eq. 3.3 was 810.52 µg papain/g adsorbent. Binding capacity with 15% ethanol on 15 min obtained from batch

adsorption is 3204.54 μg papain/g adsorbent (section 4.2.1). This value is great higher than the dynamic binding capacity. Dynamic binding capacity was influenced by applied liquid velocity and viscosity of the mobile phase (Chase and Draeger, 1992). In this case, low dynamic binding capacity is due to insufficient time for solid-liquid phase to reach equilibrium. The flow rate used in the test of breakthrough curve is 936 cm/h and the settled bed height is 5 cm. The bed was expanded to a degree of 2 with the bed height of 10 cm, thus, the residence time of feedstock in the column is only 38 s. The result of equilibrium binding time have obtained in the batch adsorption is 15min (Figure 4.2 B). Apparently, 38 s is not enough to reach the adsorption equilibration. This kind of phenomenon was also observed by other researchers (Ng *et al.*, 2007; Li and Chase, 2009b; Ramos *et al.*, 2011). The adsorption of papain by the EBA process could be improved by increasing the settled volume of adsorbent used to form the expanded bed with a consequential increase in the residence time of liquid in contact with the bed. The increasing of the adsorption by increasing the bed height was performed by some researches and they observed that adsorption of target protein was significantly improved (Li and Chase, 2009b; Ramos *et al.*, 2011). Nevertheless, the maximum expanded height of Fastline™ 10 EBAC column is limited to 35 cm, it is not possible to run a bed height in expanded mode which can be adapt to the requirement of 15 min equilibration in order to obtain a high adsorption. Generally, high residence time is correlated to high protein adsorption (Vergnault *et al.*, 2004; Ng *et al.*, 2007), however, long residence time may still not favor the purification of papain due to competitive binding between the contaminants and papain to the adsorbent. A high residence time may allow more contaminant proteins bound to the adsorbents. Although the improvement of adsorption by increasing the residence time in this research cannot be fulfilled, the front analysis about the breakthrough curve and bed height is of instructive significance for the future work.

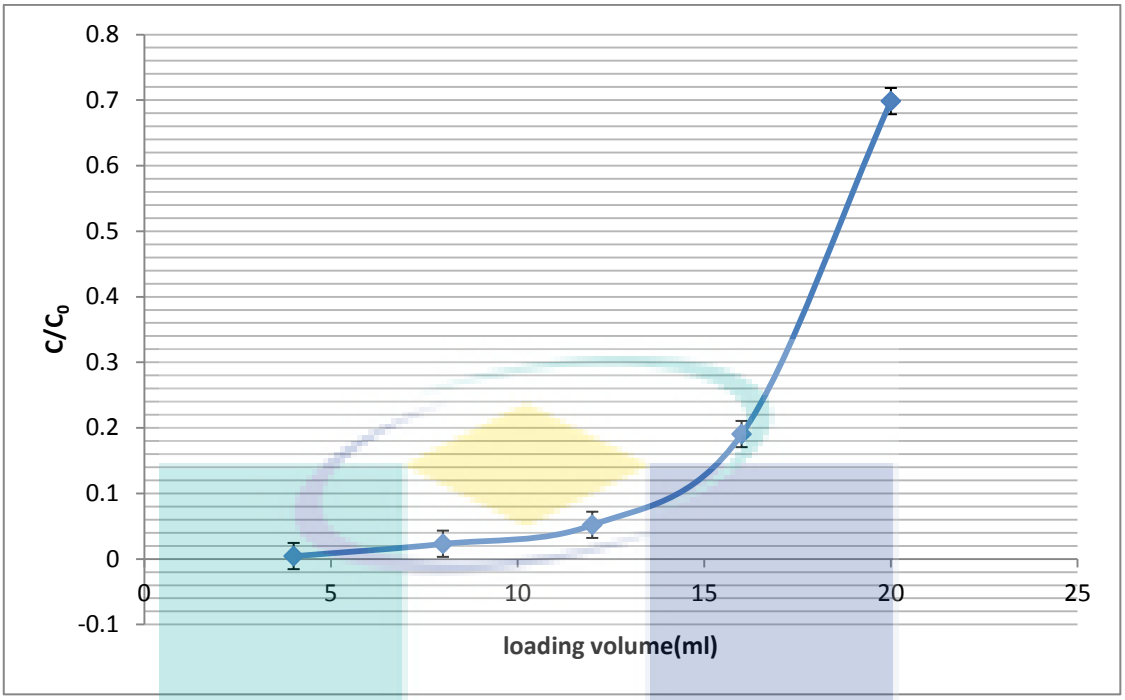


Figure 4.8: Breakthrough curve of the papain from unclarified feedstock onto Amberlite™ XAD7HP in RP-EBAC

4.3.2 Operation of RP-EBAC

The EBAC process for the adsorption and elution of papain from feedstock suspension was then investigated. The settled bed height was fixed to 5cm as used in the frontal analysis of breakthrough curve. The results of the comparative EBAC profile are shown in Figure 4.9. The performance of the RP-EBAC for the recovery of papain from feedstock suspension is summarized in Table 4.4. When the bed expansion was stable at a degree of 2, the unclarified feedstock suspension with 243.15 µg/ml of papain was applied onto the column. The solid particles flowed through the bed voids and left the column through the outlet pipe. The purity of the papain in the elution is 58.39% with a purification factor through this process is 4.96. The purity of papain in the stage of flowthrough and washing is 2.06% and 2.02% respectively. Nevertheless, the papain purity in the unclarified feedstock is 10.92%. This again confirmed that papain was adsorbed on the Amberlite™ XAD7HP during RP-EBAC process. Figure 4.10 is the Graphical illustration of the SDS-PAGE analysis for pooled protein fractions collected in RP-EBAC on a 15% polyacrylamide gel. Compare lane 5 with lane 2 of Figure 4.10, it's clearly showing that lane 5 has less bands than lane 2 and refer to the result of

papain purity in table 2 at same time, it concludes that papain was purified during this purification and less contaminant proteins exist in the elution fraction after purification. It indicates the process is a favorable.

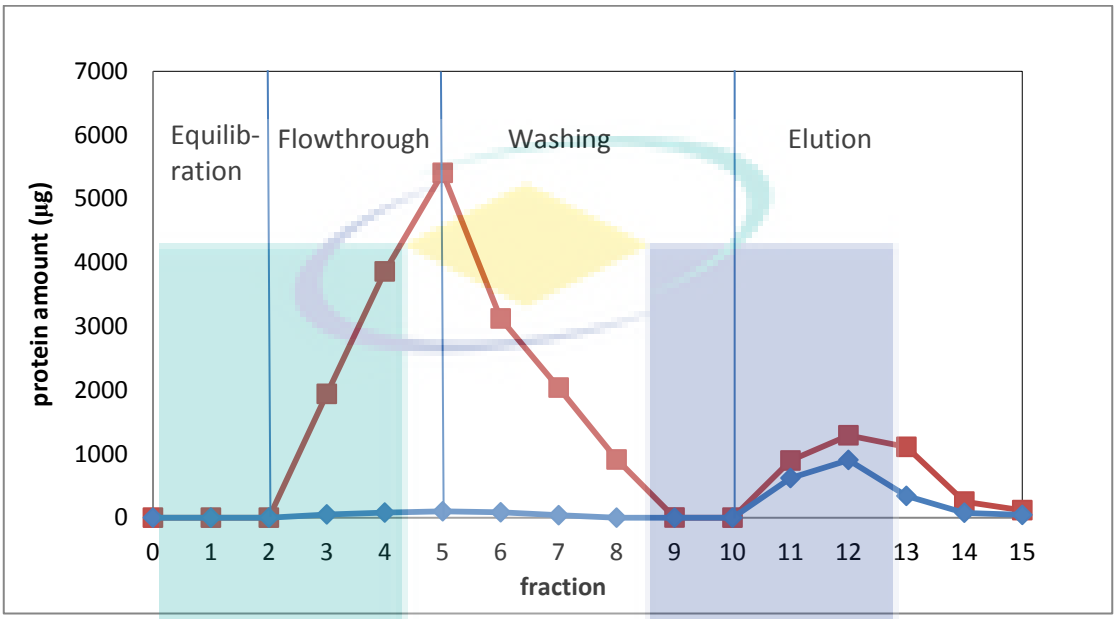


Figure 4.9: Profile of separation of 14ml feedstock suspension on Amberlite™ XAD7HP in EBAC at a superficial velocity of 936cm/h during feeding. Faction 1, 2 are the equilibrium buffer, fraction 3-5 are the flowthrough; fraction 6-10 are washing, fraction 11-15 are elution. Papain amount (◆) and (■) total protein amount.

Table 4.4: The performance of papain purification from feedstock suspension using RP-EBAC. The data were obtained as duplicate analysis

Purification stage	**Feedstock suspension**	**Flowthrough**	**Washing**	**Elution**
Total volume (µg)	14	15	25	25
Total protein (µg)	31169.77 ±363.15	11209.83 ±134.88	6078.14 ±116.72	3667.09 ±69.25
Amount of papain (µg)	3404.21 ±0	230.7 ±16.12	122.81 ±2.48	1987.72 ±99.24
Papain purity (%)	10.92 ±0.1	2.06 ±0.1	2.02 ±0.1	54.2 ±1.7
Yield (%)				58.3 ±2.90
Purification factor				4.96 ±0.21

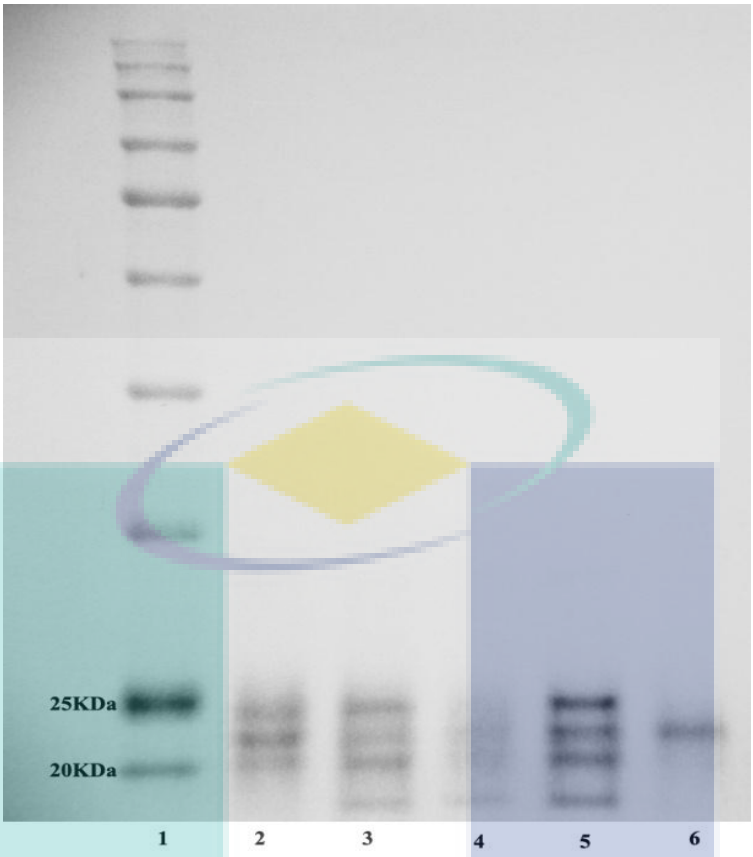


Figure 4.10: Graphical illustration of SDS-PAGE of the pooled protein fractions collected in RP-EBAC operation. Lane 1: protein molecular weight marker. Lane 2: elution. Lane 3: flowthrough. Lane 4: washing. Lane 5: feedstock. Lane 6: papain standard.

4.4 ENHANCEMENT OF PAPAIN PURIFICATION BY TWO-STEP ELUTION IN EBAC

The two-step elution protocol was modified on the basis of the results shown in Table 4.4 in order to achieve a higher efficiency of purification process. A successful elution scheme for this duty can consist of two major elution steps: the first step is elution of impurities at a lower ethanol concentration followed by elution of papain with 40% ethanol since it is appropriate to elute most of the papain as shown in the result of section 4.7.

The result of Table 4.5 shows that two-step eltuion with 25% ethanol as the first-step elution buffer result in the highest purity of papain in the second-step elution,

the purity and purification factor can high to 74.98% and 7.04 respectively, and the yield of papain is 35.39%. The use of one-step elution (Section 4.7) with 40% ethanol elution only gives purity of 54.2% and yield of 58.39%. The optimization of first-elution step can be carried out in two ways: (1) the use of 20% ethanol to obtain a higher yield of papain or (2) the use of 25% ethanol to obtain a higher purity of papain. It should be emphasized that it is considered more important to achieve a highly pure papain rather than a high yield since the *Carica papaya* is an abundant cheap starting material, thus, favoring the second of the two options described above.

Table 4.5: The purification performance of papain from feedstock suspension using RP-EBAC with two-step elution. The data were obtained as triplicate analysis.

Elution conditions	Purification stage	Volume (ml)	Yield of Protein (%)	Yield of papain (%)	Purity (%)	Purification factor	Loss of protein (%)	Papain lose in whole process (%)
20%, 40%	Flow through	15	35.21 ±0.45	6.96 ±0.30	2.14 ±0.06			
	15% washing	25	19.88 ±0.29	3.92 ±0.10	2.13 ±0.02			
	20% elution	15	2.65 ±0.21	6.52 ±0.06	26.7 ±1.87			
	40% elution	25	8.32 ±0.52	48.37 ±0.23	62.95 ±7.05	5.81 ±0.65	33.94 ±0.10	34.23 ±1.99
25%, 40%	Flow through	15	35.65 ±0.62	6.65 ±1.23	1.99 ±0.40			
	15% washing	25	19.21 ±0.74	3.89 ±0.33	2.16 ±0.10			
	25% elution	15	6.74 ±0.20	23.89 ±0.01	37.78 ±1.13			
	40% elution	25	5.03 ±0.34	35.39 ±023	74.98 ±5.67	7.04 ±0.53	33.37 ±0.42	30.18 ±1.79
30%, 40%	Flow through	15	37.36 ±0.81	6.03 ±0.92	1.84 ±0.24			
	15% washing	25	20.04 ±0.06	4.07 ±0.25	2.31 ±0.13			
	30% elution	15	9.73 ±0.06	35.95 ±0.01	42.07 ±0.24			
	40% elution	25	6.54 ±0.67	25.56 ±0.08	44.5 ±4.49	3.91 ±0.39	26.33 ±0.01	28.39 ±0.56
35%, 40%	Flow through	15	35.78 ±0.42	6.49 ±0.46	1.98 ±0.11			
	15% washing	25	18.66 ±0.58	4.41 ±0.12	2.57 ±0.15			
	35% elution	15	14.61 ±0.52	59.45 ±1.2	44.38 ±0.69			
	40% elution	25	3.44 ±0.05	9.78 ±0.32	30.97 ±1.5	2.84 ±0.14	27.51 ±1.59	19.87 ±1.2

The RPLC allows the adsorption of hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase (ethanol solution). Decreasing the mobile phase polarity by adding more organic solvent reduces the hydrophobic interaction between the solute and the solid support resulting in desorption. The more hydrophobic the molecule the more time it will spend on the solid support and the higher the concentration of organic solvent that is required to promote desorption (Buszewski and Noga, 2012).

Figure 4.11 is the graphical illustration of SDS-PAGE of the pooled protein fractions collected in RP-EBAC operation using two-step elution. Lane 2 of Figure 4.10 is the pooled fraction of elution. Lane 2 of Figure 4.11 is the pooled fraction of elution using stepwise elution strategy. Compare Figure 4.10 and Figure 4.11, it can be seen that band below the papain band was eluted down and band over papain band is left. This demonstrates that 25% ethanol is favoring to elute the protein which has a smaller molecular weight than papain, and also because of this, the papain purity was enhanced. This phenomena is caused by the mechanism of RPC in which smaller proteins are resolved earlier and proteins above about 40kDa may not be readily removed from the column (Scopes, 1993).

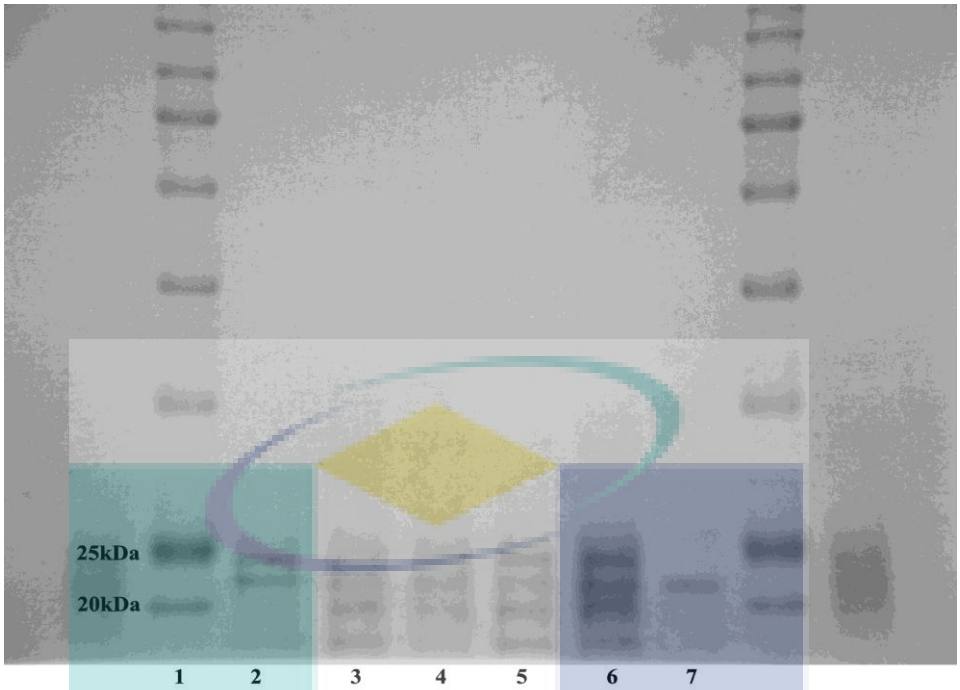


Figure 4.11: Graphical illustration of SDS-PAGE of the pooled protein fractions collected in RP-EBAC operation using two-step elution. Lane 1: protein molecular weight marker. Lane 2: second-step elution by 40% ethanol. Lane 3: first-step elution by 25% ethanol. Lane 4: washing with 15% ethanol. Lane 5: flowthrough. Lane 6: feedstock. Lane 7: papain standard.

4.5 CLEAN-IN-PLACE (CIP)

Normally the columns were used in the chromatography must be cleaned before every run to avoid contamination from microbial growth or cross contamination. This is especially important for EBA where very crude starting materials are used (Sofer and NystoÈm, 1989). In this research, papaya juice was used as the feedstock, thus, CIP is necessary to maintain the long life and the good performance of the adsorbent. The aim of this work is to study the effectiveness of proposed CIP protocol as described in Section 3.5.5 for Amberlite^{TM} XAD7HP in the use of EBAC.

Table 4.6 is the result of the CIP of Amberlite^{TM} XAD7HP in EBAC. In the first CIP, about 10403.52 μg protein was washed out from the adsorbent. It takes the percentage of around 85% from the total retained protein and still has 1826.41 μg (15%) retained protein was not washed out. Total protein was washed out in the second CIP process is 10605.28 μg, it takes the percentage of 97% from the total retained protein,

and it demonstrates that 100% ethanol is effective in washing out the contaminants from the adsorbent. The adsorbent was cleaned and can be reused for the next adsorption process.

Table 4.6: Protein amount in each stage of EBAC process. The data were obtained as duplicate analysis

	FIRST CIP	SECOND CIP
Initial protein (µg)	33552.75 ±60.55	34271.75 ±160.24
Bound protein (µg)	15710.49 ±544.36	14713.06 ±432.75
Elution 1 (25% ethanol) (µg)	1910.49 ±8.03	2141.59 ±26.50
Elution 2 (40% ethanol) (µg)	1570.06 ±25.35	1637.93 ±34.35
Retained protein (µg)	12229.9 ±577.75	10933.5 ±371.89
CIP (µg)	10403.52 ±420.66	10605.28 ±410.55
Still-Retained protein (µg)	1826.41 ±157.08	328.25 ±38.65

Furthermore, using of 100% ethanol as the cleaning solvent is of some advantages such as use a single buffer would not introduce any other buffer residues into the final product, moreover, no introduction of other solvent would no need to consider the effect of the solvent to the chemical or physical structure of the adsorbent. This kind of problems actually happened in many CIP processes (Gétaz *et al.*, 2010). Thus, in this research only chose ethanol as the cleaning solvent.

CHAPTER 5

OVERALL SUMMARY, CONCLUSION AND FUTURE PERSPECTIVES

5.1 OVERALL SUMMARY

Papain is an enzyme of high value which has been applied in many areas such as food industry and pharmaceutical. Nevertheless, the application of papain in the pharmaceutical area requires the papain of high purity. Papain's purification has been done by many methods such as salt precipitation, aqueous two phase extraction and chromatography. The most effective method on the promotion of the purity was used is chromatography. Unfortunately, chromatography has its disadvantage that is need to prior clarify the feedstock in order to avoid the particulate inside of the feedstock may block the chromatography bed and lower the efficiency of the bed, it may also reduce the frequency of use of the adsorbent. This is not help to the industrious application. The selection of appropriate purification method in order to achieve high purity of papain and high efficiency of purification is important due to the industrious requirement. Expanded bed adsorption chromatography (EBAC) is a novel method which allows the feeding of the particulate-containing feedstock. This feature solved the problem of bed blocking that happens in the conventional chromatography. At the same time, it forms a direct recovery of proteins by a very efficient purification process which integrates the clarification, concentration and purification in one unit operation with many advantages such as generates robust, easy to scale up and biocompatible. Reversed phase liquid chromatography (RPLC) is a separation based on the hydrophobicity of the protein. RPLC is an important technique of the characteristic of high purification resolution, which exploits the separation of proteins based on hydrophobic interactions between the stationary phase ligands and hydrophobic regions on the protein surface. Papain has high hydrophobic region on the surface which enabled the application of RPLC. In this

research, EBAC and RPLC was combine together to purify papain from crude papaya fruit juice which shows a great efficiency. The overall steps involved in this research are depicted in Figure 5.1 and Figure 5.2.

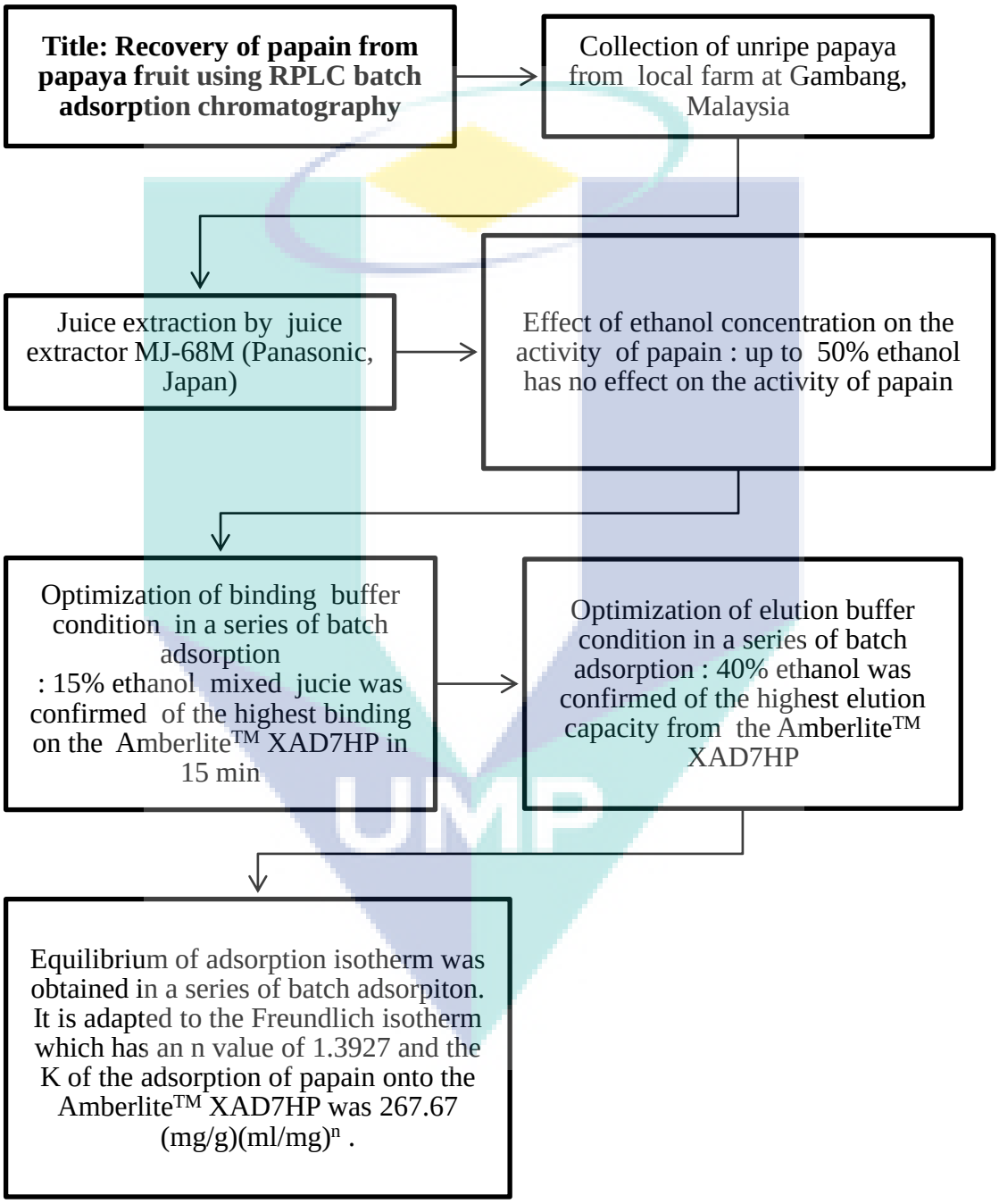


Figure 5.1: Process scheme for the recovery of papain from papaya fruit juice using RPLC batch adsorption chromatography

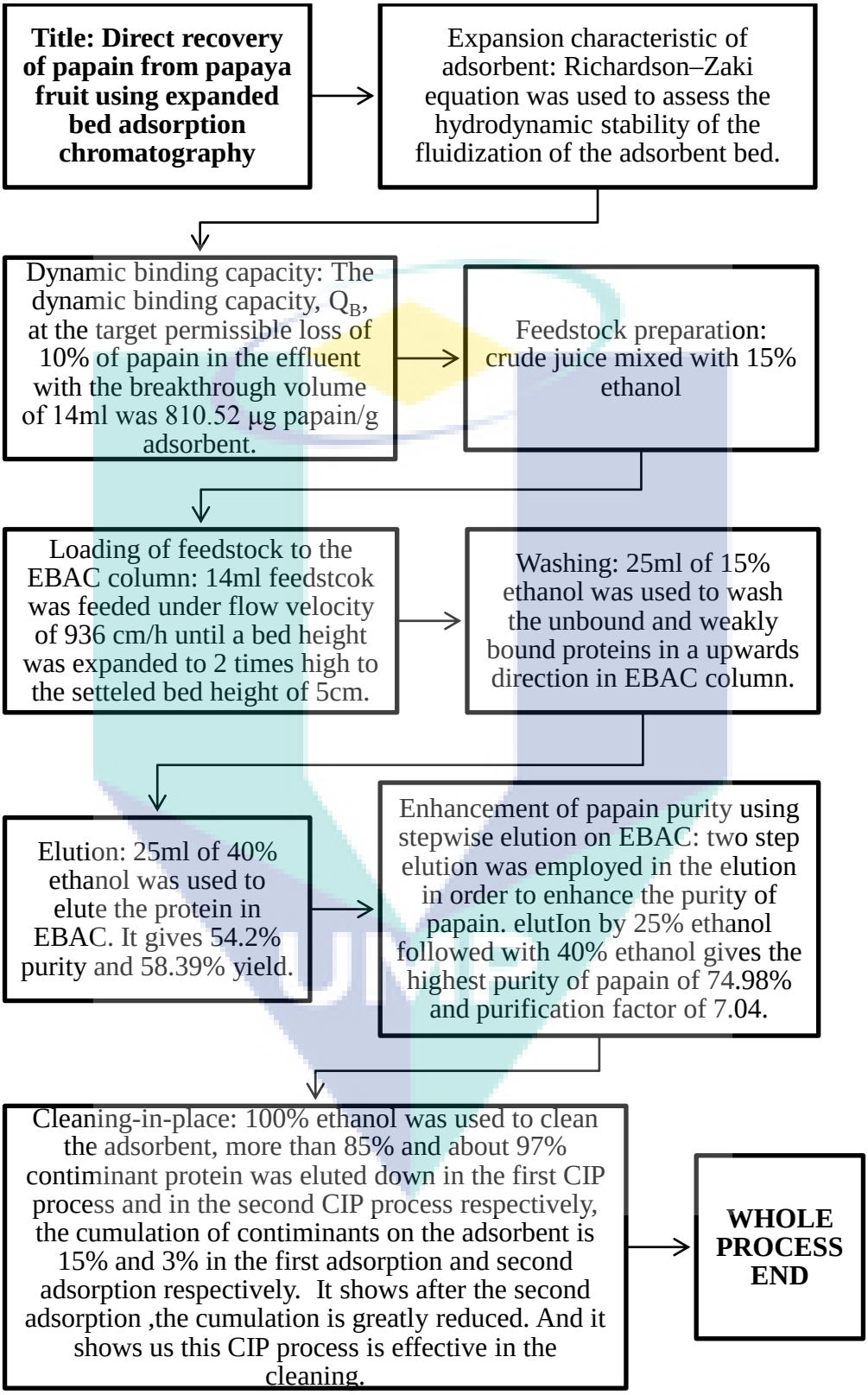


Figure 5.2: Process scheme for the recovery of papain from papaya fruit juice using expanded bed adsorption chromatography

5.2 CONCLUSION

Therefore, it can conclude that:

1. The batch adsorption reversed phase liquid chromatography on Amberlite™ XAD7HP in this study demonstrates a good capability of direct capture and purification of papain enzyme from *Carica papaya* crude extraction.
2. The papain purity of 50.5% and the yield of 65.6% were obtained in a batch adsorption reversed phase liquid chromatography and papain activity was preserved in the buffer of ethanol solution.
3. Reversed phase expanded bed adsorption chromatography (RP-EBAC) has been applied successfully to purify the papain from an unclarified *Carica papaya* juice. The EBAC mode combined clarification, capture and purification of the papain in a united process.
4. In RP-EBAC, the use of one-step elution with 40% ethanol (v/v) gives 54.2% purity and 58.39% yield of papain.
5. In RP-EBAC with two-step elution strategy, the elution of the papain using ethanol solution of 25% and 40% (v/v) separately which led to a papain purity of 74.98% and purification factor of 7.04. The first-step elution of 25% ethanol removed the contaminant proteins efficiently and thus the purity of papain was enhanced.
6. 100% ethanol was used to clean the adsorbent in the Cleaning-in-place (CIP) process and more than 85% contaminant protein was eluted down. It shows the CIP process is effective.

5.3 FUTURE PRESPECTIVES

In the present study, the purification of papain from crude papaya juice using expanded bed adsorption chromatography has been successfully performed with a mechanism of reversed phase chromatography. It demonstrates that EBAC is a powerful method of purifying the protein from the unclarified feedstock of high efficiency and shortened processing time. However, room is still available for the further improvement.

1. No study has been reported about large-scale production of papain from the papaya fruit. Our present research proved the feasibility of purifying papain using EBAC in

laboratory scale. Further study can be performed to confirm the EBAC with Amberlite™ XAD7HP whether available in the large-scale manufacturing.

2. The efficiency of the protein purification using EBAC is different with the diverse chromatography mechanisms. There are still many different mechanisms available to the adsorption of papain. These mechanisms of strong potential to be used in the purification of papain in EBAC. This would be a good point to be studied in future.
3. The application of papain already broadly studied, nevertheless, the application of papain on the antifungal and anti-melanin purpose is not well researched. The study about antifungal and anti-melanin characteristics of papain which purified from papaya fruit using the EBAC would be a very good aspect which may lead to a large scale using of papain in the pharmaceutical area and in the cosmetics.

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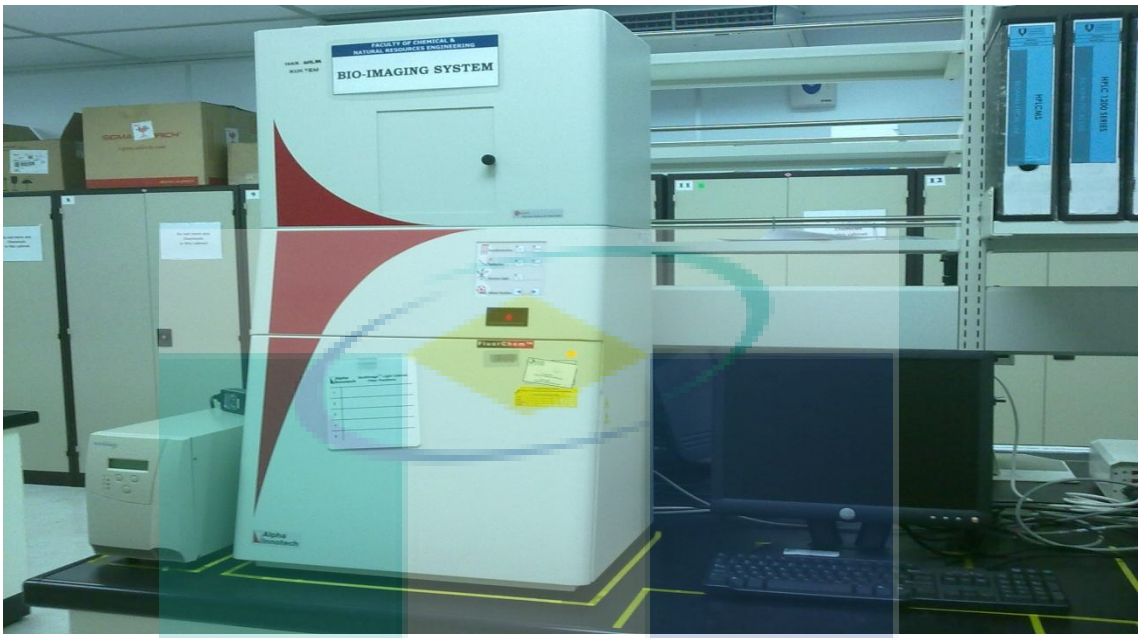
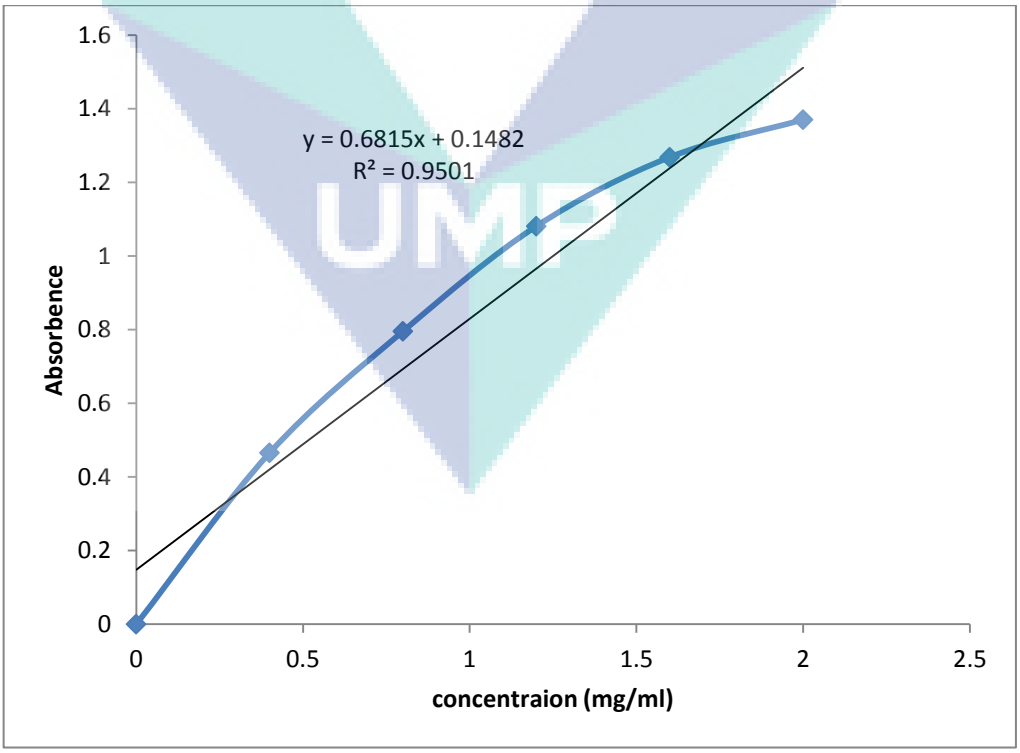
APPENDICES



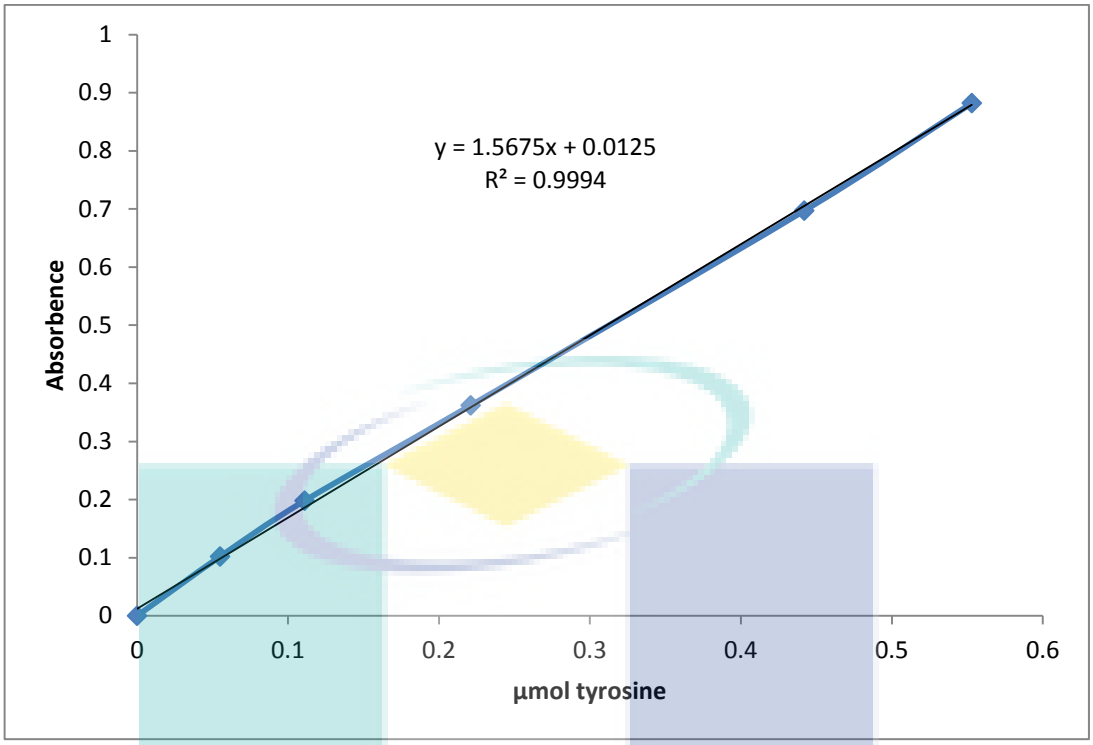
A: Bio-imaging system (Alpha Innotech): This device was used for the analyzing of SDS-PGAE gel

B: EBAC system (Upfront^{TM} 10 EBAC column, Watson Marlow peristaltic Pump)

C: DV-III Viscometer (Brookfield Eng. Lab., USA)



D: BSA standard curve by Bradford assay



E: Tyrosine standard curve