STUDIES OF ESTERIFICATION USING *CARICA* PAPAYA LIPASE

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ABSTRACT

In search for newer esterification method in industry, application of *carica* papaya lipase (CPL) satisfies that need. CPL is an enzyme that catalyses the esterification and hydrolysis. Thus it is classified as biocatalyst. This lipase is found in the unripe papaya fruit latex. The aim of this study is to study the lipolytic activity by using *carica* papaya lipase. To study this lipolytic activity of CPL, the latex from the unripe papaya fruit is obtained by making longitudinal incision on the fruit and collecting the latex in a beaker then dried to form latex powder. Then a series of four different alcohols and four different carboxylic acids, both ranging from three carbons to six carbons are used to in the esterification reaction. To analyze the esters formed gas chromatography (GC-FID) is used. GC can be used to perform both qualitative analysis to identify the ester formed and quantitative analysis to determine the yield. Esterification using different alcohols and different carboxylic acids will hopefully show different efficiency depending on chain length. The results show that CPL can conduct lipolytic activity (esterification) with various alcohols and various carboxylic acids in industry.

ABSTRAK

Dalam pencarian untuk kaedah pengesteran baru dalam industri, pelaksanaan lipase carica papaya (CPL) memenuhi keperluan itu. CPL adalah enzim yang mengkatalisis pengesteran dan hidrolisis. Jadi, diklasifikasikan sebagai biokatalis. lipase ini ditemui dalam getah buah betik mentah. Tujuan kajian ini adalah untuk mempelajari aktiviti lipolitik dengan menggunakan lipase carica pepaya. Untuk kajian ini aktiviti lipolitik CPL, getah dari buah betik mentah diperolehi dengan membuat sayatan memanjang pada buah dan mengumpul lateks dalam sebuah gelas kimia kemudian dikeringkan untuk membentuk serbuk lateks. Kemudian rangkaian empat alkohol yang berbeza dan empat asid karboksilik yang berbeza, baik mulai dari tiga hingga enam karbon karbon digunakan dalam reaksi pengesteran. Untuk menganalisis ester terbentuk kromatografi gas (GC) digunakan. GC boleh digunakan untuk melakukan analisis kualitatif untuk mengenal pasti ester dibentuk dan analisis kuantitatif untuk menentukan hasilnya. Esterifikasi menggunakan alkohol yang berbeza dan asid karboksilik yang berbeza diharapkan akan menunjukkan kecekapan yang berbeza bergantung pada panjang rantai. Keputusan kajian menunjukkan bahawa CPL boleh melakukan aktiviti lipolitik (pengesteran) dengan berbagai alkohol dengan asid karboksilik pelbagai industri.

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
mol	mole(s)
g	Gram(s)
mg	Milligram(s)
mM	Millimole(s)
mL	Milliliter(s)
µL	Microliter(s)
g/mL	Gram(s) per milliliter
g/mol	Gram(s) per mole
mg/L	Milligram(s) per liter
°C/min	Degree Celsius per minute

LIST OF ABBREVATIONS

CP	*Carica* papaya
CPL	*Carica* papaya latex lipase
GC	Gas Chromatography
GCFID	Gas Chromatography-Flame Ionization Detector
HPLC	High Performance Liquid Chromatography
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The purpose of presenting this chapter is to present the main ideas and facts regarding the background of the study, problem statement, objectives of the study and justification. All these aspects would be a foundation in order to further discover the research.

1.2. BACKGROUND OF THE STUDY

The esters are a group of organic compounds. They are naturally found in plants, fruits, fats, oils and waxes of various forms. They are responsible for the smell and taste of the material they are found in. Esters have many uses in industry, some of the intermediary products, as solvents, as softeners, and in the form of a polymer (Poly-ester) as fibres. (Schönenberger and Talamona, 2001)

Carboxylic acid and various kind of alcohol are used in industry to produce various kinds of esters. Small catalytic quantities of mineral acids are used to facilitate this process. During the reaction the educts and products (esters and water) exists at equilibrium. This equilibrium can be shifted to favour producing more esters by selectively removing the ester or water from the reaction mixture. Still there are some unreacted reactants remains in the mixture. (Schönenberger and Talamona, 2001)

In order to increase the product (ester) yield, carboxylic acid is used as alkanoyl chloride (acyl chloride or acid chloride). Through this way the formation of balance at the end of the reaction is reduced. Although by using alkanoyl chloride produces sulphur dioxide, hydrochloric acid and alkanoyl chloride residue, they can be removed by using light stream of nitrogen gas and neutralizing using base or distilled off by heating (Schönenberger and Talamona, 2001).

Besides the esterification method stated above, esterification also can be done by using enzymes from plants. Such enzyme is the lipase enzyme from papaya (*carica* papaya) latex named *carica* papaya latex lipase (CPL). CPL is known to have very good lipolytic activity, and have been classified as a biocatalyst (Mukherjee and Kiewitt, 1996). Previous researches have been done on CPL's lipolytic activity such as esterification, transesterification and interesterification.

In a previous study *carica* papaya latex was researched for specificity in the esterification of fatty acids with 1-butanol. In this research, CPL have shown selectivity of fatty acid among various types of unsaturated fatty acids and fatty acids having hydroxy, epoxy, and cyclopentenyl groups in esterification process with 1-butanol (Mukherjee and Kiewitt, 1996).

In another research of CPL and its lipolytic activity, the effectiveness of CPL as biocatalyst to synthesis octyl and decyl caprylate ester was compared with other enzymes such as immobilized lipases from *Candida antarctica* (Lipase B, Novozym, NOV) and *Rhizomucor miehei* (Lipozyme, LIP). From this research CPL have shown higher esterification activity compared to two other microbial lipase enzymes (Gandhi and Mukherjee, 2001).

Along with the researches stated above, more studies on esterification using CPL were conducted in the yesteryears in order to identify the full potential of CPL in industrial application. One such research was to increase the specific activity of CPL. CPL was immobilized on silica gel, thus forming immobilized CPL (imb-CPL). The comparison of the specific activity of free-CPL and imb-CPL was researched by studying the synthesis of methyl laurate from coconut oil using these

both forms of CPL. The results of this study shows the imb-CPL's specific activity have increased by thirteen times compared to free-CPL. Further researches have shown that imb-CPL can be reused for seven times (Azucena-Topor, 2006).

1.3 PROBLEM STATEMENT

In many industry such as food industry, dairy industry and oleochemistry industry lipase enzyme is used to hydrolyze milk fat, synthesize structured triglycerides and in polymers and surfactants production. Other than the reaction and process stated above, lipase enzyme also can be use to do esterification and transesterification (Paques et al., 2008).

But the application of enzymes in industry for esterification is not widely applied due to their high production cost and inadequate availability. Thus intense researches have been conducted lately to identify inexpensive source of lipase, which can be obtained in bulk quantities (Gandhi and Mukherjee, 2000a). There are various sources to obtain lipase enzyme such as from vegetables, seeds and latex. The yield of lipase enzyme from vegetables and seeds is much lower compared to the yield from latex such as *carica* papaya latex. This is because *carica* papaya latex is commercially available in bulk scale (Paques et al., 2008).

Thus in search for newer esterification ways in industry, application of *carica* papaya lipase (CPL) satisfies that need (Paques et al., 2008). CPL is an enzyme that catalyses the esterification and hydrolysis. Thus it is classified as biocatalyst. This lipase is found in the unripe papaya fruit latex. The aim of this study is to study the lipolytic activity by using *carica* papaya lipase.

1.3 OBJECTIVES OF THE STUDY

1.0 The aim of this study is to study the lipolytic activity by using *carica* papaya lipase.

2.0 Determine the different rate of CPL esterification when different types of substrate and reaction medium (solvent) is used.

1.4 JUSTIFICATION

Carica papaya latex (CP latex) is rich with enzymes with high potential values. Enzymes which are present in latex mostly are four types of cysteine endopeptidases papain, chymopapain, glycyl endopeptidase and caricain (Azarkan et al., 2003). And recently discovered enzyme was *carica* papaya latex lipase (CPL).

Proteinases from papaya (*Carica papaya*) are well known and known as proteolytic enzymes. These proteolytic enzymes are present in fresh latex from fruit, stems, petioles, and leaves. It has been found out previous study that, data on the composition of enzymes from latex of the different parts are not the same. Proteolytic enzymes have variety of application namely, in scientific research, medicine, and food. Medicinal preparations based on papaya proteinases is well known due to their exclusive therapeutic activity on damaged tissues and has no serious complications and is effective. Preparations of medicines can be done through isolation and purification of enzymes from latex of papaya fruit (Mezhlumyan et al., 2003).

Meanwhile, the newly discovered CPL is attracting more and more interest of researcher's in recent years. CPL is a hydrolase which is tightly bonded to the water-insoluble part of crude papain. Thus CPL is said as a "naturally immobilized" biocatalyst. There are several application of CPL have already been identified up to this date. These applications are fats and oils modification, derived from the sn-3 selectivity of CPL as well as from its preference for short-chain fatty acids, esterification and inter-esterification reactions in organic media, accepting a wide range of acids and alcohols as substrates and more recently, the asymmetric resolution of different non-steroidal anti-inflammatory drugs (NSAIDs), 2-(chlorophenoxy)propionic acids, and non-natural amino acids. Taking in consideration of all these various applications of this enzyme, more research groups would be prompt to further investigate the full potential of CPL in the future (Domínguez de María et al., 2006). Furthermore, the research on CPL's lipolytic activity has not been done in Malaysian region because it is a new field of research.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

In this chapter several different past studies were reviewed and classified accordingly to its aspects so that all these information's could be related to the present study on esterification of *Carica* Papaya Lipase.

2.2 *CARICA* PAPAYA LIPASE

Carica papaya latex lipase (CPL) was reported for the first time in the year 1935, however it was not until the early 1990's where this enzymes activity was classified when different types of triglycerides were used as substrates for this enzyme to catalyse reaction. The source of CPL is from the latex of aerial parts of the female or hermaphrodite *Carica* papaya plant. This latex can be obtained by making incisions at those aerial parts. But the plants part that gives the optimum amount and quality of latex is the unripe fruit of *Carica* papaya plant. This latex is trixotropic fluid with milk like appearance. This latex contains 85% of water and 15% of dry matter which comprise of various enzymes including CPL. Furthermore the composition of the latex may vary according to the plants age, sex and the time of the day where the latex tapping is conducted.

In the latex CPL is tightly bonded to the water-insoluble part of crude papain (dry matter matrix). Thus CPL is traditionally considered as a "naturally immobilized" biocatalyst. Even though the CPL is tightly attached to the dry matter

matrix, the activity of this enzyme is completely independent of other proteases present in the latex and the natural role of CPL in the plant is still unknown.

In the recent years, researches have been done to separate the CPL from the dry matter matrix but the research was not fruitful. Instead partial purification of the latex was able to be conducted by dissolving the latex in water and removing the aqueous phase after centrifugation. CPL would be left in the solid matter because it is not water soluble. Even though in last decades, many researches have been conducted and biotechnological applications have been reported regardless of the difficulty in purification of CPL. Examples of those research and applications are, fats and oils modification (based on the CPL sn-3 stereoselectivity), esterification and interesterification in organic media leading to the synthesis of several alkyl esters, and more recently, its efficiency in the asymmetric resolution of different chiral acids.

Furthermore, resent researchers have identified the capability of CPL to catalyse reactions in organic media and accepts a wide range of substrates for both alcohols and fatty acids. But there still some compounds which are not so well accepted as substrate by CPL. Some such compounds are cis-4, cis-6 or cis-8 unsaturated fatty acids. On the other hand CPL showed a noteworthy esterification activity towards cis-9 derivatives, as well as toward epoxydated or hydroxylated fatty acids and the highest activity when C18 saturated fatty acid were used as substrate. And the optimum temperature of CPL to catalyse esterification is 60°C. For the hydrolytic activity of triglycerides, CPL showed maximum activity when C4-C8 fatty acid were used. Wide substrate acceptability of CPL makes it a very good catalyst in industrial application. (Domínguez de María et al., 2006)

2.3 *CARICA* PAPAYA PROTEASES

Papain a cysteine protease is one of an enzyme which is contained in the latex of *carica* papaya. Papain enzyme is extracted and purified from the latex extracted from the unripe fruit of *carica* papaya, but the enzyme also can be extracted from other parts of the tree such as from roots, stem, petiole and leaves. This enzyme

haves similar actions to pepsin enzyme found in gastric juice. In year 1935 isolation and purification process of papain from fresh *carica* papaya latex was developed and later on this method was modified and the modified method became the traditional way of preparing commercial papain from dry *carica* papaya latex. Various industrial applications of papain have been identified, such as in food industry for clarifier in beers, as meat tenderizer and in preparation of protein hydrolysates; and in pharmaceutical industry in treatments for osteoporosis, arthritis, vascular diseases and cancer. Along with papain there are three more different cysteine proteases found in *carica* papaya latex, which are chymopapain, caricain, and papaya proteinase IV (Thomas et al., 2009). All these four proteases are characterized by sulfhydryl groups in active site. In previous study papain was used in hydrolysis of a protein to produce amino acids (Zhang et al., 2010). Table 2.1 lists the Characteristics of papain.

Table 2.1: Characteristics of papain

Properties	Description
Molecular Weight	21,000 daltons
Structure	A chain of 159 amino acids including three cysteine bridges. The presence of cysteine at the active site is required for activity. The SH group of cysteine easily reacts with oxygen or heavy metals.
Isoelectric Point	pH 8.5-9.0 (for autolytic processes)

Source: Uhlig (1998)

2.4 CONCLUSION

The information's obtained from different journals could be related to the present study of esterification. All the obtained information could be used to justify and compare the results obtained in the present study with those of past.

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

The aim of presenting this chapter was to present the research materials and methods related to the present study on the esterification of *Carica* Papaya Lipase. It revolves around the equipment's, sample preparation and procedures. This analysis on the methods were done so that this present study attempt can be appropriately adapted to the latest method in order to fulfil the scope and direction of the present research attempt. Figure 3.1 shows the flow chart of research methodology.

3.2 EQUIPMENTS

All equipments that are used in the experiments are standard laboratory size.

3.3 COLLECTION OF LATEX

Carica papaya tree with unripe fruit was identified. The tree was found at various locations in Gambang town, Kuantan. Latex from the plant was extracted by making some longitudinal incisions on the unripe fruit while the fruit is on the tree. The flowing latex is collected in a glass beaker. The extraction is done on bright sunny day for optimum osmotic pressure in the plant thus resulting higher latex extraction. The obtained latex is then sealed using aluminium foil to avoid contamination.

3.4 PREPARATION OF DRY LATEX

Partial purification of latex is done in the lab. The latex from the fruit is diluted in distilled water and centrifuged to form two separate layers, aqueous layer and solid matrix layer. The aqueous layer or the supernatant of the centrifuge is removed and collecting the solid matrix of the latex. The CPL is present in the solid matrix because CPL is insoluble in water. Then the solid matrix is dried in oven under 60°C. Then the dried latex is powdered and stored in Petri dish inside desiccators to avoid moisture absorbing by the latex powder.

3.5 ESTERIFICATION PROCESS

The esterification process involves three main processes such as the enzymatic esterification, synthetic esterification and synthetic esterification of internal standard.

3.5.1 Enzymatic Esterification

In first enzymatic esterification experiment, 300 µL of n-butanol, 176 mg lauric acid and 2 mL of hexane, as reaction medium, was mixed in volumetric flask. 10 mg of CPL was added to catalyst the esterification process. The mixture was reacted in a water bath and constant stirring was applied using magnetic stirrer. The temperature was set at around 60°C. Samples were taken after 5 hours' time and overnight. The samples were kept in different scintillation vials. The vials were kept in refrigerator at 4°C.

In second enzymatic esterification experiment, 6 mL of 2-butanol and 0.25 g lauric acid was mixed in volumetric flask. 0.06 g of CPL was added to catalyst the esterification process. The mixture was reacted in a water bath and constant stirring was applied using magnetic stirrer. The temperature was set at around 60°C. Samples were taken after 5 hours' time and overnight. The samples were kept in different scintillation vials. The vials were kept in refrigerator at 4°C.

In third enzymatic esterification experiment, 2 mL of n-butanol and 176 mg lauric acid was mixed in volumetric flask. 10 mg of CPL was added to catalyst the esterification process. The mixture was reacted in a water bath and constant stirring was applied using magnetic stirrer. The temperature was set at around 60°C. Samples were taken after 5 hours' time and overnight. The samples were kept in different scintillation vials. The vials were kept in refrigerator at 4°C.

3.5.2 Synthetic Esterification

4 mL of n-butanol, 352 mg of lauric acid and 5 drops of sulphuric acid was mixed in round bottom flask. The mixture was heated and reacted using reflux method at 75°C to 80°C for 90 minutes. The reacted sample was kept in the flask.

3.5.3 Synthetic Esterification (Internal Standard)

A mixture of methanol, benzene and concentrated sulphuric acid was prepared in the ratio of 20:10:1 by volume.4 mL of this mixture was transferred into a clean scintillation vial and 5.1 mg of palmitic acid was added. The mixture was heated and reacted using reflux method at 75°C to 80°C for 90 minutes. This standard must be done a day before instrumental analysis. Thus separation must be done in the same day.

3.6 LIPID ANALYSIS

The lipid analysis involves the separation of samples, qualitative as well as quantitative analysis of the sample content.

3.6.1 Separation

2.5g of sodium bicarbonate was dissolved in 50mL of ultrapure water to prepare 5% sodium bicarbonate solution.

The samples were separated using separatory funnel. A sample was poured from the scintillation vial in the separatory funnel. The vial was washed with 4 mL of hexane and poured back into the separatory funnel. First 2 mL of 5% sodium bicarbonate solution was added into the separatory funnel and closed. The funnel is then shaken less vigorously and the valve is opened time after time to release the pressure. The 5% sodium bicarbonate solution was added to neutralize any acid remains in the sample. After shaking the below aqueous layer is removed and tested for acidity using pH paper. If the layer is acidic more 5% sodium bicarbonate solution was added until the aqueous layer has turned neutral. The remaining organic layer is kept in a new clean scintillation vial and anhydrous sodium sulphate is added to remove any water present in the sample. All the samples were separated using the same method.

3.6.2 Qualitative Analysis

All the samples were filtered using micro filters and transferred in same amount, 1 mL, into different 2 mL screw top GC/HPLC vials. For analysis Agilent Technologies 7890 GC System fitted with FID detector and HP INNOWAX column (length, 30 m; internal diameter, 0.25 mm; film thickness, 0.25 μm; temperature range, 40°C to 260°C) was used. The main parameters were: injection temperature, 250°C; FID temperature, 250°C; nitrogen as carrier gas flow, 3.5 mL/min; sample splitting, split less; temperature programming, 80°C as starting temperature and temperature increment at 5°C/min, temperature was hold at 210°C for 3 minutes and at 250°C for 2 minutes. Sample list analyzed are listed as below. The retention time of sample A (butyl laurate) is identified and compared with other samples result to identify the presence of butyl laurate. Table 3.1 shows the list of all types of sample prepared for GC-FID analysis.

Table 3.1: The types of samples for GC-FID analysis

Sample vial	Sample Content
A	1 mL of synthetically produced butyl laurate via reflux method.
B	1 mL of synthetically produced butyl laurate via reflux method plus 10 μL internal standard.
C	1 mL of internal standard, methyl palmitate.
E	1 ml of sample taken after 5 hour for enzymatic esterification of n-butanol with lauric acid in hexane as solvent plus 10 μL internal standard.
F	1 ml of sample taken after over night for enzymatic esterification of n-butanol with lauric acid in hexane as solvent plus 10 μL internal standard.
G	1 ml of sample taken after 5 hour for enzymatic esterification of 2-butanol with lauric acid plus 10 μL internal standards.
H	1 ml of sample taken after overnight for enzymatic esterification of 2-butanol with lauric acid plus 10 μL internal standards.
J	1 ml of sample taken after 5 hour for enzymatic esterification of n-butanol with lauric acid plus 10 μL internal standard.
K	1 ml of sample taken after over night for enzymatic esterification of n-butanol with lauric acid plus 10 μL internal standard.

3.6.3 Quantitative Analysis

For quantitative analysis, the results of the nine samples are compared. Firstly the amount of methyl palmitate formed is calculated (assuming all of palmitic acid is reacted) using the chemical stoichiometry below. The retention time of sample C (methyl palmitate) is identified first. Then the presence of methyl palmitate in samples E, F, G, H, J and K is identified using the retention time from sample C.

1 Methanol + 1 Palmitic Acid → 1 Methyl Palmitate + 1 Water

Then the area under the peak is compared for each samples with the under the peak of internal standard (methyl palmitate) and the yield of sample is calculated. Or the yield percentage is obtained from the GC-FID results as peak area percentage after removing the solvent peak.

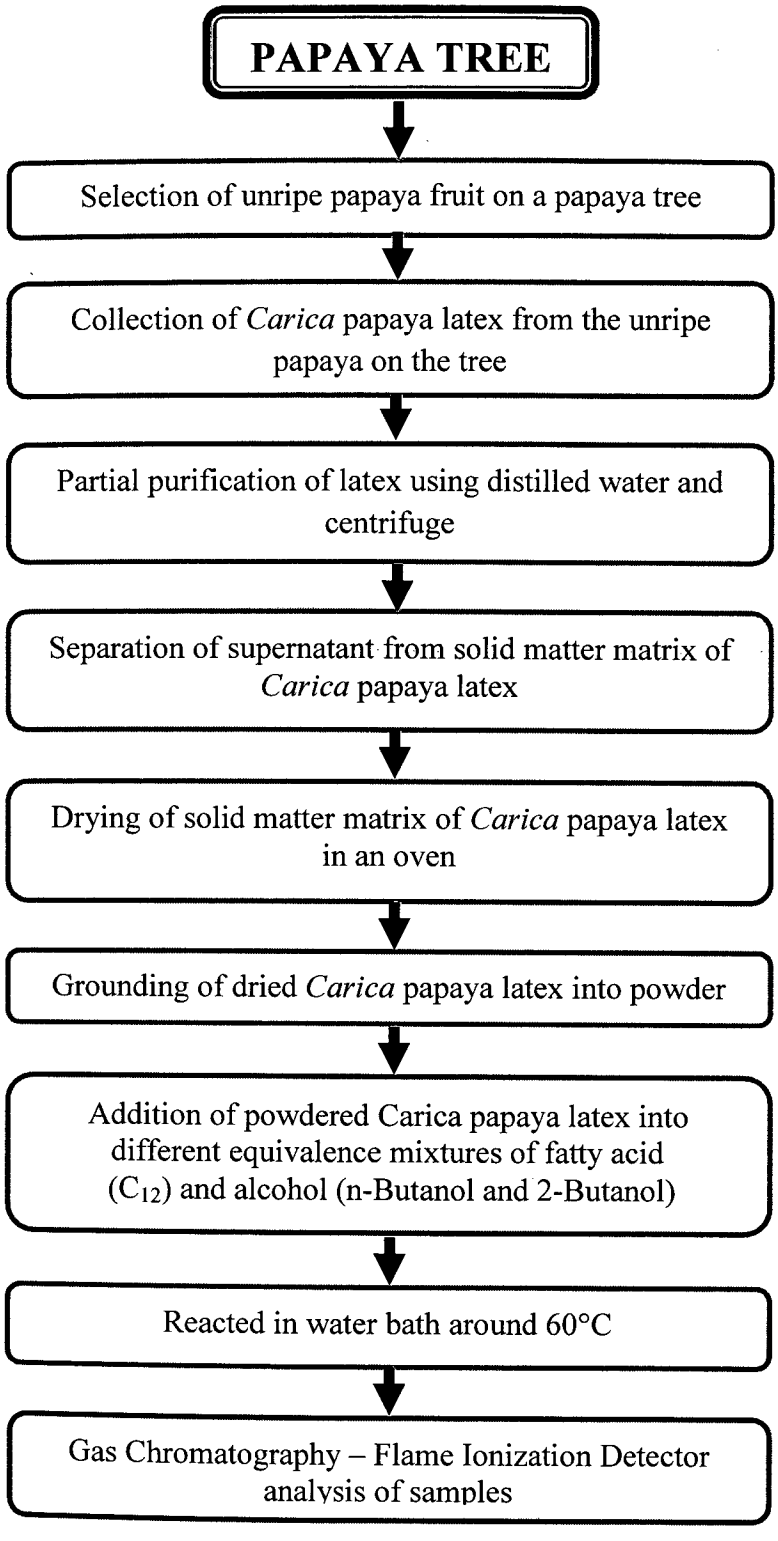

Figure 3.1: Flow Chart of Research Methodology

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

The results from first and third enzymatic esterification are compared and studied to analyse the effect of different solvent used as reaction medium on CPL's esterification activity. While results from second and third enzymatic esterification are compared and studied to analyse the effect of different substrate used on CPL's esterification activity.

4.2 RESULTS

In this analysis the presence of butyl laurate ester is determined. From the GC-FID results obtained, as in Appendices A1 to A11, it can be concluded that the analysis have produced negative results. The standard and standard mixture peaks (appendices A1 and A2) are not well separated, forming fronting peak.

4.3 DISCUSSION

4.3.1 Qualitative analysis

The presence of esters formed could not to be determined. The peaks in results A and B (Appendices A1 and A2) are inappropriate for analysis because the peaks are not well separated. This is due to the concentration of the sample A and B are too high. The concentration of the sample analysed should be 5 mg/L to 10 mg/L. Thus it is difficult to identify the retention time of butyl laurate and methyl palmitate

esters from sample A and B. Hence it is difficult to analyse the result peaks from D, E, F, G, H, I, J and K (Appendices A4, A5, A6, A7, A8, A9, A10 and A11) without the retention time of those two analytes. Furthermore there are too many foreign substances or contamination peaks (refer Appendices A1 and A2) in the sample further making it difficult to analyse. The contamination might be due to the insufficient amount of HPLC grade hexane wash in GCFID instrument. Other than that, the sample preparation and GCFID analysis of sample should be done in the same days. This will prevent any loss of analytes.

4.3.2 Quantitative analysis

In this analysis the yield of the ester is calculated. Table 4.1 shows the theoretical yield of the esterification experiment conducted (alcohol was used in excess in all experiment and assuming all the fatty acid was used in esterification process). Calculations are shown in Appendix B.

Table 4.1: Theoretical yield of esterification

Reactant	Theoretical yield (mass)
300 μL n-butanol (excess) and 176 mg lauric acid	0.23 g
6 mL 2-butanol (excess) and 0.25 g lauric acid	0.32 g
2 mL n-butanol (excess) and 176 mg lauric acid	0.23 g
4 mL n-butanol (excess) and 352 mg lauric acid; butyl laurate standard	0.45 g
Methyl palmitate; internal standard	5.38×10^{-3} g

Internal standard, methyl palmitate peak in each sample D, E, F, G, H, I, J and K is used to compare and calculate the amount of butyl laurate has formed in each sample. As the peak of methyl palmitate is failed to be determined, the amount of esters formed are also failed to be determined.