QUATERNARY AQUEOUS BIPHASIS MIXTURE FOR ENZYME EXTRACTION FROM BANANA

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UNIVERSITI SAINS MALAYSIA

2022

QUATERNARY AQUEOUS BIPHASIS MIXTURE FOR EXTRACTION FROM BANANA

by
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Thesis submitted fulfillment of the requirement for degree of Bachelor of Chemical Engineering

JULY 2022

ACKNOWLEDGEMENT

First, I would like to express my deepest gratitude to School of Chemical Engineering, Universiti Sains Malaysia for giving me this opportunity to learn and enhance my fundamental knowledge and skills to become a chemical engineer in the future. My thesis titled 'Quaternary Aqueous Biphasic Mixture For Enzyme Extraction From Banana' is the final fulfilment in obtaining the Bachelor of Engineering (Honours) in Chemical Engineering. This thesis would not have been completed without the support and guidance from certain parties.

Secondly, I would like to thank Dr. Fadzil, my supervisor for EKC 499 Final Year Project, for being very patient and supportive in providing me with the needed guidance and constructive criticism throughout the year. His willingness to discuss the research and its progress throughout the year to keep me in track is something that I deeply appreciate. Moreover, I want to acknowledge with much gratitude the important role of the course coordinator, Professor Dr. Mohd Roslee Othman for his invaluable help in stimulating suggestions and knowledge throughout the course.

I owe School of Chemical Engineering a huge debt of gratitude for allowing me to use the facilities in completing my project. The technical staffs and colleagues deserve special recognition for their encouragement, direction, and kind assistance, as well as intelligent remarks and recommendations which have made my project a success. Last but not the least, I would like to thank my family especially my mother, my friends, as well as my course mates for their never-ending moral support and immense encouragement throughout my studies.

Chen Yi

July 2022

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LIST OF SYMBOLS

Symbol	Description	Unit
V_t	Volume of top phase of ABS	µL
V_b	Volume of bottom phase of ABS	µL
C_t	Concentrations of proteins in the top phase of ABS	M
C_b	Concentrations of proteins in the bottom phase of ABS	M
ΔG_{hyd}	Free energy of hydration	kJ/mol
E	Extraction efficiency	%
ΔH	Enthalpy	J/g
R	Phase volume ratio	
K	Partition coefficient of total protein	
K_e	Partition coefficient of enzyme	

LIST OF ABBREVIATIONS

IL	Ionic Liquid
ATPS	Aqueous Two-Phase System
ABS	Aqueous Biphasic System
DES	Deep Eutectic Solvents
NADES	Natural Deep Eutectic Solvents
NPs	Natural Products
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
PEG	Polyethylene Glycol
UV-Vis	Ultraviolet-visible
DSC	Differential Scanning Calorimeter
BSA	Bovine Serum Albumin

KUAR BIPHASIS CAMPURAN YANG BERKESAN UNTUK EKSTRAK DARI PISANG

ABSTRAK

Pengekstrakan enzim dengan menggunakan pelbagai pelarut seperti cecair ionik telah digunakan pada masa lalu. Baru-baru ini, pelarut hijau yang merupakan pelarut eutektik mendalam telah mendapat perhatian masyarakat kerana kesederhanaan dan kos yang berpatutan dalam mengekstrak protein dengan sistem fasa dua - berair (ATPS). Melalui kajian ini, empat kolin klorida - (HBD) DES dengan nisbah molar yang berbeza dikaji untuk mengekstrak albumin serum sapi (BSA) dan protein dalam pisang. Penyelesaian DES yang paling stabil dengan membentuk campuran homogen dengan nisbah molar untuk kolin klorida: gliserol (ChCl: Gly) adalah 1: 2, kolin klorida: asid laktik (ChCl: Lac) adalah 1: 2, kolin klorida: urea (ChCl: Urea) adalah 1: 2 dan kolin klorida: glukosa (ChCl: Glu) adalah 2: 1.Dari kajian ini, kelikatan komponen individu mempengaruhi hidrogen intensif - ikatan antara campuran DES dalam membentuk campuran homogen yang stabil. Dengan pembentukan DES, kesan kepekatan dan isipadu untuk polietilena glikol (PEG), natrium sulfat (Na2SO4) dan DES dikaji. 1000 μL 1.0 M Na_2SO_4, 1000 μL PEG dan 100 μL DES dipilih kerana memerlukan masa terpendek untuk pembentukan dua fasa yang 97.33 minit. Kepekatan protein pisang dibandingkan dengan keluk penentukuran dengan DES yang berbeza. Hasil kajian menunjukkan bahawa ATPS dengan ChCl: Lac mengekstrak protein paling banyak iaitu 3678.33 μg / ml dengan kecekapan pengekstrakan tertinggi 89.60 %. Ini mungkin dipengaruhi oleh kumpulan fungsi HBD, dan kelikatan rendah ekstrak mentah pisang berinteraksi dengan DES di mana ikatan hidrogen mempengaruhi pergerakan molekul bebas dalam sistem seperti pergerakan protein dalam pelarut membawa kepada pemindahan protein dengan cepat.

QUATERNARY AQUEOUS BIPHASIS MIXTURE FOR EXTRACTION FROM BANANA

ABSTRACT

Enzyme extraction by using various solvent such as ionic liquids was used in the past. Recently, a green solvent which is deep eutectic solvents (DESs) have gained attention from the community because of its simplicity and the affordable cost in extracting the protein with aqueous two-phase system (ATPS). Through this study, four choline chloride-(HBDs) DES with different molar ratio was studied to extract bovine serum albumin (BSA) and protein in banana. Molar ratio for choline chloride: glycerol (ChCl: Gly) at 1:2, choline chloride: lactic acid (ChCl: Lac) at 1:2, choline chloride: urea (ChCl: Urea) at 1:2 and choline chloride: glucose (ChCl: Glu) at 2:1 will form a clear and transparent homogenous mixture at room temperature. With the formation of DES, the effect of the concentration and volume for polyethylene glycol (PEG), sodium sulphate (Na_2SO_4) and DES were studied. 1000 μL of 1.0 M Na_2SO_4, 1000 μL of PEG and 100 μL of DES was chosen because it took the shortest time for the formation of two-phase which is 97.33 mins. A calibration curve of different DES with BSA reagent which the corrected absorbance against protein concentration is then plotted. The protein concentration of banana was compared with the calibration curve with different DES. The results showed ATPS with ChCl: Lac (top phase) extracted the most protein which is 3678.33 μg/ml with the highest extraction efficiency of 89.60 %. This might be affected by the functional group of the HBDs, and low viscosity of banana crude extract interacts with the DES where the hydrogen bonds affect the mobility of free molecules within the system such as the movement of protein within the solvent leads to fast mass transfer of protein.

CHAPTER 1 INTRODUCTION

1.1 Research Background

Biomolecules is numerous substances produced by cells or either from living organisms and plants. Biomolecules have a variety type of molecular structure and functional group with different chemical characteristics. There are four major types of biomolecules which are carbohydrates, lipids, nucleic acids and proteins (Rogers et al., 2020). In recent decades, extraction techniques of biomolecules from natural sources such as vegetables and fruits, have increased due to their potential applications for food and nutraceutical purposes. For example, proteins are the source of amino acids for human muscle tissue and important for various type of biological functions. Each protein has their own unique function such as haemoglobin is a protein which tasked to carry oxygen throughout our body through the bloodstream. Besides that, protein produces antibodies that able to fight off infections, illnesses and keep cells healthy. Some of the most important proteins includes enzymes and hormones (Haurowitz & Lotha, 2020).

Protein enzyme is among the important biomolecules for plants and animal cells which could benefit the human. Enzymes are biological catalysts to accelerate chemical reactions in our body such as digestion, liver function and many other. For example, bananas are fruits that contains natural digestive enzymes to digest complex starches into easily absorbed sugars. The enzymes in the banana will be more active when bananas start to ripen (Raman, 2018). This is proven by a two-month study with 34 women at the connection between eating bananas and the growth of healthy gut bacteria. The result in eating bananas showed daily experienced a modest, non-significant rise in healthy gut bacteria but it experienced less bloating (Mitsou et al.,

2011). Therefore, yellow bananas are much sweeter than unripe green bananas, yet it is good for digestion (Raman, 2018)

The enzyme can be extracted from plant or fruit extract via liquid extraction and precipitation process. This process usually requires the help of aqueous mixture which is either the aqueous binary, ternary or quaternary two-phase systems (ATPS). Ternary mixture such as deep eutectic solvent (DES) and natural deep eutectic solvent (NADES) have gain much attention for biomolecules extraction including the protein's enzyme. The natural-based term emphasizes the use of natural derived compounds such as urea, citric acid, glucose, amino acids and many other.

Ternary mixture such as those known as DES and NADES are eutectic mixture. The term eutectic is actually coming from the Greek 'eutektos' which means easily melted. (Kay, 2012) Eutectic mixture is made up of at least two solid components that change phase from solid to liquid at a certain composition due to depression of it melting temperature. DES are eutectic mixtures in which the melting temperature of the mixtures at the eutectic point is significantly lower than the melting temperature of the pure components (Alhadid et al., 2020). Currently deep eutectic mixture (commonly known as DES) has been developed for extraction and purification process because they have superior properties than the well-known ionic liquids due to the biodegradability, non-toxicity, and low cost. Similarly, NADES are bio-based DES which composed of two or more compounds that is plant based primary metabolites, such as organic acids, sugars, alcohol, amines and amino acids (Liu et al., 2018). The utilization of natural components in the former example is the main distinction between DES and NADES, although the nature of their interactions is similar.

Greener solvents are recommended in chemical industries for a more sustainable world, thus new technologies to sustain our environment and minimizes the energy are introduced and researched. Several research had been done recently on the application of DES or NADES for enzyme extraction. For examples, extraction of bromelain from pineapples, extraction of amylases from bananas and extraction of hydroxytyrosol from olive leaves using NADES (Zurob et al., 2020).

In my opinion, development of effective enzyme extraction plays an important role in recent years. The uses of enzymes can improve the value, quality and effectiveness of food products in existing applications and expand the uses in feed and food industries which able to improve the health of humans in terms of nutrient consumption. Besides that, DES and NADES which also known as 'green solvents' are preferred in enzyme extraction because it has the potential of green approach due to their higher biodegradability, lower toxicity and lower environmental impact.

1.2 Problem Statement

Bioseparation is a process of separation and purification of a biological molecule from crude bulk solution. In particular, protein-based bioseparation is a very complex process that includes, precipitation, ultracentrifugation, ultrafiltration and size exclusion chromatography. For example, alcohol usually used in recovery process and precipitation in biphasic ternary mixture of aqueous salt-polymer for ionic protein and organic solvent sensitive protein. Alcohol includes methanol, ethanol and isopropanol, and excessive dehydration by salts will affect and reduce the bioactivity of the protein due to excessive change of their native structure. For an enzyme, its biological activity is priority for catalytic application. Therefore, a ternary mixture is proposed to provide stabilization via hydrogen bond fixation on enzyme protein structure during the precipitation process.

Proteins in three-dimensional structure have weak interactions and very sensitive molecules. The hydrogen bond interaction and disulfide bonds of the amino acid side chain are responsible in stabilizing the protein and play an important role in protein cryopreservation and formulation. Aqueous biphasic systems (ABS) formed by polymers such as PEG are used in extraction and purification of proteins. Tons of researches has proven that ABS system able to maintain the structure and functional stability of protein. Each of the component has its own purposes, where water as a solvent, salt or polymers as phase forming agent while HBD roles are either as phase additive or properties modifier.

There are two types of hydrogen bonding which is the intramolecular hydrogen bonding and intermolecular hydrogen bonding. During the enzyme extraction, there are various types of HBDs to be chosen. Thus, we need to study the stability of protein structure with the HBDs in the ABS by understanding its correlations with their

physical and chemical properties. NADES is a combination of two or three natural, renewable, biodegradable and inexpensive natural substances with specific interactions such as ionic, polar and hydrogen bonding. NADES is a very promising aqueous solution for bioseparation that can stabilize the protein instead of using alcohol. Thus, understanding each of the roles (bioseparation, proteins, HBD, HBA, etc.) may enhance the extraction process of protein-based compounds such as enzymes, DNA, antibodies, and many others.

1.3 Research Objectives

The objectives of this research are to extract by using a polymer/salt/water system. The objectives include the following detailed studies.

i. To investigate the effect of HBD and DES on the formation of ATPS.
ii. To study the feasibility of ATPS for enzyme protein extraction from banana.

CHAPTER 2 LITERATURE REVIEW

2.1 Biomolecules

Biomolecules is numerous substances produced by cells or either from living organisms and plants. Biomolecules have a variety type of molecular structure and functional group with different chemical characteristics. Carbohydrates, lipids, nucleic acids, and proteins are the four major categories of biomolecules (Rogers et al., 2020). Biomolecule extraction techniques from natural sources such as vegetables and fruits have grown in popularity in recent decades as a result of their potential applications in food and nutraceuticals purposes. Proteins, for example, provide a supply of amino acids for human muscle tissue and are essential for a variety of biological processes. Each protein serves a specific purpose, for example, haemoglobin is a protein that is responsible for transporting oxygen throughout our bodies via the bloodstream. Protein also generates antibodies, which are capable of fighting infections, diseases, and maintaining cell health. Enzymes and hormones are two of the most important proteins (Haurowitz & Lotha, 2020).

Protein enzyme is one of the most important macromolecules for plant and animal cells, and it has the potential to assist humans. Enzymes are biological catalysts that help our bodies speed up chemical reactions like digestion and liver function. Bananas, for example, are fruits that contain natural digestive enzymes that help to break down complicated carbohydrates into sugars that are easily absorbed. When bananas begin to ripen, the enzymes in them become more active (Raman, 2018). This is proven by a two-month study with 34 women at the connection between eating bananas and the growth of healthy gut bacteria. The result in eating bananas showed daily experienced a modest, non-significant rise in healthy gut bacteria but it experienced less bloating (Mitsou et al., 2011).

Liquid extraction and precipitation can be used to extract the enzyme from plant or fruit extract. This procedure normally necessitates the use of an aqueous mixture, which can be either binary, ternary, or quaternary systems. Ternary mixtures like DES and NADES have gotten a lot of attention for extracting biomolecules, including protein enzymes.

2.2 Ternary Mixture

In the last few decades, enzyme extraction used ionic liquids (ILs) as solvent. ILs was constrained by their high cost due to their poor biodegradability, biocompatibility, and production sustainability (A. Paiva et. al., 2014). A new generation of solvents, called deep eutectic solvents (DES) was introduced and it is more environmentally friendly and lower toxicity compared to ILs. After 2010, NADES was introduced and showed even more environmentally friendly compared to DES due to their easy renewal (Y.H. Choi et. al., 2011).

Ternary mixture such as those known as DES and NADES are eutectic mixture. The term eutectic is coming from the Greek ‘eutektos’ which means easily melted. (Kay, 2012) Eutectic mixture is made up of at least two solid components that change phase from solid to liquid at a certain composition due to depression of it melting temperature. DES are eutectic mixtures in which the melting temperature of the mixtures at the eutectic point is significantly lower than the melting temperature of the pure components (Alhadid et al., 2020). Currently deep eutectic mixture (commonly known as DES) has been developed for extraction and purification process because they have superior properties than the well-known ILs due to the biodegradability, non-toxicity, and low cost. Similarly, NADES are bio-based DES which composed of two or more compounds that is plant based primary metabolites,

such as organic acids, sugars, alcohol, amines, and amino acids (Liu et. Al, 2018). The major difference between DES and NADES is the use of the natural components in the former case but the nature of the interactions between DES and NADES is similar.

2.3 Deep Eutectic Solvent (DES)

DESs are widely known as a new class of ionic liquid (IL) analogues because they share many characteristics and properties with ILs. A large variety of anionic and cationic species can be found in DESs, which are eutectic mixes of Lewis or Bronsted acids and bases. Abbot and co-workers released a paper on the study in 2001 that a mixture of a choline chloride and a metal salt could form a liquid at temperature below 100 °C (Abbott et al., 2001). Two years later, the same group created DES, a combination of ChCl and a hydrogen bond donor (HBD). In the years since, it has been reported that DES is produced by combining ChCl with various carboxylic acids (Abbott et al., 2004).

DES are easily produced without any complicated purification steps by mixing two or more compounds and heating them to around 80 °C (Abbott et al., 2003 & Imperato et al., 2005). DES contain large, nonsymmetric ions that have low lattice energy which has leads to low melting points. A quaternary ammonium salt is frequently complexed with a metal salt or HBD to produce DES. The charge delocalization that results from hydrogen bonding, such as between a halide ion and the HBD moiety, lowers the melting temperatures of the separate components. In 2001, a study by Abbott et al. where quaternary ammonium salts were heated with zinc chloride and the resulting liquids measured was found the lowest melting point, 23-25 C, when choline chloride was used as the ammonium salt (Abbott et al., 2011).

One of the most important characteristics of DES is their ability to be employed as an extracting solvent for a variety of chemically distinct solutes (Zhang et al., 2012). The physical properties of these solvents, such as viscosity, density, miscibility, and polarity, determine their function as extraction solvents. Viscosity of the individual components of the intensive hydrogen-bonding between the DES mixture and the interfacial tension force between the components have an important role to form the homogenous mixture. The abilities of DES in phase-forming are associated with their affinity for water molecule. DES with a lower lattice energy will tend to have smaller interactions with the chloride anion. Besides that, functional group of HBDs play an important role in the formation of two-phase where formation hydrogen bond it affects the abilities of DES in phase-forming is associated with their affinity for water molecule. HBDs with lower hydrophilicity had a significant contribution on the two-phase region whereas HBDs with a higher relative hydrophilicity have a small effect on the two-phase formation.

Choosing solvents with a low viscosity to facilitate mixing and a significant density difference from the matrix to permit phase separation (Huddleston, J. G. et al., 2015). The melting points of each of the components that make up the DES will typically be lower than the melting point of the DES mixture. The freezing point is determined by the DES components (HBD and HBA type) as well as the molar ratio. Aside from that, because to its high viscosity, DES has low conductivity. However, when the temperature rises, the viscosity of the material decreases, which causes the conductivities of DES to increase sharply (Zhang et al., 2012, Tang et al., 2015 & Abbott et al., 2011).

2.4 Natural Deep Eutectic Solvent (NADES)

The viscosities of the NADES produced from glucose-citric acid-water, fructose-citric acid-water, betaine-citric acid, and choline chloride (ChCl)-CA are substantially greater, ranging from 14,480 to 437,768 cP. Glucose forms a stronger hydrogen bond with ChCl will cause a higher viscosity which affects the properties of the NADES. Moreover, the influence of NADES on the reaction kinetics shows that the conductivity of a binary NADES system can considerably increase as the kinetic energy rises the intermolecular cohesive forces contribute to liquid viscosity (Stefanovic, et. al., 2017). So, viscosity of a NADES species is very sensitive to kinetic energy (Zhao et al., 2015).

Greener solvents are recommended in chemical industries for a more sustainable world, thus new technologies to sustain our environment and minimizes the energy are introduced and researched. Several research had been done recently on the application of DES or NADES for enzyme extraction. For examples, extraction of bromelain from pineapples, extraction of amylases from bananas and extraction of hydroxytyrosol from olive leaves using NADES (Zurob et al., 2020).

2.5 Applications of NADES

In recent years, NADES applications have been introduced. However, the fundamental structural component of NADES media depends on the interactions between their constituent molecules, which are regulated by several variables, including water content, temperature, and component ratio. NADES as extraction media, includes the separation of the target analytes for the extraction of proteins. NADES have a high ability to extract phenolic compounds which relates to the hydrogen bond interactions that are established between the phenolic compounds and

the NADES molecules. For example, a study shows that the extraction of phenolic compounds from safflower by using different NADES, lactic acid: glucose, glucose: choline chloride and fructose: glucose: sucrose (Paiva et al., 2014). NADES species have demonstrated good chromatographic selectivity for the separation of natural products (NPs), they are capable of selectively extracting natural products NPs in high quantities.

The need for green solvents in the worldwide scientific and industry communities has led to a fast increase in NADES investigations in the chemical and natural product literature since 2011. For certain NPs, NADES shows strong solubilizing selectivity, which poses both possibilities and difficulties in the applications. This describes how Nature creates chemicals that are lipophilic without the use of organic solvents. These NADES species might not be suitable solvent candidates for a general extraction purpose in natural product research (Liu Y. et al., 2018).

2.6 Aqueous Two-Phase System (ATPS)

In the purification or separation of biological materials, the ATPS is an excellent alternative to organic-water systems (Gu. et. al., 2014). ATPS is made up of water and two hydrophilic components, which are commonly two incompatible polymers, a polymer, and a salt, or two salts, that when dissolved in water create a biphasic system (Asenjo et. al., 2012). Beijerinck was the first to introduce ATPS in 1896 (Beijerinck et. al. 1896). The formation of two-phase is greatly affected by the properties of the HBA and HBDs in terms of its polarity, viscosity, density, miscibility, and extraction efficiency. (Mehariya *et al.*, 2021) The functional group of HBDs play an important role in the formation of two-phase where the formation of

the number and the strength of the hydrogen bond in ATPS. For example, glycerol and glucose have hydroxyl group (-OH) and aldehyde group while lactic acid has functional group of carboxylic group (-COOH) and urea has a carbonyl group (-C=O).

Mass transfer occurs quickly in these systems due to the low interfacial tension. Another benefit of ATPS is its selectivity, which allows it to be used to separate a certain component from the rest of the system (Roosta et. al., 2016). Because of these characteristics, ATPS is now used in continuous operations and large-scale processes (Selber et. al., 2004). The potential of various ATPS in the separation and purification of biomolecules, such as proteins, antibiotics, enzymes, and many others, has recently been studied.

2.6.1 Effect of type salts in ATPS

Polymer or a polymer and a salt are the most typical biphasic systems. ILs and short-chain alcohols are two further examples (Grilo, et al., 2016, Molino, J. V. D., et al., 2013 & Ruiz-Ruiz, F., et al., 2012). The presence of salt alters phase behaviour, resulting in variations depending on the type and concentration of salt while the only disadvantage of the polymer-salts system is its high ionic strength (Albertsson, 1961).

BSA's salt-out effect enhanced the hydrophobicity of the bottom phase in the presence of relatively high salt concentration, which significantly reduced the solubility of BSA in the bottom due to competition from the huge number of salt ions with BSA for water molecules. The salting-out power of the cations was examined utilising the binodal curve data as the anion of these salts (SO_4^{2-} and H_2PO^4) where Na_2SO_4 had the highest value compared to Li_2SO_4 and K_2SO_4 due to the lower polymer concentrations. Because Na^+ has a greater salting-out effect than the cations K^+ and Li^+, the Na^+ cations were more successful in the creation of ATPS. As the free energy

of hydration (ΔG_{hyd}) value for K^+ was -295 kJ/mol while the (ΔG_{hyd}) value for Na^+ was -365 kJ/mol. So, when the value of (ΔG_{hyd}) lower , the larger the hydration shell which will contribute to the salting-out effect by reducing the amount of water available to hydrate the polymer.

The existence of neutral salts like NaCl has no significant impact on ATPS' liquid-liquid equilibrium data. However, at high salt concentrations (more than 1M), the phase diagram changes. Because of the variable distribution of salt ions across the phases, the presence of NaCl in ATPS changes the partition coefficient. Different hydrophobicity ions are present in the added salt. Hydrophobic ions force the partitioning of their counter ions to the more hydrophobic phase, and vice versa (Saravanan, S. et al., 2006). Biomolecules go through a salting-out process that changes their state from salt-rich to PEG-rich. Since the structure and function of proteins were maintained by the hydrogen bonding linkage between the amino acid residues and water, the rising salt concentration resulted in the reduction of the water content of the DES-rich top phase, and the protein tended to enter the salt-rich phase.

2.6.2 Effect of different PEG Concentrations in ATPS

PEG, ($HO\text{-}(CH_2CH_2O)_n\text{-}H$), with molecular weights ranging from 200 to 10,000. At room temperature, the water-soluble and hygroscopic polymer is a waxy, white solid with a molecular weight of 800 and an indistinct, viscous liquid with a molecular weight of 600 (Raja, S. et al, 2011). Solid PEG is extremely soluble in water, and liquid PEG is miscible with water in all proportions. For example, PEG-2000 has a solubility of roughly 60% in water at 20 °C. With or without the addition of water, lower molecular weight liquid PEG can be employed as solvents on their own. Aside from that, PEG also has low flammability and it is biodegradable.

PEG plays an important role on partitioning the protein in ATPS because the partitioning of biomolecules in the PEG/Salt system is driven by the volume exclusion effect (polymer-rich) and the salting-out effect (salt-rich). The influence of both volume exclusion and salting out effect would result in partitioning of biomolecules in the interphase in systems with high concentration or high molecular weight polymer and high salt concentration (Raja, et al, 2011). The lower and higher critical solution temperatures, which are suggestive of a miscibility gap in aqueous PEG solutions, are the temperatures at which the polymer and water split into polymer-rich and polymer-poor phases. Theoretically, this behaviour is truly explained by the polymer chain's amphipathic properties, which include hydrophobic ethylene groups interspersed with ether groups that can mimic the hydrogen-linked structure of water. The salting effect of the salts caused the cloud point to decrease, which led to PEG phase separation.

PEG is inexpensive and has the ability to form a two-phase system with other neutral polymers and salts. PEG is so frequently employed in ATPS as one of the phase-forming polymers. PEG has also been demonstrated to significantly enhance protein refolding and activity recovery (Cleland, et al, 1992). As a result of the hydrophobic interaction between the biomolecule and PEG, a high PEG concentration increases the number of polymer units involved in bio-molecular partitioning, which leads to a higher number of biomolecules partitioning into the PEG phase (Paiva, et al., 2014).

2.6.3 Effect of the concentration of DES in ATPS

The efficiency of the enzyme extraction and ATPS formation are greatly influenced by the type and concentration of DES. For instance, an ATPS using ChCl-G/K2HPO4 (0.9 g mL1, 2.0 mL) and 10 mg BSA demonstrates that the extraction efficiency increased significantly when the amount of DES changed between 1.0g and 1.3g, then dropped as the amount of DES increased. The extraction efficiency could be as high as 95.67 %. The ability of the DES and protein to combine in top phase was the mechanism for this alteration. However, because of the significantly increased concentration of DES and the resulting high viscosity of the top phase solution, BSA was unable to move into the top phase.

As a result, there is a chance that DES and protein molecules will coalesce in the ABS top phase, causing more bovine serum albumin to be aggregated by more DES micelles. As the concentration of DES increases, the top phase becomes more viscous and there is little space for protein partitioning, which lowers extraction efficiency. Additionally, increasing the DES concentration from 1.0 g to 1.5 g causes the phase volume ratio to rise from 0.5 to 0.73 because more DES molecules bind to the water in the salt phase, which causes the top phase volume to rise. This is because DES's affinity for water was increased by its high concentration. (Xu *et al.*, 2015)

2.6.4 Application of ATPS

ATPS has been used to recover proteins, enzymes, amino acids, antibiotics, plasmids, DNA, and nanoparticles, among other biomolecules. ATPS is now widely employed in the production of high-value biopharmaceuticals such as monoclonal antibodies, growth factors, and hormones. Recently, researchers investigated the feasibility of employing ATPS to purify biopharmaceuticals as an alternative to

traditional chromatography techniques (Azevedo, et al., 2009 & Rosa, et al., 2009). Some of the most recent applications include aqueous two phase flotation (ATPF), membrane supported ATPS (MEMEX), and extractive fermentation.

In terms of cost, downstream processing is a critical step in the purification and separation of biomolecules. Protein processing several purification processes due to the complexity of the starting material (Grilo, et al, 2016 & Silva, & Franco, 2000). As a result, the demand for a high-yielding and cost-effective purification process is growing. Due to batch processing and high pressure drops, protein chromatography is not a viable technology for use on a wide scale. Because of their limited solubility in these systems, aqueous solutions containing organic solvents are not recommended for protein purification (Asenjo & Andrews, 2011). ATPS is being researched to solve these constraints. As a result, ATPS has been used to recover protein from crude feedstocks on a large scale, and this use of ATPS has sparked the most attention (Hatti-Kaul, 2001). The components of ATPS and their surface characteristics play a major role in protein partitioning in both stages (Asenjo & Andrews, 2011). Protein will often collect in the upper, hydrophobic, and less polar phase, which is normally PEG. With the aid of an extra salt, proteins can be separated by modifying the molecular weight of the polymer, ion type, or ionic strength in the salt phase (NaCl). The partition coefficient of protein partitioning will be affected by these modifications.

2.7 Differential Scanning Calorimetry (DSC)

DSC is a powerful analytical tool for the identification of various physical properties and thermal transitions of polymeric materials. DSC is used to estimate the melting and mesomorphic transitions along with their entropy and enthalpy.

Differential scanning calorimetry (DSC) measures the energy transferred to or from a sample undergoing a physical or chemical change. The pretransition baseline represents the change in heat capacity of the hairpin form with temperature, and the excess heat capacity during unfolding (the peak) provides a profile of the melting process to unfolded form. In theory, the unfolding data in the lower curve represent the true heat capacity of the DES sample. Higher DES sample concentrations are necessary to see the transition differences. With an increase in the proportion of high molecular mass polymers, the greatest temperature endotherm shifts upwards, but no correlation between the parameters has been found.

CHAPTER 3 MATERIALS AND METHODS

3.1 Research Plan

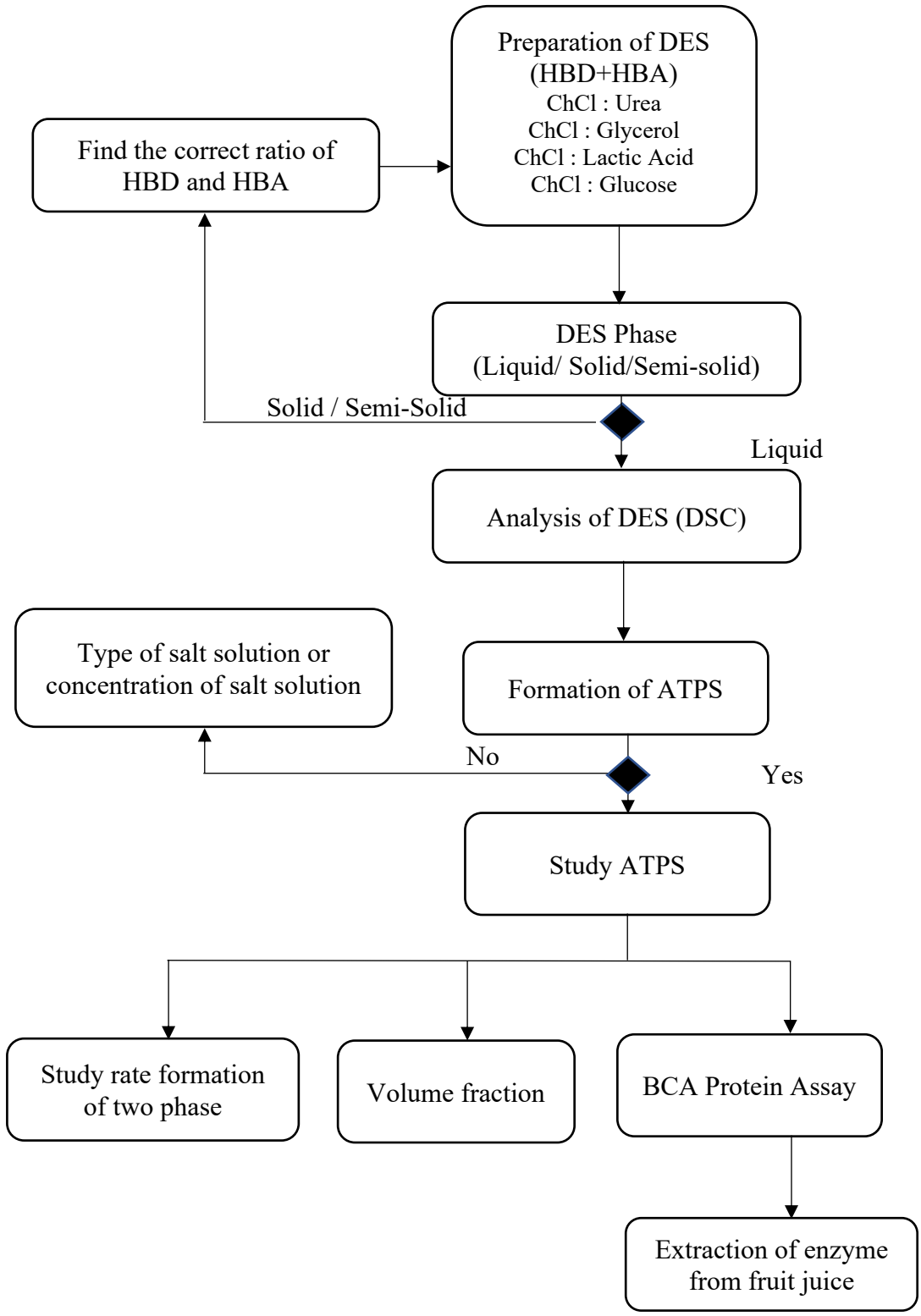

Figure 3- 1: Experimental Flow Chart.

3.1 Materials and Chemicals

Juice made from fresh bananas was purchased at the neighbourhood market. Choline chloride (ChCl), glycerol (Gly), lactic acid (Lac), urea and glucose (Glu), sodium sulphate (Na_2SO_4), potassium diphosphate (K_2SPO_4), ammonium sulphate (NH_4SO_4), and PEG 2000 were acquired from Sigma-Aldrich, Co. (United States). The Novagen BCA protein assay kit, available from Fisher Scientific, contains a standard protein with a concentration of 2 mg/ml, such as bovine serum albumin, along with a BCA solution and 4% cupric sulphate (BSA). The BCA solution has a pH of 11.25 and is composed of bicinchioninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate. Deionized water was obtained in the laboratory.

3.2 Apparatus

2 ml cuvettes, 100µL-1000µL pipette, 20µL-200µL pipette, 5µL-50µL pipette, beakers, graduated test tubes, test tube racks, containers for DES, laboratory weighing balance, stirrer, heater plate, conical flasks, filter funnel, incubators, cuvette and UV-Vis spectrometer were prepared and used in the experiment.

3.3 Preparation of Deep Eutectic Solvents (DES)

The preparation of DES was carried out by mixing the quaternary ammonium salt (Choline Chloride) and HBDs (Lactic Acid, Urea, Glycerol, and Glucose) at a specific molar ratio. The NADES was prepared according to the procedure described by Duran et. al. (Durand, et. al., 2013). The ChCl and lactic acid dried in a vacuum oven at 0.5 bar and 60°C before further use. The amount of ChCl and lactic acid is calculated and was measured on laboratory balance. The dried choline chloride and urea (ChCl: Urea) was mixed at a molar ratio of 1:1 in a sealed container.

Subsequently, the mixture was heated at 100°C and slowly stirred to form a colorless liquid after approximately two hours. The time to form the liquid depends on the total volume, temperature, and homogeneity of the mixture was recorded. This step is repeated ChCl: Urea with different molar ratio of 1:2 and 2:1 respectively.

Then, steps are repeated as above in preparing different types of NADES which are choline chloride and lactic acid (ChCl: Lactic Acid), choline chloride and glycerol (ChCl: Glycerol) and choline chloride and glucose (ChCl: Glucose) with molar ratio of 1:1 and 1:2 and 2:1 respectively.

3.4 Formation of ATPS

ATPS formed by mixing PEG, salt, and DES. Salt solution (1.0 M of Na_2SO_4) was prepared by mixing the sodium sulfate and water. 0.3M of PEG-2000 was prepared by mixing PEG with deionized water. 200uL of Na_2SO_4 and 200uL of PEG was mixed. Then, the glass tube gently was shook and placed in rack until it forms two phases. After shaking the glass tube, the time take of the formation of two phases will be recorded by using stopwatch. The height of top phase and the bottom phase was recorded by using a ruler. Next, DES was added into two phase system, shake gently, and placed the tube glass in the rack until two phases are form.

3.4.1 Effect of different type and concentration of salt in ATPS

The type of salt used were Na_2SO_4, K_2SO_4, $(NH_4)_2SO_4$. Phase ratio and rate of formation of ABS were the parameters observed to determine the best type and concentration of salt to be used in the ABS formation. ABS was prepared in a graduated test tube by mixing 0.25M of PEG and different type of salt using pipette at the volume ratio of 1:1. The different concentration of Na_2SO_4 used were 0.5M, 1.0M,

1.5M and 2.0M, while the concentration of K_2SO_4, and $(NH_4)_2SO_4$ usedwere 1.0M, 1.5M, 2.0M, 2.5M and 3.0 M respectively.1000 µL of 0.25 M of PEG was mixed with 1000 µL of 0.5 M Na_2SO_4 using pipette in a graduated test tube. The mixture was then shaked vigorously until it become cloudy. The mixture was left to be stagnant at room temperature until the formation of transparent two-phase systems was observed clearly. The rate of formation and the volume of each phase were recorded. The procedure was then repeated for different types and concentration of salt.

3.4.2 Effect of DES concentration in ATPS

The PEG 2000 concentration used were 0.1 M, 0.15 M, 0.2 M, 0.25 M, and 0.3 M. Phase ratio and rate of formation of ABS were the parameters observed to determine the optimum PEG concentration to be used in the ABS formation. ABS was prepared in a graduated test tube by mixing optimum concentration of desired salt that was determined from previous steps and different concentration of PEG using pipette at the volume ratio of 1:1. 1000 µL of optimum concentration of desired salt was mixed with 1000µL of 0.1M PEG using pipette in a graduated test tube. The mixture was then shook vigorously until it become cloudy. The mixture was left to be stagnant at room temperature until the formation of transparent two-phase systems was observed clearly. The rate of formation and the volume of each phase were recorded. The procedure was then repeated for different concentration of ABS.

3.5 Preparation of BSA standard

3.5.1 Dilutions for standard assay

The green BCA working reagent was prepared by added 400 µL of 4% cupric sulphate solution into 20 ml of BCA solution in a 50ml beaker. The general protein assay procedure was started with the preparation of a BSA standard with known concentration of protein for constructing the calibration graphs. The guidelines for preparing diluted BSA standards was illustrated in table below where the BSA was the protein used while the diluent was the DES used.

Table 3- 1: Dilution for standard assay.

Tube	Volume of BSA	Volume of diluent	Final BSA concentration
1	250 µl from 2mg/ml solution	250 µl	1000 ug/ml
2	250 µl from tube 1	250 µl	500 ug/ml
3	250 µl from tube 2	250 µl	250 ug/ml
4	300 µl from tube 3	300 µl	125 ug/ml
5	100 µl from tube 4	400 µl	25 ug/ml
6	0	400 µl	0 ug/ml

Each step will be repeated by pipette top phase and bottom respectively of the DES.

3.5.2 Preparation of BCA working reagent

Table 3- 2: BCA working reagent.

	Each sample	x 20
Test tube assay		
BCA Solution	1 ml	20 ml
4 % Cupric Sulphate	20 µl	400 µl

3.6 Protein Extraction

50 µl of each standard or protein sample was pipetted from the dilutions for standard assay's test tubes into labeled new test tubes. Add 1.0 ml BCA working reagent. Mix by gently vortexing. Then, incubate reactions at 37 °C for 30 min or at room temperature 2-16 h. Then, allow tubes to cool to room temperature. Add 1 ml water to a clean cuvette and adjust the absorbance reading at 562 nm to zero. Sample was transferred to clean cuvettes. The absorbance of all reactions within 10 mins was measured by using UV-vis spectrophotometer. The blank's absorbance from the measurements of the other standard substrated with protein samples' absorbances to get the corrected absorbances. A graph of the corrected absorbance versus the known mass of the BSA standards was plotted. The recorded corrected absorbance reading for the samples assayed which fall within the linear range of the standard curve was interpolated by using the standard curve. The amount of protein present in the original sample by correcting for the dilution and sample volume was calculated. Each step will be repeated for top phase and bottom of the DES respectively (Choline chloride: Lactic acid, Choline chloride: Glycerol and Choline chloride: Urea).

3.7 Enzyme Extraction from Banana

Bananas was purchased and blended into juice before using it. The banana's residue in the juice was filtered by using filter funnel. Salt solution was added into the banana juice to obtain 1.0 M. Then, 1000 µl was pipetted into 6 different test tubes. Next, 100 µl of DES (Top phase and bottom phase of ChCl: Gly, ChCl: Lac and ChCl: Urea respectively) was added into the solution.

50 µl each standard or protein sample was pipetted from the prepared solution of the 6 test tubes into labeled new test tubes. Add 1.0 ml BCA working reagent. Add

1.05 ml of deionized water to dilute the concentrated protein sample. Mix by gently vortexing. Then, the reactions were incubated at 37 °C for 30 mins. Then, allow tubes to cool to room temperature. 1 ml water was added to a clean cuvette and adjust the absorbance reading at 562 nm to zero. Sample was transferred to clean cuvettes. The absorbance of all reactions was measured and recorded within 10 mins. The blank's absorbance from the measurements of the other standard substrate with protein samples' absorbances to get the corrected absorbances. The corrected absorbance versus the top phase and bottom of the DES was plotted. The concentration of protein will be obtained by comparing with the calibration curve. The partition coefficient and extraction efficiency will be calculated based on the protein concentration of the top phase and bottom of ATPS.

Formula of partition coefficient, K:

$$K = \frac{Concentration\ of\ Protein\ (Top)}{Concentration\ of\ Protein\ (Bottom)}$$

Formula of extraction efficiency, E:

$$Phase\ Volume\ Ratio, R = \frac{V_t}{V_b}$$

$$E = \frac{KR}{1 + KR}$$