AQUEOUS TWO-PHASE SYSTEM OF POLYETHYLENE GLYCOL 6000 AND NACL FOR PARTITIONING BEHAVIOR OF L-CYSTINE

By

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CERTIFICATION OF APPROVAL

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Abu Raihan bin Mohamad

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CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

ABU RAIHAN BIN MOHAMAD

UNIVERSITI
TEKNOLOGI
PETRONAS

FINAL YEAR PROJECT

DISSERTATION

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Abstract

The aim of this study is to investigate the feasibility of extracting of natural rubber protein, named hevein; represented by its amino acid, cysteine (L-cystine) by using aqueous two phase system (ATPS) of polyethylene glycol of molecular weight 6000 (PEG 6000) and sodium chloride. ATPS has been proposed as an alternative method as it preserved biological activity, nontoxic, and cost-effective. Purification and extraction by using polyethylene glycol/salt ATPS is very suitable since hevein can tolerate very well with inorganic solvent. Feasibility of ATPS of PEG 6000 with sodium chloride is studied where the phase diagram is developed. A precise composition of PEG 6000 with sodium chloride from the binodal curve is mixed to form two phases system to study the partitioning behavior of L-cystine where the concentration of each component and L-cystine on both phases will be analyzed. The partition behavior is recorded with respect to PEG 6000 and NaCl concentration. The highest percentage recovery of L-cystine is observed.

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Chapter 1: Introduction

1.1 Background

1.1.1 Natural rubber

Most natural rubber in the world is obtained from a single plant species, *hevea brasiliensis* (rubber tree). Natural rubber is in bulk source as Malaysia is the world's third biggest natural rubber producer. In Southeast Asia only, from three countries which are Thailand, Indonesia and Malaysia contribute 72% of world natural rubber production. World natural rubber is forecast to rise 4.3% annually to 12.5 million metric tonne in 2013. (Agropedia, 2010). Economically, other than conventional use of natural rubber such as tires, tubes and other rubber goods, there is high value protein lies in the natural rubber latex, called *hevein*.

1.1.2 Hevein

One of the most predominant proteins found in the latex is hevein, which is compartmentalized in lutoids, the vacuolar structures of the laticifer cells. Hevein is a single chain protein of 43 amino acids, rich in *glycine* and *cysteine*. (Gidrol et al, 1993). Rubber latex contains 5-10 g/l protein, about one third of which occurs in the lutoids-body fraction. More than one half of the latter is hevein. It is a small cysteine-rich protein with a polypeptide chain length of 43 residues. Hevein is a very stable protein. (Soedjanaatmadja et al, 1995). Out of 43 residues of amino acids in hevein, 8 residues per mole is half-cystine. (Walujono et al, 1975). In this study, cysteine (L-cystine) as the most dominant amino acid in hevein is used to represent hevein as its amino acid molecules is simpler than larger protein molecules.

Hevein acts as multivalent bridge in bringing together the rubber particles by interacting with the glycosylated protein of 22 kDa (i.e. via sugar linkage) and forms aggregates. (Gidrol et al, 1993). This indicates that hevein stimulates the coagulation of latex. Furthermore, a newer study proved that hevein has a stabilizing effect on suspensions of rubber particles. (Soedjanaatmadja et al, 1999). This finding further explains higher latex yields from rubber clones containing higher hevein contents in the lutoid-body fractions. Besides that, hevein was also found to inhibit the growth of fungal. (Parijs et al, 1991).

Table 1: Amino acid analysis of hevein. (Walujono et al, 1975)

Amino acid	Residue per moles			
	Determined by Archer, (n.d)	This paper	Nearest Integer	From amino acid sequence
Aspartic acid	5.1	6.2	6	7
Thearonine	0.9	0.9	1	1
Serine	5.0	3.7	4	4
Glutamic acid	3.9	5.5	5-6	6
Proline	2.1	1.9	2	2
Glycine	3.9	4.9	5	5
Alanine	0.8	1.0	1	1
Half-cystine	**8.0**	**7.9**	**8**	**8**
Leucine	2.1	1.9	2	2
Tyrosine	1.9	1.0	1	1
Lycine	2.1	2.0	2	2
Histidine	1.4	1.4	1-2	1
Arginine	1.9	1.0	1	1
Tryptophan	2.3	2.0	2	2
Total residues	42.1	42.2	41-43	43

1.1.3 Aqueous Two-phase System (ATPS)

There are a few possible ways to recover hevein. B.L Archer (1960) has done an elementary analysis of precipitation and paper electrophoresis to isolate and characterizes crystalline hevein. A year later, A. Kanukaran et al (1961) proposed starch gel electrophoresis and ion exchange chromatography techniques to separate the protein and analyst amino acid contents. This method of extraction and purification was also adopted by Walujono and co-workers (1975) to investigate the sequence of amino acid in hevein. S. J. Tata (1980) isolated the protein by using fractional precipitation with ammonium sulphate and by ion exchange chromatography. However, the techniques mentioned above are most required expensive chemicals and equipments. The current protein extraction technique, chromatography has practical and cost limitation for high value protein production where it requires rapid turn-over of the packing materials and high maintenance cost of the column. (Harrison et al, 2003)

ATPS is a liquid-liquid extraction technique that consists of two liquid phases of two immiscible and hydrophilic phase-forming agents which are added together in a certain proportions or conditions that exceed a critical and are less than a maximum concentration, temperature or pH. The agents can be two polymer solutions in which the phase extraction phenomenon is induced by incompatibility; or a-polymer-and-a-lyotropic-salt solutions by Coulombic interactions (Kenkare, Hall, & Caccamo, 1995); or two thermoseparating polymer solutions by thermal energy; or two pH-responsive polymer solutions by pH changes. Each of these water-rich phases dominantly contains one of the species. ATPS holds has many advantages as an inexpensive yet efficient large-scale liquid-liquid extraction technique for protein extraction: ATPS provides mild environment for proteins, can be integrated with upstream processes, it can handle high throughput, highly selective hence offering high yield, and most importantly, it costs cheaper than any other conventional techniques. (Ahmad, Hauan, & Przbycien, 2011)

1.1.4 The phase diagram

The *phase diagram* shows the potential working area for a particular two-phase system and works as "fingerprint" unique to that system under set conditions of, for example, pH, temperature and polymer or salt concentration. Information can be generated from such a diagram (refer Figure 1) includes: the concentration of phase-forming components necessary to form a system with two-phases that are in equilibrium, the subsequent concentration of phase components in the top and bottom phases, and the ratio of phase volumes. Present on the diagram is a *binodal* curve, which divides a region of component concentrations that will form two immiscible aqueous phases (i.e., above the curve) from those that will form one phase (i.e., at and below the curve). (Kaul, 2000)

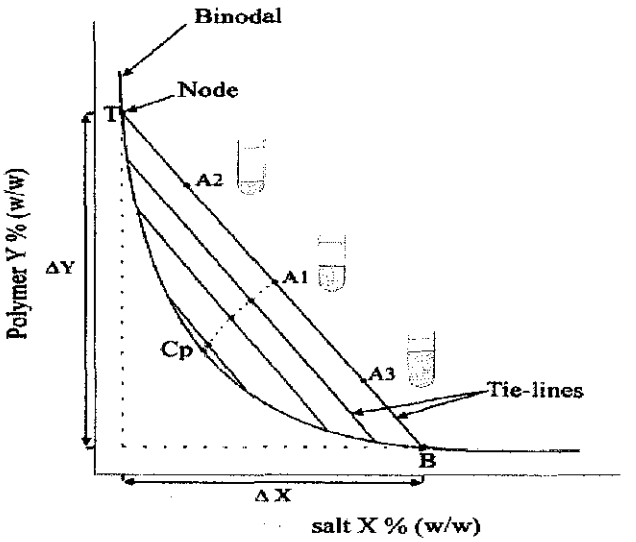


Figure 1: The Phase Diagram. (Kaul, 2000)

Bottom phase salt X (% w/w) is plotted on the abscissa and top phase polymer Y (% w/w) is plotted on the ordinate, where A2 and A3 represent the total compositions of three systems lying on the same tie-line with different volume ratios. The final composition of the top and bottom phase is represented by nodes T and B, respectively. The ratio of the segments AB (top phase) and AT (bottom phase) can be approximated graphically by the volume ratio of the two-phases. The critical point. Cp, is where the tie-line length (TLL) = 0. (Kaul, 2000).

1.2 Problem Statement

As the existing high-value protein extraction techniques have limitations practically and costly, we need to come up with an inexpensive yet efficient protein extraction method. ATPS is an efficient and inexpensive large scale liquid-liquid extraction technique for protein extraction. We want to investigate the feasibility of polymer-salt ATPS of PEG 6000 and sodium chloride where a precise composition of PEG 6000 with sodium chloride from the binodal curve is mixed to form two phases system for recovery of L-cystine which represents hevein. The concentration of each component on both phases will be analyzed to determine the L-cystine partitioning behavior where L-cystine is mixed in the solution of the ATPS. The partition coefficient is recorded with respect to PEG 6000 and NaCl concentration. The highest percentage recovery of L-cystine will be observed.

1.3 Objectives

This study embarks on the following objectives:

1. To extract hevein from natural rubber available in Malaysia.
2. To characterize and measure hevein amount and compositions in natural rubber.
3. To investigate the feasibility of PEG 6000-NaCl aqueous two-phase system in protein recovery and the composition required for two phases to occur using the phase diagram.
4. To investigate the partitioning behavior of L-cystine in terms of polymer and salt concentration.

1.4 Scope of the study

In order to achieve the objective of this study, the scope consists studying the feasibility of aqueous two-phase system of PEG 6000 and NaCl for partitioning behavior of the most dominant amino acid in hevein, cysteine. In this study, cysteine is represented by cystine (L-cystine) which is a crystalline, sulfur containing amino acid, formed by two molecule of amino acid cysteine. ATPS will be formed by adding polyethylene glycol with molecular weight 6000 and sodium chloride at different compositions to get the binodal curve. The recovery performance in terms of partition coefficient will be recorded with respect to the effect of PEG 6000 and sodium chloride concentration.

1.5 Significance of the project

This study is expected to develop research-scale experiments setup to investigate the recovery of high-value byproducts from industrial waste using liquid-liquid extraction based on common polymer and salts. Characterization protocol and analytical equipment setup for polymer, salt and L-cystine is developed in this study to produce expertise in liquid-liquid extraction technique based on common polymers and salts.

Chapter 2: Literature Reviews

2.1 Hevein

Table 2: Composition of protein in natural rubber latex and amino acids of hevein.

References	Findings	Analysis
Walujono et al, 1975	Out of 43 residues of amino acids in hevein, 8 residues per mole is cysteine, 5 residues per mole is glycine while 7, 6 and 4 residues per mole are aspartic acid, glutamic acid and serine, respectively	The most amino acids residues in hevein are cystiene.
Gidrol et al, 1993	One of the most predominant proteins found in the latex is hevein, which is compartmentalized in lutoids, the vacuolar structures of the laticifer cells. Hevein is a single chain protein of 43 amino acids, rich in glycine and cysteine.	Hevein is the most dominant protein in natural rubber, rich in glycine and cysteine
Soedjanaatmadja et al, 1995	Rubber latex contains 5-10 g/l protein, about one third of which occurs in the lutoids-body fraction. More than one half of the latter is hevein. It is a small cysteine-rich protein with a polypeptide chain length of 43 residues.	Hevein is a cysteine rich protein with 43 amino acid residues

2.2 Protein recovery via aqueous two-phase system

Table 3: Previous study on protein recovery via ATPS

References	ATPES System	Findings	Analysis
Klomklau et al, 2005	PEG 1000 - various salt	The best salt for ATPS of tuna protein extraction is magnesium sulphate: optimum concentration at 20% PEG and 15% salt.	One of the best salts for protein extraction is $MgSO_4$.
Nitsawang et al, 2006	PEG 6000 – ammonium sulphate	Optimum condition for cysteine recovery : 8% PEG, 15%salt, 20 or 40mg /ml protein, and pH 5	Optimum polymer, salt and protein concentration for cysteine recovery.
Khadpoon et al, 2010	PEG 1000 – ammonium sulphate	Optimum condition for *Calotropic procera* protease recovery: 10% PEG, 20% salt, pH at 8 and temperature is 60°C	Optimum polymer, salt and protein concentration for cysteine recovery.
Li M et al, 2010	PEG 6000 – NaH_2PO_4/K_2HPO_4	Optimum condition for cysteine recovery: 14.33-17.65% PEG, 14.27-14.42% salt, pH 5.77-6.30 and temperature 20°C.	Optimum polymer, salt and protein concentration for cysteine recovery.

2.3 PEG – NaCl binodal curve

Ho-Gurierrez et al (1994) have conducted experiment to determine the phase diagram of water, polyethylene glycol molecular weight 8000 and sodium chloride at 333 K where they have found that the top phase were rich in PEG and low salt, while the bottom phase contains most of the salts and little polymer. This study proved that aqueous two-phase system of PEG 8000 – sodium chloride is feasible at 333K. Region A is one liquid phase, region B is two-liquid phases while region C is liquid and solid phase.

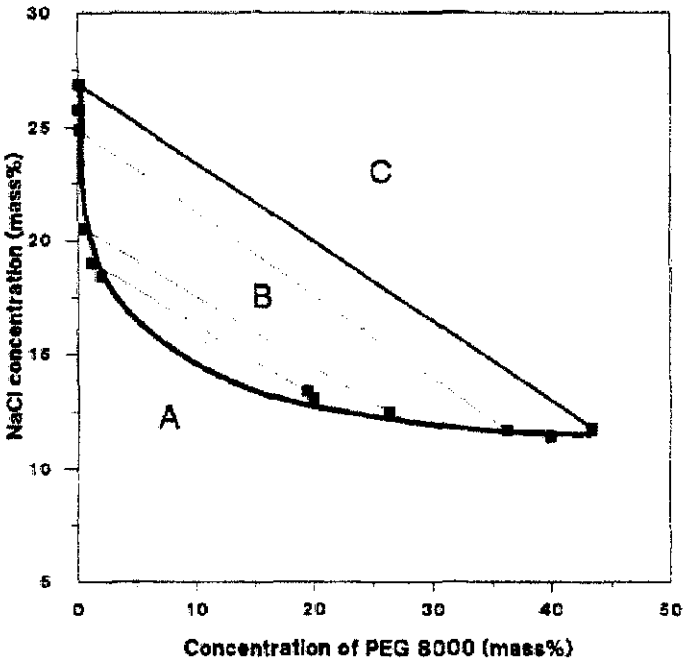


Figure 2: Binodal curve of water + PEG 8000 + NaCl at 333K

Chapter 3: Methodology

3.1 Research Methodology

Stage 1: Project planning and literature reviews

- Planning for the project is done and literature review on important aspects of the project such as natural rubber, protein extraction and ATPS briefly done.
- Identification other possible high-value protein extraction techniques currently used in the market and their limitations to compare with aqueous two-phase system method.

Stage 2: Study on natural rubber and hevein composition

- Collect data on protein composition in natural rubber and amino acid composition of hevein to identify the most dominant protein in natural rubber and amino acid in hevein.
- Select the amino acid of hevein to represent hevein for protein extraction. (L-Cystine selected).

Stage 3: Screening and identifying the ATPS

- Screen and identify the suitable polymer and salt for ATPS system; which give high efficiency of protein recovery via literature reviews.
- Study of factors affecting the aqueous two-phase system such as polymer, salt or protein concentration, mixture pH and temperature.

Stage 4: Preparation of tools, equipment and stock solutions

- Identification of tools which will be used in the experiments.

- Identification of equipments and methods of quantifying to be used to analyze the standard solution and protein.
- Stock solutions of PEG 6000 (50 % w/w) and NaCl (25% w/w) are prepared to be used for experiments. PEG solution is stored at 4°C while NaCl solution stored at room temperature. Solution for L-cystine is prepared fresh before used.

Stage 5: Development of PEG 6000, NaCl standard curve

- Standard (calibration curve) of PEG 6000 and NaCl solutions are prepared through dilution method from the stock solutions.
- PEG 6000 solutions (0.05 – 45% w/w) is prepared and analyzed using refractometer.
- NaCl solutions (0.01 – 0.20% w/w) is prepared and analyzed using conductivity meter.

Stage 6: Development of PEG 6000 – NaCl binodal curve

- Choosing correct method to develop the binodal curve of PEG 6000 - NaCl at 298.15 K.
- Binodal curve of PEG 6000 – NaCl is developed to determine the phase where the mixture forms two liquid phases at certain PEG 6000 and salt compositions.
- PEG 6000 and NaCl feasible concentration range for ATPS is determined.

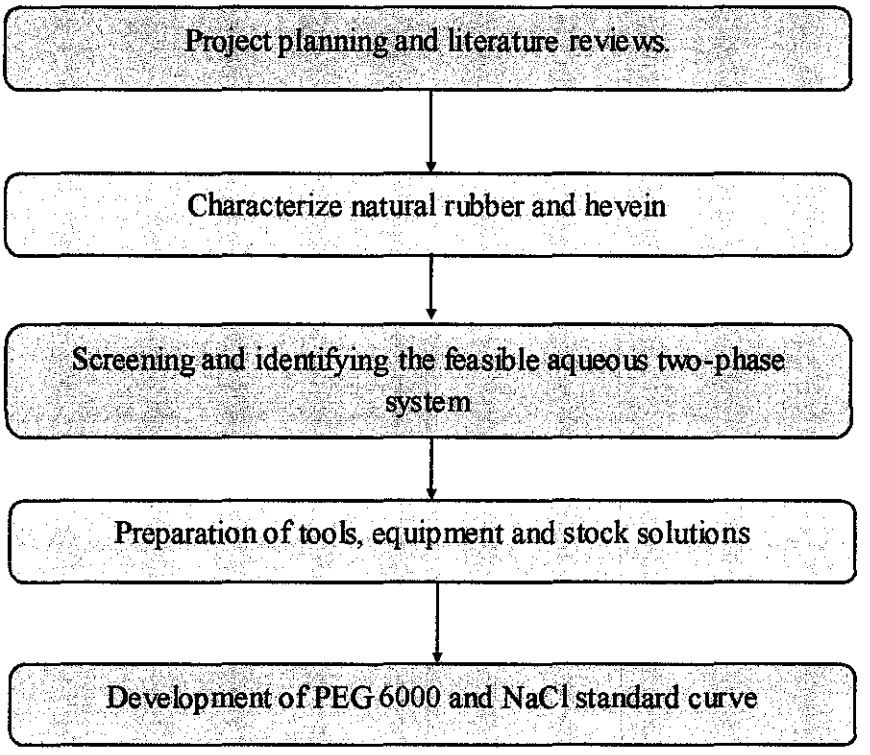
Stage 7: Development of L-cystine standard curve for partitioning behavior

- Standard curve for L-cystine is prepared by reacting solution of L-cystine hydrochloride with ninhydrin reagent.
- Solutions of L-cystine from 300 to 1800 ppm are prepared fresh, each mixed with ninhydrin reagent before analyzed using UV-vis spectrophotometer at peak wavelength.

Stage 8: Investigation of partitioning behavior of L-cystine

- Partitioning behavior of L-cystine will be studied based on different parameters, PEG 6000 and NaCl concentration. Range where the mixture become two-phase is obtained from the binodal curve. Experiment is at room temperature and pH is maintained using pH buffer.
- Partitioning behavior of L-cystine under different polymer and salt concetration will be studied.

The summary of research methodologies will be summarized in Figure 2.



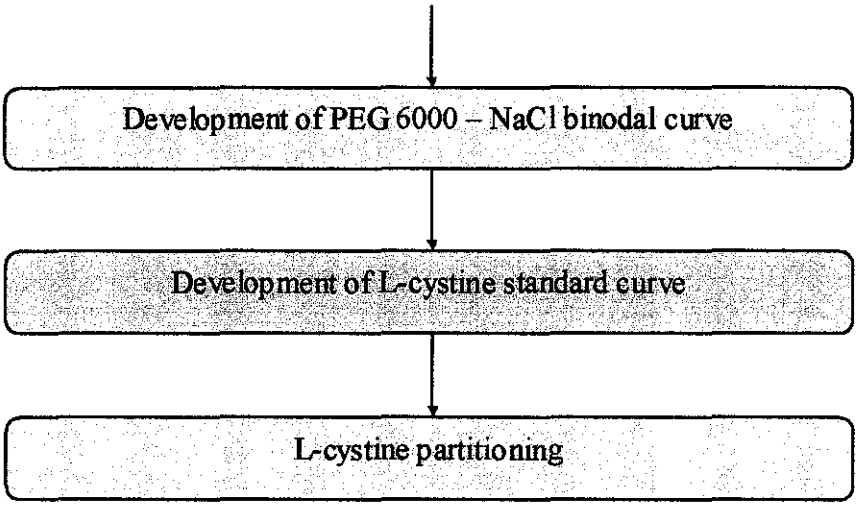


Figure 3: Research methodologies.

3.2 Experimental Procedures

3.2.1 Preparation of PEG 6000 standard curve.

1. To prepare 50% (w/w) PEG solution, 50 g of PEG 6000 is weighted into a volumetric flask and water is added until the total weight is 100g.

2. Dilution method is used to prepare PEG solution at different weight percentage.

Table 4: Dilution table for different wt% of PEG solution

PEG 6000			
wt %	Desired volume (ml)	Volume from stock solution (ml)	Volume from stock solution (µl)
40	5	4.0000	4000.0000
30	5	3.0000	3000.0000
25	5	2.5000	2500.0000
15	5	1.5000	1500.0000
10	5	1.0000	1000.0000
5	5	0.5000	500.0000
3	5	0.3000	300.0000
1	5	0.1000	100.0000
0.5	5	0.0500	50.0000
0.1	5	0.0100	10.0000
0.05	5	0.0050	5.0000

3. The refractive indexes of all solutions are measured using refractometer and all results is recorded and analyzed.

3.2.2 Preparation of NaCl standard curve

1. To prepare 25% NaCl salt solution;

$$25NaCl \times \frac{58.44NaCl}{58.44NaCl} = 25gNaCl$$

2. 25 g of NaCl weighted into a volumetric flask and water is added until the total weight is 100g.

3. Dilution method is used to prepare salt solution at different concentration.

Table 5: Dilution table for different wt % of NaCl solutions

Salt (Nacl)			
wt %	desired volume (ml)	volume from stock solution (ml)	volume from stock solution (µl)
20	10	8	8000
15	10	6	6000
10	10	4	4000
5	10	2	2000
3	10	1.2	1200
1	10	0.4	400
0.1	10	0.04	40
0.05	10	0.02	20
0.01	10	0.004	4

4. All solutions are measured using conductivity meter (Hach Sesion5) and all results are recorded and analyzed.

3.2.3 Preparation of PEG 6000 – NaCl binodal curve

1. Node determination method is used to develop the binodal curve of PEG 6000 – NaCl.
2. A series of system is prepared and the phase composition at the top and bottom phase is analyzed.
3. Starting with 6% (w/w) of PEG 6000 and 6% (w/w) NaCl. If the resultant mixture forms one phase, another system is prepared with 2% weight percentage increment until two-phases formed.
4. The systems with two phase observed are separated and samples from the top and bottom phase is collected and put into sample containers.
5. All samples are measured using refractometer and conductivity index and results for concentration of each sample are tabulated.
6. Graph for concentration of NaCl versus concentration of PEG 6000 is plotted and the tie-line for each wt% is connected.
7. Each composition at top and bottom phase is connected and best-fit binodal curve is drawn. The Cp is taken from the graph, where the Tie-line Length (TLL) is equal to zero.

3.2.4 Preparation of L-Cystine standard curve

1. Acid ninhydrin reagent is prepared by adding 250 mg of ninhydrin into the mixture of 6ml of acetic acid and 4ml of concentrated hydrochloric acid. Mixing done at room temperature for duration of 30 minutes.
2. L-cystine is diluted with acid hydrochloric to prepare L-cystine solution. Six solutions with concentration of 300 ppm, 600 ppm, 900 ppm, 1200 ppm, 1500 ppm and 1800ppm are prepared.
3. 1ml of L-cystine each solution is mixed thoroughly in a test tube with 1ml of acetic acid and 1 ml of acid ninhydrin reagent.

4. All tubes is heated in a water bath at 95°C for 20 minutes then immediately cooled in ice bath. All samples are then diluted with 95% ethanol to 10ml and sent to analyze using UV-vis spectrophotometer.

5. A blank ethanol reagent was made without L-cystine solution under the same condition to compare the absorbance with all the samples prepared before.

3.2.5 Aqueous two-phase system for L-cystine partitioning behavior

1. PEG 6000 solution with concentration 16% (w/w) is prepared. NaCl solutions with concentration of 14% (w/w), 16% (w/w), 18% (w/w) and 20% (w/w) are prepared using the stock solution.

2. L-cystine solution at 1200 ppm is prepared fresh. Defined amounts of PEG and NaCl solution are added to L-cystine solution and total mixture was made up to 50g with water. pH of mixture is regulated by adding pH buffer (pH = 7).

3. Mixture is gently shaken for 15 minutes then left for stabilizing for 20-24 hours at room temperature.

4. Experiment is repeated using fixed concentration of NaCl (16 % w/w) but different concentration of PEG 6000 (14, 16, 18 and 20 % w/w). Step 2 and 3 are repeated.

5. After stabilizing, all the samples are reacted with acid ninhydrin reagent and the compositions of protein at top and bottom phases are analyzed using UV-vis spectrophotometer and compared with the L-cystine standard curve.

3.3 Tools, Equipments and Materials.

3.3.1 Equipments

1. Refractometer
2. Conductivity meter
3. UV-vis spectrophotometer
4. pH meter
5. Cooling/heating water bath
6. Micropipette
7. Plastic syringe

3.3.2 Materials

1. Polyethylene glycol (PEG) 6000
2. Sodium chloride.
3. L-cystine
4. Ethanol
5. Hydrochloric acid
6. Acetic acid
7. pH buffer

Chapter 4: Results and Discussions

4.1 Standard curve for PEG 6000

Table 6: Refractive index reading for PEG solutions

PEG 6000 (Refractive Index)					
wt %	Reading 1	Reading 2	Reading 3	Average	2nd Trial
40	1.39118	1.39187	1.39207	1.39171	1.390227
30	1.37388	1.37397	1.37402	1.37396	1.374953
25	1.36880	1.36875	1.36875	1.36877	1.367833
15	1.35747	1.35773	1.35756	1.35759	1.354137
10	1.35091	1.35090	1.35091	1.35091	1.347673
5	1.34693	1.34698	1.34703	1.34698	1.341193
3	1.34370	1.34347	1.34371	1.34363	1.338597
1	1.34103	1.34103	1.34104	1.34103	1.336047
0.5	1.33723	1.33723	1.33728	1.33725	1.335440
0.1	1.33628	1.33628	1.33628	1.33628	1.334990
0.05	1.33620	1.33621	1.33620	1.33620	1.334930

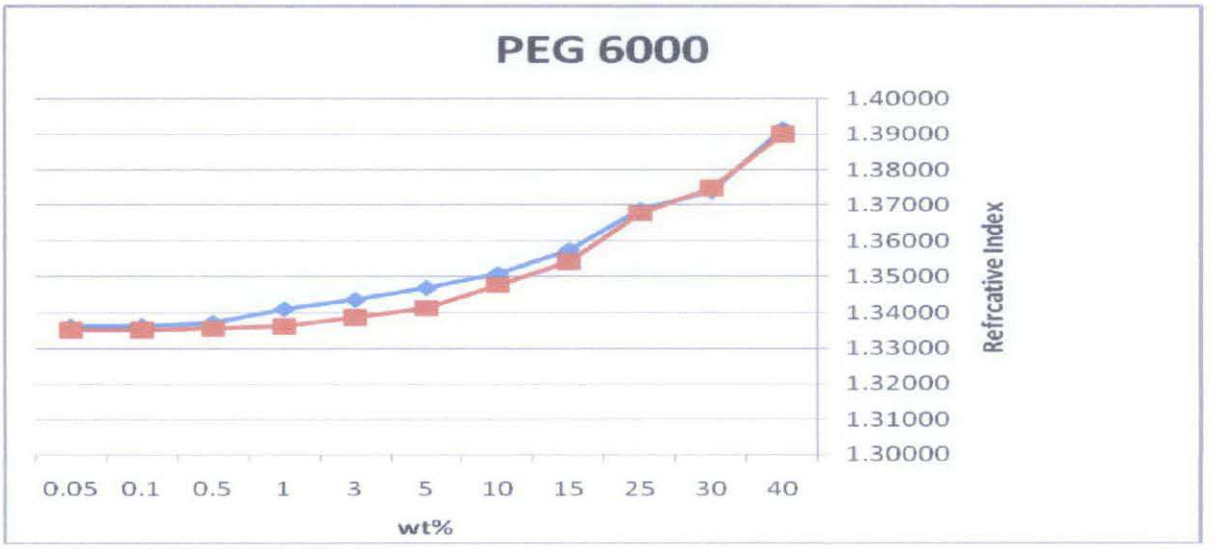


Figure 4: Standard curve of PEG (first trial)

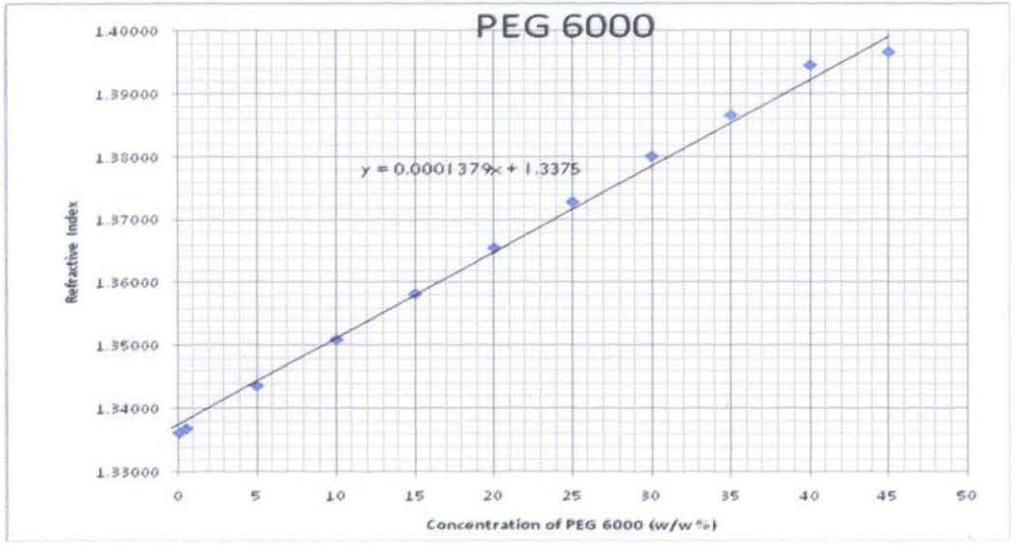
- Since the graph obtained was not a linear and the correlation was low, the experiment was repeated. The standard solution was re-prepared through dilution method. Then, the analysis for PEG 6000 was done and better result was obtained as shown below.

Standard curve PEG 6000 (Second trial)

PEG 6000 (Refractive index)										
wt%	desired volume (ml)	Volume from previous solution, ml M1V1=M2V2	volume needed,ml	Volume to prepare, ml	Volume from previous solution, ml M1V1=M2V2	Result				
						D. water	1	2	3	Average
45	3	2.70	5.67	50	45.00	1.33613	1.39651	1.39681	1.39626	1.39653
40	3	2.67	5.63	50	44.44	1.33613	1.39442	1.39444	1.39455	1.39447
35	3	2.63	5.57	50	43.75	1.33615	1.38602	1.38676	1.38675	1.38651
30	3	2.57	5.50	50	42.86	1.33614	1.37986	1.38009	1.38006	1.38000
25	3	2.50	5.40	50	41.67	1.33615	1.37289	1.37295	1.37252	1.37279
20	3	2.40	5.25	50	40.00	1.33613	1.36552	1.36551	1.36550	1.36551
15	3	2.25	5.00	50	37.50	1.33612	1.35818	1.35822	1.35825	1.35822
10	3	2.00	4.50	50	33.33	1.33613	1.35091	1.35090	1.35091	1.35091
5	3	1.50	3.30	50	25.00	1.33614	1.34355	1.34357	1.34354	1.34355
0.5	3	0.30	3.30	50	5.00	1.33613	1.33684	1.33682	1.33683	1.33683
0.05	3	0.3000	3.00	50	5.00	1.33612	1.33615	1.33615	1.33614	1.33615

Table 7: Readings for second trial of PEG standard curve

Figure 5: Standard curve of PEG 6000



- From the second trial, we have obtained a better results and a linear graph is obtained using linear-fit method. The relationship for the standard curve of PEG 6000 is:

$$\mathbf{y = 0.001379x + 1.33775}$$

Where y = refractive index

x = concentration of PEG 6000 (% w/w)

4.2 Standard curve for sodium chloride (NaCl)

Table 8: Result on conductivity of NaCl solutions (First trial)

Salt (NaCl) (Conductivity meter)				
wt %	Reading 1	Reading 2	Reading 3	Average
20	N/A	N/A	N/A	N/A
15	N/A	N/A	N/A	N/A
10	N/A	N/A	N/A	N/A
5	N/A	N/A	N/A	N/A
3	N/A	N/A	N/A	N/A
1	N/A	N/A	N/A	N/A
0.1	84.5	84.8	84.6	84.6
0.05	40.7	40.8	50.0	40.8
0.01	17.7	17.8	17.9	17.8

- Since the conductivity meter showed error reading for values of more than 1% weight of salt solution, the reason is because the equipment can only measure conductivity of ionic solutions up to 299 mS/cm.

- For second trial, smaller concentration of NaCl solution is prepared using dilution from the 25% (w/w) stock solution.

Standard curve NaCl (Second trial)

Table 9: Result on conductivity of NaCl solutions (Second trial)

Weight % (w/w)	Conductivity (mS/cm)			
	1st reading	2nd reading	3rd reading	Average
0.01	17.7	17.8	17.6	17.7
0.05	41.0	40.9	40.8	40.9
0.10	84.3	84.4	84.2	84.3
0.15	121.4	121.8	121.6	121.6
0.20	156.6	156.8	156.7	156.7
0.25	193.1	193.6	193.8	193.5

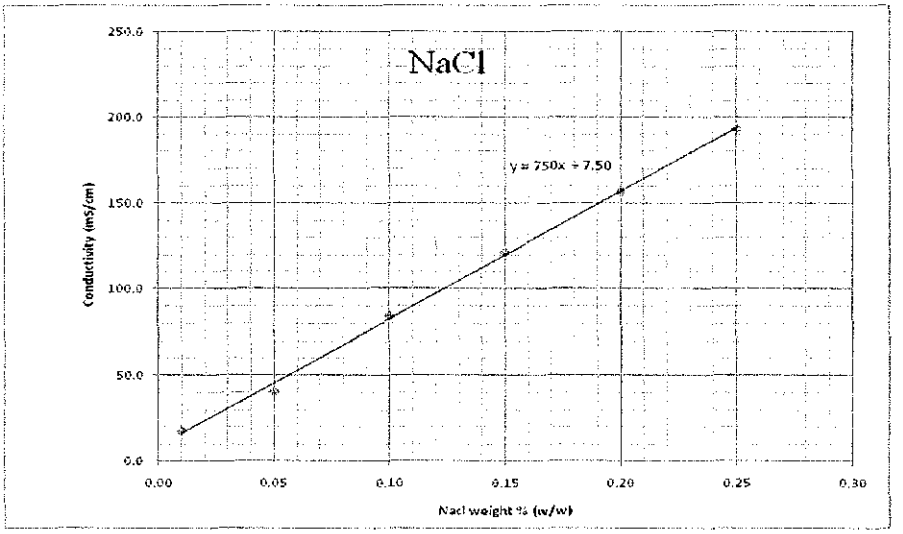


Figure 6: Standard curve of NaCl

- For second trial, we obtained a linear graph. The relationship for NaCl standard curve is:

  y = 750x + 7.50

  Where y = conductivity (mS/cm)

  x = concentration of NaCl (% w/w)

- To determine the concentration of NaCl samples, the samples collected will be diluted to part per hundred and compared with the graph to get the actual concentration. The value of concentration then multiplied by 100.

4.3 Binodal Curve of PEG 6000- NaCl

Table 10: Observation of phases from node determination method

PEG and NaCl (% w/w)	Phases
6	One
8	One
10	One
12	One
14	Two
16	Two
18	Two
20	Two
22	One
24	One

Table 11: Composition at the top phase

	Top phase	
wt %	PEG (wt %)	NaCl (wt%)
14	19.82	11.71
16	28.91	10.46
18	36.72	9.91
20	42.52	9.56

Table 12: Composition at the bottom phase

	Bottom phase	
wt %	PEG (wt %)	NaCl (wt%)
14	2.51	18.85
16	1.03	22.25
18	0.50	25.31
20	0.01	28.40

Binodal Curve

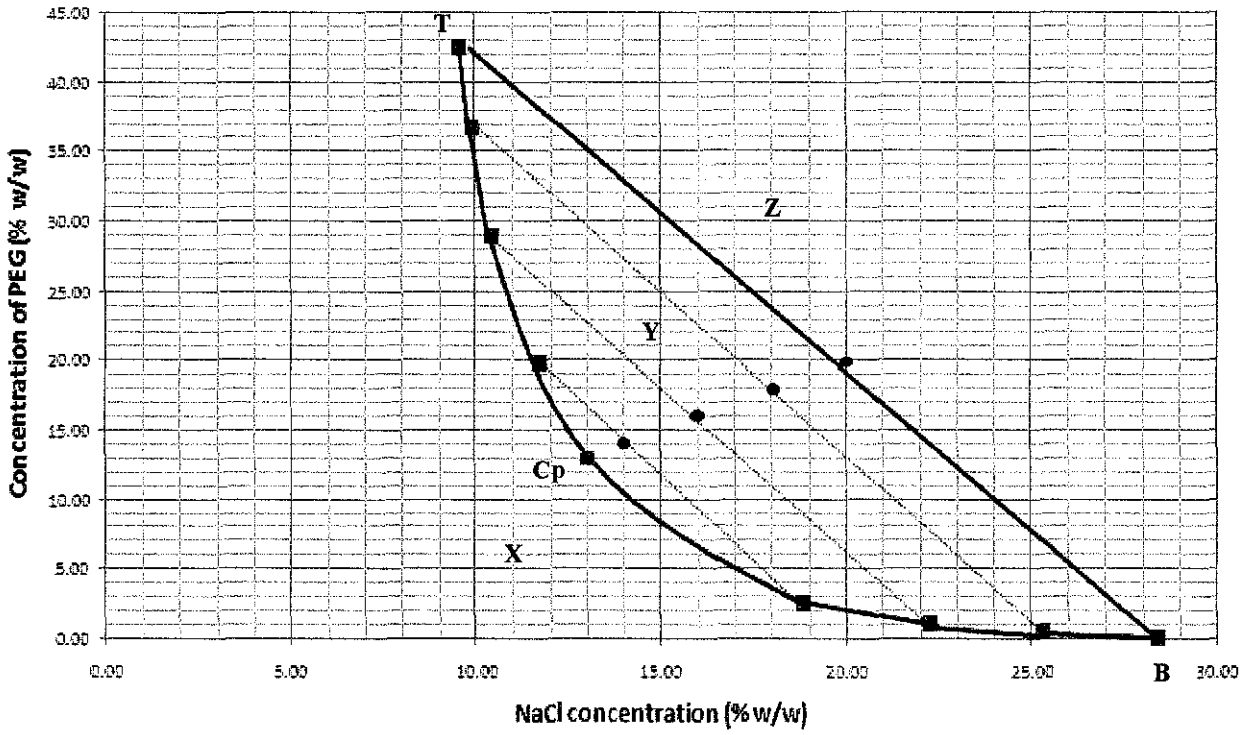


Figure 7: Binodal curve of PEG 6000 – NaCl at 293.15K

- From the binodal curve, the mixture of PEG 6000 and NaCl start to form two phases system at the Cp node. From the binodal curve, we have proved that aqueous two-phase system for PEG 6000 and NaCl is a feasible ATPS. The region of X and Z are the composition where the mixture of PEG and NaCl form one phase while region Y is where the mixture becomes two-phases corresponding to the concentration of both components.

- The final composition of the top and bottom phase is represented by nodes T and B, respectively.

- The Cp value is plotted on the curve where TLL = 0; where the composition of PEG and NaCl at both top and bottom phases are same.

- From the curve, we obtained the critical point where Cp is approximately 13% (w/w).

4.4 Standard curve for L-cystine

- A blank reagent without L-cystine solution is prepared under the same condition. Firstly two blank reagent blank is analyzed side by side in the UV-vis spectrophotometer, then one reagent blank and one L-cystine are measured, then other L-cystine solutions are measured one by one.
- The peak on the reading is observed and two peaks can be seen at 455 and 482 nm wavelengths. The amount of absorbance at both peaks is obtained and graphs are plotted.

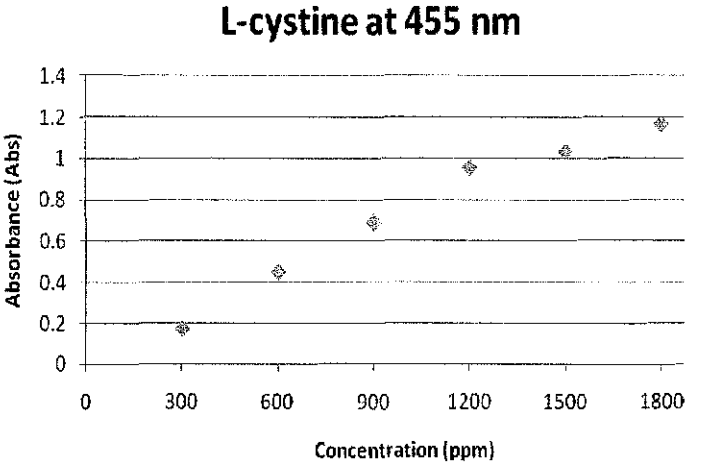


Figure 8: Amount of absorbance at different concentration of L-cystine at 455nm

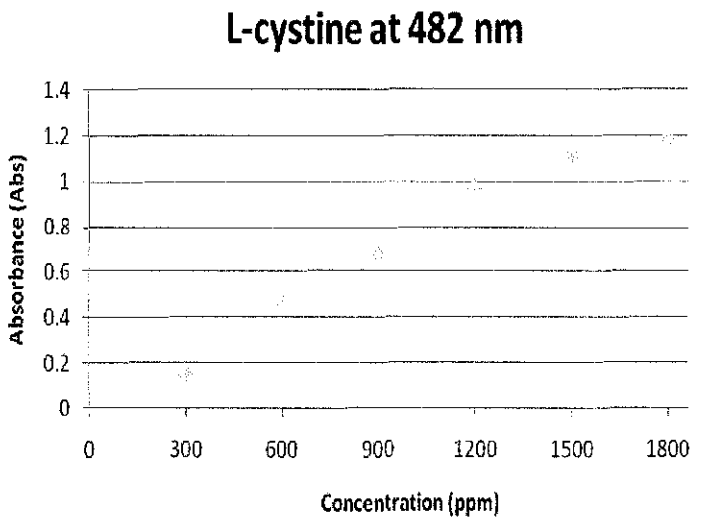


Figure 9: Amount of absorbance at different concentration of L-cystine at 482nm

- Since the amount of absorbance at wavelength of 482 is higher, this wavelength is selected as indicator for L-cystine composition and standard curve for L-cystine is plotted.

Table 13: Amount of absorbance at different concentration of L-cystine

Concentration (ppm)	Absorbance
300	0.151
600	0.463
900	0.682
1200	0.972
1500	1.115
1800	1.187

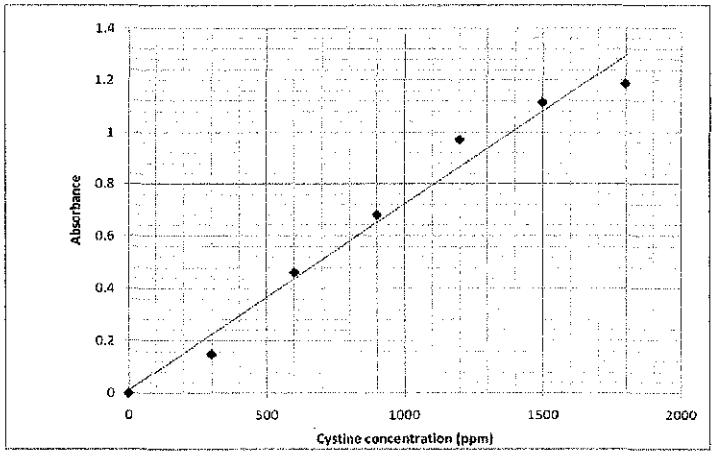


Figure 10: Standard curve of L-cystine

From the graph, as we obtained a linear graph from best linear-fit method, where the relationship between concentration of L-cystine and amount of absorbance is $y = 0.0007142x$ where y is the amount of absorbance and x is the concentration of L-cystine in ppm. However as we observed that the data are starting to decline after 1500 ppm, we can conclude that using bigger range of concentration than 1800 ppm, we can get the optimum value for L-cystine concentration thus the linear relationship above is only valid from concentration range of 0 ppm to 1500 ppm and the concentration for L-cystine partitioning behavior is taken within this range.

4.5 Aqueous two-phase system for L-cystine partitioning behavior

Table 14: Effect of polymer concentration to partitioning behavior of L-cystine

X% PEG, 16 % NaCl			
PEG (wt %)	Amount of Absorbance	Concentration of L-cystine at top phase	% extracted from top phase
14	0.603	816.30	68.02
16	0.655	889.11	74.09
18	0.685	931.11	77.59
20	0.622	842.90	70.24

Table 15: Effect of salt concentration to partitioning behavior of L-cystine

16% PEG, X% NaCl			
NaCl (wt %)	Amount of Absorbance	Concentration of L-cystine at top phase	% extracted from top phase
14	0.592	800.90	66.74
16	0.655	889.11	74.09
18	0.667	905.91	75.49
20	0.648	879.31	73.28

Results on partitioning behavior of L-cystine (initial concentration of 1200 ppm) by different polymer and salt concentration showed that L-cystine preferentially partitioned to the top phase (PEG rich). This may be because cysteine is a polar chain side with no charge. Salts can change the electrostatic charge of ATPSs and influence the distribution ratios of charged amino acids. For example, in Shang et al (2004) study, electrostatic interaction between lysine cation and salt anion is big so lysine prefers to be in the salt-rich phase. Cysteine did not prefer

the bottom phase because the electrostatic interaction between cysteine and salt anion is low. Amount of cysteine recovered is increased with increase in PEG concentration. This may be because hydrophobicity of amino acids. The bigger the PEG composition, the more hydrophobicity the top phase. It is one of the important influences for cysteine preferring the PEG rich phase. However, in general there is no significant effect with increasing or decreasing concentration for both PEG 6000 and NaCl concentration to the partitioning behavior of L-cystine but the highest L-cystine recovery is achieved at 18% (w/w) PEG 6000 and 16% (w/w) NaCl with amino acid recovery of 77.6%.

Conclusion

This study demonstrate the partitioning of amino acid by using aqueous two-phase system so that further understanding of fundamental partitioning behavior of more complex proteins in these systems can be obtained. ATPS of polyethylene glycol with molecular weight of 6000 and sodium chloride is a feasible liquid-liquid extraction technique to recover hevein from natural rubber latex. However, no significant effects in increasing or decreasing of PEG 6000 and NaCl concentration are observed for the partitioning behavior of L-cystine.

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