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Optimisation of Ammonium Sulfate for Protein Purification from Kedah-Kelantan Cattle (*Bos Indicus*) Placenta

Nurul Athirah binti Mohd Zuki@Rosli

F15A0181

A report submitted in fulfillment of the requirements for the degree of Bachelor of Applied Science (Animal Husbandry) with Honours

Faculty of Agro-Based Industry

Universiti Malaysia Kelantan

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

____

Student:

Name: Nurul Athirah Binti Mohd Zuki @ Rosli

Matric No: F15A0181

Date:

Approved by:

____

Supervisor

Name: Dr Khairiyah Binti Mat

Date

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OPTIMISATION OF AMMONIUM SULFATE FOR PROTEIN PURIFICATION FROM KEDAH-KELANTAN CATTLE (*Bos Indicus*) PLACENTA

ABSTRACT

The optimisation of ammonium sulfate, ($(NH_4)_2SO_4$) was applied to purify protein based on solubility from Kedah-Kelantan cattle (*Bos Indicus*) placenta. Double precipitation was carried out by using different concentration of $(NH_4)_2SO_4$. First precipitation used 0% (initial concentration), 20%, 40%, 60% and 80% of $(NH_4)_2SO_4$ as final concentration. Meanwhile, second precipitation was started with 20%-40%, 40%-60%, 40%-80% and 60%-80% of $(NH_4)_2SO_4$. In first precipitation, the extracted supernatant was added with different concentration of $(NH_4)_2SO_4$. An incubation was applied for 2 hours at 4°C. The sample were centrifuged for 1 hour at 15,930 rpm. The supernatant from first precipitation was used in second precipitation. The $(NH_4)_2SO_4$ was added to achieve required concentration. The sample was incubated for overnight at 4°C. Centrifugation was applied for 1 hour at 15,930 rpm. Dialysis was carried out to remove the salts from the sample. The precipitates from second precipitation were dissolved in 1ml of 0.01 M Tris-HCl buffer (pH 7.5) and extensively dialyzed against same buffer. The samples were centrifuged for 1 hour at 12,000 rpm. The supernatant was undergoes Bradford protein assay. In the first precipitation, the concentration of protein was highest (22.92 mg/ml) at 0%-60% of $(NH_4)_2SO_4$ with 40% concentration of unknown protein sample. The concentration of protein was significantly highest (25.09 ml/mg) at 40%-60% of $(NH_4)_2SO_4$ with 20% concentration of unknown protein sample in second precipitation. These data showed that the concentration of $(NH_4)_2SO_4$ play important role in purification protein based on solubility.

Keyword: placenta, ammonium sulfate, precipitation, dialysis, Bradford protein assay

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PENGOPTIMUMAN AMONIUM SULFAT UNTUK MENULENKAN PROTEIN DARI PLASENTA LEMBU KEDAH-KELANTAN (*Bos Indicus*)

ABSTRAK

Pengoptimuman amonium sulfat, ($(NH_4)_2SO_4$) plasenta daripada lembu Kedah-Kelantan (Bos Indicus) bagi menjalankan penulenan protein berdasarkan konsep keterlarutan. Presipitasi berganda telah dijalankan dengan menggunakan konsentrasi $(NH_4)_2SO_4$ yang berbeza. Presipitasi yang pertama dimulakan dengan 0% (konsentrasi awal), 20%, 40%, 60% and 80% of $(NH_4)_2SO_4$ sebagai konsentrasi akhir. Sementara itu, presipitasi yang kedua dimulakan dengan 20%-40%, 40%-60%, 40%-80% and 60%-80% konsentrasi $(NH_4)_2SO_4$. Di presipitasi pertama, (NH4)2SO4 dengan konsentrasi yang berbeza ditambah ke dalam supernatan yang diambil dari proses ekstraksi. Sampel disimpa selama 2 jam pada suhu 4℃. Sampel diempar selama 1 jam pada kelajuan 15,930 rpm. Supernatan dari presipitasi pertama digunakan di dalam presipitasi kedua. $(NH_4)_2SO_4$ ditambah untuk mencapai konsentrasi yang diperlukan. Sampel disimpan pada suhu 4℃ selama 24 jam. Sampel disentrifus pada selama 1 jam pada kelajuan 15,930 rpm. Dialisis telah dijalankan bagi menyingkir garam dari sampel. Hasil presipitat dari presipitasi kedua dilarutkan ke dalam 1ml of 0.01 M Tris-HCl (pH 7.5) dan didialisiskan dengan larutan yang sama. Sampel diempar selama 1 jam pada kelajuan 12,000 rpm. Supernatan digunakan dalam *Bradford protein assay*. Untuk presipitasi pertama, protein konsentrasi tinggi (22.92 mg/ml) pada 0%-60% $(NH_4)_2SO_4$ bersama 40% konsenstrasi sample protein yang tidak diketahui. Konsentrasi protein paling tinggi (25.09 ml/mg) pada presipitasi yang kedua dengan 40%-60% $(NH_4)_2SO_4$ bersama 20% konsentrasi sampel protein yang tidak diketahui. Data ini menunjukkan bahawa konsentrasi $(NH_4)_2SO_4$ memainkan peranan yang penting dalam penulenan protein berdasarkan konsep keterlarutan.

Kata kunci: plasenta, amonium sulfat, presipitasi, dialisis, *Bradford protein assay*

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LIST OF SYMBOLS

g	g-force
rpm	revolutions per minute
°C	degree Celcius
%	percentage
g	gram
nm	nanometer
µg	microgram
µL	microliter
µg/ml	microgram per milliliter
mg/ml	milligram per milliliter
$(NH_4)_2SO_4$	ammonium sulfate

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LIST OF ABBREVIATIONS

PSPB	pregnancy-specific protein B
PSPA	pregnancy specific protein A
PAG-1	pregnancy-associated glycoprotein-1
bPAG	bovine pregnancy-associated glycoprotein
CT	cotyledon tissue
AS	ammonium sulfate
BSA	bovine serum albumin
RIA	radioimmunoassay
CBBG	coomassie brilliant blue G
STD	standard deviation
pH	potential hydrogen
pI	isoelectric point
TNCs	trinucleate cells
SyPs	syncytial plaques
MTCs	mononucleate trophoblast cells
BNCs	binucleate cells
hCG	human chorionic gonadotropin
PPL	pregnancy percentage loss
ELISA	enzyme-linked immunosorbent assay
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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CHAPTER 1

INTRODUCTION

1.0 RESEARCH BACKGROUND

According to (Hochuli, 1992), the purification is a necessary part of biotechnological manufacturing in biological product including antibiotic, a vitamin, or a recombinant protein which was coming from fermentation broth or cell culture supernatant. Purification step was important in downstream processing industry. Besides, the purification of protein known as an essential first step in molecular biological studies in order to figure out the properties and its biological roles such as molecular weight, charges, hydrophobicity and solubility. These properties can be exploited to purify a protein from a mixture (Strategy, 2005)

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In order to investigate the protein separation based on solubility, there were variety of salts could help in purification of protein. In previous study, ammonium sulfate ($(NH_4)_2SO_4$) was used as it had high ionic strength that can break down protein and hydrogen bond (Sattayasai, 2012). The optimum usage of $(NH_4)_2SO_4$ can be determined according to Green & Hughes (1955) or by using online ammonium sulfate calculator. As mentioned in Huang, Cockrell, Stephenson, Noyes, & Sasser, (1999) and (Garbayo et al., 1998), the protein desired molecules were precipitated between 40%- and 80%- $(NH_4)_2SO_4$.

In this study, the placenta of Kedah-Kelantan (KK) cattle (*Bos Indicus*) was used as an experimental subject where the various types of protein. Recently, there were variety of proteomic studies involving mammalian placenta cell had been investigated. The placenta was experienced a few steps before purification process including isolation and extraction. There were a few types of protein in the placenta that play their important roles. Tefera, Jeanguyot, Thibier, & Humblot (2001) reported that there was one special protein named as pregnancy specific protein B (PSPB) which always presented at an earlier stage of the pregnancy which known as an early gestation period. It was located in the placenta. PSPB or can be reclassified as pregnancy-associated glycoprotein-1 (PAG-1) is located in the cotyledons of mammalian placenta. According to Hamilton et al. (1982), they were operated as placental antigens detectable in the maternal circulation of pregnant mammals. Pregnancy-associated glycoprotein (PAG) or PSPB expressed in the outer epithelial cell layer which was known as trophectoderm of ungulate placenta in several eutherian species (Etiatin, Ajuthi, Urwantara, & Alib, 2009; J. A.; Green et al., 2000).

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The occurrence of limitations in this study had been restricting better result in future. There were many study materials especially chemical reagents were used in order to run the experiment. This situation limited the experiment due to over price of those chemical reagents. Besides, technical difficulties and inexperience in handling several processes like purification process leaded to the problems. Furthermore, the facilities and equipment were inadequate for conducting the experiment. Therefore, possibilities to obtain accurate results are lower due to these limitations.

1.1 PROBLEM STATEMENT

Purification had been one of a focal tool in identifying protein purity which consequently involved a few of procedures including separation based on solubility. According to Sattayasai (2012), separation protein based on solubility can be done by salting out or salt precipitation which usually using $(NH_4)_2SO_4$ since it has a high ionic strength. Conversely, optimising the usage of $(NH_4)_2SO_4$ due to the protocols involve during purification of protein had become an issue. In addition, the amount of dry $(NH_4)_2SO_4$ is not fixed in obtaining an optimum percentage of saturated solution because it is depending to the volume of supernatant from extraction process. Therefore, the focus of the study was to identify the best percentage of $(NH_4)_2SO_4$ for purification protein solubility of protein from Kedah-Kelantan cattle (*Bos Indicus*) placenta.

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1.2 HYPOTHESIS

H_O = Different percentage of ammonium sulfate ($(NH_4)_2SO_4$) does not give significant effect in protein purification from Kedah-Kelantan cattle (*Bos Indicus*) placenta.

H_1 = Different percentage of ammonium sulfate ($(NH_4)_2SO_4$) gives significant effect in protein purification from Kedah-Kelantan cattle (*Bos Indicus*) placenta

H_O is rejected when p-value is less than 0.05 ($p<0.05$).

1.3 OBJECTIVES

The objectives of the study were:

1.3.1 To identify the best percentage of ammonium sulfate ($(NH_4)_2SO_4$) for protein purification from Kedah-Kelantan cattle (*Bos Indicus*) placenta.

1.3.2 To evaluate the concentration of crude protein from placenta of Kedah-Kelantan cattle (*Bos Indicus*) at best percentage of ammonium sulfate ($(NH_4)_2SO_4$) by using Bradford protein assay.

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1.4 SCOPE OF THE STUDY

The scope of this study was to purify the protein from placenta of Kedah-Kelantan cattle. This purification process was assisted with usage of $(NH_4)_2SO_4$. In this study, the usage of $(NH_4)_2SO_4$ in protein solubility was determined in order to achieve the best percentage of $(NH_4)_2SO_4$. The best percentage of $(NH_4)_2SO_4$ was identified before purifying process starts. It was significant in this study due it was used in purifying the protein. Furthermore, the concentrations of protein are evaluated at the end of the experiment. The protein precipitate was used in dialysis process with buffer solution in order to remove salts and harvest a bulk of proteins from placenta.

1.5 SIGNIFICANCE OF THE STUDY

The implication of this study reinforced the knowledge on purification techniques and how to apply all the procedures. In addition, this study improved the understanding of purification of protein especially based on solubility. Furthermore, this study gave more exposure on the function of $(NH_4)_2SO_4$ and it proved that the amount of $(NH_4)_2SO_4$ can influence in obtaining the best percentage of $(NH_4)_2SO_4$ for purification of protei

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CHAPTER 2

LITERATURE REVIEW

2.1 PLACENTA

The placenta in the body was known as unique creature that have special function with variety type of proteins, peptide and steroid hormones (Rama & Rao, 2003). It differentiated and grew from an embryonic tissue until reaching maturity in a period of only weeks or months depending on the species. Placental had special roles which acts as nutrient supplier directly towards the foetus for developing. According to Miyazawa (2014), during placentation in mammals, they operated different strategies as to nourish and support the development of foetus. The family of the animals that have cloven-hoofed especially in ruminants known as Bovidae.

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They promoted more maternal attachment points on the placenta called as cotyledons and hybrid cells named trinucleate cells (TNCs) or syncytial plaques (SyPs). There were different in 3 types which are known as mononucleate trophoblast cells (MTCs), binucleate cells (BNCs) and TNCs. Whereas BNCs were designed by differentiation of MTCs by endoreduplication.

The placenta act as the middle-man between the maternal organism and non-self of proteins for foetus development and also to complete the immune functions and activity in the body during pregnancy period (Buse et al., 2014). The placenta that located in ruminant bodies were characterised by separating attachment areas which was including the placentomes which were needed due to affectionate interaction between uterine caruncles and chorionic cotyledons (U M Igwebuike, 2009; Schlafer, Fisher, & Davies, 2000). Udensi M Igwebuike & Ezeasor (2013) stated that placentomes which were figured during implantation process which known as the attachment of the conceptus to the maternal endometrium could lead to the founding of placental structures. Bowen & Burghardt (2000) explained the beginning of implantation and placentation were starting on Day 15 to 16 of gestation in sheep, unfortunately it was not completely develop until Day 50 to 60 of pregnancy.

2.2 PROTEIN

According to Semester (2000) proteins are long chains which were build up from 20 types amino acids as building block. Previously, a research paper had been published by University of North Dakota (2014) noted that proteins known as a complex organic

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compounds that constructed by chain of amino acids as a fundamental structure of protein. Proteins plays important roles as they were required to provide energy for the body and also important component of every cell in the body. According to Radivojac (2013), proteins known as biological macromolecules that have responsibilities on activities in our body including cells, tissue and organs as they play a central roles in the structure and function of cells.

Garbayo et al., (1998) stated that the placenta concealed the proteins at the peripheral circulation of the mother which was detected as a special protein act as useful indicator of both pregnancy and foetus development. According to Huang et al. (1999), proteins produced by placenta such as human chorionic gonadotropin (hCG). The protein was expressed in the ruminant placenta and also used as to diagnose pregnancy (Sousa, Ayad, Beckers, & Gajewski, 2006). The BNCs of ruminant in the placenta aggregated varies types of proteins including placental lactogen (Lee, Wooding, & Brandon, 1981), pregnancy specific protein B (PSPB) (Huang et al., 1999) and pregnancy-associated glycoprotein (PAG) (Zoli, Guilbault, Delahaut, Benitez-Ortiz, & Beckers, 1992).

According to Humblot (2001), pregnancy-specific protein is related to early gestation or late embryonic mortality that can lead to abortions that had been discussed in several ruminant species. PSPB can also be reclassified as pregnancy-associated glycoprotein-1 (PAG-1) which is located in the cotyledons of mammalian placenta. Patel, Takahashi, Imai, & Hashizume, (2004) explained that bovine pregnancy-associated glycoprotein-1 (bPAG-1) also had the priority during pregnancy that was labelled as a potential biochemical marker of pregnancy status. These glycoprotein can be noticed in

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the maternal circulation by week 3 after breeding and have been used as pregnancy markers in cattle (Zoli et al., 1992). (Sasser, Ruder, Ivani, Butler, & Hamilton, 1986) stated that PSPB was demostrated in the trophoblastic binucleate giant cells of ruminant placenta which act as major secretory product of placenta in ruminants once implantation has begun.

PSPB was distinguished in maternal peripheral circulation from the beginning of placentation and had been widely practiced in pregnancy diagnosis of animals especially in ruminants (Alan K . Wood , Robert E . Short , Ann-Elaine Darling , Gary L . Dusek, 2016; Humblot et al., 1990; Sasser et al., 1986; Stellflug, 1988). PSPB is a protein that was first isolated from bovine placenta at an extra-embryonic membranes (Hamilton et al., 1982) and also was revealed in the serum of pregnant cattle by Sasser et al. (1986). In previous study shows pregnancy-specific protein which are PSP A and PSP B from bovine placental membranes by Hamilton et al., (1982) isolated two. PSPA was known as α-fetoprotein which is not strictly limited to pregnancy diagnosis while PSPB was confirmed as placenta-specific (Sasser et al., 1986).

In previous study by (Abdulkareem et al., 2012), PSPB had been used as to recognize the early gestation in Iraqi water buffalo. In addition, 60 calving Holstein cows was used to investigate the relationship between placental retention, hormone progesterone and bPSPB by Tefera et al. (2001). Previously, Gábor et al. (2016) carried out an investigation on dairy cattle in order to observe the associations between percentage pregnancy loss (PPL) in dairy cattle. The investigation started with pregnancy diagnosis by ultrasonography followed by pregnancy diagnosis by serum PSPB

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concentrations by using serum progesterone concentrations and the production and environmental factors that could give significant effects. Thus, this study discovered 149 822 pregnancy diagnoses to Holstein-Friesian cows in Hungarian dairy herds with ages are over 13 years in. Huang et al. (2000) exposed the development a specific double-antibody radioimmunoassay (RIA) for elk and moose PSPB that would help in quantifying PSPB in animal placental. Gajewski et al., (2009) carried out an experiment to discover a new method for determining the PAG in milk and blood by RIA test and then compared the results with the other method for pregnancy diagnosis in the cows. At the end of that study, the data showed that the RIA method was rigid enough to be used in order to measure PAG concentrations in the maternal blood and also milk of cows.

According to Sasser, Ruder, Ivani, Butler, & Hamilton (1986), they conducted the study in order to establish a double antibody RIA for PSPB and use the RIA to measure PSPB serum of pregnant cattle and to determine the accuracy of measurement of PSPB for detecting pregnancy. All the studies above that had been conducted previously shows that PSPB can be used and applied in any branch of studies which are related to animal pregnancy. Previous study by D . B . Houston , C . T . Robbins (2016) used 10 captive and 8 wild mountain goats to carry out pregnancy diagnosis by RIA for identifying of PSPB.

2.4 PURIFICATION PROCESS

According to Kumar & Sharma (2015) concluded that proteins are widely used as well as in organic chemistry in the area of enzymatic catalysis in various type of industrial

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process including food processing, leather, cosmetics and pharmaceutical industries. The purification process had been seen as one of the important steps in the protein processing industry. This is because proteins are required in purified form to be applied in this industry. Hedhammar, Karlstrom, & Hober (2006) mentioned that the objective of a purification process is to identify the concentration of the desired protein and the transfer to an environment at stable state which could be applied further to other process instead of only removal of salts or unwanted contaminants from the protein sample.

2.4.1 PURIFICATION OF PROTEIN

Huang et al. (1999) and Garbayo et al. (1998) studies presented the purification process had been applied in order to purify PSPB. Both of these studies were conducted separation based on solubility in purifying PSPB. There were different separation techniques as following desired properties such as solubility, size or shape, isoelectric point (pI) or charge, binding to small molecules and hydrophobicity (Song, 2006). Sattayasai (2012) concluded that there are seven steps could be practiced in protein purification: (1) extraction of crude protein; (2) identification of the desired protein; (3) separation into a few fractions; (4) identification of the desired protein; (5) separation after protein purification; (6) identification of the desired protein; and (7) evaluation of purity involve in protein purification but it is not necessary to follow all the steps or techniques in the process for every protein.

Some properties should be concerned in purification of proteins such as charge, hydrophobicity, affinity, solubility and stability and molecular weights (Sattayasai,

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2012). Hunte, Jagow, & Schagger (2003) advised that in purification protocol, it was better to work at cold room temperature which is 4℃ in order to minimize proteolysis activity occur. Thus, protein will be more stable at 4℃.

2.4.2 SEPARATION BASED ON SOLUBILITY

Protein were separated on the different properties which including the molecular size, solubility, charge and specific binding-affinity. To identify molecular size, a few of separation methods can be practiced included two process, chromatography and electrophoresis which all of them were applied after salt precipitation.

Solubilisation and precipitation methods provide some degree of purification. There were two necessary steps in purification including differential extraction and centrifugation which were commonly practiced occasionally followed by differential precipitation techniques. These methods could be applied on a large scale and also afford low to medium resolution at low cost expenses (Hunte et al., 2003). Scopes (2013) explained that the solubility of proteins at high salt concentration level could be affected by temperature which triggered the hydrophobic interaction. The solubility of proteins generally decrease with increasing temperature in salting out process. Working with protein in cold room were advised.

The current of study were applied separation based on solubility only that involve salt precipitation. According to Sattayasai (2012), separation based on solubility could be

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investigate in two ways that known as isoelectric precipitation and salt precipitation. This is because the solubility of hydrophilic protein depends on the charges and hydrogen bonding with water molecule. Isoelectric precipitation were applied when net charge of a protein is zero at its isoelectric pH (pI) due to properties of protein molecule that was easy to accumulate and then precipitate at this condition. Salt precipitation or salting out is different because this process were carried out due to unknown pI. Hydrogen bond between protein and molecule would be broke down at the higher concentration of some salt. The addition of organic solvents or polymers, or variety of the pH or temperature would give significant effects on precipitation process (Hedhammar et al., 2006).

2.4.3 SALT PRECIPITATION OR SALTING OUT

Salting out of proteins was described as dissolving the salt in solution containing the proteins (Scopes, 2013). According to Ryan (2011), differential protein precipitation was known as classical method which was a faster and economical step in protein purification. It was discovered by exploiting the inherent physico-chemical properties of the polypeptides. This method included precipitating of protein which was lysed from the host cell that commonly used to concentrate the desired protein before further polishing steps with different ways of purification. Protein precipitation occurred spontaneously due to differential solubility between a protein-rich soluble phase and a solid chemical precipitant which is salts. Insolubilisation of proteins can be proceed by interaction with a suitable precipitant that can decreases the interest of protein to the solvent and increases the protein's interest to other protein molecules. This can promote protein accumulation and eventually precipitation. Protein solubility decreases gradually as ionic strength

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increases in the process at a very high ionic strength known as salting-out (Duong-Ly & Gabelli, 2014).

Salting out of proteins were carried out by using high concentrations of salts such as ammonium sulfate, ($(NH_4)_2SO_4$) is one of the oldest and cheapest methods of protein concentration that can be applied (Moore & Kery, 2009). $(NH_4)_2SO_4$ normally used in salting out due to it has high ionic strength (Sattayasai, 2012). $(NH_4)_2SO_4$ could help in stabilizing proteins and prevent the protein from denatured (Ab, 2011). Page & Thorpe (2002) noted that $(NH_4)_2SO_4$ precipitation is the most widely practiced and adaptable procedure due the ability of yielding a 40% pure protein. $(NH_4)_2SO_4$ applied due to the first choice salt for initial development of a salting-out program in order to precipitate desired protein as they could yield high concentration of protein according to Hermodson (1996). According to Wingfield (2016), due to low protein solubility, it could lead to more precipitation at higher salt concentrations. This proved by reducing of solubility of proteins, so the stability of the native conformation can be enhanced.

Stabilization of proteins that occurs was evaluated as beneficial method by practicing ammonium sulfate fractionation over other techniques (Scopes, 2013). Proteolysis and bacterial action could be avoid by applying high salt concentration. It was quite good to have a stage in purification, where the sample can be left overnight without any bacterial infection. Ammonium sulfate was used in fractionation of rabbit muscle extract by Czok & Bucher, (1940) which five fractions were divided by ammonium sulfate precipitation which each of them contained enzymes. Recently, ammonium sulfate $(NH_4)_2SO_4$ had been used in purification of process of papain crude extract (Purwanto,

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2016). The roles of ammonium sulfate $(NH_4)_2SO_4$ as precipitating agent is common in purification of protein. Ammonium sulfate $(NH_4)_2SO_4$ also had been used in a others purification process (Silva, Kuhn, Moraes, Burkert, & Kalil, 2009; Page & Thorpe, 2002; and Gräslund et al., 2008).

Ammonium sulfate $(NH_4)_2SO_4$ will be used in current study in order to purify protein from Kedah-Kelantan cattle (*Bos Indicus*) placenta. The optimum concentration of ammonium sulfate saturated was aimed to be between 40%- and 80%- saturated ammonium sulfate in order to purify large amount of protein from bovine placenta that located the PSPB in this current study. A study exposed that the PAG-related molecules were precipitated between 40%- and 80%-saturated ammonium sulfate (857 mg) (Garbayo et al., 1998). Other study explained that in results which is most immune-reactive proteins retained in extracted supernatant were precipitated between 40%- and 75%- saturated ammonium sulfate (Huang et al., 1999) after homogenization and pH treatment.

2.4.4 DIALYSIS

Dialysis was one of the important procedure before completing purification process. This method is simple and consumed a longer period than others due to the separation which was depending on diffusion process (Sattayasai, 2012). Luo, Wu, Xu, & Wu (2011) explained the concept of dialysis which related to a semi-permeable membrane had been used in order to separate relatively small molecules from large ones

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with. Dialysis was known as a spontaneous separation process since the driving force for the separation process is mainly concentration gradient.

Whitford (2007) explained that the dialysis is important in removing low molecular weight of contaminants or salts and also to exchange buffer in a solution which were containing protein. Dialysis is closely related with osmotic pressure that allowed to get rid of most salt ions but it is impossible to remove the salts completely (Cseke, Kaufman, Kirakosyan, & Westfall, 2011).

2.5 BRADFORD PROTEIN ASSAY

The binding of Coomassie Brilliant Blue G-250 (CBBG) to protein was applied in order to evaluate the concentration of protein which was described by Bradford (1976). The binding of the dye to protein was produced to the shift in the maximum absorption of the dye from absorbance 465nm to 595 nm. This assay is an easiest method which was required approximately 2 minutes of measurement and incubation for 1 hour in order to have good colour stability. This methods was very reproducible and quick with the dye binding process virtually complete in. By using CBBG, it is fast, inexpensive, and sensitive (Kunze, Medizin, & Republic, 1989). However, the Bradford method had limitation which was only of limited use when proteins had to be quantitated from samples with less than 10 µg/ml.

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According to Sedmakand & Grossberg (1977), the absorbance of the mixture could be measured as soon as the dye solution is mixed with a protein sample. The absorbance of the solution is stable by standing them for 60 to 90 minutes at room temperature. The test could be used to observe many types of proteins and polypeptides with molecular weights greater than 3000. The assay has high reproducibility by detecting albumin that less than 1.0 µg.

Bradford (1976) CBBG was used to estimate protein concentration in the sample. However, this approach seemed to be more sensitive as the Lowry method but it was very easy to perform in the experiment. The composition of the protein which were amino acids could be high sensitivity in this test. This assay might lead to bias due to presence of detergent. This can be a major problem when the detergent concentration cannot be determined and corrected (Hunte et al., 2003)

In current study, the mixture of Bradford reagent and other solution was measured by using spectrophotometer at absorbance 595nm. This idea involved making a series of standard solutions by using Bovine Serum Albumin (BSA) that are then assayed for their value. These values can be used to generate a standard curve graph, which can then be used to determine the concentration of unknown protein samples.

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CHAPTER 3

MATERIALS AND METHODS

3.1 LOCATION

The placenta was obtained from Kedah-Kelantan cattle (*Bos Indicus*) at Agropark, University Malaysia Kelantan, Jeli Campus, Kelantan. The study was conducted at Post-graduate Laboratory, Faculty of Agro Based Industry, University Malaysia Kelantan Jeli Campus, Kelantan.

3.2 COLLECTION OF PLACENTA

The placenta was collected after the cow undergone parturition process. Fresh foetal cotyledons tissue (CT) were detected away from caruncular tissues. CTs was washed with 0.9% NaCl and stored in -20°C (Garbayo et al., 1998). It was used in extraction process in order to obtain crude proteins from cotyledons tissues.

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3.3 AMMONIUM SULFATE PRECIPITATION

The supernatant containing crude protein was collected from extraction process and stored in -20℃ until use. In this study, double precipitation were applied in order to identify the optimum usage of ammonium sulfate $(NH_4)_2SO_4$ and the best percentage to purify pregnancy-specified protein B (PSPB). The addition of $(NH_4)_2SO_4$ in each beaker was determined by using online ammonium sulfate calculator (Sigma, 2004).

3.3.1 FIRST AMMONIUM SULFATE PRECIPITATION

Crude protein had been precipitated with different concentration of $(NH_4)_2SO_4$. Dry $(NH_4)_2SO_4$ was slowly added to the supernatant to obtain 20%-ammonium sulfate solution (Huang et al., 1999) and stirred until it was completely dissolved. The solution was allowed to precipitate in 2 hours at 4℃ (Page & Thorpe, 2002). After incubating for 2 hours, centrifugation was applied at 15,930 rpm for 60 minutes (Etiatin et al., 2009). The pellets were discarded and supernatant was used in second precipitation process. Different concentrations of $(NH_4)_2SO_4$, 40%-, 60%- and 80%- were repeated with same procedure.

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Table 3.1: The weight of $(NH_4)_2SO_4$ in each sample for first precipitation

Types of sample	Weight of $(NH_4)_2SO_4$ (g)
0% - 20%	0.424
0% - 40%	0.904
0% - 60%	1.444
0% - 80%	2.064

Figure 3.1: The solution after incubating for 2 hours at 4°C with different concentration of AS, 0%-20%, 0%-40%, 0%-60%, 0%-80% (from left to right)

3.3.2 SECOND PRECIPITATION OF AMMONIUM SULFATE

The supernatant were collected from first precipitation of $(NH_4)_2SO_4$. Additional of dry $(NH_4)_2SO_4$ was similarly added into supernatant with 20%-ammonium sulfate

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solution to achieve a 40%-ammonium sulfate solution. The solution was allowed to stand overnight at 4℃ (Page & Thorpe, 2002). Centrifugation was applied at 15,930 rpm for 60 minutes (Etiatin et al., 2009). The precipitate was retained. The procedures were repeated to achieve different concentration.

Table 3.2: The amount of the percentage need to achieve in second precipitation of $(NH_4)_2SO_4$

Percentage of $(NH_4)_2SO_4$ solution in first precipitation	Percentage of $(NH_4)_2SO_4$ achieve in second precipitation
20%	40%
	60%
	80%
40%	60%
	80%
60%	80%

Table 3.3: The weight of $(NH_4)_2SO_4$ in each sample for second precipitation

Types of sample	Weight of $(NH_4)_2SO_4$ (g)
20% - 40%	0.113
20% - 60%	0.241
20% - 80%	0.387
40% - 60%	0.120
40% - 80%	0.263
60% - 80%	0.129

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Figure 3.2: After incubating overnight at 4℃ with different concentration of AS, 20%-40%, 20%-60%, 20%-80% (from left to right)

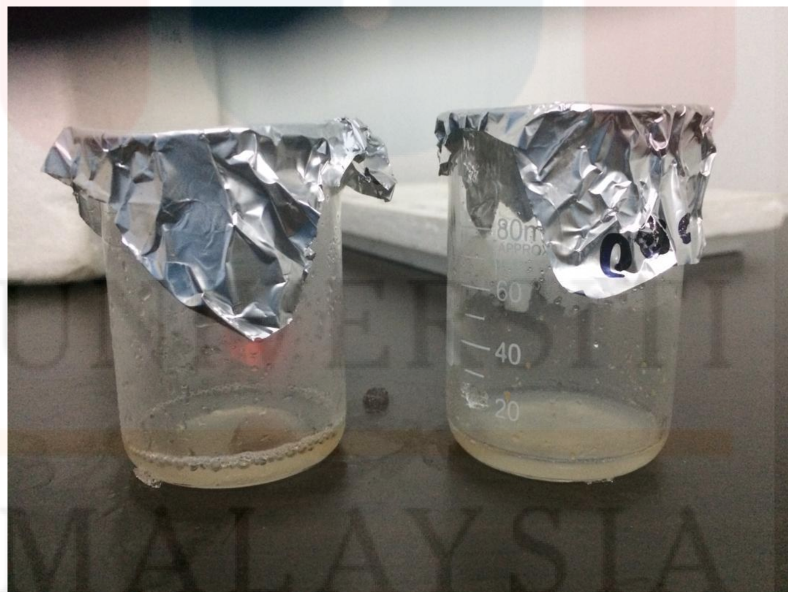

Figure 3.3: After incubating overnight at 4℃ with different concentration of AS, 40%-60%, 40%-80% (from left to right)

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Figure 3.4: After incubating overnight at 4℃ with 60%-80% concentration of AS

3.4 Dialysis

The precipitates from first and second precipitation were collected and dissolved in approximately 1 ml of 0.01 M Tris-HCl buffer (pH 7.6) (Garbayo et al., 1998). The samples were placed inside a visking tube and extensively dialyzed against same buffer at 4℃ for 24 hours with 2 times of changing buffer in every 6 hours. The solution was then centrifuged at 12,000 rpm for 60 minutes. At the end of the experiment, the pellets were discarded and the supernatant was used in Bradford protein assay in order to determine the concentration of crude protein.

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Figure 3.5: Dialysis for 24 hours at 4°C

3.5 Quantification of crude protein

As for protein assay, preparation of protein standard according to Bradford (1976) from 1 mg/ml bovine serum albumin (BSA) with different concentration which were started as 0 (the blank), 20, 40, 60, 80, and 100 µL.

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Table 3.4: The volume of reagents used in preparation of protein standard

Concentration of BSA (%)	Volume of BSA (µL)	Volume of phosphate buffer (µL)	Bradford reagent (µL)
20	100	2.40	2.5
40	200	2.30	2.5
60	300	2.20	2.5
80	400	2.10	2.5
100	500	2.00	2.5

Table 3.5: The volume of reagents used in measuring crude protein in each sample

Concentration of unknown protein sample (%)	Volume of supernatant (µL)	Volume of phosphate buffer (µL)	Bradford reagent (µL)
20	100	2.40	2.5
40	200	2.30	2.5
60	300	2.20	2.5
80	400	2.10	2.5
100	500	2.00	2.5

After all solution were prepared, 1 hour of incubation were applied at 40℃ (Bradford, 1976). The solution was measured at 595nm by spectrophotometer.

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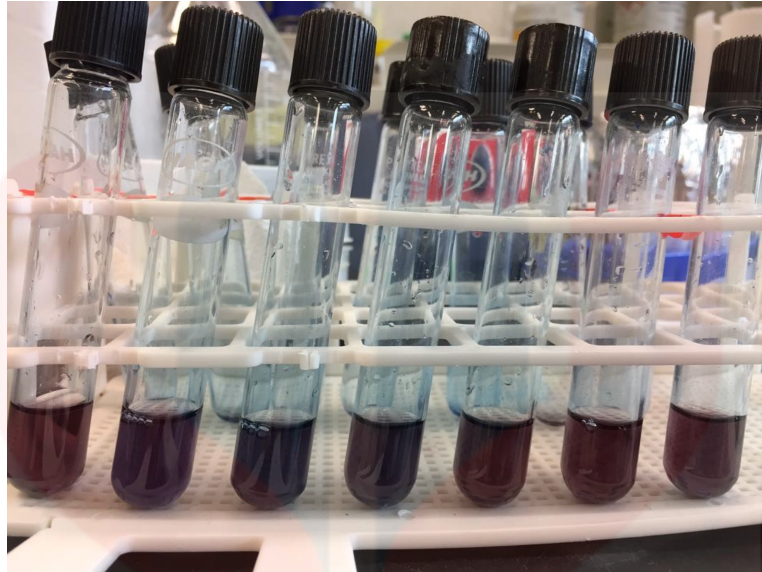

Figure 3.6: After 1 hour of incubation at 40℃

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 QUANTIFICATION OF CRUDE PROTEIN

The standard curve graph (R^2=0.9569) was prepared as reference in order to identify the protein concentration in particular volume was presented in Figure 4.1.

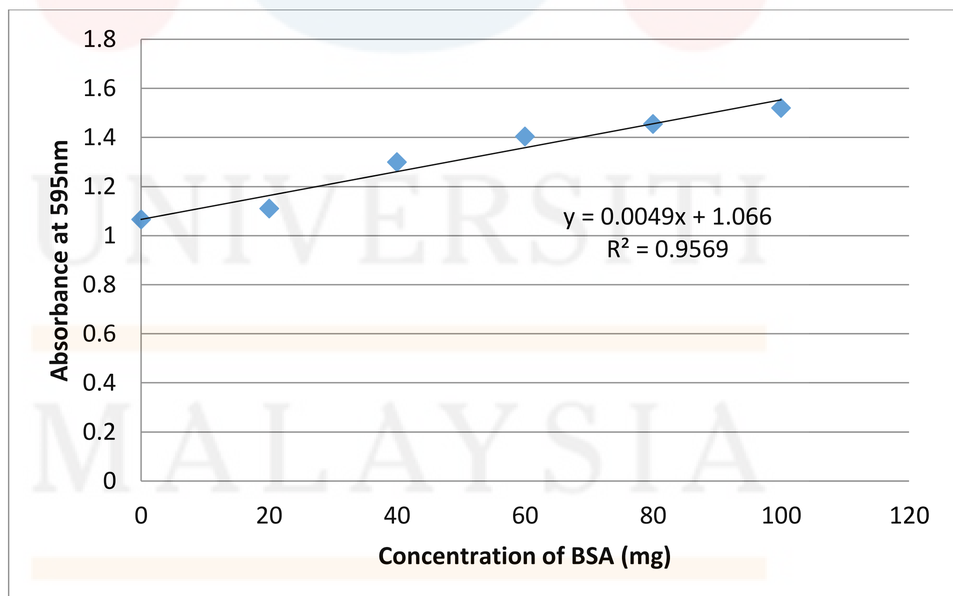

Figure 4.1: Standard curve graph

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4.1.1 FIRST AMMONIUM SULFATE PRECIPITATION

The quantity of crude protein in with different percentage of ammonium sulfate and concentration of protein used in Bradford test was presented in Table 4.1.

Table 4.1: Concentration of protein in first precipitation of ammonium sulfate (mg/ml)

Ammonium sulfate concentration	Concentration of unknown protein sample (%)	Concentration of protein (mg/ml)
0% - 20%	20	10.09 ± 1.36^{a}
	40	10.29 ± 0.17^{a}
	60	10.00 ± 1.66^{a}
	80	10.47 ± 0.22^{a}
	100	11.26 ± 0.59^{a}
0% - 40%	20	22.39 ± 0.40^{bc}
	40	21.40 ± 0.16^{bc}
	60	20.47 ± 1.77^{bc}
	80	18.60 ± 0.26^{b}
	100	19.67 ± 0.13^{bc}
0% - 60%	20	21.68 ± 1.50^{bc}
	40	22.92 ± 1.05^{c}
	60	22.02 ± 1.02^{bc}
	80	20.22 ± 0.97^{bc}
	100	21.27 ± 1.13^{bc}

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0% - 80%	20	20.65 ± 0.85^{bc}
	40	20.21 ± 0.87^{bc}
	60	20.16 ± 1.75^{bc}
	80	20.71 ± 0.59^{bc}
	100	21.14 ± 0.25^{bc}

[1]Values are means±STD of two replicates. Values in the same row with different superscripts are significantly different (p<0.05)

In first precipitation of ammonium sulfate, 20% of ammonium sulfate concentration with different concentration of unknown protein sample which were 20%, 40%, 60%, 80% and 100% respectively. The 20% of ammonium sulfate concentration with 100% concentration of unknown sample had highest protein concentration (11.26 mg/ml) compared to others. There were no significant differences ($p<0.05$) in mean 20% of ammonium sulfate concentration.

From the Table 4.1, it can be concluded that 0% - 20% of ammonium sulfate with different concentration of unknown sample harvested the lowest concentration of protein compared to other treatments. This happen due to low concentration of ammonium sulfate which was unable to purify protein based on solubility. It was agreement with (Duong-Ly & Gabelli, 2014) stated that salting out can be used to separate proteins based on their solubility in the presence of a high concentration of salt. Due to low concentration, hydrogen bond between protein and water molecule was not destroy. This situation had been explained by Sattayasai (2012), high concentration of some salt can precipitate protein by destroying hydrogen bond between protein and water molecule.

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The solution with 40% of ammonium sulfate concentration with 20% concentration of unknown protein concentration contained second highest protein concentration (22.39 mg/ml) compared to other treatments. Meanwhile, the 40% of ammonium sulfate with 80% concentration of unknown protein sample contained lowest protein concentration (18.60 mg/ml). There were significant differences ($p<0.05$) between 20% and 80% concentration of unknown protein sample with 40% of ammonium sulfate.

According to Table 4.1, the 60% of ammonium sulfate concentration with 40% concentration of unknown protein sample contained significantly highest protein concentration (22.92 mg/ml) compared to other treatments. The 80% concentration of unknown protein sample showed lower (20.22 mg/ml) of protein contained compared to others. The results showed that there were significant differences ($p<0.05$) between 40% and 80% concentration of unknown protein sample with 60% of ammonium sulfate concentration.

Table 4.1 showed that 40% of ammonium sulfate concentration had second highest of protein concentration while 60% of ammonium sulfate concentration significantly highest in first ammonium precipitation. According to Scopes (2013) that stated in previous study, the first trial fractionations with range 0% to 48% of ammonium sulfate could produce 25% of protein precipitated followed by second and third trial, 32% and 35%. In previous study, in first precipitation with 40% of ammonium is the optimum percentage to harvest highest concentration of protein (Etiatin et al., 2009; Garbayo et al.,

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1998; Fan Huang et al., 2000). It can be concluded that there was a slightly difference between this current study and previous.

From the Table 4.1, the 80% of ammonium sulfate concentration showed that there were no significant differences ($p<0.05$) in mean. The highest protein contained in this treatment was with 100% concentration of unknown protein sample (21.14 mg/ml) while the lowest was with 60% concentration of unknown protein sample (20.16 mg/ml). The concentration of protein with 80% of ammonium sulfate concentration precipitated high concentration of protein with range 20 mg/ml to 22 mg/ml.

4.1.2 SECOND PRECIPITATION OF AMMONIUM SULFATE

Table 4.2 presented the quantity of crude protein in with different percentage of ammonium sulfate and concentration of protein used in Bradford test.

Table 4.2: Concentration of protein in second precipitation of ammonium sulfate (mg/ml)

Ammonium sulfate concentration	Concentration of unknown protein sample (%)	Concentration of protein (mg/ml)
20% - 40%	20	12.82 ± 0.29[a]
	40	13.94 ± 0.53[a]
	60	14.18 ± 0.42[a]
	80	14.57 ± 0.56[a]
	100	14.77 ± 0.53[a]

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40% - 60%	20	25.09 ± 1.53^{b}
	40	24.86 ± 1.70^{b}
	60	24.77 ± 1.98^{b}
	80	23.58 ± 1.21^{b}
	100	24.30 ± 1.11^{b}
40% - 80%	20	12.44 ± 0.20^{a}
	40	13.33 ± 0.00^{a}
	60	12.97 ± 0.61^{a}
	80	12.86 ± 0.29^{a}
	100	12.94 ± 0.10^{a}
60% - 80%	20	12.57 ± 0.36^{a}
	40	12.64 ± 0.03^{a}
	60	12.85 ± 0.27^{a}
	80	12.79 ± 0.27^{a}
	100	12.97 ± 0.58^{a}

[1]Values are means±STD of two replicates. Values in the same row with different superscripts are significantly different ($p<0.05$)

After centrifugation process, there were some of samples were not precipitate. The sample with percentage 20% - 40%, 40% - 60%, 40% - 80%, and 60% - 80% precipitated at the wall of centrifuge tubes. The 20% - 40% of ammonium sulfate with 100% concentration of unknown protein sample contained highest (14.77 mg/ml) protein concentration among others concentration while 20% concentration of unknown protein sample contained low of protein concentration (12.82 mg/ml).

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For 40% - 60% of ammonium sulfate concentration with 20% concentration of unknown protein sample, it contained significantly highest protein concentration (25.09 mg/ml) compared to other treatments. Meanwhile, the 40% concentration of unknown protein sample contained second highest (24.86 mg/ml) protein concentration compared to other treatments.

The solution with 40% - 80% of ammonium sulfate concentration and 40% concentration of unknown protein sample contained higher (13.33 mg/ml) protein concentration than others. The lowest (12.44 mg/ml) protein concentration is identified in 20% concentration of unknown protein sample compared to other treatments.

The 60% - 80% of ammonium sulfate with 100% concentration of unknown protein sample had highest (12.97 mg/ml) protein concentration while 20% concentration of unknown protein sample contained low protein concentration (12.57 mg/ml). In second precipitation, it could be concluded that there were no significant differences ($p<0.05$) in mean of each concentration of ammonium sulfate (Table 4.2).

From the Table 4.2, for second precipitation, it could be concluded that there were no significant differences ($p<0.05$) in mean of each ammonium sulfate concentration. The 40% - 60% showed to have highest protein concentration among other treatments. According to Scopes (2013) based on trial fractionations with ammonium sulfate indicate that saturation range 40% - 60% shows a lower percent protein precipitation compared to 60% - 80% of ammonium sulfate. Thus, these findings were significantly different to the current study.

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From both experiment, first and second precipitation, there were possible errors that could be affected the results. One of them including the temperature. The temperature of cold room was not in required temperature with range 5°C to 8°C. According Hunte et al. (2003), the early stages of a purification protocol it is generally advisable to work at cold room temperature which is 4°C in order to minimize proteolysis activity and the protein will be more stable at 4°C.

The proper method in addition of ammonium sulfate could be one of the possible errors this current study. As mentioned by (Etiatin et al., 2009; Garbayo et al., 1998; Fan Huang et al., 2000), the ammonium sulfate should be added into stirred supernatant in order to minimise the dissolving process and to make it dissolve completely before incubation was applied. In this current study, the ammonium sulfate was added into supernatant without immediately stirring. Thus, the time to dissolve the ammonium sulfate was increased.

Moreover, the centrifugation process could be affected the results. For both first and second precipitation in this current study used similar procedure which is the sample was centrifuged at 27,000 g for 1 hour. This method could give some effects to the sample due would not precipitated enough at the wall of centrifuge tube in second precipitation. In previous study, Etiatin et al. (2009) noted that first precipitation with 40% concentration of ammonium sulfate applied 27,000g at 60 minutes while for second precipitation with 80% concentration of ammonium sulfate applied with 27,000g at 90 minutes.

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Furthermore, the Bradford protein assay could generate some errors in this current study as it was sensitive test. It was full agreement from (Kunze et al., 1989) that noted Bradford by using CBBG was very sensitive although it was high reproducibility and could be used to detect less than 1.0 µg of albumin. (Bradford, 1976) also noted that this method was sensitive to the amino acid composition of the protein. This assay also could be biased by the present of detergent (Hunte et al., 2003).

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CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The objectives of current experiment had been achieved by obtaining the best percentage of $(NH_4)_2SO_4$ for protein purification and the highest concentration of crude protein were evaluated from placenta of Kedah-Kelantan (*Bos Indicus*) cattle. It seemed, the best percentage of ammonium sulfate concentration also can be decided (40% - 60%) with highest among of protein concentration (25.09 mg/ml) after conducting first and second precipitation process. Therefore, this study can enhance the better selection of ammonium sulfate concentration when working out in the precipitation of protein which can be used in PSPB isolation.

Protein purification which was involving separation based on solubility required two important steps which were salting out or salt precipitation and dialysis. In salting out, the optimisation of salt concentration is important in order to breakdown the hydrogen bond between protein and water molecule. Dialysis was the most important part as needed to remove salts before proceeding to the next step.

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Optimisation of ammonium sulfate were required in order to purify proteins. Supernatant were undergone double precipitation as to identify the best percentage of ammonium sulfate that can be used to purify proteins. Furthermore, optimisation in incubation period also important. In this study, the solution with different concentration of ammonium sulfate were undergone two incubation with different time period, which was one hour for first precipitation and 24 hours for second precipitation.

5.2 Recommendation

In the future, researcher can apply different methods in purification protein solubility of protein with advance technology by optimising others parameters such as temperature, pH and type of salts. Besides, purification of protein separation based on size and density can be applied. Separation based on size and density were need some important procedures including centrifugation, dialysis and molecular filtration.

If the desired protein is a water-soluble molecule, the centrifugation of the supernatant can be applied with high speed (10,000 g to 20,000 g) in order to exclude cell debris and large organelles from the crude protein extract. Then, organelles were fractionated by using density gradient centrifugation can be used in order to separate according to their sizes and density into fractions by stepwise increasing of the centrifugal force. Various materials can be used to make gradients such as sucrose and Ficoll. Meanwhile, dialysis used to remove out salts or contaminants from sample by osmotic pressure. Molecular filtration played same role as dialysis. In order to fractionate proteins,

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a membrane with specific pore size is used. The molecules smaller than the pore only can pass through the membrane by using pressure or centrifugation force

The quantification of protein can be further in the future by characterization of the protein. It can be done by enzyme-linked immunosorbent assay (ELISA) test. As mentioned, PSPB or PAG-1 located in the placenta which act as pregnancy marker. These glycoprotein can be measured by using ELISA. Besides, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) can be applied in order to separate protein based on their molecular weight. SDS-PAGE and PI can be used to identify molecular weight of bovine PSPB (bPSP). Estimation of the molecular size of bPSPB varied considerably, with values ranging from 37 kDa to 78 kDa, and isoelectric points (pI) ranged from 4.0 to 4.4. This method can be applied for pregnancy detection as pregnancy diagnosis is an important part in reproduction management of ruminant industry. Early identification of pregnancy is important in order to improve the breeds in ruminant industry. The improvement of the breeds can lead to the successful in beef and dairy industries in Malaysia.

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REFERENCES

Ab, E. (2011). Protein Purification. *Strategy*, *10*(1), 93. https://doi.org/10.1186/1475-2859-10-93

Abdulkareem, T. A., Eidan, S. M., Ishak, M. A., Al-Sharifi, S. A. M., Alnimer, M. A., Passavant, C. W., … Sasser, R. G. (2012). Pregnancy-specific protein B (PSPB), progesterone and some biochemical attributes concentrations in the fetal fluids and serum and its relationship with fetal and placental characteristics of Iraqi riverine buffalo (Bubalus bubalis). *Animal Reproduction Science*, *130*(1–2), 33–41. https://doi.org/10.1016/j.anireprosci.2012.01.002

Alan K . Wood , Robert E . Short , Ann-Elaine Darling , Gary L . Dusek, R. . G. S. and C. A. . R. S. (2016). Serum Assays for Detecting Pregnancy in Mule and White-Tailed Deer Author: The Journal of Wildlife Management , Vol . 50 , No . 4, *50*(4), 684–687.

Bowen, J. A., & Burghardt, R. C. (2000). Cellular mechanisms of implantation in domestic farm animals, *11*, 93–104. https://doi.org/10.1006/scdb.2000.0155

Bradford, M. M. (1976). A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *254*, 248–254.

Buse, E., Häeger, J., Svensson-arvelund, J., Markert, U. R., Faas, M. M., Ernerudh, J., … Pfarrer, C. (2014). Toxicologic Pathology. https://doi.org/10.1177/0192623313482208

Cseke, L. J., Kaufman, P. B., Kirakosyan, A., & Westfall, M. V. (2011). Isolation and Purification of Proteins. *Handbook of Molecular and Cellular Methods in Biology and Medicine*, 291–312.

Czok, B. Y. R., & Bucher, T. (1940). CRYSTALLIZED ENZYMES FROM THE MYOGEN OF RABBIT SKELETAL MUSCLE ’, (1939).

D . B . Houston , C . T . Robbins, C. . A. . R. and R. . G. . S. (2016). Pregnancy Detection in Mountain Goats by Assay for Pregnancy-Specific Protein B: Published by : Wiley on behalf of the Wildlife Society Stable URL : http://www.jstor.org/stable/3800993 Accessed : 23-05-2016 01 : 16 UTC, *50*(4), 740–742.

Duong-Ly, K. C., & Gabelli, S. B. (2014). Salting out of Proteins Using Ammonium Sulfate Precipitation. *Methods in Enzymology*, *541*, 85–94. https://doi.org/10.1016/B978-0-12-420119-4.00007-0

Etiatin, E. T. S., Ajuthi, D. S., Urwantara, B. P., & Alib, C. T. (2009). Ekstraksi dan Isolasi Ovine Pregnancy Associated-Glycoprotein (ovPAG) dari Kotiledon Plasenta Domba Garut pada Saat Melahirkan, *14*(3), 208–215.

Gábor, G., Kastelic, J. P., Abonyi-Tóth, Z., Gábor, P., Endrődi, T., & Balogh, O. G. (2016). Pregnancy Loss in Dairy Cattle: Relationship of Ultrasound, Blood Pregnancy-Specific Protein B, Progesterone and Production Variables. *Reproduction in Domestic Animals = Zuchthygiene*, *51*(4), 467–473. https://doi.org/10.1111/rda.12703

Gajewski, Z., Sousa, N. M. De, & Pawlinski, B. (2009). Concentration of bovine

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pregnancy associated glycoprotein in plasma and milk : Its application for pregnancy diagnosis in cows GLYCOPROTEIN IN PLASMA AND MILK : ITS APPLICATION, (May 2014).

Garbayo, J. M., Remy, B., Alabart, J. L., Folch, J., Wattiez, R., Falmagne, P., & Beckers, J. F. (1998). Isolation and partial characterization of a pregnancy-associated glycoprotein family from the goat placenta. *Biol Reprod*, *58*(1), 109–115. https://doi.org/10.1095/biolreprod58.1.109

Gräslund, S., Nordlund, P., Weigelt, J., Hallberg, B. M., Bray, J., Gileadi, O., … Gunsalus, K. C. (2008). Protein production and purification. *Nature Methods*, *5*(2), 135–146. https://doi.org/10.1038/nmeth.f.202.Protein

Green, A. A., & Hughes, W. L. (1955). [10] Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. *Methods in Enzymology*, *1*(C), 67–90. https://doi.org/10.1016/0076-6879(55)01014-8

Green, J. A., Xie, S., Quan, X., Bao, B., Gan, X., Mathialagan, N., & Roberts, R. M. (2000). Pregnancy-Associated Bovine and Ovine Glycoproteins Exhibit Spatially and Temporally Distinct Expression Patterns During Pregnancy 1, *1631*, 1624–1631.

Hamilton, W. C., Hass, G. M., Butler, J. E., Ruder, C. A., Sasser, R. G., & Williams, R. J. (1982). Detection and Partial Characterization of Two Proteins " 2 Bovine to be bovine placental, 925–933.

Hedhammar, M., Karlstrom, A., & Hober, S. (2006). Chromatographic methods for protein purification. *Stockholm: Royal Institute of Technology*, 1–31. Retrieved from http://www.biotech.kth.se/courses/gru/courselist/BB2040/Forelasningar/ChromMethods.pdf

Hermodson, M. (1996). Current protocols in protein science, edited by J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, and P.T. Wingfield. New York: Wiley, 1995, 864 pages (core)   576 pages (4 supplements)/year (updated looseleaf), print and CD-ROM. *Proteins: Structure, Function, and Genetics*, *24*(3), 409–409. https://doi.org/10.1002/prot.340240303

Hochuli, E. (1992). Purification Techniques for Biological Products. *Pure & Appl. Chem*, *64*(1), 169–184. https://doi.org/10.1351/pac199264010169

Huang, F., Cockrell, D. C., Stephenson, T. R., & Noyes, J. H. (2000). A Serum Pregnancy Test with a Specific Radioimmunoassay for Moose and Elk Pregnancy-Specific Protein B Garth Sasser Published by : Wiley on behalf of the Wildlife Society Stable URL : http://www.jstor.org/stable/3803246. *The Journal of Wildlife Management*, *64*(2), 492–499. https://doi.org/10.2307/3803246

Huang, F., Cockrell, D. C., Stephenson, T. R., Noyes, J. H., & Sasser, R. G. (1999). Isolation, purification, and characterization of pregnancy-specific protein B from elk and moose placenta. *Biology of Reproduction*, *61*(4), 1056–1061. https://doi.org/10.1016/B978-0-12-804729-3.00012-2

Humblot, P. (2001). Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. *Theriogenology*, *56*(9), 1417–1433.

FYP FIAT

https://doi.org/10.1016/S0093-691X(01)00644-6

Humblot, P., De Montigny, G., Jeanguyot, N., Tetedoie, F., Payen, B., Thibier, M., & Sasser, R. G. (1990). Pregnancy-specific protein B and progesterone concentrations in French Alpine goats throughout gestation. *Journal of Reproduction and Fertility*, *89*, 205–212. https://doi.org/10.1530/jrf.0.0890205

Hunte, C., Jagow, G. Von, & Schagger, H. (2003). *Membrane Protein Purification and Crystallization A Practical Guide*. Retrieved from http://books.google.com/books?hl=en&lr=&id=qosOyZ7hUW8C&oi=fnd&pg=PP2&dq=Membrane+Protein+Purification+and+Crystallization+A+Practical+Guide&ots=QAL-S5gol6&sig=wV6Zmv7wqEiaP197K4UXS-rF0Oo

Igwebuike, U. M. (2009). A review of uterine structural modifications that influence conceptus implantation and development in sheep and goats, *112*, 1–7. https://doi.org/10.1016/j.anireprosci.2008.12.010

Igwebuike, U. M., & Ezeasor, D. N. (2013). The morphology of placentomes and formation of chorionic villous trees in West African Dwarf goats (Capra hircus), *83*(3), 313–321.

Kumar, P., & Sharma, S. M. (2015). An overview of purification methods for proteins. *Ijar*, *1*(12), 450–459.

Kunze, H., Medizin, E., & Republic, F. (1989). Refinement Quantitative of the Coomassie Brilliant Blue G Assay for Protein Determination, *102*, 100–102.

Lee, C. S., Wooding, F. B. P., & Brandon, M. R. (1981). Immunogold co-localization of ovine placental lactogen and the antigen recognized by the SBU-3 monoclonal antibody in sheep placental granules.

Luo, J., Wu, C., Xu, T., & Wu, Y. (2011). Diffusion dialysis-concept , principle and applications. *Journal of Membrane Science*, *366*(1–2), 1–16. https://doi.org/10.1016/j.memsci.2010.10.028

Miyazawa, T. (2014). AN ENDOGENOUS RETROVIRUS INVOLVED IN PLACENTAL MORPHOGENESIS IN BOVINAE: DIVERSITY OF MAMMALIAN PLACENTAS AND ENDOGENOUS RETROVIRUSES. *Placenta*, *35*(10), A4. https://doi.org/10.1016/j.placenta.2014.08.013

Moore, P. A., & Kery, V. (2009). High Throughput Protein Expression and Purification, *498*, 309–314. https://doi.org/10.1007/978-1-59745-196-3

Page, M., & Thorpe, R. (2002). Purification of IgG by precipitation with sodium sulfate or ammonium sulfate. *The Protein Protocols Handbook*, 721–722. https://doi.org/10.1385/1-59259-169-8:983

Patel, O. V., Takahashi, T., Imai, K., & Hashizume, K. (2004). Generation and purification of recombinant bovine pregnancy associated glycoprotein. *Veterinary Journal*, *168*(3), 328–335. https://doi.org/10.1016/j.tvjl.2003.09.012

Purwanto, M. G. M. (2016). The Role and Efficiency of Ammonium Sulphate Precipitation in Purification Process of Papain Crude Extract. *Procedia Chemistry*, *18*(Mcls 2015), 127–131. https://doi.org/10.1016/j.proche.2016.01.020

Radivojac, P. (2013). A (not so) Quick Introduction to Protein Function Prediction, 1–

FYP FIAT

20.

Rama, S., & Rao, A. J. (2003). Regulation of growth and function of the human placenta, 263–268.

Ryan, B. J. (2011). Differential Precipitation and Solubilization of Proteins. *Methods in Molecular Biology (Clifton, N.J.)*, *681*, 203–213. https://doi.org/10.1007/978-1-60761-913-0_11

Sasser, R. G., Ruder, C. A., Ivani, K. A., Butler, J. E., & Hamilton, W. C. (1986). Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. *Biol Reprod*, *35*(4), 936–942. https://doi.org/10.1095/biolreprod35.4.936

Sattayasai, N. (2012). Protein Purification. *Chemical Biology*. https://doi.org/10.5772/35425

Schlafer, D. H., Fisher, P. J., & Davies, C. J. (2000). The bovine placenta before and after birth : placental development and function in health and disease, 145–160.

Scopes, R. (2013). *Protein Purification*. (C. R. Cantor, Ed.) (Third Edit).

Sedmakand, J. J., & Grossberg, S. E. (1977). A Rapid , Sensitive , and Versatile Assay for Protein Using Coomassie Brilliant Blue G250, *552*, 544–552.

Semester, F. (2000). 8 . 1 Protein Structure Introduction, (January 1999), 1–19.

Sigma. (2004). Technical bulletin ammonium sulfate solution, 4.1M, 1–4.

Silva, L. A., Kuhn, K. R., Moraes, C. C., Burkert, C. A. V., & Kalil, S. J. (2009). Experimental Design as a Tool for Optimization of C-Phycocyanin Purification by Precipitation from Spirulina platensis. *Journal of the Brazilian Chemical Society*, *20*(1), 5–12. https://doi.org/10.1590/S0103-50532009000100003

Song, Y. (2006). Protein Purification.

Sousa, N. M., Ayad, A., Beckers, J. F., & Gajewski, Z. (2006). Pregnancy-associated glycoproteins (PAG) as pregnancy markers in the ruminants. *Journal of Physiology and Pharmacology*, *57*(SUPPL. 8), 153–171. https://doi.org/10.13614/j.cnki.11-1962/tu.2014.03.002

Stellflug, C. A. R. J. N. (1988). ApriZ 25, 1987 23, 1988, *29*(8741), 905–912.

Strategy, G. C. (2005). Purification of Protein, 133–192. https://doi.org/10.1533/9781845695088.1.26

Tefera, M., Jeanguyot, N., Thibier, M., & Humblot, P. (2001). Pregnancy-Specific Protein B (bPSPB) and Progesterone Monitoring of Post-Partum Dairy Cows with Placental Retention. *Journal of Veterinary Medicine Series A: Physiology Pathology Clinical Medicine*, *48*(6), 331–336. https://doi.org/10.1046/j.1439-0442.2001.00359.x

The University of North Dakota. (2014). Eggs and Dairy. *Protein Factsheet*, 1–5.

Wingfield, P. T. (2016). Protein precipitation using ammonium sulfate. *Current Protocols in Protein Science*, *2016*(April), A.3F.1-A.3F.9. https://doi.org/10.1002/0471140864.psa03fs84

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Zoli, A., Guilbault, L., Delahaut, P., Benitez-Ortiz, W., & Beckers, J. (1992). Radioimmunoassay of a Bovine Its Application Pregnancy-Associated Glycoprotein for Pregnancy in Serum :, 46: 83-92.

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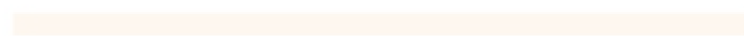

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APPENDICES

A.1: One-way ANOVA of first ammonium sulfate precipitation

ANOVA

Volume

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	860.057	19	45.266	45.285	.000
Within Groups	19.992	20	1.000		
Total	880.049	39			

Table A.2: Post Hoc Analysis using Duncan Multiple Test for 0% - 20% ammonium sulfate concentration

Treatment	N	Subset for alpha = 0.05		
		1	2	3
20%	2	10.0886		
40%	2	10.2927		
60%	2	9.9967		
80%	2	10.4661		
100%	2	11.2620		
Sig.		.998	.80	.215

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

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Table A.3: Post Hoc Analysis using Duncan Multiple Test for 0% - 40% ammonium sulfate concentration

Treatment	N	Subset for alpha = 0.05		
		1	2	3
20%	2		22.3947	22.3947
40%	2		21.4049	21.4049
60%	2		20.4661	20.4661
80%	2		18.5988	
100%	2		19.6702	19.6702
Sig.		.998	.80	.215

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Table A.4: Post Hoc Analysis using Duncan Multiple Test for 0% - 60% ammonium sulfate concentration

Treatment	N	Subset for alpha = 0.05		
		1	2	3
20%	2		21.6804	21.6804
40%	2			22.9151
60%	2		22.0171	22.0171
80%	2		20.2212	20.2212
100%	2		21.2722	21.2722
Sig.		.998	.80	.215

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

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Table A.5: Post Hoc Analysis using Duncan Multiple Test for 0% - 80% ammonium sulfate concentration

Treatment	N	Subset for alpha = 0.05		
		1	2	3
20%	2		20.6498	20.6498
40%	2		20.2110	20.2110
60%	2		20.1600	20.1600
80%	2		20.7110	20.7110
100%	2		21.1396	21.1396
Sig.		.998	.80	.215

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

A.6: One-way ANOVA of second ammonium sulfate precipitation

ANOVA

Volume

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	972.691	19	51.194	74.729	.000
Within Groups	13.701	20	.685		
Total	986.393	39			

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Table A.7: Post Hoc Analysis using Duncan Multiple Test for 20% - 40% ammonium sulfate concentration

Treatment	N	Subset for alpha = 0.05	
		1	2
20%	2	12.8233	
40%	2	13.9355	
60%	2	14.1804	
80%	2	14.5682	
100%	2	14.7722	
Sig.		.406	.930

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Table A.8: Post Hoc Analysis using Duncan Multiple Test for 40% - 60% ammonium sulfate concentration

Treatment	N	Subset for alpha = 0.05	
		1	2
20%	2		25.0886
40%	2		24.8641
60%	2		24.7722
80%	2		23.5784
100%	2		24.3029
Sig.		.406	.930

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

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Table A.9: Post Hoc Analysis using Duncan Multiple Test for 40% - 80% ammonium sulfate concentration

Treatment	N	Subset for alpha = 0.05	
		1	2
20%	2	12.4355	
40%	2	13.3335	
60%	2	12.9661	
80%	2	12.8534	
100%	2	12.9355	
Sig.		.406	.930

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Table A.10: Post Hoc Analysis using Duncan Multiple Test for 60% - 80% ammonium sulfate concentration

Treatment	N	Subset for alpha = 0.05	
		1	2
20%	2	12.5682	
40%	2	12.6396	
60%	2	12.8539	
80%	2	12.7927	
100%	2	12.9661	
Sig.		.406	.930

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

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Figure A.11: Separation between supernatant and pellet after centrifugation

Figure A.12: Preparation of dialysis

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Figure A.13: Bradford reagent

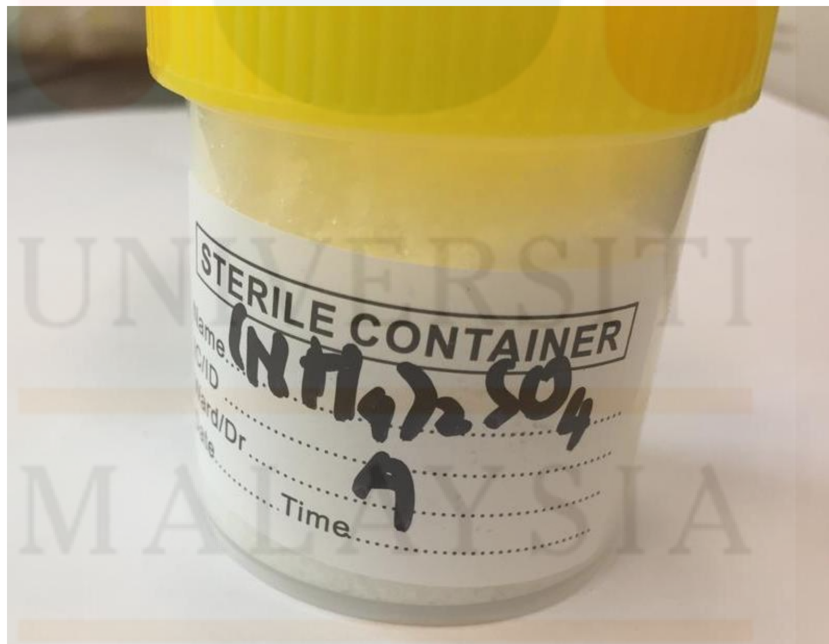

Figure A.14: Dry ammonium sulfate

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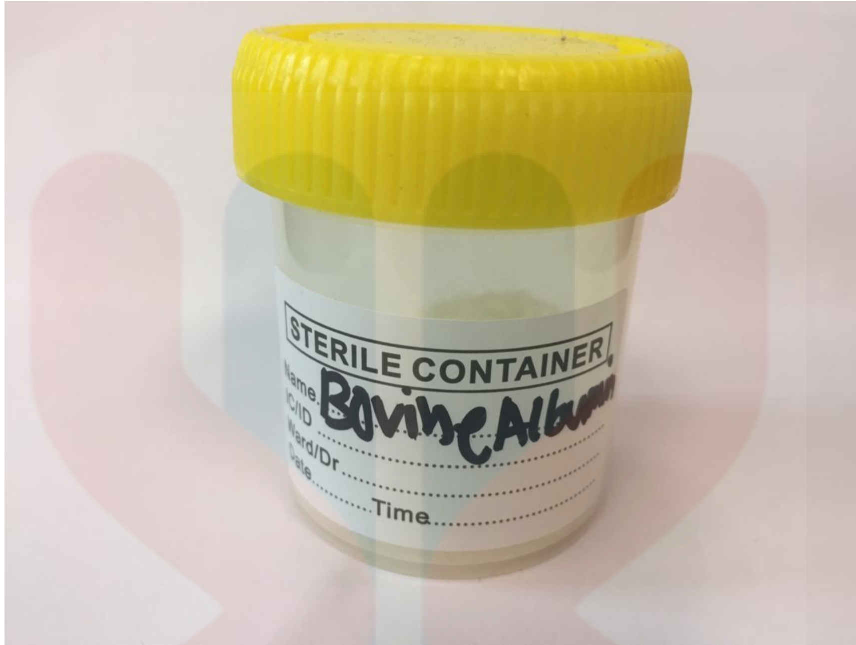

Figure A.15: Dry bovine serum albumin (BSA)

Figure A.16: Visking tube

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Figure A.17: Centrifuge 5418 R

Figure A.18: Supra 22K

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Figure A.19: Spectroquant Pharo 300

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