Optimisation of Total Protein Concentration Based on Buffer Type and Ratio in Placental Protein Extraction of Bovine Kedah Kelantan

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

Faculty of Agro-Based Industry

University 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 institution

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Signature

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I certify that the report of this final year project entitled "Optimisation of Total Protein Concentration Based on Buffer Type and Ratio in Placental Protein Extraction of Kedah Kelantan Cattle (*Bos Indicus)*" by Tee Yung Yee, matric number F15A0234 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honours, Faculty of Agro-Based Industry, University Malaysia Kelantan.

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LIST OF SYMBOLS

%	Percentage
µg	Microgram
µl	Microlitre
g	Gram
kDA	Kilodaltons
mg	Milligram
ml	Millilitre
ºC	Degree Celsius

LIST OF ABBREVIATION

$-NH_2$	Amine group
-COOH	Carboxyl group
bPAG	Bovine pregnancy associated protein
DOC	Sodium deoxycholate
EDTA	Ethylenediaminetetraacetic acid
H^+	Hydrogen ion
HCl	Hydrochloric acid
pI	Isoelectric point
PAG	Pregnancy associated protein
PBS	Phosphate buffer saline
PSPB	Pregnancy-specific protein B
BCN	Binucleate cell
PGE	Prostaglandin E
PMSF	Phenylmethylsulphonyl fluoride
RIPA	Radioimmunoprecipitation assay
Pi	Protease inhibitor
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
NaCl	Sodium chloride
-OH	Hydroxyl group
SDS	Sodium dodecyl sulfate

Optimisation of total protein concentration based on buffer type and ratio in placental protein extraction of Kedah Kelantan cattle

ABSTRACT

Proteomic study related with placental protein has been an interest topic to the researcher over decades to determine the function and mechanism of particular protein that perhaps to improve pregnancy related disorder or pregnancy diagnosis in human and livestock industry. Protein extraction is a preliminary step of protein purification which mainly focus on maximisation of total protein yield. The heterogenous properties causes diversification of protein, therefore there is no absolute protocol in protein extraction, the type of buffer and ratio could give different protein concentration in different type of mammalian tissue, hence lead to the study of optimisation of types of buffer and buffer ratio to obtain better total protein yield. The objectives of this study are to compare the total protein yield based on three types of buffer with three different ratios. Three types of buffer included phosphate buffer saline (PBS), radioimmunoprecipitation assay (RIPA) buffer and RIPA buffer with addition of protease inhibitor (Pi) whilst sample to buffer ratio, 1:1, 1:3, and 1:5 were used in the series of protein extraction process that involved mechanical disruption, incubation, sonication, and centrifµgation. Bradford assay was carried out to determine the total protein yield based on the standard curve of bovine serum albumin (BSA). From this study it is concluded there is a significant interaction between buffer type and ratio ($P<0.05$) where the use RIPA buffer with 1:1 ratio gave the best total protein yield (194.880 ± 15.089 mg/g). This study is perhaps to be a useful reference to future study in term of protein extraction of mammalian tissue especially placental tissue.

Keyword: Placenta protein extraction, Foetal cotyledon, total protein yield, PSPB

Pengoptimuman kepekatan protein keseluruhan berdasarkan jenis dan nisbah penimbal dalam pengekstrakan protein plasenta daripada lembu Kedah Kelantan

ABSTRAK

Kajian proteomik yang berkaitan dengan protein plasenta telah menjadi topik minat kepada penyelidik selama beberapa dekad dalam penentuan fungsi dan mekanisma protein tertentu yang diharapkan dapat memperbaiki gangguan berkaitan kehamilan atau diagnosis kehamilan dalam industri manusia dan ternakan. Pengekstrakan protein adalah langkah awal dalam purifikasi protein yang terutama dalam menumpukan peningkatan dalam jumlah hasil protein. Ciri-ciri heterogen menyebabkan kepelbagaian protein, oleh itu tidak ada protokol yang mutlak dalam pengekstrakan protein, jenis penimbal dan nisbah dapat memberikan kepekatan protein yang berlainan bergantung kepada jenis tisu mamalia yang berbeza, sehingga menyebabkan pengoptimalan jenis penimbal dan nisbah untuk mendapatkan hasil protein yang lebih baik. Objektif kajian ini adalah untuk membandingkan jumlah hasil protein berdasarkan tiga jenis penimbal dengan tiga nisbah yang berbeza. Tiga jenis penimbal termasuk phosphate buffer saline (PBS), penimbal radioimmunoprecipitation assay (RIPA) dan RIPA dengan penambahan perencat protease manakala nisbah sampel kepada penimbal adalah 1:1, 1:3, dan 1:5 telah digunakan dalam proses pengekstrakan protein yang melibatkan gangguan mekanikal, inkubasi, sonikasi, dan pengemparan. Ujian Bradford dijalankan untuk menentukan jumlah hasil protein berdasarkan keluk standard bovine serum albimin (BSA). Kesimpulan daripada kajian ini ada terdapat interaksi yang signifikan antara jenis penampan dan nisbah ($P < 0.05$) di mana penggunaan penimbal RIPA dengan nisbah 1:1 memberi hasil protein yang terbaik (194.880 ± 15.089 mg / g). Kajian ini diharapkan dapat menjadi rujukan yang berguna terhadap kajian masa depan dari segi pengekstrakan protein tisu mamalia terutamanya tisu plasenta.

Kata kunci: Pengekstrakan protein plasenta, cotyledon janin, hasil protein total, PSPB

CHAPTER 1

INTRODUCTION

1.0 Research background

The proteomic study is always being the interest in life science research as it is the most diverse group of biologically essential molecules as well as for cellular structure and function. The protein purification was invented for precisely studying the protein of interest. Protein purification has more than 200 years of history and it was first attempted by Antoine Fourcroy in 1789, who had prepared substances from plants which had similar properties as albumin which coagulates upon heating and dissolution in alkalis (Carpenter, 1994). In the early of 20th century, the separation technologies of protein have developed into several methods such as filtration, precipitation, and crystallisation. During the eruption of world war II where there was an acute need for blood proteins, the fractionation method was developed to purify albumin and other plasma proteins (Tan & Yiap, 2009). The method comprises multiple precipitation steps which are pH, ethanol concentration and temperature, each step will result in a different concentration of protein. Back in 1906, Mikhail Tswett the botanist introduced chromatography and Theodor Svedberg presented that centrifµgation able to separate protein in 1924. A few decades later, several protein separation methods were developed. This included electrophoresis

and affinity chromatography in the 1930s and ion exchange chromatography in 1940s (Biologics International, n.d.). Protein extraction plays an important role in ensuring protein yield before proceeding to the purification of protein and further study of protein. A lot of protocols have been invented by scientists to extract more total protein from the sample. However, due to the diversity of protein in term of structure and properties and its heterogeneousness, there is no exact extraction method that able to produce a similar result in different type of protein (Kapoor, 2006).

The study of protein particularly in pregnancy related protein has become interest to some researcher which intention not only targeted to human, but act as important tool in improving the livestock production. The study of placental protein helps determine the function and mechanism of specific protein in regulating the pregnancy. Pregnancy-specific protein B (PSPB) also known as pregnancy-associated glycoproteins B, that produced during the pregnancy stage in maternal (Dunbar, Wong, Ruder-Montgomery, Chew, & Sasser, 1990). It was described as placental antigens that presented in maternal blood serum after implantation and are produced by mono and binucleate trophoblastic cells in the placenta cotyledon tissue (Austin, King, Vierk, Sasser, & Hansen, 1999). In the present study, this protein has been used in pregnancy diagnosis in reproduction management of ruminant (Gajewski et al., 2009). Pregnancy diagnosis has become indispensable element in breeding system of ruminant where early detection of pregnancy helps identify the successfulness of the reproductive management, and determination of problem at the early stage (Bekele, Addis, Abdela, & Ahmed, 2016).

1.1 Problem statement

Several researches in relates with the proteomic study have been published with a variety of protein extraction protocols (Chourey et al., 2010; Horvath & Riezman, 1994; Méchin, Damerval, & Zivy, 2007; Velapatiño, Zlosnik, Hird, & Speert, 2013; Zakharchenko, Greenwood, Alldridge, & Souchelnytskyi, 2011). The choice of protein extraction method is highly depending on the preference, availability of material and facilities, as well as financial to conduct the study of desired protein. Nevertheless, it is also depending on the source of the material, the location of the cell of the desired protein as well as the downstream application of the protein after being studied. Hence, there is no exact method or reagent that is optimal for general protein extraction due to the heterogeneous properties of the protein. A different source of mammalian tissue may require different buffer solution to maximise the total protein yield. Hence, in the study of PSPB, a variety of buffer such as Tris-HCl, radioimmunoprecipitation assay (RIPA) buffer, phosphate buffer saline (PBS) was involved in protein extraction as well as the ratio of buffer volume to sample weight.

1.2 Hypothesis

H null: There is no significant difference of total protein yield between types of the buffer used, and the ratio of the buffer.

H one: There is a significant difference of total protein yield between types of the buffer used, and the ratio of the buffer.

H null: There is no significant interaction between types of the buffer and the ratio of the buffer.

H one: There is a significant interaction between types of the buffer and the ratio of the buffer.

1.3 Objectives

1. To compare the yield of protein concentration from foetal cotyledon of the bovine placenta using different types of buffer.

2. To optimise the ratio of the buffer used in the protein extraction process.

1.4 Scope of study

The scope of the study is about the molecular study of protein extraction of the bovine placenta by using a different kind of buffer and ratio.

1.5 Significance of the study

The optimisation of the extraction method in foetal cotyledon could possibly increase the total protein yield. Furthermore, through the evaluation of several selected reagents can aid in the identification of a suitable method to be performed to improve total protein yield. Therefore, this research can be used as a reference in the protein of

mammalian tissue to improve the data result and avoid additional cost in the future experiment. Protein extraction plays an important role in deciding the success in a subsequent step as the total protein yield is improved indicates the targeted protein yield can be possibly increased as well. Therefore, isolation of desired protein PSPB can be studied, in term of its activity, properties and even its role, which could help in downstream application to develop an advanced technology to improve the ruminant production system in Malaysia.

1.6 Limitation of study

By performing a suitable method to extract as much total protein as possible which including the target protein which is pregnancy-specific protein B However, there is lack of research focus on protein extraction of mammalian tissue which gives rise to lack of information available. Some equipment might be needed in the published method, however, due to the lack of facilities provided hence the method of protein extraction is limited. This may affect the result in this research. Furthermore, the price of the reagent required in this research is high, which increase the overall budget of whole research.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein

Protein is an essential molecule in cells as it takes part in a variety of function from cellular support to cell signalling and cellular. The chemical properties of the proteins are counted on the amino acid units presented and sequence in the polymer chain (Kinsinger, 2002). The proteins are biosynthesised by a variety of molecules in the cytoplasm which broadly classified as alpha-amino acids. The alpha-amino acid is represented by the formula in Figure 2.1:

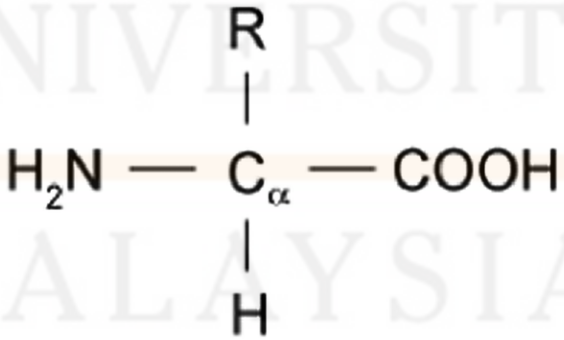


Figure 2.1: The general structure of an alpha-amino acid

Source: Dickinson, Parker, Schoutsen, & Charlton (2014)

R group attached to the carbon atom responsible in the distinction of one alpha-amino acid from one another. The R group can be either pure hydrocarbon or containing other elements such as amino groups, oxygen, or nitrogen carboxylic acid. Proteins express amphoteric nature due to the existence of basic substituent ($-NH_2$) and the acidic substituent (-COOH) in one molecule whereby they turn anion in basic solution and cation in acidic solution (Kinsinger, 2002). The acid strength of protein in aqueous solution depends on the structure and composition of the R group in the amino acids. This resulted in either the carboxylic acid or amine group of the protein interact with water and gives out weak acids, basic, or neutral properties. Figure 2.2 represented the charged form of alpha-amino acid.

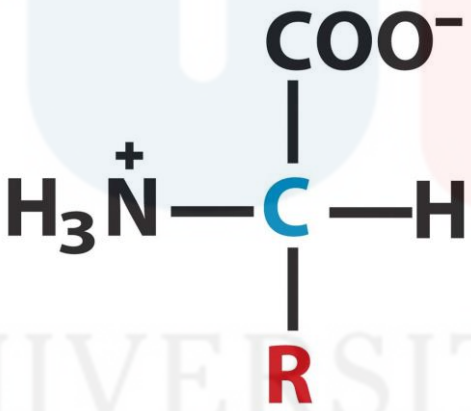


Figure 2.2: The zwitterionic form of the amino acid

Source: Biochemportal (2013)

Each protein has its own iso-electric point (pI) in which the protein will have an equal number of cations and anions implies zero net electric charges at the particular pH (Jain, Jain, & Jain, 2005). At the isoelectric point, the osmotic pressure and viscosity and

solubility of the protein solution are the least, therefore it is easier to get precipitated out. This also implies the solubility increases as increasing acidity or alkalinity. Denaturation indicates the loss of biological activity of protein which may cause the protein to decrease in solubility; size and shape; and cessation of biochemical activity (Jain et al., 2005).

Denaturation of protein involves disruption of bond and destruction causes change in secondary and tertiary structures, while the primary structure will still remain the same as the denaturation reaction not strong enough for breaking up the peptide bonds (Chaplin, 2001; Ophardt, 2003). The denaturation of protein occurs due to the disruption of bonding interaction that responsible to hold the secondary and tertiary structure. It can be caused by both physical and chemical agents. Physical agents including mechanical action, heat treatment, freezing operation, irradiation while chemical agent including acids, alkalis, heavy metal salts, guanidine detergent, urea etc (Shortle, 1996; Taneja, n.d.). Effect of protein denaturation including decreasing solubility, loss of biological activity and crystallising activity; and susceptible to enzymatic hydrolysis (Neurath, Greenstein, Putnam, & Erickson, 1944).

There are seven types of proteins including antibodies, enzymes, contractile protein, structural protein, transport protein, storage protein, and hormonal protein. Protein is synthesised in the body through the process of translation which occurs in the cytoplasm and it involves translation of genes into proteins (Regina Bailey, 2017). Protein structure determines the function of a protein and there is four levels of structure: primary, secondary, tertiary and quaternary. The levels of protein structure are categorised based on the degree of complexity of the structure in the polypeptide chain. In some case, protein can attach with carbohydrate during protein translation or during glycosylation and form glycoprotein.

2.2 Glycoprotein

Carbohydrate (Oligosaccharide, monosaccharide, polysaccharides) chain covalently bonded to the polypeptide side chains of the protein formed a glycoprotein. This process is known as protein glycosylation which takes place in endoplasmic reticulum's lumen and Golgi complex (Berg, Tymoczko, & Stryer, 2002). The carbohydrate is a short molecule, branched, and may make up of simple sugars, amino sugars, as acidic sugars (Anne Marie Helmenstine, 2018). Glycoprotein is more hydrophilic than simple protein due to the hydroxyl groups of the sugar. As a result, the glycoprotein is more soluble in water than normal proteins and leads to the proper folding of the tertiary protein structure (Saraswathy & Ramalingam, 2011). Furthermore, the thermodynamic and kinetic stability is enhanced where thermodynamic stability refers to the increase in melting temperature and free energy difference among unfolded and folded state (Shental-Bechor & Levy, 2009).

There are two major groups of glycoproteins called N-linked glycosylated protein and O-linked glycosylated protein. They are categorised based on the attachment site of the carbohydrate to amino acid of the protein. In N-linked glycoprotein, the glycan moiety is attached to asparagine via N-acetylglucosamine linkage (Saraswathy & Ramalingam, 2011). It gains sugar from endoplasmic reticulum membrane and being proceeded to Golgi complex for further modification (Anne Marie Helmenstine, 2018). Whereas O-linked glycoprotein has N-acetyl galactosamine residue at reducing termini linked with the hydroxyl group of serine or threonine of polypeptide backbone (Cole, Butler, & Kobata, 2015). This process only takes place exclusively in Golgi complex (Berg et al., 2002). The structure difference between N-linked glycoprotein and O-linked glycoprotein are represented in Figure 2.3. This type of protein bound to sμgar in Golgi complex (Anne

Marie Helmenstine, 2018). In this topic will mainly focused on N-linked glycoprotein as the desired protein of this study is pregnancy-specific protein B (PSPB).

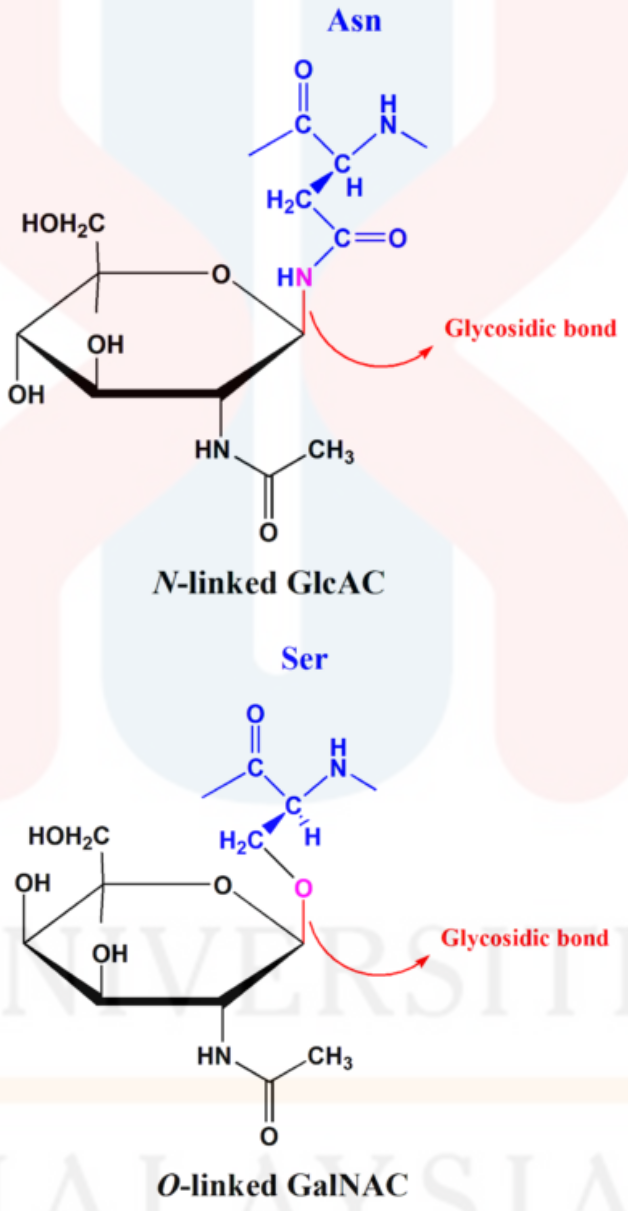


Figure 2.3: N-linked and O-linked Glycoprotein

Source: Wikibook (2018)

In the endoplasmic reticulum, the oligosaccharide that is meant for attaching the asparagine residue of a protein will attach to the terminal phosphate group in dolichol phosphate and transferred and linked to the asparagine residue of growing polypeptide chain (Berg et al., 2002). When the glycoprotein is transported to the Golgi complex, it undergoes modification and is then transferred to either secretory granules, plasma membrane, or lysosome based on the signals that have encoded in the sequence of amino acid and the three-dimensional structure (Berg et al., 2002).

2.3 Placenta, foetal cotyledon, binucleate cells

Placenta is an extra-specialised temporary organ that plays an essential role in establishment and maintenance of pregnancy. It performs as multi-functional organ which functions as respiratory, depurative, nutritional, endocrine as well as the immunological role (Perényi, 2012; Strachan, 1925). Following the fertilisation, the embryo goes throμgh stages of division into the morula stage. In blastocyst stage, trophoblast cell formed as an outer wall which surrounding the blastocoel, the fluid-filled central cavity so-called inner cell mass (ICM) starts rapid growth of embryo (Cross, 1998; Rindler, n.d.). Trophoblast cells were assumed to have specialised functions and remain as outermost layer of cells which will cover the placenta. Trophoblast cells form an epithelial layer that covers the chorion. The size expansion of the embryo is resulted by more fluid within blastocoel produced and become the cavity of yolk sac (Schlafer, Fisher, & Davies, 2000). The third layer of cells in inner cell mass, “mesoderm”, extending from the embryonic disc and form a layer between trophectoderm and endoderm. As the embryo grows and elongate, trophoblast cells fold with somatic mesoderm to form amnion. Figure 2.4 show the foetus in the placenta.

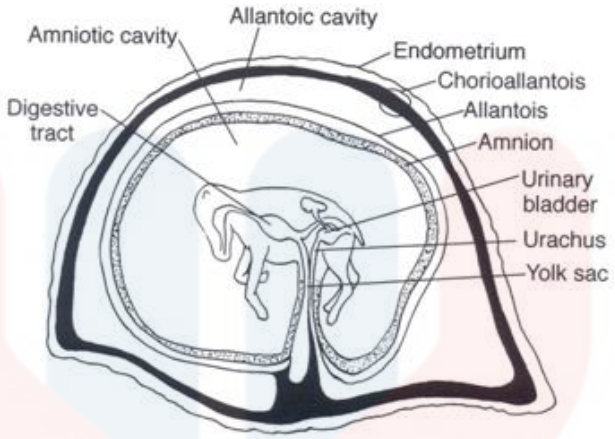


Figure 2.4: foetus in the placenta

Source: Husvéth (2011)

As the embryo is forming into a foetus, the fusion between allantois and the chorion form chorioallantois and invaded the endometrium of the gravid uterus at 4 weeks of gestation in the cow and become more intimate over specialised areas of the endometrium called caruncle (Schlafer et al., 2000). Over the time followed with the growth and expansion of extraembryonic foetal membranes, the flat surface of the chorioallantois become irregular over these caruncles, and those areas are grossly recognized as cotyledons. The combination of cotyledon and caruncular tissue formed the placentome (Perényi, 2012; Schlafer et al., 2000). Figure 2.5 representing the bovine foetus and placenta with cotyledons. The number of cotyledons in the bovine placenta is around 70-150 and are convex and pedunculated rather than concave shape seen in sheep (Perényi, 2012; “Placentation in Ruminants,” n.d.).

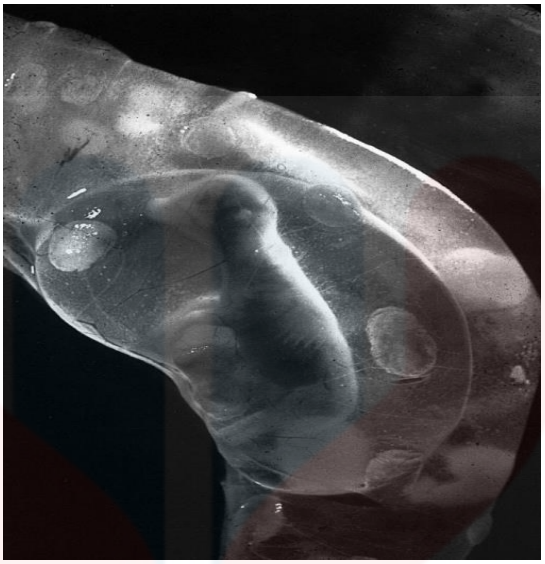


Figure 2.5: Bovine foetus and placenta with cotyledons

Source: Schlafer et al., (2000)

The fascinating feature of the ruminant placenta is the population of binucleate cells (BCN) (Carter, 2005). Foetal binucleate cells constitute 15-20% of the trophectodermal epithelial cells (Schlafer et al., 2000; Wooding, 1992). These cells are derived from the mononucleate cells that undergone nuclear mitosis process without subsequent cytokinesis. Initially, it located deep in trophectoderm before maturation and will migrate to the maternal surface during maturation (Perényi, 2012; Wooding, 1992). Young BNC contains a small volume of dense ribosome-filled cytoplasm and will later reorganize into an oval or spherical cell that has no contact with the basement membrane or apical trophectodermal tight junction. Followed by the cell growth, an extensive array of rough endoplasmic reticulum and large Golgi body will be developed (Wooding, 1992). The aforementioned organelles will later produce a considerable number of characteristic granules which occupy more than 50% of the volume of BNC at maturity. Wooding (1992) suggested that these granules contain numerous protein and glycoprotein constituents

including the placental lactogen hormone. It produces hormones and growth factors such as progesterone, pregnancy-associated glycoproteins (bPAG-1, bPAG-2, bPAG-3) (Schlafer et al., 2000).

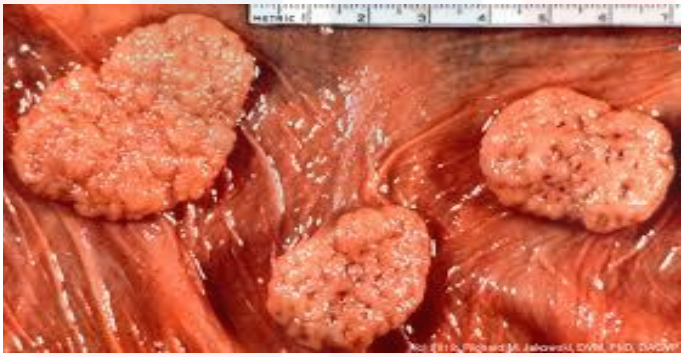


Figure 2.6: Bovine cotyledon placenta

Source: µGA veterinary medicine

2.4 PSPB properties and function

PSPB (pregnancy-specific protein B) is a pregnancy-associated glycoprotein (PAG) that produced by binucleate cells of the trophectoderm of ruminants, it can be detected started from the third week of pregnancy until parturition in the serum of pregnant cows (Dunbar et al., 1990). It belongs to aspartic peptidases, a class of proteolytic enzymes that works well in acidic condition (Davies, 1990). Research related to PAG reported that despite the PAG amino sequence was greater than 50% similar to pepsin, cathepsin D, and cathepsin E, but they are enzymatically inactive due to the critical amino acid substitutions at the active site regions (Green, Xie, & Roberts, 1998; Xie et al., 1991).

PSPB can only be detected during the pregnancy stage, therefore, Dunbar et al. (1990) speculated that its origin came from the placenta. Rocket immunoelectrophoretic were used to measure the concentration of PSPB that circulating throμghout the pregnancy and postpartum in arbitrary unit proven that PSPB concentration was low in the first five months of pregnancy and increase gradually from 5th to 8th month but inclined from 8th to 9th month (Sutcliffe, 1985). PSPB possessing several molecular masses, from 43 to 66 kDa, pI of 4.0 to 4.4, and migrated on immunoelectrophoretic like alpha-2 globulin (Garbayo et al., 1998a; Sasser, Ruder, Ivani, Butler, & Hamilton, 1986; Willard, White, Wesson, & Stellflug, 1995). Furthermore, this protein was very labile and become denatured when stored at -20c.

Weems et al. (2007) suggested that PSPB regulates progesterone by regulating the placental PGE secretion. The PSPB initiates secretion of PGE at the 200th day in bovine placenta. Weems et al. (1999) stated that oestradiol- 17B regulates placental secretion of PSPB, while PSPB, in turn, controls the placental secretion of PGE, while PGE regulates placental secretion of progesterone at 90 days of pregnancy in sheep. The result of Xie et al. (1991) proposed that PAGs may be able to carry out endocrine function where they bind with specific cell surface receptors that located on the maternal target cells with their binding clefts ((Perényi, 2012).

Pregnancy-associated glycoprotein (PAG) function as inhibiting the growth of bovine myeloid bone marrow cells at 2400 to 3000 ng/ml concentration ((Perényi, 2012). Additionally, Dosogne et al. (1999) proposed the involvement of PAG in local immunosuppression which is necessary for early pregnancy to prevent miscarriage due to host rejection by the maternal immune response.

2.5 Protein purification

Purification of protein was first attempted and reported by Fourcroy in 1789, who isolated substances from the plant which has similar properties as egg white. The purification of plant protein was conducted in the nineteenth century. However, most were not considered pure according to modern standard (Scopes, 2001). Initially, the aims of protein purification were mainly for academic whereby the protein needed was only for studying the structure and test the rival theories of the pre-DNA days until the eruption of world war II where the urgent need for blood protein has turned out to be the pushing factor in the development of protein purification. As a result, this became the inception of large-scale protein purifications for commercial purposes such as food supplements, pharmaceuticals etc (Kumar & Sharma, 2015; Scopes, 2001). The preliminary step of protein purification is aimed at a concentration of the protein sample which in term of processing volume and increase the portion of the target protein in total protein (Healthcare, 2010). Hence, the careful choice of starting material is pivotal in simplifying the problem of purification which is the volume of the desired protein (Beeley, 1985).

2.6 Protein extraction

Protein extraction is a critical step before proceeding protein purification. Selection of the appropriate protocol is crucial to ensure high total protein yield due to diverse properties of the protein. The principle aim of any extraction method is to disrupt the tissue to the higher degree, with minimum force utilised, and be reproducible (Doonan, 1996). Besides that, the nature of the sample, the location of the interest protein, and

protein stabilisation during extraction (Luís, Alexandre, Oliveira, & Abreu, 2016; Thermo Scientific, 2015). Cell lysis is the first steps for protein extraction to release the cellular protein whereby it can be achieved through several methods (Mao, Huang, & Ho, 2010). The extraction of protein from animal tissue, in compared with plant tissue, is relatively straightforward because it is enclosed soft plasma membrane instead rigid cell wall (Doonan, 1996).

Generally, cell lysis can be classified into two major groups which are mechanical and non-mechanical method. In mechanical lysis, the cell membrane will be broken down by shearing force such as homogenization, sonication, grinding with alumina or sand, grinding with glass beads, as well as mortar and pestle (Ahmed, 2005).

Whereas non-mechanical lysis can be further divided into three groups, which is physical, chemical and enzymatic. Physical disruption is a non-cell-contact method which ruptures the cell membrane through external force such as thermal lysis, cavitation and osmotic shock (Islam, Aryasomayajula, & Selvaganapathy, 2017). While chemical cell disruption involves in the use of lysis buffer to interrupt the cell membrane by changes of pH which including alkaline lysis and detergent lysis. Enzymatic cell lysis utilizes enzyme such as lysozyme, cellulase, or protease to break the cell membrane.

Normally, researchers often combine both mechanical and non-mechanical method in protein extraction for better total protein yield. Garbayo et al., (1998a) combined both mechanical and non-mechanical in the extraction of PAG from goat placenta by adding PBS lysis buffer during the homogenization of the sample. A similar concept was applied by Huang, Cockrell, Stephenson, Noyes, & Sasser, (1999) in the extraction of PSPB from elk and moose placenta, where the sample was initially undergone mechanical lysis which is thawed and frozen for three times, cut into smaller pieces and stirred with sand in lysis buffer.

2.6.1 Factors affecting protein yield

Protein is a heterogenous biological macromolecule whereby its properties can be severely affected by even small changes in hydrogen ion concentration which is pH (Ahmed, 2005). Therefore, certain factors must be taken into consideration to avoid or minimise any possible contamination or degradation of protein from the sample.

2.6.1.1 Buffering component

The maintenance of pH in the protein solution is very important since the tremendous change of pH can cause the protein to change in shape due to the changes in the force of attraction between the groups in the side chains of the protein causes the protein to unfold and lead to protein dysfunction (Ernest, n.d.). During the cell disruption process, the mechanical force causes the breakdown of the internal compartments including lysosome to be liberated out resulted in a sudden change of pH, which in turn leading to degradation and denaturation of protein to happen. (Nguyen, n.d.). This is due to the fact that in animal cells, lysosome plays role in the digestion of material that been taken in by the cell through endocytosis, and it containing approximately 50 different degradative enzymes that will be involved in the hydrolysis of proteins, DNA, lipids, RNA, and polysaccharides (Cooper, 2000). Hence, breaking down the cell membrane during the extraction process can result in the release of these aforementioned lysosomal hydrolase into the cell cytosol environment and causes a sudden drop in pH since the concentrated amount hydrogen ion (H^+) were liberated through the compartment as well.

Therefore, the addition of buffer is important for ensuring a proper pH environment for the protein of interest as it can suppress minor changes of pH by either taking up or providing H^+ in protein solution to achieve equilibrium (Ahmed, 2005; Ops Diag, n.d.). Buffer solution can be categorised into acid buffer and alkaline buffer, where below pH 7 are acid buffer and above as alkaline buffer (Clark, 2015). Acid buffer is made of a strong acid with the weak conjugate base while the alkaline buffer is a combination between a strong base and a weak acid.

In this study, where the protein of interest is PSPB which is cytosolic protein, it is important to mimic the pH environment of cytosol which is around 7.2 to protect the protein (Boron, Boulpaep, & Walter, 2004). In the research related to PSPB, Tris-HCl, potassium phosphate buffer was used for the protein extraction (Garbayo et al., 1998b; Huang et al., 1999; Kiewisz et al., 2008; Zoli et al., 1991). However, the ratio of buffer to the tissue may differ depends on the method designated, which become the objective in this study. It can be seen from a study on the effect of buffer volume on the plant leaves gave a significant result where 3 ml of Tris-buffered phenol gave overall higher protein concentration (mg/g) in compared with 15 ml of buffer (Abdullah, Chua, & Rahmat, 2017). This, however, could be different when it comes to animal cells.

2.6.1.2 Temperature

The recommended temperature in protein extraction is 4 ºC and below (The Protein Man, n.d.). Meglič, Levičnik, Luengo, Raso, & Miklavčič (2016) reported that the temperature has a great effect on protein extraction, and the best temperature post-treatment is 4 ºC. A study conducted on the extraction of *Pleurotus citrinopileatus*

showed that extraction in cold water has relatively higher protein yield compare in hot water (Chen, Weng, Yu, Koo, & Wang, 2016). As the extraction temperature increased, the solubility and stability decreased (Deak & Johnson, 2007). When the temperature extraction exceeded the optimum temperature of protein, denaturation will occur.

2.6.1.3 Protease

Protease can be released into buffer environment where they do not normally access during the cellular disruption (Ops Diag, n.d.; The Protein Man, 2016.) In normal circumstances, the proteases are normally will be confined in specialised organelles to minimise the chance of undesirable proteolytic activity to other protein, within these organelles, protease would be associated with specific regulators to control the action of the protease (Ryan, 2011 as cited in Vanaman & Bradshaw, 1999). When the cells are disruption during protein extraction, the protease will be separated from their regular molecules and exposed to the protein of interest and leading to undesired proteolysis. This risk can be minimised by performing the cellular disruption at low temperature, where the enzyme activity is restricted; the use of protease inhibitor should be taken into consideration as well (Ericsson & Nistér, 2011).

Protease can be divided into four groups based on the catalytic active site and the mode of action: serine proteinase, cysteine proteinase, aspartic proteinases, and metalloproteinases (Rawlings & Barrett, 1999). As such, protease inhibitors can be classified based on the type of protease they inhibit and the mechanism which they inhibit the enzyme (Ritchie, 2013). For example, EDTA is metalloproteases whereas PMSF is a serine protease inhibitor. The usage of certain protease inhibitor such as

phenylmethylsulphonyl fluoride (PMSF) must be handled carefully due to its sensitivity towards temperature, where only have a half-life of 35 minutes after taken out from the stock solution (Van Venrooij & Maini, 2012).

2.6.1.4 Method of cell disruption

Cell disruption can be categorised into mechanical and chemical method. Prechilled mortar and pestle are one of the mechanical disruptions of the cell for the little amount of sample. The tissue sample should be cut into a smaller size to facilitate the grinding process. However, when mortar and pestle are chosen for tissue disruption in protein extraction, the yield of protein is depending on the force applied to the tissue. Hence, this can be a limiting step for a large number of the sample which cannot be ground adequately using mortar and pestle (Kasem, Rice, & Henry, 2008). Protein denaturation and aggregation can occur due to the heat generated during the process, to encounter this problem, it should be conducted in 4 degree Celsius of temperature condition.

Reagent-based methods in cell lysis are claimed to be rapid, gentle, efficient and could lead to a high protein yield. It functioning as disruption of lipid membrane and cell wall. However, the component within the reagent might affect the protein assays and mass spectrometry (Thermo Fisher Scientific, n.d.).

2.7 Quantification of protein

Accurate measurement of protein concentration is essential since the results will be used in other calculations such as determining the enzyme activities (Olson & Markwell, 2007). In this case, instead of the determination of enzyme activities, the accuracy of measurement is essential in comparing the effectiveness of the type of buffer and buffer ratio towards the total protein yield. However, there is no protein assay that could give absolutely accurate results as each method of protein assay has its own difference advantage and limitations.

2.7.1 Bradford assay

Bradford assay is one of the various methods in protein quantification. It is a rapid and reasonably accurate method in determining the concentration of an unknown protein concentration as it able to produce a maximum absorbance within 5 minutes (Essays, 2013; Stoscheck, 1990). The free dye in Bradford reagent can exist in 3 basic colours which are red, green and blue forms with maximum absorbance at 470nm, 650nm, and 590nm respectively. Red and green form is more protonated (pH 1), in contrast, blue form of dye (pH 2-11) is more anionic (Chial, Thompson, & Splittgerber, 1993; Kruger, 1996).

Bradford assay is recommended for use to determine protein content of disrupted cells compared with Lowry method and Biuret method. Biuret method has relatively low sensitivity compare to Bradford assay as it is less efficient for protein concentrations below 5mg/ml. (Martina & Vojtech, 2015). Despite Lowry method is more sensitive than

Biuret method, but it requires a long duration of incubation time of around 40 minutes and involves two steps, which less efficient in compared with Bradford assay.

Bradford assay is highly sensitive as it could detect protein as little as 0.5 µg of bovine serum albumin (Stoscheck, 1987). But, it is also sensitive to interference by other compounds such as detergent and depends on the sequence of protein where it binds efficiently with arginine and an aromatic residue (Martina & Vojtech, 2015). However, the interference by detergent in Bradford assay can be eliminated by running the proper buffer control (Bradford, 1976).

The anionic form of blue dye called Coomassie Brilliant Blue G250 (CBBG) reacts with the cationic amino acids or side chain of amino acids to form both hydrophobic and ionic interactions resulted in visible colour changes (Essays, 2013; Experimental Bioscience, n.d.). The blue colour intensity increases as the of protein concentration in the reagent increase. This implying the more protein-bound complexes are formed throµgh the reaction (Chial et al., 1993). The dye-protein complex has an absorbance maximum at 595nm (Compton, S. J., and Jones, C. G., 1985 as cited in Chial et al., 1993). The absorbance reading of a range of known protein concentration will be plotted and perform regression to for obtaining linear fashion. The concentration of the unknown protein will be then quantified by using Beer's law.

2.7.2 Bovine serum albumin

Generally, Bovine Serum Albumin (BSA) works well for a protein standard as its wide availability in high purity, relatively inexpensive and parades the best linearity in the protein assay (Doumas, 1975). It is a serum albumin protein isolated from cows

("Bovine Serum Albumin," n.d.). Its application includes ELISA, immunoblots, and immunocytochemistry, additionally, it can be used as a nutrient in cell and microbial culture, stabilize some restriction enzyme during digestion of DNA, prevent the adhesion of enzyme to apparatus such as reaction tubes, pipette tips, as well as a vessel. BSA also commonly used in protein quantification such as BCA assay and Bradford assay by comparing an unknown quantity of protein to known quantity of BSA.

CHAPTER 3

METHODOLOGY

3.0 Experimental design

In this study, the buffer solutions needed prior to protein extraction were prepared. The experiment was repeated for three times to minimise the error and improve the overall accuracy in this study.

3.1 Preparation of buffer solution

Three types of buffer which is phosphate buffer saline (PBS), radioimmunoprecipitation assay (RIPA) buffer and phosphate buffer were prepared.

3.1.1 Preparation of 500 ml of phosphate buffer saline (1X)

400 ml of distilled water was prepared, added with 4g of sodium chloride (NaCl), 0.1 g of potassium chloride (KCl), 0.72 g of disodium hydrogen phosphate (Na_2HPO_4), 0.12 g of potassium dihydrogen phosphate (KH_2PO_4). The pH of the solution was adjusted to 7.4 with 1M hydrochloric acid (HCl). The distilled water was then added to total volume of 500ml.

3.1.2 Preparation of 500 ml of radioimmunoprecipitation assay (RIPA) buffer

400ml of deionised water was prepared, added with 4.4 g of sodium chloride (NaCl), 0.75 g of EDTA, 5g of Triton X-100, 0.5 g of sodium dodecyl sulphate (SDS), 5 g of sodium deoxycholate (DOC), and 2.5 ml of 1M Tris-HCl with pH 7.6. The solution was then added with deionised water up to 500 ml. The pH of the solution is then adjusted with 1M hydrochloric acid (HCl) to 7.6. The solution was left to stir with magnetic stirrer until no precipitate is visible and stored under temperature 4 degree Celsius.

3.1.4 Preparation of 500 ml of 0.1M phosphate buffer

500 ml of 0.1M dipotassium hydrogen phosphate (K_2HPO_4) solution was prepared by adding 8.709 g K_2HPO_4 powder into 500 ml distilled water and mixed well. On the other hand, 0.1M potassium dihydrogen phosphate (KH_2PO_4) was prepared by adding 6.8045 g of KH_2PO_4 powder into 500 ml distilled water and mixed well. 307.5 ml of 0.1M

K_2HPO_4 solution was mixed with 192.5 ml of 0.1M KH_2PO_4 solution in stock bottle (DeAngelis, 2007). The pH of the phosphate buffer was checked and adjusted to pH 7.

3.2 Collection of samples

The placenta was collected from post-parturition cattle Kedah Kelantan in Agropark, University Malaysia Kelantan Jeli Campus. The foetal cotyledons were removed from the placenta and stored at -20 ^oC until use (Kiewisz et al., 2008).

3.3 General protein extraction

General protein extraction comprises mechanical disruption with addition of buffer, incubation, sonication and centrifugation were done after preparation of sample and buffer.

3.3.1 Sample preparation

The foetal cotyledon samples were thawed and cut into smaller pieces approximately 2-cm^2 (Huang et al., 1999; Sasser et al., 1986; Willard et al., 1995). The cutting process was done in the container surrounded by ice in cold room UMK.

3.3.2 Preparation of buffer solution ratio

The volume for each buffer except RIPA buffer with addition of Pi was prepared accordance with sample weight to buffer volume ratio 1:1, 1:3 and 1:5 as shown in table 3.1. The volume of RIPA buffer with addition of Pi was prepared as presented in table 3.2.

Table 3.1: The volume of buffer and sample weight based on the ratios

Ratio (w:v)	Sample weight (g)	Volume of buffer (ml)
1:1	1	1
1:3	1	3
1:5	1	5

Table 3.2: Volume of RIPA buffer and protease inhibitor based on the ratio

Ratio (w:v)	Sample weight (g)	Volume of buffer (µl)	Volume of protease inhibitor (µl)
1:1	1	990	10
1:3	1	2970	30
1:5	1	4950	50

3.3.3 Protein extraction with addition of PBS

1 g of foetal cotyledon pieces were weighed and transferred into pre-chilled mortar. 1ml of phosphate buffer saline (PBS) was added into mortar containing sample and ground until fully dissolved with the buffer. The mixture was then transferred into beaker and incubated for 2 hours in the cold room UMK. The same procedure was repeated to ratio 1:3 and 1:5.

3.3.4 Protein extraction with addition of RIPA buffer

1 g of foetal cotyledon pieces were weighed and transferred into pre-chilled mortar. 1ml of RIPA buffer was added into mortar containing sample and ground until fully dissolved with the buffer. The mixture was then transferred into beaker and incubated for 2 hours in the cold room UMK. The same procedure was repeated to ratio 1:3 and 1:5.

3.3.5 Protein extraction with addition of RIPA buffer with protease inhibitor

1 g of foetal cotyledon pieces were weighed and transferred into pre-chilled mortar. 10 ul of protease inhibitor cocktail was added into 990 ul of RIPA buffer. The solution is mixed well and added into mortar containing sample and ground until fully dissolved with the buffer. The mixture was then transferred into beaker and incubated for 30 minutes in the cold room UMK. The same procedure was repeated to ratio 1:3 and 1:5.

3.3.6 Sonication

Figure 3.1 represented the sonicator in PG lab. The lysate was kept on ice as shown in figure 3.2 and sonicated with amplitude 30%, 3 seconds of pulse on and off for three minutes.

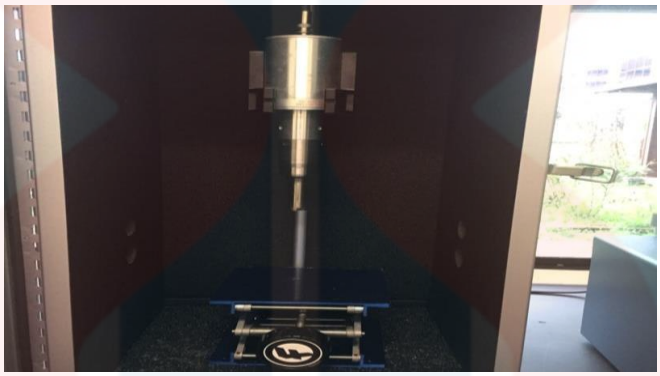


Figure 3.1: Sonicator

Figure 3.2: Protein sample kept on ice for sonication process

3.3.7 Centrifugation

The lysate was centrifuged with Eppendoft Centrifuge 5415 R (Figure 3.3), with revolution per minute (rpm) of 12000 for one hour. The supernatant was harvested while the pellet was discarded (Figure 3.4).

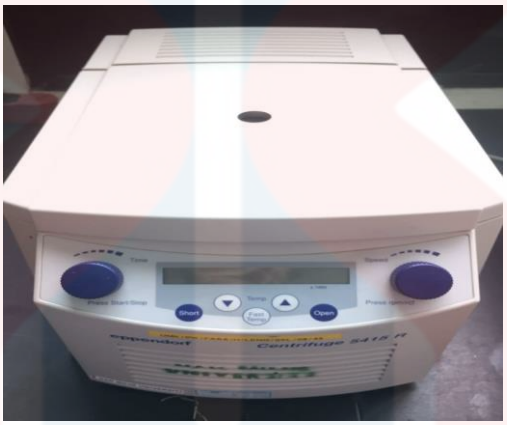


Figure 3.3: Eppendoft Centrifuge 5415R

Figure 3.4: supernant (liquid) and pellet (bottom of microtube) after centrifµgation

3.4 Quantification of protein concentration

In this study, Bradford assay was chosen as protein quantitation method. Bovine serum albumin (BSA) was chosen as standard protein in this assay for creating a standard calibration graph.

3.4.1 Preparation of bovine serum albumin (BSA) with known concentration

Bovine serum albumin (BSA) master stock was prepared by adding 0.5g into 2ml of deionized water to produce 250 mg/ml of BSA concentration. 2 µl was taken out from BSA master stock into 500 µl of 0.1M phosphate buffer to produce 1 mg/ml of BSA concentration and labelled as tube 1. 250 µl of phosphate buffer was added into each of 6 microtubes labelled with tube 2 to tube 7. 2-fold dilution was carried out by transfer 250 µl into tube 2, mixed well, and take same amount and transfer into tube 3. Same steps were repeated to subsequent tubes. Table 3.3 represented the BSA protein concentration in each tube.

Table 3.3: The BSA protein concentration from 1000 to 16.125 µg/ml by 2-fold dilution

Tube	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Protein Concentration (µg/ml)	1000	500	250	125	62.5	31.25	16.125

3.4.2 Collection of absorbance reading from standard protein

Eight test tubes containing 1 ml of Bradford reagent were prepared on the tube rack with label “blank” and tube 1 to tube 7. 100 µl of phosphate buffer was added into “blank” tube and mixed well. 100 µl of BSA protein standard was taken from microcentrifµge tube 1 into test tube 1 and mixed well, same steps applied to tube 2 to tube 7, left 10 minutes before transferred into 1ml cuvette. The absorbance reading was taken from spectrophotometer (Thermo Scientific Gynesis 20) as shown in figure 3.5 with wavelength 595nm in chemistry lab.

Figure 3.5: Spectrophotometer Thermo Scientific Gynesis 20

3.4.3 Protein standard curve

The readings of absorbance value of protein standard were tabulated and plotted into graph with Microsoft excel. The equation (y=mx+c) and R square value (R^2) was obtained from the Microsoft excel. The whole assay will be repeated until R^2 obtained is between 0.9 to 1.

3.4.4 Optimisation of unknown protein amount in Bradford assay

6 tubes were prepared on the tube rack added with labelled 10X, 20X, 50X, 100X, 200X and 500X. Table 3.4 represented the volume of protein (µl) and volume of phosphate buffer (µl) required in each dilution factor. Each label stands for dilution factor. Bradford assay was carried out on each dilution factor and the absorbance value of each sample were recorded (Figure 3.6). The absorbance value from different dilution factor taken were compared with range of absorbance value of standard protein standard curve. In this study, 200X dilution factor were chose to dilute the unknown protein sample with phosphate buffer and proceeded to Bradford assay.

Table 3.4: The dilution factor of unknown protein concentration

Dilution factor	Volume of protein sample (µl)	Volume of phosphate buffer (µl)
10X	10	90
20X	5	95
50X	2	98
100X	10	990
200X	5	995
500X	2	998

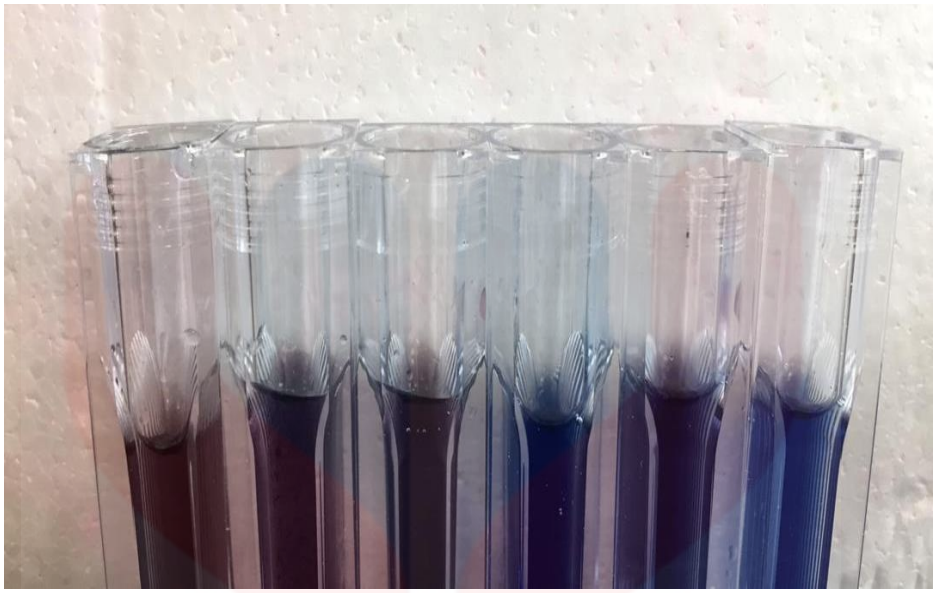


Figure 3.6: Dilution factor of 500X, 200X, 100X, 50X, 20X, and 10X in Bradford reagent

3.4.5 Identification of protein concentration

The protein concentration of sample with 200X dilution factor was calculated by using equation (y=mx + c) obtained from the standard protein curve where y = absorbance value, m = gradient of standard curve, x = protein concentration, and c = y-intercept. After that, the original protein concentration was obtained by multiplying the protein concentration with 200 using Microsoft Excel 2016.

3.5 Statistical analysis

Data for protein concentration yield was analysed by using Statistical Package for the Social Sciences (SPSS) version 20. One-way analysis of variance was conducted separately on type of buffer and buffer ratio at 95% confidence level. Post hoc test were conducted on the parameter that showed significant difference ($p<0.05$).

Two-way analysis of Variance was conducted to investigate the interaction between type of buffer and buffer ratio at 95% confidence level. Post hoc test were conducted when the result is significant ($p<0.05$).

CHAPTER 4

RESULT AND DISCUSSION

The present study was an attempt to find out the effectiveness of three types of buffer with different of sample to buffer ratios. As stated in the previous study, the experiment was repeated three times and the data collected are proceed to statistical analysis to determine the significant difference among buffer and ratio; and whether there is interaction between type of buffer and buffer ratio towards total protein yield. For better understanding the results were divided into four heads, namely standard curve of BSA, Type of buffer, Buffer ratio, interaction between buffer type and ratio, and comparison with previous study.

4.1 Standard curve of BSA

Table 4.1 represented the absorbance reading of standard protein solution within the range of 0 to 1000 µg/ml. The graph (Figure 4.1) was plotted based on the reading of absorbance value. R-value and equation were measured based on the trendline revealed 0.9815 and y=0.0005x + 0.6058.

Table 4.1: Absorbance value for standard protein solution of Bradford assay

Protein concentration (µg/ml)	Absorbance value (AU)	Standard error of mean
0	0.584	0.036
16.125	0.603	0.037
31.25	0.617	0.036
62.5	0.638	0.035
125	0.661	0.030
250	0.766	0.032
500	0.900	0.046
1000	1.088	0.042

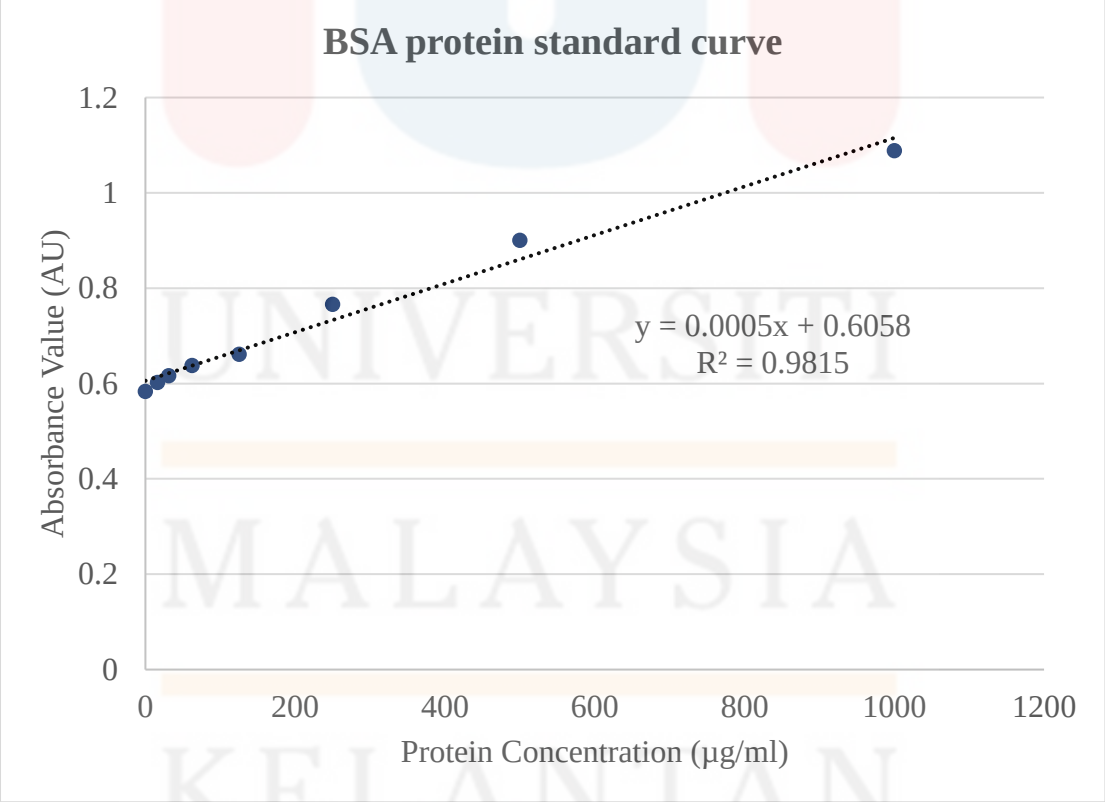


Figure 4.1: standard curve for protein standards for Bradford assay

4.2 Type of buffer

Table 4.1 representing the ANOVA score for the buffer ratio 1:1, 1:3, and 1:5 in buffer PBS, RIPA, and RIPA with addition of protease inhibitor (RIPA + Pi). Based on the table, it shown that there was statistically significant different between buffer ratio ($p<0.5$) in buffer RIPA and RIPA with addition of protease inhibitor whereas there was no significant different ($p>0.5$) between buffer ratio in PBS. A Tukey post hoc test revealed that the protein concentration in RIPA buffer was statistically significantly highest at ratio 1:1 (194.880 ± 15.089^{b}) compared to buffer ratio 1:3 (52.747 ± 1.313^{c}) and ratio 1:5 (19.680 ± 3.274^{c}). Another Tukey post hoc test been conducted on buffer ratio in buffer RIPA with addition of protease inhibitor reported that the ratio 1:1 (106.747 ± 12.616^{d}) was significantly differed with ratio 1:5 (36.213 ± 1.733^{e}), while 1:3 (67.280 ± 13.547^{de}) is not significantly different with ratio 1:1 and 1:5.

Based on the result (Table 4.2), the protein yield is highly affected by the type of buffer ($p<0.05$). The overall total protein concentration was relatively higher in RIPA buffer in compared with PBS while it is not significantly differed with RIPA added with protease inhibitor. This probably due to the composition within the buffer significantly resulted in more cell disruption which caused the release of protein from the cell compartment. RIPA buffer is one of the famous buffer as choice for protein extraction from mammalian tissue (Kurien & Hal Scofield, 2015). The significant protein yield could be resulted by the presence of Triton X-100, sodium deoxycholate, and sodium dodecyl sulphate as detergent to solubilise the poorly soluble protein and break up the membrane structure of the cell. Hence, this allow efficient rupture of the trophoblastic cell and release of protein from the cell compartment. SDS and sodium deoxycholate are an ionic detergent, which containing anionic hydrophilic head group, possessing harsh

properties that tend to denature protein as they disrupt both inter and intra molecular protein-protein interaction (Cockley, 2007). The presence of this detergent in buffer in protein extraction might cause massive protein denaturation and reduce protein yield due to the aforementioned properties, however, the total protein yield was relatively the highest among three buffers. This probably due to the addition of Triton X-100 within in the buffer, which relatively mild compared to SDS. Triton X-100 characterized as uncharged hydrophilic head groups, is non-denaturing because they disrupt protein-lipid and lipid-lipid interactions instead of protein-protein interaction. Therefore, it can help maintaining the stability of enzyme and efficiently solubilise the protein. Whist, the disruption of cell can be effective undergone with presence of these two detergents in disruption of interaction. Study conducted on membrane protein extraction, turned out the presence of SDS given out the highest total protein yield compared with non-ionic and zwitterionic detergent (Arachea et al., 2012). The addition of EDTA in the buffer act as chelator helps reduce oxidation damage of protein particularly by metallo-protease through formation of a stable complex with enzyme complex (Auld, 1995; European Molecular Biology Laboratory [EMBL], n.d.).

In compared with PBS buffer, the lack of detergent and chelator in the buffer causes the disruption of cell had to depends solely on mechanical disruption to break the cell membrane for the release of protein. In addition to this point, the absence of protease inhibitor causes the protein were vulnerable to the protein degradation followed by the release of enzyme from the cell compartment after cell disruption. Hence, the low total protein could be due to the degradation of protein by the activation of enzyme. Phosphate and Tris-HCl act as buffering agent that preventing protein denaturation by protecting protein against a huge fluctuation of the pH after the release of substance during cell disruption. There should be no difference of impact on total protein yield between the

choice of either phosphate or Tris-HCl as suggested by Sepehrimanesh M (2015), where there is no significant difference between the choice of phosphate and Tris-HCl on protein concentration (Sepehrimanesh & Kazemipour, 2015).

Hypothetically, the total protein yield of the RIPA with addition of protease inhibitor should relatively higher than RIPA without protease inhibitor since protease inhibitor function as protecting the protein samples from being degraded by the protease that liberated from membrane fragment and cellular compartment (G-Biosciences, 2012, 2018). However, the current experiment had given negative outcome where there is no significant difference between RIPA with protease inhibitor and RIPA without protease inhibitor. This could be because of the poor handling causes inactivation of protease inhibitor solution as protease inhibitors are unstable for long duration of time, either in stock solution or working concentration (Ritchie, 2013). The mishandling could be happened from beginning in making of protease inhibitor, or contamination occur during in the process of transferring part of stock solution into unsterilized container. Inactivation of protease inhibitor could be happened due to the inappropriate storage problem as protease inhibitors are sensitive to fluctuation of temperature. The allocated incubation time for RIPA buffer added with protease inhibitor can be one of the possible reasons for lower protein yield than RIPA buffer, due to the incomplete protein solubilisation in compared to RIPA buffer alone.

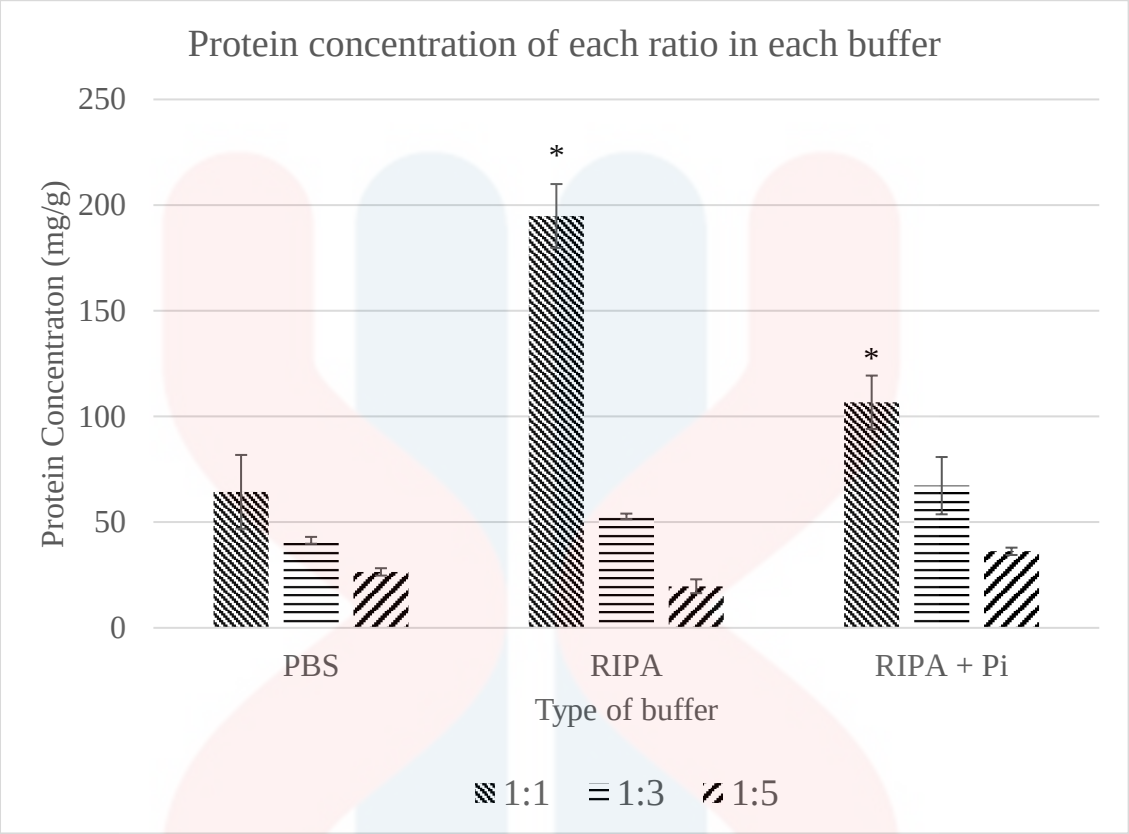
Pregnancy-specific protein B is a type of glycoprotein and also belongs to aspartic enzyme family (Huang et al., 1999; Klisch et al., 2006; Xie et al., 1991). Protease inhibitor cocktail containing variety type of inhibitors including aspartic protease inhibitor such as pepstatin, act as a reversible protease inhibitor. This resulted the binding of aforementioned targeted protein and causes the protein failed to form dye-protein

complex with Coomassie Brilliant Blue in Bradford assay caused erroneous in data collection.

Table 4.2: Protein concentration of each ratio in each buffer

Type of buffer	Buffer ratio	P-value	Total Protein concentration (mg/g) Mean ± Standard Error Mean
PBS	1:1	0.98	64.347 ± 17.458^{a}
	1:3		41.413 ± 1.622^{a}
	1:5		26.480 ± 1.744^{a}
RIPA	1:1	0.00	194.880 ± 15.089^{p}
	1:3		52.747 ± 1.313^{q}
	1:5		19.680 ± 3.274^{q}
RIPA + Pi	1:1	0.01	106.747 ± 12.616^{x}
	1:3		67.280 ± 13.547^{xy}
	1:5		36.213 ± 1.733^{y}

Different superscript letter within the same column is significantly differed according to Tukey HSD test ($P < 0.05$)



* indicates the most significant among three ratios

Figure 4.2: Protein concentration of each ratio in each buffer

4.3 Buffer ratio

Table 4.2 representing the ANOVA result for protein concentration based of type of buffer in ratio 1:1, 1:3, and 1:5. The table reported there is significant difference of protein concentration ($p<0.5$) between type of buffer in ratio 1:1 and 1:5, in contrast, there is no significant difference of protein concentration in ratio 1:3. Post hoc test has been conducted separately on ratio 1:1 and 1:5. In ratio 1:1, buffer PBS (321.733 ± 87.288) and RIPA with addition of protease inhibitor (106.747 ± 12.616^{b}) do not differ significantly in term of their mean protein concentration but were significantly lower than RIPA buffer (194.880 ± 15.089^{a}). In ratio 1:5, it is reported that the mean number of

protein concentration of PBS (26.480 ± 1.744^{de}) do not significantly differed to the mean number of RIPA added with protease inhibitor (36.213 ± 1.733^{d}) but statistically higher than RIPA buffer (19.680 ± 3.274^{e})

Currently, there is limited study related to the optimisation of buffer ratio in protein extraction. Theoretically, 1:3 ratio could produce more concentrated extract while the volume of 5 to 10 of buffer able to yield more soluble protein and less viscous extract (Grabski, 2009).Study conducted on protein extraction on banana reported that buffer ratio 1:3 could yield protein compare with 1:1 and 1:5 (Mayil Vaganan, Sarumathi, Nandakumar, Ravi, & Mustaffa, 2015). This statement was partially proven by current experimental data where the total protein concentration in 1:3 were significantly higher than 1:5 ($P<0.05$) but were lower than ratio 1:1. While the statement of ratio sample to buffer 1:5 were found to be optimal in protein extraction of monoclonal antibodies (Gottschalk, 2014), seems against the result of this experiment. Geissler et al., 2011 agreed that ratio of 5 ml buffer per gram can be the starting point to avoid the loss of protein activity or nonspecific binding to containers. But then again, the ratio of buffer should be lower down if concentrated sample is essential, such as 1:1 or 1:2. Golemis & Adams (2005) suggested that unnecessary dilution of cells in lysis buffer should be avoided as this might causes instability in structure and activity, therefore 1:1 buffer ratio was supported. Based on the data result, 1:1 ratio was apparently the highest among three ratios, proven that more protein solubilised in compared with other ratio.

The low protein yield in 1:3 and 1:5 buffer ratio could be due to the excessive content within each lysis buffer that causes protein denaturation. The denatured protein aggregated and formed layer at the bottom of the tube after centrifugation. In RIPA buffer, the increase of buffer to sample ratio implied the increased detergent level compared to protein level within the sample. Supposedly the increased of non-ionic Triton-X 100

detergent level could aids in solubilisation of protein which can improve the protein yield by disruption of membrane protein through detergent-lipid interaction. However, the increased of SDS and sodium dodecyl cholate as ionic detergent has disrupted the protein-protein interactions, and brought protein into the denatured state (Stetsenko & Guskov, 2017).

The presence of salt helped in maintaining the ionic strength of the medium, and increase the total concentration of solutes outside the cell (Brennan, 2018). In PBS, the total protein yield decreased as increased of buffer to sample ratio possibly due to raising of salt concentration of the tissue surrounding, inactivated some of the protein (Chandra & Endow, 1993). In term of RIPA added with protease inhibitor, the increased amount of protease inhibitor followed by the increased of buffer to sample ratio resulted in more formation of protease inhibitor-protein complex causes poor yield of protein. Some protease inhibitor may carry out irreversible function which causes denaturation of the protein and affect the result of total protein yield.

There are no significant differences of total protein yield ($p=0.147$, $p>0.05$) among three buffers in ratio 1:3 suggested either of these three buffers can be used in 1:3 ratio and will not bring huge difference in term of total protein yield.

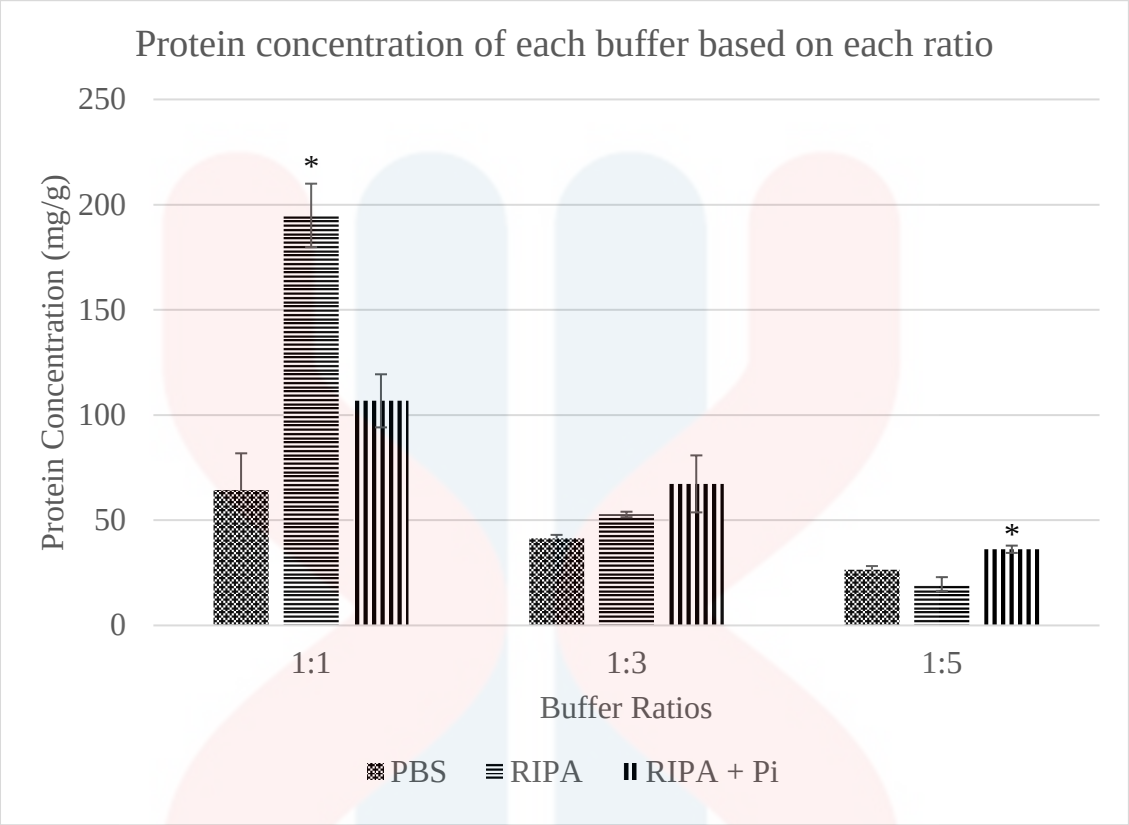
In 1:5 buffer ratio, RIPA added with protease inhibitor was statistically higher than RIPA and PBS, which suggested RIPA added with Pi ratio was could yield better in 1:3 and 1:5 compared with other buffers with same ratio. This may possibly because of too much detergent within RIPA has caused denaturation to the protein and resulted low total protein yield. While incomplete protein solubilisation may happen in PBS due to the lack of additive except salt, resulted the cell disruption had to depends on the mechanical force in to break the cell membrane. Additionally, this may contribute to incomplete cell membrane disruption in PBS and the proteins were remained in the cellular compartment.

Moreover, the viscosity decreases as increase of buffer ratio implies the decrease of total protein yield was verified in the current research. This was supported by the study of Goncalves et al (2016) on the effect of protein concentration on the viscosity of a recombinant albumin solution formulation revealed that the viscosity of protein increase as the increase of protein concentration in the solution. Few studies had suggested that this phenomenon was governed by the protein-protein interaction including electrostatic, hydrophobic, steric, hydrogen-bonded interactions and dipole-dipole interaction (Mao et al., 2010; Saluja, Badkar, Zeng, Nema, & Kalonia, 2007).

Table 4.3: protein concentration of each buffer in each ratio

Buffer Ratio	**Type of buffer**	**P-Value**	**Total Protein Concentration (mg/g) Mean ± Standard Error Mean**
1:1	PBS	0.002	64.347 ± 17.458^{b}
	RIPA		194.880 ± 15.089^{a}
	RIPA + Pi		106.747 ± 12.616^{b}
1:3	PBS	0.147	41.413 ± 1.622^{p}
	RIPA		52.747 ± 1.313^{p}
	RIPA + Pi		67.280 ± 13.547^{p}
1:5	PBS	0.007	26.480 ± 1.744^{xy}
	RIPA		19.680 ± 3.274^{y}
	RIPA + Pi		36.213 ± 1.733^{x}

Different superscript letter within the same column is significantly differed according to Tukey HSD test ($P < 0.05$)



***** indicates the most significant value among buffers

Figure 4.3: Protein concentration of each buffer based on ratio

4.4 Interaction between buffer type and ratio

Table 4.3 represented the result of two-way ANOVA test of protein concentration between type of buffer and buffer ratio. It was revealed that there was significant different between type of buffer ($p=0.000$, $p<0.05$), buffer ratio ($p=0.000$, $p<0.05$), and most importantly, there was a significant interaction ($p=0.000$, $p<0.05$) between type of buffer and buffer ratio on protein concentration. A test of simple effect was conducted and found that there is significant difference ($p=0.000$, $p<0.05$) among the average protein concentration of 1:1 in buffer type, while no significant difference was detected in 1:3 ($p=0.210$, $p>0.05$) and 1:5 ($p=0.509$, $p>0.05$). Based on the pairwise comparison table in

buffer ratio 1:1 reported that PBS, RIPA, and RIPA added with Protease inhibitor were significantly different from each other. In term of the effect of buffer ratio of each type of buffer towards concentration, it was stated that all buffer ratio of each buffer type has significant effect ($p<0.05$) on protein concentration. Based on the pairwise comparison, in PBS, only buffer ratio 1:1 has significantly different with 1:5. In RIPA buffer and RIPA added with protease inhibitor, there is significant difference when compare 1:1 with 1:3 and 1:5.

4.5 Comparison of result with previous study

Surprisingly, in the comparison with the result of previous study, it was found out that the current experimental design has greatly enhance the total protein yield whereby the total protein yield in several present researches related with extraction of placental protein could yield about 13 to 16 mg/g of total protein from either bovine or ovine. (Arima & Bremel, 1983; Azadmanesh et al., 2012; Barbato, Melo de Sousa, Barile, Canali, & Beckers, 2013; El Amiri et al., 2003; El Amiri, Remy, Melo de Sousa, & Beckers, 2004; Garbayo et al., 1998b; Kiewisz et al., 2008). However, the content of PAG presented could not be compared with previous study due to the detection of PAG was not conducted in this study. Furthermore, some of the researches were using Lowry assay instead of Bradford assay, which also causes further weaken the effectiveness of the result.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The yield of protein concentration from foetal cotyledon extraction with three types of buffers has been compared and analysed, turned to be radioimmunoprecipitation assay buffer (RIPA) gives out the highest protein concentration ($p<0.05$) among three buffers could be due to the presence of detergent improve the solubilisation of protein. However, the addition of protease inhibitor into RIPA were unexpectedly yield less total protein in compared with RIPA could be due to the presence of aspartic protease inhibit the binding of the desired protein, pregnancy specific protein B as well as lack of incubation time. The ratio of the buffer had been optimised shown that 1:1 produced the best result among three ratios (1:1, 1:3, and 1:5) suggested that increase dilution of tissue sample with lysis buffer could yield less protein due to the increase of content level to tissue causes denaturation of protein In this study, there were significant interaction between buffer type and buffer ratio on the total protein yield has highlighted the importance of the presence of detergent and effect of protease inhibitor in protein extraction process. In conclusion, 1:1 ratio of RIPA buffer to tissue sample (194.880 ± 15.089) yield the highest total protein followed by the RIPA added with protease inhibitor

(106.747 ± 12.616 mg/g). Furthermore, the total protein yield in this study was significantly higher than the total protein yield in the previous study, however, the pregnancy-associated glycoprotein (PAG) content were failed to validate since the detection of PAG was not included in this experiment protocol.

5.2 Recommendation

As recommendation, the optimisation on other parameters including the amplitude and duration of sonication, duration and the speed of centrifugation, the buffer composition aiming on specific protein and pre-treatment of the sample prior to extraction could be further investigated to improve the total protein yield. Furthermore, the choice of buffer can be further optimised such as urea buffer, citrate buffer, tris phenol buffer, SDS buffer etc.

The detection of pregnancy associated glycoprotein (PAG) such as SDS-PAGE or western blot shall be carried out in the future study to detect the presence of desired protein particularly PSPB to prove the effectiveness of this experimental protocol in compared with the previous study.

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APPENDIX A

Table A.1: BSA protein concentration from 1000 to 16.125 µg/ml by 2-fold dilution

Protein Concentration (µg/ml)	Absorbance Reading (AU)				
	Replication 1	Replication 2	Replication 3	Average	Standard Error Mean
0	0.65	0.527	0.574	0.584	0.036
16.125	0.672	0.545	0.591	0.603	0.037
31.25	0.68	0.557	0.613	0.617	0.036
62.5	0.705	0.586	0.623	0.638	0.035
125	0.713	0.608	0.663	0.661	0.030
250	0.825	0.716	0.758	0.766	0.032
500	0.965	0.812	0.924	0.900	0.046
1000	1.13	1.004	1.131	1.088	0.042

Table A.2: The three replication of protein concentration of 200X diluted protein sample tested with different buffer and ratio by Bradford assay

Treatment	Ratio	Protein concentration (µg/g)				
		Replication 1	Replication 2	Replication 3	Average	Standard error mean
PBS	1:1	148.4	426.4	390.4	321.733	87.288
	1:3	220.4	192.4	208.4	207.067	8.110
	1:5	118.4	130.4	148.4	132.400	8.718
RIPA	1:1	1080.4	828.4	1014.4	974.400	75.445
	1:3	276.4	260.4	254.4	263.733	6.566
	1:5	130.4	76.4	88.4	98.400	16.371
RIPA + PI	1:1	618.4	410.4	572.4	533.733	63.080
	1:3	214.4	448.4	346.4	336.400	67.735
	1:5	196.4	166.4	180.4	181.067	8.667

Table A.3: The three-replication data of original total protein concentration of 1 g protein sample tested with buffer type and ratio

Treatment	Ratio	Protein concentration (mg/g)				
		Replication 1	Replication 2	Replication 3	Average	Standard Error Mean
PBS	1:1	29.68	85.28	78.08	64.347	17.458
	1:3	44.08	38.48	41.68	41.413	1.622
	1:5	23.68	26.08	29.68	26.480	1.744
RIPA	1:1	216.08	165.68	202.88	194.880	15.089
	1:3	55.28	52.08	50.88	52.747	1.313
	1:5	26.08	15.28	17.68	19.680	3.274
RIPA + PI	1:1	123.68	82.08	114.48	106.747	12.616
	1:3	42.88	89.68	69.28	67.280	13.547
	1:5	39.28	33.28	36.08	36.213	1.733

Table A.4: Two-way ANOVA of buffer type and ratio

Tests of Between-Subjects Effects					
Dependent Variable: protein concentration					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	70865.399[a]	8	8858.175	29.651	.000
Intercept	123946.593	1	123946.593	414.885	.000
type	9194.536	2	4597.268	15.388	.000
ratio	42838.127	2	21419.064	71.696	.000
type * ratio	18832.735	4	4708.184	15.760	.000
Error	5377.493	18	298.750	-	-
Total	200189.485	27	-	-	-
Corrected Total	76242.892	26	-	-	-
a. R Squared = .929 (Adjusted R Squared = .898)					

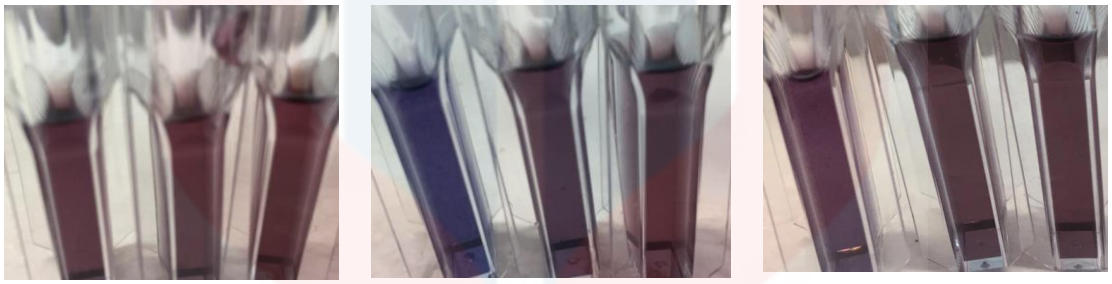
Table A.5: Pairwise comparison of buffer ratio* buffer type

Pairwise Comparisons							
Dependent Variable: protein concentration							
buffer ratio	**(I) buffer type**	**(J) buffer type**	**Mean Difference (I-J)**	**Std. Error**	**Sig.[b]**	**95% Confidence Interval for Difference[b]**	
						Lower Bound	**Upper Bound**
1:1	**PBS**	**RIPA**	-130.533*	14.113	.000	-167.779	-93.288
		Pi	-42.400*	14.113	.023	-79.645	-5.155
	RIPA	**PBS**	130.533*	14.113	.000	93.288	167.779
		Pi	88.133*	14.113	.000	50.888	125.379
	Pi	**PBS**	42.400*	14.113	.023	5.155	79.645
		RIPA	-88.133*	14.113	.000	-125.379	-50.888
1:3	**PBS**	**RIPA**	-11.333	14.113	1.000	-48.579	25.912
		Pi	-25.867	14.113	.250	-63.112	11.379
	RIPA	**PBS**	11.333	14.113	1.000	-25.912	48.579
		Pi	-14.533	14.113	.950	-51.779	22.712
	Pi	**PBS**	25.867	14.113	.250	-11.379	63.112
		RIPA	14.533	14.113	.950	-22.712	51.779
1:5	**PBS**	**RIPA**	6.800	14.113	1.000	-30.445	44.045
		Pi	-9.733	14.113	1.000	-46.979	27.512
	RIPA	**PBS**	-6.800	14.113	1.000	-44.045	30.445
		Pi	-16.533	14.113	.770	-53.779	20.712
	Pi	**PBS**	9.733	14.113	1.000	-27.512	46.979
		RIPA	16.533	14.113	.770	-20.712	53.779

Table A.6: Pairwise comparison of buffer type*buffer ratio

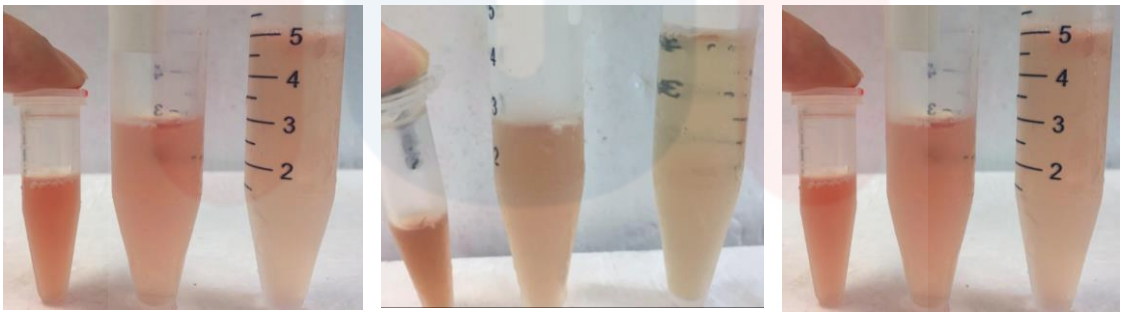
Pairwise Comparisons							
Dependent Variable: protein concentration							
buffer type	**(I) buffer ratio**	**(J) buffer ratio**	**Mean Difference (I-J)**	**Std. Error**	**Sig.[b]**	**95% Confidence Interval for Difference[b]**	
						Lower Bound	**Upper Bound**
PBS	**1:1**	**1:3**	22.933	14.113	.365	-14.312	60.179
		1:5	37.867*	14.113	.046	.621	75.112
	1:3	**1:1**	-22.933	14.113	.365	-60.179	14.312
		1:5	14.933	14.113	.912	-22.312	52.179
	1:5	**1:1**	-37.867*	14.113	.046	-75.112	-.621
		1:3	-14.933	14.113	.912	-52.179	22.312
RIPA	**1:1**	**1:3**	142.133*	14.113	.000	104.888	179.379
		1:5	175.200*	14.113	.000	137.955	212.445
	1:3	**1:1**	-142.133*	14.113	.000	-179.379	-104.888
		1:5	33.067	14.113	.092	-4.179	70.312
	1:5	**1:1**	-175.200*	14.113	.000	-212.445	-137.955
		1:3	-33.067	14.113	.092	-70.312	4.179
Pi	**1:1**	**1:3**	39.467*	14.113	.036	2.221	76.712
		1:5	70.533*	14.113	.000	33.288	107.779
	1:3	**1:1**	-39.467*	14.113	.036	-76.712	-2.221
		1:5	31.067	14.113	.123	-6.179	68.312
	1:5	**1:1**	-70.533*	14.113	.000	-107.779	-33.288
		1:3	-31.067	14.113	.123	-68.312	6.179

APPENDIX B



PBS **RIPA** **RIPA + Pi**

Figure B.1: Bradford assay of protein sample with buffer PBS, RIPA, and RIPA + Pi and ratio 1:1, 1:3 and 1:5 (from left)



PBS **RIPA** **RIPA + Pi**

Figure B.2: Supernatant of protein sample with buffer PBS, RIPA and RIPA + Pi and ratio 1:1, 1:3, 1:5 (from left)

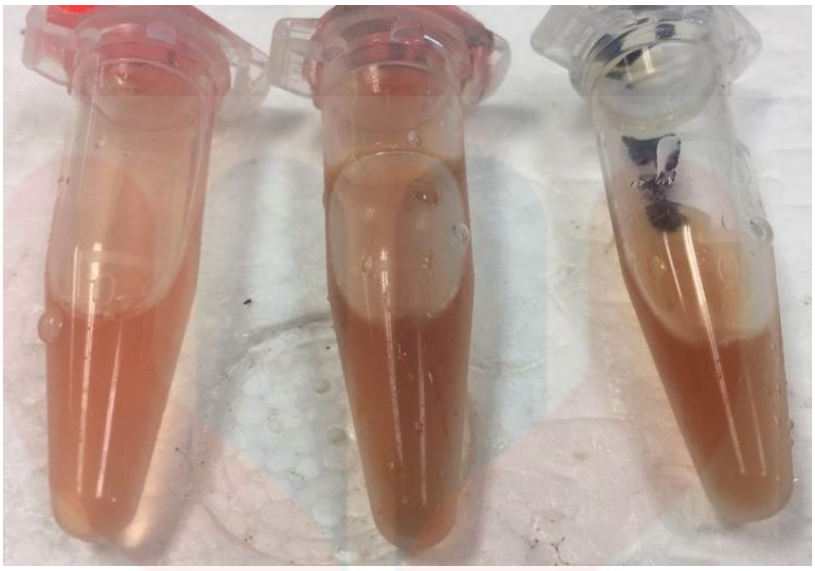


Figure B.3: The supernatant of protein sample 1:1 ratio of PBS, RIPA + Pi, and RIPA (from left)