Aim of the homogenisation process

ASSIGN BUSTER

Introduction:

Eukaryotic cells such as liver cells enclose a variety of different types of membrane bound structures called organelles (nuclei, mitochondria) as well as macromolecules (ribosomes) (Padh, 1992).

Subcellular fractionation is an invaluable technique allowing scientists and researchers alike to successfully isolate and separate specific subcellular components within the cell (Becker et al, 2009). This allows researchers to study the different organelles (using biochemical techniques) in a greater degree of detail therefore increasing our knowledge about the many different types of organelles and macromolecules, thus leading to new scientific advances in this ever advancing era of science and technology (Bonney, 1982; Berns, 1986).

It is this very method which in the past allowed Christian de Duve to discover the lysosomes and peroxisomes for which he shared a Nobel Prize with Albert Claude and George Palade in 1974 (Becker et al, 2009).

Subcellular fractionation can be safely divided under 3 major headings: Homogenisation proceeded by fractionation and finally purification.

Homogenisation:

The aim of the homogenisation process is to effectively and efficiently disrupt and break the cells outer membrane thereby releasing their subcellular components (nuclei, mitochondria). This disruption and breaking of the cells must be achieved in a manner that will leave the delicate

organelles of interest undamaged and morphologically intact (Loewen, 2003).

The cells to be homogenised are kept in an isotonic buffer (0. 25M sucrose, 1mM EDTA and 1mM of Tris at pH 7. 0). This is to protect the fragile organelles from osmotic damage due to osmotic unbalance as well as environmental instability such as pH interference (Guteriezze, 2010).

Many different homogenisation techniques exist and are available, some such include mechanical grinding using Potter-Elvehjen glass homogeniser, cutting methods using warren blender, ultrasonic vibrations in a process called sonication and utilising high pressure such as in the French Press (Loewen, 2003).

The Potter-Elvehjen glass homogenizer was used in this experiment. The Potter-Elvehjen glass homogeniser consists of a Teflon pestle which is closely fitted into a glass homogeniser. The homogenising machine moves the Teflon pestle in a verticle up-down motion while simultaneously rotating within the glass homogeniser containing the cells to be homogenised (Mangiapane, 2010).

The space between the Teflon pestle and glass homogeniser is incredibly small (0. 004″-0. 006″). Therefore as the Teflon pestle moves throught the glass homogeniser a shear force is generated which causes disruption of the cells. The organelles which are released by this process pass undamaged, safetly through the gap between the pestle and glass homogeniser (Loewn, 2003; Mangipane, 2010).

The shear forces produced can sometime be destructive to the organelles causing irreversible damage and therefore shear forces need to be controlled. This can be controlled by adjusting the gap width between the pestle and glass homogeniser. A bigger width can protect organelles from damage but the negative side effect of this is that the generated shear forces will not be strong enough to disrupt the cells and therefore few or none organelles will be present in the homogenate. Therefore a careful balance between cell disruption and organelle damage must be maintained.

Chemical, physical and structural damage can be caused to organelles due to shear forces which can cause errors when purifying the organelle using biochemical techniques due to enzymes specific to the particular organelle being damage or rendered inactive and these problems must therefore be overcome. Some such precautions which when utilised can overcome or minimise unnecessary damage includes the use of different homogenisation techniques which are more suitable for the cells being homogenised (osmotic disruption, chemical disruption may be considered). Carefull usage of the homogenising equipment (Lowen, 2003).

Fractionation:

Once the homogenate has been formed, it is ready to be placed in a centrifuge and undergo centrifugation which will separate the different fractions/organelles. Centrifugation generates a centrifugal force which separates the different types of organelles based on their size and density as well as the density and viscousity of the solution the homogenate is in. Therefore the the higher the molecular weight of the organelle the greater the distance I will travel down the centrifuge tubes or the higher its

sedimentation rate and consequently the smaller the molecular weight of the organelle the smaller the distance it will travel down the centrifuge tube or the lower its sedimentation rate (Becker et al, 2008; Mangipane, 2010). The greater an organelles sedimentation rate is the greater the organelles sedimentation coefficient (in Svedberg units, named after Theodor Sveber who developed the ultracentrifuge) will also increase (Becker et al, 2009).

Centrifugal forces can be calculated using

For example, if a homogenate containing nuclei, mitochondria and ribosomes is subjected to a centrifugal force, logically the nuclei will be near the bottom of the tube, the ribosomes at the top part of the tube and the mitochondria somewhere in between the nuclei and ribosomes.

There are 2 main type of centrifugation methods: Differential centrifugation and density gradient centrifugation.

Differential centrifugation

This type of centrifugation works on the principles that large dense molecules (nucei) will have a higher sedimentation rate compared to small and less dense molecules (ribosomes) (Becker et al, 2009). During low centrifuge speeds and short times the heavy and dense organelles sediment and can be collected, while as high centrifuge speeds and longer timer the lighter and less dense molecules will sediment and can also be collected (manipulative techniques).

Therefore in the homogenate used in the experiment, by using appropriate centrifuge speeds and times the nuclei and mitochondria can be separated using 1500g for 10min and 20000g for 10min respectively.

Density gradient centrifugation

The method used in density gradient centrifugation, also known as rate-zonal centrifugation works on the principle of separating molecules based on their densities and is achieved by using a density gradient in the centrifuge tube (manipulative techniques; Becker et al, 2009). The density gradient is normally provided by a concentrated sucrose solution which increases in density towards the bottom of the centrifuge tube. The sample requiring fractionation is placed in a layer over the density gradient sucrose solution (Becker et al, 2009). As the centrigugation process proceeds, the different molecules or organelled of different densities are separated based on their densities and that of the increasing sucrose density. When the fractionation bands have been formed are are distinctive the fraction may be remover via a syringe or separation methods. This type of centrifugation can be used to further separate mitochondria from lysosomes and peroxisomes since each of them has a different density.

Measurements of enzyme activity and macromolecular composition of fractions. “ purity” of fractions.

During the centrifugation processes, such as in the differential centrifugation the various types of organelles and macromolecules form gelatinous pellets at the end of each consecutive centrifuge (Dyson, 1979). The different pellets produced contain a variety of different fractions of subcellular

organelles and macromolecules and is not specific for just a single type of organelle or macromolecule. As an example in the first centrifugation process to form the nuclei fraction at 1500g for 10mins, the nuclei is pelleted along with other molecules of similar size and molecular weight such as unbroken cells, cell debris and pieces of the cell membranes (Bonney, 1982). In the second centrifugation to form the mitochondrial fraction at 20000g for 10min the pellet contains mitochondria, lysosomes and peroxisomes due to their similar sizes and molecular weight. In the final centrifugation process at 20000g for 10min a supernatant fraction was formed containing many small and low molecular weight molecules such as the endoplasmic reticulum, microsomes and ribosomes (Minorsky, 2009; Berns, 1986).

As stated before fractions will not only require the organelles of interest but also other organelles and macromolecules. It is therefore necessary to be able to assess the purity of the fractions. This can be done in a variety of ways.

Microscopic analysis via the light microscope or even electron microscope can be used to identify the different macromolecules present within the fraction, therefore giving an indication whether or not the fractionation procedure has been successful. A mitochondrian therefore can be differentiated from a peroxisome or lysosome basen on its structure (Bonney, 1982). Microscopic anaylsis can also be used in assessing the biochemistry of the fraction by using various cytochemical techniques.

Biochemical techniques are a very good way of assessing the type of organelle present as well as the purity of a fraction. Measuring enzyme

activity is an excellet method sine some enzymes are very specific and found in one particular organelle.

Marker enzymes present in fractions and importance of the techniques involoved in the advancement of biochemistry and cell biology.

Marker enzyems are routinely used in subcellular fractionation to differentiate between the many different types of organelles and macromolecules present within the cell. Mitochondria for example can be detected indirectly by the presence of succinate dehydrogenase while lysosomes can be detected by Acid Phosphatase (Bonner, 2007).

The function of the mitochondria for example is to generate adenosine triphosphate (ATP) by a process called oxidative phosphorylation and an enzyme specific to the mitochondrion called succinate dehydrogenase can be used as a marker enzyme to differentiate between the presence of mitochondria and other organelles and macromolecules present in the fraction (Padh, 1992). Succinate dehydrogenase (SDH) is specific to the inner mitochondria membrane and is responsible for catalysing the oxidation reaction of Succinate, which is a component of the citric acid cycle, into fumarate which is another component of the citric acid cycle. Since flavin adenine dinucleotide (FAD) is reduced producing FADH2 (Guterize, 2010; Padh, 1992; Girolamo, 2010).

Succinate is the electron doner while FAD is the electron acceptor. The products of the above reaction are then reacted with an artificial electron acceptor called INT(a tetrazolium salt) to form a red coloured compound called formazan.

This reaction is required because both the fumarate and FADH2 produced in reaction are colourless and therefore there is no certain way of determining succinate dehydrogenase activity, therefore the intensity of the red coloured formazan produced during a specific timeframe in the second reaction can be measured using a spectrophotometer gives an indirect indication of succinate dehydrogenase activity and therefore an indication of the presence of mitochondria as well as its purity within the fraction (guterize, 2010; padh, 1992).

Electron microscopy of the isolated organelles is generally the final step in assessing the purity of the fractions as well as studying their morphology (padh, 1992).

It is these methods and techniques used in subcellular fractionation which has allowed researchers such as George Palade and Christian de Duve studying to understand and discover the structures, biochemistry and roles played by the various organelles.

Results:

Table 1 shows the volumes of the homogenate, nuclei fraction, mitochondrial fraction and supernatant fraction. The Homogenate volume was obtained after rat liver homogenisation; NF volume was obtained after 2 consecutive centrifugations at 1500g for 10min; MF volume was also obtained by 2 consecutive centrifugations at 20000g for 10min; SF volume was obtained from the supernatant of the MF centrifugation.

Table 2 shows known amounts of bovine serum albumin (BSA) which underwent the biuret reaction; the absorbance’s were measure using a

spectrophotometer at 550nm. As protein amount increases so do the absorbance's. This data was used to plot a BSA standard curve.

Figure 1 illustrates the BSA standard curve which is a line of best fit. From this graph, the protein amount is determined by using the absorbance values for the different fraction shown in table 3 below. H, NF, MF and SF correspond to homogenate, nuclei fraction, mitochondrial fraction and supernatant fraction respectively. The vertical and horizontal red, blue, green and black coloured lines represent H, NF, MF and SF respectively.

From the above graphical data Protein concentration (mg/ml), total protein amount (mg) and protein recovery for each fraction relative to the homogenate can be calculated.

Homogenate:

Nuclei Fraction:

Mitochondrial Fraction:

Supernatant Fraction:

From the above results the total percentage of protein recovery relative to the homogenate can be determined:

The above calculated results are show together in table 3.

Table 3 shows the absorbance values obtained from the spectrophotometer. Row B shows the amount of protein that was determined from the BSA standard curve. Row C showed the amount of protein present in 1ml of each fraction; the homogenate had the highest protein concentration, followed by

the SF and MF and finally by the NF containing the lowest amount of protein concentration. Row D shows the total amount of protein in each of the fraction and therefore follows the same pattern as the values for Row C. Row E shows the amount of protein recovered relative to the homogenate; The percentage of protein recovery was as follows: SF> MF> NF.

Table 4 shows the actual fraction concentrations used, obtained by diluting the original fractions (table 3) with phosphate buffer. The supernatant fraction was left undiluted.

Table 5 shows absorbance of each of the fractions (0. 2ml) which were diluted by the addition of 4ml of ethyl acetate within formazan. The average absorbance minus the control gives the corrected mean absorbance for each of the fractions. The control values for all 4 fractions were 0 because they were given as negative values by the spectrophotometer. The highest absorbance was recorded for the SF followed by the homogenate, MF and NF.

By obtaining the data collected from the previously calculations in tables 1, 3 and 5 it is possible to calculate; the total activity of Succinate Dehydrogenase (SDH), the percentage recovery of SDH relative to the homogenate, the specific activity of SDH and the relative specific activity of SDH relative to the homogenate in all 4 fractions (H, NF, MF and SF).

Below are the equations which will be used in the calculations:

Beer-Lamberts Law: The calculations below will make (concentration) the subject of the formula as well as prove that the units for = or .

âˆ´ this can be rearranged to form,

, since always equals to , the equation can now be represented as,

, the units of this new formula can be calculated as follows,

the in the bottom fraction can be cancelled out with the at the top giving,

Which âˆ´ gives which is Molarity or concentration.

The equation will be used throughout the rest of the calculations. The Formazan molar extinction coefficient= and the assay volume used will be 0. 004L (4ml).

Homogenate:

The absorbance for the homogenate in table 5 was 1. 1385 therefore,

âˆ´ since this can be arranged to give,

The volume used was which gives therefore,

Activity âˆ´

âˆ´ total activity of in -1

The answer is required in âˆ´ since

Therefore total activity for Homogenate =

âˆ´

Therefore specific activity for Homogenate

Nuclei Fraction (NF):

The absorbance for the nuclei fraction in table 5 was 0. 117 therefore,

âˆ´ since this can be arranged to give,

This gives,

Activity âˆ´

âˆ´ total activity of in

The answer is required in âˆ´ since

Therefore total activity for Nuclei Fraction =

âˆ´

Therefore specific activity for Nuclei Fraction

Mitochondrial Fraction (MF):

The absorbance for the mitochondrial fraction in table 5 was 0. 398 therefore,

âˆ´ since this can be arranged to give,

This gives,

Activity âˆ´

âˆ´ total activity in in –

The answer is required in âˆ´ since

Therefore total activity for Mitochondrial Fraction =

âˆ´

Therefore specific activity for Mitochondrial Fraction

Supernatant Fraction (SF):

The absorbance for the supernatant fraction in table 5 was 1. 485 therefore,

âˆ´ since this can be arranged to give,

This gives,

Activity âˆ´

âˆ´ total activity in in

The answer is required in âˆ´ since

Therefore total activity for Supernatant Fraction =

âˆ´

Therefore specific activity Supernatant Fraction

Calculations for the % SDH recovery and specific SDH activity relative to the homogenate;

Since the % SDH recovery and specific SDH activity is to be calculated relative to the homogenate, therefore the homogenate percentage for them both will be 100%

Nuclei Fraction:

Mitochondrial Fraction:

Supernatant Fraction:

The main findings of these calculations can be summarized in the table below:

Table 6 shows that SDH activity is highest in the SF, followed by the homogenate, MF and finally by NF. The %of SDH recovery (relative to the homogenate) was greatest in the SF, followed by the MF and the NF. The specific SDH activity was greatest in the SF followed by the MF, NF and lastly by the homogenate. The % of specific SDH activity (relative to the homogenate) was greatest in the SF, followed by the MF and NF.

Figure 2 illustrates the main findings from table 6. It can be seen that % SDH recovery increases from the Nuclei fraction to the supernatant fraction. The % of specific SDH activity steadily falls from the supernatant fraction to the nuclei fraction.

Discussion:

According to the results obtained in table 3, it was seen that 99. 25% of the protein relative to the homogenate was still present within all the fractions. This high percentage recovery indicates that very little protein was lost during the formations of the nuclei, mitochondrial and supernatant fractions by centrifugation. The 0. 75% of protein that was lost is most likely to have been lost while homogenizing the pellets formed during each consecutive centrifugation process. During the usage of hand homogenizers small

quantities of the pellet containing the proteins are stuck to the homogenizing vessel or the pestle. These small quantities of proteins being lost during each hand homogenizing process therefore contributes to the loss of proteins recovery. From this high protein recovery it can be said that the overall homogenisation process was very efficient.

During each successive centrifugation at different speeds a distinct pellet was formed, thus indicating the separation of organelles. In table 3, different amounts of proteins were present within the pellets. Since these proteins are associated with the different organelles present, this indicates that since different amounts of proteins were found in the fractions therefore various different types of organelles must also be present. But this is not always the case since proteins from other fractions could have been damaged due to the homogenization and centrifugation processes. Therefore the calculations performed on Succinate Dehydrogenase activity, recovery and specificity (table 6, figure 2) showed that that the total SDH activity was highest in the supernatant fraction. Since SDH is a specific marker enzyme to the mitochondrion organelle as explained earlier, the data suggests that the separation of mitochondria during centrifugation to be present within the suspected mitochondrial dfraction was not optimal. The supernatant also had a very high protein content of 885mg (table3) which indicated therefore that most of the organelles have separated into this freaction, thus indicating the hight amount of SDH activity within the supernatant fraction.

In a differential centrifugation process the successive increases in the centrifugal forces applied should create a gradient of the presence of different organelles, with the heaviest molecules in the centrifuge tubes with

lowed centrifugal forces, the mediam molecular weighted organelles such as mitochondria in a centriguge in the centrifuge with a medium centrifugal force is applied and small molecular weight organelles such as ribosomes in the centrigue tubes where the highest centrifugal forces are applies.

Therefore the separation of organelles has occurred but not to a great extent as seen by the reults in table 3 and table 6. Seperation of organelles could have been greatly improved by possibly refining the lab protocol. To ahieve better mitochondrial separation and therefore more accurate SDH activity measurments the centrifugation process should be done at 20000g but for 20min and not 10min as stated by Loewen (2003) and Becker et al (2009). This will help separate the mitochondria out better.

Different centrifugation methods such as density gradient centrifugation can be utilized after the intital differential centrifugation to better separate organelles of similar sizes such as mitochondria, lysosomes and peroxisomes. The new fractions produced can by the densiy gradient centrifugation can be recovered with the use of a syringe. Many other techniques such as the initail homogenisation stage could also have been changed and other techniques could have been used as described earlier.

Conclusion:

It was found by this experiment that subcellular fractionation is not a perfect method and therefore inaccuracies must be expected. But it is a process that has revolutionaised our understanding of cell structure and function.

It was found in the experiment that differential centrifugation can separate organelles to an extent to form a nucleic fraction, mitochondrial fraction and

supernatant fraction. Marker enzymes which are present in specific organelles can be used to help distinguish between different organelles as well as the fractions relative purity. SDH was used in this experiment and was found to be present higher in the supernatant, possibly due to experimental error. SDH was specific to the supernatant fraction therore again indicating the presence of mitochondria in the supernatant.

The usage of such techniques in this ever advancing era of science and technology has set the stage for future studies and techniques involved in further studying the cells and increasing our knowledge of life as each day passes.