Activity 5: Extracting chloroplasts using ultracentrifugation

Objective

- To extract chloroplasts from the leaf using ultracentrifugation
- To examine chloroplasts under a light microscope

Safety

- When cleaning the blades, the liquidiser must be turned off.
- Ensure the centrifuge lid is kept closed until the spinning stops.

Procedure

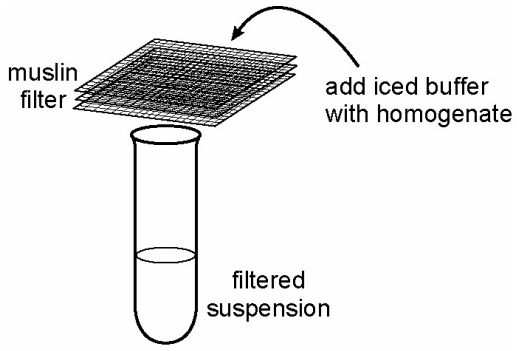
1. It is important to carry out all these steps in a cold environment, using chilled equipment to reduce any enzyme action. Therefore, using the large beaker of ice, push the smaller beaker into this ice.
2. Add 20 cm^3 of cold isotonic buffer to the small beaker and use scissors to cut up 20 g spinach leaves into the iced buffer.
3. Grind in a chilled pestle and mortar or homogenise in a liquidiser.
4. Now replace the small beaker in the ice with a centrifuge tube and filter the chilled suspension into this tube through three layers of washed muslin.
5. Place in the centrifuge at low speed for five minutes to remove cell walls, part-opened cells and starch grains.
6. Pour the supernatant into a clean centrifuge tube and now centrifuge at 500–600 g for 5–10 minutes.
7. Pour off the supernatant. The remaining sediment will contain the nuclei and chloroplasts.
8. Use a dropping pipette to remove a small sample and place onto a microscope slide and cover with a coverslip.
9. Observe under the L.P. lens of the microscope and focus before turning up to the high power.
10. Draw some of the organelles you can see.

Equipment/materials

- 200 cm^3 beaker of ice
- 50 cm^3 beaker
- Two centrifuge tubes and access to a centrifuge
- Scissors
- Chilled pestle and mortar
- Chilled funnel
- Three layers of washed muslin
- Two dropping pipettes
- 20 cm^3 ice-cold isotonic buffer
- 20 g spinach leaves
- Microscope slides and coverslips
- Access to a light microscope

Data/diagram

Figure 5



Analysis of results

From the examiner

- Ultracentrifugation is a differential technique used to separate cell organelles into different fractions.
- Chloroplasts are large organelles which are easily separated into a fraction for examination or to use in an experiment.

Questions

1. Why is it important to reduce the enzyme activity in the cells during preparation?
2. What is the basic principal of differential centrifugation?
3. Describe the appearance of the chloroplasts under the light microscope.
4. Explain why it is not possible to see any internal detail of the chloroplasts.

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Notes on procedure

Density gradient centrifugation

An alternative method to extract chloroplasts without a differential centrifuge:

1. Carry out steps 1–5 as per the student sheet.
2. Pour the supernatant into a clean tube to give a suspension of organelles.
3. Pipette the suspension of organelles into a cold continuous density gradient of sucrose in a centrifuge tube.
4. Centrifuge for 5 minutes.
5. The tube will now contain layers of different fractions of cell organelles. At the bottom will be the cell debris. The layer above will contain nuclei. The middle layer contains the chloroplasts and the mitochondrial layer is above and ribosomes on the top layer.
6. The fractions can be removed by carefully pipetting out each layer in turn, without disturbing the layer below. Or if a plastic tube is used the fractions can be removed by a small hole bored into the bottom.

From the examiner

- Ultracentrifugation is a differential technique used to separate cell organelles into different fractions.
- Chloroplasts are large organelles which are easily separated into a fraction for examination or to use in an experiment.

Answers

1. Enzyme activity in the cells during preparation would result in the breakdown of the organelles since the damage would rupture the lysosomes and release the lytic enzymes they contain.
2. The centrifuge acts on the heaviest particles or fractions first. The result is that the first fraction contains the largest, heaviest organelles and subsequent fractions contain organelles of smaller size and mass order.
3. The chloroplasts appear as small, green spherical or ovoid structures.
4. The resolution of the light microscope is not sufficient to magnify the chloroplasts enough to view the internal detail. An electron microscope is needed.

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Requirements per student*/group of students	Notes
200 cm^3 beaker of ice	
50 cm^3 beaker	
Two centrifuge tubes and access to a centrifuge	
Scissors	
Chilled pestle and mortar or liquidiser	
Chilled funnel	
Three layers of washed muslin	
Two dropping pipettes	
20 cm^3 ice-cold isotonic buffer	For 100 cm^3 buffer solution Prepare 0.15 M solution of each of K_2HPO_4 **and** KH_2PO_4 Mix 38 cm^3 of the K_2HPO_4 with 62 cm^3 of KH_2PO_4 Add 0.01 g of KCL and 6 g of glucose powder Mix and keep ice cold. Send out in a container of ice - works well in a polystyrene container.
20 g spinach leaves	Nettle leaves or dandelion leaves make good alternative sources.
Microscope slides and coverslips	
Access to a light microscope	

Notes

To set up a continuous density gradient of sucrose:

- In a centrifuge tube, add 5 cm^3 of chilled concentrated sucrose (most dense).
- Carefully add 5 cm^3 of less concentrated sucrose.
- Finally, add 5 cm^3 of dilute sucrose (least dense).
- The tube must not be shaken or stirred.

This procedure is best done just before it is needed if the technician is to prepare it in advance.