## Exercise 2

# Chloroplast Isolation and Separation of Chlorophyll–Protein Complexes by Electrophoresis

# INTRODUCTION

Photosynthesis is the process by which living organisms convert light energy into chemical energy in the form of the covalent bonds of organic molecules. All photosynthetic organisms contain specialized pigment molecules that act as light-gathering "antennae," which serve to capture photons of light, convert the energy of photons into the energy of excited electrons, and pass this "excitation energy" to an electron transport system. The electron transport system allows the excitation energy to be converted into biologically-useful forms of energy, *i.e.*, ATP and NADPH. In green plants, the principle photosynthetic pigments are **chlorophyll *a*** and **chlorophyll *b***, although accessory pigments such as the carotenoids (*e.g.*, Beta carotene and the xanthophylls) are also involved.

Chlorophyll molecules are loosely attached to specific proteins, forming **chlorophyll-protein complexes,** which are integral components of the membranes of the chloroplast thylakoids. Three major pigment-protein complexes have been characterized in most higher plants (Lawlor, 1987):

- **LHC**, the Light-Harvesting Complex, which contains both chlorophyll *a* and *b* (along with carotenoid pigments) and accounts for over 50% of the total chlorophyll in the thylakoids. LHC is located primarily in grana thylakoids, and serves to capture photons and funnel excitation energy to chlorophyll P680 in the reaction center of photosystem II.

- **CP I**, Chlorophyll-Protein Complex I, which contains the reaction center chlorophyll P700 and electron transport molecules of photosystem I, as well as some chlorophyll *a* "antenna" molecules. CP I is found primarily in stroma thylakoids (*ie*. stroma lamellae).

- **CP IV**, Chlorophyll-Protein Complex IV, which contains the reaction center chlorophyll P680 and electron transport molecules of photosystem II. CP IV is found in grana thylakoids, where it is apparently linked to LHC.

An important first step in the identification and characterization of the components of the photosynthetic apparatus of green plants is the isolation

of the various chlorophyll-protein complexes. **The objectives of this exercise are to 1) isolate chloroplasts from spinach leaves, 2) solubilize the chloroplast membranes to free the chlorophyll-protein complexes, and 3) separate chlorophyll-protein complexes by electrophoresis.**

## CHLOROPLAST ISOLATION

A typical cell contains many kinds of organelles and molecules, so a study of the function or characteristics of a specific component must usually start with a purification step. The first task is usually to lyse, or break open, the cells gently enough to leave the desired organelle or molecule intact. This can be done mechanically, chemically, or by using various biological agents; generally, the method used for a particular experiment has been determined by a series of trials to see which gives the best results. Cell lysis is usually done in the presence of a buffer of appropriate biological osmotic strength and pH, often protease inhibitors will be added, and the entire procedure is generally performed in the cold (*eg*. 4°C) in order to slow down the degradation of the cell components. The isolation of chloroplasts is typically carried out in dim light to minimize the photo-destruction of chlorophyll.

Once the cells have been lysed, the separation of the various components can start. A wide variety of techniques are available, including centrifugation, dialysis, salting out, chromatography, and electrophoresis. Typically, a combination of approaches is used that removes some of the unwanted material at each step. In this exercise, spinach leaves will be **homogenized** and the chloroplasts will be isolated from the whole plant cell lysate by **filtration** and **differential centrifugation**. Chloroplast membranes will be dispersed using the **ionic detergent sodium dodecyl sulfate (SDS)**. The final step will be the separation of the chlorophyll-protein complexes by **SDS polyacrylamide gel electrophoresis (SDS-PAGE).** Refer to appendix I for an explanation of electrophoresis.

## BACKGROUND READING

You should read the appropriate sections of your textbook or other reference books, on the structure and function of chloroplasts and the role of chlorophyll in photosynthesis. You should also have an understanding of

the basic theory behind the techniques of differential centrifugation and electrophoresis (including denaturing and non-denaturing gels).

There are several points in the lab exercise that require some careful thought— consider them **before** you come to lab:

Why does the isolation buffer contain 0.3 M sorbitol?

If you obtain an absorbance value of 0.65 OD from your chloroplast suspension, what is the chlorophyll concentration? How would you dilute the suspension to obtain a chlrophyll concentration of 1 mg/ml?

How much of the 10% SDS is needed to provide a 40:1 ratio of SDS:chlorophyll?

## PROCEDURES

### CHLOROPLAST ISOLATION

The following procedure will provide enough chloroplast suspension for 4 groups:

1) Weigh out **40** g of fresh spinach (*Spinacia oleracea*) leaves from which the major veins have been removed.

2) Tear the leaves into small pieces and place them in a blender with **200** mls of cold isolation buffer (= 0.3 M sorbitol; 0.1 M Tris-Cl, pH 7.8; 5 mM $MgCl_2$; 10 mM NaCl).

3) Blend the mixture at low speed for **10** seconds. Use a glass rod to push any tissue pieces down the sides of the jar into the solution, and blend for **10** more seconds.

4) Filter the homogenate (called a tissue 'brei') through **8** layers of cheesecloth into a beaker. Add approximately 40 ml of filtrate to each of four **40** ml centrifuge tubes, making sure they are balanced ($\pm$ 0.1 g).

5) Centrifuge at 4$^{o}$C for **5** minutes at **1000** *x g* .

6) Decant and discard the supernatant from all 4 tubes. Add **0.5** mls of cold isolation buffer each tube and gently resuspend the chloroplasts with a paint brush.

7) Store the centrifuge tube on ice, away from bright light.

## CHLOROPHYLL CONCENTRATION MEASUREMENT

1) Add **0.05** ml (= **50** $\mu$L) of the chloroplast suspension to a clean glass conical centrifuge tube.

2) Use a graduated cylinder to measure out **7.5** ml of 90% acetone, add to the centrifuge tube, cover tightly with Parafilm and invert several times to dissolve the chlorophyll. A flocculent precipitate of protein should be visible.

3) Remove the protein by centrifuging for **2** minutes at about 500 x *g*. The protein should form a pellet at the bottom of the tube. Pour the supernatant (acetone extract) into a clean tube and discard the tube containing the pellet.

4) Pour some of the supernatant into a clean spectrophotometer cuvette. Zero the Spectronic 20 using a blank of 90% acetone and read the absorbance of your sample at **652** nm.

5) If the absorbance reading is greater than **1.5**, pour the contents of the Spectronic 20 tube back into the tube containing the acetone extract, dilute the acetone extract with 90% acetone until its absorbance below **1.5**. Record how much additional acetone was added.

6) Use the equation below to determine the concentration of chlorophyll in your chloroplast sample:

$$[\text{chlorophyll}]\ \text{mg/ml} = \text{absorbance} \times \frac{\text{total vol. (ml) of acetone used}}{\text{vol. (ml) of suspension used}} \times 0.029$$

7) Once you have determined the chlorophyll concentration, **THROW AWAY THE ACETONE-CHLOROPHYLL SOLUTION AND THE ACETONE IN THE BLANK CUVETTE.**

8) If the chlorophyll concentration of your original suspension is above 1 mg/ml, it must be diluted. Remove **1** ml of the chloroplast suspension and place it in a small test tube. Add enough **isolation buffer** to dilute the chlorophyll concentration of this sample to **1** mg/ml. Gently mix the diluted suspension.

## SOLUBILIZATION OF CHLOROPLAST MEMBRANES

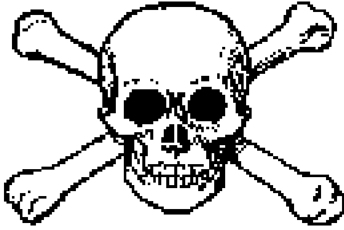
1) Place **1.0** ml of the diluted chloroplast suspension (= 1 mg chlorophyll per ml) into a clean 1.5 ml plastic Eppendorf centrifuge tube.

2) Add sufficient 10% SDS (= 100 mg SDS per ml) to provide a **40:1** (w/w) ratio of SDS:chlorophyll. Mix gently to avoid creating a foam that is impossible to get rid of.

3) Spin the tube in the "microfuge" at **12,000***xg* for **5** minutes. Carefully transfer the supernatant to a clean tube. The supernatant contains the chlorophyll-protein complexes. Add 1-2 drops of glycerin (glycerol) and mix gently with a micropipette.

4) Small volumes (10-20 µl) of your solubilized membrane protein mixture should be loaded on the electrophoresis gel.

## PREPARATION OF POLYACRYLAMIDE GELS



**UNPOLYMERIZED ACRYLAMIDE IS A NEUROTOXIN AND CAN BE ABSORBED THROUGH INTACT SKIN. BE EXTREMELY CAUTIOUS WHEN USING ACRYLAMIDE; WEAR GLOVES AND AVOID SPILLS. NEVER MOUTH PIPETTE A SOLUTION OF ACRYLAMIDE.**

1) Each group will assemble and pour one slab gel. The details on assembling the plates and how to pour will be demonstrated.

2) Prepare to polymerize the gel: To **10 mls of separating gel** (8% acrylamide/bis-acrylamide; 0.4 M Tris-HCl, pH 8.0; 0.1% SDS) add **5 μL TEMED** (N,N,N',N'-tetramethyl-ethylenediamine) and **50 μL 20% ammonium persulfate**. Swirl gently to completely mix the contents, but avoid excessive aeration.

3) Use a Pasteur pipette to completely fill the plates with gel and slide the plastic comb into the top. Avoid trapping air bubbles under the teeth of the comb.

4) Allow 30-45 minutes for polymerization of the gel. Once polymerized, the plates can be removed from the casting holder, and the combs gently removed.

5) Insert the gel apparatus into the buffer chamber. Pour **running buffer** (0.1 M Tris-Cl; pH 8.0; 0.13% SDS) into the upper reservoir, until it is midway between the short and long plates.

6) Rinse out the wells with running buffer and load 5-15 **μL** of your sample into several wells. Keep a careful record of the volume of sample in each well. Load 1-3 **μL** of the known molecular weight protein markers into the end wells.

7) When all samples have been loaded, pour running buffer into the lower chamber until it reaches the bottom of the gels. Snap the cover on the apparatus, making sure the leads are attached to the correct electrodes.

8) Once the gel apparatus is ready, plug the leads into the power supply. Turn on the power and adjust to 100 volts.

9) Apply the voltage until the solvent front, as shown by a faint greenish-yellow band of free chlorophyll, is about 1 cm from the bottom of the gel (approximately 30 minutes).

10) Turn off the power, disassemble the apparatus. Carefully remove the gels from the glass plates and place them into Petri dishes with a small volume of distilled water to keep them moist.

11) Use a plastic ruler placed behind the gel to measure the distance from the bottom of the well to the bottom of each of the visible chlorophyll-protein bands.

12) Illuminate the gel with UV light in a darkened room. Make a note of what do you see.

13) Label your plate to distinguish it from that of other groups. Your instructor will rinse the gels in distilled water, then stain them with Coomassie blue for about 1 hour. The gels will be destained overnight to remove stain from the regions of the gel lacking protein bands.

14) Tomorrow afternoon, come in and examine the destained gel. Compare the number of bands visible now with the number you could see prior to staining. NOTE: Some bands may be very faint- look carefully, using a light-table if possible. Measure the distance moved by each of the marker proteins.

## ANALYSIS OF RESULTS

1) Compare the chlorophyll-containing bands visible before staining with regard to their density, thickness, color.

• Which bands correspond to the chlorophyll-protein complexes LHC, CP I and CP IV?

2) For each of the molecular weight marker protein bands, look up its molecular weight on the information sheet provided. On graph paper plot the distance moved versus molecular weight for the each of the marker proteins. Use this standard curve to estimate the molecular weight of the chlorophyll protein complexes and any other chloroplast protein bands that are visible.

• Can you positively identify the LHC, CP I, and CP IV bands from their molecular weights?

• Which proteins might comprise the additional bands visible after staining? What specific proteins are abundant in chloroplasts of higher plants? Can you identify any of these proteins based on their molecular weights?

3) What further studies might be carried out on the chloroplast proteins isolated by SDS-PAGE?

## Appendix I. ELECTROPHORESIS

Many of the amino acids that comprise a specific protein carry either positive or negative charges (mainly as $NH_3^+$ or $COO^-$). The overall charge on a protein depends on the relative frequency of the various types of amino acids. Proteins with many acidic amino acids will tend to be acidic themselves, and thus be negatively charged, and *vice versa*. The overall charge will also depend on the pH of the surrounding liquid and on the presence of other molecules.

If such a charged protein molecule is exposed to an electric current, it will tend to move to the oppositely charged pole. The rate at which the protein moves in an electric field depends on a combination of factors, including its net charge, the strength of the electric current, the viscosity of the medium and the size and shape of the protein molecule. Normally, the protein migrates through a gel formed by polymerizing two chemicals, **acrylamide** and **bis-acrylamide**, into a three-dimensional network. This "polyacrylamide" gel can be adjusted to contain different sizes of pores by changing the concentrations of the two components; the larger the pores, the easier it is for proteins to move through the gel. Most protein analysis is done using this system of **polyacrylamide gel electrophoresis** (**PAGE**).

If a mixture of proteins are placed in the same environment and exposed to the same electrical current, their movement will depend on their individual charge, size and shape. Some proteins will move rapidly toward the positive electrode, some slowly or not at all, while others move toward the negative electrode. Since all molecules of similar charge, size, and shape should behave the same, they will form a 'band' of molecules migrating toward one of the poles. This does not mean that all proteins in the same band are identical; a given band may contain many different types of proteins, all of which happen to travel at the same rate. In a **non-denaturing PAGE** system, the protein conformation remains intact. Since this system provides little information about the properties of a protein it is seldom used, except to keep enzymes in an active form.

The vast majority of protein analysis is done using the ionic detergent **sodium dodecyl sulfate** (**SDS**). SDS binds strongly to hydrophobic regions of the proteins and, being negatively charged, causes an unfolding of the

chain (in an attempt to keep all the similar charges as far from each other as possible). This **denaturing** process is often enhanced by the addition of b-mercaptoethanol, a reducing agent that breaks disulfide bridges, allowing the complete unfolding of the polypeptide chain. Proteins in the presence of an excess of SDS are all in the form of individual linear molecules and all carry a very large net negative charge. The only variable which can then affect their speed in an electric field is their molecular size. In SDS-PAGE all molecules in the same band must also be the same size; this still does not ensure that they are all identical, but at least one factor (size) is now known. A mixture of proteins, when separated by **SDS-PAGE**, will form a series of bands, arranged in order of molecular weight, the largest being nearest the starting point (origin) and the smallest having travelled the furthest.

Cells may contain up to several thousand types of protein and most of these are colorless (chlorophyll-protein complexes and hemoglobin are obvious exceptions!). In order to visualize the bands of proteins in a gel they must be stained with an agent that will color the proteins, but not the gel. The commonest such agent is the dye **Coomassie Blue**, which can produce a visible band with as little as 100 ng of protein. In the last few years silver nitrate has become widely used, despite some problems; it can be used on bands containing as little as 5 ng of protein and produces a dark brown or black stain.

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