Zymogram of starch interacting proteins

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Objective:
The aim of the lab is to separate and qualitatively analyse starch interacting enzymes of tissues from two different plant species. Enzymes should be identified by the substrates used for incubation and the final glucan-product detected.

Background:

Description of methology
Zymography is an electrophoretic technique mainly used for the qualitative assessment of enzyme activity. The technique can be performed under native as well as denaturing conditions. When using denaturing conditions there is a subsequent need to renature the separated enzymes in order to render them active. Under native conditions the enzyme usually retains its three dimensional configuration and is active when the separation is completed. Poly acrylamid gel electrophoresis (PAGE) is commonly used in order to separate proteins for zymograms. Under denaturing conditions a detergent, sodium dodecyl sulphate (SDS), is included to denature the proteins and provide a negative charge that is proportional to the protein mass. This allows determination of protein molecular weight via comparison of migration for protein standards of known size. Under native PAGE, the migration distance depends on the protein intrinsic charge, and on the pore size of the gradient gel. Thus no prediction regarding molecular weight can be made.
Substrates for enzyme/s of interest can be provided in several ways. They can be included in the gel used for separation or included in a second gel where an imprint is made of the separating gel. A third alternative is to incubate the separation gel in a solution containing the substrate. In the last case it is important that the substrates can permeate the gel and come in contact with the enzyme contained in the gel. Commonly a chemical is used that will stain and thus visualize the enzyme product.
An array of enzymes interacts with starch in biosynthetic as well as degradative pathways which is described in Chapter 7 of the Plant Biochemistry textbook. These enzymes will in different ways change a starch structure or depending on substrate access yield various starch like polymers. Iodine will stain starch in different ways depending on starch structure. This is dependent on chain length of polymerized glucose units. Long linear glucose chains as amylose will be stained bluish-black and heavily branched molecules consisting as amylopectin will be stained reddish-brown. Starch is normally not soluble in water. However the starch we will use for this lab has been made water soluble by limited degradation.

Starch composition and biosynthesis
Starch is a plant-derived polysaccharide synthesized as an energy storage molecule which can be found in most plants. Starch is composed of two molecules, amylose and amylopectin, both of which are made up of linked glucose residues (Figure 1). Amylose is an essentially linear molecule of α-1,4 linked D-glucose residues with less than 1% of the glucose molecules attached as α-1,6 branches. Amylopectin is also made up of α-1,4 linked D-glucose residues but differs from amylose by being highly branched with approximately 5% of the glucose units linked by α-1,6 branches. The distribution of the amylose and amylopectin content in starch in many plants is approximately 1:4.

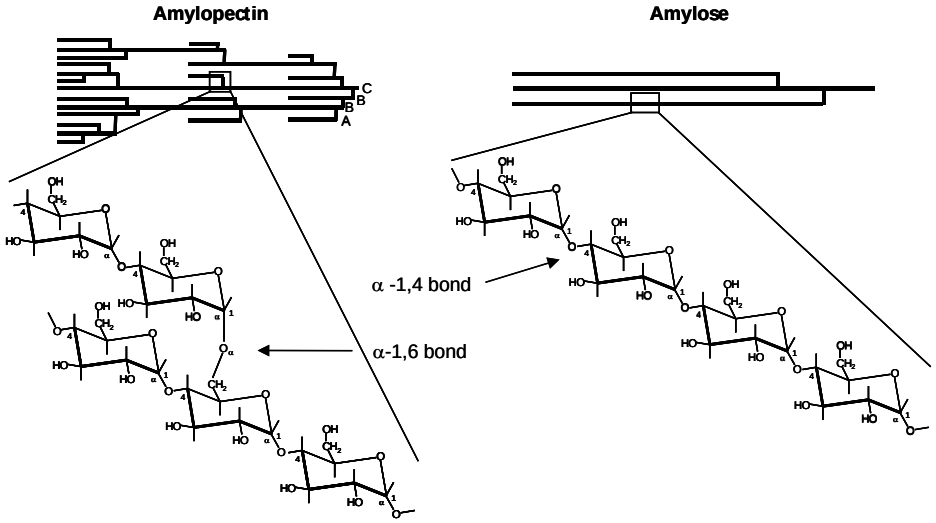

Figure 1. *Schematic illustration of amylopectin and amylose. Amylopectin is a highly branched polymer of glucose molecules linked by α-1,4 bonds and α-1,6 branches. Amylopectin chains differs in length and are called A, B, and C, depending on their location in the amylopectin molecule. Amylose is a mostly linear polymer of glucose molecules linked by α-1,4 bonds and contains only a few α-1,6 branches.*

Three major enzymes regulate the central process of starch synthesis (Figure 2); ADP-glucose pyrophosphorylase (ADPgase), starch synthases (SS) and starch branching enzymes (BE). ADPgase catalyses the formation of the glucosyl donor ADP-Glc from Glc-1-P and adenosine triphosphate (ATP). ADP-Glc molecules are linearly attached to the non-reducing end of an α-1,4-glucan chain through α-1,4- glucosidic linkages by starch synthases. Branches in the starch molecules are introduced by branching enzymes, catalysing the formation of α-1,6 glucosidic linkages The branching enzymes act by hydrolysing an α-1,4 linkage and reattaching the chain with an α-1,6 linkage. Starch phosphorylase is an enzyme involved in degradation of starch, releasing glucose-1-phosphate from the non-reducing end of the starch molecule. However, starch phosphorylase is a reversible enzyme and can act by synthesising a linkage between glucose-1-phosphate and a starch molecule.

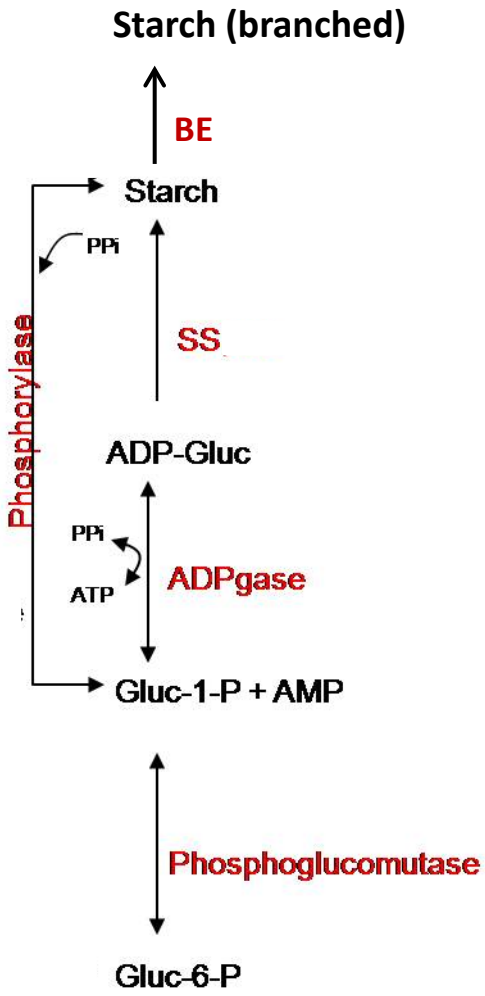

Figure 2. *Schematic figure of the **t**hree major enzymatic steps catalysing the biosynthesis of starch; ADP-glucose pyrophosphorylase (ADPgase), starch synthases (SS) and starch branching enzymes (BE).*

General flow of experiment:

- Homogenize plant tissue in buffer
- Centrifuge to remove insoluble
- Native PAGE of soluble extract
- Incubation with substrates
- Staining of gel
- Documentation

Experimental procedure:

a) Weigh 400-700 mg plant tissue from two different plant species of your choice in a pre-weighed micro-tube (maximum fill half the tube).
b) Homogenize plant tissue 1:1 (w/v) carefully in cold extraction buffer (provided) using a blue plastic pestle. Keep the sample cold, otherwise the enzymes will be destroyed.
c) Centrifuge at 10,000 g for 5 min at 4°C.
d) Immediately load 20 µl from each of the supernatant in five repeats on native 10% PAGE-gel (in total 10 wells). Load 10 µl blue loading dye in a 11^{th} well to keep track of the samples.
e) Run in coldroom 150 V for 2 hrs or until the dye has reached the bottom of the gel.
f) Use run time of electrophoresis to produce all buffers needed for washing and incubation **(this is a critical step since it is very important that the buffers are correctly made).**
g) Vertically divide the gel into five identical pieces (a ruler works fine) **and mark them 1-5**.
h) **Wash gelpieces 1, 2 and 3** in 100mM Tris-HCl pH 7.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol at room temperature 10 min.
i) **Wash gelpiece 4** in 50 mM Tricine-NaOH pH 8.5, 25 mM potassium acetate, 2mM EDTA, 2 mM DTT, 10% glycerol 10 min at room temperature.
j) **Wash gel piece 5 in** deionized water 3x5 minutes and immerse in Gel Code Blue Stain Reagent for 1 hour. Rinse with deionized water o/n with several changes of water. All steps are performed on a shaker table.

k) Incubation is done with gel pieces 1-4 in four different substrate combinations:

Same buffers as washed in, supplemented with:

1. 1% soluble starch in Tris buffer
2. 1% soluble starch + 50 mM Glucose-1-phosphate + 2,5 mM AMP in Tris buffer (same as h above)
3. 50 mM Glucose-1-phosphate + 2,5 mM AMP in Tris buffer (h)
4. 0,5 mg/ml BSA + 0,3% glycogen + 1 mM ADP-glucose in Tricine buffer (same as i above)

18 ml is enough for all groups regarding each substrate incubation.

- 2-3 hrs incubation on "shaking table" at room temperature for substrates/gels no 1-3
- Incubation o/n for substrate/gel no 4, also shaker table and room temperature.

- After incubation the gel is briefly rinsed in deionized water and then visualized in 0,04% I2, 0,4% KI 10 min at shaker table. For the overnight incubation you will need to do the staining the day after when back in the laboratory.

- Documentation by putting the gels on light-table and photographing or scanning.

Laboratory report

Introduction:
Briefly outline starch biosynthesis and degradation for storage organs as tuber and seed. Briefly describe method used. Why is it qualitative rather than quantitative?
Materials and methods:
Outline what you did and what materials were used.
Results:
Describe differences in observations depending on substrates used as well as plant material used. Note colour differences. Use photographs taken as figures and refer to them.
Discussion:
Discuss what **enzyme activities you have observed** and why you observed a certain type of activity under certain experimental conditions. Identify substrates and changes to them induced by enzymes. Explain colour difference. Use your text book to support your report.