

Product Analysis of Starch Active Enzymes by TLC

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[Abstract] Thin layer chromatography (TLC) is a useful technique for detecting the presence of monosaccharides through to oligosaccharides, though it needs to be optimized for the specific sugars that are analyzed. Here we present a method for visualizing the reaction product(s) of starch active enzymes, which can contain α -1, 4 linked and α -1, 6 linked glucose. This was first published in *Molecular Microbiology* (Cockburn *et al.*, 2015). The TLC protocol is an adapted version of that published by Robyt and Mukerjea (Robyt and Mukerjea, 1994). For a summary of the products generated by starch active enzymes see the review by Hii *et al.* (2012).

Materials and Reagents

1. TLC Silica gel 60 F₂₅₄ 20 x 20 cm plates (Note 1) (Merck Millipore Corporation, catalog number: 1.05715.0001)
2. Filter paper circles (15 cm diameter) (VWR International, catalog number: 470204-480)
3. MilliQ quality water (dH₂O)
4. Acetonitrile (anhydrous) (Sigma-Aldrich, catalog number: 271004)
5. Ethyl acetate (anhydrous) (Sigma-Aldrich, catalog number: 270989)
6. 2-propanol (Sigma-Aldrich, catalog number: I9516)
7. N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich, catalog number: 222488)
8. Methanol (anhydrous) (Sigma-Aldrich, catalog number: 322415)
9. Sulfuric acid (Fluka, catalog number: 84716)
Note: Currently, it is "Sigma-Aldrich, catalog number: 84716".
10. Dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, catalog number: D128)
11. α -D-Glucose (anhydrous) (Sigma-Aldrich, catalog number: 158968)
12. Maltose monohydrate (Sigma-Aldrich, catalog number: M9171)
13. Maltotriose (Sigma-Aldrich, catalog number: M8378)
14. Maltotetraose, DP4 (Sigma-Aldrich, catalog number: 47877)
15. Maltopentaose (TCI America, catalog number: M1023)
16. Maltohexaose (Sigma-Aldrich, catalog number: M9153)
17. Maltoheptaose (Sigma-Aldrich, catalog number: 284017)

18. Isomaltose (Sigma-Aldrich, catalog number: I7253)
19. D-Panose (Sigma-Aldrich, catalog number: P2407)
20. Glycogen from bovine liver (Sigma-Aldrich, catalog number: G0885)
21. Amylopectin from potato starch (Sigma-Aldrich, catalog number: A8515)
22. Amylose from potato (Sigma-Aldrich, catalog number: A0512)
23. Dextran from *Leuconostoc* spp. (Sigma-Aldrich, catalog number: 31389)
24. Pullulan from *Aureobasidium pullulans* (Sigma-Aldrich, catalog number: P4516)
25. Your enzyme of interest and appropriate buffer
26. Mobile phase (see Recipes)
27. Staining solution (see Recipes)
28. Oligosaccharide standards (see Recipes)
29. Glycogen solution (see Recipes)
30. Amylopectin solution (see Recipes)
31. Amylose solution (see Recipes)
32. Dextran solution (see Recipes)

Equipment

Note: All equipment needed is shown in Figure 1.

1. TLC developing chamber (VWR International, catalog number: 21432-739)
2. Chromatography sprayer (Note 2) (Sigma-Aldrich, catalog number: Z529729)
3. Oven capable of reaching 120 °C (Boekel Scientific, catalog number: 107905)
4. (Optional) Milwaukee™ Dual Temperature Heat Gun (Thermo Fisher Scientific, Fisher Scientific, catalog number: 19-313-598)
5. Corning™ Scholar™ PC-170 Hotplate (Thermo Fisher Scientific, Fisher Scientific, catalog number: 13-641-564)

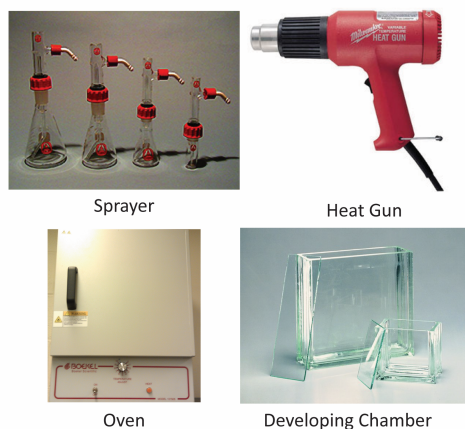


Figure 1. Equipment needed for this protocol

Procedure

Enzyme reaction

1. Prepare 20x concentrated solutions of the enzyme of interest and the appropriate buffer. The concentration of enzyme required will be enzyme and substrate specific, but 1 µg/ml final concentration (20 µg/ml stock) is likely to be a reasonable starting place.
2. Mix 90 µl of each substrate to be studied with 5 µl of concentrated buffer and pre-incubate at the desired temperature.
3. Add 5 µl of the concentrated enzyme, mix by pipetting up and down and take a 20 µl sample, immediately freezing or otherwise halting the reaction. Continue to take samples at 10 min, 20 min and 30 min (Note 3), freezing the samples until you are ready to perform TLC.

TLC

4. Add 100 ml of the mobile phase to the TLC developing chamber. Soak 1 or 2 pieces of the filter paper in the mobile phase and stick it to the wall of the chamber (see Note 4). Replace the lid of the chamber and allow 30 min for equilibration of the mobile phase with the atmosphere of the chamber.
5. With a pencil draw a line 2 cm from the bottom of the TLC plate and include evenly spaced hashmarks (at least 1 cm apart) for each sample to be loaded. These may be labelled below the line in pencil.
6. Load 1 µl of each standard and sample. Standards may be combined to save space, though at a slight cost to resolution. For less concentrated samples the initial spot can be allowed to dry prior to addition of another 1 µl drop in the same position. This can be done repeatedly as a method for concentrating the sample.
7. Once the spots have dried place the TLC plate in the developing chamber, immersing the bottom of the plate into the mobile phase at the bottom. The liquid level of the mobile phase should not come above your pencil line. Replace the cover on the chamber.
8. Incubate until the mobile phase reaches the top of the plate, approximately 3 h for a 20 cm plate.
9. To separate longer oligosaccharides (>approximately 12 monosaccharide units), remove the plate from the chamber, allow it to dry and then put it back in the chamber to perform a second ascent.

Video 1. Steps of TLC procedure



Staining

10. Allow the plate to dry and then spray the plate with the staining solution, coating it evenly.
11. Allow the plate to dry and then place it in the oven or use a heat gun at 120 °C, until spots become clearly visible, approximately 10 min in oven, somewhat less with the heat gun.

Analysis

12. Use the standards to determine the identity of the products produced. Note that sugars containing α -1, 6 linkages will run approximately equivalent to an all α -1, 4 linked oligosaccharide one monosaccharide unit longer, for each α -1, 6 linkage present. e.g. isomaltose, runs at approximately the same distance as maltotriose and panose runs at the same distance as maltotetraose (see Figure 2).

Representative data

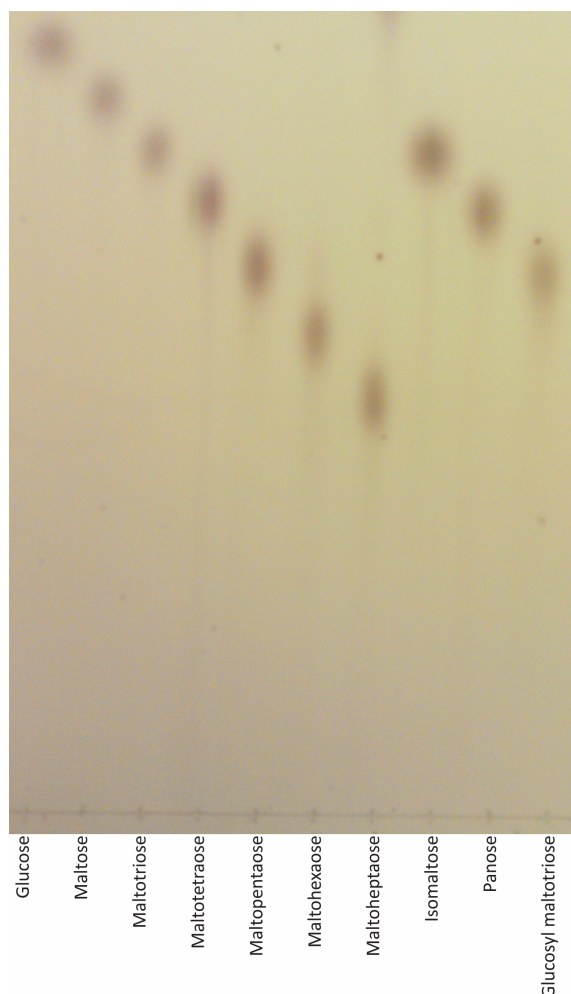


Figure 2. Sugar standards. Illustration of the run profile for sugar standards. Maltose through maltoheptaose are α -1, 4 linked polymers of glucose from DP2 to DP7. Isomaltose is glucose α -1, 6 linked to glucose, panose is glucose α -1, 6 linked to maltose and glucosyl maltotriose is glucose α -1, 6 linked to maltotriose. Each standard was at 5 mM and 1 μ l was loaded onto the plate.

Notes

1. Smaller format plates can be used, particularly if investigating the production of shorter oligosaccharides (maltoheptaose or smaller).
2. In the absence of a sprayer the plate can be dipped in the staining solution or the staining solution can be poured over the plate, though streaking is more likely to occur.
3. This procedure is designed to investigate initial reaction products. It is also useful in some cases, particularly with debranching enzymes (hydrolyzing α -1, 6 linkages), to examine the final reaction products. This can be accomplished by using a higher

concentration of enzyme and/or a longer incubation time, allowing the reaction to proceed to completion.

4. Soaking the filter paper and sticking it to the wall of the chamber is optional, but will shorten the time needed for equilibration to occur. This step is not shown in Video 1.
5. For storage of the polysaccharide solutions pass through a 0.2 μm filter and add sodium azide to 0.02% and store at room temperature (Lichstein and Soule, 1944). For oligosaccharide solutions, store frozen.

Recipes

1. Mobile phase

Mix 85 ml acetonitrile, 20 ml ethyl acetate, 50 ml 2-propanol, 60 ml water

Requires approximately 100 ml per assay (less with smaller TLC plates and chambers)

2. Staining solution

Add 10 ml of sulfuric acid to 190 ml of methanol

Dissolve 0.6 g of N-(1-naphthyl)ethylenediamine dihydrochloride

3. Oligosaccharide standards

Make to 5 mM in water

4. Glycogen solution

Dissolve 150 mg in 50 ml of water

5. Amylopectin solution

Dissolve 150 mg in 3 ml DMSO

Slowly add hot water (above 60 °C) with vigorous stirring, bringing the volume up to 50 ml

6. Amylose solution

Dissolve 75 mg in 10 ml DMSO

Slowly add very hot water (above 80 °C) with vigorous stirring, bringing volume up to 50 ml

7. Dextran solution

Dissolve 150 mg in 50 ml of water

Acknowledgments

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References

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