

Assay of Arabinofuranosidase Activity in Maize Roots

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[Abstract] Root is a perfect model for studying the mechanisms of plant cell growth. Along the root length, several zones where cells are at different stages of development can be visualized (Figure 1). The dissection of the root on these zones allows the investigation of biochemical and genetic aspects of different growth steps. Maize primary root is much more massive than the root of other Monocots and thus more convenient for such type of research. Plant cell wall, mainly consisting of polysaccharides, plays an important role in plant life. Therefore, measurement of plant carbohydrate content and glycoside-modifying enzyme activity in plant cells has become an important aspect in plant physiology. One of the well-documented changes of hemicelluloses molecules during elongation growth of monocots cells is the decrease of arabinose substitution of glucuronoarabinoxylans. This might be caused by changes in synthesis of this polysaccharide or by the action of arabinofuranosidases. Here, we describe the protocol of spectrophotometric measuring of arabinofuranosidase activity in maize root by the rate of hydrolysis of chromogenic substrate (4-nitrophenyl α -L-arabinofuranoside).

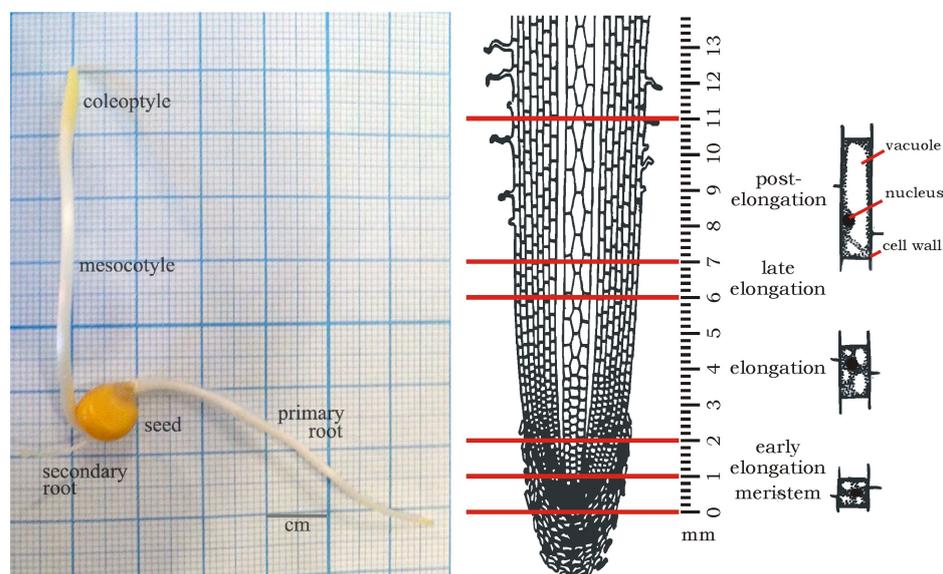


Figure 1. Scheme of plant material collection for further arabinofuranosidase assay. Four-day-old dark-grown maize seedling (left panel). Different zones of primary maize root and corresponding stages of cell development, according to Kozlova *et al.* (2012) (right panel).

Materials and Reagents

1. Microcentrifuge tubes (1.5-2 ml)
2. CarboPac PA1 analytical column (4 x 250 mm) (Thermo Fisher Scientific, catalog number: 035391)
3. CarboPac PA1 guard column (4 x 50 mm) (Thermo Fisher Scientific, catalog number:)
4. Maize seedlings
5. 4-nitrophenyl α -L-arabinofuranoside (4NPA) (Sigma-Aldrich, catalog number: N3641)
6. 4-nitrophenol (NP) (spectrophotometric grade) (Sigma-Aldrich, catalog number: 1048)
7. DL-Dithiothreitol (Sigma-Aldrich, catalog number: D0632)
8. Quick Start™ Bradford 1x Dye (Bio-Rad Laboratories, AbD Serotec®, catalog number: 5000205)
9. Sodium Acetate Anhydrous (NaOAc) (Sigma-Aldrich, catalog number: W302406)
10. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: 306576)
11. L-(+)-ARABINOSE (Sigma-Aldrich, catalog number: W325501)
12. NaN_3
13. Na_2CO_3
14. NaOCl
15. Glacial acetic acid
16. Ice
17. MilliQ water (18 M Ω -cm)
18. 5% (w/w) solution of NaOCl (see Recipes)
19. 0.03% (w/w) NaN_3 (see Recipes)
20. 50 mM NaOAc buffer (pH 6.0) with 3 mM of dithiothreitol on 0.03% (w/w) NaN_3 (see Recipes)
21. 10 mM 4NPA (see Recipes)
22. 1 M Na_2CO_3 (see Recipes)
23. Calibration solutions for NP (see Recipes)
24. 50% (w/w) NaOH solution (see Recipes)
25. 15 mM NaOH (buffer A for HPAEC) (see Recipes)
26. 1 M NaOAc in 0.1 M NaOH (buffer B for HPAEC) (see Recipes)
27. Calibration solutions for arabinose (see Recipes)

Equipment

1. Spectrophotometer (required wavelength is 405 nm)
2. Thermostat (required temperature is 27 °C)
3. Microcentrifuge (required spin rate is 10,000 x g)
4. Thermoshaker (required temperature and spin rate are 37 °C and 600 rpm, correspondingly)

Note: Thermoshaker should be appropriate for incubation of microcentrifuge tubes.

5. Mini centrifuge/vortex
6. Analytical balance with 0.1 mg readability
7. Ion chromatography system DX-500 (Thermo Fisher Scientific, Dionex) equipped by electrochemical detector ED40, gradient pump GP40 and chromatography oven LC 30
8. Refrigerator (required temperature is $-20\text{ }^{\circ}\text{C}$)
9. Automatic pipettes (100-1,000, 20-200 and 2-20 μl)
10. Mortar and pestle
11. Plastic grid and the tray of proper size

Note: The cell of grid should be approximately 1 cm^2 or slightly bigger.

Software

1. PeakNet software (Thermo Fisher Scientific, Dionex, model: version 5.21)

Procedure

Principal scheme of the experiment is shown in Figure 2.

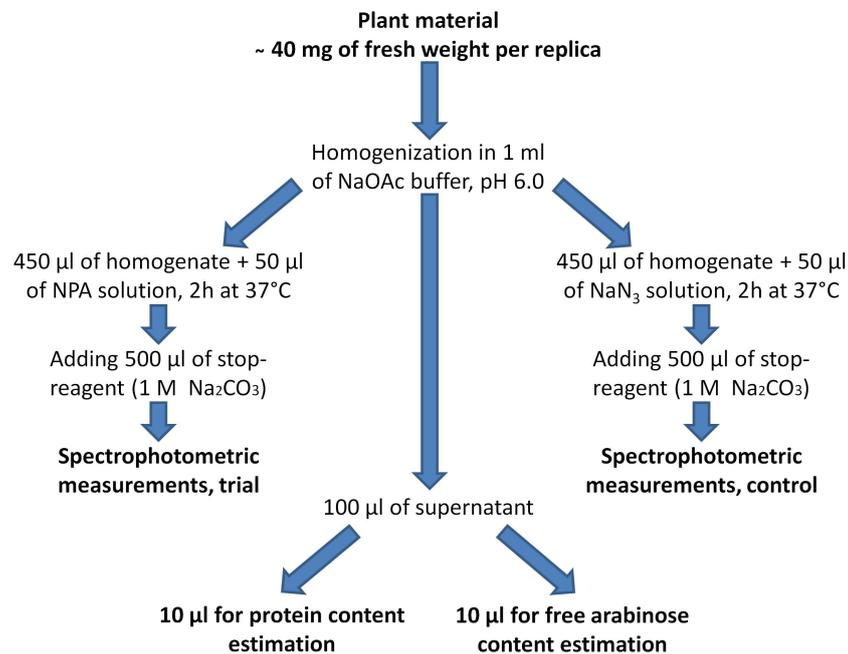


Figure 2. Scheme of the experiment

1. Seeds of maize (*Zea mays* L.) were sterilized in 5% (w/w) solution of NaOCl for 10 min then were washed twice in water. Seeds were placed in the cells of plastic grid that was mounted in a tray with water. Plants were grown in the dark in a thermostat at $27\text{ }^{\circ}\text{C}$ for

96 h. While any part of etiolated seedling can be used for the assay, we have used five zones (meristem, early elongation, elongation, late elongation, post-elongation) of primary roots (Figure 1). The recommended amount of plant material in each sample is 30-40 mg. One mm of maize root represents approximately one mg of fresh weight. Thus, root pieces of 10 mm length from three or four seedlings or pieces of 1-mm length from 30-40 seedlings are enough for one replica.

2. 30-40 mg of fresh plant material was homogenized with mortar and pestle on ice in 1 ml of 50 mM NaOAc buffer (pH 6.0) with 3 mM of dithiothreitol to prevent the intramolecular and intermolecular disulfide bonds formation and with 0.03% (w/w) NaN₃ to restrict bacterial contamination. Homogenate was transferred to microcentrifuge tube (1.5-2 ml). Homogenates that were already prepared could be stored in a refrigerator (-20 °C) while other samples are being done.
3. 1 ml of suspension was centrifuged for 10 min at 10,000 x g. Then 100 µl of supernatant were separated for further estimation of protein content according to Bradford (1976) (step 9) and free arabinose content estimation (step 10). Supernatants could be stored in a refrigerator (-20 °C). Steps 9-10 could be carried out later. The arabinose and protein contents do not change if samples are frozen.
4. Pellet was resuspended in the remaining 900 µl of buffer solution using Vortex. Plant material was not separated from supernatant because of the potential presence of wall-associated arabinofuranosidases that might be not extracted by the buffer solution. The suspension was divided into two equal parts (450 µl each) that were transferred to plastic tubes as control and trial variants of each sample.
5. 50 µl of 10 mM 4NPA in 0.03% (w/w) NaN₃ was added to the trial tubes, and 50 µl of 0.03% (w/w) NaN₃ to the control tubes. Both of the variants were incubated in the thermoshaker at 600 rpm at 37 °C for 2 h. During that time cell wall arabinofuranosidases (either soluble or wall-associated) in trial tubes removed arabinose residues from 4NPA molecules producing yellow-colored NP. Enzymes in control tubes had no chromogenic substrate for reaction.
6. The reaction was stopped by the addition of 500 µl of 1 M Na₂CO₃ both to the control and to the trial tubes. The coloration of the solution has become more pronounced at that moment due to increased pH (11.5).
7. Samples were centrifuged for 10 min at 10,000 x g. Optical densities of both trial and control supernatants were measured on a spectrophotometer at wavelength 405 nm. The control mean was subtracted from the trial mean. The result was used for the determination of the amount of liberated NP according to calibration.
8. Calibration measurements were done for NP solutions with concentrations in a range from 0.0025 mM to 0.25 mM (Figure 3). 500 µl of 1 M Na₂CO₃ were added to 500 µl of each NP solution, because it enhances the coloration.
9. Determination of protein content.

- a. 490 μ l of water was added to 10 μ l aliquots of supernatant from step 3 of each sample. 500 μ l of Bradford dye reagent was also added to each microcentrifuge tube and then mixed using vortex.
 - b. After 5 min each sample was read on a spectrophotometer at 595 nm.
10. Determination of free arabinose content.
- a. 90 μ l of water was added to 10 μ l aliquots of supernatant from step 3 of each sample.
 - b. 25 μ l of obtained solution was directly injected to the column of HPAEC system without any pretreatment.
 - c. 15 mM NaOH (buffer A) and 1 M NaOAc in 0.1 M NaOH (buffer B) were used as eluents. The column temperature was maintained at 30 $^{\circ}$ C, and the elution rate was 1 ml/min. The gradient elution was performed as follows: 0-20 min-100% A; 20-21 min-linear gradient until the A/B ratio = 90:10(%); 21-31 min-linear gradient until the A/B ratio = 70:30(%); 31-32 min-linear gradient until the A/B ratio 0:100(%); 32-42 min-100% B; 42-43 min-until the A/B ratio = 100:0(%). Column was equilibrated with eluent 100% A for 30 min.
 - d. Arabinose content was determined by the area of the corresponding peak using the PeakNet software and the calibration for arabinose standard.
11. Calibration for free arabinose was done using solutions with concentrations in a range 2-10 μ g/ml.

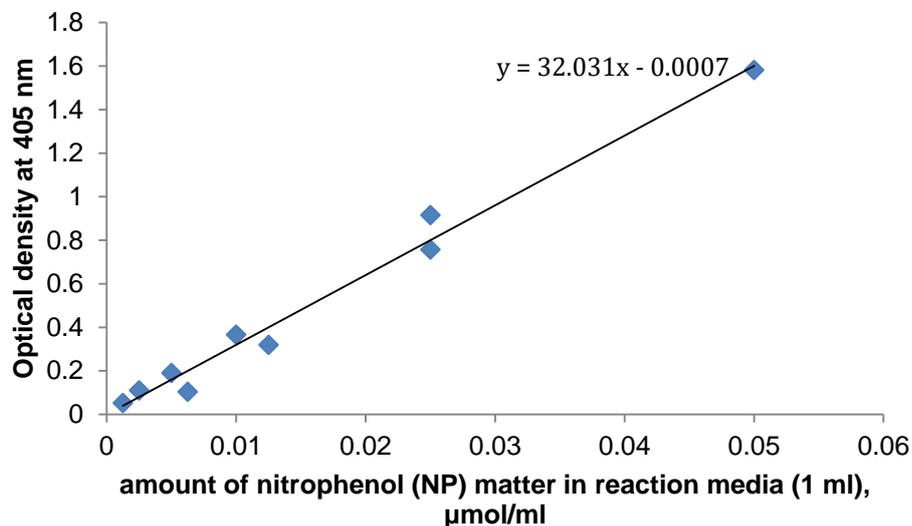


Figure 3. Calibration for different nitrophenol (NP) concentrations. Do not confuse the concentration and the amount of NP matter in reaction volume (see Table 1).

Notes

1. Experiments should be repeated three-four times for each sample.

- One unit of arabinofuranosidase activity corresponds to the amount of enzyme that liberates one μmol of NP per minute. Differences of glycosyl-hydrolase activities between samples can be defined by using the units of enzymatic activity per mg of total proteins. Exact formula for arabinofuranosidase activity calculation is given in Figure 4.

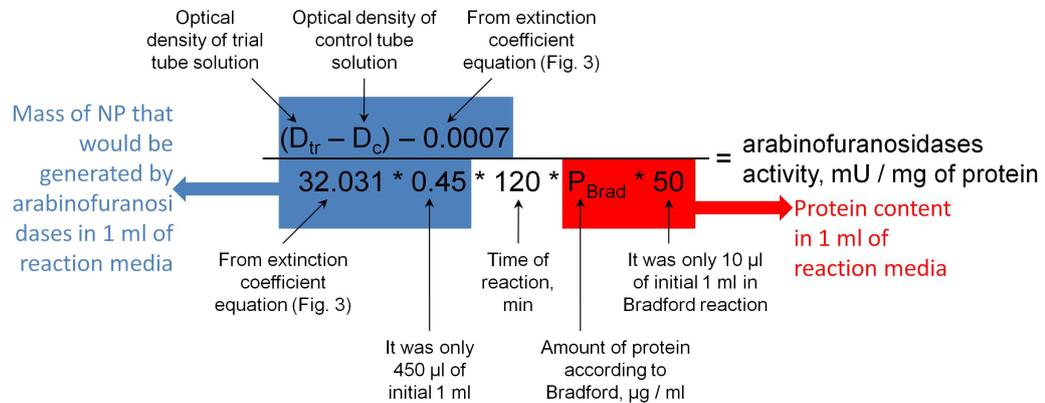


Figure 4. Formula for calculation of arabinofuranosidases activity. The result will be obtained in milliUnits of activity per mg of protein content.

- Described protocol with small modifications can be used for determination of any other glycosyl-hydrolase activity with synthetic aryl glycosides. Preliminary experiments may be useful for the optimization of incubation time and of plant material amount. However, the enzyme activity that is measured only by the rate of chromogenic substrate hydrolysis can remain the potential one and can be not realized in plant tissues (Fry, 2004). The aryl-glycoside hydrolysis coupled with the demonstration of final product presence is much more convincing. Thus, steps 10 and 11 were included in the protocol. If for any reason you cannot perform these stages, you may conclude only about the potential activity of arabinofuranosidases.

Recipes

Note: The recipes that are given below let to produce the amount of solutions that is sufficient for calibration and four repeating of the experiments with five samples in each replica.

- 5% (w/w) solution of NaOCl
Dissolve 25 g of NaOCl in 475 ml of water
- 0.03% (w/w) NaN_3
Dissolve 0.03 g of NaN_3 in 99.97 ml of water
- 50 mM NaOAc buffer (pH 6.0) with 3 mM of dithiothreitol on 0.03% (w/w) NaN_3
Dissolve 0.205 g of NaOAc and 0.0231 g of DL-dithiothreitol in 40 ml of 0.03% (w/w) NaN_3

Adjust the pH to 6.0 by the adding of glacial acetic acid

Adjust the volume to 50 ml by the adding of 0.03% (w/w) NaN₃

4. 10 mM 4NPA

Dissolve 0.0027 g of 4NPA in 0.03% (w/w) NaN₃

Adjust the volume of solution to 1 ml by additional 0.03% (w/w) NaN₃

5. 1 M Na₂CO₃

Dissolve 2.12 g of Na₂CO₃ in 0.03% (w/w) NaN₃

Adjust the volume of solution to 20 ml by additional 0.03% (w/w) NaN₃

6. Calibration solutions for NP

Dissolve 0.0139 g of NP in 1 ml of 0.03% (w/w) NaN₃ to produce 100 mM NP solution

Add 10 µl of 100 mM NP solution to 990 µl of 0.03% (w/w) NaN₃ to produce 1 mM NP solution

Prepare 9 concentrations of NP according to the table below, place 500 µl of each calibration solution to individual tubes and add 500 µl of 1M Na₂CO₃ to all.

Table 1. NP solution preparation

NP concentration, mM	Amount of NP matter in reaction volume (1 ml), µmol	Volume of 1 mM NP solution, µl	0.03% (w/w) NaN ₃ solution volume, µl
0.25	0.125	250	750
0.1	0.05	100	900
0.05	0.025	50	950
0.025	0.0125	25	975
0.02	0.01	20	980
0.0125	0.00625	12.5	987.5
0.01	0.005	10	990
0.005	0.0025	5	995
0.0025	0.00125	2.5	997.5

7. 50% (w/w) NaOH solution

HPAEC buffers should be prepared using template solution of NaOH

Add 5 ml of degasified MilliQ water (18 MΩ-cm) to 5 g of NaOH

8. 15 mM NaOH (buffer A for HPAEC)

Add 780 µl of 50% NaOH in 1,000 ml of degasified MilliQ water (18 MΩ-cm)

9. 1 M NaOAc in 0.1 M NaOH (buffer B for HPAEC)

Dissolve 82.05 g of NaOAc in degasified MilliQ water (18 MΩ-cm)

Adjust volume of solution to 1,000 ml by the adding of degasified MilliQ water (18 MΩ-cm)

Add 5.23 ml of 50% NaOH to NaOAc solution

10. Calibration solutions for arabinose

Dissolve 0.001 g of arabinose in 1 ml of MilliQ water to produce 10 mg/ml solution

Add 100 μ l of 10 mg/ml arabinose solution to 900 μ l of MilliQ water to obtain 1 mg/ml solution

Prepare 5 solutions of arabinose according to Table 2 below

Table 2. Arabinose solution preparation

Arabinose concentration, μ g/ml	Volume of 1 mg/ml arabinose solution, μ l	Volume of MilliQ, μ l
2	2	998
3	3	997
5	5	995
8	8	992
10	10	990

Acknowledgments

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