Plant Endo-β-mannanase Activity Assay

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[Abstract] Endo-β–mannanases in plants require post-translational modification, such as N-glycosylation and disulfide-linked dimerization, for their catalytic activity. Determination of the plant endo-β–mannanase activity needs to modify the assay conditions for optimizing their enzymatic reaction. Here, we describe a modified method for plant endo-β–mannanase assay. A high-salt buffer without thiol reductants is required for effective extraction of the enzyme. The enzyme is able to digest water-insoluble AZCL galactomannan to release water soluble dyed fragments, which is detected through measurement of absorbance at 590 nm wavelength. Increase in absorbance at 590 nm is correlated directly with enzyme activity.

Materials and Reagents

1. Liquid nitrogen
2. BCA Reagent (Tiangen Biotech, catalog number: PA115-01)
3. Bovine serum albumin
4. AZC L-galactomannan (Megazyme, catalog number: I-AZGMA)
5. Commercial *Aspergillus niger* endo-β-mannanase (Megazyme, catalog number: E-BMANN)
6. 100 mM phenylmethanesulfonyl fluoride (PMSF) (see Recipes)
7. 0.5 M ethylene diaminete traacetic acid (EDTA) (see Recipes)
8. Extraction buffer (see Recipes)
9. 0.1 M sodium acetate buffer (pH 5.0) (see Recipes)

Equipment

1. Mortar and pestle
2. 10,000 M_r cut-off filter (EMD Millipore, catalog number: UFC801096)
3. Centrifuge
4. Incubator shaker
5. Water bath

6. Microplate reader or Spectrophotometer

Procedure

1. Samples (~ 10 g developing xylem or leaves from one-year-old poplar) are ground in liquid nitrogen to a fine powder and homogenized at 4 °C in 1.5-volume fold of extraction buffer for 1 h (strong enzymatic activity can be detected in developing xylem).

2. The homogenate is centrifuged at 10,000 $x g$ for 30 min at 4 °C.

3. The supernatant is then passed through a 10,000 M_r cut-off filter and dehydrated to < 500 µl, then the protein is diluted to ~1 µg/µl in 0.1 M sodium acetate buffer (pH 5.0) at 4 °C.

4. The protein extraction is measured by BCA Reagent using bovine serum albumin as a standard.

5. 200 µl of reaction mixture containing 100 µl of 1% AZC L-galactomannan (w/v, in 0.1 M sodium acetate buffer, pH 5.0) and 20 µg of extracted proteins or heated inactive proteins (100 °C 10 min, as control) is incubated at 40 °C for 2 h with continuous shake.

6. The reaction mixture is boiled at 100 °C for 5 min and centrifuged at 12,000 $x g$ for 5 min.

7. The absorbance (A) of the supernatant at 590 nm is determined. The background values (A_0) obtained using heated inactive proteins are subtracted from values (A_1) obtained using active extract ($A = A_1 - A_0$).

8. Standardization

   a. Enzyme activity of a serial dilutions of a commercial *Aspergillus niger* endo-β-mannanase (E-BMANN, Megazyme) is determined under the conditions: 200 µl of reaction mixture containing 100 µl of 1% AZC L-galactomannan and E-BMANN is incubated at 40 °C for 2 h.

   b. A standard curve correlated with E-BMANN activity is shown in Figure 1. For absorbance values in a range of 0.05~0.9, these values can be calculated by reference to the equation: $Y = SX + C$. Where:

      Y = endo-β-Mannanase activity (in micro-Units/assay, *i.e.* per 200 µl)

      S = Slope of the calibration graph

      X = Absorbance of the reaction at 590 nm (A)

      C = Intercept on the Y-axis

   c. According to the manufacturer's instruction, one Unit of activity is defined as the amount of enzyme required to release one micromole of mannose reducing-sugar equivalents per minute under the defined assay conditions (1 micro-Unit = 1 pmol/min).

9. Calculation of enzyme activity: endo-β-Mannanase activity in the sample is determined by reference to the standard curve to convert absorbance values to micro-Units per assay (Y),

then further to micro-Units per µg protein (Y/[20 µg protein], pmol/min/[µg protein]).
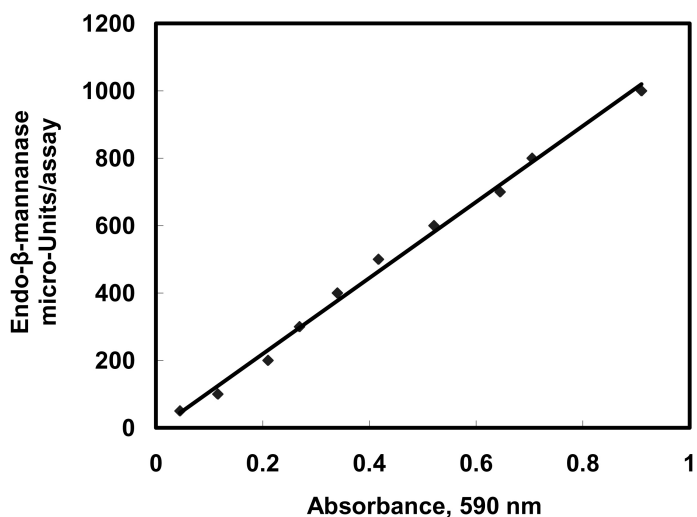

Figure 1. Endo-β-mannanase standard curve on the commercial endo-β-mannanase

Recipes

1. 100 mM Phenylmethanesulfonyl fluoride (PMSF) 10 ml
    Mix 0.174 g of PMSF with 10 ml isopropanol
    Store in small aliquots at -20 °C

2. 0.5 M Ethylene diamine tetraacetic acid (EDTA) (pH 8.0) (1 L)
    Dissolve 186.1 g EDTA-Na·2H$_2$O in 800 ml dH$_2$O
    Adjust pH to 8.0 with NaOH (~20 g NaOH particles)
    Add dH$_2$O to 1 L
    Autoclave at 121 °C for 20 min
    Store at RT

3. Extraction buffer (1 L)
    1 M sodium acetate buffer (pH 5.0)
    10 mM EDTA
    10 mM sodium azide
    3 mM PMSF
    Mix 57 ml glacial acetic acid (1.05 g/ml) and 20 ml 0.5 M EDTA with 800 ml dH$_2$O
    Adjust pH to 5.0 with NaOH
    Add 0.65 g sodium azide
    Add dH$_2$O to 1 L
    Add 30 µl of 100 mM PMSF per ml extraction buffer immediately before use

Note: Do not add the sodium azide until pH is adjusted. Acidification of sodium azide will release a poisonous gas.

4. 0.1 M sodium acetate buffer (pH 5.0)

   Mix 5.7 ml glacial acetic acid (1.05 g/ml) with 800 ml dH$_2$O

   Adjust pH to 5.0 with NaOH

   Add 0.65 g sodium azide

   Add dH$_2$O to 1 L

Acknowledgments

References

1. Zhao, Y., Song, D., Sun, J. and Li, L. (2013). Populus endo-beta-mannanase PtrMAN6 plays a role in coordinating cell wall remodeling with suppression of secondary wall thickening through generation of oligosaccharide signals. *Plant J* 74(3): 473-485.