Spectrophotometric assay for measuring mannitol oxidase activity

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Abstract

Mannitol oxidase is an enzyme present in some gastropods, converting the polyalcohol mannitol into the sugar mannose. Molecular oxygen is the hydrogen acceptor and hydrogen peroxide is produced. Therefore, this enzymatic activity can be measured by monitoring H_2O_2 production using a horseradish peroxidase coupled assay adapted from previously published methods (Malik et al., 1987; Cablé et al., 1993; Rocha et al., 2003). This assay was used to evaluate mannitol oxidase activity in the digestive gland of gastropods, but in some slugs and snails this enzyme was also detected in digestive tract tissues (Vorhaben et al., 1984; Malik et al., 1987). This enzyme can also oxidase other polyalcohols (Large & Connock, 1994)

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Before start

Prepare the following solutions:

Homogenization medium for marine gastropods

(adapted from Stewart et al. 1994 and Moyers et al. 1991)

Sucrose - 500 mM

Potassium chloride - 150 mM

Ethylenediamine tetraacetic acid (EDTA) sodium salt - 1 mM

Tris-HCl 50 mM pH 7.4

This medium, without phenylmethylsulfonyl fluoride (PMSF), can be stored at -20º C in aliquots. To avoid the inactivation of PMSF in *aqueous solution,* add 5 µl of 200 mM PMSF (in absolute ethanol) per ml of medium just before use (final PMSF concentration 1 mM).

Good results were also obtained with freshwater and terrestrial gastropods using this high osmolarity

medium.

Incubation medium

Phenol - 1.060 mM

4-aminoantipyrine - 0.082 mM

Horseradish peroxidase (» 200 U/mg) - 5 U/ml

Bovine serum albumin - 0.06 % (w/v)

Mannitol (or other polyalcohol) - 25 mM

Potassium phosphate 50 mM pH 7.4

This medium can be stored frozen (-20º C) in aliquots without loss of activity.

In control medium the substrate is omitted.

Materials

- Potter-Elvehjem homogenizer by Contributed by users
- Bath sonicator by Contributed by users
- Centrifuge by Contributed by users
- Double-bean UV-visible spectrophotometer, with temperature control in the cuvette compartment by Contributed by users

Protocol

Sample preparation

Step 1.

1. Homogenize approximately 0.05-0.06 g of digestive gland tissue per ml of cold homogenization medium using a Potter-Elvehjem homogenizer at 1,000 rpm, keeping the tube in ice.
2. Register the exact weight of digestive gland tissue used and the final homogenate volume.
3. Sonicate the homogenate during 3 x 15 s using a bath sonicator, keeping the tube in an ice-water bath.
4. Centrifuge the homogenate at 1,000 g for 5 min at 4º C, and use the supernatant to assess mannitol oxidase activity. Keep samples on ice and use immediately after preparation.

Sample preparation

Step 2.

1. In a tube, mix 960 µl of incubation medium with 40 µl of sample (sample dilution factor in the

assay: 25), agitate with a vortex, and pour into the spectrophotometer cuvette.
2. Measure the increase of absorbance at 500 nm, at 25º C, during 3-5 min (or longer for samples with very low enzyme activity). Make sure that activity is linear in time and proportional to sample concentration. If not, adjust sample dilution.
3. For control, monitor non-specific increases of absorbance at 500 nm using medium without substrate, and subtract if necessary.

Construction of a calibration line with H2O2 standards

Step 3.

1. Prepare a standard solution (≈1 mM) by diluting 1/10,000 a 30% H_2O_2 stock solution (≈10 M).
2. Measure the absorbance at 240 nm, and calculate the exact concentration of the standard solution using the extinction coefficient of H_2O_2 at 240 nm (ε = 43.6 M^{-1}. cm^{-1}).
3. Repeat the assay used for mannitol oxidase activity, using increasing amounts of H_2O_2 as indicated in the following table:

	µl per tube					
Incubation medium	900	900	900	900	900	900
H_2O	100	80	60	40	20	0
H_2O_2 standard solution	0	20	40	60	80	100
Concentration of H_2O_2 in the assay (nmol . ml^{-1})	0					
Absorbance at 500 nm	0					

Use the absorbance values and the corresponding H_2O_2 concentrations to obtain a calibration line and its equation.

Determination of enzyme activity

Step 4.

Use the equation of the calibration line to calculate the production of H_2O_2 per min.

Activity (nmol . ml^{-1}. min^{-1}) = Δ [H_2O_2] per min **x** 25*

* sample dilution factor in the assay

Calculate the activity per g of tissue (nmol . g^{-1}. min^{-1}) or per mg of protein (nmol . mg^{-1}. min^{-1}).

References

Step 5.

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