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SHORT COMMUNICATION

A FLUOROMETRIC PROCEDURE FOR DETERMINATION OF β-GLUCOSIDASE*

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Abstract—A rapid fluorometric technique has been developed for the measurement of β-glucosidase activity. The change in concentration of a non-fluorescent substrate such as 4-methylumbelliferone β-D-glucoside is measured directly by recording increases in fluorescence as the aglycone is produced. Sweet clover, corn leaf and almond emulsin were used as enzyme sources in the development of the method which resulted in good linearity in Lineweaver–Burk determinations.

INTRODUCTION

β-GLUCOSIDASE (β-D-glucoside glucohydrolase) catalyzes the hydrolysis of phenolic glucosides to their corresponding aglycones.[1] These free phenolics, which are produced after cellular disruption, are highly toxic[2] to microorganisms and are believed to play a role in disease resistance.[3–5]

There are several methods reported in the literature for the assay of β-glucosidase activity. These include colorimetric techniques which involve the use of salicin,[6] dinitrophenyl[7] and bromonaphthyl[8] glucosides; all require several steps and lack sensitivity. Fluorometric methods have been developed[9,10] but they require periods of incubation, development of fluorescence at high pH, and they do not measure the initial reaction velocity. The fluorometric procedure described herein directly measures fluorescence as the reaction proceeds, hence allowing continuous monitoring of the reaction. In this assay the change in fluorescence as the reaction proceeds is recorded against time. The free aglycone is highly fluorescent, while the glycosidic linkage with 4-methylumbelliferone gives only a slight fluorescence.[11] The amount of substrate hydrolyzed is then directly determined from a standard curve.

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METHOD

4-Methylumbelliferone β-D-glucoside (Sigma Chemical Co.) at 0·1 μmol/ml in 0·1 M acetate buffer, pH 5·0, was used as the stock substrate solution. The reaction mixture contained the following: 0·01–0·05 μmol of glucoside, 0·10–0·50 ml of enzyme extract, and 0·1 M acetate buffer pH 5·0 to a total vol. of 4 ml. Enzyme extracts were prepared from almond emulsin (Nutritional Biochemicals) and acetone powders of corn and sweet clover leaves. A standard curve of fluorescence vs. concentration was made from a 0·001 μmol/ml solution of 4-methylumbelliferone (Nutritional Biochemicals).

Measurements were made with a Turner 111 fluorometer equipped with a water-cooled door maintained at ambient temperature, and a Beckman recorder. A long wavelength, 366 nm, light source was used with a 7–60 primary filter and 2A-47B secondary filter. Sensitivity was set at 1x. Neutral density filters of 10 and 40% were used to lower the sensitivity.

RESULTS AND DISCUSSION

The increase in fluorescence after the addition of the enzyme remains linear for the length of the assay. A typical recorder trace of the assay is seen in Fig. 1. The enzyme activity is expressed as μmol of umbelliferone glucoside hydrolyzed per min by using the standard curve.

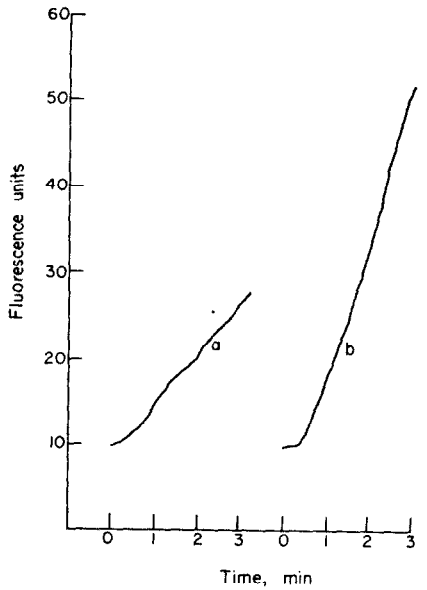

FIG. 1. RECORDINGS OF β-GLUCOSIDASE ACTIVITY FROM CORN.
Trace *a* is of 0·1 ml enzyme added to 0·1 μmol 4-methylumbelliferone glucoside at $T = 0$. Trace *b* is with 0·5 ml enzyme.

Early work utilizing umbelliferone conjugates necessitated that fresh solutions be prepared daily, due to high amounts of spontaneous hydrolysis. The non-enzymatic hydrolysis of the commercially available glucoside was therefore monitored to determine its stability. The fluorescence of a glucoside solution with a concentration of 0·05 μmol in 4 ml of 0·01 M acetate buffer, pH 5·0 was measured for 60 min. Under the conditions of

the assay, the substrate hydrolyzes at the rate of $0{\cdot}50 \times 10^{-6}$ μmol/min. Since this rate accounts for 0·091 fluorescence units/min the spontaneous hydrolysis can be considered negligible. When stored in the refrigerator, a stock solution can be kept for several weeks.

This fluorometric technique gives good linearity between enzyme concentration and activity for a fixed substrate concentration. Results from this procedure also indicated linearity in Lineweaver-Burk plots and consistent K_m values. With two levels of almond emulsin glucosidase, the K_m was determined to be $2{\cdot}88 \times 10^{-4}$ M. This is similar to the value of $2{\cdot}40 \times 10^{-4}$ M obtained by Price and Robinson who used dinitrophenyl glucoside as the substrate.[12] Other K_m values of $1{\cdot}25 \times 10^{-4}$ M and $6{\cdot}66 \times 10^{-4}$ M were obtained for corn leaf and sweet clover tissues.

EXPERIMENTAL

Acetone powders were prepared from corn leaves and sweet clover leaves and stored in a dessicator at −15°. Crude enzyme extracts were prepared by homogenizing 1 mg of powder/ml of ice-cold 0·01 M acetate buffer, pH 5·0. This homogenate was centrifuged at 20 000 *g* for 10 min at 0° and the supernatant was used as the enzyme solution.

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Key Word Index—β-Glucosidase; estimation; fluorometric procedure; 4-methylumbelliferone β-D-glucoside.