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High-Throughput Microtiter Plate-Based Chromogenic Assays for Glycosidase Inhibitors

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ABSTRACT

Rapid microtiter plate-based colorimetric assays have been developed that allow the screening of large numbers of samples for the presence of inhibitors of α-glucosidase, α-amylase, and β-galactosidase. The assays are particularly useful for screening large numbers of microbial culture filtrates.

Index Entries: Enzyme inhibitors; α-glucosidase; α-amylase; β-galactosidase.

INTRODUCTION

Glycosidase inhibitors have considerable potential for the treatment of human disease. For example, glycosidase inhibitors, particularly a α-glucosidase, α-amylase, and β-galactosidase, have potential in the management of carbohydrate imbalance diseases, such as diabetes (*1*), certain forms of hyperlipoproteinemia (*2*), and in the treatment of obesity (*3*). Since sucrose and starch are the primary saccharides consumed by humans, inhibitors of α-glucosidases would be particularly useful in the management of hyperglycemia (*4*). Glycosidase inhibitors also have potential uses in regulating glycoprotein processing in the cell. The variability in

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the carbohydrate portion of therapeutic glycoproteins has caused problems in specifically defining the therapeutic agent to the satisfaction of regulatory authorities. The use of glycosidase inhibitors could result in a population of glycoprotein molecules with identical carbohydrate moieties (*5*). Glycosidase inhibitors are also being investigated for the treatment of both cancer (*6*) and AIDS (*7*). To date, a number of low-molecular-weight glycosidase inhibitors have been identified as a result of screens of microbial culture supernatants. These include Acarbose (*8*), an α-glucosidase inhibitor produced by the Actinoplanes; Tendamistat (*9*), Trestatin (*10*), and Oligostatin (*11*), all inhibitors of α-amylase; Nojirimycin (*12*) and 1-deoxy-nojirimycin (*12*), which are inhibitors of glucoamylase; Pyridinolol (*13*) and a series of isoflavinoids (*14*), which are inhibitors of β-galactosidase; Panosialin (*15*), an inhibitor of viral neuraminidase; and Siastatins A and B (*16*), inhibitors of viral, bacterial, and mammalian neuraminidases. A comprehensive review of glycosidase inhibitors and their uses can be found in ref (*17*). We describe here rapid microtiter plate-based assays for screening for inhibitors of α-amylase, α-glucosidase, or β-galactosidase. We have successfully used these assay to screen algal extracts and culture supernatants for the presence of glycosidase inhibitors and to monitor the purification of such inhibitors (*18*).

MATERIALS

All methods require the availability of a microtiter plate reader.

α-Amylase Assay

1. Assay buffer: 0.5*M* sodium citrate buffer, pH 7.1, 0.5*M* NaCl, and 15 m*M* $MgCl_2$.
2. α-Amylase (Sigma, Dorset, U.K. Type 1-A, ex-porcine pancreas): 0.16 U/mL in 0.1*M* sodium citrate buffer, pH 7.1, 50 m*M* NaCl, and 3 m*M* $MgCl_2$.
3. Substrate solution: Prepared from α-amylase PNP kit, C system (Boehringer Mannheim, Sussex, U.K.). Dissolve one tablet in 16 mL of 0.1*M* sodium citrate buffer, pH 7.1, 0.5*M* NaCl, and 3 m*M* $MgCl_2$. (*See* Note 1.)

β-Galactosidase Assay

1. Assay buffer: 0.5*M* sodium citrate buffer, pH 7.3, 5 m*M* β-mercaptoethanol, and 9 m*M* $MgCl_2$.
2. β-galactosidase (Sigma, ex-*Escherichia coli*): 0.017 U/mL in 01.*M* sodium citrate buffer, pH 7.3, 1 m*M* β mercaptoethanol, and 1.8 m*M* $MgCl_2$.
3. Substrate solution: *p*-nitrophenyl-β-D-galactopyranoside, 1.5 mg/mL in 0.1*M* sodium citrate buffer, pH 7.3, 1 m*M* β-mercaptoethanol, and 1.8 m*M* $MgCl_2$.

α-Glucosidase Assay

1. Assay buffer: 0.5*M* sodium citrate buffer, pH 6.8, and 3 m*M* $MgCl_2$.
2. α-Glucosidase (Sigma, Dorset, U.K. Type III ex-yeast): 0.1 U/mL in 0.1*M* sodium citrate buffer, pH 6.8, and 3 m*M* $MgCl_2$.
3. Substrate solution: *p*-nitrophenyl-α-D-glucopyranoside, 0.5 mg/mL in 0.1*M* sodium citrate buffer, pH 6.8, and 3 m*M* $MgCl_2$.

METHODS

α-Amylase Assay

The assay is based on the cleavage of the substrate by α-amylase to release *p*-nitrophenyl maltotrioside. α-Glucosidase, which is present with the substrate, cleaves this product to release *p*-nitrophenol, which is detected by its absorbance at 410 nm.

1. For each sample to be tested, add the following to a microtiter plate well (*see* Note 2).
   A. Assay buffer (50 μL) (*see* Note 3).
   B. Aqueous test sample (100 μL) (*see* Note 4).
   C. Enzyme solution (50 μL).
2. Incubate the plate at 37°C for 30 min (*see* Note 5).
3. To each well, add 50 μL of substrate solution. Using a microtiter plate reader, determine the A_{410} of each well (time zero).
4. Incubate the plate at 37°C for 1 h or at room temperature for 2 h. Then determine the A_{410} for each well.
5. Determine the increase in absorbance from time zero for each well (*see* Note 6).
6. Since the assay involves the use of both α-amylase and α-glucosidase, a positive result could be owing to the inhibition of either enzyme. It is therefore necessary to ensure that the sample does not inhibit the α-glucosidase assay (Methods: α-Glucosidase Assay), thus confirming that you have an α-amylase inhibitor.

β-Galactosidase Assay

The assay is based on the cleavage of a β-1,4 glycosidic bond in the substrate to release *p*-nitrophenol, which is detected by its absorbance at 410 nm.

1. For each sample to be tested, add the following to a microtiter plate well (*see* Note 2).
   A. Assay buffer (25 μL) (*see* Note 3).
   B. Aqueous test sample (100 μL) (*see* Note 4).
   C. Enzyme solution (50 μL).

2. Incubate the plate at 37°C for 30 min (*see* Note 5).
3. To each well, add 50 µL of substrate solution. Using a microtiter plate reader, determine the A_{410} of each well (time zero).
4. Incubate the plate at 37°C for 1 h or at room temperature for 2 h. Then determine the A_{410} for each well.
5. Determine the increase in absorbance from time zero for each well (*see* Note 6).

α-Glucosidase Assay

The assay is based on the cleavage of an α-1,6 glycosidic bond in the substrate to release *p*-nitrophenol, which is detected by its absorbance at 410 nm.

1. For each sample to be tested, add the following to a microtiter plate well (*see* Note 2).
   A. Assay buffer (50 µL) (*see* Note 3).
   B. Aqueous test sample (100 µL) (*see* Note 4).
   C. Enzyme solution (50 µL).
2. Incubate the plate at 37°C for 30 min (*see* Note 5).
3. To each well, add 50 µL of substrate solution. Using a microtiter plate reader, determine the A_{410} of each well (time zero).
4. Incubate the plate at 37°C for 1 h or at room temperature for 2 h. Then determine the A_{410} for each well.
5. Determine the increase in absorbance from time zero for each well (*see* Note 6).

NOTES

1. The substrate solution contains *p*-nitrophenyl-α-D-maltoheptaoside (0.8 mg/mL) and α-glucosidase (2 U/mL). In the absence of the substrate kit, the substrate solution may be prepared from the individual components. The principle of the assay is described in Methods: α-Amylase Assay.
2. Obviously, control samples need to be run on each plate. If culture supernatants are being studied, then culture medium should be used in place of the test sample. Since in any screening program the majority of samples being tested are negative for inhibition, these negative samples should also give the same readings as the control samples.
3. For solutions that are to be added to each well on the plate, i.e., buffer, enzyme, and substrate solutions, these are most conveniently added using a multichannel pipet.
4. The assays described here can also be used to test organic solvent extracts as long as the solvents are water miscible, do not

react with the plastic of the microtiter plate, and do not significantly inactivate the enzyme. For each particular solvent, it is therefore necessary to find the maximum volume that the assay will tolerate. We have routinely studied methanol extracts of microorganisms using these assays. For each of the assays described here, we have found that the 100 μL aqueous test sample can be replaced by 100 μL of 30% methanol in the α-glucosidase and α-amylase assays, and 100 μL of methanol in the β-galactosidase assay.

5. The preincubation step is included to allow identification of noncompetitive inhibitors, e.g., compounds binding at allosteric sites on the enzyme. Such an inhibition will be time-dependent, hence, the preincubation step. However, if an inhibitor is a competitive one, then this step is unnecessary. When using this assay to monitor the purification of an inhibitor, it is therefore advisable to determine whether this preincubation step is necessary for a particular inhibitor, since elimination of this incubation step will save time.
6. Most wells should give readings in the region of 0.4 absorbance units. However, wells containing inhibitors should show zero or very little increase in absorbance.
7. These assays are particularly useful for the rapid monitoring of fractions generated during inhibitor purification.
8. These assays can also be used to screen for the *presence* of enzyme rather than inhibitors. Simply delete the enzyme from the assay mixture and delete the 30-min preincubation step. The presence of enzyme in the aqueous test sample is indicated by an increase in the A_{410} in a particular well.

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