Biotechnology Letters **24:** 1277–1280, 2002.
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Development of a coupled enzyme assay for the measurement of alternanase activity**,***

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Received 21 December 2001; Revisions requested 8 January 2002; Revisions received 22 May 2002; Accepted 23 May 2002

Key words: alternan, alternanase, coupled enzyme assay, cyclic tetrasaccharide, panose

Abstract

Alternanase, an endoglucanase that hydrolyzes the bacterial exopolysaccharide alternan, will also hydrolyze the trisaccharide, panose, to produce glucose and a disaccharide that can be formed into a novel, cyclic tetrasaccharide. The glucose can then be selectively and quantitatively measured by enzyme-based reaction which forms the basis of a coupled enzyme assay to quantitate alternanase activity. By this method a preparation of alternanase purified by affinity chromatography on immobilized isomaltose had a maximum reaction rate (V_{max}) of 0.75 μmol glucose min^{-1} and a K_m of 34 mM for panose. Two competitive inhibitors of alternanase activity were also evaluated using this coupled enzyme assay: isomaltose had a K_i of 94 mM while the cyclic tetrasaccharide had a K_i of 66 mM.

Introduction

Alternanase is an endoglucanase that hydrolyzes the α-D-Glc*p*-(1→3), α-D-Glc*p*-(1→6) linkage sequence of the bacterial exopolysaccharide alternan (Biely *et al.* 1994, Côté & Ahlgren 2001). The main products of this enzymatic hydrolysis include isomaltose, the trisaccharide α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc and the cyclic tetrasaccharide *cyclo* {→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (Côté & Biely 1994). The nature of the products of the hydrolysis of alternan have complicated the development of a convenient assay method that can be used to quantitatively determine the specific activity of this enzyme. For instance, an assay based on measuring the increase in reducing sugar formed by the hydrolysis of alternan will significantly underestimate alternanase activity due to the fact that about half of the total product is the non-reducing cyclic tetrasaccharide. This method, while convenient for some qualitative analyses, is not ideal for determining enzymatic parameters such as maximum reaction velocity because the stoichiometry of the products is not consistent and reaction rates may be variable relative to the size of the alternan. An alternate method involves Remazol Brilliant Blue-conjugated alternan (RBB-alternan) (Rinderknecht *et al.* 1967, Wyckoff *et al.* 1996). With this substrate it is possible to estimate the activity of alternan by measuring the formation of ethanol-soluble oligosaccharide fragments after precipitation of the remaining unhydrolyzed RBB-alternan. One drawback to this method is that the RBB-alternan does not always have a consistent degree of bound dye per synthesis, which leads to inconsistencies between preparations. Furthermore, the release of soluble dyed fragments is not readily convertible to standard units of activity. We have also made radiolabeled alternan by using the enzyme alternansucrase to polymerize [^{14}C]glucose derived from [^{14}C]sucrose (Côté &

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Robyt 1987), which after purification can serve as a substrate for alternanase. However this method actually measures the fraction of polysaccharide which remains insoluble in methanol by adhering to a piece of filter paper, and it is hard to calibrate this method because small oligosaccharides have intermediate solubility in this method. Typically the results obtained from this procedure are derived from relatively small changes in large amounts of radioactivity, and also the correlation of radioactivity measured to the actual number of bonds hydrolyzed is problematic. Alternanase will hydrolyze 4-nitrophenyl α-isomaltoside to isomaltose and 4-nitrophenol, the latter being quantitatable spectrophotometrically at 415 nm (Côté & Ahlgren 2001). But this substrate is not commercially available, has low solubility (below the measured K_m for this compound), and the overall reaction is very slow, which taken together limit the usefulness of this compound for this application.

The previous observation that the enzyme alternanase would hydrolyze 2 mol of the trisaccharide, panose {α-D-Glc$p$-(1→6)-α-D-Glc$p$-(1→4)-α-D-Glc} to form 2 mol of glucose and the cyclic tetrasaccharide *cyclo* {→6)-α-D-Glc$p$-(1→3)-α-D-Glc$p$-(1→6)-α-D-Glc$p$-(1→3)-α-D-Glc$p$-(1→} (Côté & Ahlgren 2001) led to the idea that an assay that would specifically quantitate glucose could be used to indirectly measure the activity of the enzyme alternanase in a coupled reaction scheme. This report describes the use of a commercially available method to quantitate glucose, based on the action of hexokinase coupled with glucose-6-phosphate dehydrogenase in a reaction that produces NADH, in a coupled enzyme assay to measure the enzymatic activity of alternanase.

Experimental

Enzymes

Alternanase was purified from crude, cell-free concentrated supernatant from 3 day old cultures of *Bacillus* sp. NRRL B-21195 using affinity chromatography on immobilized isomaltose (JA Ahlgren & GL Côté, submitted). The preparation used in the reactions described here contained 0.5 mg ml^{-1} protein in 50 mM MOPS, pH 7, containing 1 mM $CaCl_2$ and 1.5 mM NaN_3.

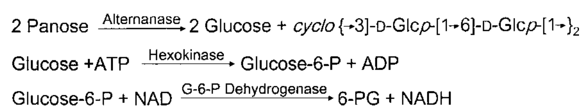

Fig. 1. Reaction scheme for the measurement of alternanase activity using panose by coupled enzyme assay with hexokinase. Panose is α-D-Glc$p$-(1→6)-α-D-Glc$p$-(1→4)-α-D-Glc.

Materials

Panose, glucose, isomaltose, immobilized isomaltose affinity chromatography resin, and the hexokinase-based glucose determination kit were purchased from Sigma-Aldrich Chemical company (St. Louis, MO). The cyclic tetrasaccharide *cyclo* {→6)-α-D-Glc$p$-(1→3)-α-D-Glc$p$-(1→6)-α-D-Glc$p$-(1→3)-α-D-Glc$p$-(1→} ($cGlc_4$) was prepared by hydrolyzing native alternan from *Leuconostoc mesenteroides* NRRL B-21297 with alternanase and purified as described (Côté & Biely 1994). All other chemicals were reagent grade or better.

Enzyme assays

Mixtures of alternanase (5 μg total protein) and panose (0–178 mM) contained in 100 μl in 0.5 ml capped plastic microfuge tubes were incubated at 45 °C. A 10 μl sample was removed immediately after the reactants were combined and thereafter every 5 min for 20 min. To each sample in a 2-ml capped plastic microfuge tube in a wet ice bath, 1.5 ml of the reconstituted hexokinase reagent (as specified by the manufacturer) was added and the mixture incubated a minimum of an additional 5 min at ambient temperature before measuring the absorbance of the solution at 340 nm. A series of incremental glucose concentrations were used to calibrate the assay method under the same conditions used for the alternanase plus panose reactions. A blank reaction consisted of 10 μl of the 50 mM MOPS buffer alone. Control reactions containing an equivalent volume of alternanase alone as well as panose alone were also prepared; the absorbance values obtained from these reactions were subtracted from the absorbance values for the complete reaction mixtures in order to determine the reaction velocity.

Protein concentration determinations

The protein concentration of alternanase solutions was determined by the bicinchoninic acid protein assay method using bovine serum albumin as a reference (Pierce Chemical Company, Rockford, IL).

Results and discussion

The coupled-enzyme assay reaction scheme (Figure 1) that measures the enzymatic activity of alternanase preparations is composed of two steps: in the first step the enzyme alternanase releases one mole of glucose from each mole of panose. This is followed by a second step that selectively and quantitatively measures the glucose concentration in solution. Therefore a unit of alternanase activity is defined as the amount of enzyme which produces a micromole of glucose from panose per minute at 45 °C. Two enzyme-based methods were evaluated for the quantitation of glucose. One is based on a hexokinase-coupled reaction and the second scheme uses a glucose oxidase-based reaction (data not shown). The advantage of using the hexokinase-based coupled assay over the glucose oxidase based assay is that the latter assay requires adhering to a 20-min incubation period for the glucose determination phase of the method, because the absorbance value continues to change slowly over time. The absorbance value from the glucose quantitation phase of the hexokinase coupled assay reaches a steady value after 5 min, which remains constant for approx. 1 h, which greatly simplifies matters when handling a large number of samples. Both methods otherwise yielded identical results.

Preliminary assays showed that it was not necessary to inactivate the alternanase after the first step of the process as long as the reaction was chilled to 0 °C or below after the desired incubation at 45 °C; control reactions held for more than 1 h at 0 °C showed no increase in glucose levels over those processed immediately. The 10 μl reaction volume was diluted to 1.5 ml for the second step of the reaction, conducted at room temperature, which diluted any residual panose by more than 150-fold so that it would not likely continue to be hydrolyzed by alternanase to any significant extent during the glucose quantitation step. The analysis of the assays showed that 5 μg purified alternanase per 100 μl reaction was suitable, as judged by yielding linear results, over a wide range of panose concentrations at 45 °C for at least 20 min. Plots were made of absorbance versus time, and the slope of the line determined for each concentration of panose tested. The slope of the line was then converted from change in absorbance units per minute to μmol glucose formed per minute. As seen in Figure 2, a double reciprocal plot of the data yielded a straight line and the values for the maximum reaction velocity (V_{max}) and Michaelis–Menton constant (K_m) can be readily

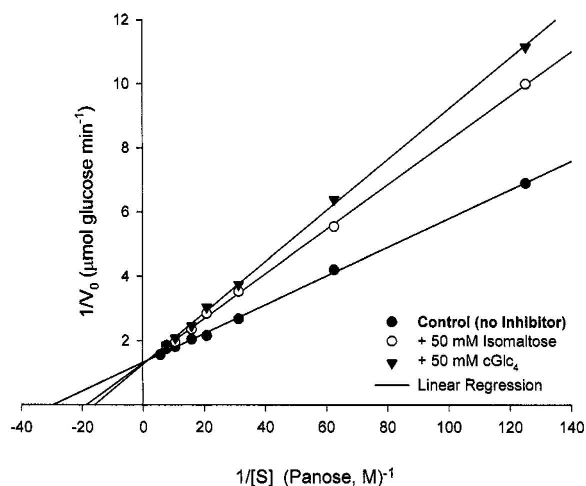

Fig. 2. Effect of panose concentration on the rate of reaction of alternanase; (●) control (no inhibitor); (○) with 50 mM isomaltose; (▼) with 50 mM cGlc$_4$.

calculated. A V_{max} value of 0.75 μmol glucose min^{-1} at 45 °C and a K_m value of 34 mM panose were derived for this preparation of alternanase. The actual highest rate measured, 0.65 μmol glucose min^{-1} when assayed with 178 mM panose for 20 min, consumed 73% of the available panose in the reaction, so for routine assay work it would be preferable to have a higher concentration of panose to maintain enzyme saturation. A starting concentration of 0.3 M panose would be reasonable, which in this case would consume only 50% of available panose at a V_{max} of 0.75 μmol glucose min^{-1} in a 20-min single time point assay.

The coupled reaction method is also useful for quantitating the effect of adding inhibitors of alternanase activity, such as isomaltose or cGlc$_4$, both of which are reaction products. In the case of isomaltose, it would not be possible to quantitate these kinetic parameters based on a reducing sugar assay because it itself is a reducing sugar. By adding a constant amount of inhibitor to a series of panose-alternanase reactions identical to those used to measure the V_{max} of the uninhibited reaction, the inhibitor constant (K_i) as well as the nature of the inhibition can be determined. As seen in Figure 2, a double-reciprocal plot of the results of reactions with and without 50 mM isomaltose showed that the V_{max} of the reaction did not change (same intercept on the y-axis) but the apparent K_m for the reaction was changed, indicating that the inhibition was competitive. The K_i value for isomaltose can be derived from the x-axis intercept and

was determined to be 94 mM. A similar experiment was performed with 50 mM $cGlc_4$, which also showed competitive inhibition of alternanase, and had a K_i value of 66 mM.

Based on these results we conclude that the coupled assay utilizing panose as the substrate and monitoring the formation of glucose provides a reliable and convenient method to quantitate alternanase activity. As more research on alternanase is carried out, such as improved production processes including cloning and potential overexpression to increase yields of the enzyme, a method involving commercially available components that provides consistent and reproducible results for the measurement of alternanase activity will be very useful and appreciated.

Acknowledgements

The authors thank Drs Terence R. Whitehead and Lubomir Kremnicky for their insightful discussions on assay development and Mr James J. Nicholson for his technical assistance.

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