Note

Rapid Assay of Glucoamylase Using a Fluorescence-labeled Glucoamylase Inhibitor, Acarbose

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Glucoamylase (α-1,4-glucan glucohydrolase EC 3.2.1.3) hydrolyzing starch to yield glucose is an important enzyme in Japanese fermentation industries, such as sake, shoyu, and miso manufacturing. In sake brewing, glucoamylase is produced by Aspergillus oryzae in sake-koji, which is a solid culture of the fungus on steamed rice. In sake brewing, the fungal glucoamylase is important in controlling the rate of alcohol fermentation by yeast. Therefore, every lot of rice-koji has to be examined for its glucoamylase activity.

Usually, glucoamylase activity was assayed by measuring the rate of release of glucose from soluble starch, using the Somogyi-Nelson method or the glucose oxidase method. However, in routine analysis, the extract from rice-koji contains a large amount of glucose and oligosaccharides, and these saccharides affect the assay of the glucoamylase activity. Therefore, the koji-extract had to be dialyzed before the enzyme assay by the conventional methods.

On the other hand, we have reported that the pseudooligosaccharides produced by Streptomyces castaneoglobisporus apparently inhibited fungal glucoamylases, and that affinity columns prepared with the immobilized glucoamylase inhibitors, the pseudo-oligosaccharides or acarbose, effectively adsorbed glucoamylase from unpasteurized sake (J. Ferment. Bioeng., in press). These observations suggested that the substantially high affinity of the inhibitor for glucoamylase (acarbose,

![Fig. 1. Coupling of 2-Aminopyridine to Acarbose by Reductive Amination.](image)

One mg of acarbose and 40 μl of a 2-aminopyridine solution (1.0 g of 2-aminopyridine, 0.8 ml of conc. HCl and 1.2 ml of distilled water) were mixed in a sealed tube, then the reaction mixture was heated at 100°C for 30 min. After heating, the reaction mixture was cooled and mixed with 3 μl of the reducing reagent (10 mg of NaBH₄CN, 20 μl of 2-aminopyridine solution, and 30 μl of distilled water). The tube was re-sealed and heated at 90°C for 15 hr. The PA-acarbose was purified successively on columns of Dowex 50W-X2 (H⁺, 200–400 mesh, 2.5 x 30 cm) and Sephadex G15 (1.5 x 100 cm) (J. Ferment. Bioeng., in press).

![Fig. 2. Protocol for Fluorometric Assay of Fungal Glucoamylase.](image)

Glucoamylase and the labeled inhibitor were dissolved with 10 mM acetate buffer (pH 5.0). One unit of glucoamylase activity is defined as the amount of enzyme that releases one milligram of glucose per hour under the conditions described by Iwano et al. One unit of inhibitory activity is defined as the amount of inhibitor that inhibits one unit of glucoamylase. Fluorescence was measured with a fluorescence spectrophotometer (Shimadzu model FU-3200) using an excitation wavelength of 315 nm and an emission wavelength of 360 nm.

![Fig. 3. Fluorometric Measurement of Glucoamylase Activity of the Purified Enzyme.](image)

A dilution series of glucoamylase were prepared with a purified glucoamylase, amyloglucosidase from A. oryzae (Sigma). Glucoamylase solution in concentration of 0–2.0 U/ml (A) and 1–6.5 U/ml (B) were incubated with 100 μl of PA-acarbose/ml and 100 μl of PA-inhibitor solution, respectively. The fluorometric assay was done by the protocol shown in Fig. 2.

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Fig. 4. Correlations between Results of the Usual Method and the Fluorometric Method in the Determination of Glucoamylase Activity.

Each 10 g of rice-koji prepared under various kinds of culture conditions was extracted with 50 ml of 10 mM acetate buffer (pH 5.0) containing 0.5% NaCl for 3 hr at room temperature, and then filtered with Toyo No. 5 paper (Advantec). The filtrate was directly analyzed by the fluorometric assay, while it was dialyzed against 10 mM acetate buffer, pH 5.0, before the assay by the usual method.

K_0 = 0.5 μM; maltose, K_m = 1.1 mM) might be applicable to the assay of the enzyme. In this study, we have developed a rapid assay method for glucoamylases using a fluorescence-labeled glucoamylase inhibitor. Acarbose⁵ and 2-aminopyrididine were of choice as a glucoamylase inhibitor and a fluorescent reagent, respectively (Fig. 1). Coupling of 2-aminopyrididine to acarbose was done by the procedure described by Hase et al.⁶ (see legend for Fig. 1) From 1 mg of acarbose, finally 300 μg of purified pyridilaminated (PA-) inhibitor was eluted as a single peak on Shim-pack CLC-ODS (M) (4.6 mm × 15 cm, Shimadzu) under monitoring the glucoamylase-inhibitory activity and the fluorescent intensity.

A protocol for glucoamylase assay using the fluorescence-labeled inhibitor is shown in Fig. 2. A glucoamylase solution to be assayed was reacted with the PA-inhibitor, and then the glucoamylase-bound PA-inhibitor was removed from the reaction mixture by an anion-exchange resin. Then, the glucoamylase activity in the sample was represented by the decrement in the fluorescent intensity (F_{flu}) that was given as the difference between the fluorescent intensity before (F_{flu}) and after (F_{flu}) the elimination of the fluorescent affinity complex.

Glucoamylase activities in dilution series of a commercially available enzyme were measured by the fluorometric method. Both at lower (0.1—2 μM) and at higher (1—65 μM) glucoamylase activities, adequate linearities were observed between the enzyme activities and the decrements in the fluorescent intensity (Fig. 3). Neither 10% (w/v) glucose nor 20% (v/v) ethanol disturbed this fluorometric assay system (data not shown).

Subsequently, rice-koji samples prepared under different conditions were extracted with 0.5% NaCl in 10 mM acetate buffer (pH 5.0), and glucoamylase activities (A) in a portion of each extract was assayed by the fluorometric method. On the other hand, the rest of extract was dialyzed against 10 mM acetate buffer (pH 5.0) and examined for the glucoamylase activities (B) by the usual assay method described by Iwao et al.⁵ As shown in Fig. 4, a good correlation (r = 0.992) was observed when A values were plotted on the abscissa and B values as the ordinate. Coefficients of variation (C.V.) showed no significant difference in both methods (7.5% in the usual and 7.7% in the fluorometric methods).

These results show that glucoamylase activity can be measured by the fluorometric assay method with the PA-inhibitor. The advantage of this assay method against the conventional methods is that (1) it does not require dialysis of enzyme solution and strictness in reaction time; (2) the handling is simple and total reaction time is brief. Since the activity in rice-koji is measured within 1 hr using the fluorometric method, we will immediately know the change in glucoamylase activity during koji-cultivation, and also it will be possible to control cultivation conditions, such as temperature, humidity, and mixing frequency, for maximum production of glucoamylase in rice-koji.

Additionally, other fungal glucoamylases from Rhizopus sp. and Aspergillus niger in commercial use could be measured by the fluorometric method, in the same manner as A. oryzae glucoamylase (data not shown). These results let us confirm an application of the fluorometric method for other fermentation industries, in which such glucoamylases are used.

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References