Microassay for Rapid Screening of α-Amylase Activity

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A microassay was developed for measuring the activity of α-amylases in the nanogram enzyme concentration range, based on the use of dye-labeled cross-linked starch as the substrate, and the release of soluble colored fragments formed in enzyme hydrolysis. Reaction conditions were optimized to generate a linear correlation between the increase in absorbance and a reaction time of 0−10 min, as well as enzyme concentrations in the range of 0−50 ng. A standard curve for the conversion of absorbance to enzyme activity units was constructed. The protocol developed was applied to monitoring the production of ultralow concentrations of recombinant barley α-amylase in yeast cells.

Keywords: *α-Amylase; barley α-amylase; microassay*

INTRODUCTION

Several colorimetric methods have been developed for the analysis of α-amylases (Asp, 1990). The enzyme activity is measured by the appearance of reducing groups formed by the hydrolysis of starch, or the increase in soluble colored products released from dyed starch substrates during enzymatic degradation. The latter method has the advantage of involving a relatively simple procedure of adding an enzyme solution to the dyed substrate, followed by incubation and measurement of the absorbance (Ceska et al., 1969). Furthermore, the reaction is unaffected by reducing substances present in the sample, because the color intensity is proportional to the soluble colored fragments released into solution from the dyed starch substrate and therefore to the enzyme activity. In our investigations on the expression and secretion of recombinant α-amylase in *Saccharomyces cerevisiae*, we found this method particularly applicable in that the glucose used in the YEPD medium did not interfere with the assay, and that the culture medium could be continuously monitored for α-amylase activity without prior removal of the glucose by dialysis or protein precipitation. The dye-labeled starch substrate method, however, has been used primarily as a qualitative, and perhaps semiquantitative, measurement of α-amylase activity.

In this paper, we describe a systematic study of the reaction parameters for the detection of very low concentrations of α-amylase using dye-labeled starch substrates, and the correlation of results obtained in this assay with those of the bicinchoninic acid method. We established linear correlations among absorbance versus time, concentration, and unit conversion. The microassay enables a rapid measurement of a large number of microvolume (200−250 μL) samples for α-amylases in ultralow concentrations (ng, 10^{-3} U), and can be readily adopted for high-throughput screening of microbial colonies for enzyme activities.

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MATERIALS AND METHODS

Materials. Dye-labeled starch substrates were obtained from Pharmacia AB (Phadebas, Uppsala, Sweden). The following chemicals were purchased from Sigma (St. Louis, MO): cupric sulfate pentahydrate, L-serine, imidazole hydrochloride, 4,4′-dicarboxy-2,2′-biquinoline (bicinchoninic acid), soluble starch.

Assay Procedure Using Dye-Labeled Starch. A solution of dye-labeled starch (Phadebas) was prepared by dissolving one starch tablet in 4.48 mL of water by stirring. Enzyme solutions containing an appropriate amount of barley α-amylase were prepared in 0.06 M sodium acetate, 1 mM $CaCl_2$, pH 4.7, in 1.5 mL microfuge tubes, and preincubated at 37 °C for 5 min in a water bath. To each enzyme solution was added 70 μL of the dye-labeled starch substrate, and the mixture was incubated at 37 °C for 5 min. The reaction was terminated by the addition of 30 μL of 1 M NaOH. The resulting mixture was centrifuged at 5,000 rpm for 5 min to remove the undigested starch, and 200 μL of the supernatant was transferred to a microsample plate for measuring the absorbance at 610 nm.

Bicinchoninic Acid (Copper−BCA) Assay of Reducing Sugars. The formation of reducing sugars was assayed according to Waffenschmidt and Jaenicke (1987) and Fox and Robyt (1991) with modifications. A 1.25% solution of soluble starch in 50 mM imidazole−HCl buffer was prepared by incubating at 80 °C for 5 min with stirring. Stock solutions A (BCA) and B (copper sulfate) were prepared according to Fox and Robyt (1991), and the working solution was made fresh by mixing equal volumes of the two stock solutions. The procedure consisted of incubating 1.25 mL of soluble starch solution (preequilibrated at 37 °C for 15 min) and 300 μL of enzyme solution in a 1.5 mL microfuge tube at 37 °C for 30 min. A 100 μL aliquot of the reaction mixture was diluted with 400 μL of H_2O, and mixed with 500 μL of the working reagent. The mixture was centrifuged at 12 000 rpm for 5 min, and 200 μL of the supernatant was pipetted into the wells of a microsample plate. The plate was incubated at 80 °C for 35 min and cooled at room temperature for 15 min, and the absorbance was measured at 560 nm. A maltose standard was run under the same incubation conditions by adding 100 μL of maltose solution (0.1−2.0 μg/100 μL) and 100 μL of working reagent to the wells of the same plate.

Purification of Barley α-Amylase 1 Isozyme. Crude enzyme from barley malt was extracted with 0.2 M sodium acetate buffer containing 1 mM $CaCl_2$, pH 5.5. The extract was heated at 70 °C for 15 min, cooled, and centrifuged at 8000*g*

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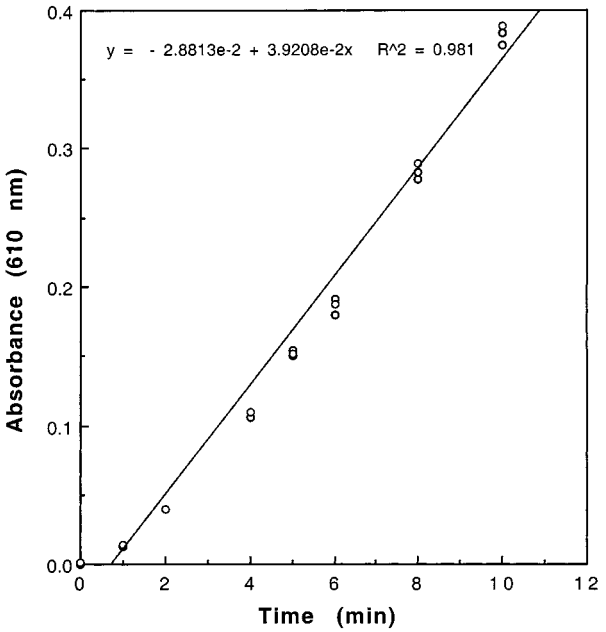

Figure 1. A plot of absorbance at 610 nm due to soluble colored fragments released from dye-labeled starch substrates during enzymatic degradation as a function of reaction time. The enzyme concentration was 25 ng.

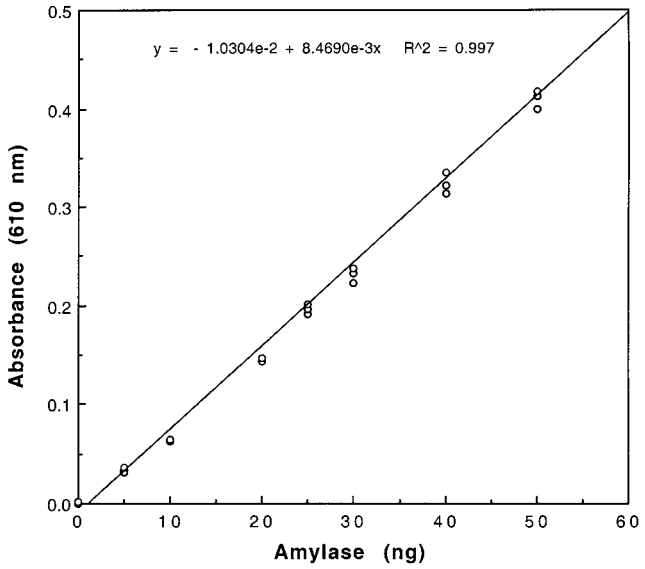

Figure 2. A plot of absorbance at 610 nm due to soluble colored fragments released from dye-labeled starch substrates during enzymatic degradation as a function of enzyme concentrations. The incubation time was 5 min.

for 15 min. The α-amylase in the supernatant was precipitated with 70% ammonium sulfate at 4 °C, dialyzed against the initial buffer (0.2 M sodium acetate, 1 mM $CaCl_2$, pH 4.70), and loaded onto a CM Sepharose CL-6B column (2 × 90 cm). The column was eluted with a linear gradient of 0.02−0.06 M sodium acetate, containing 1 mM $CaCl_2$, pH 4.70 (MacGregor and Morgan, 1992). The protein concentration in the fractions was estimated by absorbance at 280 nm, and enzyme activity was measured using dye-labeled starch substrate. The active fractions were pooled and concentrated by ultrafiltration (PM10 membrane, Millipore). The resulting crude α-amylase 1 sample was applied to a Sephacryl S-300 column using a running buffer of 0.06 M sodium acetate, 1 mM $CaCl_2$, pH 4.70. Fractions containing enzyme activity were pooled, dialyzed against the initial buffer, and further purified by CM Sepharose CL-6B as described above.

Recombinant α-Amylase Activity in Yeast Culture. *S. cereversiae* yeasts harboring the pYEX expression vector (consisting of the phosphoglycerate kinase promoter and the full-length leader sequence from *Kluyveromyces lactis*) with or without the barley α-amylase cDNA gene construct were grown in YEPD medium (1% yeast extract, 1% bactopeptone, 2% D-glucose)/2 mM $CaCl_2$. The culture was removed at various time intervals, and centrifuged to pellet the cells. A 200 μL volume of the supernatant was added to 70 μL of dye-labeled starch solution for enzyme activity measurement as described above. The relative cell density was estimated by reading the absorbance at 600 nm.

RESULTS AND DISCUSSION

Figure 1 presents a plot of absorbance at 610 nm as a function of time for the hydrolysis of insoluble dye-labeled cross-linked starch (Phadebas) by barley α-amylase. The reaction was performed using 25 ng of enzyme. The increase in absorbance versus time was linear from 1 to 10 min. Apparently, there was a lag period of ∼1 min during which the enzyme had to penetrate through and bind to the substrate before hydrolysis occurred. The reaction also proceeds linearly with increasing concentration of the enzyme (Figure 2). The linear correlation was observed when α-amylase was used in the range of 0−50 ng (in the final 200 μL sample) with the reaction time set at 5 min. In this protocol, all reactions were carried out in microfuge tubes with a reaction volume of 300 μL, and 200 μL of the final reaction mixture was transferred to microsample plates for absorbance reading. The reactions could be conducted in microplates instead of microfuge tubes, if a swinging-bucket rotor with microplate carriers was available for use in the removal of undigested dye−starch substrates after the reaction. The use of microplates for carrying out the reactions could be integrated into high-throughput screening systems with robotic dispensing mechanisms.

The dyed substrate (Phadebas) contains starch labeled covalently with Cibacron blau F3 G-A and cross-linked by 1,4-butandiolglycidether (Ceska et al., 1969; Barnes and Blakeney, 1974). Enzyme activity corresponds to the hydrolytic release of water-soluble dye-labeled fragments, the concentration of which can be measured spectrophotometrically. Other dye-labeled starch substrates, such as Remazol brilliant R blue starch, are also commercially available. However, the use of these substrates requires ethanol precipitation and filtration (or centrifugation) to remove undigested high molecular weight starch fragments (Rinderknecht et al., 1967; Linko et al., 1975). In addition, dye-labeled starch without cross-linking has been known to be susceptible to β-amylase, resulting in an assay nonspecific for α-amylase activity (Mathewson and Pomeranz, 1977; Barnes and Blakeney, 1974). In the course of our investigation, we also found that certain of these substrates showed exceedingly high background readings and prewashing of the dyed starch with buffer was needed to eliminate interferences in subsequent steps.

To compare the results obtained from the dye-labeled starch substrate protocol with those from other colorimetric methods measuring the reducing groups in hydrolysis, we modified the copper−BCA procedure developed by Waffenschmidt and Jaenicke (1987) and Fox and Robyt (1991) to accommodate the enzyme reaction in microfuge tubes. A maltose standard curve was constructed with a linear range of 0−2 μg of maltose (Figure 3). The time course of the reaction presented in

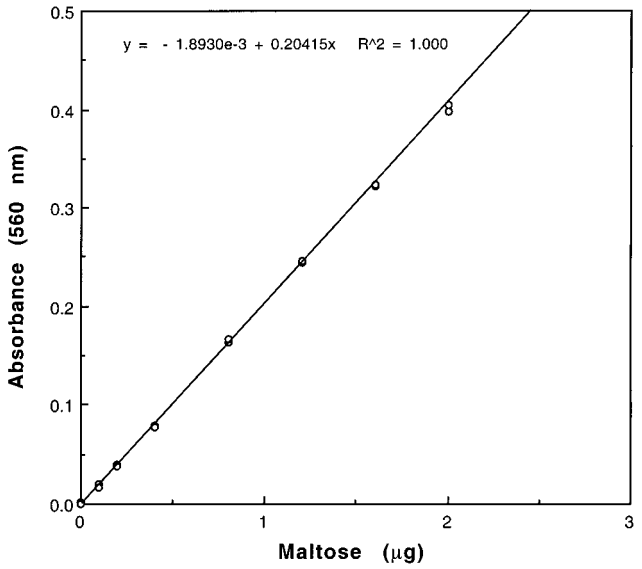

Figure 3. A maltose standard curve based on the copper–BCA method.

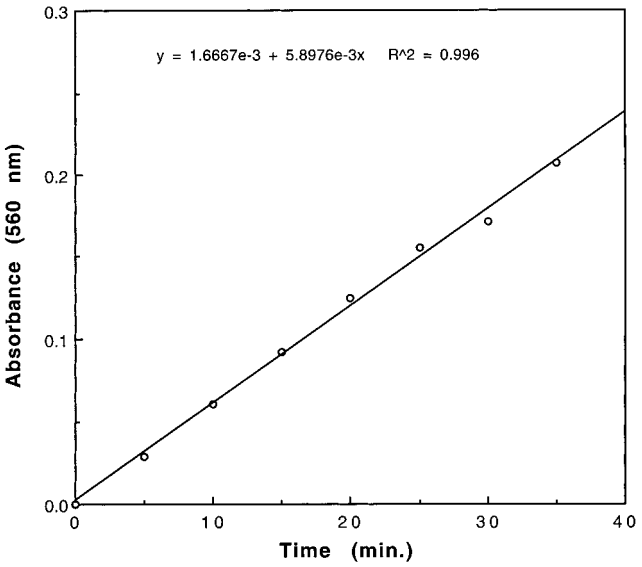

Figure 4. Absorbance at 560 nm due to the formation of reducing groups during enzymatic degradation as a function of reaction time. The enzyme concentration was 1 ng.

Figure 4 suggests a linear correlation between the formation of reducing groups in hydrolysis and the reaction time of 0–35 min, using 1 ng of α-amylase for the reactions. A linear plot was also obtained when the reaction was performed with a 5 min incubation using enzyme concentrations in the range of 0–2.5 ng (in the final 200 μL sample) (Figure 5). Enzyme concentrations higher than 2.5 ng would yield a nonlinear curve under the reaction conditions outlined for this protocol. It is evident that the copper–BCA method is more sensitive than the dye-labeled starch substrate method. However, the detection limit of the latter method is within the working range of enzyme concentrations in most experiments (see below). The procedure involves a fewer number of reagents and steps. Most important, the method is unaffected by the presence of reducing substances or other potential interferences such as high concentrations of metal ions and proteins in the sample. We found it particularly applicable in monitoring the production of α-amylase in cell cultures containing glucose in the medium (see below).

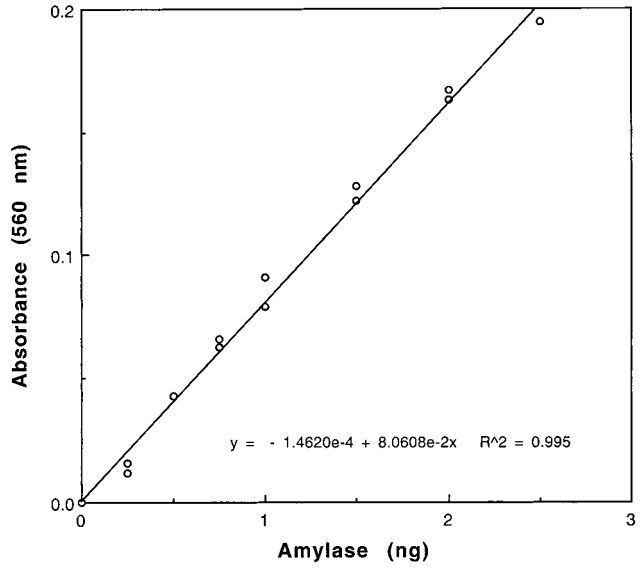

Figure 5. Absorbance at 560 nm due to the formation of reducing groups during enzymatic degradation as a function of enzyme concentrations. The incubation time was 5 min.

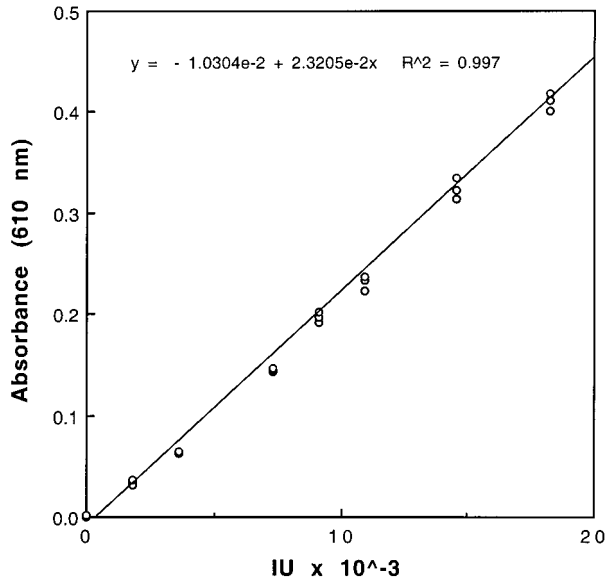

Figure 6. A linear correlation in a plot of color absorption in the hydrolysis of Phadebas versus the corresponding enzyme activity (μg of α-amylase catalyzing the hydrolysis of 1 μmol of glucosidic linkage/min at 37 °C).

Conversion of absorption to International Enzyme Units was achieved by generating a standard curve using soluble starch substrate based on the copper–BCA assay. Figure 6 shows a linear correlation in a plot of color absorption in the hydrolysis of Phadebas versus the corresponding enzyme activity (μg of α-amylase catalyzing the hydrolysis of 1 μmol of glucosidic linkage/min at 37 °C). Using the same scheme, conversion to other enzyme units, such as μmol of maltose/min, could be established. It should be noted that this standard curve was prepared using barley α-amylase 1 purified from barley malt under the conditions described above. α-Amylases obtained from other sources or preparations would cause variations in the standard curve and hence the conversion factor.

The protocol developed in this investigation was employed in monitoring the production of recombinant barley α-amylase in *S. cerevisiae*. The absorbance at 610 nm was converted to enzyme units for the plot, using

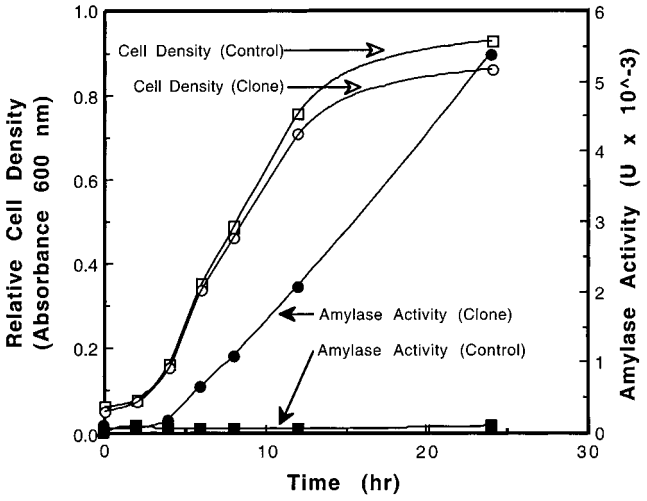

Figure 7. Recombinant barley α-amylase production in *S. cerevisiae* strain DY150 harboring the pYEX/cDNA gene. The control is the yeast strain DY150 containing the expression vector.

the standard curve described above. The increase in α-amylase activity in the culture medium with the clone harboring the barley α-amylase gene is presented in Figure 7. The control containing *Amy*$^-$ yeast cells (pYEX expression vector without the amylase gene) did not show detectable activities during similar growth. Yeast cells and *Saccharomyces* in particular, are usually grown in YEPD medium containing 10% glucose. Assay methods based on measuring reducing groups cannot be used directly for measuring α-amylase activities in yeast liquid cultures (Kumagai et al., 1990; Sogaard and Svensson, 1990), and the culture sample must be ethanol precipitated prior to analysis of activity or enzyme kinetics. The assay has been successfully adapted for semiautomation including growing the culture in a deep-well microplate, performing the reaction in a regular microplate, and reading the absorbance with a high-throughput photometer.

CONCLUSION

A rapid, simple microassay based on the hydrolysis of dye-labeled cross-linked starch substrate was developed using a microplate reader and tested for measuring the expression and secretion of nanogram quantities of recombinant barley α-amylase in yeast cells. Optimized conditions for the parameters of incubation are time, concentration, and temperature, for the detection of an ultralow quantity of α-amylase. A standard curve was constructed for relating absorbance obtained in this assay to activity units. The microassay can be applied to a large number of samples in a short time. The protocol could be integrated into high throughput systems with robotic handling capabilities, enabling the screening of thousands of samples in a single run. The underlying principle of this protocol could also be utilized for developing a similar microassay for cellulolytic enzymes.

LITERATURE CITED

Asp, E. H. Laboratory methods for alpha-amylase analyses. *Cereal Foods World* **1990**, *35*, 941−946.

Barnes, W. C.; Blakeney, A. B. Determination of cereal alpha amylase using a commercially available dye-labeled substrate. *Die Starke* **1974**, *26*, 193−197.

Ceska, M.; Hultman, E.; Ingelman, B. G.-A. A new method for determination of α-amylase. *Experientia* **1969**, *25*, 555−556.

Fox, J. D.; Robyt, J. F. Miniaturization of three carbohydrate analyses using a microsample plate reader. *Anal. Biochem.* **1991**, *195*, 93−96.

Kumagai, M. H.; Shah, M.; Terashima, M.; Vrkljan, Z.; Whitaker, J. R.; Rodriguez, R. L. Expression and secretion of rice α-amylase by Saccharomyces cerevisiae. *Gene* **1990**, *94*, 209−216.

Linko, Y.-Y.; Saarinen, P.; Linko, M. Starch conversion by soluble and immobilized α-amylase. *Biotechnol. Bioeng.* **1975**, *17*, 153−165.

MacGregor, A. W.; Morgan, J. E. Determination of specific activities of malt α-amylases. *J. Cereal Sci.* **1992**, *16*, 267−277.

Mathewson, P. R.; Pomeranz, Y. Detection of sprouted wheat by a rapid colorimetric determination of α-amylase. *J. Assoc. Off. Anal. Chem.* **1977**, *60*, 16−20.

Rinderknecht, H.; Wilding, P.; Haverback, B. J. A new method for the determination of α-amylase. *Experientia* **1967**, *23*, 805.

Sogaard, M.; Svensson, B. Expression of cDNAs encoding barley α-amylase 1 and 2 in yeast and characterization of the secreted proteins. *Gene* **1990**, *94*, 173−179.

Waffenschmidt, S.; Jaenicke, L. Assay of reducing sugars in the nanomole range with 2,2′-bicinchoninate. *Anal. Biochem.* **1987**, *165*, 337−340.

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