The Use of Reduced β-Limit Dextrin as Substrate in an Automated Amylase Assay[1]

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ABSTRACT

A sensitive automated method for determining α-amylase has been developed, using sodium borohydride-reduced β-limit dextrin as substrate and employing a Technicon AutoAnalyzer. Hydrolysis products from the reaction between reduced β-limit dextrin and enzyme were reacted with potassium ferricyanide and the decrease in absorbance measured at 420 nm. The method was illustrated with amylases from fungal, bacterial, and plant sources. β-Amylase increased the sensitivity of the assay and must be added in excess for α-amylase sources containing this enzyme.

The effects of α-amylases from various sources in altering breadmaking quality are now well established, and it is desirable to have suitable automated methods of determining the activities of such enzymes for use in flour supplementation. In this laboratory we are interested in endogenous levels of α-amylase found in wheat varieties, and this assay also can be facilitated by automation. Trachman and Saletan (1) have recently described an automated method for determining α-amylase based on the American Society of Brewing Chemists' manual procedure (2) and using a Technicon AutoAnalyzer. α-Amylase reacts with a β-limit-dextrin substrate, and the resulting decrease in iodine-dextrin color at 480 nm. is then measured. As there is not a linear decrease in absorbance with increase in enzyme concentration, a calibration curve must be prepared. At higher levels of enzyme, the nonlinearity is particularly bad, with large increases in enzyme concentration causing only small decreases in absorbance. Strumeyer and Romano (3) have developed a sensitive automated method for determination of α-amylase, utilizing reduced starch as substrate. Use of reduced starch eliminates the interfering reducing power that normal starch has on various reducing-sugar methods, with consequent increase in sensitivity of the method. Unfortunately, this substrate is equally susceptible to β-amylase attack, so that α-amylase containing β-amylase (most plant sources) cannot satisfactorily be assayed. The purpose of this paper is to illustrate that reduced β-limit dextrin can also be satisfactorily used as substrate for α-amylase-activity determination, and that the assay can be easily automated. The hydrolysis of reduced β-limit dextrin by α-amylase is measured by the ferricyanide reducing-sugar procedure. This substrate has the advantage that it is able to measure α-amylase in the presence of β-amylase, yet has the sensitivity of reduced starch.

MATERIALS

Sources of α-Amylase

The enzymes used in this study were: fungal α-amylase, 5,000 SKB units per g.;

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α-amylase from *Bacillus subtilus*, 1,187 absorbance units (AU) per mg. (Calbiochem, Los Angeles, Calif.); and sweet potato β-amylase (Sigma Chemical Co., St. Louis, Mo.). Fungal-enzyme wafers having a potency of 7,000 SKB units per g. were kindly supplied by Anheuser-Busch Inc., St. Louis, Mo.; and samples of malted wheat and barley flour, by Ross Industries, Wichita, Kans.

Alkaline Ferricyanide Solution

Potassium ferricyanide (0.625 g.) was dissolved in 1 liter distilled water containing 20.0 g. sodium carbonate, and the resulting solution filtered.

EXPERIMENTAL

Preparation of Reduced β-Limit-Dextrin Stock Solution

β-Limit dextrin was first prepared from waxy-maize starch as previously described (4,5). Large amounts of substrate are required in an automated assay, however, and the dialysis step in the procedure was greatly simplified by first concentrating the 6 liters of β-limit-dextrin solution with an Amicon ultrafiltration assembly (Amicon Corp., Lexington, Mass.), equipped with a PM-30 membrane, prior to dialysis and freeze-drying. Freeze-dried β-limit dextrin (2 g.) was dissolved in 77.5 ml. of boiling water. The solution was cooled in an ice bath, and 70 mg. of sodium borohydride, dissolved in 2.5 ml. water, added dropwise with stirring. This was followed by an additional half-hour of stirring in the ice bath and 2 hr. of standing at room temperature. Substrate prepared in this manner was stable for at least 1 week if refrigerated.

Preparation of Reduced β-Limit-Dextrin Working Solution (0.5%)

An appropriate volume of the stock solution was measured out and 0.2 ml. acetone added, with stirring, to each 10 ml. of stock solution. The pH was brought to 5.5 with dilute acetic acid, and 0.05M acetate buffer, pH 5.5, added to bring the final substrate concentration to 0.5%. In order to obtain optimum clarity of the working solution, it was centrifuged at 13,000 r.p.m. (22,000 × g) for 30 min. Substrate prepared in this manner could satisfactorily be used over a 2-day period if a small amount of toluene was added to minimize bacterial contamination.

Operational Procedures

The basic Technicon AutoAnalyzer (Technicon Corp., Chauncey, N.Y.) was used for automating the amylase assay. The system consisted of a Sampler II; Proportioning Pump II; 35°C. incubation bath with a 20-ft. glass coil; 90°C. constant-temperature bath with a standard 40-ft. glass coil; colorimeter, with a 420-nm. filter; and a recorder, using optical-density paper. The flow diagram for the analyses is similar to that of Strumeyer and Romano (3), and is shown in Fig. 1. Sample flowing at 0.32 cc. per min. meets 0.5% reduced β-limit-dextrin substrate flowing at 1.60 cc. per min., and the stream is air-segmented and passed into the incubation bath. Incubation was carried out for 7 min. at 35°C. and the reaction was terminated by the introduction of alkaline ferricyanide (1.00 cc. per min.) into the stream. Following passage of the sample through the 90°C. bath, the resulting decrease in absorbance was measured at 420 nm. Insoluble deposits slowly formed in the tubing over a period of a few days, causing problems in baseline stability. These were removed by washing the system each day with a solution of 1 ml.

Triton X per 180 ml. water, followed by a distilled-water wash. Detergent in the sampler wash was minimized at 1 drop Triton X per 1,500-ml. distilled water, to avoid possible enzyme inhibition.

RESULTS

Sodium borohydride was used to reduce the terminal glucosyl residue of β-limit dextrin into an inert glucitol residue. Figure 2 illustrates the effect of nonreduced and reduced β-limit dextrin on the ferricyanide baseline of the AutoAnalyzer stream. The nonreduced β-limit dextrin effected a substantial reduction of the alkaline ferricyanide with consequent decrease in observed absorbance, whereas no such decrease was observed with reduced β-limit dextrin. The reduced substrate allows, therefore, a greater usable range of assay with resultant increased sensitivity, and also minimizes variations in results caused by high blank values. Standard maltose samples were regularly run, and the change in absorbance vs. maltose concentration plotted on linear paper (Fig. 3). The working range of assay is seen to be about 0.6 AU.

α-Amylases from a number of different sources were chosen to illustrate the versatility of using reduced β-limit dextrin as substrate. Enzyme assays were calculated on the basis of the amount of maltose liberated, although it is realized that maltose is only one of the hydrolysis products. All assays were carried out in the presence and absence of excess β-amylase. This excess was determined by adding increasing amounts of β-amylase to the maximum concentration of α-amylase being used in the assay until no further increase in reaction rate was found. β-Amylase, acting alone, had no effect in degrading the substrate, but in the

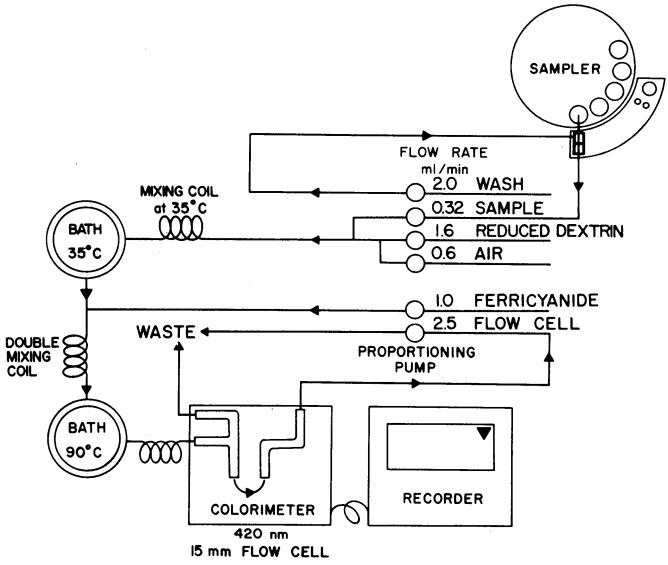
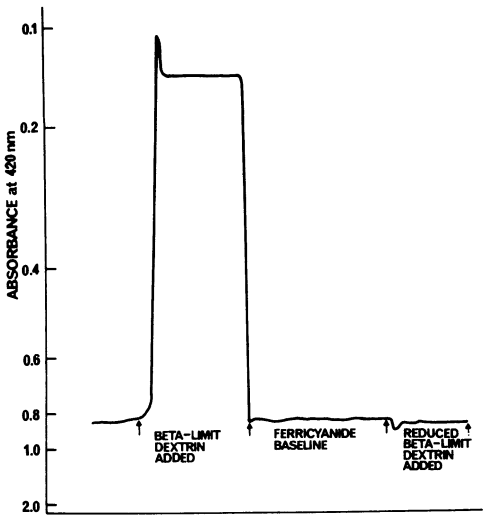


Fig. 1. Technicon AutoAnalyzer flow-sheet for determination of α-amylase, with reduced β-limit dextrin as substrate.



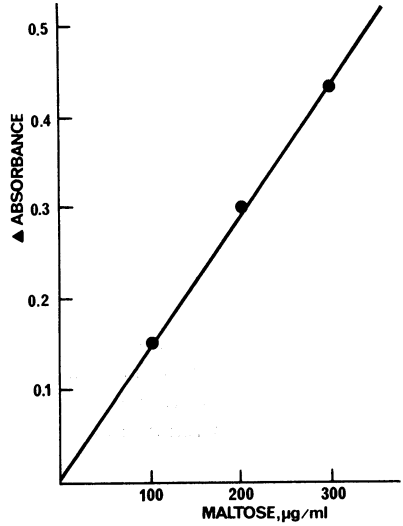


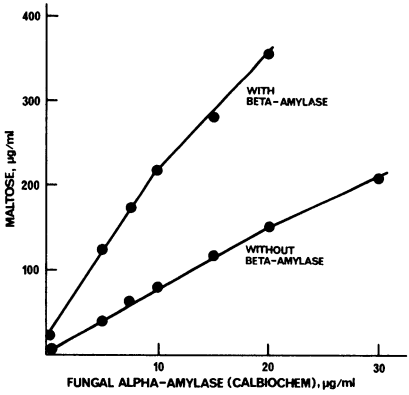
Fig. 2 (left). Effect of nonreduced and reduced β-limit dextrin on the ferricyanide baseline of the AutoAnalyzer.

Fig. 3 (right). Standard calibration curve for the reduction of ferricyanide by maltose on the AutoAnalyzer.

presence of any α-amylase source, caused a greater release of hydrolysis products with consequent increase in sensitivity of the method. For assay of α-amylases containing no β-amylases, such as those from fungal or bacterial origin, there is therefore the choice of adding β-amylase to increase the sensitivity of the method. For assay, however, of α-amylases from sources containing variable amounts of β-amylase, i.e., malted-wheat or malted-barley flours, an excess of β-amylase must be added to eliminate variability in results. Figures 4 and 5 illustrate the effect of enzyme concentration on the hydrolysis of reduced β-limit dextrin, using fungal α-amylase sources. Linear increases in hydrolysis products with increase in fungal amylase occurred with low enzyme concentrations, but slightly curvilinear behavior occurred at higher concentrations when the liberation of hydrolysis products exceeded about 150 γ per ml. of maltose. Similar behavior was found for the effect of bacterial α-amylase (Fig. 6), and malted-wheat-flour α-amylase (Fig. 7). Malted-barley-flour α-amylase exhibited linear behavior, but the concentration of hydrolysis products never exceeded 50 γ per ml. in this study. The probable explanation of the falling-off in reaction rate at higher levels of enzyme is that the substrate is no longer in excess.

In the case of some α-amylase sources, and in particular malted-barley and malted-wheat flours, it is necessary to correct for the presence of endogenous reducing substances. To determine this blank value, samples are passed through the manifold, but bypassing the incubation bath. Caution must also be used in assaying amylase sources in which the presence of amyloglucosidase is suspected, as this enzyme would also hydrolyze the reduced β-limit dextrin.

The present method could be made even more sensitive by increasing the length of the incubation time or by amplifying color development with potassium cyanide.



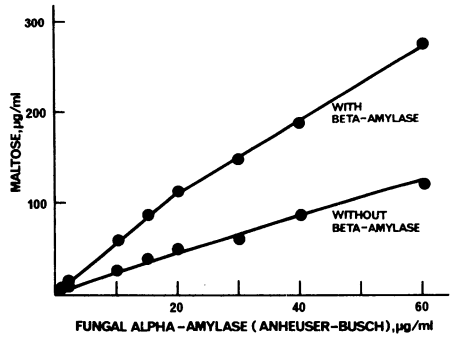
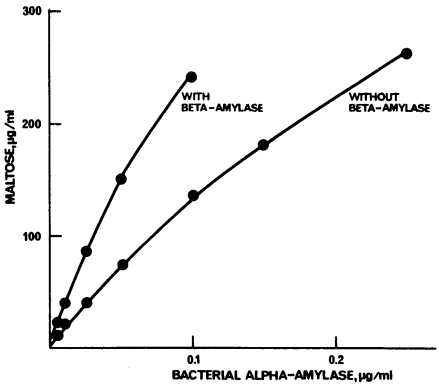


Fig. 4 (left). Hydrolysis of reduced β-limit dextrin by different amounts of fungal α-amylase (Calbiochem).

Fig. 5 (right). Hydrolysis of reduced β-limit dextrin by different amounts of fungal α-amylase (Anheuser-Busch).



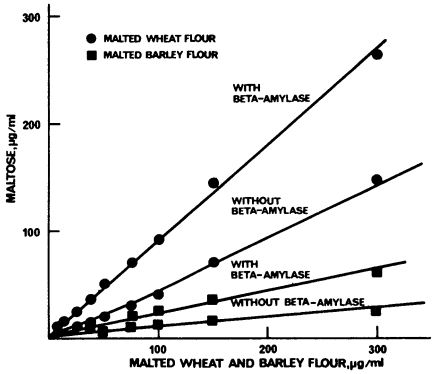


Fig. 6 (left). Hydrolysis of reduced β-limit dextrin by different amounts of bacterial α-amylase.

Fig. 7 (right). Hydrolysis of reduced β-limit dextrin by different amounts of malted-wheat- and malted-barley-flour α-amylases.

For analyzing extremely low activities such as may be found in sound wheat flours, it has been found more convenient in this laboratory to use a semiautomated method and manually incubate sample and reduced β-limit dextrin, prior to introduction into the AutoAnalyzer.

SUMMARY

Sodium borohydride-reduced β-limit dextrin has been found satisfactory as a substrate for determining α-amylase activity. The chief advantage in using this substrate is that it has a negligible blank in reducing-sugar determinations, and as a

consequence, allows a greater usable range of assay and reduces variations in results. An automated assay utilizing this substrate is described, and α-amylases from fungal, bacterial, and plant sources can be satisfactorily determined. β-Amylase increases the sensitivity of the method, and must be added in excess of α-amylase sources containing this enzyme.

Acknowledgment

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