A Sensitive Kinetic Microassay for the Determination of Wheat α-Amylase[1]

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A nephelometric method of measuring α-amylase (AACC method 22-07, Kruger and Tipples 1981) has been successfully used for several years at the Grain Research Laboratory. The enzyme-catalyzed rate of decrease in turbidity of a β-limit substrate, which is proportional to the amount of α-amylase, is monitored using a Perkin-Elmer model 191 grain amylase analyzer attached to a recorder. The sensitivity of the machine is increased to near maximum. The advantage of the method is its sensitivity for monitoring the very low levels of enzyme typically found in Canadian wheats. On the other hand, the method has limitations in that it has not been possible to semiautomate, and a substantial amount of manipulative steps and operator expertise are required for carrying out the test and interpreting the results.

This article describes a method using a kinetic microplate reader, which was assessed as a potential replacement. It is based on a principle similar to that of the modified AACC method 22-07 method but overcomes some of the limitations described above. Using this method, enzyme-catalyzed rates of decreases in turbidity of β-limit dextrin substrate were found to be satisfactorily monitored simultaneously in 96-well microtiter plates.

MATERIALS AND METHODS

Wheats and Flours

Wheats from No. 1, 2, and 3 Canada Western Red Spring individual cargo and cargo composites from 1991 were used. Samples were chosen to represent a spectrum of α-amylase values, as determined by the modified AACC method 22-07, and had extremes in falling number (FN) values of 250 and 435 sec.

Flours for Canada Western Red Spring wheat cargo samples were prepared with an Allis-Chalmers mill, using a 500-g sample of wheat with an extraction of approximately 74% (clean-wheat basis).

Enzyme Source

Fungal α-amylase, 5,000 SKB units per gram, was obtained from Calbiochem (Los Angeles, CA).

Reference α-Amylase Assay

AACC method 22-07, modified as described previously (Kruger and Tipples 1981) was used as the reference method against which the new microplate method was compared. Substrate for the assay was β-limit dextrin. Results are reported in terms of the milligrams of maltose hydrolyzed per minute × 10^{-3} per gram of sample.

Extraction of Ground Wheats and Flours

Ground wheat and flour samples were extracted as for the nephelometric α-amylase assay (Kruger and Tipples 1981). Five milliliters of 0.2*M* sodium acetate-acetic acid buffer, pH 5.5, containing 0.001*M* $CaCl_2$ was added to a 1-g sample, and the suspension was agitated continuously for 1 hr. The mixture was centrifuged at 15,000 × *g* for 20 min, and the supernatant was additionally filtered through glass wool before analyses.

Kinetic Microplate Assay for α-Amylase

The assay used a UV Thermomax microplate reader (Molecular Devices, Palo Alto, CA) attached to an IBM-compatible computer equipped with Softmax software (Molecular Devices, Palo Alto, CA). The software enabled kinetic measurements to be recorded progressively during an enzymatic reaction and conversion of reaction rates to enzymatic activities. Enzyme extracts (20 μl) and blanks consisting of 20 μl of acetate buffer were initially pipetted into individual wells of a 96-sample microplate. Reactions were initiated in the microplate wells by rapidly adding 250 μl of 0.5% β-limit dextrin solution (prepared as per Kruger and Tipples 1981) at 37°C to the wells containing enzyme and blanks. A 13-sec programmed shaking was then initiated to ensure homogeneous mixing of reagents, and the decrease in absorbance at 340 nm in all wells was measured at 6-sec intervals over 10 min. Reaction rates over a user-defined range of 0.025 absorbance were determined by the computer software, and blank values were automatically subtracted from unknown enzyme extracts. Fungal α-amylase standards of known enzymatic potency were run simultaneously, and results from ground wheat or flour samples were automatically converted to such units.

Because of the small amounts of substrate required and the ease of carrying out multiple analyses, runs were normally conducted in triplicate or quadruplicate to improve precision.

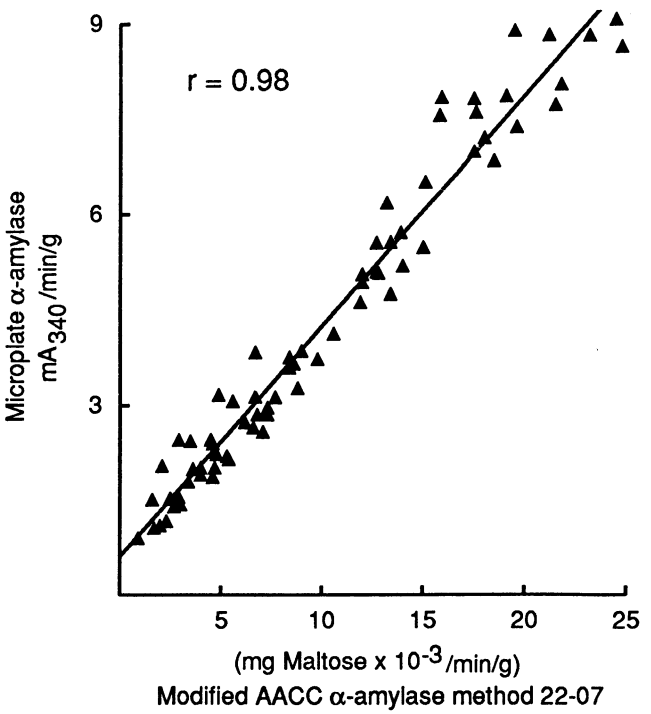

Fig. 1. α-Amylase as determined by the kinetic microplate method versus α-amylase as determined by modified AACC method 22-07 for wheats and flours. A = absorbance.

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RESULTS

The sensitivity of the present kinetic microplate assay was dependent on the concentration of the substrate used. Preliminary experimentation indicated that the level used in the AACC nephelometric assay (AACC method 22-07, Kruger and Tipples 1981) was equally suitable in the present assay for achieving the sensitivity required for measuring low α-amylase levels. In addition, the use of the same substrate concentration enabled a better comparison of the two methods.

Decreases in absorbance resulting from the wheat or fungal α-amylase catalyzed decreases in turbidity of β-limit dextrin were linear up to an absorbance change of -0.025, after which the rates became progressively curvilinear. This was selected, therefore, as the maximal allowable absorbance change for calculation of enzyme activity. For a large number of wheat samples, this absorbance change occurred in 5 min or less. Serial dilutions of ground wheat, wheat flour, and fungal α-amylase extracts were assayed and displayed linear relationships between absorbance change and enzyme concentrations (r values of 0.99, 0.99, and 1.00, respectively).

When compared at equivalent enzyme activity as determined by the nephelometric α-amylase assay, there was a very similar response for the cereal and fungal sources in terms of rate of change in turbidity. This suggests, as might be expected, that both methods detect substrate breakdown similarly.

Fungal α-amylase extracts with potencies of 0.2, 4, and 10 $\times 10^{-2}$ SKB units per liter were replicated three, six, nine, and 12 times to assess the precision of the microplate reader for carrying out this assay. Coefficients of variation ranged randomly from 1.4 to 7.1%. No improvement in precision was achieved by carrying out more than three replications.

Day-to-day machine variability was assessed by analyzing five wheat extracts varying widely in enzyme activity over a three-day period in triplicate. Overall coefficients of variation ranged from 3.2 to 6.2%, well within the precision expected of the machine. The reliable level of detection of enzyme activity by the kinetic microplate assay was found to be 3.0 mg of maltose $\times 10^{-3}$ hydrolyzed per minute per gram of sample as determined by modified AACC method 22-07 (Kruger and Tipples 1981).

The kinetic microplate reader was further evaluated as a potential replacement for the nephelometric assay by comparing the analyses of 72 ground wheat and flour samples by both methods. Wheats and wheat fours ranged in activity by the nephelometric method from 2.1 to 22.0 and 1.6 to 25.4 mg of maltose $\times 10^{-3}$ per minute per gram, respectively. As shown in Figure 1, there was a good linear correlation between the two methods, with a Pearson correlation coefficient of 0.98. With this wide range of amylase activities, it was necessary to dilute extracts several-fold with the nephelometric assay. The kinetic microplate assay, however, required no dilution within this range.

DISCUSSION

Assays for α-amylase from cereal (Sirou et al 1990) and bacterial sources (Fox and Robyt 1991) using a microplate reader have been reported. These have been fixed-point assays, however, in which the α-amylase catalyzed degradation of starch or oligosaccharide is carried out manually, followed by measurement of the resultant products with the microplate reader. This is the first report of a dynamic assay for this enzyme using this apparatus in which the degradation of substrate can be monitored continuously as the reaction progresses and for a large number of samples simultaneously. One of the biggest advantages of the method is that a standard enzyme preparation of known potency can be run concurrently with the unknowns, and the results can be calculated immediately from a plate-specific computer-generated standard curve. Any small variation in operating conditions throughout the day will not affect the results because uniform treatment ensures that both the standard and the unknowns are affected in the same way. Because up to 96 analyses can be performed in a relatively short time, this allows a larger number of replicate analyses with consequent improvement in the precision of the assay. In many cases, analysis times can be shortened to 5 min or less. Coupled with the fact that very small amounts of extract (i.e., 20 μl) are required for analyses, it is particularly suited for applications such as analyzing column effluents from enzyme purifications or where the amount of enzyme is limited (i.e., single kernels). Another advantage of the assay is the economy in use of reagents. For example, preparation of β-limit dextrin is time-consuming and expensive. The present assay uses 250 μl of a 0.5% β-limit dextrin solution per assay compared with 3,000 μl for the nephelometric grain amylase analyzer procedure. Furthermore, because dilutions are minimized, there is additional time saving and less chance for dilution errors. Research is continuing on a long-term comparison of the two methods with the eventual purpose of substituting the kinetic microplate assay because of its many advantages.

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