Modified Procedure for Use of the Perkin-Elmer Model 191 Grain Amylase Analyzer in Determining Low Levels of α-Amylase in Wheats and Flours[1,2]

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ABSTRACT

The Perkin-Elmer model 191 grain amylase analyzer was employed at its highest possible sensitivity to determine the α-amylase levels in wheats and flours containing low to moderate levels of the enzyme. β-Limit dextrin (0.5%) was used as substrate, and the rate of decrease in turbidity effected by enzyme was followed on a recorder attached to the grain amylase analyzer. Straight-line relationships were found between increases in concentration of enzyme and rates of decrease in nephelos per minute. The method was evaluated and found satisfactory for assaying α-amylase levels in wheats ranging in Hagberg falling numbers from 240 to 460 sec and in amylograph viscosities from 125 to 925 BU. The method has some advantages over an automated fluorometric procedure for α-amylase presently being used by the authors.

Of the many methods for specific determination of α-amylase in cereal products, the nephelometric method of Zinterhofer et al (1973) is one of the most promising. An instrument based on this method and marketed by Perkin-Elmer Corp., Oak Brook, IL, has been widely used in the clinical field for several years now. The assay employs amylopectin as substrate. This instrument has recently been introduced into the cereals industry and is called the Perkin-Elmer model 191 grain amylase analyzer (GAA). In cereals, β-amylase interferes with the assay because of its competitive hydrolysis of amylopectin (Kruger et al 1979). This limitation has been overcome by employing β-limit dextrin as substrate (Campbell 1980). Our own experience has been that although the procedure recommended for the instrument can readily detect various levels of α-amylase in sprout-damaged wheat, it is less satisfactory in discriminating between endogenous levels of enzyme in mature wheats containing negligible amounts (<0.5%) of sprouted kernels. This article describes changes in the instrument settings and substrate concentration in order to obtain maximum sensitivity for the assay.

The modified procedure was tested with wheats and flours containing low to moderate levels of α-amylase and compared with the amylograph and falling number methods and with a specific and sensitive automated fluorometric method for α-amylase (Marchylo and Kruger 1978).

MATERIALS AND METHODS

Wheat

A series of 64 wheats ranging in falling numbers from 240 to 470 sec and 75 flours ranging in amylograph viscosities from 125 to 925 BU were evaluated.

Enzyme Sources

Fungal α-amylase, 5,000 SKB units per gram, was obtained from Calbiochem (Los Angeles, CA). Sweet potato β-amylase, type 1-B, containing 750-1,000 Sigma units per milligram of protein, was obtained from Sigma Chemical Co. (St. Louis, MO).

Extraction of Ground Wheats and Flours

Extracts for α-amylase determinations were prepared as described previously (Marchylo and Kruger 1978). Extracts were completely free of particulate matter.

Amylograph Viscosity Determination

This was performed on a Brabender Visco/amylo/Graph, using 65 g of flour and 450 ml of water (AACC 1976). Milling was performed on an Allis-Chalmers laboratory mill, using a 500-g sample of wheat to an extraction of approximately 74% (clean wheat basis). To this was added a small percentage of flour (approximately 1.5%) obtained with a bran finisher. Amylograph peak viscosity is reported in BU.

Falling Number Determination

The standard ICC method (ICC 1967) was followed, using a 7-g sample of wheat. A 300-g sample was first ground in a Raymond hammer mill, using a 0.87-mm sieve. Results are reported in seconds.

Automated Fluorometric α-Amylase Assay

Extracts from 47 ground wheats and 47 flours in which α-amylase activity had been determined were assayed as described previously (Marchylo and Kruger 1978), using the fluorometric substrate β-limit dextrin anthranilate, and were automated with a Technicon AutoAnalyzer. Activity was expressed as milligrams of maltose liberated per minute per gram at 35°C.

Operational Procedure

Modifications. To obtain the desired sensitivity, a number of modifications were made to the normally adopted procedure (Campbell 1980): 1) the substrate concentration was decreased to 0.5%; 2) a low-speed centrifugation was carried out on the substrate before use to ensure that no particulate matter was present in the substrate; 3) the highest possible sensitivity of the model 191 GAA was employed; and 4) the change in nephelos with time was monitored with a recorder.

Preparation of β-Limit Dextrin Substrate. The procedure was similar to that described previously (Kruger 1972, Kruger and Marchylo 1972, MacGregor et al 1971). Forty grams of waxy-maize starch (American Maize Products Co., Roley, IN) was suspended in 2 L of 90% dimethyl sulfoxide and stirred overnight. The suspension was diluted to 10 L with 0.005 *M* acetate buffer, pH 4.6, containing 0.2 ml (4,760 Sigma units) of sweet potato β-amylase, and stirred for 24 hr, followed by addition of 0.2 ml (4,760 Sigma units) of sweet potato β-amylase and a further 24-hr stirring. The solution then was concentrated to a volume of approximately 1 L with an Amicon ultrafiltration apparatus (Amicon Corp., Lexington, MA) equipped with a PM-30 membrane. The concentrate subsequently was exhaustively dialyzed and the solution freeze-dried. The freeze-dried substrate was finely ground with a coffee mill and stored in a dark bottle.

The yield of substrate was 40-42.5% and the method was the most satisfactory in terms of substrate quality and batch-to-batch reproducibility. Two simplified modifications of the above preparation of substrate were also evaluated and found generally acceptable. In one procedure, the waxy-maize starch was suspended in dimethyl sulfoxide overnight (1 g per 25 ml of water) with stirring. The suspension was brought to a 1% concentration with 0.2 *M* acetate buffer, pH 4.6, and heated to boiling. After the

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mixture was cooled, 0.2 ml (4,760 Sigma units) of sweet potato β-amylase were added, and the mixture was then stirred for 4 hr. After overnight dialysis, the suspension was freeze-dried. The other procedure was as above except that the waxy-maize starch was suspended directly in buffer (1 g/ 100 ml) with boiling. In both cases the yield was higher, 67–68%, than that of the lengthier procedure. Such a large difference in yield probably cannot be accounted for by contamination with reaction by-products. Furthermore, addition of β-amylase had no effect on the substrate, ruling out incomplete breakdown of amylopectin to β-limit dextrin. An alternate explanation is that traces of α-amylase are present in the sweet potato β-amylase and very slowly decreased the size of the β-limit dextrin formed by the lengthier procedure. Two preparations of "α-amylase free" β-amylase were analyzed for α-amylase isoenzymes, utilizing a flatbed polyacrylamide and a β-limit dextrin plate technique as described previously (Marchylo et al 1980). The results confirmed that traces of α-amylase were definitely present.

Working substrate was prepared by adding 0.5 g of β-limit dextrin to 100 ml of cold 0.2*M* acetate buffer, pH 5.5, followed by boiling. The substrate then was cooled to room temperature and centrifuged at 2,000 *g* for 15 min to remove any particulate matter that would interfere with the subsequent assay. Three-milliliter aliquots were then pipetted into cuvettes and incubated at 37°C before the assay.

β-limit dextrin substrate is also available commercially from Perkin-Elmer Corp. (Oak Brook Instrument Division, 200 York Road, Oak Brook, IL 60521). Because this substrate contains buffer and possibly maltose, the concentration described above must be modified. As a guide, the 0.5% β-limit dextrin has a nephelos value of 34–40 nephelos units when compared to the factory 50-nephelos standard.

Operation of Instrument. The model 191 GAA was attached to a Unicam AR 25 linear recorder. In the present study the nephelos mode of the machine was employed. With substrate in the measuring cell, the nephelos scale was expanded to its maximum sensitivity by use of the sensitivity coarse and fine settings of the machine. The blank setting was then adjusted such that the meter scale read about 90% of full scale. The recorder span control was adjusted to give the same reading. Upon addition of 0.2 ml of enzyme extract, an immediate change in nephelos reading was seen. The coarse setting was quickly changed to again obtain a meter reading of approximately 90% of full scale. The decrease in nephelos was followed on the recorder and the change in recorder units per minute obtained. No attempt was made to calibrate the enzyme directly in terms of nephelos units because this would have necessitated that the concentration and nature of the substrate be identical from day to day. Instead, fungal α-amylase, 5,000 SKB units per gram, was chosen as a reference. The potency of the fungal α-amylase standard was checked from time to time by measuring the amount of reducing sugars in terms of milligrams of maltose liberated from reduced β-limit dextrin substrate at 35°C per minute per milligram of enzyme (Kruger and Marchylo 1972). An appropriate set of dilutions of fungal α-amylase was run at least twice during a normal working day, and changes in nephelos units per minute were converted into the amount of maltose liberated per minute from reduced β-limit dextrin. One milligram of fungal α-amylase liberated 8 mg of maltose per minute at 35°C.

The substrate was checked daily for the possible effects of β-amylase. The effect was normally negligible. With the occasional preparation of β-limit dextrin, a small change in nephelos occurred with time and was corrected for in calculation of α-amylase activities.

RESULTS AND DISCUSSION

Typical recorder traces for decreases in turbidity of β-limit dextrin substrate with time using the modified procedure are shown in Fig. 1. As illustrated, particularly by recorder trace E, small

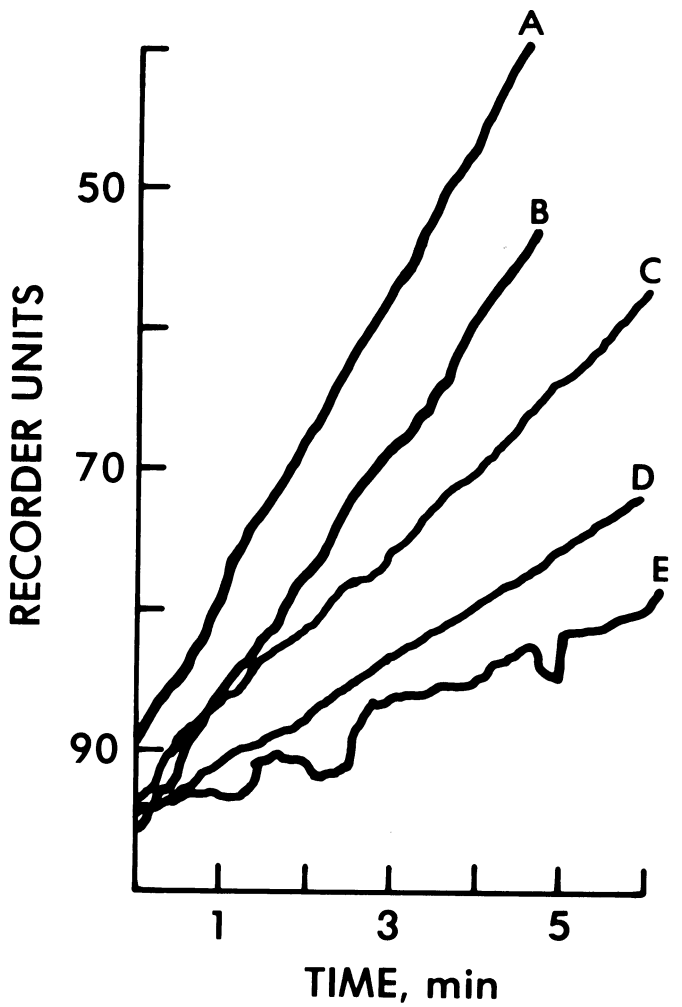

Fig. 1. Decrease in turbidity with time as expressed by change in recorder units for various concentrations of malted wheat α-amylase. **A**, 200 μl; **B**, 150 μl; **C**, 100 μl; **D**, 50 μl.

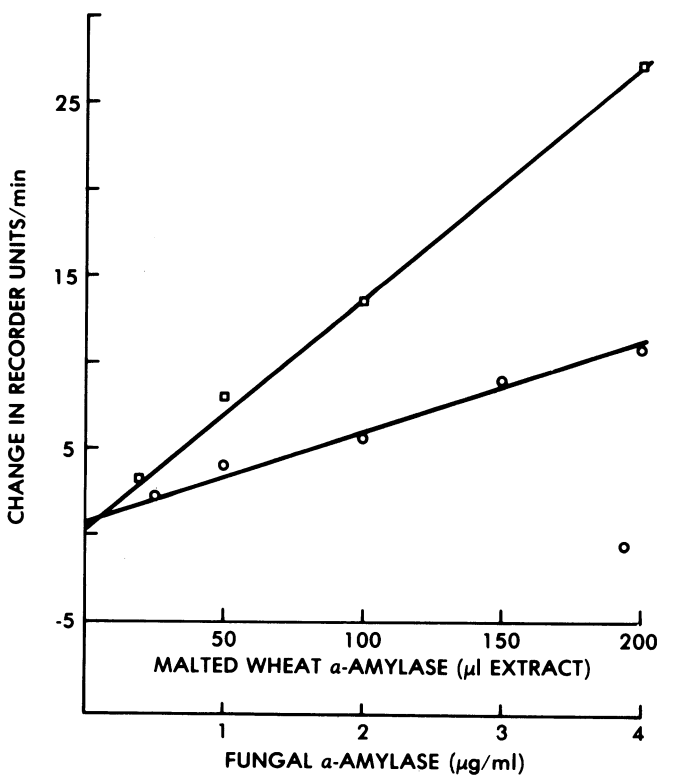

Fig. 2. Change in nephelos with increasing concentrations of fungal (□) and malted wheat (o) α-amylases.

fluctuations in nephelos readings with time occurred independently of the overall steady decrease in nephelos due to enzyme. Such variations would have caused significant error if the model 191 GAA meter and fixed time alone were employed.

Reaction times of 1–5 min were required depending upon the α-amylase activity of the extract. For both wheat and fungal α-amylase, the rate of decrease of turbidity was proportional to enzyme concentration, as shown in Fig. 2. Furthermore, replicates were in very close agreement.

A series of 64 wheats and 75 flours were evaluated using the modified model 191 GAA procedure. As curvilinear increases in α-amylase occurred for decreases in amylograph or falling number values, results were calculated as liquefaction numbers for the wheats and mobility values for the flours. Hlynka (1968) has shown that reciprocals of the aforementioned methods exhibit straight-line relationships with changes in α-amylase activity. As shown in Fig. 3, increases in α-amylase were proportional to increases in both liquefaction numbers and mobilities. The correlation coefficients of 0.93 were in the expected range because both the falling number method and the amylograph method are influenced

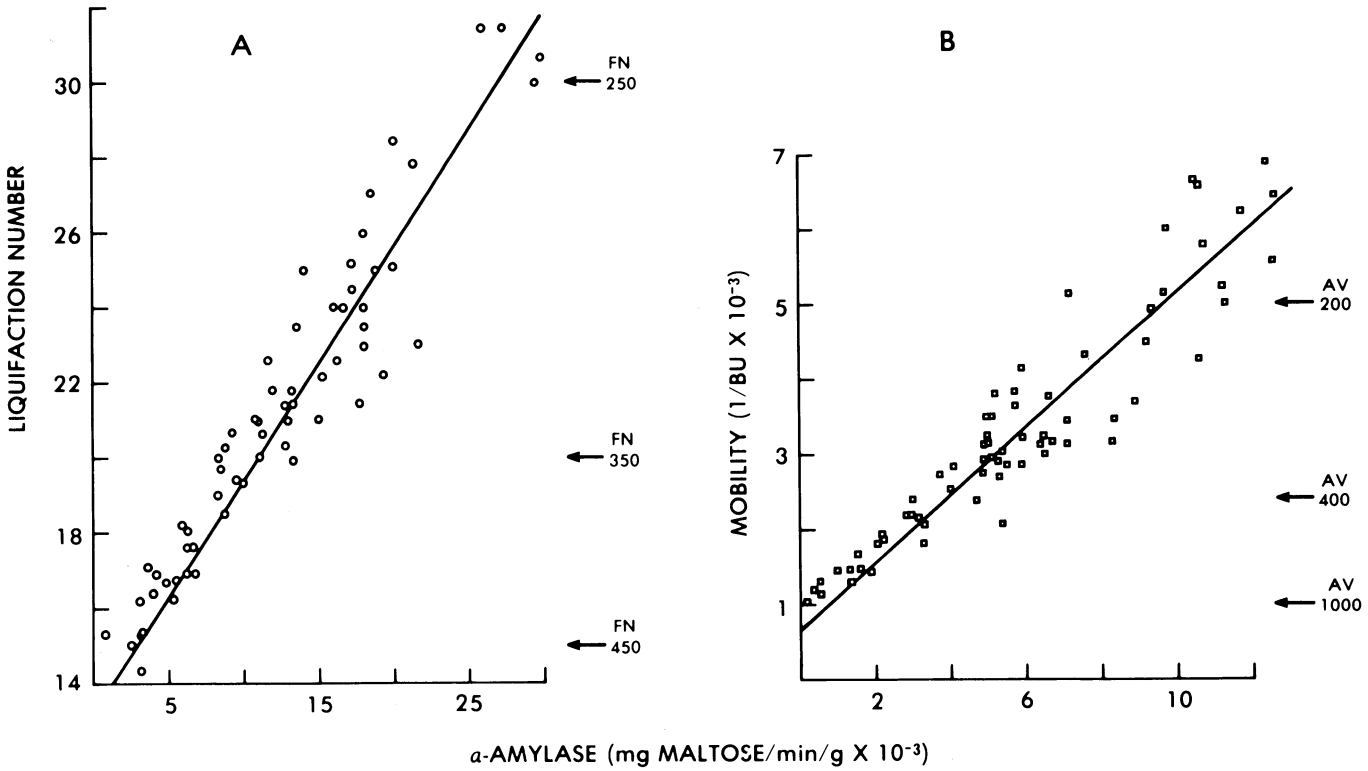

Fig. 3. α-Amylase as determined by the modified model 191 grain amylase analyzer procedure versus: **A,** liquefaction numbers of wheats; **B,** mobilities of flours. **FN** = falling number, **AV** = amylograph value. r = 0.93 for A and B.

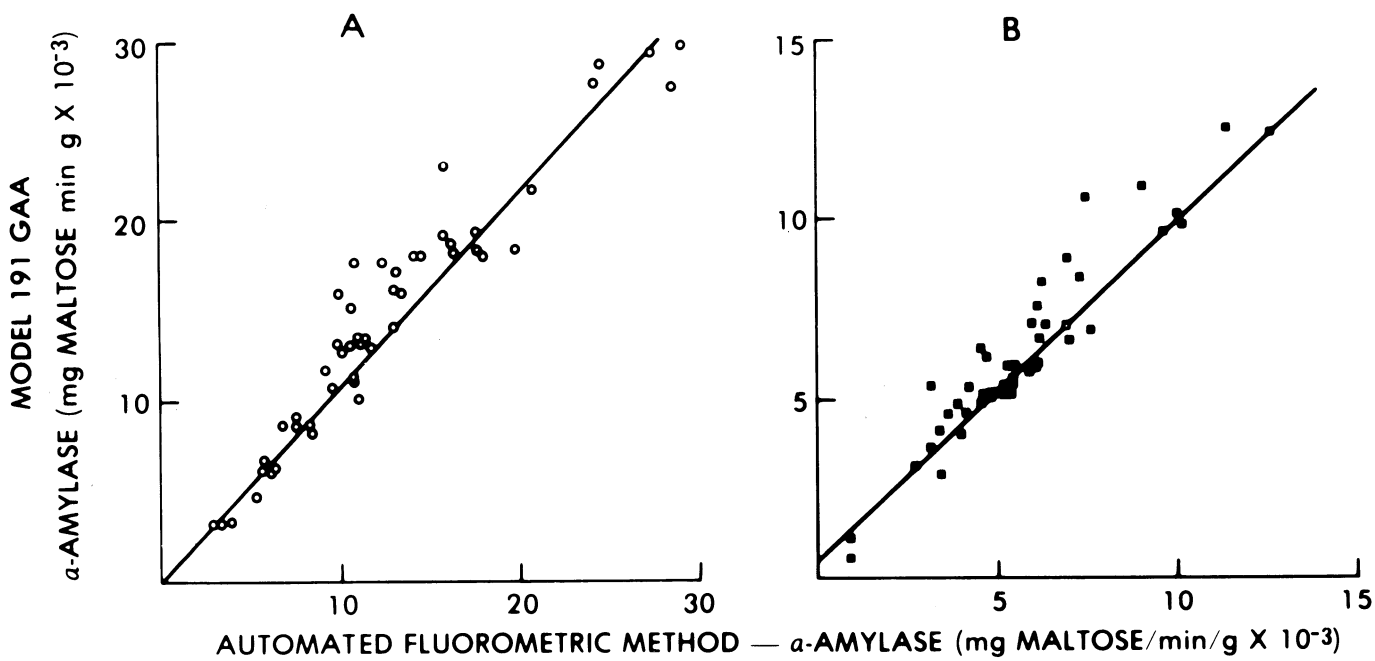

Fig. 4. α-Amylase as determined by the modified model 191 grain amylase analyzer procedure versus α-amylase as determined by the automated fluorometric procedure for: **A,** wheats; **B,** flours. r = 0.97 for A and 0.94 for B.

by factors other than α-amylase, such as starch damage and the nature of the starch.

A large number of samples had extremely low levels of α-amylase activity and, for some flours, the levels were almost negligible.

The modified procedure for the determination of α-amylase had a sensitivity equal to that of the automated fluorometric method described previously for flours (Marchylo and Kruger 1978) in that the same set of fungal dilutions could be used as standards for both methods. The modified procedure was evaluated, therefore, as a potential replacement for the automated fluorometric method, which was currently being used in the laboratory. As shown in Fig. 4, close agreement was obtained between methods for both wheats and flours even though the nature of the tests was quite different. In the nephelometric procedure, the breakdown of products with high molecular weight was being followed. The automated fluorometric procedure, on the other hand, measured the formation of sugars with low molecular weight. Because the falling number and amylograph methods also are based on the breakdown of high molecular weight products in a viscometric assay, one might expect to obtain a better parallel using the modified model 191 GAA procedure than the automated fluorometric procedure. Correlation coefficients, however, were very similar between liquefaction numbers or mobilities and α-amylase levels determined with the automated fluorometric procedure. This indicates that the two α-amylase methods were comparable in their ability to measure wheat α-amylase. The automated fluorometric procedure has the apparent advantage of a much higher throughput of samples with minimum operator assistance. The number of analyses is usually limited by the number of samples that can be extracted in a working day. The modified model 191 GAA procedure has many advantages. The cost of the equipment is significantly lower and a great deal less complex to implement. β-Limit dextrin is less expensive than β-limit dextrin anthranilate. Preparation of heat-treated blanks is not required. Preliminary examination of an unknown enzyme extract in order to assess the proper dilution for assay requires only 1 min with the model 191 GAA compared to 25 min with the automated fluorometric method. An advantage of both assays is that a very small amount of extract is required for an assay, making them particularly attractive for determining α-amylase when a limited amount of material is available for analyses, such as in plant breeding or enzyme purification procedures.

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