Evaluation of a Nephelometric Method for Determining Cereal Alpha-Amylase[1]

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ABSTRACT

A nephelometric assay adapted from a clinical method was evaluated for suitability in determining α-amylase levels in sprouted soft white wheat. In the manual mode of machine operation, the velocity of decline in light scattering was directly proportional to the amount of enzyme assayed. The accurate limits of the assay performed in the automated mode were defined as 0–720 machine units of activity, with a commercial β-limit dextrin as substrate. This substrate was more reactive than amylopectin and less subject to β-amylase interference. The automated assay yielded data that correlated highly with falling number results.

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Sprout damage in wheat cost the United States an estimated 18 million dollars in lost sales during the 10 years following the embargoes of U.S. wheat shipments to Japan in 1968 (Pacific Northwest Grain Export Association 1978). The inferiority of sprout damaged wheat for end-use products stems from the presence of α-amylase (α-1,4-glucan-4-glucanohydrolase EC 3.2.1.1.), which is synthesized de novo during germination. Most of the wheat imported by the Japanese from the Pacific Northwest is used in the production of noodles and sponge cakes. The quality of these products can be seriously impaired by levels of α-amylase that have no significant effect on the quality of American products such as bread. Because detrimental levels of the enzyme can be present in the grain before sprouting is visible, a method that would rapidly and accurately measure α-amylase content and enable accurate assessment of wheat quality in marketing channels is necessary. The assay procedure should be simple so that it can be used by personnel unfamiliar with enzyme chemistry.

A variety of assay methods for α-amylase have been published. These are based on monitoring different aspects of the amylase reaction: liquefaction of a starch gel (AACC methods 22-10 and 56-81), the decrease in color of iodinated β-limit dextrin (AACC method 22-01), the increase in reducing power of a starch solution (AACC method 22-16), the formation of dialyzable fluorescent reaction products from an anthranilate derivative of β-limit dextrin (Marchylo and Kruger 1978), and the formation of filterable dye-labeled reaction products from a particulate chromogenic substrate (Mathewson and Pomeranz 1977). Each of these types of assays has its shortcomings, including nonspecificity for α-amylase activity, low sensitivity, and lack of results that can be correlated with those obtained by other methods. Although the fluorometric method is free from these particular problems, its technique and instrumentation are complex and require a skilled operator.

Recently, Kruger et al (1979) tested a newly developed method of analyzing α-amylase with an improved Perkin-Elmer Grain Amylase Analyzer-191. They found that β-amylase interfered with the assay, which used amylopectin as substrate. By substituting β-limit dextrin for amylopectin, we have since been able to use that method to sensitively assay α-amylase activity in grain. The method is simple, like its prototype, the clinical automated procedure (Zinterhofer et al 1973).

The work we report deals with the limits of sensitivity of the nephelometer used, specificity of the substrate to α-amylase, correlation of nephelometric data with falling number data on sprouted wheat, and particular shortcomings of the commercial system.

MATERIALS AND METHODS

Alpha-Amylase Determination

Commercial Nephelometric Method–The Nephelometer. The model 191 Grain Amylase Analyzer (Perkin-Elmer Corp., Coleman Instruments Div., Oak Brook, IL 60521) was evaluated in this study. This instrument has been described previously (Campbell 1980). With the instrument in the manual mode, we measured decreases in the machine's nephelos units (NU) with time, plotted their relationship, and calculated the slope for the initial linear portion of the curve. The slope, expressed as change in NU per minute, was thus the initial velocity of enzyme activity. Nephelos measurements were standardized to the 50-NU standard supplied by the factory.

The basic principle by which the unit measures amylase activity is that the right angle scattering of light by suspended starch particles decreases as amylase cleaves the starch into dextrins. A more complete explanation of nephelometric methods is found elsewhere (Vanous et al 1978).

The Substrate. We evaluated the new commercial substrate packaged in lyophilized form by Perkin-Elmer. It consists primarily of β-limit dextrin, citrate buffer, and sodium azide (Campbell 1980). Other β-limit dextrins available in bulk from biochemical suppliers were not compared with this commercial preparation. Such a comparison could not be done fairly because little is known about the formulation of those products. The new substrate was compared with that used in the clinical technique (Zinterhofer et al 1973).

Both substrates had to be boiled (in a boiling waterbath at 100°C) for 10 min after hydration for gelatinization to occur. To

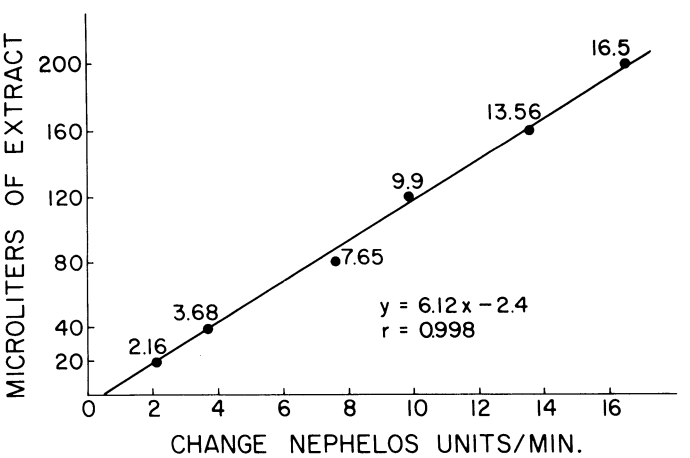

Fig. 1. The effect of enzyme concentration on change in nephelos units per minute.

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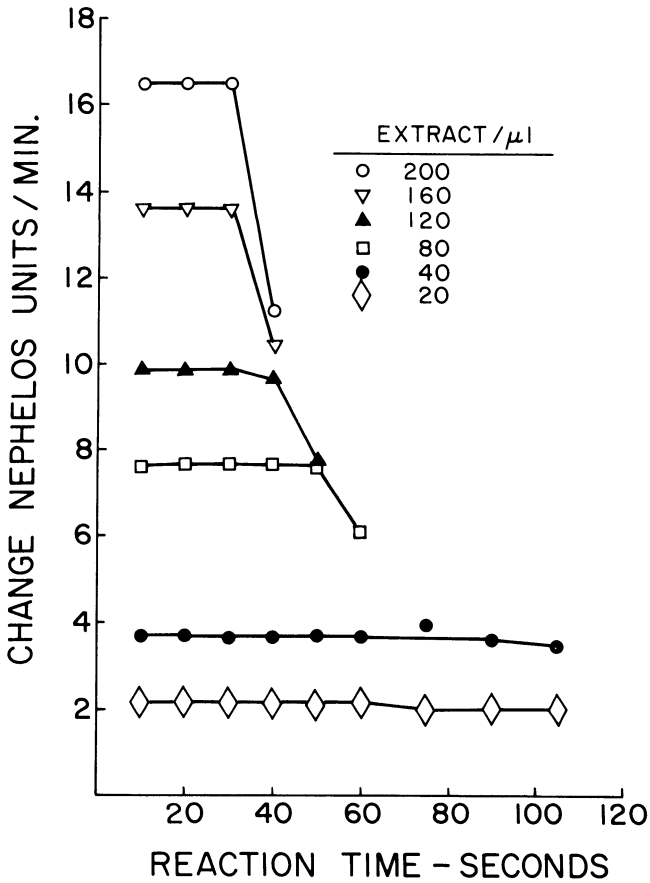

Fig. 2. The influence of enzyme concentration on maintenance of initial velocity with respect to reaction time.

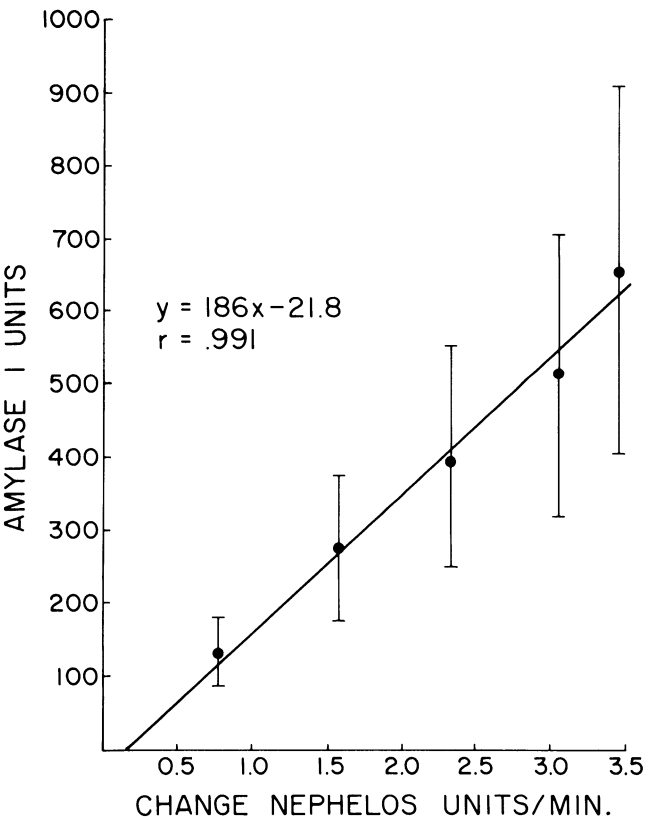

Fig. 3. Correlation between 1-min reaction velocities determined semiautomatically (amylase 1 units) and manually (nephelos units changed per minute). Vertical lines represent the adjustable range of needle deflection at various enzyme concentrations.

prevent dust contamination, hydration and boiling were done with the substrates in loosely capped screw-top Erlenmeyer flasks. Subsequently, the substrates were brought to room temperature by being allowed to cool (not by being chilled) before use. After cooling, the substrates could be refrigerated for about 24 hr without loss of quality. Light scattering of the substrates was defined against the 50-NU standard.

The Calibrator. A factory-packaged, lyophilized amylase preparation was used to standardize the system. Each of the automated modes of the unit was calibrated against a specific volume of mixture consisting of this amylase preparation and prepared substrate (Campbell 1980).

Sample Preparation and Assay Procedure. Whole grain was ground in a Udy Cyclone grinder to pass a screen with openings of 0.024-in. diameter. Four grams of the meal obtained was extracted (agitation with a magnetic stir bar) with 20 ml of a solution that was 0.5% NaCl and 0.02% $CaCl_2$. The speed of the magnetic stirrer was set so that foaming of the suspension during the 10-min extraction was kept to a minimum. After extraction, the slurry was poured into a funnel lined with Whatman 2V filter paper, and the filtrate was collected.

During the 10-min extraction process, 3.0-ml aliquots of prepared substrate were pipetted into disposable cuvettes (Evergreen Scientific, Los Angeles, CA), which were then placed in the dry block heater of the nephelometer to warm to 37°C.

Using a 200-µl SMI Micropettor, we added sample filtrate to the warmed substrate. The cuvette was capped, inverted rapidly four times, and inserted into the measurement well of the nephelometer for manual or automated assay.

The nephelos (manual) mode was also used to determine the effects of enzyme concentration, time, substrate concentration, and substrate type on initial velocity of enzyme activity. From the results, we defined the working limits for the automated assay cycles. We also investigated the interrelationship of the nephelos and automated modes of operation.

Falling Number Determination. The standard AACC method was used (AACC method 56-81).

Enzyme Source. Common soft white wheats of different sprout levels were ground and extracted. These extracts were not treated to destroy β-amylase.

One gram of ground Nugaines wheat (sprout level, 30.0%; elevator code #74-UUC-107) was extracted with 20 ml of solution

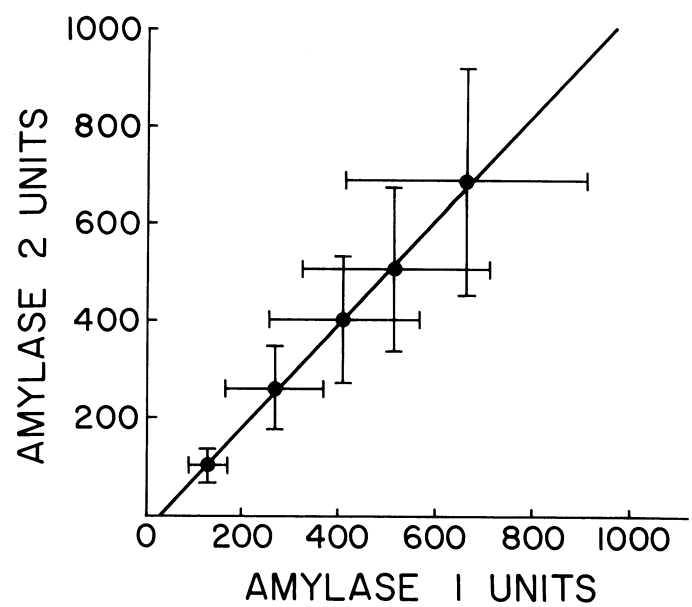

Fig. 4. Correlation of results determined with the nephelometer in the two automated assay modes, the 1-min assay (amylase 1 units) and the 2-min assay (amylase 2 units). Crosses represent the adjustable range of needle deflection at various enzyme concentrations. The values for amylase 2 above 300 are calculated.

as described above. This extract was used as a source of amylase activity in experiments to define the working limits of the assay.

A variety of soft white wheat samples were procured from the Washington State Grain Inspection Service in Colfax, WA. We used three of these, code numbered 2381, 1928, and 1818 (sprout levels 0.0, 4.5, and 9.8%, respectively), to compare reaction characteristics of β-limit dextrin and amylopectin substrates. Several other samples were selected for use in tests to determine the correlation between the nephelometric assays and falling numbers.

Treatment of Extracts with $HgCl_2$. Sample extracts were treated with $HgCl_2$ to inhibit β-amylase activity. The stock solutions of inhibitor consisted of 2, 4, 6, and 10-m*M* $HgCl_2$ in distilled water. Replicate extracts were pooled, mixed, and divided into 10-ml samples. From each of these samples, 0.1 ml of extract was removed and replaced by an equal amount of one of the stock inhibitor solutions. The mixtures were agitated on a magnetic stirrer for 30 min before assay.

RESULTS AND DISCUSSION

In assay systems in which either disappearance of substrate or appearance of product can be quantitated, initial velocity of an enzymatic reaction is directly proportional to the amount of enzyme present. The nephelometric assay, which is based on light scattering, measures neither substrate nor product quantitatively. Nevertheless, the amount of sprouted wheat extract assayed by the manual method correlated highly with the initial velocity of enzyme activity (Fig. 1).

In the semiautomatic modes, the instrument indicates only the difference between measurements made for the sample at the beginning and end of the assay cycle. The differences, therefore, are velocity terms and are expressed as arbitrary units per minute or per 2 min (amylase 1 units or amylase 2 units). The initial velocity of many of the reactions assayed manually was maintained for less than a full minute; therefore, the relationship between velocity and enzyme concentration determined semiautomatically would not be linear for high-activity samples. Because the 2-min assay is the more sensitive of the automated assays and therefore measures a smaller range of change in NU, our major concern was for the 1-min assay. Because it measures a larger range of NU change, we felt it might be particularly susceptible to error induced by substrate limitation and/or product (feedback) inhibition. Both phenomena would inhibit enzyme activity and prevent the initial reaction velocity from being maintained in the late stages of the assay period. Therefore, we performed experiments to define the maximal velocity of enzyme activity that could be sustained without change for 90 sec. This period was adequate, we felt for a substrate-extract mixing and assay time of approximately 70 sec. The extra 10 sec in the assay cycle allowed for the lag period of the unit before assaying began. Figure 2 shows that the maximal velocity of enzyme activity that fulfilled our criteria for constant activity was approximately a 4-NU decrease per minute.

The values obtained manually for enzyme activity correlated linearly with those obtained semiautomatically. Various concentrations of a sprouted wheat extract were assayed by both methods, and the results correlated well. In Fig. 3, values for the 1-min automated cycle represent the midpoint of the adjustable range of needle deflection following the assay. Those values were related to the initial reaction velocities by the following equation:

$$\text{Amylase 1 units} = (186)\,(\text{NU/min}) - 21.8.$$

By applying this equation to the data in Fig. 2, we found that amylase 1 units above 720 would not be reliable if obtained with the present, commercial β-limit dextrin. Further research by the

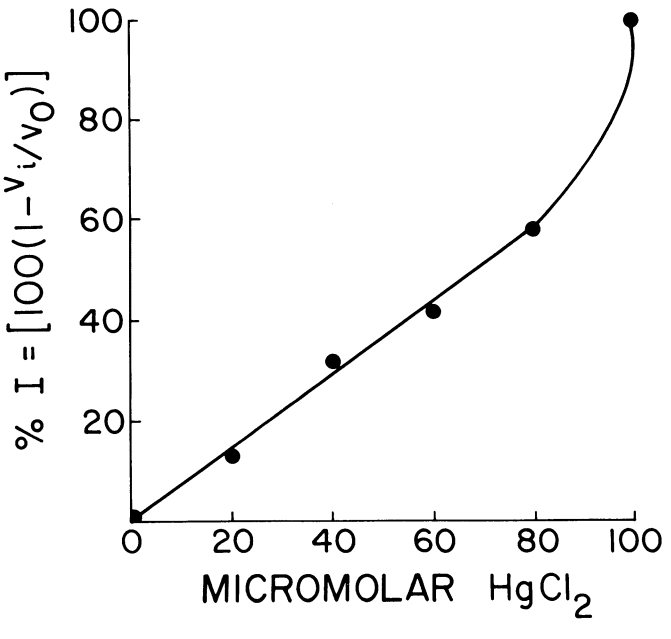

Fig. 5. Percent inhibition (I) of amylase activity from sound wheat on amylopectin substrate as a function of increasing $HgCl_2$ concentration (μ*M*). v_I = Inhibited, v_o = control.

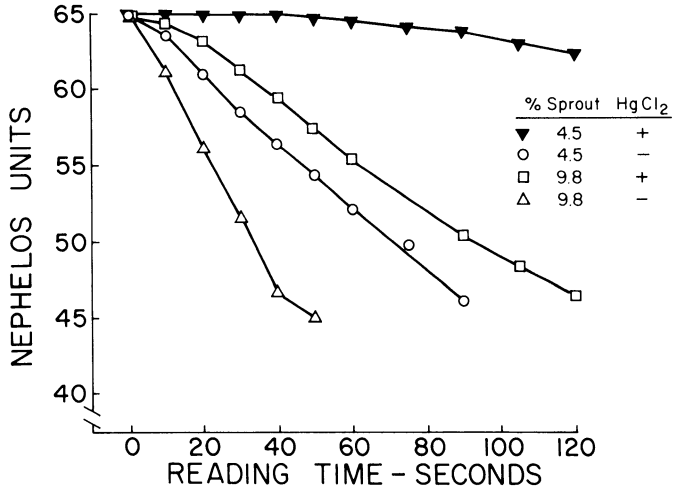

Fig. 6. Loss of amylolytic activity from wheat extracts at two different sprout levels in amylopectin substrate after extract treatment with 100 μ*M* $HgCl_2$. + = $HgCl_2$ added, − = no $HgCl_2$.

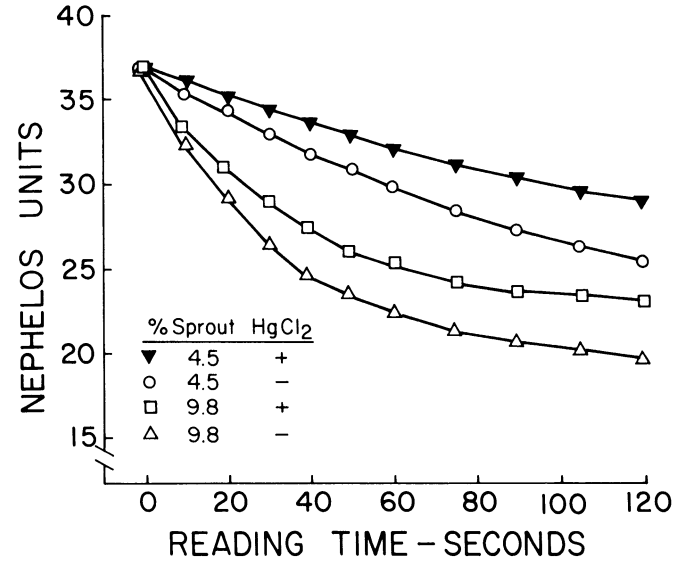

Fig. 7. Loss of amylolytic activity from wheat extracts at two different sprout levels in β-limit dextrin substrate after extract treatment with 100 μ*M* $HgCl_2$. + = $HgCl_2$ added, − = no $HgCl_2$.

manufacturer is necessary to determine the cause(s) of the decrease in reaction velocity (ie, substrate limitation or product inhibition). Although a decrease in reaction velocity within a reaction time of 1–2 min is inconvenient, it should not bar use of the technique by an operator aware of this limitation. A second assay can easily be performed using a diluted or smaller aliquot of the extract.

The 2-min automated assay is the more sensitive of the automated assays. Five dilutions of the commercial substrate preparation were run on both of the automated modes; the values obtained were means of the high and low values in the adjustment range. Comparison of the values given for each extract by the two automatic cycles proved that the units for the two cycles are identical. The 2-min cycle scale is merely an expansion of the first third of the 1-min cycle scale (Fig. 4). This continuity of unit value between the two cycles allows easy comparison of values from the assay of constant extract volumes.

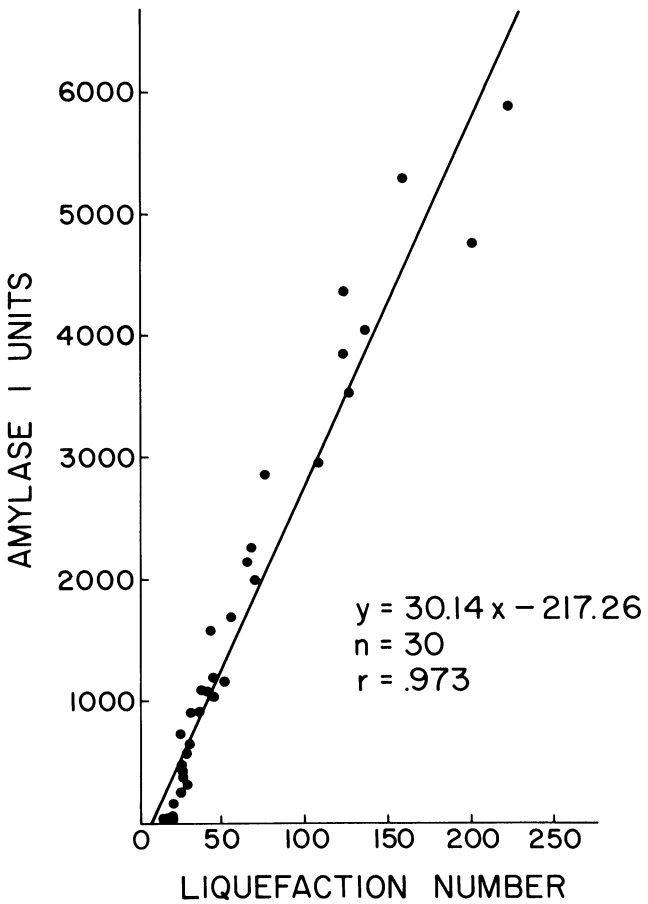

Fig. 8. Correlation of amylase 1 units with liquefaction number. All activity standardized to 200 μl of extract prepared with one part by weight of wheat and five parts by volume of extractant.

Kruger et al (1979) reported that this nephelometric technique was not sensitive and that the results did not correlate with those obtained by established methods of amylase detection. They found that sound wheat extracts and pure β-amylase preparations were very active on the clinical amylopectin substrate. In the present study, we found[4] that the addition of sound wheat extract to a preparation of β-limit dextrin substrate resulted in no measurable activity after several minutes of incubation. This type of experiment yields no data, however, concerning the contribution of β-amylase to the reaction in the presence of α-amylase. The theoretical possibility exists that the substrate will no longer limit β-amylase activity following a single cleavage by α-amylase.

We compared the contributions of β-amylase to the amylolytic activities provided by two extracts of wheat different in sprout levels. In these experiments, we used amylopectin and β-limit dextrin as substrates and $HgCl_2$ as a β-amylase inhibitor. The concentration of inhibitor was based on the results of another experiment, in which we determined the concentration of $HgCl_2$ needed to inhibit amylolysis of amylopectin substrate by an extract of sound wheat. The rapid increase in inhibition as the concentration of $HgCl_2$ was increased from 80 to 100 μm, as shown in Fig. 5, was not understood until turbidity was noted in these treated extracts. We believe that at these concentrations the inhibition was due not only to mercuric ion inactivation of β-amylase's catalytic disulfhydryl group, but also to "salting out" of enzyme.

Figures 6 and 7 show the effects of 100 μM $HgCl_2$ on enzyme activity with respect to both level of sprout damage and substrate type. Significant data from these reaction curves are summarized in Table I. Inhibition was less severe with β-limit dextrin than with amylopectin. This indicates that β-amylase contributes less error to α-amylase assays when β-limit dextrin is used as substrate. If some α-amylase had, as we believe, been precipitated by the $HgCl_2$, the contribution of β-amylase to amylolytic activity may have actually been less than indicated by our data. Decrease in inhibition with increasing sprout levels indicates selective inhibition of β-amylase; its contribution to amylolysis would have remained constant even though α-amylase activity increased.

In 100 μM $HgCl_2$, β-limit dextrin was hydrolized two to three times as rapidly as amylopectin by an identical amount of extract. As a result, small amounts of α-amylase that would not yield activity on amylopectin will cause measurable change of the β-limit dextrin when measured nephelometrically.

Amylase levels of wheat extracts analyzed by the nephelometric method, with β-limit dextrin as substrate, correlated highly with falling number data that had been transformed to liquefaction numbers by Perten's (1964) formula:

$$\text{Liquefaction no.} = \frac{6{,}000}{\text{Falling no.} - 50}$$

[4] Unpublished data.

TABLE I
Mercuric Chloride Inhibition (I) of Beta-Amylase Present in Extracts of Two Wheat Samples Differing in Sprout Damage

Enzyme Source	$HgCl_2$ Concentration (μM)	Reaction velocity (nephelos units lost per min)	v_i/v_o[a]	Percent I[b]
Amylopectin substrate				
4.5% Sprouted (No. 1928)	100	1.56	0.12	88
	0	12.90	...	...
9.8% Sprouted (No. 1818)	100	11.40	0.41	59
	0	27.60	...	...
β-limit dextrin substrate				
4.5% Sprouted (No. 1928)	100	4.74	0.67	33
	0	7.02	...	...
9.8% Sprouted (No. 1818)	100	18.00	0.77	23
	0	23.40	...	...

[a] v_i = Inhibited, v_o = control.
[b] Percent I = 100 [1 − (v_i/v_o)].

Figure 8 shows that nephelometric data could be used to accurrately predict falling number and, hence, to indicate even those lots of wheat that would be acceptable for end-product use in Japan.

The nephelometric method is a rapid technique. Furthermore, it can be applied to other cereal grains. Preliminary investigations in this laboratory indicate high correlation between millidextrinizing units (mDU's) and nephelometric data for malting barley.

ACKNOWLEDGMENT

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