A SENSITIVE AUTOMATED METHOD FOR THE DETERMINATION OF α-AMYLASE IN WHEAT FLOUR[1,2]

B. MARCHYLO and J. E. KRUGER, Canadian Grain Commission, Grain Research Laboratory, Winnipeg, Manitoba, Canada R3C 3G9

ABSTRACT

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β-Limit dextrin anthranilate was prepared from amylopectin anthranilate and employed as substrate for an automated fluorometric assay of α-amylase. α-Amylase attack upon this substrate resulted in the formation of dialyzable fluorescent products whose intensity increased linearly with increase in concentration of fungal, bacterial, and malted wheat α-amylase. β-Amylase had a negligible effect upon the assay. The method determined satisfactorily the levels of α-amylase in flours with amylograph viscosities ranging from 200 to 860 BU.

The breadmaking quality of wheat flour is strongly affected by the amount of endogenous α-amylase. Because of this, we have been interested in rapid methods of determining the levels of α-amylase present in different cultivars of wheat and their respective flours. Of the methods available, the amylograph and falling number are still the most sensitive. They have the disadvantages, however, of being time consuming and cumbersome to perform, as well as requiring a large sample for the determination. Such disadvantages may be overcome by automated methods. One method that appeared promising was the measurement of reducing sugars liberated from reduced β-limit dextrin following α-amylase action (1). The method was fully automatable and proved suitable for measuring α-amylases from cereal, fungal, and bacterial sources. It was unsatisfactory, however, for determining very low levels of α-amylase normally present in flours because of the high blank value resulting from natural levels of reducing sugars, as well as the extremely long incubation time required.

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[2]Presented at the 61st Annual Meeting, New Orleans, 1976.

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The present paper describes an automated method employing β-limit dextrin anthranilate as substrate, which eliminates such difficulties. α-Amylase attack results in the formation of dialyzable fluorescent reaction products which increase linearly with increases in enzyme concentration. The method is very sensitive and β-amylase has a negligible effect upon the assay. α-Amylase from fungal and bacterial sources, as well as from cereals can be assayed satisfactorily by this method.

MATERIALS AND METHODS

Enzyme Sources

The commercial enzymes used in this study were: fungal α-amylase, 5,000 SKB units/g (Calbiochem, Los Angeles, CA); *Bacillus subtilus* α-amylase, 1,187 absorbance units/mg (Calbiochem, Los Angeles, CA); barley β-amylase, 2,000° Lintner/g (Schwarz-Mann, Orangeburg, NY), and sweet potato β-amylase, type 1-B containing 750–1,000 Sigma units/mg protein (Sigma Chemical Co., St. Louis, MO). Each was dissolved in 0.2*M* acetate buffer, pH 5.5 containing 10^{-3} *M* $CaCl_2$ prior to use. α-Amylase was also extracted from malted Manitou wheat, heat treated at 70° C and purified by glycogen-complex formation (2).

Extraction of Wheat Flours

Flour (2 g) was added to 10 ml of 0.2*M* acetate buffer, pH 5.5, containing 10^{-3} *M* $CaCl_2$ and mixed in a VirTis Model 23 Homogenizer for 30 sec at a speed setting of 60. An aliquot of the suspension was placed in a 10-ml ultracentrifuge tube equipped with a screw cap and rotated on a Labquake rotator (Labindustries, Berkeley, CA) for 2 hr at room temperature. The mixture was centrifuged then at 96,600 × *g* for 10 min and the clear extract used for analyses.

α-Amylase Activity Using Reduced β-Limit Dextrin

Fungal α-amylase was used as reference and was assayed by measuring the reducing sugars liberated from reduced β-limit dextrin at 35° C (1). Reducing sugars were determined by an automatic neocuproin method (3) and activity was expressed as milligrams of maltose liberated per min at 35 ° C. One milligram of fungal α-amylase liberated 8 mg of maltose per min.

Amylograph Viscosity Determination

This was performed on a Brabender ViscoAmylograph using 65 g of flour and 450 ml buffer (4).

Falling Number Determination

This was carried out on a 7 g sample by the standard ICC method (5).

OPERATIONAL PROCEDURE

Preparation of β-Limit Dextrin Anthranilate

Ten grams of amylopectin anthranilate (Calbiochem, Los Angeles, CA) was suspended in a small amount of water and added slowly with stirring to 400 ml of 0.2*M* acetate buffer, pH 4.5 and the suspension brought to a boil. After cooling to room temperature, 0.2 ml of sweet potato β-amylase was added and the

mixture was stirred slowly for 1.5 days. It was then reboiled to destroy β-amylase and dialyzed against distilled water at 4° C for 2 days. After freeze-drying, the substrate was ground to a fine powder in a Moulinex electric coffee mill and stored in a dark bottle at 4° C. The yield of substrate was 50–55%.

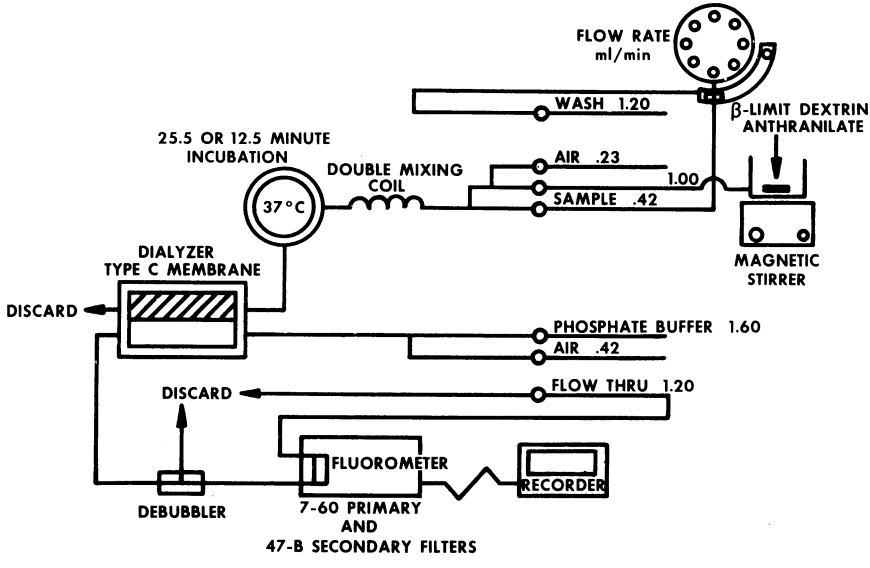

Fig. 1. Technicon AutoAnalyzer flow-sheet for determination of α-amylase, with reduced β-limit dextrin anthranilate as substrate. The sample to wash ratio was 2:1.

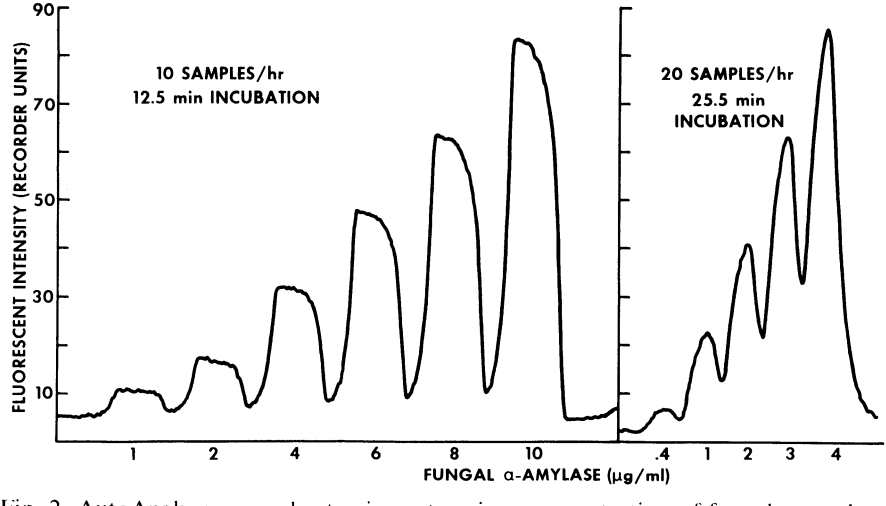

Fig. 2. AutoAnalyzer recorder tracings at various concentration of fungal α-amylase: (Left) 10 samples/hr, 12.5-min incubation. (Right) 20 samples/hr, 25.5-min incubation.

Preparation of 0.25% β-Limit Dextrin Anthranilate Working Solution

Sixty milliliters of 0.2*M* acetate buffer, pH 5.5 containing 10^{-3} *M* $CaCl_2$ was combined with 1.2 ml of 30% Brij 35 solution and brought to a boil. After removing from heat, 0.5 g β-limit dextrin anthranilate was added with vigorous stirring. The mixture was homogenized for 3 min with a VirTis Model 23 Homogenizer at a speed setting of 15 and added to 100 ml of 0.2*M* acetate buffer, pH 5.5, preheated to 90° C. After stirring for 30 min at 90° C to ensure a colloidal suspension the volume was brought to 200 ml with 0.2*M* acetate buffer, pH 5.5. The suspension was then brought to room temperature while being stirred.

Although initial experiments were carried out with 0.5% substrate a concentration of 0.25% was subsequently found sufficient to ensure linearity.

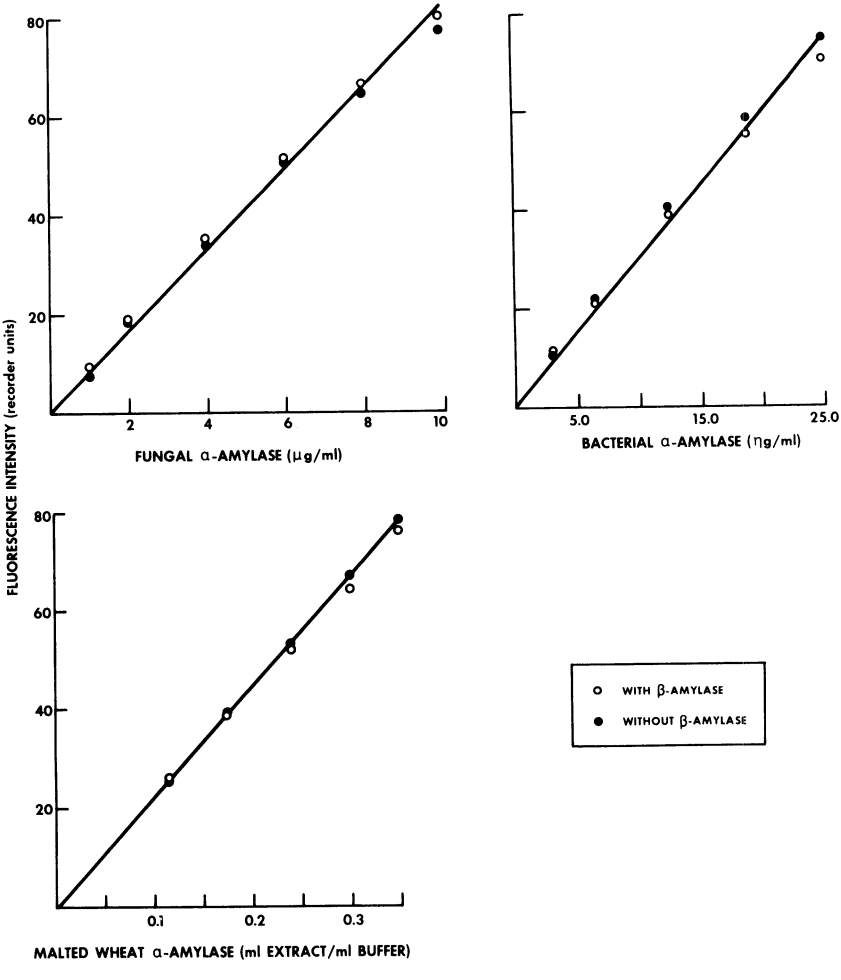

Fig. 3. Hydrolysis of β-limit dextrin anthranilate by different amounts of fungal, bacterial, and malted wheat α-amylase in the presence and absence of excess β-amylase.

Triton X-100 in place of Brij 35 proved unsuitable as a coating formed on the walls of the tubing and coils, and interfered progressively with the analyses. The substrate was stable for 2–3 days when protected from light.

Operational Procedure

The procedure was adapted from that of Rinderknecht and Marbach (6) for serum α-amylase employing amylopectin anthranilate as substrate.

The Technicon AutoAnalyzer (Technicon Corp., Tarrytown, NY) flow sheet for the assay is shown in Fig. 1. Substrate suspension was stirred continuously throughout the assay in a dark bottle. Sample, flowing at 0.42 ml/min met an air segmented stream of substrate flowing at 1 ml/min and then passed through a double mixing coil and was incubated in a 37° C bath with a 12.5- or 25.5-min mixing coil, depending upon the sensitivity required. Upon leaving the incubation bath the stream passed through a 24-in. dialyzer equipped with a type C membrane and the reaction products dialyzed into an air-segmented recipient stream of 0.2*M* Sorenson's phosphate buffer, pH 7.2 flowing at 1.60 ml/min. The recipient stream was debubbled and passed through a Model 111 Turner

TABLE I
Determination of α-Amylase of 24 Hard Red Spring Wheat Flours of Varying Amylograph Viscosity by the Automated Fluorometric Method

Amylograph Viscosity (BU)	Fluorescent Intensity of Blank (recorder units)	Fluorescent Intensity of Sample-Blank (recorder units)	α-Amylase Activity[a] (mg maltose/min/gm × 10^{-3})
200	11.0	48.5	9.8
205	22.5	48.0	10.6
260	13.0	52.5	10.0
275	15.5	38.5	8.2
350	12.0	35.0	7.6
355	12.5	35.0	7.2
400	13.0	36.5	7.0
430	14.0	29.0	6.4
430	13.0	34.5	6.6
460	11.5	29.5	6.4
470	13.0	33.0	7.3
490	12.0	30.0	6.7
530	17.0	31.0	6.4
580	13.0	28.0	5.3
585	15.5	28.5	5.4
595	11.5	25.5	5.6
615	14.5	21.5	4.8
620	13.5	33.0	6.2
635	22	25.5	4.8
645	16	28.5	5.4
650	11.0	21.5	4.6
660	12.0	28.5	6.2
725	11.0	24.5	5.4
860	12.5	21.0	4.6

[a]Converted from fluorescent intensity recorder units with a fungal α-amylase reference.

Fluorometer equipped for automated chemistry. The fluorometer contained a No. 7-60 primary and a No. 47-B secondary filter and the light sensitivity was set at 10X or 30X. Lower and upper limits of fluorescent intensity were set at 5 and 95%, respectively, by running fungal standards of the appropriate concentrations. Typical tracings, with varying concentrations of fungal α-amylase assayed at 10 and 20 samples per hr, are shown in Fig. 2. The pH of the recipient stream was very important and at a pH lower than 7.2 the fluorescent intensity decreased markedly.

RESULTS AND DISCUSSION

α-Amylase from Fungal, Bacterial, and Malted Wheat Extracts

α-Amylases from fungal, bacterial, and malted wheat extracts were tested for their abilities to break down β-limit dextrin anthranilate into dialyzable fluorescent products. The results obtained with a 12.5-min incubation at 37° C and a fluorometer aperture setting of 10X are shown in Fig. 3. In all cases fluorescent intensity increased linearly with increase in enzyme concentration. Sensitivity of the assay could also be increased considerably. For example, as little as 0.002 SKB units of fungal α-amylase activity could be detected with a 25.5-min incubation and 30X aperture setting of the fluorometer.

As fluorescent intensity is an arbitrary measure of enzyme activity, fungal α-amylase was selected as a reference. In addition to availability in large quantities, its potency decreased extremely slowly with time, if stored in the cold. The potency of the α-amylase 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 37° C per min per milligram of enzyme. An appropriate series of dilutions of fungal α-amylase such as in Fig. 3 were run at least twice during a normal working day, and fluorescent intensity values could be converted into the amount of maltose liberated from reduced β-limit dextrin.

The most useful feature of using β-limit dextrin anthranilate for determination of α-amylase was that excess barley β-amylase (20 μg) had no effect upon the assay, as observed in Fig. 3. This indicated its particular suitability for determining cereal α-amylases where β-amylases may be in excess.

α-Amylase From Wheat Flour

To analyze for the α-amylase present in wheat flours, a 25.5-min coil in the 37° C incubation bath and the 30X aperture setting on the fluorometer were chosen. This provided the required sensitivity to detect the low levels of α-amylase in flours with very high amylograph viscosities. As flour extracts contained some dialyzable fluorescent compounds, a blank was run with each sample. These were prepared by heating an aliquot of the flour extract at 90° C for 10 min to inactivate the α-amylase, cooling rapidly to room temperature and filtering through glass wool.

Twenty-four flour samples with varying amylograph viscosities selected from Canadian HRS wheats (either plant breeders' cultivars or Canadian export cargoes) were examined. The results are shown in Table I. Actual fluorescent intensities have been included to show typical values for flours and blanks. Thus blank values due to inherent dialyzable fluorescent substances in the flour accounted for 18–46% of the total fluorescent intensity valued, although the

average contribution was around 30%. Fluorescent intensity values for all of the flours fell within the scale of the recorder and dilutions were not necessary. The fluorescent intensity values of the sample minus blank were converted to α-amylase activity with a fungal α-amylase reference and a two-fold difference in activity was found between the high and low amylograph flours. As shown in Fig. 4, the α-amylase activity decreased curvilinearly with increase in amylograph viscosity. Similar behavior has been observed when the α-amylase determined by other methods such as the ICC (7), viscometric (8), or Phaedabas tablet method (9) is plotted against the amylograph viscosity. Deviations from the curve were observed for some samples and can be expected as the amylograph of a particular wheat cultivar may be affected by factors other than α-amylase such as the nature of the starch (10). A similar curvilinear behavior was observed when falling number values for a number of whole wheat flours were plotted against α-amylase activity. However, when α-amylase activity was plotted *vs.* the reciprocals of the amylograph or falling number units, as suggested by Hlynka (11), a straight line relation between the methods was observed (Fig. 5). Highly

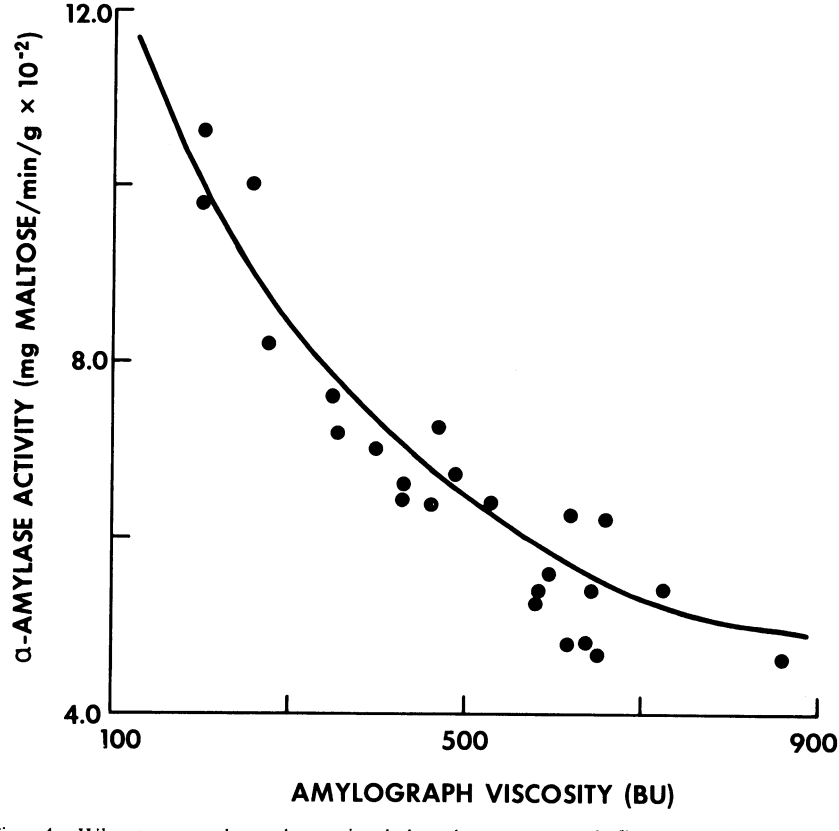

Fig. 4. Wheat α-amylase determined by the automated fluorometric method *vs.* amylograph viscosity.

significant correlations were seen between α-amylase determined by the present automated method and the reciprocals of both the amylograph and falling number values.

CONCLUSIONS

The fluorometric method described in this paper can determine the α-amylase activities from fungal, bacterial, or cereal sources. It is particularly suitable for determining the α-amylase present in wheat flour where very low levels of α-amylase may be present and where β-amylase is in large excess. In the method as described, 20 analyses per hr can be performed, with each peak attaining near maximum fluorescence intensity. Samples throughput could be increased considerably, although reduced fluorometric intensity for individual peaks would occur. We have noticed that the flow cell of the Turner Model III fluorometer is rather large. This causes undue mixing which might be minimized with other types of fluorometers. Even at 20 analyses per hr, however, more determinations can be performed in a working day than with the amylograph or falling number apparatus and with much less operator manipulation. Some operator time is required, however, in preparation of extracts. These can be prepared conveniently for the following day's analyses while the previous day's samples are being analyzed. The present method required as little as 0.84 ml of

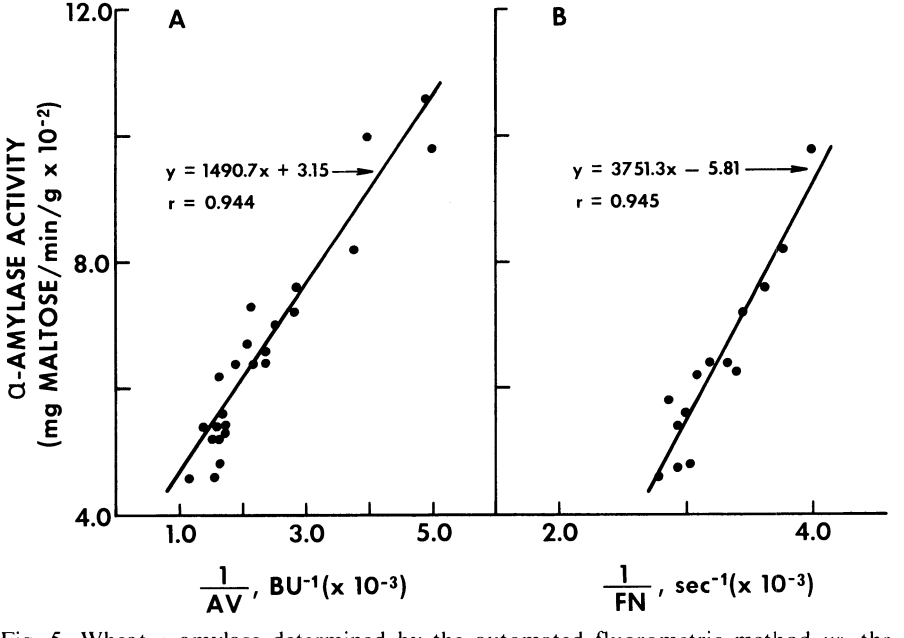

Fig. 5. Wheat α-amylase determined by the automated fluorometric method *vs.* the reciprocals of the amylograph viscosity (AV) and falling number (FN).

extract for one determination. It will be very useful, therefore, for plant breeders involved in screening large numbers of samples in which a limited amount of material is available.

Literature Cited

1. KRUGER, J. E., and MARCHYLO, B. The use of reduced β-limit dextrin as substrate in an automated amylase assay. Cereal Chem. 49: 453 (1972).
2. KRUGER, J. E., and TKACHUK, R. Wheat alpha-amylases. I. Isolation. Cereal Chem. 46: 219 (1969).
3. DYGERT, S., LI, L. H., FLORIDA, D., and THOMA, J. A. Determination of reducing sugar with improved precision. Anal. Biochem. 13: 367 (1965).
4. AMERICAN ASSOCIATION OF CEREAL CHEMISTS. Approved methods of the AACC. Method 22-10, approved May, 1960. The Association: St. Paul, Minn.
5. INTERNATIONAL ASSOCIATION FOR CEREAL CHEMISTRY. Determination of "falling number" (according to Hagberg-Perten) as a measure of the degree of alpha-amylase activity in grain and flour. ICC Standard No. 107.
6. RINDERKNECHT, H., and MARBACH, E. P. A new automated method for the determination of serum α-amylase. Clin. Chim. Acta 29: 107 (1970).
7. CARR, J. G., and SPILLANE, P. A. Some factors affecting amylolytic behaviour in Irish flours. J. Sci. Fd. Agric. 20: 638 (1969).
8. TIPPLES, K. H. A viscometric method for measuring alpha-amylase activity in small samples of wheat and flour. Cereal Chem. 46: 589 (1969).
9. BARNES, W. C., and BLAKENEY, A. B. Determination of cereal alpha-amylase using a commercially available dye-labeled substrate. Die Starke 26: 193 (1974).
10. MEREDITH, P. Inactivation of cereal alpha-amylase by brief acidification: The pasting strength of flour. Cereal Chem. 47: 492 (1970).
11. HLYNKA, I. Amylograph mobility and liquefaction number. Cereal Sci. Today 13: 245 (1968).

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