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A Viscometric Method for Measuring Alpha-Amylase Activity in Small Samples of Wheat and Flour[1]

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ABSTRACT

A viscometric method has been adapted for the determination of wheat alpha-amylase activity. A simple U-tube viscometer is used to follow the drop in viscosity of gelatinized potato-starch solution when incubated with an extract of whole wheat or flour. The reciprocal of the time taken for the viscosity to decrease to one-half of its original value is used as the basis of alpha-amylase units. The significance of the effect of beta-amylase is discussed and results are compared with those of other methods of alpha-amylase analysis. Relatively small quantities of material are required and the method may be used for analysis of single kernels. Where whole kernels are used, simultaneous grinding and extraction is carried out by shaking with extracting solution and ball bearings in stainless-steel centrifuge tubes. The method was used to show that nondormant wheat increases markedly in alpha-amylase activity when germinated, and that most of the initial increase in activity originates in the germ end of the kernel.

This Laboratory has been interested for some time in the alpha-amylase activity of wheat and its relation to dormancy, sprout damage, and flour quality. During the course of the study the need arose for a suitable method of determining low levels of alpha-amylase activity in small samples, and even in single kernels, of wheat.

Estimation of alpha-amylase activity has traditionally been based on the ability of the enzyme to attack gelatinized or "susceptible" starch. The effect of amylolytic action on starch is manifested in three main ways: a decrease in the viscosity of a gelatinized starch solution; production of smaller-molecular-weight dextrins, the formation of which causes the characteristic starch-iodine blue color to change; and an increase in the number of reducing end-groups in the system.

The selection of the best method for measurement of alpha-amylase activity, and also measurement of beta-amylase activity, depends to a large extent on the relative proportions of the two enzymes in the material under study. Each enzyme produces the three effects on starch that are listed above, but at different rates. Because beta-amylase attacks starch from the nonreducing end of the chains and removes maltose units, it reduces viscosity slowly. Beta-amylase is blocked by alpha-1,6-glycosidic linkages and produces beta-limit dextrins from amylopectin

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unless alpha-amylase is present in the system. As there is usually a great excess of beta-amylase activity in sound wheat flours, measurements of beta-amylase activity are usually based on determination of reducing power of products of digestion of flour or standard substrate. Alpha-amylase determinations may also be based on reducing-power determinations, but the effect of beta-amylase predominates and may vary with different materials. Inactivation of beta-amylase by heat-treatment is sometimes carried out prior to analysis, but this may partially inactivate alpha-amylase. Therefore, reducing-power determinations on autolytic digests or on starch digests are not usually satisfactory for determination of alpha-amylase activity.

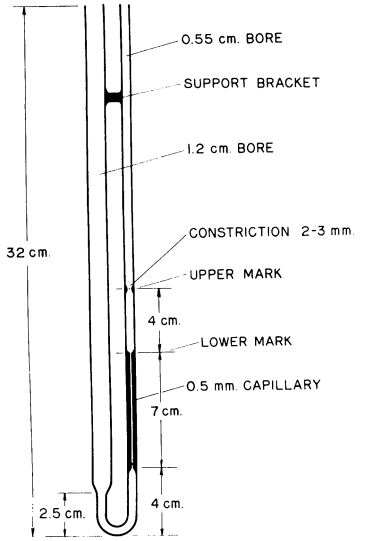
Colorimetric procedures for measurement of the rate of change in starch-iodine blue color during digestion in either autolytic or starch digests provide estimates of alpha-amylase activity, but some of these methods are quite time-consuming. In addition, they are influenced by variations in activity of beta-amylase in the extract. Some colorimetric methods are based on the use of beta-limit dextrin as substrate (1,2,3), with or without the use of excess beta-amylase (4). However, swamping of the system with beta-amylase may make the assay less sensitive to differences in alpha-amylase activity, especially if these are low. Viscosity methods such as the falling-number method (5) and amylograph procedures are autolytic procedures and, like all autolytic methods, these may be influenced by differences in starches and in damaged-starch content of the flours. Therefore, it appeared advisable to consider a viscosity method based on liquefied starch prepared from standard material for studies on alpha-amylase determination in small samples of wheat that may be low in alpha-amylase activity.

There is nothing original in using fall in viscosity of a gelatinized starch solution as a measure for determination of alpha-amylase activity. In 1935 Jozsa and Johnston(6) described an improved version of an earlier Jozsa and Gore procedure that provided a unit called the "liquefon," which was based on the percentage of starch liquefied by alpha-amylase. The proposed method is based on this procedure but is essentially on a micro scale, so that small amounts of material and solutions and a simple viscometer are used.

This paper describes this microprocedure for determination of alpha-amylase activity and reports comparisons with other methods for determination of this activity. Observations on the effect of beta-amylase on the measurements are recorded. A study of alpha-amylase development during germination of wheat is presented.

MATERIALS AND METHODS

The viscometric method finally developed requires simple apparatus, and the enzyme extracts require only small amounts of grain or flour. The sample is agitated in a steel centrifuge tube, of 25- or 60-ml. capacity, with buffer and steel balls to macerate the grist. The filtered extract, 2 ml., is added to a 3-ml. aliquot of gelatinized potato-starch solution in an Ostwald-type viscometer, and the viscosity of this solution is measured at appropriate intervals. The time required to reduce the initial viscosity to one-half of its value is the basis of calculation to viscosity reducing units.



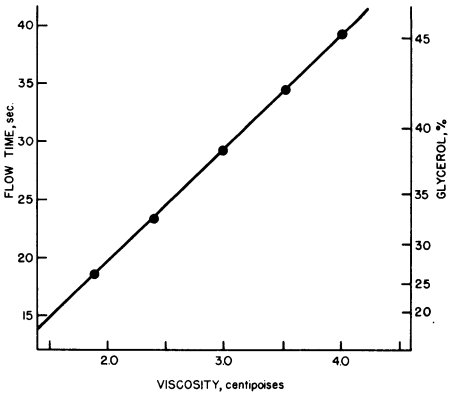


Fig. 1 (left). Viscometer dimensions.

Fig. 2 (right). Example calibration curve for a viscometer with various glycerol-water mixtures of known viscosity.

Apparatus

1. Viscometers: Figure 1 is a diagram of the Ostwald-type viscometer with dimensions. The critical dimension is the capillary bore, which should be of a size such that flow times are neither too long nor too short. The blank starch solution should preferably have a flow time of 35 to 45 sec. For purposes of standardization, each viscometer is calibrated at 25°C. against a series of 3-ml. aliquots of glycerol-water solutions ranging in viscosity from about 1.4 to 4.6 cp. Figure 2 is an example of a typical calibration curve for a viscometer. A series of glycerol-water solutions was made up according to the calibration tables of Sheely (7), and the specific gravity (25°/25°) of each solution was determined in order to calculate the exact viscosity. Figure 2 shows the range of viscometer "flow times" observed, compared with the viscosity and percentage of glycerol used. For each viscometer a straight-line calibration curve is obtained of viscosity against flow time.

2. Extraction tubes: stainless-steel centrifuge tubes with special water-tight caps with rubber washers, which are closed by tightening a central nut. For single wheat kernels, tubes of 2.7 cm. i.d. and 25-ml. capacity are used. Tubes of 3 cm. i.d. and 60-ml. capacity are used when several kernels are to be extracted.

3. Steel balls: stainless-steel balls 5/8 in. in diameter are used inside the steel extraction tubes.

4. Wrist-action shaker or equivalent, used for shaking steel centrifuge tubes with stoppered contents.

Reagents (All A.R. Grade)

1. Extracting solution: 20 g. sodium chloride and 0.2 g. calcium acetate per liter.

2. Buffer solution: Dissolve 164 g. anhydrous sodium acetate (272 g. pure crystallized) in distilled water. Add 120 ml. glacial acetic acid, and make up to 1 liter; the pH should be 4.6.

3. Gelatinized potato-starch solution (1.5%): Bring about 200 ml. distilled water to the boil. Add slowly, with stirring, a slurry of 3.75 g. BDH (British Drug Houses) potato starch in water and rinse in starch residue with water. Maintain boiling with continuous stirring for 4 min. Cover with watch-glass and cool in 25°C. water bath for 1 hr.

Add 5 ml. buffer solution and make up to 250 ml. with distilled water. Make up fresh starch solution daily.

Procedure

Preparation of Wheat or Flour Extract. Whole kernels are stored for several days prior to the determination in a desiccator over saturated sodium bisulfite solution. Wheat moisture content equilibrates to about 13.8%. This removes the necessity of carrying out a moisture determination. For single-kernel work, the kernel is weighed, placed in a 25-ml. stainless-steel centrifuge tube, and crushed with a stainless-steel rod. Two steel balls and 18 ml. of extracting solution are added; the small headspace is flushed with nitrogen and the cap secured. Where several kernels of whole wheat are to be analyzed, about 0.3 g. of kernels is taken in a 60-ml. tube, and 50 ml. of extracting solution and three ball bearings are added. Extractions are carried out on a wrist-action shaker for 1 hr. at room temperature. The tube contents are then filtered through No. 4 filter paper, the first few drops of filtrate being discarded. The extracts are kept in a 25°C. water bath until used. Flours may be extracted in the normal way by using 0.5 to 1 g. of flour, and 1 teaspoon of acid-washed dry sand plus 50 ml. extracting solution in a 125-ml. Erlenmeyer flask. The flasks, placed in a water bath at 25°C., are shaken every 15 min. and filtered after 1 hr.

Determination of Blank. A viscometer blank is prepared by adding 2 ml. extracting solution to 3 ml. starch solution at 25°C. and mixing in a 125-ml. Erlenmeyer flask. An aliquot of the mixture (3 ml.) is pipetted into a viscometer maintained in an accurate and sensitive water bath at 25°C. After 0.5 to 1 hr. the viscosity is determined by sucking the liquid up the narrow limb to above the upper mark and determining the time taken for the top meniscus to pass from the upper mark to the lower mark. The process is repeated until satisfactory replicates are obtained. The viscosity is found from the calibration curve of the particular viscometer used. A length of rubber tubing attached to a water pump is suitable for sucking up liquid by holding the end of the tubing against the end of the viscometer limb.

Determination of Test Extract Activity. Two milliliters of extract and 3 ml. starch solution are mixed at 25°C., and 3 ml. of the mixture is pipetted into a viscometer. The exact time of initial mixing of extract and starch is noted. After about 10 min. the "flow time" (i.e., time taken to fall from upper to lower mark) is determined. The reaction is continued, periodic flow-time measurements being taken until the viscosity has dropped to one-half of the blank viscosity value. A total of five or six readings is normally sufficient. A graph is then made of log reaction time against viscosity and a straight line is drawn through the points. The

TABLE I. EXAMPLE OF CALCULATION OF ALPHA-AMYLASE ACTIVITY OF FLOUR SAMPLES

Weight taken	1.0546 g.
Moisture content	14.7%
Volume of extracting solution	50 ml.
Aliquot taken	2 ml.
Blank flow time (viscometer A)	39.6 sec.
Blank viscosity (from viscometer A calibration curve)	4.06 cp.
Reaction time of test extract to reach half blank viscosity (2.03 cp.)	33.4 min.
Reciprocal of half-viscosity time (activity of aliquot)	0.0299
Activity in original extract, 0.0299 X (50/2) =	0.748 VR units[a]
Activity per g. flour, 0.748/1.0546 =	0.709 VR units
Activity per g. corrected to 14% m.b.	0.71 VR units

[a]Viscosity reducing units.

reaction time corresponding to the "half viscosity" is found. Ideally the reaction time should be between 15 and 60 min. If the reaction time is too short the determination should be repeated with a diluted extract. If the reaction time is over 2 hr., then a repeat extraction should be made on a larger sample. Where no more sample is available, or a single kernel has been used, it will suffice to note activity as being less than the value corresponding to a 2-hr. half-viscosity time.

Alpha-amylase activity is calculated as follows: The reciprocal of the half-viscosity time is taken as the activity in arbitrary "viscosity reducing (VR) units." This activity is calculated back to the original extraction solution volume (50 or 18 ml.) by multiplying by (for example) 50/2 or 18/2. The value is then divided by the weight of flour or wheat taken and corrected either to a dry-weight basis or to a constant moisture-content basis.

An example of activity calculation is given in Table I.

Determinations should be carried out in duplicate, although with single kernels only one extract will be available for replicates. In the present study the standard error of a single determination over a wide range of activities was ± 5% of the activity being measured.

Care should be taken to prevent the formation of bubbles in the test liquid, and excessive movement of the liquid up and down the narrow limb should be avoided because mechanical shear will reduce the effective viscosity.

RESULTS AND DISCUSSION

Effect of Beta-Amylase

In sound wheats and flours there is normally a large excess of beta-amylase compared with the level of alpha-amylase. Although alpha-amylase has a much more striking effect on gelatinized starch viscosity than has beta-amylase, which reduces molecular weight gradually by the progressive removal of maltose units from the nonreducing end of a starch chain, the effect of beta-amylase is still significant if the alpha-amylase activity is low.

An experiment was carried out to determine the effect of added beta-amylase on the value obtained in this test. Crystalline sweet-potato beta-amylase was used and its beta-amylase activity was checked by the 3,5-dinitrosalicylic acid test (8).

Table II shows results of adding various amounts of beta-amylase to two flours.

TABLE II. EFFECT OF ADDITION OF PURE SWEET-POTATO BETA-AMYLASE ON VISCOMETER MEASUREMENT OF ALPHA-AMYLASE ACTIVITY IN FLOUR EXTRACTS

	Level of Beta-Amylase Activity	Alpha-Amylase
		VR units[a]/ g. dry weight
Flour A	Natural	0.78
	Natural x 2	1.06
Flour B	Natural	1.37
	Natural x 1.6	1.77
	Natural x 6	4.16
	Natural x 10	5.81

[a]Viscosity reducing.

Although beta-amylase does have an effect, it is only marked when the amount present is changed greatly. In most flours and wheats the salt-extractable beta-amylase activity is fairly constant, and probably does not vary by more than about ± 20% from a mean value. For example, Table III shows beta-amylase activities for a series of flours milled from varieties of HRS wheats grown by Canadian plant breeders in 1967. Even in the most extreme case in this series the variation in activity represents a difference of only 12% from the mean.

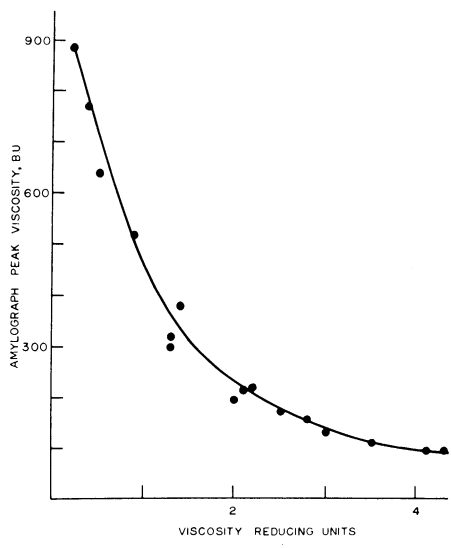


Fig. 3 (left). Amylograph peak viscosity vs. viscosity reducing units for a series of wheat flours.

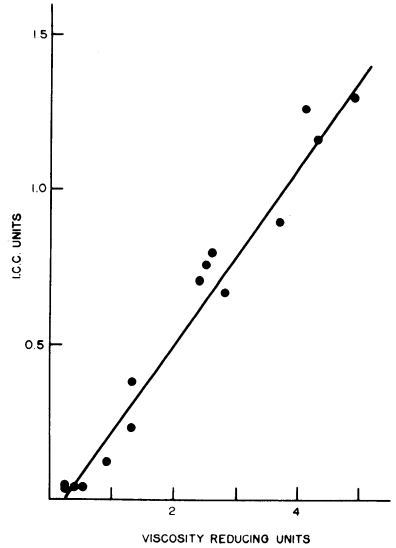


Fig. 4 (right). ICC colorimetric units of alpha-amylase activity vs. viscosity reducing units for a series of HRS wheat flours.

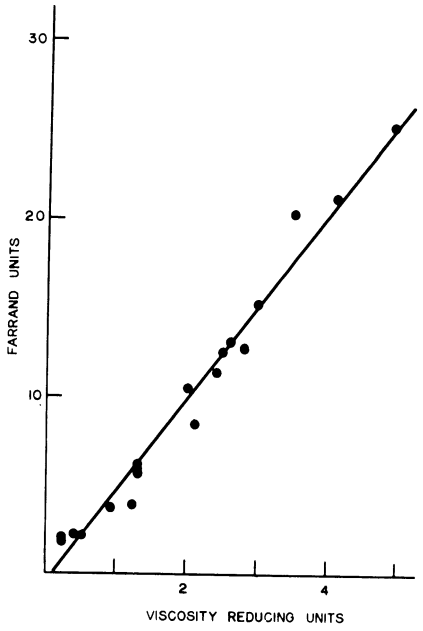


Fig. 5. Farrand alpha-amylase units vs. viscosity reducing units for a series of HRS wheat flours.

Comparison of the Method against Other Alpha-Amylase Methods

Results from this method were compared with results for the amylograph test (65 g. flour and 450 ml. water), the ICC colorimetric method (1), and the Farrand colorimetric method (2).

Figures 3, 4, and 5 show results for each of these three methods plotted against results obtained by the viscometric method for a series of Canadian flours.

Because amylograph viscosity decreases with increasing alpha-amylase activity, the relation between the peak viscosity and enzyme activity is curvilinear. Increments of alpha-amylase activity have a much greater effect on the amylograph at the low-activity end of the scale than where there is already high activity and the values are compressed. Results by the viscometric method compare well with amylograph viscosity values, particularly with sound flours. Some small discrepancies may be explained by differences in starch-damage level between flours. For flours of similar alpha-amylase activity, amylograph values drop with increasing starch damage, but the viscosity-reducing units should not be affected, owing to the use of an external substrate.

Of the two colorimetric methods examined, the Farrand method compared rather more favorably with viscosity reducing units than did the ICC method. Neither of the colorimetric methods seemed to be as sensitive as the viscometric method at low levels of alpha-amylase activity.

Study of Alpha-Amylase Activity during Germination of Wheat

The method has been applied in our Laboratory for studying the behavior of nondormant and dormant wheat during germination tests.

TABLE III. BETA-AMYLASE ACTIVITY OF A SERIES OF FLOURS FROM VARIETIES OF HRS WHEATS GROWN BY CANADIAN PLANT BREEDERS IN 1967

Variety	Beta-Amylase Activity[a] mg. maltose/g. flour	Variety	Beta-Amylase Activity[a] mg. maltose/g. flour
Marquis	555	C.T. 299	575
Manitou	606	C.T. 430	628
Park	578	C.T. 518	656
Thatcher	575	C.T. 758	584
Cypress	652	C.T. 765	525
C.T. 282	601	C.T. 768	515
C.T. 292	622	C.T. 772	627
C.T. 297	520	Mean	588

[a]By the 3.5 dinitrosalicylic acid method (ref. 8). Results expressed as mg. maltose liberated per g. flour in 5 min. at 20°C. and pH 4.6 from 0.5% gelatinized soluble starch.

For example, a series of experiments was carried out on a sample of HRS wheat (variety Selkirk) that was completely nondormant. When subjected to germination tests at room temperature on damp filter paper inside Petri dishes, all kernels germinated within 17 hr. and alpha-amylase activity increased, as shown in Table IV. Activity was determined by air-drying kernels after the specified germination period, then storing in a desiccator at constant humidity prior to analysis.

There was a rapid increase in total alpha-amylase, such that after 41 hr. of germination at 70°F. the increase in total alpha-amylase was almost 150-fold.

Most of the initial production of alpha-amylase appears to take place in the germ end of the kernel. For example, an analysis of 17- and 24-hr. germinated material gave the results shown in Table V. Even after 24 hr. of germination the distal-end activity was only 1.9 units per g. whereas the whole-kernel activity was 49.5 units per g. Hence, the initial synthesis of alpha-amylase would seem to take place in the germ end of the kernel.

There is a synthesis of alpha-amylase in the distal portions, presumably in the aleurone layer, as demonstrated by the increase in activity of separated distal ends. Whole kernels removed after 0, 7, 17, and 24 hr. of germination were cut into two parts. The cut surfaces of the distal halves were sealed with silica grease and replaced on the Petri dishes for further periods of time. After the further "germination" periods the halves were removed and air-dried, the silicone grease was removed, and the alpha-amylase activity was assayed. Results are listed in Table

TABLE IV. CHANGES IN ALPHA-AMYLASE ACTIVITY DURING GERMINATION OF NONDORMANT WHEAT

Germination hr.	Visual Appearance	Alpha-Amylase VR units/g. dry weight
0		1.6
7	No chitting evident	1.3
17	All kernels chitted	8.8
24	Chitting well advanced	50
31	Plumule 3 mm.; radicle 6 mm.	91
41	Plumule 4 mm.; radicle 10 mm.	224

TABLE V. ALPHA-AMYLASE ACTIVITY OF NONDORMANT WHEAT GERMINATED FOR 17 AND 24 HOURS

Parts Analyzed	Germination	
	17 Hours VR units/ g. dry wt.	24 Hours VR units/ g. dry wt.
Whole kernels	8.8	49.5
Germ ends	25.3	....
Distal ends	1.3	1.9

VI. The magnitude of the increase in distal-end activity is related to the length of time of the initial germination, although even with no initial germination a fourfold increase was demonstrated after 160 hr. of distal-end germination.

TABLE VI. ALPHA-AMYLASE ACTIVITY OF DISTAL HALVES OF NONDORMANT KERNELS AFTER GERMINATION OF WHOLE GRAIN (in viscosity reducing units per g. dry weight)

Initial Germination Period of Whole Kernels hr.	Additional Germination Period for Distal Halves (Hours)				
	0	24	48	72	160
0		1.8		2.2	5.2
7	1.3	1.4	2.4	3.3	
17	1.3	1.2	2.4	3.8	
24	1.9	2.0	3.2	8.8	

GENERAL DISCUSSION

The viscometric method described above was found to correlate reasonably well with other methods of alpha-amylase estimation, and it is thought to be more sensitive than colorimetric procedures for sound flours of low alpha-amylase activity. Beta-amylase, although it can influence results in other materials, does not pose a serious problem in wheat and flour owing to the relatively constant level of the salt-extractable beta-amylase normally found in these materials.

Acknowledgments

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