A COLORIMETRIC METHOD FOR THE DETERMINATION OF ALPHA-AMYLASE ACTIVITY (ICC METHOD)[1]

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ABSTRACT

A colorimetric method for the determination of alpha-amylase activity in cereals and cereal products as well as in fungal and bacterial preparations is described. The alpha-amylase activity, determined at 30°C., is expressed as a function of alpha-amylase concentration and of the velocity constant for the hydrolytic degradation of limit dextrin. The results are convertible to the well-known SKB-units and H-units.

Many modifications of the original Wohlgemuth method (1) to make the Wohlgemuth principle more suitable for the determination of alpha-amylase in wheat and rye have been suggested (2–6). Colorimetric methods have been the most satisfactory (7–10).

The method of Sandstedt, Kneen, and Blish (11), based on the Wohlgemuth procedure (1), has achieved wide acceptance for measuring alpha-amylase activity. In this method (12) alpha-amylase activity is expressed in terms of the digestion time required for the enzyme to convert limit dextrin under the influence of an excess of beta-amylase to products which give a red-brown coloration with iodine. The end point can be determined by use of a dextrin-iodine standard or a permanent glass color standard (13). The method is satisfactory for malt and other enzymatic products, but it is not sufficiently sensitive and is very time-consuming for the determination of small amounts of alpha-amylase in normal flours.

The General Assembly of ICC (International Association for Cereal Chemistry) decided in June 1964 to accept a colorimetric method for the determination of alpha-amylase activity as a preliminary ICC basic method. The method described in this paper, based on the H-unit method proposed by Hagberg (10), has been devised by the author and revised by the members of the ICC study group on "Determination of amylase activity and sprouting."

Determination of Alpha-Amylase Activity

Scope. The method is applicable to cereals or cereal products ranging from very low to very high in alpha-amylase activity. It can also

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be used for estimating the alpha-amylase activity of additives of fungal and bacterial origin.

Principle. The alpha-amylase activity of an enzyme extract is determined at 30°C. by kinetic measurement of its reaction with a standard limit dextrin substrate made from Lintner starch.

Reagents. The reagents are, as far as possible, those recommended by the American Association of Cereal Chemists, Method 22–01 (12). The chemicals used must be of analytical reagent quality.

Method

Reagents:

1. Soluble starch. Use Merck's soluble Lintner starch, special for diastatic power determination. Available from Merck & Co., Inc., Rahway, N.J.
2. Beta-amylase. Use special beta-amylase powder free from alpha-amylase, available from Wallerstein Co., Staten Island 3, N.Y., or use material of equivalent quality.
3. Stock iodine solution. Dissolve 11.0 g. of potassium iodide in a minimum of distilled water and add 5.50 g. of iodine crystals. Stir until iodine is dissolved and dilute to 250 ml. Store solution in darkness in a brown bottle. Make fresh solution monthly.
4. Dilute iodine solution for analysis. Dissolve 40.0 g. potassium iodide in distilled water, add 4.00 ml. stock solution (reagent 3), and dilute to 1,000 ml. Make fresh solution daily.
5. Buffer solution. Dissolve 120 ml. glacial acetic acid and 164 g. anhydrous sodium acetate or 272 g. sodium acetate with 3 molecules water of crystallization ($CH_3COONa \cdot 3H_2O$) and dilute to 1,000 ml.
6. Calcium chloride solution, 0.2%. Dissolve 2 g. anhydrous calcium chloride in 1,000 ml. distilled water.
7. Buffered limit dextrin substrate. Suspend 10.00 g. soluble starch (reagent 1) calculated on dry weight basis (moisture determined according to ICC standard method, i.e., drying for 1.5 hr. at 130°C.) in approx. 20 ml. distilled water, and add the suspension slowly with stirring to about 300 ml. boiling distilled water. Use wash bottle to transfer all of the starch. Boil the solution slowly while stirring for 2 min. and then cool it in running water; cover beaker with watch glass to avoid development of a "skin" on surface of starch solution. Add 25 ml. buffer solution (reagent 5) and 250 mg. beta-amylase (reagent 2), dissolved in approx. 5 ml. of distilled water. Make up volume to 500 ml. and, after addition of a few drops toluene, shake the solution. Store at room temperature for at least 20 hr. before use. A fresh solution must be prepared every 3 to 6 days. The pH of the solution should be 4.7.

Apparatus:

1. Constant-temperature bath regulated to 30° ± 0.1°C.
2. Constant-temperature bath regulated at 20° ± 0.5°C.
3. Colorimeter, provided with a yellow filter (wave length approx. 575 mμ) and with a logarithmic scale expressing absorbancy (optical density, extinction). Some colorimeters are equipped with scales showing 10 times higher values than the logarithmic values.

Sampling of cereals and cereal products: A representative sample should be taken according to the ICC standard method for sampling.

Procedure:

1. Preparation of grain sample. The quantity of grain to be ground (pulverized) must be at least 200 g.; 300 g. is better. Grind the sample (preferably in a hammer-type laboratory mill) to pass through an 0.8-mm. sieve, and then blend thoroughly.

2. Preparation of flour sample. Sift flour through an 0.8-mm. sieve to disaggregate lumps.

3. Preparation of enzyme extract. Extract 5 g. flour or finely ground grain with 100 ml. calcium chloride solution (reagent 6) for 1 hr. at 30°C. Mix sample and liquid thoroughly in the mixing tube or flask by shaking 10 times. After mixing, immediately place the tube with the sample in a water bath at 30°C. before mixing the next sample. After 15, 30, and 45 min., lift each sample from the water bath, turn upside down exactly 10 times, and reimmerse. (Continuous shaking gives different results and is not recommended.) After 60 min. of extraction, take out samples and, without shaking, immediately decant into a centrifuge tube, or pour onto a filter. Samples must not be left standing in the mixing tube. Either centrifuge the suspension 10 min. at 2,000–3,000 r.p.m. (approx. 1,000–2,000 $\times$ g), or filter until a water-clear extract is obtained. A suitable filter paper is Whatman No. 42. It is recommended that rye extracts be centrifuged since they are often difficult to filter.

4. Adjustment of colorimeter. Mix 2 ml. calcium chloride solution (reagent 6) with 10 ml. dilute iodine solution (reagent 4). Dilute with 40 ml. distilled water and attemper to 20°C. With this mixture adjust the extinction at 20°C. to the value 0 on the colorimeter (at 575 mμ). Check the adjustment at intervals during the period of measurement.

5. Substrate control. Mix 1 part of limit dextrin substrate (reagent 7) with 3 parts of 0.2% calcium chloride solution (reagent 6). Mix 2 ml. of this solution with 10 ml. dilute iodine solution (reagent 4) and dilute with 40 ml. distilled water. The extinction reading at 20°C. should be 0.55–0.60 (5.5–6.0 on certain scales).

If the extinction is too high, or too low, a tube (cuvet) with a different dimension can be used, or the dilution can be changed to use a larger or a smaller quantity of water, instead of 40 ml. The same quantity of water used in making the substrate control must also be used for determination of the zero point and for determination of all extinction values.

6. Determination of alpha-amylase activity. Attemper the standard substrate (reagent 7) to 30°C. Transfer 15 ml. of the clear enzyme extract into a test tube or flask and place in 30°C. bath. After 5–10 min., when the extract has attained a temperature of 30°C., add 5 ml. of the standard substrate, using a rapid-flow pipet, and shake the mixture vigorously. (In all measurements, the ratio between the volume of enzyme extract, or the extract diluted with 0.2% calcium chloride, and that of the substrate must be 3:1.) Simultaneously with pipetting of the substrate into the extract, start a stop watch or record the time. At intervals of 5 or 10 min. take the following actions:

(a) pipet a 2-ml. volume of this solution into 10 ml. iodine solution (reagent 4) prediluted with 40 ml. distilled water (at 20°C.);
(b) attemper to 20°C.;
(c) read the extinction on the colorimeter.

The enzyme concentration selected should be such that the final reaction time at the last extinction reading is between 15 and 40 min. for a limit dextrin conversion of 35 to 60% (i.e., 35 to 60% of the extinction of the substrate control). If the extinction decreases too rapidly, dilute the enzyme

extract with calcium chloride solution (reagent 6) and make a new determination. With very low enzyme concentrations as for some flour samples, it is necessary to extend reaction times to 60 min. or more in order to obtain accurate readings.

Expression of results:

1. Method of calculation and formulas used. Alpha-amylase activity (A) is expressed as a function of alpha-amylase concentration and of the velocity constant for the hydrolytic degradation of limit dextrin. Calculate the activity using equation 1:

$$A = \frac{500\,k}{c/f} \qquad (1)$$

where: A = alpha-amylase activity;
500 = factor to obtain more practicable values;
k = velocity constant for hydrolytic degradation of limit dextrin;
c = concentration of enzyme extract: normally c = 5 (5 g. sample extracted with 100 ml. of calcium chloride solution);
f = dilution factor in case the enzyme extract has been diluted; without dilution (5 g. sample extracted with 100 ml. of calcium chloride solution), f = 1; with dilution of 1 part of normal extract with 1 part of calcium chloride solution, f = 2.
k = $(\log E_{t_1} - \log E_{t_2})/(t_2 - t_1)$.
E_{t_1} and E_{t_2} = extinction values at times t_1 and t_2; normally the first reading made at 5 or 10 min. is chosen as E_{t_1}, although other points may be used;
t_1 and t_2 = measuring times (min.).

By substituting in equation 1, assuming c = 5,

$$A = \frac{100 \times f}{t_2 - t_1} \times (\log E_{t_1} - E_{t_2})$$

Graphical calculation of alpha-amylase activity (A).

For controlling the reliability of extinction readings, it is recommended that *log E* values be plotted on ordinary graph paper, against the reaction time in min. The points should all fall on a straight line. It is possible to calculate the alpha-amylase activity (A) from the slope (b) of this line according to equation 2:

$$A = 100 \times f \times b \qquad (2)$$

where: f = dilution factor
b = p/q (see Fig. 1, Example 2)
$p = \log E_{t_1} - \log E_{t_2}$
$q = t_2 - t_1$

Example 1. A 5-g. sample was extracted with 100 ml. of calcium chloride solution. Since the rate of the reaction with limit dextrin was too high, the enzyme extract was diluted with calcium chloride solution:
To 1 part of extract, 1.5 parts of calcium chloride solution was added.
Therefore, f = 1 + 1.5 = 2.5.
After time intervals of 5, 10, and 20 min. after mixing of the substrate and enzyme extract, the extinction readings were:

E_5 — 0.498 corresponding log value 0.6972 — 1
E_{10} — 0.425 corresponding log value 0.6284 — 1
E_{20} — 0.308 corresponding log value 0.4886 — 1

t_1 was chosen at 5 min. and t_2 at 20 min.

$$\text{Then, } A = \frac{100 \times 2.5}{(20 - 5)} \times (0.6972 - 0.4886) = 3.5.$$

Example 2. This example is shown graphically in Fig. 1.

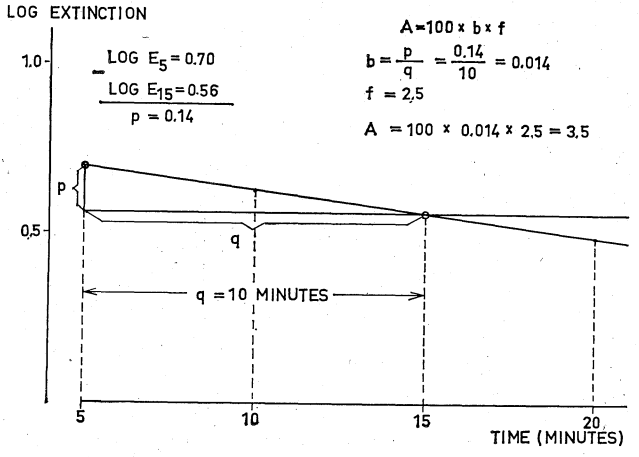

Fig. 1. Graphical calculation of alpha-amylase activity from extinction readings. (Same conditions as in Example 1.)

2. *Accuracy of the determination.* The results should be recorded to one figure beyond the decimal point. The maximum permissible difference between results of duplicate determination should not be greater than ± 5% of the mean value. For values less than 2, a difference of ± 0.1 units is permissible. The activity values for unsprouted grain are normally less than 1.

Relationships among alpha-amylase activity (A), the H-unit, and the SKB-unit. Many samples have been analyzed by the present method, the SKB method (12), and the method of Hagberg (10). Representative data are shown below (Table I).

TABLE I

Sample	A-Units	H-Units	SKB-Units	SKB-Units Calculated from A-Units
Flour 1	0.2	0.05	...	0.02
Flour 2	0.8	0.22	...	0.09
Flour 3	1.7	0.46	...	0.19
Malt 1	227	61	23	25
Malt 2	398	107	46	48
Enzyme preparations 1	1,079	291	120	119
Enzyme preparations 2	4,195	1,130	480	460

H-unit. A-values have a close relationship to H-units, which may be calculated as follows according to equation 3.

$$\text{H-units} = A \times 0.27. \quad (3)$$

SKB-unit. SKB values have a good relationship to H-units, which may be calculated as follows according to equation 4.

$$\text{one SKB-unit} = \text{one H-unit} \times 0.42. \quad (4)$$

Consequently,

$$\text{one SKB-unit} = A \times 0.11. \quad (5)$$

Discussion

The ICC colorimetric method for the determination of alpha-amylase activity is applicable to cereals and cereal products as well as to enzyme preparations used for the supplementation to balance alpha-amylase activity in flours. Since the alpha-amylase activity is determined at 30°C., a relatively low temperature corresponding to the fermentation temperature for doughs, the results are not influenced by the thermostability of the enzymes from different sources. Therefore this method is suitable to measure activity of fungal amylase supplements. The alpha-amylase of fungal origin is relatively heat-labile, and the practical viscometric methods (Amylograph, Falling Number) cannot be used to evaluate the amount of fungal amylase added to wheat flour. The ICC method is also suitable to measure alpha-amylase activity by differentiating fungal, cereal, and bacterial enzymes on the basis of differences in thermal inactivation as proposed by Fleming *et al.* (14).

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