MEASUREMENT OF TOTAL AND GELATINIZED STARCH BY GLUCOAMYLASE AND *o*-TOLUIDINE REAGENT

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ABSTRACT

A method to quantitatively determine gelatinized starch in flour- or starch-containing foods was developed. It is based on the principle that gelatinized starch is easily digested by glucoamylase to form glucose, which condenses with *o*-toluidine to give a green chromogen in glacial acetic acid. A sample of flour was dispersed in water and subjected to glucoamylase digestion under controlled conditions for 30 min; then the glucoamylase was precipitated with 25% trichloroacetic acid (TCA) solution. The reaction mixture was centrifuged, the supernatant was reacted with *o*-toluidine, and the color was measured with a spectrophotometer at 630 nm. Total starch was determined by a similar procedure, except that the intact starch in the sample was gelatinized in NaOH solution. From these two readings, the percentage of starch gelatinization could be calculated. The recovery test was satisfactory. Total starch content was calculated by multiplying glucose produced by 0.90. The correlation coefficient between this method for total starch and the official AACC polarimetric method was 0.98. The newly developed method has the advantage of rapidity and precision.

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Gelatinization changes physical properties of starches and affects their use. Methods to measure starch gelatinization are numerous. They are based on birefringence (1), absorption of dyes (2), swelling (3), solubility (4), viscosity (5–7), paste clarity (8–10), X-ray diffraction patterns (11,12), proton magnetic resonance (13), differential scanning calorimetry (14), and enzymatic susceptibility (15–17). Among the methods, loss of birefringence and enzymatic susceptibility are most sensitive and most widely used. Generally, loss of birefringence is good for determining the gelatinization of starch (1), but quantitative determination of starch gelatinization based on loss of birefringence is laborious and subject to sampling errors (18). Moreover, the method cannot be used with cooked heterogeneous samples (19).

We propose a new method to determine total starch content of cereals and degree of starch gelatinization using glucoamylase digestion and an *o*-toluidine color reaction to determine the glucose produced. *o*-Toluidine reacts selectively with glucose in acetic acid solution and yields a stable color which follows the Beer-Lambert law over a wide range of concentrations (20). Its basic advantages are simplicity, relative specificity, and economy (20,21).

MATERIALS AND METHODS

Wheat starches from soft red winter wheat were isolated by wet milling (22), purified by the method of Fellers *et al.* (23), and freeze-dried. Gelatinized wheat starches were prepared by autoclaving a 2% suspension of wheat starch at 120° C

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and 15 psi for 1 hr. The starch solution was mixed with 3 vol of methanol in a Waring Blendor at high speed and then washed twice with methanol, filtered, and dried in a desiccator over calcium sulfate under vacuum. When dry, the gelatinized starches were passed through an 80-mesh sieve. Ball-milled starches were prepared by placing 50-g samples in a 1875-cc porcelain jar and grinding at room temperature for 10 hr using 800 ceramic balls (2.5-cm diameter).

The total starch content of 12 different cereal flours was determined by both the enzymatic method described herein and the official AACC polarimetric method (24). To determine total starch, the intact starch of the sample was gelatinized in *N* NaOH; then the mixture was neutralized with *N* HCl. The total starch was converted by glucoamylase to glucose, which was then reacted with *o*-toluidine. Absorbance was measured at 630 nm.

Determining Gelatinized Starch Percentage by Glucoamylase and *o*-Toluidine

The method is applicable to cereal flours or starch preparations containing no appreciable quantities of low-molecular-weight oligosaccharides such as glucose, raffinose, and sucrose.

Reagents

o-Toluidine Reagent. Dissolve 1.5 g of thiourea in 940 ml of glacial acetic acid and add 60 ml of *o*-toluidine. Store in an amber bottle.

Sodium Acetate Buffer. Dissolve 4.1 g of anhydrous sodium acetate in 1 liter

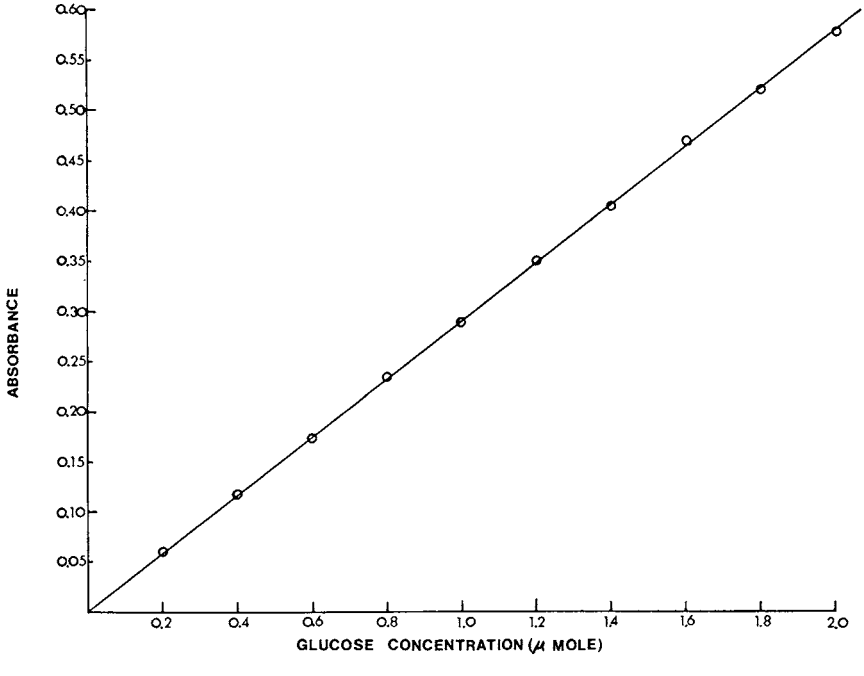

Fig. 1. Standard curve established with a glucose solution (720 μg/ml).

distilled water and adjust pH to 4.5 with acetic acid.

Glucoamylase Solution. Disperse 2 g of *Rhizopus* glucoamylase (catalog No. A-7255, Sigma Chemical Co.) in 250 ml of acetate buffer and filter rapidly through glass fiber paper (Whatman No. GF/A). Use within 2 hr. Specific activity of the glucoamylase was 28.4 μmol glucose formed/min/mg protein at pH 4.5 and 40°C.

Procedure

a.—*Preparing Sample with Partially Gelatinized Starch.* Disperse 20 mg of sample in 5 ml of distilled water in a 50-ml centrifuge tube.

b.—*Preparing Sample with Totally Gelatinized Starch.* Disperse 20 mg of sample in 3 ml of distilled water and 1 ml of 1*N* NaOH contained in a 50-ml centrifuge tube. Five minutes later, add 1 ml of 1*N* HCl.

c.—*Glucoamylase Digestion and Glucose Determination.* Add 25 ml glucoamylase solution to each tube and incubate 30 min at 40°C. Add 2 ml 25% trichloroacetic acid (TCA) to inactivate glucoamylase (and to precipitate it and other proteins), and centrifuge at 16,000 × *g* for 5 min. To 0.5 ml of supernatant solution in test tubes, add 4.5 ml of *o*-toluidine reagent. Place test tubes in boiling water for 10 min, cool with cold water, and add 5 ml of glacial acetic acid. Measure absorbance at 630 nm.

Gelatinized starch percentage was calculated as follows:

$$Y = \frac{100\,(B - k)}{A - k} \qquad k = \frac{A\,(C - B)}{A - 2B + C}$$

where

A = the absorbance of total gelatinized starch;
B = the absorbance of a mixture of partially gelatinized or intact starch after 30-min enzymatic hydrolysis;
C = the absorbance of a mixture of partially gelatinized and intact starch after 60-min enzymatic hydrolysis;
k = the absorbance of 1% of intact starch digested in 30 min, and is a constant for each variety or specially treated starch and needs to be determined only once in routine analysis; and
Y = gelatinized starch percentage.

Total Starch. A standard curve (Fig. 1) was established with a glucose solution (720 μg/ml). Samples were treated the same way as described above in steps b and c. The glucose concentration was read from the standard curve. The percentages of starch in samples were calculated by the following formula:

$$\% \text{ total starch} = \frac{\text{glucose} \times 0.90}{\text{sample wt (dry basis)}} \times 100$$

RESULTS AND DISCUSSION

Of several protein precipitants tested, 25% TCA solution was superior. TCA Solutions were added to the tubes containing gelatinized starch after digestion with glucoamylase. The data (Table I) indicate that 20% TCA solution

completely precipitated the proteins.

Effects of 25% TCA on starch were investigated by using a 10-hr ball-milled starch. The glucoamylase hydrolysate was treated with *o*-toluidine reagent 30, 60, 90, 120, 150, and 180 min after adding 25% TCA. Absorbance did not change significantly at any time (Table II), which indicated that no starch degradation occurred after adding 25% TCA.

Results from using various concentrations of several of the components of the assay system are shown in Table III. The reagents did not affect absorbance.

The effects of glucoamylase concentrations and digestion times on starch hydrolysis are shown in Fig. 2. As anticipated, variations of glucoamylase concentration and digestion time caused changes in rate of hydrolysis of susceptible starch. These data indicate that 100 mg (2.65 units) glucoamylase/ml buffer provided maximum conversion of starch to glucose in 20 min.

Color stability of the final solution was investigated by hydrolysis of a 10-hr ball-milled starch sample. The color was measured and there were no significant

TABLE I
Effect of TCA Concentration on Inactivation of Glucoamylase

TCA Concentration Solution, %	Absorbance (630 nm)	TCA Concentration %	Absorbance (630 nm)
2.0	0.500	15.0	0.130
5.0	0.502	20.0	0.000
10.0	0.502	25.0	0.000
...	...	30.0	0.000

TABLE II
Effect on Absorbance of Time when 25% TCA Solution is Added

	Time (min)					
	30	60	90	120	150	180
Absorbance (630 nm)	0.258	0.260	0.255	0.256	0.260	0.258

TABLE III
Effect of Reagents on Absorbance

Glucose ml	Water ml	NaOH ml	HCl ml	Buffer ml	Glucoamylase ml	25% TCA Solution ml	Water ml	Total Volume ml	Absorbance (630 nm)
1	2	1	1	0	25	2	0	32	0.310
1	2	1	1	25	0	0	2	32	0.310
1	4	0	0	25	0	0	2	32	0.312
1	4	0	0	0	0	0	27	32	0.310

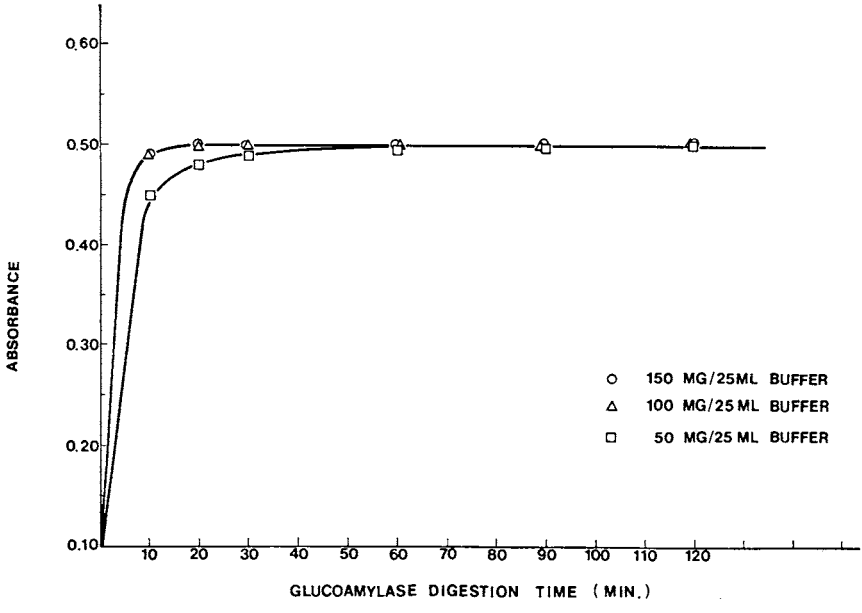

Fig. 2. Effects of glucoamylase concentrations and digestion times on starch hydrolysis.

TABLE IV
Stability of Color Produced by the *o*-Toluidine method

	Time after Heating (min)					
	30	60	90	120	150	180
Absorbance (630 nm)	0.255	0.255	0.252	0.250	0.245	0.240

TABLE V
Comparison of Known Percentages of Gelatinized Starch with Percentages Measured by the New Method

Known Percentage Gelatinized Starch	Enzymatic Method (Percentage Gelatinized)
0.0	0.4
5.0	5.4
10.0	10.8
20.0	20.0
40.0	39.8
60.0	60.2
80.0	80.6
100.0	99.7

TABLE VI
Comparison of Polarimetric and Enzymatic Methods for Measuring Starch Content

Sample	Polarimetric Method %	Enzymatic Method %
Wheat starch (soft)	96.3	94.2
Sorghum flour	86.5	84.2
Cassava flour	78.5	76.4
Barley flour	66.0	66.5
Rice flour	85.2	82.2
Rye flour	72.5	73.7
Corn meal	81.4	80.8
Wheat flour (soft)	80.6	78.3
Wheat flour (soft)	81.2	80.4
Wheat flour (hard)	77.8	75.7
Wheat flour (hard)	78.4	76.8
Wheat shorts	68.8	66.4

changes in absorbance from 30 to 180 min (Table IV). The color developed by the *o*-toluidine method was stable for at least 60 min.

The accuracy of the method was tested (Table V) by using a series of samples containing 0, 5, 10, 20, 40, 60, 80, and 100% gelatinized starch. Known starch gelatinization percentages agreed well with the percentages ascertained by this new method.

The starch contents of different cereal flours determined by both polarimetric and enzymatic methods are shown in Table VI. The correlation coefficient between the two methods was 0.98.

Literature Cited

1. LEACH, H. W. Gelatinization of starch, p. 289. In: Starch: Chemistry and technology, ed. by R. L. Whistler and E. F. Paschall. Academic Press: New York (1965).
2. JONES, C. R. The production of mechanically damaged starch in milling as a governing factor in the diastatic activity of flour. Cereal Chem. 17: 133 (1940).
3. COLLISON, R., and ELTON, G. A. Some factors which influence the rheological properties of starch gels. Staerke 13: 164 (1961).
4. RADLEY, J. A. Solubility pattern of various starches obtained refractometrically. Staerke 12: 232 (1960).
5. SANDSTEDT, R. M., KEMPF, W., and ABBOTT, R. C. The effects of salts on the gelatinization of wheat starch. Staerke 12: 333 (1960).
6. SCHOCH, T. J. Measuring the useful properties of starch. Staerke 11: 156 (1959).
7. OSMAN, E. M., and DIX, M. R. Effects of fats and nonionic surface-active agents on starch pastes. Cereal Chem. 37: 464 (1960).
8. BECKFORD, O. C., and SANDSTEDT, R. M. Starch gelatinization studies. I. Simplified equipment for the study of starch gelatinization by means of light transmission. Cereal Chem. 24: 250 (1947).
9. KITE, R. E., MAYWALD, E. C., and SCHOCH, T. J. Funktionelle eigenshaften von lebensmittelstarken. Staerke 15: 131 (1963).
10. COOK, D. H., and AXTMAYER, J. H. Determining the gelatinization temperature of starches. Ind. Eng. Chem. (Anal. Ed.) 9: 226 (1937).
11. COLLISON, R. Swelling and gelation of starch, p. 168. In: Starch and its derivatives, ed. by J. A. Radley. Chapman and Hall: London (1968).

12. HELLMAN, N. N., FAIRCHILE, B., and SENTI, F. R. The bread staling problem. Molecular organization of starch upon aging of concentrated starch gels at various moisture levels. Cereal Chem. 31: 495 (1954).
13. JASKS, E. Starch gelatinization as detected by proton magnetic resonance. Cereal Chem. 48: 437 (1971).
14. STEVENS, D. J., and ELTON, G. A. H. Thermal properties of the starch/water system. Part 1. Measurement of heat of gelatinization by differential scanning calorimetry. Staerke 23: 8 (1971).
15. SULLIVAN, J. W., and JOHNSON, J. A. Measurement of starch gelatinization by enzyme susceptibility. Cereal Chem. 41: 73 (1964).
16. SHETTY, R. M., LINEBACK, D. R., and SEIB, P. A. Determining the degree of starch gelatinization. Cereal Chem. 51: 364 (1974).
17. NOVELLE, L., and SCHUTTE, R. J. The susceptibility of sorghum starch to amylolysis. J. Sci. Food Agr. 12: 552 (1961).
18. WILLIAMS, P. C., and FEGOL, K. S. W. Colorimetric determination of damaged starch in flour. Cereal Chem. 46: 56 (1969).
19. SEIB, P. Starch gelatinization: Chemical and physical effects. Feedstuffs 43(12): 12 (1971).
20. DUBOWSKI, K. M. An *o*-toluidine method for body-fluid glucose determination. Clin. Chem. 8: 215 (1962).
21. COOPER, G. R., and McDANIEL, V. The determination of glucose by the orthotoluidine method, p. 159. Stand. Methods Clin. Chem. Academic Press: New York (1970).
22. WOLF, M. J. Wheat starch isolation, p. 6. In: Methods in carbohydrate chemistry, ed. by R. L. Whistler, Vol. IV. Academic Press: New York (1964).
23. FELLERS, D. A., PHILLIP, H. J., MOSSMAN, A. P., and SHEPHERD, A. B. Process for protein-starch separation in wheat flour. Food Technol. 23: 560 (1969).
24. AMERICAN ASSOCIATION OF CEREAL CHEMISTS. Approved methods of the AACC. Method 76-20, approved May 1969. The Association: St. Paul, Minn.

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