Starch Determination in Some Dietary Fiber Sources

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ABSTRACT

Commercially available and relatively inexpensive enzymes, single glucoamylase preparations and a high-temperature, dual system of α-amylase plus glucoamylase, were used successfully to determine starch in dietary fiber sources such as some cereal brans that contain large amounts of cellulose and hemicellulose. Gas chromatography was used to measure neutral sugars released by the enzyme preparations, glucose oxidase-peroxidase to measure released glucose only, and optical rotation to directly measure starch content in $CaCl_2$ or dimethyl sulfoxide solution. Incubation of autoclaved substrate with glucoamylase at 50°C for 2 hr or use of high temperature α-amylase (75°C for 20 min) plus glucoamylase (65°C for 35 min) appeared to minimize the effects of suspected hemicellulase and cellulase impurities. Both enzyme systems and methods of detecting glucose yielded similar starch contents of bran materials. Starch values obtained by optical rotation for wheat brans were lower than those measured by enzyme methods.

Starch content of wheat brans, dry-milled corn bran, and soy hulls became of interest to us during composition studies of these dietary fiber sources. Methods of starch analysis are reviewed by Lyne (1976), but the main application of such methods has been to materials relatively high in starch content. The use of glucoamylase to hydrolyze starch and of glucose oxidase to measure released glucose, recommended by Thivend et al (1972), is incorporated into an approved AACC method (AACC 1976). A recent manuscript by Baur and Alexander (1979) shows the utility of this method using readily available commercial glycoamylases combined with high temperature α-amylase and applied to cereal products of relatively high starch content. Recent work by Southgate, Hudson, and Englyst (1978) incorporates gas chromatography (GC) into an analysis scheme to improve the specificity of sugar measurement.

We have been concerned about the possible lack of specificity when applying colorimetric or optical rotation methods to plant tissues that contain appreciable amounts of hemicellulose and cellulose and relatively small amounts of starch. Saunders et al (1970) clearly demonstrated that discrepancies in apparent starch content may occur when different methods are used to examine wheat fractions high in dietary fiber content.

In this study we compare starch values obtained from polarimetric methods using $CaCl_2$ or dimethyl sulfoxide (DMSO) with values obtained using glucoamylase or a combination of α-amylase plus glucoamylase to hydrolyze starch. Released glucose is determined with glucose oxidase and with GC. Measurement by GC of some neutral monosaccharides (arabinose, xylose, galactose, and mannose) as a function of enzyme concentration or incubation conditions enabled us to detect degradation of hemicellulose, cellulose, and pectins by impurities in the enzyme preparations. Our intent was to establish that readily available enzymes that are not ultrapure can be used to measure starch in come substrates that contain relatively low amounts of starch in the presence of appreciable amounts of cellulose and hemicellulose.

MATERIALS AND METHODS

Cereal bran fractions of 18-30 mesh particle size distributions (~0.98—0.52 mm) were sieved from AACC-certified food grade wheat bran (a soft white winter wheat), Eagle variety hard red winter wheat bran, Waldron variety hard red spring wheat bran, and durum wheat bran. Dry-milled corn bran (milling lots 3 and 4) and soybean hulls were obtained for these studies. All bran samples were ground either in a micromill or a Udy cyclone mill before being used as substrate for enzymes. Cellulose and apparent hemicellulose contents, respectively, estimated by detergent fiber methods, were 8-11 and 35-40% for wheat brans, 52 and 33% for soy hulls, and 22 and 70% for corn bran. Starch isolated in this laboratory from dent corn (Watson 1964) and wheat starch, obtained from Commercial Solvents, served as controls.

Polarimetric determinations of starch, using $CaCl_2$ solution as a solvent, were made according to the method of Clendenning (1945) as shown in the AACC method (AACC 1976). Uranyl acetate was used to precipitate protein. Polarimetric determinations using 90% DMSO and 10% H_2O as solvent were made according to procedures of Wolf et al (1970). A specific rotation for starch was considered in $CaCl_2$ to be 203 at 589 nm and 234 at 546 nm, whereas 220 was used for starch in 90% DMSO at 546 nm.

Some neutral sugars in amyloglucosidase-substrate mixtures and in substrate blanks were measured by GC as the alditol acetates, as was done by Sloneker (1971). Enzyme blanks were run to determine whether carbohydrate associated with enzyme preparations might release sugars into solution.

Experiences of Wood et al (1977) prompted us to examine the effects of different commercial enzymes. Amyloglucosidase, grade II, from *Rhizopus* genus mold, lot 75C0247 (Sigma A) and lot 127C0505 (Sigma B) was purchased from the Sigma Chemical Co. (St. Louis, MO). Amyloglucosidase from *Aspergillus*, manufactured by B.D.H., was purchased from Gallard-Schlesinger

TABLE I
Activities of Enzyme Preparations

Enzyme Preparation	Activity (units per amount)	Listed Definition of Unit of Activity
Agidex	3,000/g	Weight of diluted enzyme that generates 25 mg of glucose in 1 hr at 65°C using a starch slurry at pH 4-5.
Sigma		
Lot A	9,660/g	
Lot B	2,200/g	Amount of enzyme that liberates 1 mg of glucose from soluble starch in 3 min at pH 4.5 at 55°C.
New-Sumyzyme	3,000/g	Amount of enzyme that liberates 10 mg of reducing sugar (calculated as dextrose) from 2% soluble starch solution at 42°C in 10 min at pH 4.0.
Diazyme L-100	100/ml	Amount of activity that catalyzes production of 1 g of dextrose from a 4% (w/v) soluble starch solution in 1 hr at 60°C and pH 4.2.
HT-1000	100/mg	Amount of activity that liberates 1 mg of reducing sugar (calculated as maltose) in 30 min at 40°C, pH 5.4, from a 4% (w/v) soluble starch solution.

[1]The mention of firm names or trade products does not imply endorsement or recommendation by the USDA over other firms or similar products not mentioned.

TABLE II
Glucose (%) in Brans Hydrolyzed with Amyloglucosidase, Colorimetrically Determined[a]

Time, Temperature, Enzyme Type, and Amount	Bran	Percent Glucose[b]
Overnight, room temperature, Sigma A,[c] 100 mg	AACC wheat	29.0 ± 1.5
	Waldron wheat	16.4 ± 0.6
Two hours, 50° C, Sigma B,[c] 50 mg	AACC wheat	26.8 ± 0.3
	Waldron wheat	15.0 ± 0.2
	Corn lot 3	3.6 ± 0.1
	Soy hulls	0.56 ± 0.1

[a] Determined with glucose oxidase-peroxidase system containing a chromogen.
[b] As-is material unless otherwise specified. Standard deviations shown.
[c] Amyloglucosidase, lots A and B, from Sigma Chemical Co.

(Carle Place, NY) as Agidex powder, lot 6234870. New-Sumyzyme 3000, a glucoamylase from *Rhizopus delamar*, was a gift from Sumitomo Corp. of America (New York). Diazyme L-100, a glucoamylase from *Aspergillus*, and HT-1000, an α-amylase from *Bacillus subtilis*, were gifts from Miles Laboratories, Inc., Elkhart, IN. These two enzymes were chosen as typical representatives of high-temperature starch hydrolysis systems now in commercial use. Activities of these enzyme preparations are shown in Table I. Glucose oxidase, grade III, and peroxidase from horseradish, grade II, were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN.

Substrate samples, 100–300 mg and 25 ml of distilled water, were placed in 250-ml Erlenmeyer flasks and immediately autoclaved at 120° C, 20 psi, for 15 min to render the starch accessible to enzyme attack. After the samples cooled, 25 ml of buffer solution was added to bring the mixtures to desired pH and ionic strength. Acetate buffer, pH 4.5, 0.2 *M*, was added to the autoclaved samples that were incubated with the enzymes (Sigma A and B, New-Sumyzyme) from *Rhizopus*, and phosphate buffer, pH 7.0, 0.2 *M*, was added to those incubated with the enzyme (Agidex) from *Aspergillus*. Effects of enzyme concentration were examined by varying the amounts of Sigma enzyme from 50 to 300 mg. Agidex,

TABLE III
Gas Chromatography Measurements (weight in percent) on Brans[a] with Glucoamylase Systems

	Conditions			Neutral Sugars			
		Enzyme					
Bran	Time	Type	Amount (mg)	Arabinose	Xylose	Mannose	Glucose
AACC wheat	···	Blank	···	tr	tr	tr	0.56 ± 0.23
	Overnight	Sigma A	150	1.3 ± 0.2	0.8 ± 0.2	1.4 ± 0.2	27.6 ± 1.3
		Sigma B	150	1.6 ± 0.2	1.4 ± 0.4	1.2 ± 0.3	27.6 ± 1.5
	2 hr	Sigma A	50	0.43 ± 0.15	tr[b]	tr	27.4 ± 0.9
		Sigma B	50	0.37 ± 0.14	tr	0.31 ± 0.15	26.3 ± 0.6
		Agidex	100	0.29 ± 0.10	tr	3.3 ± 0.2	26.2 ± 1.1
		NS[c]	60	···	···	···	35.3 ± 2.5
				···	···	···	25.7 ± 2.5[d]
Waldron	···	Blank	···	tr	tr	tr	0.74 ± 0.21
	Overnight	Sigma B	50	0.73 ± 0.06	tr	0.55 ± 0.07	17.7 ± 1.2
	2 hr	Sigma B	150	0.53 ± 0.06	0.42 ± 0.18	0.55 ± 0.30	17.6 ± 1.5
		Sigma B[e]	50	0.25 ± 0.03	tr	0.38 ± 0.11	15.6 ± 1.1
		Agidex	100	tr	tr	1.37 ± 0.05	15.3 ± 0.1
		NS[c]	60	···	···	···	20.1 ± 1.7
				···	···	···	16.2 ± 1.4[d]
Durum	···	Blank	···	tr	tr	0.23 ± 0.10	0.47 ± 0.09
	Overnight	Sigma B	50	0.64 ± 0.05	tr	0.52 ± 0.10	20.6 ± 1.9
	2 and 4 hr	Sigma B	50	0.23 ± 0.04	tr	0.29 ± 0.06	20.9 ± 0.8
Eagle wheat	···	Blank	···	tr	tr	tr	0.32 ± 0.10
	Overnight	Sigma B	50	0.77 ± 0.09	0.24 ± 0.07	0.70 ± .12	26.6 ± 0.9
	2 and 4 hr	Sigma B	50	0.21 ± 0.06	tr	0.26 ± 0.11	24.3 ± 1.8
Corn-3[f]	···	Blank	···	1.66 ± 0.09	0.35 ± 0.03	tr	0.23 ± 0.09
	2 hr	Sigma B	50	1.67 ± 0.07	0.36 ± 0.05	tr	3.75 ± 0.50
Corn-4[f]	···	Blank	···	0.95 ± 0.12	0.21 ± 0.07	tr	0.39 ± 0.12
	2 hr	Sigma B	50	1.04 ± 0.03	0.24 ± 0.06	0.29 ± 0.21	2.70 ± 0.10
Soy hull[g]	···	Blank	···	tr	tr	tr	tr
	2 hr	Sigma B	50	tr	tr	0.50 ± 0.11	0.70 ± 0.20

[a] Values are based on dry weight of as-is substrate and include contributions from enzyme preparations unless otherwise indicated. Overnight incubation was at room temperature; 2-hr and 4-hr incubations were at 50° C. tr = trace amount, ⩽ 0.2%; values significantly less than 0.2% not given.
[b] Trace.
[c] New-Sumyzyme 3000.
[d] Value corrected for glucose in enzyme preparation.
[e] And 4 hr.
[f] Dry milled.
[g] Fat and sugar extracted.

which contains kieselguhr as a diluent, was added in 100-mg portions.

Some samples were incubated overnight at room temperature with stirring, others for 2 or 4 hr at 50°C with shaking. After incubation, samples were heated at 80–85°C for 5 min to stop enzyme activity and then were transferred to 38 × 110-mm centrifuge tubes. Centrifugation at about 2,000 × *g* for 15 min clarified the supernatant.

Five-milliliter aliquots of clear supernatant were taken from the samples to be analyzed by colorimetric enzyme methods. Absolute ethanol, 20 ml, was added to the aliquots to precipitate protein and polysaccharides, and the system was stirred for 5 min and centrifuged as before. One milliliter of alcoholic supernatant was then analyzed with glucose oxidase-peroxidase and the chromogen 3,3′-dimethoxybenzidine-dihydrochloride, using the method of Bauminger (1974).

All clarified liquid was withdrawn from samples to be analyzed by GC, and the samples were washed three times with 25 ml of distilled water and centrifuged after each washing. Washings were combined with the original supernatant, and total volume was reduced in a Rotavapor (Büchi Co.) under water vacuum at 50°C. Final liquid was transferred to a 25-ml volumetric flask and brought to volume with distilled water. Neutral sugars in aliquots of these samples were processed to form alditol acetates (Sloneker 1971).

Starch and bran substrates, autoclaved as previously described, were treated upon cooling by incubation with ~5.5 mg of HT-1000 for 20 min at 75°C in the presence of NaCl, calcium ion, and acetate buffer, pH 6.5. This treatment was followed immediately by incubation with 1.0 ml of Diazyme L-100 for 35 min at 65°C in acetate buffer, pH 4.2 (Baur and Alexander 1979). AACC Method 76-11 was used with the two starch controls.

RESULTS AND DISCUSSION

Comparison of colorimetrically determined glucose obtained with glucose oxidase-peroxidase systems is shown in Table II. AACC bran and Waldron bran registered lower glucose values when incubated at 50°C for 2 hr with 50 mg of glucoamylose than at room temperature overnight with 100 mg. These results suggest that other enzyme activity exists in the sample of Sigma glucoamylase and that reaction with the substrate at an elevated temperature helps suppress undesired activity.

Table III shows GC measurements on brans used as substrates with different glucoamylases under a variety of conditions. When glucoamylase preparations were incubated by themselves at 50°C for 2 hr as blanks, the Agidex sample yielded ~3% mannose and the New-Sumyzyme 3000 sample yielded ~16% glucose (not shown). These significant amounts of mannose and glucose are reflected in the corresponding values obtained on substrate samples incubated with Agidex or New-Sumyzyme 3000. Except for these two results, amounts of the five sugars released by these glucoamylase enzyme blanks were insignificant and usually less than 0.2%. Galactose, when detected, was always less than 0.2% and, therefore, is omitted from the table.

Table III shows that reaction at 50°C for 2 or 4 hr yielded smaller amounts of arabinose, xylose, and mannose than reaction overnight at room temperature. Additional glucose was released from the two red wheat brans, Waldron and Eagle, by Sigma glucoamylase, as indicated by higher values from room temperature runs and by statistical analysis of results. However, within our experimental error, significant additional glucose was not measured from AACC or Durum brans incubated at room temperature, even though higher amounts of arabinose, xylose, and mannose were measured and a higher concentration of glucoamylase was used on AACC bran. These glucose results could reflect differences in contents of nonstarch glucans.

Glucoamylase concentrations must be kept at a minimum and incubated at elevated temperatures. Fifty milligrams of Sigma B incubated with Waldron bran overnight at room temperature released the same amount of glucose as did 150 mg of enzyme incubated at 50°C for 2 hr, whereas 50 mg of Sigma B at 50°C for 2 hr released less glucose but the same amount as did 100 mg of Agidex or 60 mg of New-Sumyzyme 3000. The New-Sumyzyme 3000 sample, both as an enzyme blank and when reacted with substrate, released noticeably less arabinose, xylose, and mannose than did the other glucoamylases. Sigma A and Sigma B did not differ significantly.

The corn brans and soy hull sample appeared to be little affected by glucoamylase impurities when incubated at 50°C for 2 hr. Amounts of arabinose and xylose in corn brans were the same in both the blank and the glucoamylase-treated samples. A small amount of mannose and glucose was found in the soy hull sample, although one cannot tell whether the glucose was the result of starch hydrolysis or was released along with mannose, perhaps as a result of a slight attack upon the substrate by enzyme impurities.

Some GC measurements on the high-temperature enzymes HT-1000 and Diazyme L-100 incubated with Eagle and Waldron brans are presented in Table IV. Significant amounts of neutral sugars are observed in the blank for the dual enzyme combination, and the amount of glucose in the blank is a significant fraction of the total glucose released upon incubation with the substrate. The sugar ribose was also present, but not measured, in the dual enzyme blank. Samples of AACC, Waldon, and Eagle brans, which had been extracted three times at 50°C with 80% ethanol-20% H_2O to remove free sugars and then air dried and incubated with the dual enzyme system, yielded glucose values equivalent to those obtained from as-is samples (data not shown).

Starch values are presented in Table V. Starch content is calculated as

$$\text{Starch} = 0.9 \times [(\text{glucose measured after glucoamylase attack upon substrate}) - (\text{free glucose in blank}) - (\text{free glucose in glucoamylase})]$$

Values for the two starch controls are essentially 100% when measured by optical rotation or the dual enzyme system, and they average 93% when measured by the single glucoamylase systems. However, these single glucoamylase systems appear to extract at least as much starch from the wheat brans as does the high-temperature dual enzyme system. A possible mechanism to rationalize these results is that even if the single glucoamylase system does not completely degrade starch in the brans, active hemicellulase and cellulase impurities may be sufficient to compensate for incomplete starch hydrolysis. In view of the very low amounts of xylose, arabinose, and mannose released by glucoamylase at 50°C, we believe a better suggestion is that the single glucoamylase systems do essentially degrade native starch to glucose and, therefore, the isolated starch controls contain sufficient impediments to prevent hydrolysis by a single glucoamylase system. This proposal also relies upon the expected temperature sensitivity (Fleet and Phaff 1974) of β-glucanases.

Optical rotation values on as-is wheat brans show some variation between measurements done in $CaCl_2$ and in DMSO and are often lower than values obtained with the enzymes. Our lower starch values are consistent with the work of Saunders et al, who attribute such results to the presence of negatively rotating hemicellulosic components.

Our conclusions from this study are that 1) commercial

TABLE IV
Gas Chromatography Measurements on Brans[a] with High-Temperature, Dual Enzyme[b] System

Conditions	Neutral Sugars (mg)				
	Arabinose	Xylose	Mannose	Galactose	Glucose
Enzyme blank	3	0.8	22	7	16
Enzymes incubated with					
Waldron bran (270 mg)	4	1	23	6	56
Eagle bran (93 mg)	3	0.6	21	6	37

[a] Values are based on dry weight of as-is substrate and include contributions from enzyme preparations unless otherwise indicated. Incubation temperature 50°C.

[b] HT-1000 (5.6 mg) and Diazyme L-100 (1.0 ml).

TABLE V
Starch Content, (% dry weight) Measured with Alternate Systems

Substrate and Enzyme	Enzyme Methods[a]			Optical Rotation Measured in	
	Glucoamylase and		High-Temperature System[b] and Gas Chromatography	DMSO[c]	$CaCl_2$
	Gas Chromatography	Glucose Oxidase			
Starch[d]					
Dent corn	...	...	100.0 ± 3.1	100.4 ± 2	...
Sigma B	...	92.7 ± 1.5[e]	...	...	...
Agidex	...	93.8 ± 0.7[e]	...	...	...
New-Sumyzyme 3000	90.5 ± 2.5	...	...	...	...
Wheat	...	...	100.2 ± 3.2	98.8 ± 2	...
Sigma B	...	93.6 ± 2.8[e]	...	...	...
Agidex	...	94.6 ± 1.7[e]	...	...	...
New-Sumyzyme 3000	92.8 ± 0.91	...	...	...	...
Bran					
Wheat					
AACC	...	...	20.7 ± 1.6	20.7 ± 0.4	20.1 ± 0.5
Sigma B	23.5 ± 0.8	23.6 ± 0.3	...	...	...
Agidex	23.1 ± 1.0	...	...	...	...
New-Sumyzyme 3000	23.1 ± 2.3	...	...	...	...
Waldron	...	...	11.9 ± 0.8	10.0 ± 0.4	11.9 ± 0.6
Sigma B	13.5 ± 0.95	12.8 ± 0.3	...	...	...
Agidex	13.1 ± 1.0	...	...	...	...
New-Sumyzyme 3000	13.9 ± 1.1	...	...	...	...
Durum	...	...	17.6 ± 0.5	14.4 ± 0.2	15.6 ± 0.3
Sigma B	18.5 ± 1.0	...	...	...	...
Eagle	...	...	21.6 ± 1.3	17.9 ± 0.3	17.7 ± 0.3
Sigma B	21.5 ± 1.7	...	...	...	...
Corn[f]					
Lot 3, Sigma B	3.2 ± 0.55	2.9 ± 0.1	3.04 ± 0.14	3.0 ± 0.4	...
Lot 4, Sigma B	2.1 ± 0.15	...	...	1.8 ± 0.2	...
Hulls, Soy[g]	0.6 ± 0.2	0.5 ± 0.2	0	0[h]	...

[a] Amounts: Sigma B, 50 mg; Agidex, 100 mg; New-Sumyzyme 3000, 60 mg. Conditions: 50° C for 2 or 4 hr.
[b] HT-1000 and Diazyme L-100.
[c] Dimethyl sulfoxide.
[d] Some starch controls examined using 30-mg sample incubated with 20 mg of New-Sumyzyme 3000. The ratios of enzyme concentrations to starch controls were within the limits used with the brans.
[e] Measured according to AACC method 76-11.
[f] Dry milled.
[g] Fat and free sugars extracted.
[h] Slight negative rotation.

glucoamylase preparations can be successfully used for starch determinations, provided proper incubation conditions of temperature, time, and enzyme concentrations are first examined to minimize effects of cellulase and/or hemicellulase impurities and interference from nonstarch glucans (the high-temperature dual enzyme system used here is also suitable for starch determinations on bran materials); 2) GC is a good method of measuring glucose released from plant tissues and, in addition, yields information on the effects of potential impurities in enzyme preparations by measuring other neutral sugars released from the substrate; 3) however, the use of GC is time consuming and unnecessary if only a starch value (glucose) is desired because suitable glucose oxidase-peroxidase systems can be used successfully to measure glucose from starch in bran materials; and 4) optical rotation methods are not generally suitable for measuring starch contents of bran materials.

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