

# Enzyme Purity and Activity in Fiber Determinations

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Dietary fiber is mainly composed of plant cell wall polysaccharides such as cellulose, hemicellulose, and pectic substances, but it also includes lignin and other minor components (1). Basically, it covers the polysaccharides that are not hydrolyzed by the endogenous secretions of the human digestive tract (2,3). This definition has served as the target for those developing analytical procedures for the measurement of dietary fiber for quality control and regulatory considerations (4).

Most procedures for the measurement of total dietary fiber (TDF), or specific polysaccharide components, either involve some enzyme treatment steps or are mainly enzyme-based. In the development of TDF procedures such as the Prosky method (AOAC International 985.29; AACC 32-05)(5), the Uppsala method (AACC 32-25)(6), and the Englyst method (7), the aim was to remove starch and protein through enzyme treatment, and to measure the residue as dietary fiber (after allowing for residual, undigested protein and ash). Dietary fiber was measured either gravimetrically or by chemical or instrumental procedures. Many of the enzyme treatment steps in each of the methods, particularly the Prosky (5) and the Uppsala (6) methods, are very similar.

As a new range of carbohydrates is being introduced as potential dietary fiber components, the original assay procedures will need to be reexamined, and, in some cases slightly modified, to ensure accurate and quantitative measurement of these components and of TDF. These "new" dietary fiber components include resistant, nondigestible oligosaccharides; native and chemically modified polysaccharides of plant and algal origin (galactomannan, chemically modified celluloses, and agars and carrageenans); and resistant starch. To measure these components accurately, the purity, activity, and specificity of the enzymes employed will become much more important. A particular example of this is

the measurement of fructan. This carbohydrate consists of a fraction with a high degree of polymerization (DP) that is precipitated in the standard Prosky method (5,8), and a low-DP fraction that is not precipitated and consequently is not measured (9). Resistant starch poses a particular problem. This component is only partially resistant to degradation by  $\alpha$ -amylase, so the level of enzyme used and the incubation conditions (time and temperature) are critical.

In this article, some of the enzyme-related problems that are being experienced in the measurement of dietary fiber components are addressed. The importance of enzyme activity and purity have not been sufficiently stressed in the past. In this article, procedures for the standardization of enzyme activity and for checking important enzyme contaminants are described. Also, the effect of enzyme concentration on measured dietary fiber levels is demonstrated.

## METHODOLOGY AND SUBSTRATE PREPARATION

### Total Dietary Fiber Determinations

TDF was measured using AACC Method 32-05 (AOAC International Method 985.29), employing amyloglucosidase (E-AMGDF), thermostable  $\alpha$ -amylase (E-BLAAM), and protease (E-BSPRT) (Megazyme). The conditions of the method were followed exactly. The effect of cellulase contamination in amyloglucosidase on recovery of  $\beta$ -glucan in dietary fiber determinations was studied using highly purified amyloglucosidase spiked with different levels of the cellulase removed from a crude source of amyloglucosidase (and purified chromatographically). The effect of varying levels of thermostable  $\alpha$ -amylase or amyloglucosidase on dietary fiber values for resistant starch samples was determined using AOAC International Method 985.29. The effect of heat-treated crude *exo*- and *endo*-inulinase mixture preparation (Fructozyme [9]; Novo Nordisk, Bagsvaerd, Denmark) and of a purified fructanase mixture (E-FRMXLQ, Megazyme) on recovery of fructan and other polysaccharide components was studied using the standard AOAC International dietary fiber method with inclusion of the fructanase treatment

concurrent with the amyloglucosidase treatment (3,9).

### Substrate Solutions

**Starch Solution.** Two grams of soluble starch (S-9765, Sigma) was added to a 50-ml beaker and slurried in 20 ml of cold water. This slurry was poured into boiling and vigorously stirring water (150 ml), and stirring was continued for 5 min. The solution was cooled to room temperature, treated with 10 ml of 2M sodium acetate buffer (pH 4.5) and 10 ml of 200 mM calcium chloride, and adjusted to pH 4.5 and 200 ml (for amyloglucosidase assay). Alternatively, a slurry of soluble starch (2 g in 20 ml of sodium maleate buffer [100 mM, pH 6.5]) was added directly to 150 ml of boiling sodium maleate buffer (100 mM, pH 6.5) containing 10 mM calcium chloride using the above procedure. The pH was adjusted to 6.5, and the volume was then adjusted to 200 ml with sodium maleate buffer. This starch was used for the assay of bacterial thermostable  $\alpha$ -amylase. These solutions were stored in air-tight containers, and a few drops of toluene were added to prevent microbial contamination.

**$\beta$ -Glucan Solution.** One gram of  $\beta$ -glucan (P-BGBM, medium viscosity; Megazyme) was accurately weighed into a 120-ml dry Pyrex beaker. The sample was wet with 6 ml of 95% ethanol. A magnetic stirrer bar was added, followed by 90 ml of distilled water. The slurry was immediately placed on a magnetic stirrer-hotplate and heated at a setting of 120°C with vigorous stirring. The beaker was loosely covered with aluminium foil and stirred vigorously. When the solution began to boil, the heat was turned off, but stirring was continued until the  $\beta$ -glucan completely dissolved (about 10 min). The solution was treated with 5 ml of 2M sodium acetate buffer (pH 4.5), the pH adjusted to 4.5 (with 1M HCl or 1M NaOH), and the volume adjusted to 100 ml. The solution was stored in a well-sealed glass storage bottle, and a few drops of toluene were added to prevent microbial contamination. If the  $\beta$ -glucan started to self-associate and precipitate from solution (after storage for several days), the solution was heated to 90–95°C for a few minutes and then shaken vigorously.

**Citrus Pectin Solution.** Two grams of polygalacturonic acid (P-CITPN, Mega-

zyme) was added to a 500-ml beaker, and 4 ml of ethanol was added to wet the polysaccharide. Distilled water (150 ml) was then added with vigorous stirring; the beaker was loosely covered with aluminum foil; and the solution was heated to boiling on a magnetic stirrer/hotplate. The heat was turned off, and the solution was stirred for 20 min (until all of the pectin had dissolved). On cooling to room temperature, sodium borohydride (0.2 g) was added and dissolved by stirring. The borohydride reduction was allowed to proceed at room temperature overnight. The solution was then treated with 10 ml of 2M sodium acetate buffer (pH 4.5); the pH was adjusted to 4.5 and the volume to 200 ml. This solution was stored in a well-sealed bottle at 4°C, and a few drops of toluene were added to prevent microbial contamination.

#### Nelson/Somogyi Reducing Sugar Method

The following solutions must be prepared as described (10).

**Solution A.** Dissolve 25 g of anhydrous sodium carbonate, 25 g of sodium potassium tartrate, and 200 g of sodium sulfate in water (800 ml). Dilute to 1 L, and filter if necessary.

**Solution B.** Dissolve 30 g of copper sulfate pentahydrate in 200 ml of water containing four drops of concentrated sulfuric acid.

**Solution C.** Dissolve 50 g of ammonium molybdate in 900 ml of water, and add 42 ml of concentrated sulfuric acid. Dissolve 6 g of sodium arsenate heptahydrate separately in 50 ml of water, and add this to the ammonium molybdate solution. Dilute the whole to 1 L and warm to 55°C to get complete dissolution (if necessary).

**Solution D.** Add 1 ml of solution B to 25 ml of solution A.

**Solution E.** Dilute solution C fivefold (50 ml to 250 ml) with distilled water. (The solution is stable at 4°C for about a week).

In typical enzyme assays, 0.2 ml of buffered enzyme solution was added to 0.5 ml of buffered substrate solution (10 mg/ml) and incubated at 40°C for up to 20 min. The reaction was terminated by adding 0.5 ml of solution D with vigorous mixing. The mixture was cooked in a boiling water bath for 20 min and then cooled to room temperature (5 min). These solutions were stirred for 10 sec (until carbon dioxide was completely released), and 3.0 ml of solution E was added with vigorous stirring (10 sec on a vortex mixer). The tubes were allowed to stand for 10 min and mixed again. The absorbance was measured at 520 nm. Blanks were prepared by adding enzyme preparation (0.2 ml) to 0.5 ml of solution D. The substrate solution (0.5 ml) was then added. Standards were prepared by adding reduc-

ing sugar solution (e.g., glucose, 0.2 ml, 10–50 µg) to 0.5 ml of substrate solution before addition of solution D. It is essential that the standards, blanks, and reaction solutions all be cooked concurrently in the boiling water bath.

#### MEASUREMENT OF ENZYME ACTIVITY

##### β-Glucanase Activity

**Spectrophotometric Method.** The substrate employed was tablets that contain dyed and cross-linked β-glucan (Beta-Glucazyme, Megazyme) (11). This substrate hydrates in water but is water insoluble. Hydrolysis by cellulases (*endo*-1,4-β-glucanase), malt β-glucanase, or bacterial 1,3:1,4-β-glucanase (lichenase) produces water-soluble dyed fragments, and the rate of release of these (the increase in absorbance at 590 nm) is directly related to enzyme activity.

The assay for cellulase was performed as follows: enzyme was extracted and suitably diluted in 25 mM sodium acetate buffer (pH 4.5). A substrate tablet containing dyed, cross-linked β-glucan was added to an aliquot (0.5 ml) of this enzyme (preequilibrated in a glass test tube; 16 × 120 mm) and incubated at 40°C for 10 min, without stirring. The reaction was terminated by adding 10 ml of 2% Trizma

base (pH ~8.5) with vigorous stirring on a vortex mixer. After 5 min at room temperature, the tube contents were filtered through Whatman No. 1 filter paper (9 cm), and the absorbance at 590 nm was measured against a substrate blank.

The substrate blank was prepared by adding a substrate tablets containing dyed, cross-linked  $\beta$ -glucan to 0.5 ml of extraction buffer, incubating at 40°C for 10 min, adding 10.0 ml of Trizma base (2% w/v), and filtering after 5 min at room temperature. The standard curve relating the activity of purified *Aspergillus niger* cellulase to international units of enzyme activity on barley  $\beta$ -glucan is shown in Figure 1. Activity on barley  $\beta$ -glucan was determined at a substrate concentration of 10 mg/ml in 100 mM sodium acetate buffer (pH 4.5) at 40°C using the Nelson/Somogyi reducing sugar procedure. One unit of activity is defined as the amount of enzyme required to release 1  $\mu$ mol of glucose reducing-sugar equivalents per minute under the defined assay conditions.

Activity was calculated as follows:

$$\beta\text{-glucanase (mU / ml of original solution)} = \frac{\text{mU / assay} \times 2 \times \frac{1}{1,000} \times \text{dilution}}{1,000}$$

where mU/assay (i.e., per 0.5 ml) is obtained by reference to the standard curve; 2 is a factor to convert from 0.5 ml as assayed to 1.0 ml; 1/1,000 is a factor to convert from milliunits to units; and dilution is the dilution of the original enzyme preparation.

**Viscometric Method.**  $\beta$ -Glucan solution (10 ml, 1% w/v) in 100 mM sodium acetate buffer (pH 4.5) was added to an Ubbelohde suspended level viscometer (size C) suspended in a glass-front water bath at 40°C. After equilibration of the solution (5 min), an initial viscosity reading was taken. An aliquot (0.2 ml) of enzyme was added, and the time to flow was immediately measured. Similar readings ( $t_d$ ) were taken over the next 20–30 min. The time to flow for the untreated  $\beta$ -glucan solution should be 7–8 min. The time to flow (in seconds) of the solvent ( $t_0$ ) (100

mM sodium acetate buffer, pH 4.5) was also determined. Specific viscosity was calculated as  $(t_d - t_0)/t_0$ , and this was plotted against incubation time (minutes). International units of enzyme activity can be calculated from the inverse reciprocal plots of viscosity against incubation time, but for the purpose of this exercise, the data is simply presented in graphical form with curves for given concentrations of *A. niger* endo-cellulase.

#### $\alpha$ -Amylase Activity

##### Nelson/Somogyi Reducing Sugar

**Method.** Preequilibrated enzyme preparation (0.2 ml) in 100 mM sodium maleate buffer (pH 6.5) was added to an aliquot (0.5 ml) of soluble starch (10 mg/ml) in sodium maleate buffer (pH 6.5) and incubated at 40°C. Reaction was terminated at 5, 10, 15, and 20 min by adding 0.5 ml of Nelson/Somogyi copper solution (solution D) with vigorous mixing. Reaction blanks were prepared by adding solution D (0.5 ml) to the starch substrate before the addition of the enzyme preparation. Glucose standards were prepared by adding 50 and 100  $\mu$ l of glucose standard solution (1 mg/ml) to 0.5 ml of the starch substrate solution, followed by copper solution D (0.5 ml). The volume in each tube was adjusted to 1.2 ml with sodium maleate buffer before incubation in a boiling water bath. One unit of enzyme activity is defined as the amount of enzyme required to release 1  $\mu$ mol of glucose reducing-sugar equivalents per minute under the defined assay conditions.

Enzyme activity was calculated as follows:

$$\alpha\text{-Amylase (U / ml of orig. preparation)} = \frac{\Delta E}{\text{time}} \times F \times 5 \times \frac{1}{180} \times \text{dilution}$$

where  $\Delta E/\text{time}$  is the increase in absorbance at 520nm divided by time of incubation;  $F$  is a factor to convert absorbance values to micromoles of glucose equivalents (50  $\mu$ g of glucose divided by the absorbance for 50  $\mu$ g of glucose); 5 is the conversion from 0.2 ml as assayed to 1.0 ml; 1/180 is a factor to convert from micrograms to micromoles; and dilution is the dilution of the original enzyme preparation.

**Ceralpha Method.**  $\alpha$ -Amylase was routinely assayed using the Ceralpha method (12) incorporating a reagent that contains end-blocked  $p$ -nitrophenyl maltoheptaoside in the presence of excess quantities of thermostable  $\alpha$ -glucosidase. On hydrolysis of the blocked nitrophenyl-oligosaccharide by  $\alpha$ -amylase, a  $p$ -nitrophenyl-oligosaccharide is released, which is immediately hydrolyzed to glucose and free  $p$ -nitrophenol by the excess quantities of  $\alpha$ -glucosidase present. The solution is made alkaline, and the  $p$ -nitrophenol color is measured at 410 nm. In practice, enzyme preparation (0.2 ml) in 100 mM sodium maleate buffer (pH 6.5) was incubated with

substrate solution (0.2 ml) at 40°C for 10 min. The reaction was terminated and color developed by the addition of trisodium phosphate (1% w/v, 3.0 ml, pH ~11.5).

One unit of enzyme activity is defined as the amount of enzyme required to release 1  $\mu$ mol of  $p$ -nitrophenol per minute at pH 6.5 and 40°C. For *Bacillus licheniformis*  $\alpha$ -amylase, 1 U of activity on the reagent is equal to 3.3 U on soluble starch at the same temperature and pH.

Enzyme activity was calculated as follows:

$$\alpha\text{-Amylase (U / ml of orig. preparation)} = \frac{\Delta E_{410}}{10} \times \frac{\text{total vol. in cell}}{\text{aliquot assayed}} \times \frac{1}{E_{mM}} \times \text{dilution}$$

where  $\Delta E_{410}$  is the absorbance of the reaction minus the blank absorbance; 10 is the incubation time in minutes; total volume in cell is 3.4 ml and aliquot assayed is 0.2 ml;  $E_{mM}$  of  $p$ -nitrophenol in 1% trisodium phosphate is 17.8; and dilution is the dilution of the original enzyme preparation.

#### Amyloglucosidase Activity

##### Starch/Glucose Oxidase-Peroxidase

**Method.** Preequilibrated enzyme preparation (0.2 ml) in 100 mM sodium acetate buffer (pH 4.5) was added to an aliquot (0.5 ml) of soluble starch (10 mg/ml) in sodium acetate buffer (pH 4.5) and incubated at 40°C. Reaction was terminated at 5, 10, 15, and 20 min by incubation of the reaction tube at 100°C for 5 min. Reaction blanks were prepared by incubation of the enzyme preparation at 100°C for 5 min before addition of the starch substrate solution. Glucose standard solutions were prepared by the addition of 0.1 ml of glucose stock solution (1 mg/ml in 0.2% benzoic acid) plus 0.1 ml of sodium acetate buffer to 0.5 ml of starch substrate solution. Glucose oxidase/oxidase reagent mixture (GOPOD, 3.0 ml) was added to each tube, and the tubes were incubated at 40°C for 20 min. The absorbances of all solutions were read against the reaction blank at 510 nm. One unit of enzyme activity is defined as the amount of enzyme required to release 1  $\mu$ mol of glucose per minute under the defined assay conditions.

Enzyme activity was calculated as follows:

$$\text{Amyloglucosidase (U / ml of orig. preparation)} = \frac{\Delta E}{\text{time}} \times F \times \frac{1.0}{0.2} \times \frac{1}{180} \times \text{dilution}$$

where  $\Delta E/\text{time}$  is the increase in absorbance at 510 nm divided by time of incubation;  $F$  is a factor to convert GOPOD absorbance values to micrograms of glucose (i.e., 100  $\mu$ g of glucose divided by the absorbance for 100  $\mu$ g of glucose); 1/180 is a factor to convert from micrograms to micromoles; and dilution is the dilution of the original enzyme preparation.

##### Amyloglucosidase Assay Reagent.

Amyloglucosidase activity was routinely assayed using a reagent mixture that contains  $p$ -nitrophenyl  $\beta$ -maltooside and excess

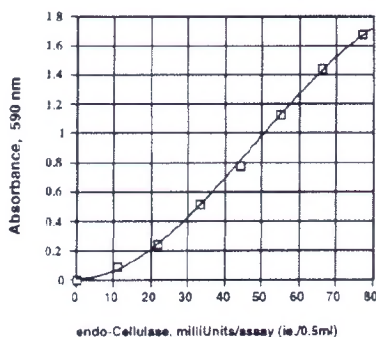


Fig. 1. Standard curve for *Aspergillus niger* cellulase on substrate tablets containing dyed, cross-linked  $\beta$ -glucan. The assay was performed exactly as described in the text.

$\beta$ -glucosidase (13). When amyloglucosidase hydrolyzes the terminal  $\alpha$ -linked D-glucosyl residue, the second  $\beta$ -linked D-glucosyl residue is immediately removed by  $\beta$ -glucosidase, releasing free *p*-nitrophenol. In practice, enzyme preparation (0.2 ml) in 100 mM sodium acetate buffer (pH 4.5) was incubated with an aliquot (0.2 ml) of substrate solution (unbuffered) at 40°C for 10 min. The reaction was terminated by the addition of 3.0 ml of Trizma base solution (2% w/v) with vigorous stirring. The *p*-nitrophenol color was measured at 410 nm. One unit of enzyme activity is the amount of enzyme that, in the presence of excess levels of  $\beta$ -glucosidase, will release 1  $\mu$ mol of *p*-nitrophenol from *p*-nitrophenyl  $\beta$ -maltoside per minute under standard assay conditions. One unit of activity on *p*-nitrophenyl  $\beta$ -maltoside is equal to 16.3 U on soluble starch at the same temperature and pH. In this communication, activity is expressed as units on *p*-nitrophenyl  $\beta$ -maltoside (PNP units) unless otherwise stated.

Enzyme activity was calculated as follows:

$$\text{Amyloglucosidase (PNP - U/ml of orig. prep.)} = \frac{\Delta E_{410}}{10} \times \frac{\text{total vol. in cell}}{\text{aliquot assayed}} \times \frac{1}{E_{mM}} \times \text{dilution}$$

where  $\Delta E_{410}$  is the absorbance of the reaction minus the blank absorbance; 10 is the incubation time in minutes; total volume in cell is 3.4 ml and aliquot assayed is 0.2 ml;  $E_{mM}$  of *p*-nitrophenol in 1% Trizma base is 17.8; and dilution is the dilution of the original enzyme preparation.

### Polygalacturonanase and Pectin Transeliminase Activity

**Reducing Sugar Assay.** Polygalacturonanase preparation (0.2 ml) was incubated with a solution of borohydride-reduced citrus pectin (0.2 ml, 10 mg/ml) in 100 mM sodium acetate buffer (pH 4.5) at 40°C for 3, 6, 9, and 12 min. The reaction was terminated by the addition of Nelson/Somogyi solution D (0.5 ml) and the color developed as described above. The assay was standardized with galacturonic acid. Enzyme activity was calculated using the same equation used to calculate  $\alpha$ -amylase activity with the Nelson/Somogyi assay procedure (10). One unit of enzyme activity is defined as the amount of enzyme required to release 1  $\mu$ mol of galacturonic acid reducing-sugar equivalents per minute under the defined assay conditions.

**Viscometric Method.** Citrus pectin solution (1% w/v) in 100 mM sodium acetate buffer (pH 4.5) was centrifuged (12,000 rpm, 10 min) to remove any insoluble particles. An aliquot (10 ml) of this solution was added to an Ubbelohde suspended level viscometer (size C) suspended in a glass-front water bath at 40°C. After equilibration of the solution (5 min), an initial viscosity reading was taken. An aliquot (0.2 ml) of enzyme was added, and the time to flow was immediately meas-

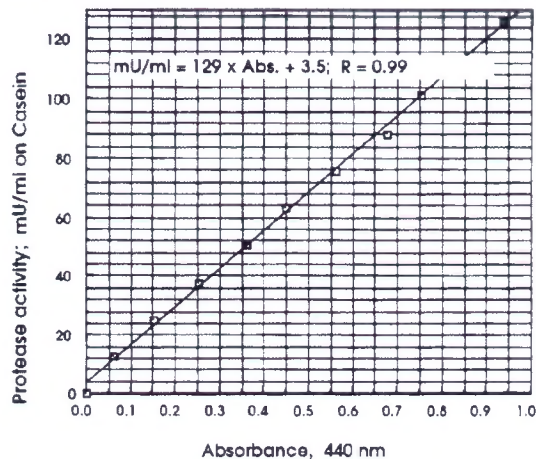


Fig. 2. Standard curve for subtilisin A on azo-casein. The assay was performed as described in the text at pH 8.0 and 40°C.

ured. Similar readings ( $t_d$ ) were taken over the next 20 min. The time to flow for the untreated pectin solution should be 5–6 min. The time to flow (in seconds) of the solvent ( $t_0$ ) (100 mM sodium acetate buffer, pH 4.5) was determined. Specific viscosity was calculated as  $(t_d - t_0)/t_0$ , and this was plotted against incubation time (minutes).

### Protease Assays

**Casein-Based Assay.** Two grams of casein was added to 180 ml of 0.1M Tris-HCl buffer (pH 8.0) and dissolved by stirring. The pH was adjusted to 8.0 with 1M HCl or 1M NaOH (if necessary) and the volume to 200 ml. The substrate, when stored in a well-sealed bottle, with a few drops of toluene added to prevent microbial contamination, was stable for at least one week at 4°C. Enzyme preparation (1 ml) was diluted to 50 ml with 100 mM Tris-HCl buffer (pH 8.0) and then further diluted to obtain a dilution suitable for assay. For assay, preequilibrated enzyme solution (2.0 ml) was added to casein substrate solution (5.0 ml) in a series of 20-ml test tubes and incubated at  $40 \pm 0.1^\circ\text{C}$  for exactly 10 min. For each enzyme being assayed, four tubes were used, three for the sample and one for the enzyme blank. Exactly 10 min from the time of addition of the enzyme to the tube, the reaction was terminated by adding 3 ml of 30% trichloroacetic acid (TCA) to each sample tube. The blanks were prepared by adding 3 ml of 30% TCA to the casein substrate (5 ml) followed by 2 ml of the diluted enzyme. All tubes were stirred vigorously and left at room temperature for 30 min to allow complete coagulation of the precipitated casein. After 30 min, the contents of each tube was filtered through Whatman No. 1 filter paper (9 cm diameter), and the absorbance of the clear filtrate was measured at 280 nm on a UV spectrophotometer (using quartz cuvettes). The assay was standardized using a tyrosine standard curve (0–1,000  $\mu$ g of tyrosine per tube; i.e.,

per 10 ml). One protease unit is defined as the amount of enzyme required to hydrolyze (and solubilize in TCA) 1  $\mu$ mol of tyrosine equivalents per minute from soluble casein at pH 8.0 and 40°C.

Enzyme activity was calculated as follows:

$$\text{Protease activity (tyrosine U/ml of orig. prep.)} = \frac{\Delta E}{10} \times F \times \frac{1}{2} \times 50 \times DF \times \frac{1}{181.17}$$

where  $\Delta E$  is the absorbance change in the enzyme reaction ( $= E_{10\text{min}} - E_{0\text{min}}$ ); 10 is the incubation time in minutes;  $F$  is a factor to convert absorbance for tyrosine (1,000  $\mu$ g) to micrograms ( $= 1,000/[\text{absorbance for } 1,000 \mu\text{g of tyrosine}]$ ); 1/2 is conversion from 2 ml as used in the assay to 1 ml; 50 is the initial extraction/dilution volume (1 ml of original preparation was diluted to 50 ml with buffer);  $DF$  is the further dilution of the original extract solution; 1/181.17 is the conversion from micrograms to micro-moles of tyrosine equivalents.

**Azo-Casein Assay.** Two grams of azo-casein (S-AZCAS, Megazyme) in a 120-ml beaker was treated with 4 ml of ethanol or industrial methylated spirits and stirred on a magnetic stirrer to remove all "lumps." This was then treated with 96 ml Tris/HCl buffer (100 mM, pH 8.0) and vigorously stirred on a magnetic stirrer until the substrate was completely dissolved (about 10 min). Azo-casein that stuck to the edge of the beaker was dislodged with a small spatula. The pH was adjusted to 8.0 with 1M HCl or 1M NaOH (if necessary) and the volume to 100 ml. The solution was stored in a well-sealed glass Duran bottle and was overlain with two drops of toluene to prevent microbial contamination. The enzyme preparation (1 ml) was diluted to 50 ml with 100 mM Tris-HCl buffer (pH 8.0) and then further diluted to obtain a dilution suitable for assay (approximately 100-fold). Preequilibrated enzyme solution (1.0 ml) in 100 mM Tris/HCl buffer (pH 8.0) was added to preequilibrated substrate solution (1.0 ml, 2% w/v) in 100 mM

Tris/HCl buffer (pH 8.0), stirred vigorously on a vortex mixer, and incubated at 40°C for 10 min. The reaction was terminated and nonhydrolyzed azo-casein was precipitated by the addition of 5% TCA (6.0 ml) with vigorous stirring on a vortex mixer for 5 sec. The reaction tubes were allowed to equilibrate to room temperature for 5 min, and then the contents were filtered through a Whatman No. 1 (9-cm) filter circle. The absorbances of all filtrates were read against the reaction blank at 440 nm. Reaction blanks were prepared by adding the TCA to the substrate solution immediately before the enzyme preparation was added. A standard curve relating the activity of subtilisin A (E-BSPDF, Megazyme) on azo-casein at pH 8.0 and 40°C to protease activity on casein (pH 8.0 and 40°C) is shown in Figure 2. One unit of *endo*-protease activity is defined as the amount of enzyme required to hydrolyze (and solubilize in TCA) 1 μmol of tyrosine equivalents per minute from soluble casein at pH 8.0 and 40°C.

Enzyme activity was calculated as follows:

$$\text{Protease activity (tyrosine U/ml of orig. prep.)} = \text{mU/assay} \times 50 \times \frac{1}{1,000} \times \text{dilution}$$

where mU/assay (i.e., per 1.0 ml) is obtained by reference to the standard curve

or to the relevant regression equation; 50 is the initial extraction/dilution volume (i.e., 1 ml of enzyme added to 49 ml of buffer); 1/1,000 is the conversion from milliunits to units; and dilution is further dilution of the original enzyme extract/dilution.

#### STANDARDIZATION OF ENZYME ACTIVITY IN DIETARY FIBER DETERMINATIONS

Measurement of TDF by AACC methods 32-05, 32-07, and 32-21 (AOAC International methods 985.29, 991.43, 993.19) requires the use of three enzymes: thermostable α-amylase, amyloglucosidase, and protease. α-Amylase and protease (subtilisin A) are from *B. licheniformis*, and amyloglucosidase is from *A. niger*. The effectiveness and purity of these enzymes for dietary fiber analyses is usually determined by analyzing specific control samples and monitoring recovery. The actual activity of the individual enzymes and of contaminants of importance is usually not provided or measured. Traditional methods for the measurement of each of these activities were evaluated, and new rapid and quantitative methods for the major activities and important contaminants are presented.

#### Standardization of α-Amylase

Two methods have been evaluated for the measurement of α-amylase, one based on the Nelson/Somogyi reducing sugar method with soluble starch as substrate, and a second that uses a defined *p*-nitrophenyl-maltosaccharide as substrate, in the presence of a thermostable α-glucosidase. These methods have been used to assay the thermostable α-amylase preparations that are available commercially for the measurement of dietary fiber. The activity of preparations currently available from major suppliers is similar, i.e., ~10,000 U/ml (soluble starch) or ~3,000 U/ml *p*-nitrophenyl-maltosaccharide (Ceralpha method). However, the activity levels of preparations of this enzyme supplied for use in AACC and AOAC International dietary fiber procedures over the past five years have ranged from 2,000 to 10,000 U/ml on soluble starch. Since the level of activity of this enzyme significantly affects the measured dietary fiber levels of certain samples, particularly resistant starch (Table I), the level of activity of this preparation should be more clearly defined than has previously been the case. From Table I, it can be seen that, for four commercial starch materials (resistant starches A–C and high-amylose maize starch), the level of α-amylase used in the assay has a very significant effect on the measured dietary fiber level. Standardization of the concentration of this enzyme is necessary to reduce inter-laboratory variations in reported values for resistant starch samples. Both described assays for the measurement of α-amylase give reliable and consistent results.

Also shown in Table I are the TDF values obtained for casein and pectin. The dietary fiber value obtained for casein was as expected, but the value for the control pectin samples was much lower than expected. This was shown not to be due to pectinase in the enzyme preparation, as the levels of this activity in the amyloglucosidase used were negligible (Fig. 3). Consequently, this must be due to incomplete precipitation of all of the pectin in the control samples. The reason for this is not clear, because the pectins from both sources were quite pure, high molecular weight materials.

Table I. Effect of Concentration of Enzymes on Dietary Fiber Content Determinations

Polysaccharide	Enzyme Preparation	Quantity (U/assay)	Total Dietary Fiber (%)
High-amylose maize starch	Thermostable α-amylase	600U	15.2
		300	23.4
		150	29.3
		75	34.2
		38	34.6
		19	38.3
Resistant starch A	Thermostable α-amylase	600U	29.5
		300	32.6
		150	34.8
		75	35.5
		38	35.8
		19	38.7
Resistant starch B	Thermostable α-amylase	150U	53.8
		75	55.5
		38	59.5
		19	61.0
Resistant starch C	Thermostable α-amylase	600U	28.1
		300	38.4
		150	43.3
Regular maize starch	Amyloglucosidase	40	0.1
		20	1.3
High-amylose maize starch	Amyloglucosidase	40	29.3
		120	28.8
Casein	Standard enzymes		1.4
Pectin <sup>b</sup>	Standard enzymes		89.0
Pectin <sup>c</sup>	Standard enzymes		86.5

<sup>a</sup> α-Amylase activity is expressed as Ceralpha units. For all samples, an aliquot (50 μl) of enzyme is used, and the activity of the preparations are 380–12,000 U/ml. In the standard AOAC procedure, the concentration of the α-amylase is 3,000 U/ml (or 150 U/assay).

<sup>b</sup> For example, from Sigma Chemical Co.

<sup>c</sup> For example, from Megazyme.

#### Standardization of Amyloglucosidase

Two methods for the assay of amyloglucosidase are described. One of these methods is more conventional, employing soluble starch as substrate and measuring the release of glucose using glucose oxidase/peroxidase reagent. The second method employs *p*-nitrophenyl β-maltoside (PNPBM) as substrate in the presence of saturating levels of β-glucosidase (13). In this latter method, as amyloglucosidase hydrolyzes the terminal α-linked D-glucosyl residue, the excess levels of β-glucosidase give immediate hydrolysis of the β-linked D-glucosyl residue, releasing free *p*-

nitrophenol, which is detected by adding an alkaline solution. Both methods give quantitative measurement of amyloglucosidase. Amyloglucosidase preparations supplied for use in TDF assay procedures have traditionally had an activity of about 130 U/ml on PNPBM (or ~2,000 U/ml on soluble starch), with 0.3 ml of enzyme being used in standard AOAC International methods (i.e., 40 U/test sample). Higher levels of activity ensure that all  $\alpha$ -limit dextrans released on hydrolysis of starch by  $\alpha$ -amylase are hydrolyzed to glucose in the standard assay format. The effect of using lower or higher levels of amyloglucosidase on the measured dietary fiber levels is shown in Table I. With approximately half of the recommended level of amyloglucosidase, the TDF fiber values for regular maize starch increase only marginally (from <0.1 to 1.3%). Increasing the level of amyloglucosidase to three times the recommended level has an insignificant effect on measured TDF in resistant starch samples, i.e., values for high-amylose maize starch decrease by less than 1%.

### Standardization of Protease

It appears that problems have not been experienced with variations in concentration of the protease used in dietary fiber determinations. There are two probable reasons for this. First, the level of enzyme used is more than adequate to give near complete hydrolysis of all susceptible bonds in proteins in the samples being analyzed. Second, protein that is resistant to hydrolysis by the protease employed (subtilisin A), and that thus remains in the recovered fiber sample, is chemically quantified and then subtracted in the calculations. However, it is still prudent to have accurate and reliable protease assays available for in-house quality control, particularly if problems are experienced. Two

methods have been developed and evaluated. One is a slight modification of traditional methods employing casein. This method is relatively easy to use and gives a linear standard curve over one absorbance unit. The second method employs azocasein and a TCA precipitation step. This assay has the advantage of being more sensitive, and the reaction products absorb in the visible range (440 nm). The standard curve for this assay is also linear over about one absorbance unit (Fig. 2). Activity units are related to the assay employing casein, and units are expressed as micromoles of tyrosine equivalents per minute (tyrosine units). In evaluating enzyme preparations currently used in dietary fiber analyses, an activity of 6–8 tyrosine units per milligram was obtained. In the standard AOAC International method, a protease concentration of 50 mg/ml is recommended (i.e., ~300–400 U/ml). Consequently, we recommend that a concentration of  $350 \pm 50$  U/ml be generally used for dietary fiber determinations.

### Contaminating Activities in Enzymes

Of the three enzymes used in dietary fiber determinations, the only enzyme that has been found to be significantly contaminated with interfering activities is amyloglucosidase. The thermostable  $\alpha$ -amylase and protease supplied commercially for dietary fiber determinations are generally free of interfering enzymes. Low levels of  $\beta$ -glucanase (*endo*-1,3;1,4- $\beta$ -D glucanase; lichenase) have been detected in protease preparations, but these were well below the level that would interfere with the dietary fiber assay (i.e., result in loss of  $\beta$ -glucan). The major contaminant in amyloglucosidase preparations (e.g., *A. niger*)

was shown to be *endo*-cellulase, and this resulted in *endo*-depolymerization of mixed-linkage  $\beta$ -glucan from barley and oat, with a resultant underestimation of this component. The effect of cellulase contamination in amyloglucosidase preparations on the viscosity of barley  $\beta$ -glucan solutions is shown in Figure 4, and the effect on analyzed dietary fiber levels of pure barley  $\beta$ -glucan is shown in Table II. It is evident that cellulase concentrations of above 1.0 U/ml in the amyloglucosidase preparations (i.e., 0.2 U/assay) result in significant depolymerization of barley  $\beta$ -glucan (Fig. 4) and loss of this component in dietary fiber analyses (Table II). Levels of cellulase equivalent to or higher than this concentration have been measured in some preparations of amyloglucosidase offered commercially for use in dietary fiber analyses. This contaminant can be readily detected and quantified to ensure that the enzyme preparation is of adequate purity to ensure quantitative recovery of  $\beta$ -glucan, a major cereal-based soluble dietary fiber component.

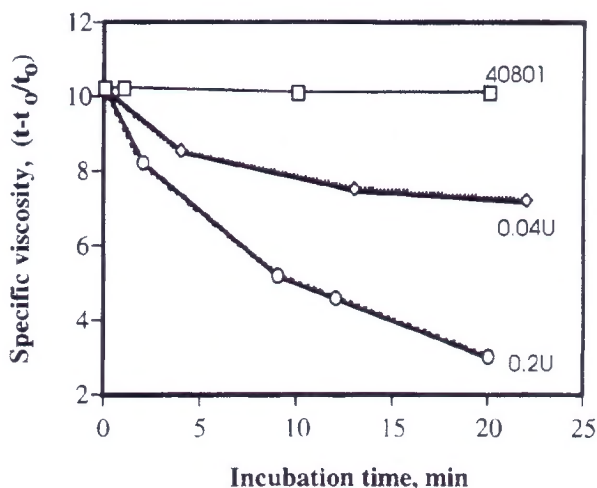


Fig. 3. Effect of pure polygalacturonase on the viscosity of citrus pectin. Enzymes used were amyloglucosidase (0.2 ml) (40801) and pure polygalacturonase at 0.04 and 0.2 U per incubation, i.e., per 10 ml of pectin (10 mg/ml at pH 4.5).

Table II. Effect of Concentration of Cellulase Contamination in Amyloglucosidase on Determined Dietary Fiber Contents of Pure Barley  $\beta$ -Glucan (100 mg)

Added Cellulase (U/assay) <sup>a</sup>	Total Dietary Fiber (%)
0.0	99.9
0.05	99.2
0.10	99.1
0.20	98.4
0.40	90.0
0.80	69.2

<sup>a</sup> i.e., per 0.2 ml AMG.

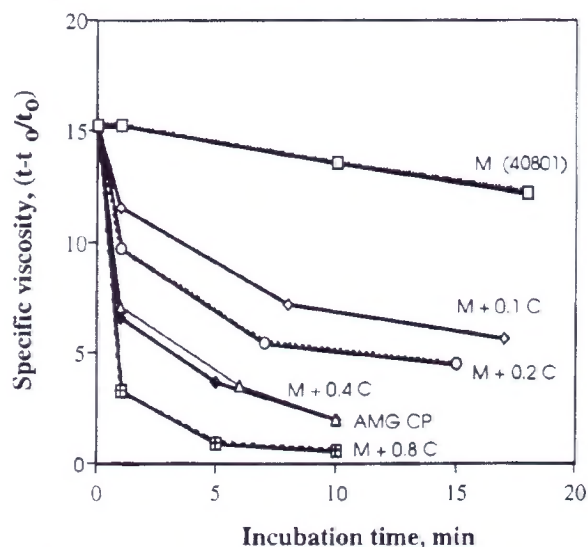


Fig. 4. Effect of *Aspergillus niger* cellulase on the viscosity of solutions of barley  $\beta$ -glucan. Assays were performed exactly as described in the text. To highly purified *A. niger* amyloglucosidase (40801), cellulase was added at concentrations of 0.1, 0.2, 0.4, and 0.8 units per 0.2 ml of amyloglucosidase, and 0.2 ml of these amyloglucosidase preparations were added to 10 ml of  $\beta$ -glucan solution (10 mg/ml, pH 4.5) in the viscometer.

Table III. Enzyme Levels in Three Fructanase Preparations<sup>a</sup>

	Commercial Fructanase Preparation	Heat-Treated Commercial Fructanase Preparation <sup>b</sup>	Pure Fructanase Mixture
exo-Inulinase	2,000	1,200	2,000
endo-Inulinase	200	200	200
Pectinase	40	4.1	0.4
Cellulase	4.0	4.0	0.1
α-Galactosidase	284	10.2	0.05

<sup>a</sup> One unit of activity is the amount of enzyme required to release 1 μmol of product per minute under standard assay conditions (40°C, pH 4.5).

<sup>b</sup> Preheated at 60°C for 120 min.

Table IV. Effect of Addition of Crude and Purified Fructanases on Recovery of Inulin and Fructan in the AOAC International Total Dietary Fiber Procedure<sup>a</sup>

Fructanase Mixture	Quantity (ml)	Recovery, %	
		Inulin (Fructan)	β-Glucan
Heat-treated	0.2	0.2	11.4
	0.1	0.2	52.0
	0.05	7.2	80.0
Pure <sup>c</sup>	0.2	0.2	99.0
	0.1	0.2	101.0
	0.05	3.5	98.5

<sup>a</sup> The levels of enzyme activities in the heat-treated and pure fructanase mixtures are as shown in Table I. The amount of β-glucan used in assays was 100 mg.

<sup>b</sup> One unit of activity is the amount of enzyme required to release 1 μmol of product per minute under standard assay conditions (40°C, pH 4.5).

<sup>c</sup> E-FRMXLQ, Megazyme.

Another enzyme activity that could cause significant underestimation of dietary fiber, particularly of fruit origin, is polygalacturonase. This enzyme is most readily assayed using a viscometric method employing high-viscosity citrus pectin as substrate. In analyzing for this activity in amyloglucosidase preparations that are commercially available for use in dietary fiber determinations, it was found that the levels were extremely low (Fig. 3). On incubation of 0.2 ml of amyloglucosidase (as used in dietary fiber analyses with citrus pectin), there was only minor viscosity reduction over the 20-min incubation period. This viscosity reduction is equivalent to a polygalacturonase concentration of less than 0.01 U/assay (i.e., per 0.2 ml of amyloglucosidase), which is below the level that would result in pectin loss in the AOAC International dietary fiber assay.

Some fructans precipitate with alcohol in the TDF procedure, but others remain soluble. A fructanase enzyme preparation completely hydrolyzes and removes fructan, allowing measurement of TDF, less fructan, by AOAC International Method 985.29. The fructan is then measured separately (3,4,9, 14). For this modification to give reliable results, the fructanase mixture used to hydrolyze the fructan must be devoid of enzymes active on other dietary fiber components. Quemener and co-workers (9) recommended heat treatment of the commercial fructanase preparation

(Fructozyme) to effectively inactivate most of the pectin-degrading activities in the preparation. This treatment gave effective inactivation of pectinase (Table III) but no removal of a contaminating cellulase (β-glucanase). The level of cellulase present is sufficient to catalyze a rapid depolymerization of β-glucan and give a significant underestimation of this component in the AACC and AOAC International dietary fiber assay procedures (Table IV). The cellulase can, however, be removed chromatographically, allowing the use of the enzyme as proposed by Quemener and co-workers (9).

## CONCLUSIONS

Dietary fiber is a mixture of polysaccharide components, so care must be exercised when introducing any changes to the standard analytical procedure, particularly if these changes involve the use of different or new enzyme preparations. The need for modification and updating of the standard format is likely to be raised as a range of "new" dietary fiber components is introduced. Problems associated with doing this have been highlighted in this article by looking at specific problems associated with the measurement of resistant starch, β-glucan, and fructan. Even in standard AACC and AOAC International dietary fiber assays, it is essential that the levels of the three enzymes employed, namely, α-amylase, amyloglucosidase, and protease, be properly standardized to ensure complete degradation of the target component.

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