Dietary Fiber Content of Cross-linked Phosphorylated Resistant Starch (RS4) Determined by the Prosky and McCleary Methods
Part II. Comparison of Assay Data

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As defined by Codex Alimentarius (40,41), dietary fiber is nondigestible carbohydrate that is principally made up of nonstarch polysaccharides, resistant starch (RS), and resistant oligosaccharides with a molecular size that equals or exceeds that of a trisaccharide. Furthermore, the nondigestible carbohydrate must elicit a beneficial physiological response, which could be 1) a bulking effect (laxation); 2) a reduction in postprandial glucose level in the blood (glycemia); or 3) fermentation in the colon that produces an increase in desirable bacteria, a decrease in pathogenic bacteria, and the release of beneficial short-chain (C-2, C-3, and C-4) fatty acids (prebiotic and laxation) (47).

Consuming nondigestible carbohydrate in whole grain and as dietary fiber is associated with a reduced risk of diseases such as obesity, diabetes, coronary heart disease, colon cancer, inflammatory bowel syndrome, and diverticulitis (63). Moreover, prebiotics interact with immune cells and help prevent allergies and infections. Finally, the phytochemicals (phenolics, carotenoids, sterols, and lignans) and minerals found in whole grain, as well as those associated with dietary fiber, also provide health benefits.

The current intake of dietary fiber in Western countries is below the recommended levels of 25 and 38 g/day for women and men, respectively, as stated by the Institute of Medicine (46). Efforts to raise intake levels have resulted in an ever-increasing number of processed foods that contain whole grain and/or ingredients rich in dietary fiber. Dietary fiber in foods is assayed to ensure proper labeling and regulation and to track human intake (62). Regulatory agencies are keen to prevent an overestimation of dietary fiber content.

AACC International (AACC) has a long history of contributions to the definition of dietary fiber and its analysis. (Scientists who have served on AACC dietary fiber committees include David Lineback, Jonathan DeVries, Julie M. Jones, Stuart Craig, Leon Prosky, Sungsoo Cho, Dennis Gordon, Mary Ellen Camire, Betty Li, Barry McCleary, and Bryan Tungland.)

Codex Alimentarius, established in 1963 by the United Nations Food and Agriculture Organization and World Health Organization, deals with issues of international food trade. The Codex Committee on Methods of Analysis and Sampling (CCMAS) held its 33rd session in Budapest, Hungary, in March 2012 (60). One item on the committee’s agenda was the in vitro assay of dietary fiber. CCMAS discussed the desirability of a “decision tree” to assist analysts in choosing from among the 17 in vitro methods of assaying dietary fiber that the committee endorsed at its 32nd session (15). With so many methods available for the assay of dietary fiber, the committee attempted to clarify their uses (65). A decision tree was found unacceptable, however, and a table was drawn up instead that contains 14 of the 17 Codex-endorsed methods (9,65). The table delineates which fiber components each method does and does not measure (16) and was adopted by CCMAS in March 2012.

Of the 17 Codex-endorsed methods (15), 8 are general assay methods for dietary fiber and have been assigned Codex Type I status; 6 others are designed for specific sources of dietary fiber and have been assigned Type II status; and the remaining 3 methods have been assigned Type IV status. A Codex Type I method is an empirical method in which the quan-
Dietary fiber in food and was specified by the U.S. Food and Drug Administration for labeling requirements (17,24). The Prosky method does not account for low molecular weight dietary fiber that is soluble in ≈75% ethanol. As a consequence, total dietary fiber determined by the Prosky method is termed high molecular weight dietary fiber (44), which is the sum of water-insoluble dietary fiber (also insoluble in ≈75% ethanol) and water-soluble dietary fiber precipitated in ≈75% ethanol.

When RS and resistant oligosaccharides were recognized as dietary fiber in the 1990s, it was discovered that some of these forms were partially captured or lost in the Prosky assay (6,38,43). Therefore, a number of AACCI and AOAC methods were devised to assay specifically for various sources of dietary fiber, including fructans, mixed β-glucans, trans-galacto-oligosaccharides, polyglucose, resistant maltdextrin, and RS. The inclusion of RS in dietary fiber presents a special challenge. Five types of RS are recognized: RS1, starch enveloped in cell walls or strong protein matrix; RS2, RS granules; RS3, retrograded starch; RS4, chemically modified starch; and RS5, amylose-lipid complex (6,14,27). Except for RS4, RS is chemically equivalent to digestible starch; however, its physical form limits the diffusion of α-amylase to starch molecules. Chemically modified starches, wherein hydroxyl groups or glycosidic bonds have been altered, also contain RS because the modified starch molecules do not fit in the amylase catalytic site. Cross-linked phosphorylated (CLP) RS4 wheat starch (64) is a food-grade starch with such a low level of substitution that most of its resistance can be attributed to its physical structure.

A food that has been fortified with dietary fiber may be assayed by both a specific and general method of determining dietary fiber, which may lead to double counting of the dietary fiber. To avoid an overestimation of some forms of dietary fiber, McCleary developed an integrated procedure (38) to assay foods for total dietary fiber. This integrated procedure, which consists of two main parts, is designed to account for nonstarch polysaccharides, RS, and nondigestible oligosaccharides. The first part is an enzymatic-gravimetric assay that measures high molecular weight dietary fiber, which includes high molecular weight nonstarch polysaccharides and RS. The second part is a high-performance liquid-chromatographic method that measures nondigestible oligosaccharides that have a degree of polymerization ≥3. The McCleary method has been designated AACCI Approved Method 32-45.01 and AOAC Method 2009.01 for measuring total dietary fiber (1,4,40), and its sequel has been designated AACCI Approved Method 32-50.01 and AOAC Method 2011.25 for measurement of insoluble, soluble, and total dietary fiber (1,4,41).

In 2010, Codex Alimentarius granted AACCI observer status, and in early 2012 AACCI sent a letter (2) to CCMAS stating that AACCI supports adoption of AACCI Approved Method 32-45.01 (AOAC Method 2009.01) for measuring total dietary fiber, along with its extended version AACCI Approved Method 32-50.01 (AOAC Method 2011.25) for measuring insoluble, soluble, and total dietary fiber (1,4). The letter states that these methods should be granted Type I status by the Codex Alimentarius Commission and that all remaining methods for dietary fiber should be moved to Type II or Type III status. The letter suggests that such an endorsement by Codex would result in “the analytical community… [migrating] to the AACCI/AOAC methods to comply with the CODEX definition, and the other methods will become obsolete with regard to DF labeling.” To date, both AACCI Approved Methods 32-07.01 (AOAC Method 991.43) and 32-45.01 (AOAC Method 2009.01) have been adopted as Type I (empirical) in vitro methods by Codex Alimentarius (1,4,15). AACCI Approved Methods 32-45.01 and 32-50.01 (1), which are equivalent to AOAC Methods 2009.01 and 2011.25 (4), are termed here the McCleary method, and AACCI Approved Methods 32-05.01 and 32-07.01 (1), which are equivalent to AOAC Methods 985.29 and 991.43 (4), are termed here the Prosky method.

The purpose of this article is to provide data showing that compared with the Prosky method the McCleary method recovers low amounts of total dietary fiber from CLP RS4 wheat and tapioca starches and from one sample of RS5 starch. We present information that supports a high level of dietary fiber in these RS4 starches, as determined by the Prosky assay. We suggest that the strong starch digestion conditions of the McCleary method result in an underestimation of RS in RS5 starch and in CLP normal RS4 cereal starches. In vivo data are needed to corroborate the in vitro data of the
Assay Methods for Measuring Total Dietary Fiber

AACC/Approved Methods 32-07.01 and 32-45.01 (AOAC Methods 991.43 and 2009.01) (1,4) were performed by Medalion Laboratories to assay total dietary fiber. These methods were also performed at Covance Laboratories to assay total dietary fiber in 13 starch samples. Four of the thirteen starches were CLP wheat, tapioca, or potato starches containing ≈0.4% P and 10% moisture.

Total Dietary Fiber Results for McCleary and Prosky Methods

The levels of total dietary fiber in 11 samples of RS and 2 samples of normal starches were determined using the Prosky and McCleary methods (Table 1). Negligible levels of total dietary fiber were found in the control samples of normal wheat and corn starches using both methods, whereas the levels in the RS samples ranged between 22.2 and 87.8%. Additionally, there appeared to be no correlation between the two sets of dietary fiber levels determined for the 11 RS samples. As discussed earlier, if a food is devoid of RS and low molecular weight soluble dietary fiber, then either the Prosky or McCleary method may be used to measure total dietary fiber (i.e., high molecular weight dietary fiber) (28,44).

As shown in Table 1, the RS2, RS4, and RS5 starches, except for high-amylose maize starches 1 and 4, contained 6–63% less total dietary fiber when assayed by the McCleary method versus the Prosky method. However, the three samples of RS3 (nongranular, processed, and retrograded starches) trended in the opposite direction by ≈10%. These data contradict the view (44) that the McCleary method may be used to measure RS2 and RS3 starches (and presumably any value can be obtained for a particular sample, depending on the incubation conditions used) (43). The RS4 wheat starches in Table 1 may be especially susceptible to the strong-starch digestion conditions of the McCleary method because these granular starches contain pores and channels (discussed below). The dietary fiber (in this case RS) levels in the three RS2 granular maize starches and three RS3 retrograded starches in Table 1 are known to increase when amylase content increases from ≈25 to 50% or higher (6,33,58). Variation in amylase levels in the high-amylose maize samples in Table 1 may explain some of the variation in their levels of total dietary fiber. However, the principal difference may be caused by structural variances that increase the packing density of solids inside the maize granules, which increases RS content (58; Cai and Shi, “Preparation, Structure, and Digestibility of Crystalline A- and B-Type Aggregates from Short α-1,4 Glucans,” submitted for publication). One additional factor may increase the level of RS determined in the McCleary assay compared with the Prosky method. During amyolysis at 37°C in the McCleary method, some of the short-chain amylase molecules released by α-amylase, even in the presence of glucoamylase, retrograde and become resistant to digestion (6,58). This phenomenon has been noted, for example, during starch digestion at 37°C and pH 6.0 for up to 25 hr in a medium containing ≈10–60 U of pancreatic α-amylase activity/mL (≈200–1,200 U/g of starch) (calculated) and ≈1 U of glucoamylase activity/mL (14 U/g of starch) (calculated) (26). Retrogradation of short-chain amylase molecules does not occur at the high digestion temperature (≈97°C) used in the Prosky method.

Starch Digestion Conditions in Prosky and McCleary Assays

RS is important in food because of its physiological functions, i.e., in vivo assay of RS gives its true content in a food. RS is

<table>
<thead>
<tr>
<th>Starch</th>
<th>Granules Below 40°C in Water</th>
<th>RS Class</th>
<th>Total Dietary Fiber (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prosky Method&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wheat or corn starch</td>
<td>Yes</td>
<td>RS4</td>
<td>84.9</td>
</tr>
<tr>
<td>CLP wheat starch (≈0.4% P)</td>
<td>Sample A</td>
<td>Yes</td>
<td>RS4</td>
</tr>
<tr>
<td>CLP tapioca starch</td>
<td>Yes</td>
<td>RS4</td>
<td>83.8</td>
</tr>
<tr>
<td>Amylose-stearic acid complex</td>
<td>No</td>
<td>RS5</td>
<td>48.1</td>
</tr>
<tr>
<td>High-amylose maize starch 1 (HMT)</td>
<td>Yes</td>
<td>RS2</td>
<td>36.3</td>
</tr>
<tr>
<td>High-amylose maize starch 2 (HMT)</td>
<td>Yes</td>
<td>RS2</td>
<td>59.7</td>
</tr>
<tr>
<td>High-amylose maize starch 3 (HMT)</td>
<td>Yes</td>
<td>RS2</td>
<td>54.8</td>
</tr>
<tr>
<td>High-amylose maize starch 4 (Hylon VII)</td>
<td>Yes</td>
<td>RS2</td>
<td>25.6</td>
</tr>
<tr>
<td>Novelose 330</td>
<td>No</td>
<td>RS3</td>
<td>32.7</td>
</tr>
<tr>
<td>CrystaLean</td>
<td>No</td>
<td>RS3</td>
<td>31.3</td>
</tr>
<tr>
<td>Promitor RS</td>
<td>No</td>
<td>RS3</td>
<td>55.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> CLP = cross-linked phosphorylated; HMT = heat-moisture treated.
<sup>b</sup> AACC/Approved Method 32-07.01 or AOAC Method 991.43 (1,4).
<sup>c</sup> AACC 32-45.01 Approved Method or AOAC Method 2009.01 (1,4).
defined as “the sum of starch and products of starch degradation [glucose, malto-oligosaccharides, α-dextrins] that is not absorbed in the small intestine of healthy individuals” (5). In vivo measurements are accomplished most often using the ileostomy model or intubation techniques (6,14). However, in vitro assays for dietary fiber in samples containing RS, e.g., AACCI Approved Methods 32-07.01 and 32-45.01 (AOAC Methods 991.43 and 2009.01) (1,4), are performed much more frequently than in vivo assays due to time constraints, costs, and ethics (6). Unfortunately, in vitro conditions for starch digestion differ from those in vivo (26), the latter of which include heterogeneous hydrolysis in a concentrated medium with a different concentration of amylase, and a different degree of agitation (56). Dietary fiber assays like AACCI Approved Methods 32-07.01 and 32-45.01 (AOAC Methods 991.43 and 2009.01) (1,4) have been designated Type I empirical methods by Codex Alimentarius. So, what explains the differences seen in Table I between levels of total dietary fiber in CLP RS4 samples determined by AACCI Approved Methods 32-07.01 and 32-45.01 (AOAC Methods 991.43 and 2009.01), and which in vitro value is closer to the in vivo level for an RS4 sample?

The AACCI Approved Method 32-07.01 (AOAC Method 991.43) (1,4) assay, which is the most widely used Prosky method, is performing as follows. The sample (1.0 g) of food or starch (<10% fat) is placed in a tall beaker with 40 mL of MES-Tris buffer (pH 8.2) containing heat-stable α-amylase (Bacillus licheniformis). No CaCl₂ is added to the digest, in keeping with the original Prosky method in which a phosphate buffer is used (1,4). The level of α-amylase activity at the start of digestion is 13 U/mL (500 U/g of sample), where the activity is determined at 40°C and pH 6.5–7.0. The beaker is covered with aluminum foil and heated in a shaken water bath (set at 98°C ± 2°C) until the digest reaches ~97°C (usually in ~15 min). Although the method does not state an agitation speed, the original Prosky method (AACCI Approved Method 32-05.01 or AOAC Method 985.29 (1,4)) states that the digest should be shaken gently every 5 min during the digestion period. The hot α-amylase digestion step is allowed to proceed for 30 min to convert digestible starch into water-soluble maltooligosaccharides and α-dextrins. Following cooling of the α-amylase digest, the protein in a sample is digested for 30 min with protease at 60°C to produce soluble peptides and amino acids. Finally, the α-amylase and protease digest is adjusted to pH 4.3 and digestion proceeds for 30 min at 60°C with added glucoamylase (to a level of 18 U/mL) to convert maltooligosaccharides and α-dextrins into glucose. The final digest is cooled, and 4 vol of ethanol is added to precipitate dietary fiber, which is collected by filtration, dried, and weighed. The dried solid is corrected for residual protein and ash, and the corrected mass is termed total dietary fiber or, more recently, high molecular weight dietary fiber (44).

In the McCleary method (AACCI Approved Method 32-45.01 and AOAC Method 2009.01 (1,4)), a sample (1.0 g) is placed in a sealed bottle with 40 mL of pH 6.0 maleate buffer containing CaCl₂ and a mixture of purified porcine pancreatic α-amylase and fungal glucoamylase. The digest is incubated at 37°C for 16 hr on a reciprocating or orbital shaker at a speed sufficient to maintain a suspension. The uncorrected activity of α-amylase in the digest at t = 0 (Table II, entry 3 [Table I in Maningat et al. (36)]), measured at pH 6.5 and 40°C, is 170 U/mL of digest (6,800 U/g of sample), and the uncorrected glucoamylase activity, measured at t = 0, pH 4.5, and 40°C, is 3.4 U/mL of digest (136 U/g of sample). Digestion is carried out at pH 6.0, and the activities of the added amylases change at that pH. McCleary and Monaghan (42) found that porcine (crude) pancreatic α-amylase at 40°C has relative activities of 100, 77, and 8.3% at pH 6.9, 6.0, and 5.2, respectively, in the presence of CaCl₂. Moreover, their results (42) indicate that at pH 6.0 and 37°C, pancreatic α-amylase is gradually denatured such that its enzymatic activity declines in 2 and 16 hr to 30 and 10%, respectively, of its initial activity.

From these data, the estimated (Table II, entry 3) α-amylase activity in the digest (at pH 6.0) obtained from AACCI Approved Method 32-45.01 (AOAC Method 2009.01) is initially (t = 0) equal to ~77% of 170 or 130 U/mL (5,200 U/g), which declines to 65 and 13 U/mL, respectively, after 2 and 16 hr of digestion. McCleary and Monaghan (42) also found that the enzymatic activity of glucoamylase at pH 6.0 and 37°C is degraded to 20% of its activity at pH 4.5 and 37°C and that glucoamylase does not lose activity upon storage for 24 hr at pH 6.0 and 37°C. Therefore, the initial t = 0 and the final t = 16 hr glucoamylase activity in the digest at pH 6.0 and 37°C is estimated to be 20% of 3.4 U/mL or ~0.7 U/mL (Table II).

After the starch digestion step in AACCI Approved Method 32-45.01 (AOAC Method 2009.01) (1,4), the pH of the digest is adjusted from pH 6.0 to 8.2, and the mixture is heated for 20 min at 100°C to denature the protein. Subsequently, the protein is digested with protease at 60°C for 30 min. Finally, the digest is brought to pH 4.3 by adding 2 M acetic acid and 4 vol of ethanol. The solids are collected by filtration, dried, and corrected for residual protein and ash to obtain high molecular weight dietary fiber. Resistant maltodextrins may not totally precipitate upon addition of ethanol (38). If the sample contains low molecular weight soluble dietary fiber, the filtrate is passed through a mixed-bed ion-exchange resin, and the deionized eluate is examined by liquid chromatography to determine resistant oligosaccharides.

AACCI Approved Method 32-45.01 (AOAC Method 2009.01) for determining dietary fiber evolved from AACCI Approved Method 32-40.01 (AOAC Method 2002.02) for determining RS (1,4), which was first described by McCleary and Monaghan (42). In these methods, samples are digested at pH 6.0 and 37°C by a mixture of α-amylase and glucoamylase for 16 hr with agitation on a reciprocating or orbital shaker. However, the initial (t = 0) uncorrected concentrations of α-amylase and glucoamylase activities in digests in AACCI Approved Method 32-45.01 (AOAC Method 2009.01) (1,4) were increased by 93% (170 U/mL versus 88 U/mL [Table II, entry 4 versus entry 3]) and 26% (3.4 U/mL versus 2.7 U/mL), respectively, compared with levels in AACCI Approved Method 32-40.01 (AOAC Method 2002.02) (1,4). AACCI Approved Method 32-45.01 (AOAC Method 2009.01) (1,4) was extended recently to differentiate between soluble, insoluble, and total dietary fiber. The extended method, AACCI Approved Method 32-50.01 (AOAC Method 2011.25), and the original method (1,4) both describe the same conditions for removing digestible starch from a sample.

**Contrasting Starch Digestion in Prosky Versus McCleary Methods**

The starch digestion conditions in the Prosky method differ markedly from those in the McCleary method. In the Prosky method (1,4), two separate digestion steps are carried out with amylolytic enzymes. In the first step, the sample is...
digested with a heat-stable bacterial \(\alpha\)-amylase at pH 7–8 in a brief, hot, and relatively tranquil medium. In the Prosky method, the first digestion step at \(\approx 97^\circ\text{C}\) could theoretically increase \(\alpha\)-amylase activity by \(\approx 2\) orders of magnitude above the level (13 U/mL) in the starting digest, but at that temperature and without added Ca\(^{2+}\) ion, almost complete denaturation of the bacterial \(\alpha\)-amylase occurs (49) at the end of the digestion step. The second amylytic digestion step in the Prosky method, which likely reduces recovery of RS (8,42), is the final digestion step with glucoamylase. Because the second digestion is carried out at 60°C, the activity of glucoamylase could theoretically quadruple to \(\approx 72\) U/mL (3,200 U/g) as opposed to 18 U/mL at 40°C.

In the McCleary assay (1,4) for total dietary fiber, digestible starch in a sample is removed by a single, long (16 hr) digestion period at 37°C in a shaken buffer that contains Ca\(^{2+}\) and Cl\(^-\) ions plus both \(\alpha\)-amylase (starting at a calculated activity of 130 U/mL and ending at 13 U/mL) and glucoamylase (starting and ending at 0.7 U/mL) (Table II, entry 3). Glucoamylase has been combined with \(\alpha\)-amylase in many in vitro assays for dietary fiber to prevent product inhibition of \(\alpha\)-amylase by maltose (14,42). Glucoamylase serves another purpose in the McCleary method. In this method, glucoamylase converts most starch hydrolysis products to glucose so they are not counted with any resistant oligosaccharides in the subsequent liquid-chromatographic step. Even so, some branched \(\alpha\)-dextrins persist in the soluble dietary fiber fraction of the McCleary digest of high-starch foods (10,44), causing an overestimation of the low molecular weight dietary fiber in these foods. An additional digestion step with high levels of glucoamylase eliminates this overestimation.

It should be pointed out that bacterial \(\alpha\)-amylase contains 9–10 subsites in its catalytic site compared with 5 subsites in pancreatic \(\alpha\)-amylase (23). These subsites bind to sequences of glucose units in a single starch molecule during the hydrolytic step. The shorter length of subsite binding in pancreatic \(\alpha\)-amylase compared with that in \(B.\ licheniformis\) suggests that pancreatic \(\alpha\)-amylase is a “pointier” scissors for cutting \(\alpha\)-dextrin.
and maltose molecules away from RS, as well as from slowly digestible starch, given that the molecular sizes of the two amy- lases are similar. In addition, pancreatic α-amylase attacks a starch molecule (sol- uble) multiple times before dissociating from its complex with the substrate, whereas the bacterial α-amylase shows no multiple attack (23). Kimura and Robyt (31) reported that pancreatic α-amylase is more eff ective in digesting native starch than bacterial α-amylase.

Native cereal starches, except those with high levels of amylose, are complete- ly digested by both the Prosky and McCleary methods and yield negligible RS, as illustrated in Table I. However, samples of different resistant starches sur- vive differently when subjected to these two in vitro methods. Potato and banana starches, both RS2 but possessing gelatini- zation temperatures much below 95°C in excess water, are lost during high-temperature digestion in the Prosky method but not during 37°C digestion in the McCleary method. For the same reason, resistant maltodextrin (RS3) produced from enzyme-treated tapioca starch (ActiStar) yields no RS in the Prosky method but yields 48.8% RS in the McCleary method (38,44). On the other hand, the McCleary method may overestimate the level of RS in RS1 starch, in which starch is encased in protein, because a protein digestion step does not precede the starch digestion step as is done in the Englyst method. In McCleary (38), two samples of dry, ground beans yielded 52–55% total dietary fiber in the McCleary assay versus 17–23% in the Prosky method. Mahasukhonthachat et al. (35) found that amylolytic (α-amylase plus glucoamylase) digestion of ground sorghum is limited by diffusion of the enzymes through the protein or cell walls surrounding the starch. Sang et al. (55) found that when determining RS in sorghum flour, a protein digestion step before the starch digestion step reduces the level of RS. They (55) found 20% RS in a sorghum flour using the Englyst pro- cedure (59), which incorporates a pepsin-digestion step (pH 2.0) before starch digestion at pH 5.2 and found 32% RS when the protease step was omitted. The original Englyst assay (20) did not include a pepsin digestion step; however, McCleary and Monaghan (42) showed that crude pancreatic used in the starch digestion step is contaminated with proteases, including pepsin activity.

The McCleary assay (1,4) recovers low levels (~25% in Table I) of total dietary fiber from CLP wheat starch compared with the level (~85%) recovered by the Prosky assay (1,4). The same trend was observed for CLP tapioca starch (61.7 versus 83.8%) and amylose-stearic acid complex (RS5) (32.5 versus 48.1%). At Iowa State University, food scientists obtained an ≈48% lower estimation of total dietary fiber content in amylose- stearic acid complex (RS5) using the McCleary assay (1,4) versus the Prosky assay (J.-L. Jane, personal communication, 2013). The starch digestion conditions employed in the McCleary method appear to be harsher than those in the Prosky method, which likely explains the low recovery of total dietary fiber content in amylose-stearic acid complex (RS5) using the McCleary versus the Prosky method (J.-L. Jane, personal communication, 2013). The starch digestion conditions employed in the McCleary method appear to be harsher than those in the Prosky method, which likely explains the low recovery of total dietary fiber content in amylose-stearic acid complex (RS5) using the McCleary versus the Prosky method (J.-L. Jane, personal communication, 2013).
amylose (Table II, entry 3). These conditions promote diffusion in the heterogeneous reaction between amylopectin and granular starch and in the release of hydrolysis products and regeneration of the enzyme. Enhanced diffusion, high amylase activities, and prolonged digestion in the McCleary assay combine to produce strong hydrolysis conditions for starch. In contrast, the Prosky method specifies a brief (~30 min), albeit hot (~97°C), starch digestion step. However, during the hydrolysis reaction, the bacterial (B. licheniformis) α-amylase simultaneously undergoes denaturation, which decreases its activity. Plank et al. (48) reported a half-life of 8.6 min for the bacterial α-amylase when it was held at 95°C in MES-Tris buffer without added Ca+2 ion, which are the conditions used in AACCI Approved Method 32-07.01 (AOAC Method 991.43) (1,4). In spite of the eventual denaturation of most of the α-amylase in the Prosky digest, negative control samples of normal starches are completely digested (Table I). Plank et al. (48) speculated that the MES-Tris buffer used in the Prosky digest promotes the denaturation reaction, but the data in Table III suggest that the absence of Ca+2 ion in the digest may be the principal cause of the destabilization of α-amylase. Table III shows the total dietary fiber determined for nine samples of RS using the revised Prosky method (AACCI Approved Method 32-07.01 or AOAC Method 991.43) (1,4), which specifies use of an inorganic phosphate buffer for starch digestion, and the Prosky procedure (20) yielded RS levels of ~70%, which was ~45% higher than that of the McCleary method and ~15% lower than the total dietary fiber level determined using the Prosky method (Table I). However, if the RS and slowly digestible starch fractions determined using the Englyst procedure (20) are added together, their total for CLP wheat starch equals or exceeds the total dietary fiber determined using the Prosky method (4,51). Sang and Seib (53) calibrated the 1992 Englyst in vitro method (20) of measuring RS using potato starch and wheat flour. Moreover, the Englyst in vitro assay of RS was ultimately calibrated to in vivo data gathered from ileostomy patients by Englyst et al. (21, 59). Following calibration of the Englyst assay, Sang et al. (54) found that five samples of CLP (0.30–0.37% P) wheat starch contained 87–89% total dietary fiber when analyzed according to AACCI Approved Method 32-07.01 (AOAC Method 991.43) (1,4) and 63–69% RS plus 24–28% slowly digestible starch when analyzed according to the Englyst method. Research at the University of Arkansas (Y. -J. Wang, personal communication, 2011) found that two samples of CLP (~0.4% P) wheat starch contained 73–78% RS plus 16–19% slowly digestible starch when analyzed according to the Englyst method (20). It appears that much of the slowly digestible starch fraction in CLP wheat starch determined by the Englyst assay is recovered as dietary fiber by the Prosky assay. On the other hand, the slowly digestible starch in CLP wheat starch and more than one-half of its RS fraction are lost in the strong digestion conditions of the McCleary

Table III. Total dietary fiber of starches determined at a commercial laboratory using the Prosky Method with phosphate buffer versus MES-Tris buffer

<table>
<thead>
<tr>
<th>Starch</th>
<th>Moisture (%)</th>
<th>Total Dietary Fiber (% as is)</th>
<th>Phosphate Buffer</th>
<th>MES-Tris Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>9.5</td>
<td>1.0</td>
<td>&lt;0.75</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Tapioca</td>
<td>11.1</td>
<td>1.0</td>
<td>&lt;0.75</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Potato</td>
<td>11.6</td>
<td>1.0</td>
<td>&lt;0.75</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Normal corn</td>
<td>11.8</td>
<td>1.0</td>
<td>&lt;0.75</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>CLP wheat 1</td>
<td>10.1</td>
<td>1.0</td>
<td>82.2</td>
<td>83.4</td>
</tr>
<tr>
<td>CLP wheat 2</td>
<td>9.6</td>
<td>1.0</td>
<td>78.1</td>
<td>81.9</td>
</tr>
<tr>
<td>CLP tapioca</td>
<td>10.9</td>
<td>1.0</td>
<td>79.4</td>
<td>70.1</td>
</tr>
<tr>
<td>CLP potato</td>
<td>14.3</td>
<td>1.0</td>
<td>78.6</td>
<td>69.8</td>
</tr>
<tr>
<td>Amylose-stearic acid complex</td>
<td>10.4</td>
<td>1.0</td>
<td>38.8</td>
<td>46.5</td>
</tr>
<tr>
<td>High-amylase maize starch 1 (HMT)</td>
<td>10.3</td>
<td>1.0</td>
<td>41.5</td>
<td>39.2</td>
</tr>
<tr>
<td>High-amylase maize starch 2 (HMT)</td>
<td>10.2</td>
<td>1.0</td>
<td>58.4</td>
<td>57.9</td>
</tr>
<tr>
<td>High-amylase maize starch 3 (HMT)</td>
<td>10.1</td>
<td>1.0</td>
<td>52.2</td>
<td>47.9</td>
</tr>
<tr>
<td>High-amylase (~70%) corn starch 4 (Hylon VII)</td>
<td>11.2</td>
<td>1.0</td>
<td>15.9</td>
<td>12.7</td>
</tr>
</tbody>
</table>

a CLP = cross-linked phosphorylated; HMT = heat-moisture treated.

b Prosky method with phosphate buffer (AACCI Approved Method 32-05.01 or AOAC Method 985.29) (1,4).

c Prosky method with MES-Tris buffer (AACCI Approved Method 32-07.01 or AOAC Method 991.43) (1,4).
method. Both slowly digestible starch and RS are thought to be desirable starch fractions in foods. Incidentally, α-amylase activity in the Englyst digest (20) declines rapidly because of its acidity (pH 5.2) (42), which is comparable to the loss of α-amylase activity due to heat and the absence of Ca\(^{2+}\) ion in the Prosky digest (discussed above). On the other hand, ≈10% of α-amylase activity appears to remain (42) after 16 hr in the McCleary digest.

Prosky Assay Used to Follow Fate of RS in Ileostomy Study. Total dietary fiber, including RS, has been assayed successfully by the Prosky method in meals and effluents from ileostomy patients (57). In the study, seven ileostomy patients were administered meals that contained instant potato flakes over a 2 day period. During the next 2 days, they were provided meals that contained instant kidney bean flakes. Total dietary starch intake in both diets averaged ≈160 g/day, with 40% from potato or bean flakes and the rest from wheat (bread) and rice. The intake of total dietary fiber per patient in the potato-flake diet was 20.5 g/day, of which 1.8 g/day was RS, and the output of total dietary fiber was 21.6 g/day (105%), of which 1.9 g/day (105%) was RS (determined by dissolution and exhaustive glucoamylase digestion). In contrast, the intake of total dietary fiber in the bean-flake diet was 43.4 g/day (Prosky method), of which 7.0 g/day was RS, and the output of total dietary fiber was 44.3 g/day (102%), of which 7.0 g/day (100%) was RS (RS1 plus RS3). These data verify that RS measured by the Prosky method in kidney bean flakes passed through the mouth, stomach, and intestinal tract of humans with no in vivo digestion. Furthermore, in the case of bean flakes, with total dietary fiber that included ≈16% RS, the in vitro level of RS in the total dietary fiber measured by the Prosky method equaled the in vivo level.

Rapid Multiplication of Bifidobacteria by CLP Wheat Starch in Human Microbiome. Bifidobacteria associated with improved gut health in humans multiplied more than threefold when subjects consumed CLP (≈0.4% P) wheat starch (RS4 type with 85% dietary fiber, dry basis, in Prosky method), whereas an RS2 high-amylase maize starch (60% dietary fiber in Prosky method) showed a slower, twofold increase (37). Moreover, the RS4 wheat starch, but not the RS2 maize starch, induced phylum-level changes, significantly increasing *Bacteroidetes* and *Actinobacteria* and decreasing *Firmicutes*. Obese individuals typically have an elevated ratio of *Firmicutes* to *Bacteroidetes* in their gut. Ten human subjects consumed fiber-enriched (RS) crackers and negative control (native wheat starch) crackers for 3 weeks each in a double-blind crossover design study. A culture-independent method (multiplex sequencing of 16S rRNA tags) was used to measure the effect of RS on the in vivo composition of gut microbiota. The subjects consumed 100 g of crackers daily; the control, RS2, and RS4 crackers yielded 4.5, 33.2, and 30.5 g of total dietary fiber, respectively, as measured by the Prosky assay. It was noted in the study that the response of gut microbiota to the intake of RS2 and RS4 varied between individuals, as has been shown in other such studies. Individuals chew and drink differently, secrete different levels of enzymes, and have different gut transit times (12).

In other human nutrition studies on CLP wheat starch, Al-Tamimi et al. (3) found that the glycemic index of a nutrition bar was low when the bar was formulated with CLP wheat starch instead of an equal level of puffed wheat. Subjects consuming 15 g of dietary fiber (assayed using the Prosky method) in the form of CLP (=0.4% P) wheat starch per test meal displayed a glycemic index of 20 compared to 60 for the negative control of puffed wheat (glucose meal has a glycemic index of 100). The subjects consumed 50 g of available carbohydrate in both meals of nutrition bars formulated with corn syrup (20%), brown sugar (11%), and puffed wheat (34%) or CLP wheat starch (34%). The low glycemic index for the nutrition bar containing CLP wheat starch indicated the bar contained a high level of dietary fiber originating from the RS4 wheat starch.

Digestion of Normal Corn and Wheat Starches in McCleary Assay. Recently R. Shukri and Y. C. Shi at Kansas State University (personal communication, 2012) subjected CLP wheat starch (Fibersym) and unmodified wheat starch to the starch digestion conditions encountered in the McCleary method (AACC Approved Method 32-45.01 or AOAC Method 2009.01 [1,4]). Both wheat starchyes are industrial grade, which means both are predominantly composed (by weight) of large granules. They followed the digestion of the two granular starches for up to 24 hr and found a first-order digestion curve for granular wheat starch with a half-life of ≈0.8 hr and 95 and 98% digestion in 5 and 6 hr, respectively. Butterworth et al. (11) argued that the digestion of starch by α-amylase follows a first-order rate equation and that slowly digestible starch is an artifact due to the depletion of substrate. However, Blazek and Gilbert (7), who studied the digestion of six granular starches in the presence of pancreatic α-amylase and fungal glucoamylase (Table II, entry 1), observed a rapid release of glucose (5–15% digestion) during the first 2 hr, which could be modeled by a first-order rate equation. The rapid digestion of starch was followed by a slower, linear release of glucose with time, which they stated could reach a limiting plateau at long digestion times or remain linear depending on digestion conditions. The latter study is consistent with granular starch containing rapidly and slowly digestible starches. The digestion curve of the CLP wheat starch recorded by R. Shukri and Y. C. Shi (personal communication, 2012) displayed rapid digestion to 18% during the first hour and then plateaued out to ≈3 hr. Scanning electron micrographs showed predominantly surface erosion during the first 4 hr of digestion. Over the next 14 hr, between 3 and 17 hr of digestion, the rate increased and reached a rate of digestion similar to that of unmodified wheat starch, during which time the granules were extensively pitted. The extent of digestion of the CLP wheat starch at 17 hr was 75% and at 24 hr was 82%. At 5 hr the extent of digestion of the CLP wheat starch was 30% and at 6 hr was 40%, while over the same time period 95–98% of native wheat starch (control) was digested. The 70% RS found at 5 hr of digestion of CLP wheat starch by the McCleary method is consistent with the level (≈70%) of RS found by the Englyst method (20). Complete digestion of the control wheat starch in 5–6 hr suggests that carrying out digestion for 16 hr in AACC Approved Method 32-45.01 (AOAC Method 2009.01) (1,4) may not be necessary for CLP normal cereal starches. McCleary et al. (44) recently reported ≈92% digestion of normal corn starch after 6 hr in the starch digestion step of the McCleary method and ≈40% digestion of CLP wheat starch (Fibersym), indicating ≈60% RS in Fibersym.

Use of Amylolitic Enzymes to Remove Digestible Starch. In recent studies on the digestion of starch in Australia (26,35), researchers conducted in vitro digestion of starch for 4–25 hr at pH 6.0 (acetate buffer) and 37°C in shaken
digests (reciprocating at 85 oscillations/min) containing a mixture of porcine pancreatic α-amylase and glucoamylase. The researchers (26,35) first digested a sample for 10–20 sec in artificial saliva (a step that was discounted here). The concentration of α-amylase activity added to the digests was much less than that used in AACC Approved Method 32-45.01 or AOAC Method 2009.01 (1,4) (Table II, entry 3). The Australians added pancreatic as their source of α-amylase in the starch digestion step; however, α-amylase activity was not reported. The α-amylase activity in pancreatic reported by Englyst et al. (20) was ≈80 U/mg, while that reported by McCleary and Monaghan (42) was ≈10 U/mg (3 Ceralpha units/mg), reported by McCleary and Monaghan (20) was ≈80 U/mg, while that activity in pancreatin reported by Englyst et al. (35) also added pancreatin to a digest to yield an α-amylase activity (with CaCl₂) at pH 6.9 and 37°C. In one Australian study, Hasišć et al. (26) started digestion (t = 0) with uncorrected α-amylase activity (with CaCl₂) at 10–80 U/mL (200–1,600 U/g of starch) plus Aspergillus niger glucoamylase at 14 Sigma units/mL (280 U/g), where the Sigma unit is μmol/min of glucose released from starch at pH 4.5 and 55°C. In another Australian study, Mahasukhonthachat et al. (35) also added pancreatin to a digest to yield an α-amylase activity at t = 0 of 2.5–20 U/mL (=200–780 U/g), as well as glucoamylase activity at 28 Sigma units/mL (1,150 U/g). In comparison, AACC Approved Method 32-45.01 or AOAC Method 2009.01 (1,4) (Table II, entry 3) calls for initial α-amylase activity (uncorrected), with CaCl₂ in a digest of 170 U/mL (6,800 U/g) plus 3.4 Megazyme units (136 U/g) of glucoamylase, where the α-amylase unit of activity is the same as that described in the Australian work, except that the glucoamylase activity is given in Megazyme units measured at pH 4.5 and 40°C. In spite of the ≈10-fold lower concentration of α-amylase used by Mahasukhonthachat et al. (35), regular corn starch granules were almost completely digested in 8 hr (18). The Australian results for digestion of corn starch indicate that corn starch, like wheat starch, would be almost completely digested in 5–6 hr in the starch digestion step of AACC Approved Method 32-45.01 or AOAC Method 2009.01 (1,4). Therefore, it seems possible to use the method to determine RS in CLP (≈0.4% P) wheat starch if the digestion period is reduced from 16 to 5–6 hr. The 16 hr digestion period of the McCleary method may have been established in foods containing RS in which digestion reaches a plateau only after an extended digestion time. One more study can be cited to emphasize the high levels of α-amylase used in the starch digestion step of AACC Approved Method 32-45.01 (AOAC Method 2009.01) (1,4). Compared with this method, Mishra and Monro (45) used 1/40 the level of α-amylase activity (3 U/mL versus 130 U/mL) for 1/4 of the time (4 hr versus 16 hr) in a stirred (magnetic bar at 130 rpm) digest to compare the digestibility of starch in 24 food products. The other conditions of the starch digestion step (i.e., pH, glucoamylase activity, temperature, and addition of Ca²⁺ ion) were practically the same as those specified in AACC Approved Method 32-45.01 (AOAC Method 2009.01) (1,4). The digestion curves for two food products containing gelatinized starch in a weak food matrix (i.e., corn flakes and white bread) resulted in 80% digestion of starch in just 20 min and a digestion plateau higher than 80% in 60 min. It is worth mentioning that α-amylase activity in the lumen of the small intestines after a meal has been estimated to be 13 IU/mL at pH ≈7 and 37°C (52) and that the residence time of digests in the small intestines is 6–8 hr (57). As a summary, Figure 1 shows two circles that depict the dietary fiber components measured by AACC Approved Methods 32-45.01 and 32-50.01 (AOAC Methods 2009.01 and AOAC Method 2011.25). In Figure 1, the diagram on the left, taken from Bridges (9) and Zielinski et al. (65), has been modified to the diagram on the right to reflect the proposed underestimation of dietary fiber in amylose-stearic acid complex (RS5) and CLP wheat and tapioca starches (RS4), as well as the possible overestimation in RS1 starch encased in protein. It is notable that CCMAS in its 33rd session (16) recognized that “some RS4 resistant starches, dependent on type,” are not measured by AACC Approved Method 32-45.01 (AOAC Method 2009.01) (1,4,65).

Conclusions

In vitro assay of total dietary fiber in foods containing appreciable levels of RS is challenging because the five classes of RS display different barriers to amylase digestion. These barriers respond differently to the various starch digestion con-

![Fig. 1. Diagrams depicting the dietary fiber components measured by AACC Approved Methods 32-45.01 and 32-50.01 (AOAC Methods 2009.01 and AOAC Method 2011.25) (1,4). The diagram on the left was redrawn from Bridges (9) and Zielinski et al. (65). The diagram on the right depicts the proposed underestimation of dietary fiber components in amylose-stearic acid complex (RS5) and cross-linked phosphorylated wheat and tapioca starches (RS4) and the possible overestimation in RS1 starch encased in protein.](image-url)
ditions specified in general assays for total dietary fiber. Thus, the in vitro levels of RS measured in foods are method-dependent. It may not be possible to design a single assay procedure for measuring total dietary fiber in foods that will recover all forms of RS, such that in vitro levels correspond to in vivo levels. Zielinski et al. (65) reported that “the reality is that currently no single [dietary fiber] method is perfect for all situations and product types.” A similar situation exists for determination of the total antioxidant capacity of foods, where a single reaction has not been uncovered (30). When a food is fortified with RS, the analyst may need to know the type of RS present before an appropriate assay for total dietary fiber can be selected. This article shows that the Prosky method is the appropriate general method for recovering total dietary fiber from CLP RS4 starches.

Acknowledgments

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References

1. AACC International. Method 32-05.01, Total Dietary Fiber; Method 32-07.01, Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products; Method 32-40.01, Resistant Starch in Starch Samples and Plant Materials; Method 32-45.01, Total Dietary Fiber (Codex Alimentarius Definition); Method 32-50.01, Insoluble, Soluble, and Total dietary Fiber (Codex Definition) by an Enzymatic-Gravimetric Method and Liquid Chromatography. Approved Methods of Analysis, 11th ed. Published online at http://methods.aaccnet.org. AACC International, St. Paul, MN.


29. Huang, D., Ou, B., and Prior, R. L. The chemistry behind antioxidant capacity


