Dietary Fiber Content of Cross-linked Phosphorylated Resistant Starch (RS4) Determined by the Prosky and McCleary Methods

Part I. Factors Affecting In Vitro Digestion of Starch in a Food Sample

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Porcine pancreatic α-amylase and Aspergillus niger glucoamylase are used often in dietary fiber assay of foods. In this article (part I) we review important variables that influence the removal of digestible starch from a food sample by these two enzymes. A second companion article (part II) provides a comparison of the starch digestion conditions of the Prosky versus McCleary methods, assay data for resistant starch (RS) samples, and a hypothesis for why the McCleary method produces a relatively low recovery of dietary fiber from cross-linked phosphorylated RS4 (17). The McCleary method is detailed in AACC Approved Methods 32-45.01 and 32-50.01 (1), which are equivalent to AOAC Methods 2009.01 and 2011.25 (2). The Prosky method is detailed in AACC Approved Methods 32-05.01 and 32-07.01 (1), which are equivalent to AOAC Methods 985.29 and 991.43 (2).

Variables Affecting Enzymatic Digestion of Starch

Table I displays data pertinent to important variables that impact the in vitro digestibility of starch, as illustrated using starch granules from normal maize, high-amylose maize (Hylon VII, National Starch and Chemical Co. [now Ingredion]), and potato. As shown in Table I, starches were digested by a mixture of α-amylase and glucoamylase at pH 6.0 for 16 hr (entries 1–4) and at pH 5.2 for 2 hr (entry 5) or by α-amylase alone at pH =7.0 for 8 or 16 hr (entries 6 and 7).

All digestions were performed with porcine pancreatic α-amylase at 37°C, except for entry 6, which was performed with human salivary α-amylase. The source of glucoamylase in all entries was A. niger. The variables displayed in Table I include 1) method of agitation of digest; 2) presence or absence of glucoamylase with α-amylase in digest; 3) concentrations of amylolytic activity; 4) pH level; 5) addition of Ca²⁺ in the form of CaCl₂; and 6) length of digestion period. Other factors known to affect in vitro starch digestibility include enzyme source, particle (granule) size, crystalline pattern, presence of pores and channels, percent amylose, presence of lipids and protein, and especially granule architecture at a length scale ≥100 nm, but not at 1–10 nm (7,16,27; Cai and Shi, “Preparation, Structure, and Digestibility of Crystalline A- and B-Type Aggregates from Short α-1,4 Glucans,” submitted for publication).

The enzyme activities detailed in Table I were calculated from data provided by suppliers and from the literature; the starches were assumed to be 100% pure. Throughout this article, 1 unit (U) of α-amylase activity is the amount of enzyme that produces reducing power equivalent to 1 μmol maltose/min from soluble starch, which is often measured at pH 7 and 37°C. One unit of glucoamylase activity is the amount of enzyme that releases 1 μmol glucose/min from soluble starch measured at 40°C and pH 4.5. In Table I differences between α-amylase activity at 37 and 40°C, pH 6.5 and 7.0, and human salivary and porcine pancreatic α-amylase were ignored. One Ceralpha unit of α-amylase activity measured...
at pH 6.5 and 40°C, which was determined on end-blocked p-nitrophenol α-maltol-heptaoside in excess α-glucosidase, was converted to 3.3 U on soluble starch at pH 6.0 to 37°C, whereas one glucoamy-lase unit determined on p-nitrophenol β-maltoside in excess β-glucosidase was converted to 16.3 U on soluble starch at the same pH (4.3) and temperature (37°C) (18).

**Details about Variables**

**Agitation of Digest.** The impact of agitation on the digestibility of raw potato and corn starches by a mixture of α-amylase and glucoamylase at pH 6.0 can be discerned from the data in Table I. McCleary and Monaghan (21) (entry 4) found that 96% of potato starch was digested when the digest (pH 6.0 at 37°C for 16 hr) was stirred with a magnetic stir bar, whereas 23% was digested when the digest was shaken. Using the same switch in agitation method, the digestibility of normal corn starch did not change and remained at 99%, whereas the digestion of Hylon VII increased from 46 to 64% with stirring (entry 4). Englyst et al. (10) reported that the digestibility of potato starch granules increased fourfold when the stroke speed on a reciprocal shaker was increased from 46 to 64% with stirring (entry 4). Englyst and Cummings (9) reported that 50% of the starch in raw potato was digested in 16 hr at 37°C when treated with aqueous (presumably near pH 7) pancreatic α-amylase with enzymic activity at 5,000 U/g of substrate. They did not report the agitation method or substrate concentration.

When agitation of a starch digest was accomplished at low shear using peristaltic pumping (4) (entry 1) and the concentrations of α-amylase and glucoamylase activity per gram of starch were reduced ≈6–10-fold (≈500 U/g at pH 6.0 and 37°C [entry 1] versus ≈3,000–5,000 U/g levels in digests that contained guar gum with in vivo RS levels determined in ileostomy patients (10,28). In another study, Englyst and Cummings (9) reported that 50% of the starch in raw potato was digested in 16 hr at 37°C when treated with aqueous (presumably near pH 7) pancreatic α-amylase with enzymic activity at 5,000 U/g of substrate. They did not report the agitation method or substrate concentration.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate and Source</th>
<th>Sample Size (g) and Digest Vol (mL)</th>
<th>α-Amylase</th>
<th>Calculated Activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Waxy (99%) potato starch, AVEBE; Hylon VII, National Starch and Chemical Co.; maize, Penford Australia</td>
<td>30 and 70</td>
<td>Crude porcine pancreatic, Sigma-Aldrich A-3176, 287 U/mL, 670 U/g, No Ca⁺²</td>
<td>221, 111, 22 U/mL, 516, 259, 51 U/g</td>
</tr>
<tr>
<td>2</td>
<td>Potato starch, AVEBE; Hylon VII and maize starches, Penford Australia</td>
<td>1 and 40</td>
<td>Crude porcine pancreatic, Megazyme, 135 U/mL, 5,400 U/g Ca⁺²</td>
<td>104, 52, 10 U/mL, 4,160, 2,080, 416 U/g</td>
</tr>
<tr>
<td>3</td>
<td>Resistant starch ingredient, source not reported</td>
<td>1 and 40</td>
<td>Purified porcine pancreatic, Megazyme, 170 U/mL, 6,800 U/g Ca⁺²</td>
<td>130, 65, 13 U/mL, 5,200, 2,600, 520 U/g</td>
</tr>
<tr>
<td>4</td>
<td>Potato starch, AVEBE; Hylon VII, National Starch and Chemical Co.; maize, Penford Australia</td>
<td>0.1 and 4.5</td>
<td>Crude porcine pancreatic, Sigma-Aldrich A-3176, 88 U/mL, 3,960 U/g Ca⁺²</td>
<td>68, 34, 7 U/mL, 3,050, 1,025, 305 U/g</td>
</tr>
</tbody>
</table>

**Mixture of α-amylase and glucoamylase at pH 6.0 for 16 hr**

<table>
<thead>
<tr>
<th>Entry</th>
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<th>Sample Size (g) and Digest Vol (mL)</th>
<th>α-Amylase</th>
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</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Potato and high-amylase maize starches and white wheat flour, sources not reported</td>
<td>0.6 and 25</td>
<td>Crude porcine pancreatic, Paines &amp; Byrne Ltd. Pancrex V, 2,000 U/mL, 83,300 U/g Ca⁺²</td>
<td>166, 0 U/mL, 6,300, 0 U/g</td>
</tr>
</tbody>
</table>

**Mixture of α-amylase and glucoamylase at pH 5.2 for 2 hr**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate and Source</th>
<th>Sample Size (g) and Digest Vol (mL)</th>
<th>α-Amylase</th>
<th>Calculated Activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Potato starch, AVEBE; Hylon VII, National Starch and Chemical Co.; maize, Cerestar USA</td>
<td>0.04 and 1</td>
<td>Purified human salivary, Sigma (2,500 Berndorf U/mg or 3,250 IU/mg), 13 U/mL, 325 U/g No Ca⁺²</td>
<td>13, 13, 13 U/mL, 325, 325, 325 U/g</td>
</tr>
<tr>
<td>7</td>
<td>Potato starch, AVEBE; Hylon VII, National Starch and Chemical Co.; maize, Penford Australia</td>
<td>0.1 and 4.5</td>
<td>Crude porcine pancreatic, Sigma-Aldrich A-3176, 88 U/mL, 3,960 U/g Ca⁺²</td>
<td>68, 34, 7 U/mL, 3,050, 1,025, 305 U/g</td>
</tr>
</tbody>
</table>
of starch [entries 2, 3, and 4]) and ≈25-fold (1 U/g [entry 1] versus 24–27 U/g [entries 2, 3, and 4]), respectively, only ≈50% of normal corn starch was digested in 18 hr versus 99% for entries 2, 3, and 4.

**Presence of Calcium and Chloride Ions.** It should be noted that the digest used in entry 1 contained no added Ca\(^{2+}\) ion (CaCl\(_2\)). Calcium ion is a well-known stabilizer of \(\alpha\)-amylase against its denaturation, whereas chloride ion is an allosteric activator of vertebrate \(\alpha\)-amylase (11). Rendleman (24) (entry 6) found that when starch digestion was performed without added CaCl\(_2\) for 8 hr at pH 7 using salivary \(\alpha\)-amylase activity at 325 U/g of starch and no glucoamylase was present, only 50% of normal corn starch and 11% of potato starch were digested.

**Amylolytic Enzyme Concentration.** In vitro assays of dietary fiber invariably entail removal of digestible starch by a relatively low concentration of amylase compared with starch, which means that the rate of starch degradation increases with increasing concentration of \(\alpha\)-amylase and glucoamylase (8).

### Starch Granule Damage and Surface Pores

Potato starch granules are thought to resist amylase digestion at 37°C because the granules do not contain surface pores (5–400 nm) or channels that radiate to the inside of the granule (5, 14, 23, 27). Thus, pancreatic \(\alpha\)-amylase with a molecular mass of ≈50,600 Da (26) and \(A. niger\) glucoamylase with a molecular mass of ≈70,000–80,000 Da (29) cannot penetrate the tightly packed surface of raw potato starch granules.

The rate of heterogeneous enzymolysis of starch granules is expected to increase with agitation, yet physical damage to granules causes an even higher rate of digestion. Stirring starch granules with a magnetic bar in a low-viscosity, aqueous suspension can create fissures in starch granules, as exemplified by potato starch. These fissures allow amylases to breach the well-organized “outer shell.” Thickening a starch digest by adding 0.2% guar gum has been shown to increase the resistance of potato starch granules from 65 to 75% during digestion by a shaken mixture of \(\alpha\)-amylase and glucoamylase at pH 5.2 and 37°C (10). It is interesting that the estimated enzyme activities presented in Table I suggest the RS fraction determined by the Englyst method (10) is likely due as much to the resistance of starch granules to high levels of glucoamylase activity in the digest as to \(\alpha\)-amylase activity (5, 21).

Kimura and Robyt (15) have shown that potato (Hylon VII) and shoti starches are much more resistant to \(A. niger\) glucoamylase than are barley, maize, and tapioca starches, which correlated with the same order as their resistance to \(\alpha\)-amylase. The cereal starches from maize, sorghum, millet, wheat, barley, and rye (large A-type granules of the three Tri- ticeae starches) contain pores (<1,000 nm) on their surfaces that lead to channels inside their granules, whereas tuber and root starches lack pores (14, 23). Digestion of soluble starch by glucoamylase is much slower than by \(\alpha\)-amylase: the turnover number per molecule of glucoamylase enzyme is ≈30 molecules of \(d\)-glucose/sec, whereas that for \(\alpha\)-amylase is ≈1,000 molecules of maltose/sec (12, 25).
Probably because of their surface pores (4,7), the digestion of granular wheat and corn starches (especially those with a high amylose content) by porcine pancreatic α-amylase proceeds by a mechanism termed “granule-by-granule” digestion (6,13,27,30). In other words, once the surface of a wheat or corn starch granule is breached by α-amylase that granule generally is digested before another granule is digested, and so on. Granule-by-granule digestion is characterized by the residual starch having an unchanged molecular weight distribution, degree of crystallinity, and gelatinization properties, although some increases in crystallinity and onset temperature of gelatinization have been reported (4,5). Granule-by-granule digestion by pancreatic α-amylase emphasizes the idea of a resistant “outer shell” on a granule that restricts α-amylase from entering the inside of the granule. In moistened starch, the products of α-amylase digestion remain inside the “shell” of a granule (31). A physical force, such as prolonged shaking or stirring of a fluid starch digest, can damage the granule surface, allowing the entrance of α-amylase inside the granule, where rapid enzymic digestion then takes place (3,13,27). In addition to stirring, fine grinding of a low-moisture (<5%) sample may cause mechanical damage to starch granules. The starch in low-moisture foods, created by freeze- or vacuum-drying as prescribed in assays for dietary fiber (1,2), is likely present in a glassy friable state, and the damage caused is akin to that caused by cryogenic grinding of grain with a 10% moisture content (16).

Amylolytic Digestion of High-Amylose Maize Starch

The digestibility of Hylon VII (RS2) shown in Table I generally is much lower than that of normal maize and varies widely (10–66%), perhaps to as high as 83%, depending on digestion conditions. Only 10% of Hylon VII was digested after 8 hr at pH 7 and 37°C by a relatively low amount of pancreatic α-amylase (325 U/g) with no added CaCl₂ and without gluco-amylase (entry 6). However, 64% of Hylon VII was digested after 16 hr at pH 6.0 and 37°C in a stirred digest with α-amylase initially at 3,050 U/g of starch plus gluco-amylase at 24 U/g of starch (entry 4) and containing added CaCl₂. Using the Englyst method (entry 5), in which the level of amylose was probably 50–70%, 83% of a high-amylose maize starch was digested by the two amylases after 2 hr.

Conclusions

Data in the literature show that the digestibility of starch at 37°C by porcine-pancreatic α-amylase increases with agitation of a fluid digest, a pH level near 7, addition of glucoamylase and CaCl₂, a high concentration of enzymic activity, a prolonged digestion period, and surface pores on or mechanical damage to granules. These variables influence the RS values found by in vitro assays of total dietary fiber.

References

1. AACCI International. Method 32-05.01, Total Dietary Fiber; Method 32-07.01, Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products; Method 32-45.01, 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://methodss.aaccnet.org. AACCI International, St. Paul, MN.

2. AOAC International. Method 985.29, Total Dietary Fiber in Foods—Enzymatic-Gravimetric Method; Method 991.43, Total, Insoluble and Soluble Dietary Fiber in Foods—Enzymatic-Gravimetric Method, MES-TRIS Buffer; Method 2009.01, Total Dietary Fiber; Method 2009.02, Total Dietary Fiber; 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. Official Methods of Analysis of AOAC International, 18th ed. The Association, Gaithersburg, MD, 2011.


8. Dona, A. C., Pages, G., Gilbert, R. C., and Kuchel, P. W. Digestion of starch: In vivo and in vitro kinetic models used to char-


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