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In vivo digestibility of cross-linked phosphorylated (RS4) wheat starch in ileostomy subjects

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ABSTRACT

An intervention study was conducted to determine the *in vivo* digestibility of a commercial Type 4 resistant starch, namely, cross-linked phosphorylated (0.4% P) wheat starch (CLP wheat starch). Commercial unmodified (native) wheat starch was the negative control. Eleven ileostomy subjects participated in a randomized, double-blinded, cross-over design with a one-week washout period between test meals. Subjects consumed a plant-free breakfast including 26.8 g CLP wheat starch which was determined to contain 25.0 g of Prosky dietary fiber. The control breakfast included 26.9 g of commercial wheat starch. The subjects collected 2 h effluents over the next 24 h, and the wet effluents were assayed for total starch by AOAC Method 996.11. That assay was estimated to recover an average of 80.0% of the total starch in effluents when the subjects consumed CLP wheat starch. The *in vivo* level of RS in the commercial sample of raw CLP wheat starch (0.4% P) was determined to be 84.0%, whereas that of raw native wheat starch was 10.8%. The effective *in vivo* dietary fiber of CLP wheat starch was 89.0% compared to native wheat starch. When determining *in vivo* RS using the ileostomy model, if the origin of resistance to digestion in the starch is not robust, the Prosky assay will likely underestimate the ileal output of dietary fiber (RS).

1. Introduction

In broad terms, dietary fiber is non-digestible polymeric carbohydrate, larger than a dimer, that escapes the small intestine and reaches the colon. As such, it often elicits a physiological response of benefit to human health. At a dietary fiber conference in 2010, five physiological effects were strongly supported as being beneficial (positive votes from 80% of the ~ 150 participants from industry, academia, and government) (Howlett et al., 2010). These included (1) reduction in blood total and /or LDL cholesterol, (2) reduction in postprandial blood glucose and/or insulin levels, (3) increase in stool bulk/laxation, (4) reduction in transit time, and (5) fermentability by colonic microflora. Other physiological effects that were discussed included reduced blood pressure, positive modulation of colonic microflora/production of short chain fatty acids, weight loss/reduction in adiposity, and increased satiety. A specific dietary fiber may elicit one or several of those benefits.

The current legal definitions of dietary fiber in the USA and in Australia/New Zealand are aligned with the 2009 CODEX ALIMENTARIUS definition (Jones, 2013).

The CODEX definition is as follows:

> "Dietary fiber means carbohydrate polymers with 10 or more monomeric units that are not hydrolyzed by the endogenous enzymes in the small intestine of humans and belong to the following categories: (1) edible carbohydrate polymers naturally occurring in the food as consumed; (2) carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic, or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities; and (3) synthetic carbohydrate polymers, which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities."

Abbreviations: AOACI, Association of Official Analytical Chemists International; CLP, cross-linked phosphorylated; DF, dietary fiber; DMSO, dimethylsulfoxide; DS, dry solids; LDL, low density lipoprotein; PPA, porcine pancreatic α-amylase; RS, resistant starch

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The legal definitions in the two countries are:

USA (FDA 2016, see p. 33852); "The … definition…include[s] (1) non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units) and lignin that are intrinsic and intact in plants; (2) isolated and synthetic non-digestible carbohydrates (with 3 or more monomeric units) that we have granted be included in the definition of dietary fiber, in response to a citizen petition we received demonstrating that such carbohydrates have a physiological effect(s) that is beneficial to human health; or (3) isolated and synthetic non-digestible carbohydrates (with 3 or more monomeric units) that are the subject of an authorized health claim";

Australia/New Zealand (Australian Government, 2016); "Dietary fiber means that fraction of the edible part of plants and their extracts, or synthetic analogs that: (a) is resistant to digestion and absorption in the small intestines; (b) promotes one or more of the following beneficial effects; (i) laxation, (ii) reduction in blood cholesterol, and (iii) modulation of blood glucose; and includes (c) polysaccharides or oligosaccharides that have a degree of polymerization greater than 2; and (d) lignin".

In many countries the dietary fiber content of packaged food is included within a nutritional declaration. In the USA the Food and Drug Administration (FDA, 2016) issued rules to update the nutritional labeling of conventional foods and dietary supplements (Salmas, DeVries, & Plank, 2017). The labeling rules are scheduled to be implemented in July 2018, absent new extensions by the FDA. In the newly proposed regulations, dietary fiber is determined by *in vitro* assay. In addition, the level of dietary fiber declared in a food must be reduced for fiber present that does not afford a beneficial physiological effect. Manufacturers are responsible to maintain records of the sources and levels of dietary fiber added to food.

Dietary fiber in foods or ingredients is ultimately determined by *in vivo* assays in humans. But an *in vivo* assay is time-consuming, costly, and ethically challenging. Consequently, *in vitro* assays are used to measure dietary fiber. The FDA (2016, see p. 33864) proposed that compliance with the newly proposed labeling of dietary fiber can be achieved with an appropriate *in vitro* AOAC Method. Presently there are 8 general and 6 specific AOAC methods to measure total or specific forms of dietary fiber (see reference 16 in Maningat Seib and Bassi (2013)). The classical AOAC Method 985.29 for total dietary fiber devised by Prosky and co-workers in the mid-1980's has undergone several revisions since 2010 in order to capture not only non-starch polysaccharides, but also resistant oligosaccharides and resistant starch (RS). In general, an *in vitro* assay for total dietary fiber is achieved starting with removal of fat and sugar from a food by solvent extraction followed by removal of digestible protein and carbohydrate by enzymatic hydrolysis. High molecular-weight dietary fiber, which includes most RS, is recovered gravimetrically in many methods, and resistant oligosaccharides (dp ≥ 3) are counted by high-performance liquid chromatography.

The presence of RS in food complicates the *in vitro* assay of total dietary fiber. The enzymatic hydrolysis conditions in the assay must be set carefully so as to remove digestible starch and leave behind RS. Five types of RS (RS1-RS5) are recognized, and they persist against α-amylase digestion because of different barriers to enzymatic action. The barriers differ in structure and size-scale, from granules to molecules. The general AOAC enzymatic methods to assay for total dietary fiber are empirical, and they specify different conditions to remove digestible starch. The different starch-digestion conditions can result in different recoveries of RS for a type that lacks robustness. In the case of cross-linked phosphorylated (CLP) wheat starch with a phosphorus level of ~ 0.4%, which is a food ingredient useful for increasing dietary fiber content, the various AOAC assays give a range of ~ 25–92% for its dietary fiber content (Table 1). Native corn and wheat starches contain negligible dietary fiber by those assays. A recent rapid-integrated total dietary fiber assay (McCleary, Sloane, & Draga, 2015) gave a level of 60.2% of total dietary fiber (RS) in CLP wheat starch. The appreciable variability in the recovery of *in vitro* indigestible starch (RS) from CLP wheat starch is attributable to its sensitivity towards vigorous *in vitro* digestion conditions (Maningat et al., 2013). In human studies, when CLP wheat starch was incorporated in diets using the value of ~ 85% total dietary fiber, this high-fiber value was confirmed by significant beneficial physiological effects (Al-Tamimi, Seib, Snyder, & Haub, 2010; Haub, Hubach, Al-Tamimi, Ornelas, & Seib, 2010; Martinez, Kim, Duffy, Schlegel, & Walter, 2010; Nichenametla et al., 2014; Upadhyaya et al., 2016). In order to resolve which *in vitro* level of dietary fiber in CLP wheat starch is nearest to the *in vivo* level, we determined the *in vivo* digestibility of CLP wheat starch in ileostomy subjects.

2. Materials and methods

2.1. Materials

Starches were provided by MGP Ingredients Inc., Atchison, KS. Most experiments were done on two samples of commercial CLP wheat starch with the trade name Fibersym® RW. One contained 9.03% moisture, 0.4% phosphorus, and 85.0% ("as is") total dietary fiber (AOAC Method 985.29, the original Prosky assay) (AOAC Int'l 2012), while the other contained 11.6% moisture, 0.4% phosphorus, and 86.7% total dietary fiber ("as is"). A third sample contained 89.1% (ds basis) dietary fiber. Native wheat starch, with the commercial name Midsol™ 50, contained 8.4% moisture and < 0.5% ("as is") total dietary fiber. *In vitro* total dietary fiber was also determined by the Lee modification (AOAC Method 991.43) (AOAC Int'l 2012) of the original Prosky assay.

2.2. Nutrition study

Ethical clearance for the study was obtained from Monash University Human Research Ethics Committee. Volunteers were included if they were aged 18–75 yr, did not have diabetes, Crohn's or Coeliac disease, were not pregnant or breastfeeding, were not taking antibiotics and did not have any intolerance to carbohydrates. All study meals were plant-free and provided by Monash University investigators. Meals were prepared and cooked at Monash University in a commercial kitchen located at Notting Hill, Melbourne, Australia. Commencing with the evening meal on the night before the study, subjects were provided a plant-free meal which included either a meat-based soup, or a meat dish consisting of salmon, chicken or beef. Serving sizes were sufficient to meet each individual's estimated energy requirements and participants were advised to eat according to appetite. No vegetable, fruit or cereal was provided.

Subjects were asked to fast overnight. On the morning of the study prior to a test breakfast, each subject was asked to empty their ostomy bag and place the effluent in a sealed container and store at −20 °C in a portable freezer supplied by researchers. This sample represented time zero. Breakfast consisted of an omelette and lactose-free milk. Five hard-boiled sweets were also provided for consumption over the day. The test breakfast of CLP wheat starch, which contained 25 g of dietary fiber based on AOAC Method 985.29 (AOAC Int'l 2012) for total dietary fiber, amounted to 29.4 g of "as is" CLP wheat starch, or 26.8 g on a "dry basis". The negative control test breakfast contained 29.4 g ("as is") of native wheat starch, or 26.9 g (DS). The raw starches were slurried in milk prior to consumption. A Sitzmarks® capsule (Konsyl Pharmaceuticals, ICC Industries, New York), which contains 24 radiopaque markers, was consumed in a test breakfast with the intent of identifying the breakfast-effluent using x-ray. Compliance of ingestion to the study diet, CLP wheat starch, native wheat starch and Sitzmarks® capsule was measured using a provided food diary and checklist.

Following a test breakfast, subjects collected and stored ileostomy effluent every 2 h for the next 16–24 h in separate sealed containers and

Table 1
Total dietary fiber (% of dry solids) in native wheat and corn starches and in cross-linked phosphorylated (CLP) wheat starch with 0.4% phosphorus, a Type 4 resistant starch.

Starch	Assay method	Dietary fiber (%)	Reference
Wheat	Prosky, AOAC 985.29	< 0.8	Maningat et al. (2013)
Wheat	Lee-Prosky AOAC 991.43	0.4	Maningat et al. (2013)
Wheat	McCleary, AOAC 2009.01	< 0.5	Maningat et al. (2013)
CLP wheat	Prosky, AOAC 985.29	89	Maningat et al. (2013)
CLP wheat	Lee-Prosky, AOAC 991.43	91.8	Maningat et al. (2013)
Corn	McCleary, AOAC 2009.01	0.7	McCleary, Sloane, Draga, and Lazewska (2013)
CLP wheat	McCleary, AOAC 2009.01	24.9	Maningat et al. (2013)
CLP wheat	Resistant St., AOAC 2002.02	31.0	McCleary et al. (2013)
CLP wheat	McCleary, AOAC 2009.01	28.7	McCleary et al. (2013)
CLP wheat	Prosky, AOAC 985.29	84.0	McCleary et al. (2013)
CLP wheat	Rapid Integrated	60.2	McCleary, Sloane, and Draga (2015)

immediately placed 2 h-effluents in the provided portable freezer. The first sample was collected over 0–2 h, the second sample over 2–4 h, and so forth. All 2 h-effluents were weighed to determine wet-weights, then they were freeze-dried and dry weights recorded. The individual wet samples were analyzed for total starch using a Megazyme kit (Catalog K-TSTA, AOAC Method 996.11). The kit specifies that samples containing RS should be pretreated to dissolve RS in either cold (4 °C) 2 M potassium hydroxide or in hot (~ 100 °C) DMSO. AOAC Method 996.11 specifies the use of DMSO. After adding buffer to obtain pH 5 or 7, the starch in a sample is converted to glucose with thermostable α-amylase and glucoamylase at elevated temperature. The amount of glucose released, according to directions in the kit, is quantitated by glucose oxidase/peroxidase/dye reagent. When analyzing wet effluents from our subjects for total starch, we assayed those effluents using the DMSO protocol. In a separate experiment, seven freeze-dried 2 h-effluents were selected and ground gently with a mortar and pestle. The ground samples were homogenized by hand, then assayed for total starch levels by both the DMSO protocol and the 2 M potassium hydroxide protocol, as well as by the method of Shukri, Zhu, Seib, Maningat, and Shi (2015) which is an assay for the direct determination of CLP wheat starch.

Total dietary fiber in all 2-h freeze-dried effluents was determined by the original Prosky assay (AOAC Method 985.29) in a commercial analytical laboratory, GrainGrowers Ltd (Sydney, NSW Australia). Data on the freeze-dried samples is given assuming the samples contain no moisture.

2.3. Microscopy

CLP wheat starch and a freeze-dried effluent from the test breakfast of CLP wheat starch were imaged with light microscopy (Olympus BX61, Olympus Optical Co., Tokyo, Japan) under bright-field or polarized-light illumination as well as with confocal microscopy (LSM, Carl Zeiss, Germany) under reflective mode.

A sample (~ 5 mg) of freeze-dried effluent, which had been collected by Subject 7 in the 4–6 h period after the test meal, was mixed with distilled water (50 μl) to facilitate dispersion of particulates. A drop of the mix was placed on a glass slide and observed with light and confocal microscopes.

2.4. Model studies were conducted with various treatments of CLP wheat starch to determine whether those treatments affected the recovery of Prosky dietary fiber and/or the recovery of total starch from a treated sample

2.4.1. Testing of variables in "sample preparation" for Prosky dietary fiber assay of CLP wheat starch

CLP wheat starch (1 part) was stirred in water (10 parts) at room temperature, and the slurry allowed to stand for 24 h. The slurry was centrifuged and the sedimented starch was freeze-dried. The dried starch was assayed for total dietary fiber at GrainGrowers Ltd according to AOAC Method 985.29, with instructions to assay one portion of the sample without mechanical grinding. AOAC Method 985.29 states to mill a dry sample to 0.3–0.5 mm mesh.

2.4.2. Exposure of CLP wheat starch to pancreatic α-amylase and the resulting extent of digestion, plus the effect of that exposure of the cross-linked starch to pancreatic α-amylase on the recovery of Prosky dietary fiber and total starch (AOAC Method 996.11) from the undigested residual starch

Solutions of α-amylase were prepared in 50 mM maleate buffer (pH 7.0) containing 0–280 IU of enzymatic activity, where one IU is the amount of enzyme that releases 1 μmol maltose/min from soluble starch at 37 °C and pH 7. The activity of the commercial sample of powdery porcine pancreatic α-amylase (Cat No E-PANAA from Megazyme International, Bray, Ireland) was reported to be 150,000 Ceralpha Units/g at 40 °C and pH 6.9, which was converted to 405,000 IU/g by multiplying by 2.7 (McCleary et al., 2015).

The amylase solution (30 ml) was placed in a 40 ml centrifuge tube with a screw-top lid, and warmed to 37 °C. CLP wheat starch (1.5 g) was added, and the tube was positioned horizontally in a shaking-water bath at 37 °C and the reciprocating-shaker speed was set at 90 strokes/min. After 4 h the digest was centrifuged, and the supernatant was assayed for total carbohydrate using phenol-sulfuric acid reagent (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with reference to a standard curve derived from glucose.

The sedimented residual starch was washed thrice with water, dried at 35 °C in a forced-draft oven, and ground with a mortar and pestle. The residual dried starch was assayed for Prosky dietary fiber and for total starch using AOAC Method 996.11 (DMSO and KOH protocols).

2.5. Statistics

A per-protocol analysis was conducted. Two sample *t*-test with paired samples were used to determine if the treatment altered the mean measurements before and after treatment. All t_{crit}, t_{stats} and P-values are outputs of two tailed *t*-tests.

3. Results

3.1. Subjects

Eleven volunteers with a well-established ileostomy were recruited from either the Ostomy or Ileostomy Association of Victoria, Australia. Of the eleven volunteers there were 6 women and 5 men of average age 63 (range 52–73 yr) and 60 (range 54–67 yr), respectively, and the weight of the women averaged 69.3 kg (range 53–103 kg) and the men 90.6 kg (range 68–120 kg). One male participant was found to be non-compliant to the ingestion of Sitzmarks® capsule with the breakfast meal and, therefore, excluded from study's per-protocol analysis. For the supplied study diet, analysis of food diaries found that participants were 100% compliant to the provided study diet. Participants either reported having an adequate amount to eat or a generous amount.

Table 2
Total starch[a] and total dietary fiber[b] in effluents collected up to 24 h by ileostomy subjects after eating a meal containing 26.8 g (dry solids) of CLP wheat starch or 26.9 g (dry solids) of unmodified wheat starch.

	Wet weight (g)		Dry weight (g)		Total starch (g)		Total dietary fiber (g)	
Subject	CLP wheat	Wheat	CLP wheat	Wheat	CLP wheat	Wheat	CLP wheat	Wheat
1	333.6	406.8	42.3	7.80	19.8	1.0	5.8	0.9
2	420.3	177.7	52.5	26.6	17.5	0.1	9.5	3.2
3	508.0	453.0	64.4	21.1	22.4	0.2	7.0	1.2
4	346.4	340.5	46.6	34.3	3.5	0.3	7.8	2.8
5	636.2	517.4	79.6	70.0	13.0	8.3	11.7	7.8
6	601.9	557.2	51.0	49.0	28.8	14.5	12.2	5.8
7	494.8	392.8	67.1	4.3	13.5	0	16.1	0.8
8	476.7	439.0	48.0	6.0	35.4	1.6	13.7	2.0
9	282.3	–	42.3	24.4	6.2	0	11.7	5.1
10	406.7	417.2	58.8	47.7	19.8	3.4	11.4	4.1
Mean	450.7	411.3	55.3	29.1	18.0	2.9	10.7	3.3
Std. Deviation	115	109	12.1	20.3	9.2	4.8	3.2	2.2

[a] Total starch determined by AOAC Method 996.11 using a commercial kit.
[b] Total dietary fiber determined in a commercial laboratory by AOAC Method 985.29, the original assay of Prosky and co-workers (AOAC International, 2012).

3.2. Wet weight of output

The wet weights of the 24 h-effluents of the 10 subjects averaged 450.7 g and 411.3 g, respectively, after the breakfast with CLP wheat starch and wheat starch (Table 2 and Fig. 1, top). The corresponding freeze-dried weights averaged 55.3 g and 29.1 g (Table 2 and Fig. 1, bottom).

3.3. Starch in output

The solid lines in Fig. 2 show, for the test meal of CLP wheat starch, the total starch determined by AOAC Method 996.11 in each 2-h

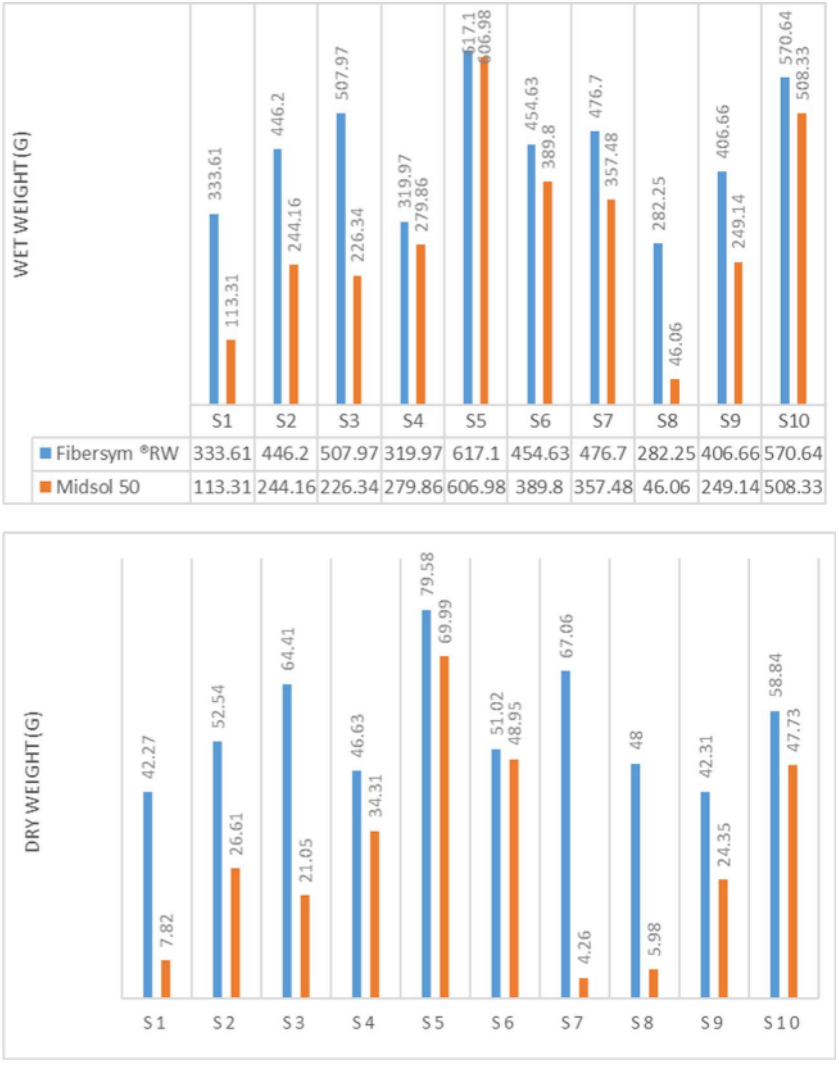

Fig. 1. Wet weight (top graph) and dry weight (bottom graph) of total effluent collected over 24 h from each subject (S1-S10) following a meal with 26.8 g (ds) CLP (0.4% phosphorus) wheat starch (left-hand bar of a pair), which contained 25.0 g of Prosky (AOAC Method 985.29) dietary fiber. The negative control diet contained 26.9 g (ds) of unmodified wheat starch (right-hand bar of a pair) with < 0.5% *in vitro* dietary fiber. For wet-weights; P-value = 0.004, t_{crit} = ± 2.26, t_{stats} = 3.83, and for dry weights; P-value = 0.008, t_{crit} = ± 2.26, t_{stats} = 3.38.

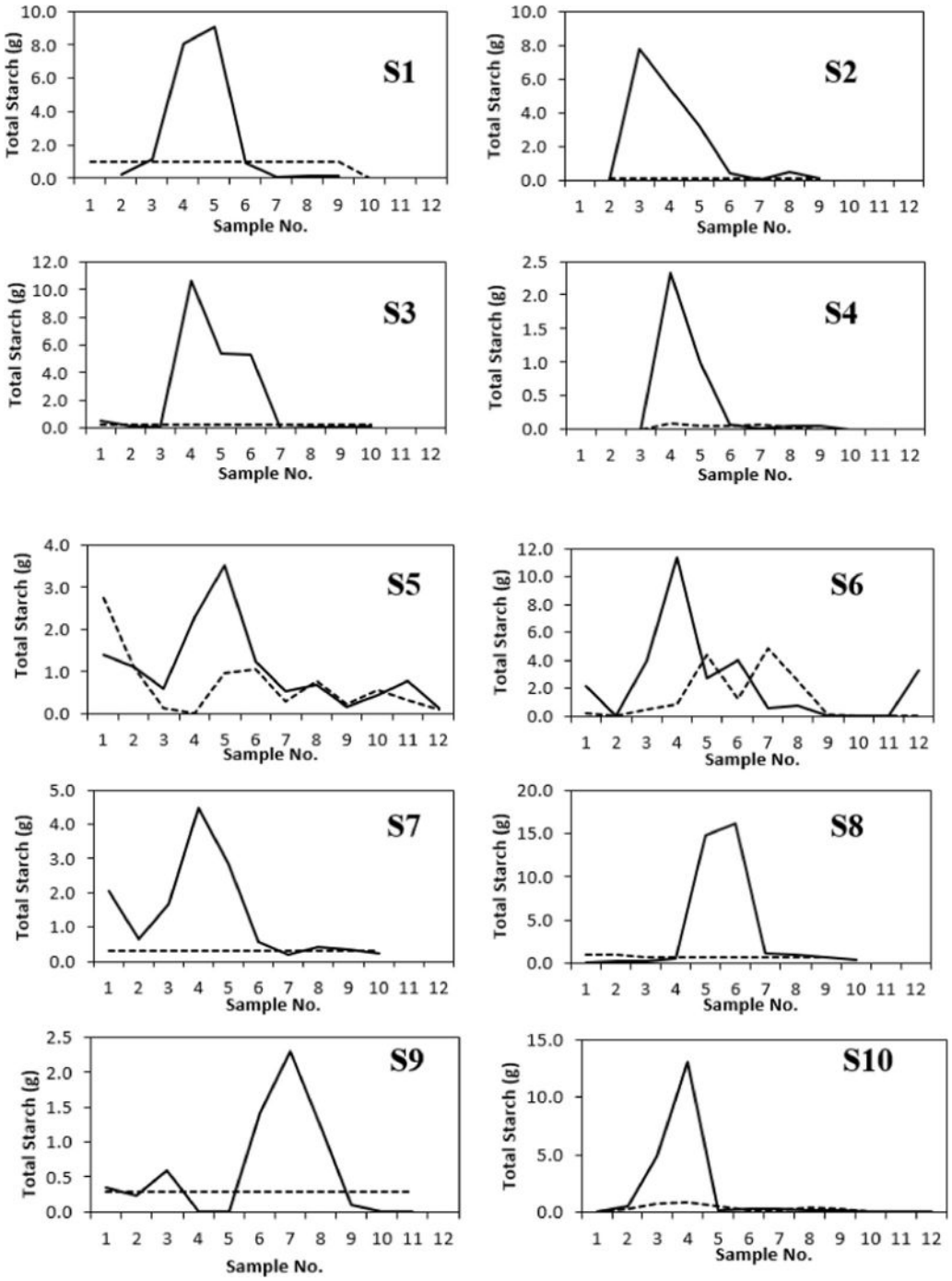

Fig. 2. Total starch in effluents (each point on a curve is for a 2 h-collection period) collected over 24 h for ten subjects (S1-S10) who consumed a meal of either 26.8 g (ds) CLP wheat starch (solid line) or 26.9 g (ds) wheat starch (dashed line).

effluent (wet) collected over the 18–24 h period after the meal. The radiopaque markers consumed with the breakfast failed to exit with all the starch-containing fractions. The initial time for significant amounts of starch to reach the ostomy bag for a subject who consumed CLP wheat starch, was generally 6–8 h, except for subjects S8 and S9 who showed initial times of 10 and 12 h. The orocecal transit time of a meal for healthy adults, determined by initiation of breath hydrogen, was reported to be 4.9 ± 1.0 h (Geypens et al., 1999), or 3.9 ± 0.4 h with a maximum breath hydrogen at 6.3 ± 0.4 h (Read et al., 1980). In our work, the time for clearance of starch in the output was 10–14 h for 6 subjects (Fig. 2). Two subjects (S2 and S9) showed a clearance time of 18 h, whereas two others (S5 and S6) gave the longest times of 24 h or more.

The dotted lines in Fig. 2 show the data points for total starch in 2-h effluents over the same period of time for the 10 subjects after they crossed-over to the unmodified, commercial wheat starch. When they consumed wheat starch the ileal output of starch was too low for its determination in every 2 h-collection for six subjects (S1-S3, and S7-S9). In their cases, each subject's 2 h-effluents were pooled prior to starch assay. The dotted line in Fig. 2 for each of those six subjects represents the mean starch level in the pooled sample.

3.4. Dry weight of output

Table 2 gives the sum of the dry solids and the total starch determined in all the 2 h-effluents after the subjects consumed CLP wheat starch or native wheat starch. The total dry solids in the 24-h ileal output by the subjects who consumed 26.8 g (ds) of CLP wheat starch

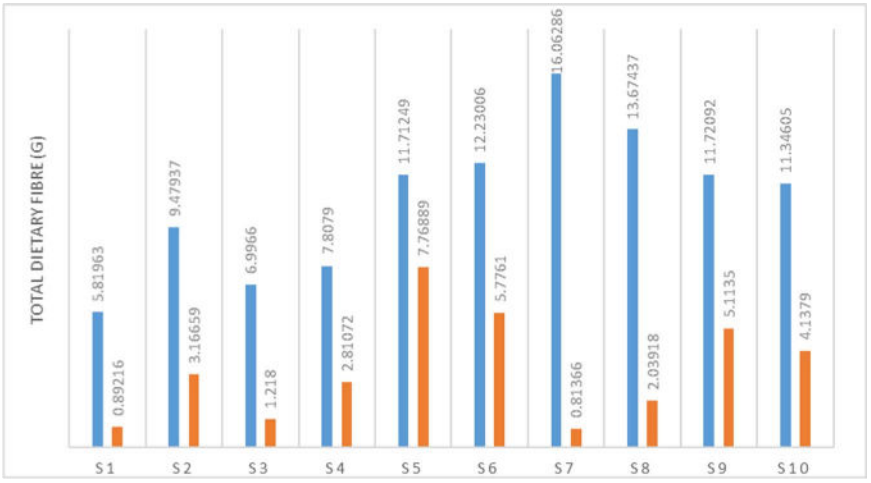

Fig. 3. Total dietary fiber in the total effluent (collected over 24 h) from each subject (S1-S10) following a breakfast containing 26.8 g (ds) CLP wheat starch (left bar) or 26.9 g (ds) unmodified wheat starch (right bar). P-value = 2.22×10^{-5}, $t_{crit} = \pm 2.12$, $t_{stats} = 5.90$.

ranged between 42.3 and 79.6 g with a mean and standard deviation of 55.3 ± 12.1 g (ds), while their 24 h-starch (AOAC 996.11) output ranged between 3.5 g and 35.4 g with a mean and standard deviation of 18.0 ± 9.2 g (Table 2). When consuming native wheat starch (26.9 g), the 24 h-output of dry solids for the subjects ranged between 4.3 and 70.0 g with a mean and standard deviation of 29.1 ± 20.3 g, and their output of total starch ranged between 0.0 and 14.5 g with a mean and standard deviation of 2.9 ± 4.8 g.

3.5. Prosky dietary fiber

Table 2 and Fig. 3 give the Prosky dietary fiber (AOAC Method 985.29) in the 24 h-output after the subjects consumed a starch-containing breakfast. The dietary fiber data in Table 2 for the subjects consuming 26.8 g of CLP wheat starch is the sum of dietary fiber determined in the individual 2 h-effluents over the 24 h-period after the test meal, and the sum ranged from 5.8g to 16.1 g with a mean and standard deviation of 10.7 ± 3.2 g. When each subject consumed 26.9 g of native wheat starch, the total dietary fiber excreted in 24 h ranged from 0.8g to 7.8 g with a mean and standard deviation of 3.4 ± 2.2 g. The dietary fiber excreted by the subjects consuming native wheat starch was determined on the pooled 2 h effluents for 6 subjects (S1-S3 and S7-S9), but was determined by summing the amounts in the individual 2 h-effluents for the 4 other subjects (S4-S6, and S10).

3.6. Model studies

Experiments were conducted in which CLP wheat starch was subjected to various treatments to determine their effects on the recovery of dietary fiber by the Prosky assay (AOAC Method 985.29), and on the recovery of total starch by AOAC Method 996.11.

3.6.1. Sample preparation of CLP wheat starch for Prosky DF

The original Prosky assay for total DF (AOAC Method 985.29) includes the steps of freeze-drying a moist sample followed by mechanically grinding the dried sample to 0.3–0.5 mm. In our experiment, the untreated sample of CLP wheat starch, as received, gave 85.8% dietary fiber (Table 3). When that sample was hydrated in excess water at ~ 25 °C and freeze-dried, but not mechanically ground, the dietary fiber content increased to 88.9% ("as is"). But when the same sample was hydrated, freeze-dried, and mechanically ground before assay, its level of dietary fiber decreased to 83.9% ("as is") (Table 3).

3.6.2. Exposure of CLP wheat starch to pancreatic α-amylase prior to Prosky DF assay

In a second model study CLP wheat starch was exposed to pancreatic α-amylase (4 h at 37 °C and pH7) to determine what effect that exposure exerted on the residual starch's Prosky DF content (AOAC Method 985.29) and on its total starch content (AOAC Method 996.11). Table 4 shows the untreated sample of CLP wheat starch used in this series of experiments contained 89.1% (DS) of Prosky dietary fiber and 68.0% total starch by AOAC 996.11. However, the total starch in the sample was 95.0% as measured by the direct method (Shukri et al., 2015) of assaying for CLP wheat starch. In our experiment a blank sample with no α-amylase treatment (0 IU/ml, Table 4) was prepared by stirring the CLP wheat starch in 50 mM maleate buffer at pH 7 and 37 °C for 4 h, then isolating and gently drying (convection oven at 35 °C) the starch. This negative control gave no detectable carbohydrate in the buffer medium, and its Prosky dietary fiber declined from

Table 3
Total dietary fiber content by the Prosky assay (AOAC Method 985.29) of CLP wheat starch (0.4% P) after hydration, freeze-drying and grinding.

Sample[a]	Dietary fiber, % ("as is")
As received from manufacturer	85.8
Hydrated, freeze-dried, not ground by analyst	88.9
Hydrated, freeze-dried, ground through 0.3–0.5 mm screen	83.9

[a] Sample of wheat starch (as is) gave 0.3% total dietary fiber.

Table 4
Exposure of CLP wheat starch (0.4% P) to pancreatic α-amylase (PAA) at 37 °C and pH 7 for 4 h; digestion by PAA, and levels of dietary fiber and starch in the undigested fraction (residual starch). All percentages reported on dry solids basis.[a]

		Residual starch[b]			
Pancreatic α-amylase[c]	Digestion by pancreatic α-amylase	Prosky dietary fiber (%)			Starch content AOAC 996.11
(IU/ml)	(%)	Calc'd[d]	Found	Δ	(%)
Untreated	–	–	89.1 ± 0.0	–	68.0 ± 0.4
0	0 ± 0.1	89.1	85.4 ± 0.4	3.7	65.6 ± 0.2
10	3.6 ± 0.3	92.4	84.1 ± 0.1	8.3	65.9 ± 0.1
50	5.8 ± 0.2	94.6	80.6 ± 0.5	14.0	70.8 ± 0.5
70	9.3 ± 0.3	98.2	75.1 ± 0.6	23.1	70.8 ± 0.8
140	19.9 ± 0.9	> 100	72.3 ± 0.8	> 25	67.7 ± 0.6
280	31.5 ± 0.2	> 100	71.3 ± 0.9	> 25	65.9 ± 0.6

[a] Cassava starch as a control gave 95.8% total starch by AOAC Method 996.11. The sample of CLP wheat starch gave 95.0% total starch content when assayed according to Shukri et al. (2015).
[b] Residual starch is that recovered after exposure of CLP wheat starch to pancreatic α-amylase.
[c] Buffer(30.0 ml) containing α-amylase shaken with CLP wheat starch(1.5 g).
[d] Theoretical level of dietary fiber in residual starch after removal of digestible starch

Table 5
Total starch determined by three assays on freeze-dried 2 h-effluents[a] from ileostomy subjects consuming 26.8 g (dry solids) of CLP wheat starch.

		Total starch[b] content (%)			Ratio of two assays	
Subject	Collection period[c] (h)	KOH	DMSO	Shukri et al. (2015)	DMSO/Shukri	DMSO/KOH
2A	6–8	6.9 ± 0.3	5.2 ± 0.4	7.2 ± 0.2	0.7	0.8
2A	8–10	26.2 ± 0.4	19.1 ± 0.2	21.9 ± 0.2	0.9	0.7
7A	10–12	21.4 ± 1.2	15.6 ± 0.4	21.3 ± 0.5	0.7	0.7
8A	8–10	43.4 ± 0.8	31.0 ± 0.4	40.0 ± 0.6	0.8	0.7
11A	6–8	34.1 ± 0.2	28.1 ± 0.9	32.2 ± 0.1	0.9	0.8
5A	10–12	6.9 ± 0.0	5.6 ± 0.8	6.6 ± 0.2	0.9	0.8
6B Wheat starch (26.9 g)	10–12	(0.9 ± 0.1)	(0.6 ± 0.2)	(0.7 ± 0.0)	–	–
Mean ± SD		23.1 ± 14.6	17.4 ± 10.9	21.5 ± 13.3	0.8 ± 0.1	0.8 ± 0.1

(c) in 2 M potassium hydroxide at 4 °C or sample preparation (d) in DMSO at ~ 100 °C. AOAC Method 996.11 for total starch specifies sample preparation in hot DMSO.
[a] Freeze-dried samples ground gently with mortar and pestle, and then homogenized by hand mixing.
[b] Total starch determined according to Shukri et al. (2015), and according to a Megazyme Kit (Catalog K-TSTA) with sample preparation.
[c] Time in hours from ingestion of starch to the start and completion of collecting the 2 h effluent.

89.1% to 85.4% (Table 4), consistent with the ~ 5% reduction caused by sample preparation (Table 3). At the same time, the total starch assay according to AOAC Method 996.11(DMSO protocol) gave 65.6% which was slightly lower compared to the untreated CLP wheat starch at 68.0%.

Exposure of CLP wheat starch to increasing levels 10–280 IU/ml) of pancreatic α-amylase resulted in 3.6–31.5% digestion of the cross-linked starch (Table 4). With that same pre-digestion, the residual fraction of cross-linked starch declined in Prosky dietary fiber content from ~ 85% to 71%, while its total starch remained between 66% and 71% as determined by AOAC Method 996.11 using the stipulated solvent DMSO. The modification of AOAC Method 996.11 using 2 M KOH as solvent for the residual starch was not attempted.

3.7. Total starch in seven effluents determined by three different assays

The recoveries of total starch from seven freeze-dried, 2 h-effluents were determined using three assay methods for total starch. Six freeze-dried, 2 h-effluents from the 10 subjects who consumed 26.8 g of CLP wheat starch were chosen that contained varying levels of starch determined by AOAC Method 996.11(Table 5). Their levels of total starch were determined also by the KOH (2 M) modification of AOAC Method 996.11, and by the method of Shukri et al. (2015). AOAC Method 996.11 gave the lowest recovery of starch (mean and s.d. of 17.4 ± 10.9%) followed by the Shukri et al. (2015) method (mean and s.d. of 21.5 ± 13.3%) (Table 5). The KOH modification of AOAC 996.11 gave the highest recovery (mean and standard deviation of 23.1 ± 14.6%).

3.8. Micrographs

Light photomicrographs of CLP wheat starch and ileostomate effluent are presented in Figs. 4 and 5. The confocal laser scanning micrographs are shown in Fig. 6.

4. Discussion

4.1. Wet and dry weight of subject output

Consuming CLP wheat starch with 0.4% phosphorus increased both wet and dry weight of ileostomy effluents (Fig. 1, and Table 2). The increase in dry weight was positively related to wet weight, showing that CLP wheat starch increased luminal water content. The water-holding property of CLP wheat starch in the upper gastrointestinal tract ranged from 5.9 to 9.7 g H_2O/g of dietary fiber, with an average of 8.0 g of H_2O/g of fiber. McBurney (1991) reported the potential water-holding capacity of psyllium at 8.6 g H_2O/g fiber.

The wet 24 h-output in this study (mean values of 411and 451 g/d for 10 subjects consuming ~ 27 g of either native wheat starch or CLP wheat starch, Table 2) was near the low end of the 635 ± 215 g/d wet-output found in another study of 26 ileostomy subjects (13 females and 13 males, age 36 ± 9 yr) who had suffered from ulcerative colitis (McNeil, Bingham, Cole, Grant, & Cummings, 1982). It should be noted that for the current study the volunteers were given a diet low in background carbohydrates, which is probably different compared to the study in England where the volunteers had been eating normally (McNeil et al., 1982). The ileostomy subjects in England recorded the foods and amounts consumed over a 7-day period. McNeil et al. (1982) reported the 26 subjects excreted an average of 17.6 g/d of dietary fiber, which was estimated to be ~ 15% below that (20.9 g/d) of control subjects. Besides dietary fiber, the 24 h dry-solids isolated from the 26 ileostomy subjects (McNeil et al., 1982) consisted of an additional 29.4 g of solids, including 14.4 g protein (N × 6.25), 5.5 g sugar, 4.7 g fat, 4.2 g ash and 0.6 g starch. Those components in the effluent plus the 17.6 g of dietary fiber summed to 47.0 g/d, accounting for 86% of the average 54.5 g/d of dry solids isolated. In our study involving 10 subjects, after they consumed a breakfast of native wheat starch together with protein and fat but no dietary fiber, they produced an average 24 h output of 29.1 g (Table 2) of dry solids, but with a large standard deviation of ± 20 g caused by subject variability. Judging from the data of McNeil et al. (1982), healthy ileostomy subjects on a diet devoid of dietary fiber could be expected to excrete ~ 36.9 g/d (54.5–17.6 g/d) of dry solids, which is similar in magnitude to the mean 29.1 g/d of dry solids excreted by our subjects when they consumed native wheat starch with other digestible nutrients. On the other hand, the mean output of dry solids for our subjects consuming 26.8 g (DS) of CLP wheat starch was 55.3 g/d, almost double the output (29.1 g/d) of the group when consuming 26.9 g (DS) of native wheat starch (Table 2). Ileostomy subjects on a typical Western diet usually excrete 2–3 times the amount of dry solids in their output compared to dry solids in feces of control subjects (McNeil et al., 1982). Unlike feces, ileostomate output is low (1–2% of total dry mass) in bacteria as opposed to 55% in feces, but dietary fiber is high in ileostomate output because it is not fermented in the large intestine.

4.2. In vivo RS in CLP wheat starch and in wheat starch determined by Prosky assay of DF

In our work with ileostomate subjects consuming 26.8 g (DS) of CLP wheat starch (0.4% P), or 26.8 g (DS) of unmodified wheat starch, initially we calculated *in vivo* RS from the Prosky dietary fiber (AOAC 985.29) contents in ileostomy effluents. The intake of total DF (Prosky) by a subject was 25.0 g for the CLP wheat starch and 0.14 g for the unmodified wheat starch. The freeze-dried solids in the individual 2-h effluents of each subject were assayed for Prosky DF and summed to give the total output of a subject (Table 2). Using the Prosky DF data

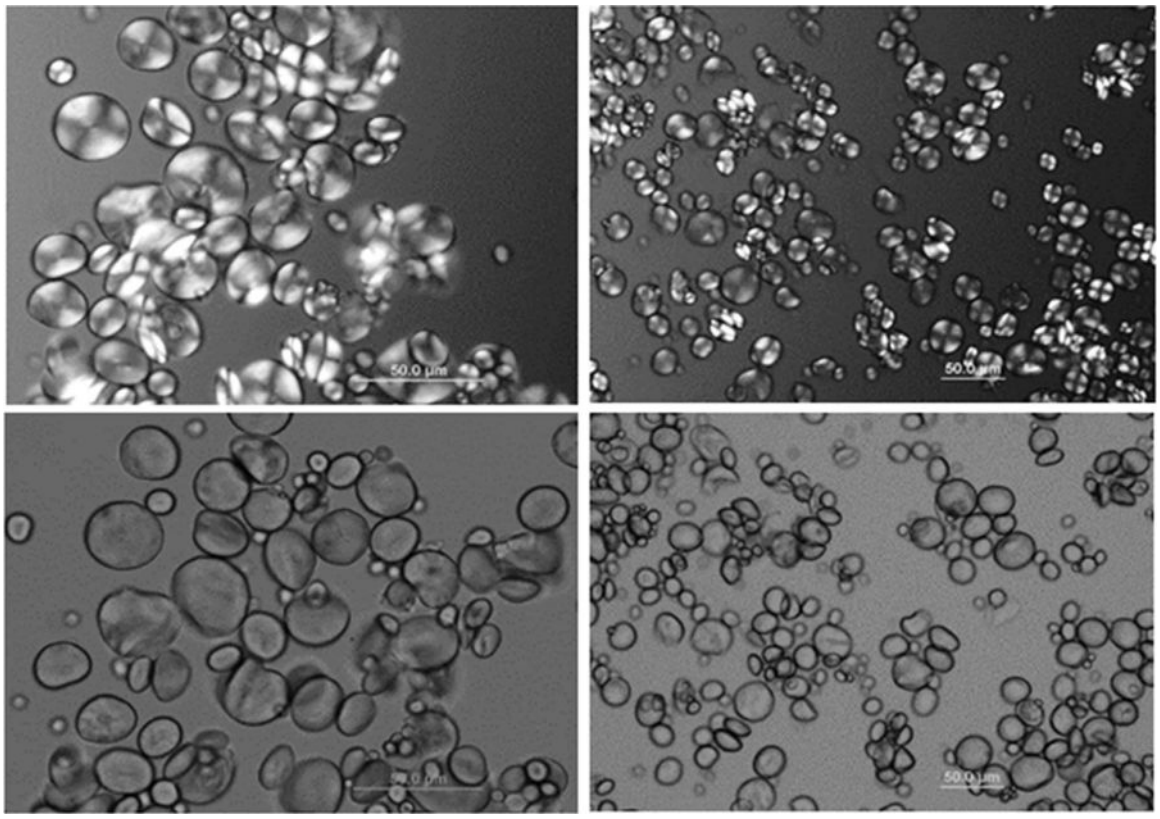

Fig. 4. Photomicrographs of CLP (0.4% phosphorus) wheat starch at two magnifications (left side 500× and right side 250×, scale bars = 50 μm). The bottom images were observed under bright-light illumination and the top images under polarized light.

and the mean output for the 10 subjects in Table 2, one calculates the *in vivo* RS level in CLP wheat starch is (10.7 × 100)/26.8 = 40.0%, while that of wheat starch is (3.3 × 100)/26.9 = 12%, both on a dry solids basis. Englyst, Kingman, Hudson, and Cummings (1996) previously reported wheat starch in flour contained ~ 0.6% *in vivo* RS when fed as biscuits to five ileostomy subjects. The higher level (12%) of *in vivo* RS we found for wheat starch may be explained by its raw state (no heating in the presence of moisture) and by its source. We used a commercial native wheat starch comprised of predominantly large granules, whereas the starch in wheat flour contains ~ 30 wt% of small granules. Commercial wheat starch also undergoes mild heat-moisture treatment during its isolation.

Langkilde and Andersson (1995) reported, in an Abstract, the *in vivo* levels of RS in three starches, high-amylose (~ 70%) corn starch (raw), potato starch (raw), and pregelatinized potato starch. Those workers used the ileostomy model and determined the RS in a subject's effluent in two steps, previously detailed in Schweizer, Andersson, Langkilde, Reimann, and Torsdottier (1990). In the first step, the alcohol-insoluble dietary fiber is recovered from ileal output according to the Prosky assay for total dietary fiber (AOAC Method 985.29). In the second step, the α-glucan in the insoluble dietary fiber is dissolved in alkali, the mixture is neutralized and then subjected to amylolytic digestion to give glucose, which is quantitated by glucose oxidase/peroxidase/dye reagent. The levels of *in vivo* RS reported by Langkilde and Andersson (1995) were, respectively, for high-amylose corn starch, potato starch and pregelatinized potato starch, 43.7%, 67.9%, and 0.8% ("as is"), or 50.3%, 78.8%, and ~ 0.9% presumably on a dry-solids basis as reported by Champ, Kozlowski, and Lecannu (2001).

Resistant starch is defined to be "the sum of starch and products of starch digestion not absorbed in the small intestines of healthy individuals" (Asp, 1992). We suggest that the classical Prosky assay for dietary fiber will give practically quantitative recovery of a Type 1 RS in ileostomate effluents from subjects consuming, for example, a legume flour as in the work of Schweizer et al. (1990). Type 1 RS is protected from amylolytic digestion in the Prosky assay by plant cell walls. However, in the case of a Type 2 RS, such as potato and high-amylose corn starch, where the starch granules are in direct contact with amylase but resist digestion because of a structural feature in their granules, the RS which is isolated as high-molecular weight dietary fiber according to Prosky could often be less than quantitative because of losses of low molecular-weight dextrins in the alcohol-precipitation step. The *in vitro* recovery of RS2, RS3 and RS4 in the Prosky or any other gravimetric assay for total dietary fiber depends on the resilience of the resistant fraction to the *in vitro* amylolytic digestion conditions designed to remove digestible starch from a sample.

4.3. Loss of DF from CLP wheat starch resulting from sample preparation

We wondered if sample preparation for the Prosky DF assay may have reduced the recovery of dietary fiber in an ileostomate output for a subject consuming CLP wheat starch. CLP wheat starch is a granular RS, similar to raw potato starch. Mechanical damage was imparted to granules of potato starch when that starch was slurried and agitated in water, and the damage decreased appreciably the recovery of RS (Englyst, Kingman, & Cummings, 1992; McCleary & Monaghan, 2002). Similarly, high-speed impact grinding of freeze-dried bread containing CLP wheat starch destroyed three-fourths of Prosky dietary fiber that was recovered otherwise from freeze-dried, gently ground bread (19.2% reduced to 4.7%, DS) (Yeo & Seib, 2009). In the present study we conducted a model experiment on CLP wheat starch to determine the effects of freeze-drying and mechanical grinding through a 0.3–0.5 mm screen on the recovery of Prosky dietary fiber. Table 3 shows that freeze-drying followed by grinding (presumably in a centrifugal mill) during sample preparation for the Prosky assay could cause a loss of as much as five percentage points in total dietary fiber recovered in the effluent of subjects consuming CLP wheat starch.

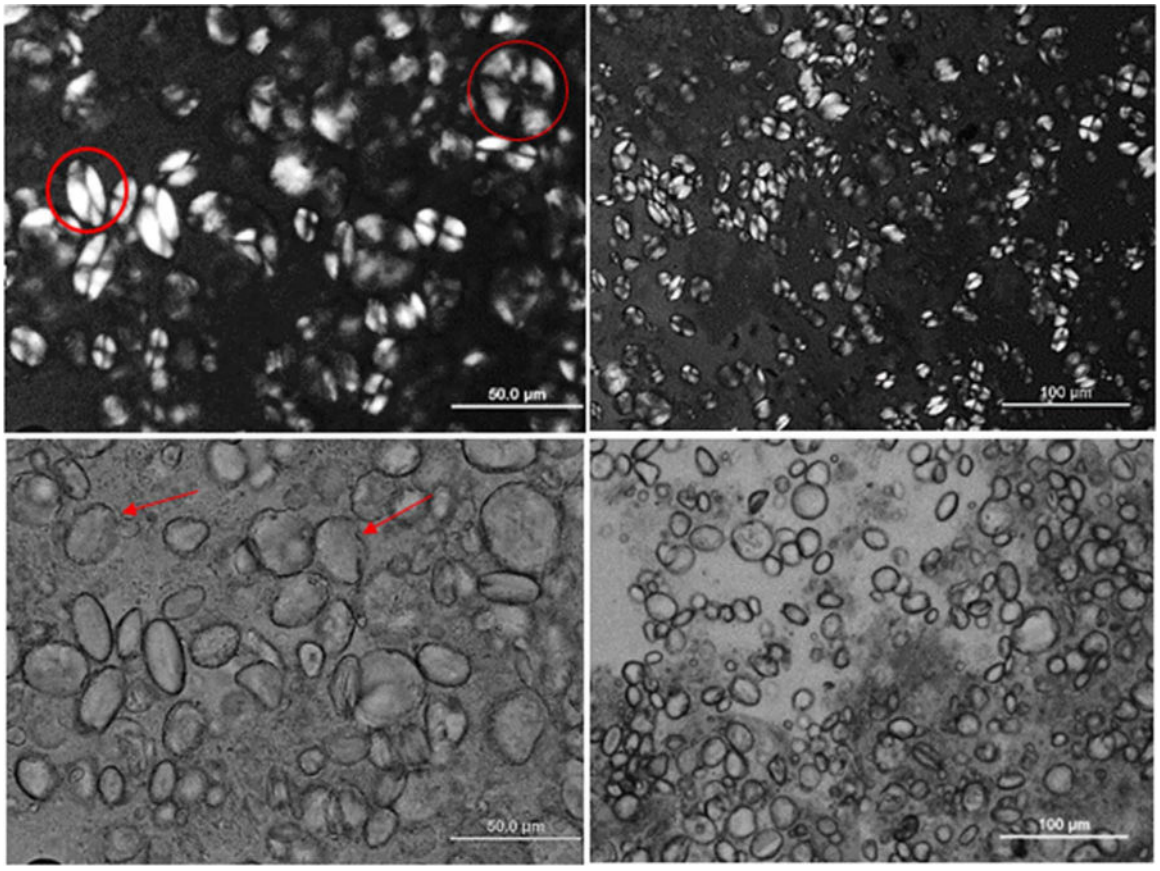

Fig. 5. Light photomicrographs at two magnifications of a freeze-dried effluent from ileostomy subject 7, collected 4–6 h after consumption of a breakfast containing 26.8 g of CLP wheat starch along with egg and milk. Bright light illuminated the sample shown at the bottom of the figure, and polarized light at the top. Scale bar = 50 µm (left side) and 100 µm (right side). Arrows point to the damaged surface of starch granules viewed under bright light. The circles in the top left photomicrograph enclose one granule retaining the Maltese-cross birefringence pattern under polarized light, whereas the other identifies a granule with a disrupted birefringence pattern.

4.4. Exposure of CLP wheat starch to pancreatic α-amylase: effect on Prosky DF

We conducted a second model study which shows the 40% *in vivo* RS for CLP wheat starch determined by the Prosky assay to quantitate outgoing fiber in ileostomy subjects, is erroneously low. Whereas mechanical damage to granules during sample preparation for Prosky assay may cause up to a 5% reduction in the recovery of dietary fiber from CLP wheat starch, we believe the main cause of the low recovery is due to α-amylase damage to granules of CLP wheat starch in a subject's

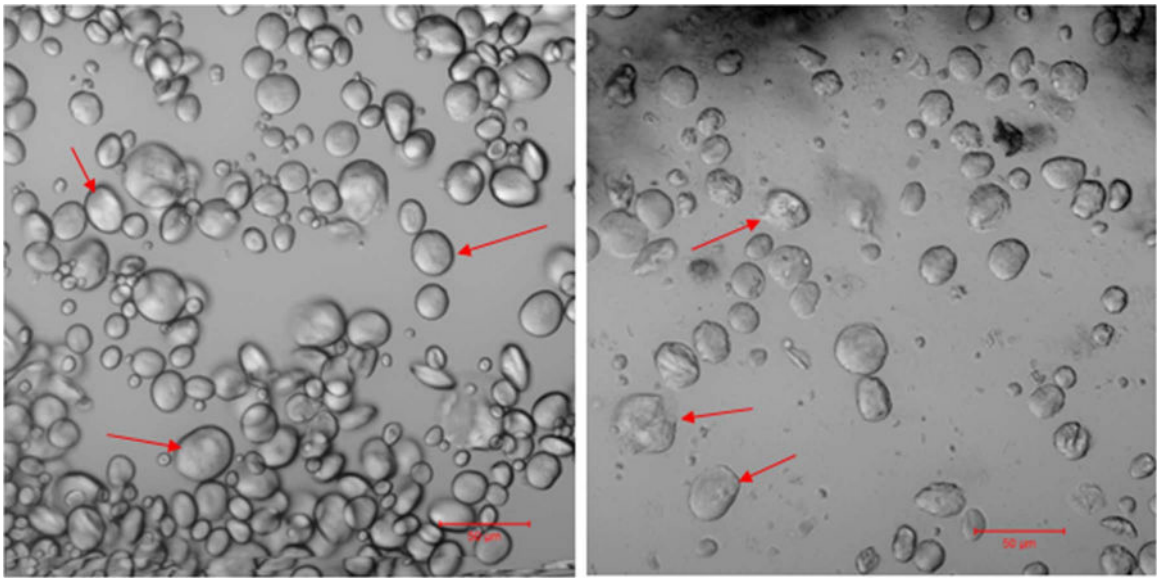

Fig. 6. Confocal micrograph of CLP wheat starch (left) and ileostomy effluent recovered from subject 7 four-six hours after the test meal (right). Scale bar = 50 µm. Arrows point to the distinct surface of granules seen in the control sample (left image), and to disrupted (digested) surfaces in granules that passed through the small intestines (right image).

small intestines.

In a model experiment CLP wheat starch (0.4% P) was exposed to differing levels of porcine pancreatic α-amylase, and the residual granules were isolated and subjected to the Prosky dietary fiber assay. The activity of pancreatic α-amylase in the duodenum after a meal has been estimated to be 13 IU/ml (Rendleman, 2000). Most of that activity persists throughout the length of the small intestines (Layer & Keller, 1999). The average residence time of food in the small intestines averages ~ 4–5 h (McCleary et al., 2015), but varies between individuals (Gerson, 2000; Geypens et al., 1999; Read et al., 1982). In our study, we chose to shake CLP wheat starch at 37 °C for 4 h in maleate buffer at pH 7 containing 0–280 IU/ml of pancreatic α-amylase activity. The residual starch was isolated, gently dried (35 °C) and gently ground, then assayed for Prosky dietary fiber.

At α-amylase activity of 10 IU/ml, the digestion of CLP wheat starch amounted to 3.6%, and the residual starch gave 84.1% Prosky dietary fiber compared to 89.1% for the untreated sample (Table 4). Removal of 3.6% digestible starch from CLP wheat starch by the mammalian α-amylase pre-treatment would theoretically increase the level of dietary fiber in the residual starch to 92.4%, a value 8.3% above that found (Table 4). It appears that the pancreatic amylase decreases the average molecular-size of starch inside the granules of CLP wheat starch such that more α-dextrins (and phosphorylated α-dextrins) are formed during the Prosky starch-digestion step. Those dextrins are too low in molecular weight to precipitate in ~ 75% ethanol, and they are not recovered in the gravimetric accounting of dietary fiber by the Prosky method. Furthermore, pancreatic α-amylase digestion of the exterior and interior of the granules (as will be shown later in photomicrographs) creates weaknesses in the granules and increases their vulnerability for increased digestion in the Prosky assay resulting in a reduced yield of indigestible residues. Increasing the activity of pancreatic α-amylase in the pre-treatment of CLP wheat starch to 50–280 IU/ml caused digestion to increase to 5.8–31.5%, and the Prosky dietary fiber level in the residual starch declined more than 25% below the calculated level of over 100% (Table 4). The results of this model experiment indicate that the Prosky dietary fiber assay is inappropriate for determining the fraction of CLP wheat starch exiting in the effluent of an ileostomy subject.

4.4.1. Exposure of CLP wheat starch to pancreatic α-amylase: effect on starch assay of undigested fraction

Whereas the exposure of CLP wheat starch to increasing levels of pancreatic α-amylase at pH 7 and 37 °C for 4 h increased its digestibility (decreased its resistance) markedly in the Prosky assay, the increase in digestibility was much less noticeable in the total starch assay by AOAC Method 996.11 (Table 4). Untreated CLP wheat starch gave 68.0% total starch by AOAC Method 996.11, and pre-treatment with 10–70 IU/ml of α-amylase caused a slight increase in recovery of total starch from ~ 67% to 71% in the undigested fraction. However, pre-treatment with 140 and 280 IU/ml reduced recovery of total starch back to ~ 66%. It is well known that the cross-links inhibit the digestion of the starch under the conditions (dissolution of sample in hot DMSO) used in AOAC Method 996.11. It should be mentioned that all samples of CLP wheat starch, regardless of α-amylase pretreatment, gave ~ 95% total starch according to the method of Shukri et al. (2015). The Shukri method specifies especially strong digestion conditions for CLP wheat starch using heat-stable α-amylase. In addition, CLP wheat starch with 0.4% P has only ~ 3% of its glucose units phosphorylated (Shukri et al., 2015). The strong digestion conditions produce low-molecular weight α-dextrins and phosphorylated α-dextrins from CLP wheat starch. Phosphodextrins of low- *versus* high-molecular weight, are more readily converted to glucose by glucoamylase. Glucoamylase is an exo-acting hydrolase that catalyzes removal of terminal glucose units from the non-reducing ends of starch chains. A single phosphate group on the non-reducing end of a long starch-chain has the potential to protect the entire chain of glucose units from digestion. The shorter the average chain-length in the phosphodextrin, the fewer the glucose units protected from glucoamylase.

Photomicrographs in Figs. 4 and 5 reveal the damage to granules of CLP wheat starch caused by the amylases in the gut of an ileostomy subject. Starch particles constituted the predominant particulate in the effluents of subjects consuming CLP wheat starch. A large proportion of the granules in the output retained birefringence (Fig. 5), but the birefringence pattern was not sharp compared to the untreated control (Fig. 4), which indicates some hydrolytic damage occurred inside the granules during their passage through the small intestines. This is consistent with the “inside out” digestion mechanism common for A-type crystalline cereal starches such as wheat, maize and rice. Those starches contain submicron-sized surface pores and channels that facilitate the diffusion of enzymes towards the hilum, a less organized area, hydrolyzing the starch in a centrifugal pattern (Dhital et al., 2010). Damage at the surface of granules in the effluent is seen in the light micrographs in Fig. 5, and is especially distinct in the confocal micrographs in Fig. 6.

4.5. Starch in subject output incompletely quantitated by conventional total starch assay

Radiopaque markers were consumed by the subjects with the starch breakfast to allow pooling of the 2 h-effluents into a single sample for convenient assaying of the total output from a starch breakfast. However, those markers failed to track the starch as it exited in the 2 h effluents as determined by AOAC Method 996.11 (Fig. 3). Method 996.11 for assaying of total starch specifies solubilization of the α-glucans in a sample by heating in dimethyl sulfoxide (DMSO). We used AOAC Method 996.11 to determine the amount of starch in each wet 2-h effluent, even though we realized the recovery of starch from CLP wheat starch by that Method is less than quantitative since the cross-linked starch resists dissolution in DMSO (Woo & Seib, 2002). AOAC Method 996.11, however, was appropriate to determine total starch excreted by the ileostomy subjects when they consumed native wheat starch. The amounts of starch in the individual 2 h-effluents, determined by Method 996.11, were summed to give the total starch in the output for each of the 10 subjects. Those sums and means are given in Table 2. The mean amount of starch (Method 996.11) leaving the small intestine was 18.0 g for the subjects consuming 26.8 g of CLP wheat starch, and 2.9 g when they consumed 26.9 g of native wheat starch. From those amounts one calculates that CLP wheat starch, on a dry basis, contains a minimum of 67.1% *in vivo* RS. The *in vivo* level found for native wheat starch was 10.8%. In those calculations, the glucose counted by AOAC Method 996.11 was assumed to originate from the ingested starch, although some may have originated from the hard candy. Englyst and Cummings (1987) reported that the amount of glucose from endogenous sources in ileostomy output is small ($\leq$ 0.7 g/d).

4.6. In vivo RS in CLP wheat starch

In order to correct for the incomplete recovery of starch in ileostomy output by AOAC Method 996.11 when the subjects consumed CLP wheat starch, we derived a “recovery correction factor”. The “recovery correction factor” was derived by assaying six, freeze-dried, 2-h effluents for their total starch levels according to AOAC Method 996.11, and comparing those levels to the ones determined by the quantitative method of Shukri et al. (2015) (Table 5). The data in Table 5 shows that Method 996.11 recovered, on average, 80% of the starch in the 2 h-effluents compared to that recovered by the method of Shukri et al. (2015). Using the “recovery correction factor”, the average starch output of the subjects when they consumed 26.8 g of CLP wheat starch is corrected from 18.0g to 22.5 g (18.0 g/0.8 = 22.5 g). The corrected value for the *in vivo* RS in CLP wheat starch becomes (22.5 g) (100)/26.8 g = 84.0%. The 22.5 g of average RS in the effluent of the 10

subjects represents 90% of the 25 g of ingested RS determined by the Prosky assay.

The "recovery correction factor" could also be derived from the total starch levels (Table 5) determined on the same six 2 h-effluents by comparing assay data from AOAC Method 996.11 using cold 2 M KOH (Table 5). The first step in the assay according to the official Method 996.11 is to heat a sample in DMSO, whereas in the KOH modification the first step is to mix a sample with cold 2 M KOH. The data in Table 5 shows that mixing the six, freeze-dried, 2 h-effluents in cold 2 M KOH gave greater recovery of total starch than mixing in hot DMSO, and the recovery of starch by the KOH method was comparable to that of the Shukri et al. (2015) assay. The mean recovery by the DMSO protocol was 80% ("recovery correction factor") of that using 2 M KOH. Recovery of total starch from ileostomy output from subjects consuming CLP wheat starch is increased in the KOH modification of the assay probably because many, if not all, the phosphate cross-links are hydrolyzed in 2 M KOH. Sang, Seib, Herrera, Prakash, and Shi (2010) reported that exposure of CLP wheat starch (0.37% P) to mild alkali (0.01 M sodium hydroxide, 40 °C for 4 h) caused removal of 22% of phosphorus from CLP wheat starch.

4.7. Effective dietary fiber in CLP wheat starch

The effective *in vivo* dietary fiber (RS) contribution of CLP wheat starch compared to native wheat starch is of interest when the cross-linked starch is added to a wheat-based food to bolster dietary fiber content. The effective dietary fiber content of CLP wheat starch can be calculated from the data in Table 2, which shows that consuming practically equal amounts, 26.8 g and 26.9 g of CLP wheat starch and native wheat starch, respectively, results in 18.0 g and 2.9 g of *in vivo* RS. Upon applying the "recovery correction factor" of 0.80, the level of RS contributed by the 26.8 g of CLP wheat starch is calculated to be 22.5 g. The effective *in vivo* dietary fiber content of CLP wheat starch compared to wheat starch is 22.5(100)/(22.5 + 2.9) or 89%. Thus, a person eating equal amounts of CLP wheat starch and native wheat starch would realize 89% of RS (dietary fiber) originating from the cross-linked starch.

5. Conclusions

Cross-linked phosphorylated (CLP) wheat starch with ~ 0.4% P is a slightly modified (~ 3% of glucose units) starch whose granules are highly resistant to heat-stable bacterial α-amylase used in the dietary fiber assay according to Prosky (AOAC Method 985.29) or to Lee-Prosky (AOAC Method 991.43). The low degree of modification of the cross-linked starch indicates it derives most of its resistance to digestion at the granule level. Recovery of RS from ileostomy subjects consuming CLP wheat starch cannot be accomplished by the Prosky assay. The Prosky assay (AOAC 985.29) entails gravimetric recovery of insoluble high-molecular weight RS. The Prosky total dietary fiber assay seems appropriate to recover *in vivo* RS from the effluent of ileostomy subjects consuming Type 1 RS or a resilient RS of Type 2–5 that is high in molecular weight. Our ileostomy studies on a meal containing ~ 26.8 g of CLP wheat starch (0.4% P), and with determination of ileal-starch output by AOAC Method 996.11, suggested that the CLP wheat starch contains a minimum of 67.1% *in vivo* RS, whereas unmodified wheat starch contains 10.8% *in vivo* RS. The recovery of total starch from ileostomy output of CLP wheat starch by AOAC Method 996.11 is approximately 80% compared to the quantitative recovery by the Shukri et al. (2015) assay. When one corrects for the 20% incomplete recovery of starch by the AOAC Method 996.11 in ileostomy output from subjects consuming CLP wheat starch, the corrected *in vivo* level of RS in CLP wheat starch (0.4%P) is 84%.

Conflict of interest statement

C.C. Maningat is vice-president of research and development and chief scientific officer of MGP Ingredients, Inc. the manufacturer of CLP wheat starch (Fibersym® RW). P.A. Seib is a consultant for the company. The remaining authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bcdf.2017.08.002.

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