FOOD COMPOSITION AND ADDITIVES

In Vitro and In Vivo Digestibility of Native Maize Starch Granules Varying in Amylose Contents

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Digestibility of maize starch granules with different amylose content (AL-0, 22, 54, 68, 80, or 90%) was investigated. Measurement of the in vivo resistant starch (RS) content of the starches was performed using surgically prepared ileorectostomized rats. The rats were fed a purified diet containing one of the starches at 652.5 g/kg diet. The in vivo RS content was determined based on the fecal starch excretion. The dietary fiber (DF) value increased as a function of the amylose content in the starch and showed a positive linear correlation with the gelatinization temperature of the granules. In contrast, the in vitro RS content was likely to depend on both the surface area and amylose contents of the starch granules. The maximum in vitro RS content was obtained with AL-68 (54.4%). In vivo RS content showed a significant correlation with the amount of in vitro RS but not in respect to the DF detected. The in vivo RS content of AL-68 (43.4%) was higher than that found in AL-90 (37.8%). A profound gap was observed for AL-54 between the amount of DF (6.4%) and RS (in vitro = 46.6% and in vivo = 40.9%) present. The results suggest that both in vitro and in vivo digestibility of maize starch is affected by the amylose content and surface area of the granules. The current evaluation suggests that the physiological occurrence of RS from maize starch might be predictable by reference to the in vitro RS value.

Starch is a glucose homopolymer usually found in 2 main forms, amylose and amylopectin. The former is an essentially linear structure where the glucose units are joined by α (1–4) glycosidic links, whereas amylopectin consists of linear α (1–4) linked chains with α (1–6) linked branch points. Amylose has degrees of polymerization of 100–10 000 monomer units, whereas the molecular weight of amylopectin can exceed 10^7 daltons (1). The compact α helical structure of amylose makes this type of conformation more difficult to digest than the open-branched structure of amylopectin (2). Amylose can also be associated with lipid, as an inclusion complex, and in this form it appears to be digested incompletely in the small intestine (3). This was demonstrated in vitro when the susceptibility of amylose–lipid complexes to porcine α-amylase was found to be significantly reduced when compared with the degree of amylolysis of free amylose (4). High-amylose maize starches (HAMS; those from various cultivars with an amylose content of >40%) are intrinsically more resistant to digestion than those with a high amylopectin content. These HAMS are classified as type-2 resistant starch (RS; 4) and have been widely used in research investigating the nutritional benefits of RS, such as the regulation of carbohydrate and lipid metabolism and in the production of short-chain fatty acids through bacterial fermentation in the colon (5, 6). Clearly, the amylose content in cornstarch granules may be used to increase the quantity of dietary RS consumed by animals and humans. HAMS offers a means of providing a range of physiological benefits while assisting in the elucidation of the underlying mechanisms of action.

Generally the susceptibility of starch granules to hydrolysis by α-amylase appeared to vary in relation to their amylose content (7, 8) but may also be affected by the granule size in terms of the available surface area per mass for enzymatic action both in vivo and in vitro (9, 10). Indeed, Kong et al. (10) showed that maize starch granules of smaller size were more susceptible to digestion by α-amylase than were potato starch granules of larger size. There is also an association between size of the maize starch granules and their amylose content, with smaller granules having a higher amylose content (11). These observations indicate that higher levels of amylose in the starch granules per se may not be the

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Table 1. Chemical composition of maize starch granules with different amylose content[a]

	Weight, %					
Sample	AL-0[b]	AL-22	AL-54	AL-68	AL-80	AL-90
Carbohydrate[c]	86.4	89.3	89.5	88.5	89.1	88.5
Moisture	12.4	10.3	9.8	10.7	10.0	10.5
Protein	0.6	0.3	0.5	0.5	0.6	0.7
Lipids	0.3	0.1	0.1	0.1	0.2	0.1
Ash	0.1	0.1	0.1	0.1	0.1	0.2
Starch[d]	83.9	83.2	86.8	83.9	83.0	84.3
Amylose[e]	0.0	21.8	53.8	68.4	79.6	90.0

[a] Values are expressed as means from 2 to 3 determinations.
[b] AL = Amylose.
[c] Determined by subtracting moisture, protein, lipids, and ash from the total.
[d] Calculated from glucose content after α-amylase digestion.
[e] Determined by iodine-colorimetric method.

only factor to affect digestibility. However, to our knowledge precise information is limited in relation to the in vitro and in vivo digestibility of HAMS differing in amylose content. Moreover, many nutritional and clinical studies still seem to use the Prosky method [dietary fiber (DF) measurement] to predict the amount of indigestible starch and to use the terms "dietary fiber" and "resistant starch" interchangeably (12, 13). This practice may lead to a profound misunderstanding of the dietary effects of consuming RS and the potential physiological consequences of HAMS ingestion.

In the present study, we fed native maize starches with different amylose contents (amylose content of 0, 22, 54, 68, 80, or 90%) to ileorectostomized rats whose ileal terminal is directly anastomosed to the rectum. In these rats, the amount of fecal excretion of starch was expected to correspond to that of undigested starch coming from the small intestine into the large intestine in normal rats. The in vivo RS values were compared with both in vitro DF and RS values obtained by the respective methods of Prosky (14) and McCleary and Monaghan (15). These in vitro values were reviewed with respect to the physicochemical properties of the starch, such as the gelatinization temperature, granule size, and the surface area of granules. Each of these parameters was considered in terms of its relevance to the prediction of the physiological occurrence of RS (in vivo RS).

METHODS

Materials

Starches with varying amylose contents (0, 22, 54, 68, 80, or 90%) from conventional maize hybrids were donated by National Starch and Chemical Co. (Seven Hills, NSW, Australia). These maize starches were assigned as AL-0, AL-22, AL-54, AL-68, AL-80, and AL-90. The chemical composition of starches is shown in Table 1. Except for their amylose content, the chemical composition of the different starches was virtually the same. Protein and fat contents were determined using the Kjeldahl method (16) and the method of Folch et al. (17), respectively. Moisture was determined as a loss in weight after drying at 105°C for 24 h. Ash content was determined by a direct ignition method at 525°C overnight. Carbohydrate content was calculated by the difference (i.e., by subtracting protein, fat, ash, and moisture from total starch granules). Net starch content was determined using a commercially available kit (Total Starch Assay Kit, Megazyme, Bray Co., Ireland) with a modification that involved preheating the samples in dimethylsulfoxide at 100°C for 30 min (18). Amylose content was determined by an iodine-colorimetric method (19).

The Rate of Pancreatic α-Amylase Digestion

Comparison of the rate of pancreatic α-amylase digestion among starches with different amylose content was made using the assay procedure described previously (20). Briefly, 100 mg starch was suspended in 20 mL 0.025 M sodium–maleate buffer (pH 6.0) and preincubated at 37°C for 5 min. A 1 mL volume of α-amylase solution (30 U, A3176, Sigma, St. Louis, MO) containing 2 mM $CaCl_2$ was added to the starch suspension and further incubated at 37°C. At each time-point, a sample (0.5 mL) was withdrawn from the incubation medium and passed through a membrane filter (0.22 μm). Portions (0.25 mL) of the filtrate were mixed with 2.25 mL 0.4 M sodium acetate buffer (pH 4.75) and incubated with 10 μL amyloglucosidase (Roche, Mannheim, Germany) at 60°C for 30 min. Liberated glucose was determined using a commercial kit (Glucose B Test Wako, Wako Pure Chemical Industries, Tokyo, Japan) and converted to the amount of digested starch.

General Procedures

Total DF was determined by the method of Prosky et al. (14) using a thermally stable α-amylase (Termamyl-120L) in phosphate buffer (pH 6.0). RS was determined according to

Table 2. In vitro dietary fiber and resistant starch values, gelatinization temperature, size, and surface area of the respective starch granules[a]

Sample	AL-0[b]	AL-22	AL-54	AL-68	AL-80	AL-90
Dietary fiber, wt %[c]	<0.5	<0.5	6.9	23.0	37.8	44.3
Resistant starch, wt %[d]	<1.0	<1.0	46.6	54.4	47.6	45.4
Gelatinization temp., °C[e]						
Start (T_S)	63.3	64.5	67.3	68.0	70.5	80.5
Peak (T_P)	70.0	69.3	73.6	76.5	80.0	86.7
End (T_E)	81.1	86.2	92.8	95.6	95.8	106.8
Median diameter, μm[f]	11.9	13.4	9.69	9.15	7.39	7.53
Surface area, m^2/g[f]	0.35	0.32	0.42	0.44	0.54	0.53

[a] Data are expressed as means from 3 determinations.
[b] AL = Amylose.
[c] Measured by the Prosky method (14).
[d] Measured by the method of McCleary and Monaghan (15).
[e] Measured using a differential scanning calorimetry.
[f] Measured using a particle size distribution analyzer.

the method of McCleary and Monaghan (15). The gelatinization temperatures of the starch samples were measured using a differential scanning calorimetry (DSC; DSC 220, Seiko Instruments Inc., Chiba, Japan). The 30% (dry-based) starch slurry was transferred to a DSC aluminum pan that was hermetically sealed. The samples were heated from 40 to 140°C with a heating rate of 5°C/min. The onset (T_o), peak (T_p), and conclusion (T_r) temperatures were recorded. Starch granule size distributions of samples were measured using a particle size distribution analyzer (CAPA-700, Horiba Ltd, Kyoto, Japan). Starch samples were dispersed in distilled water, and the starch concentration was adjusted to provide an initial absorbance about 0.7–0.9. The starch slurry was irradiated for 10 min by ultrasonic waves. After that, starch slurry was immediately transferred to the analysis cell that was placed in the particle size distribution analyzer. The samples were centrifuged at 500 rpm and the granule size was measured in the range of 2–30 μm. Microscopic examination (Olympus BH2) for starch granules was made after iodine staining using Lugol solution (Merck, Darmstadt, Germany).

Starch Digestibility in Ileorectostomized Rats

Male rats of the Wistar strain from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan) were used. They were housed individually in standard wire-bottomed cages in a room with controlled temperature (23 ± 1°C) and lighting (lights on at 0800–2000 h). The rats were fed a fiber-free purified diet with water during the experimental period. This diet (21) was formulated from 250 g/kg casein, 652.5 g/kg standard maize starch (corresponding to AL-22), and 50 g/kg corn oil. The remainder of the diet was essential vitamins and minerals. All aspects of animal care were under the oversight of the institutional animal ethics committee of Shizuoka University under accepted guidelines.

After overnight fasting, 25 rats weighing 92–123 g were subjected to an ileorectostomy in which the ileum is connected directly to the rectum according to the method of Lambert (22) with some modifications (23). The surgery was performed for 3 consecutive days (7–9 rats/day were operated). This method allows direct collection of undigested food from the terminal small intestine. Postoperatively the rats were not allowed food and water for the first 24 h, and they were then fed the fiber-free purified diet for 15–18 days. Rats received a daily intramuscular injection of antibiotics (24) at surgery and for 5 days thereafter. Rats lost about 15 g body weight during immediate postoperative recovery. However, they then gained weight on feeding, and constant growth rates (4–5 g body weight gain/day) were achieved after 5 days post surgery.

The rats, weighing 154–185 g, were divided into 5 groups of similar mean body weight and were freely fed one of 5 diets for a further 7 days. The fiber-free purified diet contained one of the starches with different amylose content (22, 54, 68, 80, or 90%) as the sole carbohydrate source. Feces (ileal excreta) was collected for the last 3 days of the experimental period, freeze-dried, and stored at –20°C. The starch content of the feces collected from rats fed each of the diets was measured using the Total Starch Assay Kit (Megazyme) with minor modifications as described above.

After the digestibility study, in order to measure the small intestinal transit time in the ileorectostomized rats, all rats consumed the same fiber-free purified diet, including standard maize starch (corresponding to AL-22) as the sole carbohydrate source at 0800–0900 and 1900–2000 for 7 days. After adaptation to a meal-feeding, the rats weighing 200–230 g were fed 3 g of the same diet, including 5% carmine (water-insoluble and unabsorbable dye) at 1900. The

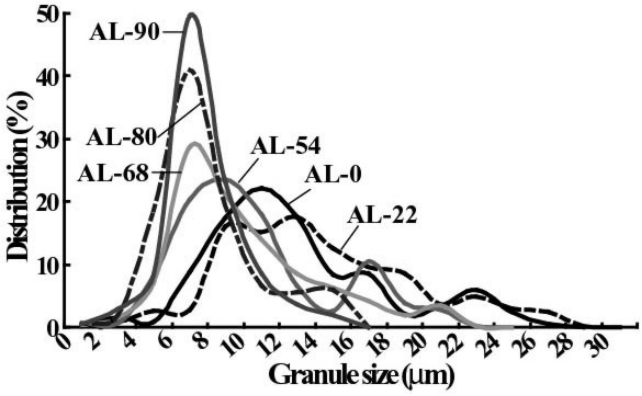

Figure 1. Granule size of maize starches with different amylose content. Particle size analyzer was used, as indicated in *METHODS* and *Materials.*

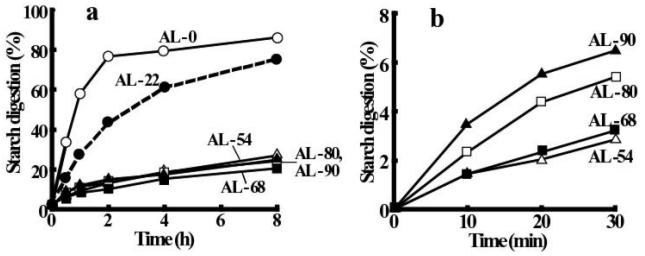

Figure 3. Rate of pancreatic α-amylase digestion of starch granules: (a) long digestion up to 8 h and (b) initial digestion rate up to 30 min. Starch (100 mg) was suspended in 20 mL 0.025 M sodium–maleate buffer (pH 6.0) and preincubated at 37°C.

feces were monitored every 15 min for the first appearance of the red dye.

Statistical Analyses

Data were initially analyzed by analysis of variance (ANOVA) and any significant differences among the means were separated by Tukey-Kramer. When variances were not homogeneous by the Bartlett test (25), data were transformed logarithmically and then analyzed by ANOVA followed by multiple comparisons. When variances varied even after logarithmic transformation of data, the results were analyzed by Kruskal-Wallis ANOVA followed by the Kolmogorov-Smirnov 2-sample test (25). Normally, results were expressed as a mean ± standard error and all statements of significant differences show the 5% level of probability. The statistical calculations were carried out using Stat View 5.0 computer software (SAS Institute, Cary, NC). Regression analyses were performed using the Stat Cel 2 program (Tokyo Shoseki, Tokyo, Japan).

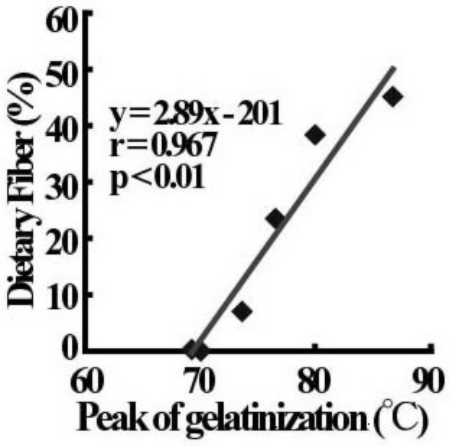

Figure 2. Correlations between dietary fiber content and gelatinization temperature. Differential scanning calorimetry (gelatinization temperature) was used, as indicated in *METHODS* and *Materials.*

Results

Dietary Fiber and Resistant Starch Values

DF was detected at AL-54, and the amount of DF continued to increase in a concomitant manner with the amylose content of the various HAMS samples. The highest DF value was obtained with AL-90 (Table 2). In contrast to DF, a significant and much larger amount of RS was detected at AL-54. For HAMS, the RS content was the highest with AL-68 and thereafter decreased in AL-80 and AL-90. Differential scanning calorimetry analysis showed that the onset (T_o), peak (T_p), and conclusion (T_r) temperatures for the granule gelatinization process increased as a function of amylose content for the respective starches (Table 2). Light micrographs of the starch granules from each sample showed that compared with AL-0 and AL-22, the granule size became smaller and the shape became irregular in AL-80 and AL-90 (data not shown). Accordingly, the particle size distribution was analyzed (Table 2, Figure 1). The median diameter of the starch granules was the greatest in AL-22 and tended to be reduced as the amylose content increased. This trend was also reflected in the surface area (mm/g) of the starch granules, with the largest in AL-80 and AL-90, lowest in AL-0 and AL-22, and intermediate in AL-54 and AL-68. Regression analyses showed that there were significant and positive correlations between DF values and T_o ($r = 0.884$, $P < 0.05$); T_p ($r = 0.967$, $P < 0.01$); and T_r ($r = 0.889$, $P < 0.05$) temperatures, but the highest correlation was observed with the T_p temperature (Figure 2). Such a correlation was not observed between RS values and the various gelatinization temperatures (data not shown). Rather, the RS contents of starch granules were likely to depend on both the surface area and amylose contents of the granules showing a parabolic fashion against the surface area with its peak of 0.44 m^2/g.

Rate of Pancreatic α-Amylase Digestion

The rates of pancreatic α-amylase digestion of AL-54, -68, -80, and -90 were much slower than those observed for AL-0

and -22, indicating that the HAMS had a greater resistance to the digestion and an associated larger RS content (Figure 3a). As expected from the results of the RS determination, differences in starch digestibility among AL-54, -68, -80, and -90 were very small at 8 h after the reaction. However, the initial digestion, up to 30 min after the commencement of the reaction, showed that a higher digestion rate was manifest for AL-90 and AL-80 compared with that observed for AL-54 and AL-68 (Figure 3b).

Starch Digestibility in Ileorectostomized Rats

Food intake was highest in AL-68 and lowest in AL-22, but there were no significant differences due to the large variation in results. Body weight gain was just the opposite, with the highest recorded when AL-22 was consumed and lowest for AL-68. The other starches resulted in intermediate weight gains. These differences were significant. Starch intake among the groups showed the same trend as for food intake. Fecal dry matter and starch differed among the groups and were significantly higher in AL-54, -68, -80, and -90 than in AL-22. Significant difference was also observed between AL-68 and AL-90. Inversely, starch digestibility was lower in AL-54, -68, -80, and -90 than in AL-22. These results correspond to in vivo RS values for the respective starches, and the in vivo RS values of AL-54, -68, -80, and -90 were significantly higher than that of AL-22. A significant difference was also observed between AL-68 and AL-90 (Table 3). To verify the homogeneity of the intestinal movement after surgery, the small intestinal transit time was measured. The first appearance of red dye in the feces was distributed between 3.0 and 4.0 h in all groups, and no differences were observed among the groups.

Discussion

One interesting feature of the present study was that the DF value of maize starch showed a positive correlation to the amylose content and the gelatinization temperatures of starch granules (Figure 2), whereas in vitro values for these starches were likely to depend on both the surface area and amylose contents of the granules showing a parabolic fashion against the surface area. DF measurement (Prosky method) was performed in boiling water to stimulate the gelatinization of the starch granules and promote its subsequent digestion by a thermally stable α-amylase. The Prosky method was designed to remove starch, which at that time was not considered to be physiologically active in the lower gastrointestinal tract, when quantifying the amount of DF. The effectiveness of this method is dependent on the gelatinization temperature of the starch granules.

The method of McCleary and Monaghan (in vitro RS measurement) is performed at the physiologically relevant temperature of 37°C and does not seek to gelatinize the starch granules. Higher levels of amylose in the starch granules appear to hinder digestibility, possibly due to a densely packed α helical structure (2) and a formation of amylose-lipid complexes (3, 4). Higher levels of amylose were also associated with significant increases in the surface area of the starch granules (Table 3), which could facilitate the enhanced adsorption of α-amylase onto the granule surface and the subsequent promotion of catalytic activity in the HAMS, especially for AL-80 and AL-90. This observation was supported by the experimental data, which showed that the initial rates of starch digestibility were almost double in AL-80 and -90 compared with those in AL-54 and -68 (Figure 3b), although the differences in digestibility after 8 h

Table 3. Food intake, body weight gain, fecal variables, and starch digestibility in ileorectostomized rats fed the respective diets for 7 days[a]

	AL-22[b]	AL-54	AL-68	AL-80	AL-90
Total food intake, g/7 days	107 ± 4	127 ± 9	131 ± 7	105 ± 10	114 ± 6
Total body weight gain[c], g/7 days	27 ± 3[1]	5 ± 3[2]	–6 ± 6[3]	–3 ± 4[2, 3]	3 ± 3[2, 3]
Starch intake[d], g/3 days	26 ± 1[2]	32 ± 2[1, 2]	34 ± 1[1]	27 ± 2[1, 2]	28 ± 2[1, 2]
Fecal dry matter, g/3 days	1.5 ± 0.2	17.3 ± 1.2[e]	19.5 ± 0.6[e]	15.0 ± 1.3[e]	14.4 ± 0.9[e, f]
Fecal starch[g], g/3 days	0.1 ± 0.0	12.9 ± 0.8[e]	14.6 ± 0.4[e]	11.1 ± 0.9[e]	10.5 ± 0.6[e, f]
Starch digestibility, %	99.5 ± 0.1	59.1 ± 0.3[e]	56.6 ± 2.3[e]	59.4 ± 0.8[e]	62.2 ± 1.0[e, f]
Resistant starch, %	0.5 ± 0.1	40.9 ± 0.3[e]	43.4 ± 2.3[e]	40.6 ± 0.8[e]	37.8 ± 1.0[e, f]

[a] Data are expressed as mean ± standard error (n = 5); values in a row not sharing a common superscript (1–3) are significantly different (P < 0.05). When variances were not homogenous even after logarithmic transformation of data, these data were analyzed by Kruskal-Wallis ANOVA followed by Kolmogorov-Smirnov 2-sample test.

[b] AL = Amylose.

[c] Mean initial body weight, 174 g (154–185 g).

[d] Calculated from dietary intake for the last 3 days of the experimental period.

[e] P < 0.05 vs AL-22.

[f] P < 0.05 vs AL-68.

[g] Feces were collected for the last 3 days of the experimental period.

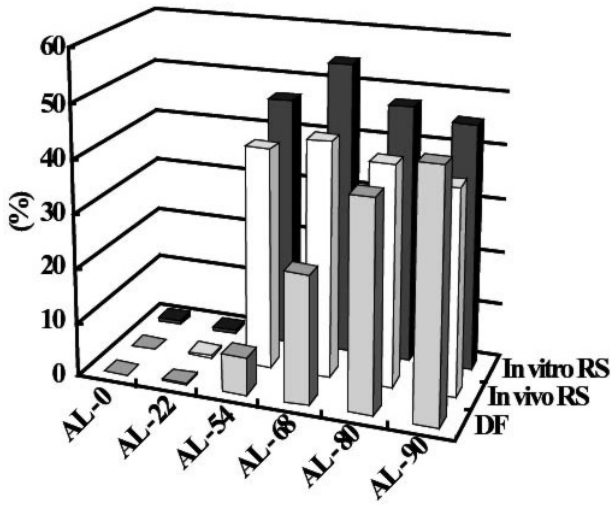

Figure 4. Comparison of dietary fiber and in vitro RS contents with in vivo RS content. In vitro RS content, determined by the method of McCleary and Monaghan (15); in vivo RS content, determined using ileorectostomized rats; DF content, determined by the method of Prosky et al. (14).

of incubation were relatively small among these starches. Clearly, in vitro digestibility of HAMS is affected by 2 major factors, namely, the amylose content (and the inherent effect on granule conformation) and the surface area of the granule. This may explain why the maximum value of in vitro RS was obtained with AL-68 and not with AL-80 or AL-90.

More importantly, the present study showed that the small intestinal digestibility of HAMS (in vivo RS) was not correlated with in vitro DF values. Rather, in vivo RS was correlated with in vitro RS values ($r = 0.997$), although the data distributed in 2 clusters of points in this situation. The largest amount of in vivo RS was obtained with AL-68. This suggests that not only the in vitro but also the in vivo RS values are similarly affected by both the amylose content and the surface area in maize starch granules. The ileorectostomized rats used in this study showed a consistent transit time, as assessed by the first appearance of dye-marked feces (3–4 h in all the rats), indicating that there were no profound fluctuations on the intestinal movement due to surgery that may result in undue retardation of the small intestinal contents. In vivo RS values of the respective HAMS were constantly lower by 7–10% than those obtained in vitro (Figure 4). At present, we have no direct evidence to explain this discrepancy. If bacterial fermentation did occur in the distal ileum, the in vivo RS content would be underestimated to some extent, although fecal sterol analysis showed that the coprostanol/cholesterol ratio was extremely low and fecal pH was around 7.4, suggesting low bacterial activity (data not shown). Therefore, it is possible that RS metabolism in rats and humans will show some differences.

At present there is no physiologically accurate in vitro measure of the RS content in human foods, although a number of methods have been proposed that still require further validation when complex food matrixes are analyzed. Any analytical procedure needs to allow for the presence of inhibitory food components (DF, lipids, etc.) as well as physiological variables, including chewing and individual variation in transit (26, 27). However, the present results clearly showed that as far as starch ingredients are concerned, the physiological occurrence of RS (in vivo RS) is predictable by extrapolation of in vitro RS value of the starch granules.

Another important observation was that there were extreme differences between the DF and the in vitro RS values when maize starch with an amylose content of 54 and 68% were tested. This was prominent in AL-54, in particular, where the DF value was only 6.4% but the in vitro RS value was 46.6% with corresponding in vivo RS value of 40.9%. Brown et al. (28) reported that the area under the plasma insulin curve in rats fed the raw maize starch granules with amylose content of 60% was significantly lower than in those fed the waxy maize starch granules, even though the DF value of this high-amylose maize starch was only approximately 6% (5). This finding is in accordance with the present results, which showed that in vivo RS values of AL-54 and AL-68 were much higher than those expected from the corresponding DF values. Studies in animals have compared the impact of high-amylose versus high-amylopectin maize starches on postprandial glycemic and insulinemic excursions. These works adopted the Prosky method to predict the amount of indigestible starch when HAMS with 60% amylose was fed to experimental animals (29–31), but the energy restriction and the supply of fermentation substrate into the colon in those animals fed the HAMS should have been much greater than the predicted values (6.0%) by DF measurement. Accordingly, these underestimations of indigestible starch content might have profound effects in understanding the mechanisms responsible for the observed physiological outcomes. Many nutritional and clinical studies still seem to use the Prosky method (DF measurement) to predict the amount of indigestible starch and to use the terms "dietary fiber" and "resistant starch" interchangeably (12, 13). Therefore, caution must be exercised when native HAMS, or other starches that potentially contain RS, are used in nutritional and clinical experiments.

In conclusion, the present results suggest that both in vitro and in vivo digestibility of maize starch with different amylose content is affected by 2 factors, namely, amylose content and the surface area of maize starch granules, and that the physiological occurrence of RS might be predictable by extrapolating in vitro RS value.

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