Butyrylated starch is less susceptible to enzymic hydrolysis and increases large-bowel butyrate more than high-amylose maize starch in the rat

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Large-bowel fermentation of resistant starch produces SCFA that are believed to be important in maintaining visceral function. High-amylose maize starch (HAMS) and acylated starches are sources of resistant starch and are an effective means of increasing colonic SCFA. Cooking increases digestibility of starches but its effects on the capacity of these starches to raise large-bowel SCFA are unknown. We have examined the effects of cooking of HAMS and butyrylated HAMS (HAMSB) on amylolysis in vitro and their capacity to raise caeco-colonic SCFA in rats. The starches were boiled in excess water and microwaved, followed by drying at 100°C. Cooking increased in vitro glucose release for both starches but significantly less from HAMSB. Rat growth rates were unaffected when fed cooked resistant starch. Digesa pH was increased in the caecum and proximal colon of rats fed cooked HAMS. Distal colonic pH was highest in rats fed cooked HAMSB. Factorial analyses (2 × 2) of caecal SCFA pools showed significant differences between HAMS and HAMSB, and that cooking significantly lowered caecal butyrate pools. Portal venous butyrate concentrations were higher in both HAMSB groups than those fed HAMS. The data suggest that HAMSB is less susceptible to in vitro amylolysis than HAMS following cooking and delivers more butyrate to rat caecum than HAMS. This attribute may be useful in food applications for specific delivery of SCFA to the colon. Preparation of carbohydrates to simulate human food in animal experiments may be important to assess nutritional and physiological effects accurately.

Resistant starch: Processing: Short-chain fatty acids: Large bowel

Increased luminal SCFA are believed to improve large-bowel health through a variety of mechanisms. These include increasing colonic blood flow (Topping & Clifton, 2001), improving mineral and water absorption and the maintenance of low luminal pH, particularly in the proximal colon, which may be beneficial in the prevention and overgrowth of pH-sensitive pathogenic bacteria (Kleessen et al. 1997). Butyrate, in particular, has been implicated in specific functions involving the epithelial metabolism (Ahmad et al. 2000) and cell cycling (Dehghan-Kooshkghazi & Mathers, 2004; Scheppach & Weiler, 2004), immune response (Andoh et al. 2003; Inatomi et al. 2005) and intestinal motility (Rondeau et al. 2003). The importance of carbohydrate fermentation in increasing large-bowel SCFA has been established (Topping et al. 1997; Young et al. 2005). It has been suggested that SCFA produced from fermentable dietary carbohydrates are higher in populations with low risk of serious bowel disease (Ahmed et al. 2000). It appears that one of the major sources of dietary carbohydrate available for fermentation is resistant starch (RS) (Andoh et al. 2003; Topping et al. 2003). RS is defined as ‘the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals’ (Asp, 1992). RS is an important dietary component and is found in a number of foods, but it appears that intakes are low in affluent Westernised countries at risk of serious large-bowel disease (Champ et al. 2003). The RS content of foods may be increased through the addition of specific ingredients such as high-amylose starches (Brown et al. 1995). However, dietary studies with such products in animals have used ungelatinised, i.e. raw, products which are not truly representative of human foods. Maize-starch mixtures that have been processed by extrusion display different physico-chemical properties such as increased water solubility, decreased gel viscosity and a greater percentage of smaller-molecular-weight oligosaccharides (Ozcan & Jackson, 2005). This could result in increased small-intestinal amylolysis, and is supported by in vitro studies that demonstrated a significant change in the structure of chestnut flour following roasting and a corresponding increased in vitro amylolysis (Pizzoferrato et al. 1999). Processing has been found to increase in vitro amylolysis in rice, pearl barley and foxtailed millet compared with the raw cereals (Lee & Chang, 2004). A recent study confirmed a 50 % reduction in the fermentative capacity in vivo of a RS (80 % amylose) in volunteers...
Materials and methods

Animals

Adult male Sprague–Dawley rats were sourced from the Animal Resource Centre, Murdoch University, Western Australia and allowed 7 d to stabilise before the experimental period. Animals were housed in wire-based cages in a room of controlled temperature (22–24°C) and lighting (lights on between 08.30 and 19.30 hours). The rats were allowed free access to a standard pelleted diet during the adaptation period. Animals were housed in a room of controlled temperature (22–24°C) and lighting (lights on between 08.30 and 19.30 hours). The rats were allowed free access to a standard pelleted diet during the adaptation period. The plate was incubated at room temperature for 24 h. Following incubation samples were centrifuged and the supernatant fraction was assayed for glucose. Briefly a 10 µl sample was pipetted in triplicate onto a ninety-six-well plate followed by 200 µl glucose oxidase reagent (Thermo Electron Corporation, Victoria, Australia). The plate was incubated at room temperature for 2 min and the supernatant fraction was assayed for glucose. Briefly a 10 µl sample was pipetted in triplicate onto a ninety-six-well plate followed by 200 µl glucose oxidase reagent (Thermo Electron Corporation, Victoria, Australia). The plate was incubated at room temperature for 2 min and the supernatant fraction was assayed for glucose.

In vitro analysis of starches

The susceptibility of the starches to amylolysis was measured in vitro to determine the likely resistance of the starches in vivo. Raw and cooked standard maize starch was included as a positive control. Each starch (100 mg) was dispersed in 0.2 M-acetate buffer (6 ml; pH 6.0), containing 10 mg α-amylase (speedase PNA8; Halcylon Proteins Pty Ltd, Australia) and 0.86 mg amyloglucosidase (Sigma Chemical Co., St Louis, MO, USA) and incubated in a shaking incubator at 37°C for 24 h. Following incubation samples were centrifuged (50 g for 2 min) and the supernatant fraction was assayed for glucose. Briefly a 10 µl sample was pipetted in triplicate onto a ninety-six-well plate followed by 200 µl glucose oxidase reagent (Thermo Electron Corporation, Victoria, Australia). The plate was incubated at room temperature for 2 min and the supernatant fraction was assayed for glucose. Briefly a 10 µl sample was pipetted in triplicate onto a ninety-six-well plate followed by 200 µl glucose oxidase reagent (Thermo Electron Corporation, Victoria, Australia). The plate was incubated at room temperature for 2 min and the supernatant fraction was assayed for glucose.

Table 1. Composition of experimental diets (g/kg diet) based on the AIN-93G and containing 10% resistant starches

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>HAMS</th>
<th>'Cooked' HAMS</th>
<th>HAMSB</th>
<th>'Cooked' HAMSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
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<tr>
<td>Standard maize starch</td>
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<td>430</td>
<td>430</td>
<td>430</td>
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<tr>
<td>HAMS</td>
<td>100</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>'Cooked' HAMS</td>
<td>–</td>
<td>100</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HAMSB</td>
<td>–</td>
<td>–</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>'Cooked' HAMSB</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sunflower-seed oil</td>
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<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Fibre (α-cellulose)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mix†</td>
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<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix†</td>
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<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>L-Cystine</td>
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<tr>
<td>Tert-butylhydroquinone</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*HAMS, high-amylose maize starch; HAMSB, butyrylated high-amylose maize starch.
†AIN-93G mineral and vitamin mixes were obtained pre-made from MP Biomedicals Inc. (Solon, OH, USA).
20 min and read on a microplate reader at 505 nm. Glucose concentrations were determined using a standard curve and percentage release by calculating the glucosyl units liberated from 100 mg starch.

Sample collection and analytical procedures

Thirty-two rats (body weight 200–260 g) were divided into four equal groups and fed one of the four diets detailed in Table 1 for a period of 10 d. Individual rat body weights and food intakes for each cage were measured daily. At the conclusion of the experimental period rats were exsanguinated under halothane anaesthesia. The lengths of the small and large intestine and caecal, colonic, liver and spleen weights were measured post mortem.

Separate digesta samples from the caecum, proximal and distal colon were collected for SCFA analysis and DM determinations. Briefly, digesta samples were weighed and diluted 1:3 in solvent containing 1.68 mM-heptanoic acid (Sigma Chemical Co.) internal standard (pH 7.0), homogenised, pH measured, then centrifuged at 2000 g for 15 min. The supernatant fraction (150 μl) was added to 30 μl 10% phosphoric acid and purified by freeze transfer sublimation. The sublimate was analysed using GLC (6890N network GC system; Agilent Technologies Inc., Palo Alto, CA, USA); the column was FFAP 30 m × 0.53 mm internal diameter (Phenomenex, Torrance, CA, USA). The solid fraction was then dried in an oven at 80 °C for 24 h to determine the DM fraction of the digesta samples. DM samples were then hydrolysed to determine the amount of SCFA remaining esterified to starch in the digesta. DM digesta (100 mg) was hydrolysed using 1·25 ml 0·45 M-sodium hydroxide for 2 h, diluted 1:3 (w/w) in solvent containing 100 mM-2-ethylbutyric acid, then purified and analysed using GLC.

Portal vein plasma SCFA were determined by diethyl ether extraction as described previously (Murase et al. 1995).

Statistical methods

Statistical analysis was performed using GraphPad Prism version 4.0 computer software (GraphPad Software, San Diego, CA, USA). Where appropriate, the effects of starch type (HAMS and HAMSB) and processing (raw or cooked) and their interactions were evaluated by two-way ANOVA. One-way ANOVA using Tukey’s post hoc test was used to determine differences among all test diets. All data are expressed as mean values with their standard errors represented by vertical bars. * Mean values with unlike letters were significantly different by one-way ANOVA (P<0.001). Analysis by two-way ANOVA showed a significant effect of starch type and cooking (P<0.0001).

Animal feeding trial

There were no significant differences in food intakes or growth rates of rats fed any of the diets (data not shown). Caecal digesta wet weights were similar in rats fed both ungelatinised starches (Fig. 2). However, statistical analysis showed that there were significant effects of treatment on digesta weight. Cooking decreased (P<0.02) and butyrylation increased (P<0.05) digesta wet weight in the caecum. Neither starch type nor cooking significantly affected digesta wet weight in the proximal or distal colon.

In the caecum and proximal colon, digesta pH was lower (P<0.01) in rats fed raw HAMS or raw or cooked HAMSB than in those fed cooked HAMS (Fig. 3). In contrast, distal

Results

In vitro hydrolysis

Only a small fraction of glucose (6%) was released from uncooked HAMSB in vitro compared with uncooked HAMS, where 26% of glucose was liberated (Fig. 1). Cooking resulted in a very significant (P<0.0001) increase in glucose release from both starches (75% for HAMS and 43% for HAMSB) but HAMSB remained more resistant to amylolysis than HAMS (Fig. 1). When standard maize starch was cooked, glucose release increased significantly from 76% to 92%.

Animal feeding trial

There were no significant differences in food intakes or growth rates of rats fed any of the diets (data not shown). Caecal digesta wet weights were similar in rats fed both ungelatinised starches (Fig. 2). However, statistical analysis showed that there were significant effects of treatment on digesta weight. Cooking decreased (P<0.02) and butyrylation increased (P<0.05) digesta wet weight in the caecum. Neither starch type nor cooking significantly affected digesta wet weight in the proximal or distal colon.

In the caecum and proximal colon, digesta pH was lower (P<0.01) in rats fed raw HAMS or raw or cooked HAMSB than in those fed cooked HAMS (Fig. 3). In contrast, distal

Fig. 1. The percentage glucose release from 100 mg starch following amylolysis in vitro of raw and cooked standard maize starch (SMS), high-amylose maize starch (HAMS) and butyrylated HAMS (HAMSB). Each starch was assayed in triplicate and values are means, with their standard errors represented by vertical bars. a,b,c,d,e,f Mean values with unlike letters were significantly different by one-way ANOVA (P<0.001). Analysis by two-way ANOVA showed a significant effect of starch type and cooking (P<0.0001).

Fig. 2. Digesta weights from the caecum, proximal colon and distal colon from rats fed high-amylose maize starch (HAMS; □), ‘cooked’ HAMS (●), butyrylated HAMS (HAMSB; ▧) and ‘cooked’ HAMSB (■). Data are means (n 8) with their standard errors represented by vertical bars. * Mean values were significantly different by one-way ANOVA (P<0.05). A significant effect of cooking was observed in the caecum by two-way ANOVA (P<0.02).
colonic digesta pH was higher in rats fed cooked HAMSB compared with raw HAMSB ($P<0.05$) although pH remained $<7.0$ in all groups.

SCFA pools (Figs. 4, 5 and 6) were higher in the caecum than in either the proximal or distal colon. Two-way ANOVA showed that butyrylation of HAMS raised caecal acetate pools ($P<0.04$) and that the decrease due to cooking was not significant ($P=0.09$). There were no significant differences in caecal acetate or propionate pools among the dietary groups; however, HAMSB (raw and cooked) had greater acetate pools than HAMS (raw and cooked). Caecal butyrate pools of the rats fed raw and cooked HAMSB were higher than the pools of both the raw and cooked HAMS groups ($P<0.001$). Cooking also reduced the butyrate pools regardless of starch type ($P<0.05$). No significant differences in SCFA pools were observed in the proximal colon among groups; however, in the distal colon both HAMS groups displayed more acetate ($P<0.001$) and propionate ($P<0.05$) but not butyrate. Although there were differences in percentage glucose release in vivo of raw and cooked HAMS (Fig. 1), there were no significant reductions due to cooking in total caeco-colonic SCFA (data not shown).

Alkaline hydrolysis of dried caeco-colonic digesta samples releases SCFA esterified to the starch molecules that can then be measured. The amount of butyrate released from 100 mg digesta remained similar throughout the large bowel (Fig. 7). The amount of butyric side chains remaining esterified to the starch in the caeco-colonic digesta samples was significantly less than the estimated consumption over the previous 24 h period of the experiment as shown in Fig. 7 (diet butyrate pool = butyrate liberated by alkaline hydrolysis x 24 h food intake).

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**Fig. 3.** Digesta pH from the caecum (n 8), proximal colon (n 4–7) and distal colon (n 7–8) from rats fed high-amylose maize starch (HAMS; □), ‘cooked’ HAMS (■), butyrylated HAMS (HAMSB; ◇) and ‘cooked’ HAMSB (●). Data are means with their standard errors represented by vertical bars. a,b Mean values with unlike letters were significantly different due to cooking in the caecum and distal colon ($P<0.05$).

**Fig. 4.** Acetate pools in the liquid phase of the digesta from the caecum (n 8), proximal colon (n 4–7) and distal colon (n 7–8) from rats fed high-amylose maize starch (HAMS; □), ‘cooked’ HAMS (■), butyrylated HAMS (HAMSB; ◇) and ‘cooked’ HAMSB (●). Data are means with their standard errors represented by vertical bars. * Mean values were significantly different by one-way ANOVA in the caecum ($P<0.02$) and distal colon ($P<0.001$).

**Fig. 5.** Propionate pools in the liquid phase of the digesta from the caecum (n 8), proximal colon (n 4–7) and distal colon (n 7–8) from rats fed high-amylose maize starch (HAMS; □), ‘cooked’ HAMS (■), butyrylated HAMS (HAMSB; ◇) and ‘cooked’ HAMSB (●). Data are means with their standard errors represented by vertical bars. Significant differences in starch type were observed by two-way ANOVA in the distal colon ($P=0.026$).

**Fig. 6.** Butyrate pools in the liquid phase of the digesta from the caecum (n 8), proximal colon (n 4–7) and distal colon (n 7–8) from rats fed high-amylose maize starch (HAMS; □), ‘cooked’ HAMS (■), butyrylated HAMS (HAMSB; ◇) and ‘cooked’ HAMSB (●). Data are means with their standard errors represented by vertical bars. * Mean values with unlike letters were significantly different by one-way ANOVA ($P<0.001$). Significant differences in starch type ($P<0.0001$) and cooking ($P<0.05$) were observed in the caecum as analysed by two-way ANOVA.
Portal vein plasma butyrate concentrations were significantly greater in rats fed HAMSB than in those fed HAMS ($P<0.05$) (Fig. 8); however, there was no significant effect of cooking on the plasma butyrate concentrations of either the HAMS or the HAMSB groups. No significant differences were observed in acetate or propionate concentrations among any of the diets or due to cooking.

**Discussion**

The present studies confirm that uncooked unmodified HAMS and HAMSB resist small-intestinal digestion in vitro and raise large-bowel digesta mass and SCFA compared with published values on standard maize starch (Morita et al. 2005). In the case of unmodified HAMS the increase was in total and individual SCFA compared with published standard maize starch (Morita et al. 2005) but with HAMSB there was an additional increase in the acid which had been esterified (Annison et al. 2003; Morita et al. 2005). To our knowledge, the effects of cooking on the performance of butyrylated starch have not been reported previously. As expected, in vitro amylolysis showed that uncooked HAMSB was extremely resistant to digestion. Cooking increased the amount of glucose released, which is consistent with greater gelatinisation, but approximately 57% of the starch remained undigested in vitro. Raw HAMS also resisted in vitro amylolysis but cooking decreased the starch remaining to less than 25% of the total glucosyl units available. Differences in the rate of amylolysis between acylated and unmodified starches have been observed over time periods of 0 to 30 min (Annison et al. 1995) and between raw and cooked starches over 2 to 6 h (data not shown). The in vitro amylolysis data obtained for the raw starches are comparable with the percentage starch digestibility observed in colectomised rats of HAMS and acetylated HAMS (Morita et al. 2005).

The reduction of in vitro resistance to amylolysis of cooked HAMS by approximately 50% is consistent with reported in vivo results by $^{13}$C breath test analysis in human subjects (Symonds et al. 2004). These authors showed a marked shift from large-bowel production of $^{13}$CO$_2$ in human subjects consuming uncooked HAMS to small-intestinal digestion when they ate HAMS as pancakes.

The significant decrease in the caecal digesta mass of rats fed HAMS following cooking is consistent with the increase in amylolysis observed in vitro. Although the caecal digesta weight of rats fed HAMSB was not significantly different compared with rats fed HAMS, the disproportionate increase in the butyrate pool is consistent with the bioavailability of the esterified SCFA and accords with previous studies with acylated starches (Annison et al. 2003; Morita et al. 2005). This demonstrates the ability of acylated starches to deliver specific acids to the large bowel in significantly greater amounts than the HAMS.

The majority of the free SCFA are found in the caecum as it is the main site of bacterial fermentation of carbohydrates in the rat. In the proximal and distal colon, the butyrate pools of the rats fed HAMSB (both raw and cooked) were not significantly different from those observed. Despite the reduction in resist- ance of cooked HAMS following the cooking.

**Fig. 7.** Butyrate pools released from the digesta solid phase by alkaline hydrolysis from the diet (diet for 24 h period), caecum (n 7–8), proximal colon (n 3–7) and distal colon (n 7–8) from rats fed high-amylose maize starch (HAMS; □), ‘cooked’ HAMS (●), butyrylated HAMS (HAMSB; △) and ‘cooked’ HAMSB (◆). Data are means with their standard errors represented by vertical bars.

**Fig. 8.** Acetate, propionate and butyrate concentrations in portal vein plasma samples from rats fed high-amylose maize starch (HAMS; □), ‘cooked’ HAMS (●), butyrylated HAMS (HAMSB; △) and ‘cooked’ HAMSB (◆). Data are means (n 8) with their standard errors represented by vertical bars (except HAMS; n 7). a,b Mean values with unlike letters were significantly different by one-way ANOVA ($P<0.05$). Analysis by two-way ANOVA showed significant effect of starch type on portal butyrate concentrations ($P<0.0001$).
et al. 2002). In the present study, cooking HAMS significantly reduced the ability of the starch to lower caecal and proximal colonic pH, which may reduce the uptake and use of SCFA produced in the lumen by colonocytes (Ritzhaupt et al. 1998) and enter the circulation. HAMSB that had been cooked could maintain acidic pH throughout the large bowel. The maintenance of acidic pH in the distal colon despite no differences in SCFA content may result from decreased digesta ammonia which is known to occur when RS intake is increased (Birkett et al. 1996). However, the reason for the significantly higher pH for cooked HAMS in the proximal colon is unclear although may be due to a lower amount of RS remaining in the digesta.

A previous study demonstrated survival of acylated starches through the small intestine of coeliacised rats (Morita et al. 2005). Approximately 90–95% of the esterified butyrate was liberated by the time the digesta had left the caecum. The present study demonstrates that starch esterified with butyrate can significantly increase luminal free butyrate by amounts far in excess of those produced HAMS.

Hydrolysis of diet samples and daily food intakes allows an estimation of the amount of esterified butyrate consumed over the last 24 h period of the experiment. Using 10 ml/min as an estimate of the average 24 h portal vein flow in the rat (including sleep and postprandial flow rates) (Mansbach et al. 1991; Mansbach & Dowell, 1993), we suggest that the majority of the butyrate liberated from the HAMSB was absorbed through the gut wall. This is supported by the suggestion that approximately 95% SCFA produced in the large bowel are absorbed through the epithelium (Topping & Clifton, 2001).

Caecal digesta SCFA, particularly butyrate, remain low in rats fed raw or cooked HAMS compared with HAMSB following 10 d feeding at 10% of the diet. This may be a result of insufficient time for bacterial adaptation to the high-amylose starch substrate (Henningsson et al. 2003). In contrast, both HAMSB diets generated significantly more butyrate in the same experimental period, demonstrating that esterification of butyrate to starch provides a rapid delivery system for specific SCFA that do not require a period of bacterial adaptation. Once the esterified SCFA have been released, the starch molecule is available for fermentation similar to native RS such as HAMS, which will further increase luminal SCFA.

In conclusion, the present study has demonstrated that butyrylated starch is less susceptible to small-intestinal amyloysis than HAMS in vitro. Cooked butyrylated starches deliver significantly greater amounts of esterified butyrate than raw or cooked HAMS, which are available for the large bowel of rats. The present study also demonstrates that cooking high-amylose starches such as HAMS significantly increases susceptibility to small-intestinal amyloysis in vitro. However, this did not translate to a significant reduction in SCFA pools in the large bowel of the rat although the luminal pH was significantly higher following cooking, which may increase the risk of luminal exposure to carcinogens. Preparation of carbohydrates to simulate human food for use in animal experiments may be important to assess nutritional and physiological effects accurately. These results suggest the butyrylated starches are an effective delivery system for specific SCFA to the large bowel and have potential to aid in the maintenance of large-bowel heath.

Acknowledgements

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References


