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A study on the *in vivo* digestibility of retrograded starch

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The digestibility of different forms of starch was examined in an ileostomy model. Six otherwise healthy ileostomists were fed a controlled polysaccharide-free diet for four days, on three of which a test starch was added at breakfast. 50 g starch was fed as either whole or homogenized chick peas or as a retrograded starch gel. A readily digestible wheat starch biscuit was used as a control. Ileostomy effluent was collected every 2 hours over a 16 hour period and a final collection made at 24 hours after the test meal. The monosaccharide composition and glycosyl linkages of the residual carbohydrate in the 2 hour peak period following the test meal was determined. Following consumption of the starch gel, poly- and oligosaccharides from mucin and starch were identified in the effluent. At the peak of effluent production following the test meal, the average ratio of starch polysaccharide to mucin was 1:0.4. Of the 50 g of starch consumed, 7% of the starch escaped digestion in this fraction. Following consumption of cooked, cooled chick peas, which were fed whole or homogenized, polysaccharides deriving from starch, mucin and the cell wall were detected in the effluent. It was estimated from comparison of the composition of the food and effluent that 14% and 16% of the ingested starch in the form of homogenized and whole chick peas had escaped digestion in the small intestine. Linkage analysis showed the chemical structure of the starch escaping digestion after feeding the whole and homogenized chick peas was similar to that obtained after feeding the starch gel.

INTRODUCTION

At present there is debate on the contribution of dietary polysaccharides and low molecular weight carbohydrates to the substrate for fermentation in the human colon. Although it is recognised that non-starch polysaccharides of plant cell wall origin could make a major contribution, the recent demonstration (Englyst & Cummings, 1985, 1986, 1987; Schweizer *et al.*, 1990; Faisant *et al.*, 1993) that not all of the starch that we eat is digested and absorbed in the small intestine has led to examination of the potential contribution from dietary starch.

The starch in our diet occurs in a number of different physical forms. Although some plant foods contain native granular starch, the majority of starch-rich foods have been processed by a heat/moisture treatment which results in disruption of the native granular structure and a partial solubilization of the starch polysaccharides. Granular starches which have been gelatinized by heating in water are readily hydrolysed by amylolytic enzymes. On cooling to room temperature the solubilized polysaccharides can reassociate or retrograde (Miles *et al.*, 1985). During this retrogradation the starch becomes increasingly resistant to mammalian amylolytic enzymes. Retrograded amylose is substantially (> 70%) resistant to amylolysis *in vitro* (Leloup *et al.*, 1992; Ring *et al.*, 1988; Eerlingen *et al.*, 1993; Jane & Robyt, 1984), and its presence in starch gels restricts access of the enzyme to the starch substrate. The resistant product of the amylolysis of an amylose gel is a short linear dextrin with a degree of polymerization ranging from 20–80 (Leloup *et al.*, 1992; Eerlingen *et al.*, 1993; Jane & Robyt, 1984). Factors affecting the rate and extent of amylolysis of starch solids *in vitro* have been determined, and include the pore size of the substrate which can both slow the rate of diffusion and restrict access of the enzyme, and the chain conformation of starch (Colonna *et al.*, 1992).

In addition, in many starch-rich foods of plant origin, the starch is enclosed by the plant cell wall forming a

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barrier preventing access of amylolytic enzymes to the substrate. The mechanical properties of the cellular matrix will also influence the changes in particle size of the food material during eating and passage through the gastrointestinal tract. It has been shown that increased particle size leads to a reduced rate of amylolytic attack, as the time for the enzyme to diffuse to its substrate increases (Colonna *et al.*, 1992).

In a recent study (Faisant *et al.*, 1993), which used ileostomists and an incubation technique with healthy volunteers to sample the starch during its passage through the gastrointestinal tract, it was found that three fractions derived from starch escaped digestion in the small intestine. The main resistant fraction was a partially crystalline fragment of retrograded amylose. This was accompanied by an oligosaccharide fraction and a high molecular weight fraction both of which were potentially digestible. For bean and potato flakes (Faisant *et al.*, 1993), it was found that the resistant amylose fraction constituted between 50 and 65% respectively of the starch-derived carbohydrate which escaped digestion in the small intestine.

There is a need for more information on the factors which affect the digestibility of starch *in vivo*. In this study we examine the *in vivo* digestibility of starch, particularly the physico-chemical properties, using an ileostomy model. 50 g of starch was fed as a retrograded starch gel, and as part of a plant tissue–chick pea–which were cooked and stored to permit retrogradation of the starch. Chick pea was chosen firstly, because a legume starch has a relatively high content of amylose which readily retrogrades after gelatinization, and secondly the cell wall polysaccharides contain little non-cellulosic glucose containing polysaccharide which could potentially interfere with the analysis of starch.

EXPERIMENTAL

Subjects

Six otherwise healthy ileostomy subjects (5 female) average age 57 (range 46–74 years) took part in the study. All had had their large bowel removed for ulcerative colitis and were studied 20 years (11–31 years) after surgery. None had evidence of small bowel disease and as far as could be ascertained has no more than 20 cm ileum resected. One subject was a diabetic and was taking glibenclamide and metformin during the study. No other medications were being taken. None had a recent history of antibiotic use.

Protocol

Throughout the study a polysaccharide-free diet was consumed by the volunteers providing a balance of protein, fat and carbohydrate. The volunteers were also asked to stick to a polysaccharide-free diet the day before they started the study.

At breakfast, either a test meal of starch was fed or the control meal containing readily digestible wheat starch. The control meal allowed the assessment of the contribution of mucin to fermentable substrate available to the colon. The order in which the test meals were fed was randomised. The starch test meals contained 50 g starch and included a retrograded chick pea starch gel and whole cooked or homogenised chickpeas, details of the preparation of which are given below. The amount of fat in the control meal was balanced by increasing the amount of fat in the diet on the control day.

Materials

Chick peas (*Cicer arietinum*) of Turkish origin were obtained from a local supermarket. Chick pea starch preparation involved homogenisation to disrupt the cellular structure following overnight soaking in water at 4°C. The homogenate was passed through a nylon sieve lined with muslin to remove cell wall debris and the starch was collected by sedimentation. Protein was removed from the sediment by washing with 0.1 M NaCl and water and the sediment was air dried at room temperature.

Preparation of cell wall material from whole chick peas involved cellular disruption of soaked chick peas using an Ultra Turrax homogeniser. The homogenate was extracted sequentially with 0.1 M NaCl and 90% DMSO to remove soluble proteins, low molecular weight solutes and starch (Ring & Selvendran, 1978). The residue was finally washed with water to remove the DMSO and then freeze dried.

Meal preparation

The three test meals consisted of cooked whole chick peas, cooked homogenised chick peas and a chick pea starch gel. For the whole and homogenised chick pea meals, chick peas which had been soaked overnight at 4°C, were boiled in excess water for one hour, cooled to room temperature and were then stored for 18 h at 4°C prior to consumption. After storage, a fraction of the cooked chick peas were homogenised in a food processor for 2 minutes. A 30% w/w starch gel was prepared by heating a mixed starch/water slurry at 90°C until a thick paste was formed. The paste was heated for a further 5 minutes, allowed to cool to room temperature and then stored for 18 h at 4°C. Immediately prior to consumption the retrograded starch gel was mashed and mixed with 5 ml lemon juice and 2 g sucrose.

Effluent

Ileostomy effluent was collected by the subjects either emptying or changing their bag every 2 hours during the

day from 7 am until 11 pm, the final collection made the following morning at 7 am. Each sample of effluent was immediately frozen over solid CO_2 and weighed. Prior to analysis, the ileostomy effluent was thawed and extracted with 3×5 ml of 80% (v/v) aqueous methanol in a centrifuge to remove soluble low molecular weight carbohydrate. Aqueous methanol was used to avoid structural alteration to the starch residue. The solid residue was dried over P_2O_5 at room temperature prior to analysis.

CHEMICAL METHODS

Sugar analysis

The polysaccharide residue in the isolated cell wall material of chick peas including hulls (testas) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120°C for 2 h to determine the non-cellulosic polysaccharide component. Additionally, hydrolysis of the cellulosic component was achieved after an initial solubilization of the isolated cell wall and hull material in 72% (w/w) H_2SO_4 at room temperature for 3 h, followed by dilution of the acid to 1 M and hydrolysis for 2.5 h at 100°C. The sulphuric acid method was also used to hydrolyse the polysaccharide residue in the ileostomy effluent. The neutral sugars in the hydrolysates were determined as their alditol acetates as previously described (Englyst & Cummings, 1984).

Methylation analysis

Aliquots (mg) of the dried chick pea feed and the ileostomy effluent were sonicated in DMSO, under argon, at room temperature for 3 hours prior to alkoxide formation and subsequent methylation (Ciucanu & Kerek, 1984). The methylated polysaccharides were hydrolysed and converted to partially methylated alditol acetates (PMAA) as previously described (Needs & Selvendran, 1993).

Gas chromatography of alditol acetates and PMAA

Alditol acetates and PMAA were separated on a capillary column (30 m × 0.32 mm ID, Thames OV-225 silicone) over a temperature gradient in a Hewlett Packard 5890 series II chromatograph equipped with a flame ionisation detector. The pattern of methyl substitution of the PMAA was determined by combined GC–MS using a Vectographic Trio 1S mass spectrometer. The identity of the PMAA was determined from this data and the retention time relative to 2,3,4,6 tetra-O-methyl glucose.

Determination of nitrogen content

Samples (*ca* 5 mg) of dry, aqueous alcohol (80% v/v) insoluble effluent residue were carefully weighed into tinfoil capsules on a microbalance. Samples of the feeds were dried at 105°C overnight and reweighed before analysis. Total nitrogen was determined with a Carlo Erba ANA 1400 Automatic Nitrogen Analyzer using cyclohexanone, 2, 4-dinitrophenylhydrazone as nitrogen standard. The nitrogen to protein conversion factor used was 5.86 (Yamaguchi, 1992).

RESULTS

The amount of effluent, collected over two hour periods, peaked between 4 and 10 h after consumption of the test breakfast. Transit time for the whole and homogenised chick pea was slightly longer than for the chick pea starch but on average, effluent production peaked at 6 h. The fractions representing this peak in production following the test breakfast were analyzed. The dry weight and the nitrogen content of the peak fractions analyzed are shown in Table 1. After feeding the starch gel, the mean dry weight of the peak fraction was 5.4 g, increasing to ~9 g when whole or homogenized chick pea were fed. The nitrogen content of the effluents obtained after feeding the starch and chick pea test meals were ~5.7 and 3.2% with an estimated protein content of 33 and 19% respectively (Yamaguchi, 1992) assuming that the nitrogen present is protein in origin. In practice this will be a slight over estimate due to the presence of nitrogen containing (mucin) glycoproteins. Low molecular weight carbohydrate in the effluent was extracted during the aqueous alcohol (80% v/v) treatment and was found to represent less than 2% of the total carbohydrate content in the effluent.

The average neutral sugar composition of the peak effluents examined for chick pea starch, whole and homogenized chick pea test meals are shown in Table 2 and expressed as mole %. In the effluent collected from the six subjects after consumption of the chick pea starch test meal, the main neutral sugars found were fucose, galactose and glucose, with glucose being present in the largest amount (79 mole %). When the retrograded chick pea starch was fed as part of a tissue, either as whole or homogenized cooked chick pea, non-starch polysaccharides of cell wall origin were identified. The major neutral sugar present in both types of efflu-

Table 1. Dry weights (mean values with their standard errors for six subjects) and nitrogen content of the peak in effluent production following the test meals of retrograded chick pea starch (CPS), homogenized chick pea (HCP) and whole chick pea (WCP)

Effluent	Dry weight (g)	Nitrogen (% w/w)
CPS	5.4±2.7	5.7
HCP	9.1±3.5	3.3
WCP	8.5±4.7	3.2

Table 2. Neutral sugar composition (mean values for six subjects expressed as mole %) of ileostomy effluent at the peak of production, obtained after feeding the test meals of retrograded chick pea starch (CPS), homogenized chick pea (HCP) and whole chick pea (WCP)

Test meal	Rha	Fuc	Ara	Xyl	Gal	Glc
CPS	nd	6.6	tr	nd	9.3	78.7
HCP	2.9	2.6	31.0	4.1	6.5	52.2
WCP	3.1	3.4	29.4	3.3	7.7	52.9

nd: not detected.
tr: trace.

ent was glucose which originated from both starch and cellulose. The arabinose present must be of cell wall origin and the fucose from mucin.

The carbohydrate fraction of the effluent was investigated in more detail using a methylation analysis to determine the linkages between the monosaccharides. Table 3 shows the polysaccharide linkages found in the ileostomy effluent following consumption of each test meal, expressed as mole %. The fucosyl and galactosyl linkages found – terminal fucose and galactose, 1-3, 1-2 and 1-3-6 linked galactose are characteristic of intestinal mucins (Slomiany *et al.*, 1984). The glucosyl linkages found were those of starch, terminal glucose, 1-4 linked glucose, and a 1-4-6 linked branch point, since the methylation procedure used does not methylate the cellulose (Ring & Selvendran, 1978), although a small contribution from a cellulosic xyloglucan cannot be excluded. Also, in the effluents collected following the whole and homogenised chick pea meals, glycosidic linkages characteristic of a pectic arabinan (*t*-, 1-4/1-5, 1-2-3-5/1-2-3-4 Ara) were present. However, from the methylation analysis it was not possible to identify unambiguously the rhamnosyl and xylosyl linkages. For the effluents obtained from feeding whole and homogenized chick peas, the average measured characteristics–dry weight, nitrogen content (Table 1), neutral sugar composition (Table 2) and glycosidic linkages (Table 3), were similar.

Analysis of the neutral monosaccharide composition of a cell wall preparation from chick pea is shown in Table 4. Two methods of hydrolysis were used to liberate the neutral sugars. Firstly, hydrolysis with trifluoroacetic acid (TFA) which hydrolyzed the non-cellulosic polysaccharides, and secondly, a sulphuric acid hydrolysis, involving a pretreatment with 72% (w/w) sulphuric acid, which additionally hydrolyzed cellulose. The dominant neutral sugar in the TFA hydrolysis was arabinose, with relatively small amounts of rhamnose, xylose, galactose and glucose being present. Rhamnose, arabinose and galactose are common neutral sugar constituents of the cell wall pectic polysaccharides, while xylose is often a component of the hemicelluloses. The glucose present could come from a small amount of contaminating starch or a hemicellulosic xyloglucan. The sulphuric acid hydrolysis of the cell wall preparation increased the content of glucose in the hydrolysate as a result of the hydrolysis of cellulose. The absence of fucose in the cell wall hydrolysate means that the presence of fucose in effluent hydrolysates is diagnostic for intestinal mucin. The data obtained on the neutral sugar composition of the cell wall are in close agreement with earlier published research (Champ *et al.*, 1986).

Table 3. Linkage analysis, expressed as mole %, of the polysaccharides in the ileostomy effluent at peak production, following consumption of the test meals of retrograded chick pea starch (CPS), homogenized chick pea (HCP) and whole chick pea (WCP)

Sugar linkage	CPS	WCP	HCP
t-Fuc	4.4	1.9	1.4
t-Gal	3.2	4.6	3.3
1-3 Gal	6.0	4.2	3.2
1-2 Gal	4.6	3.2	2.0
1-3-6 Gal	1.9		
t-Glc	5.1	3.3	2.9
1-4 Glc	69.2	43.6	45.3
1-4-6 Glc	6.0	4.9	4.7
t-Ara	nd	16.0	16.3
1-4/1-5 Ara	nd	13.8	14.1
1-2-3-4/5 Ara	nd	4.6	6.8

Table 4. Neutral sugar composition, expressed as mole %, of cell wall hydrolysates following TFA hydrolysis and H_2SO_4 hydrolysis, and a hydrolysate of the whole chick pea

Hydrolysate	Rha	Fuc	Ara	Xyl	Gal	Glc
TFA	6.3	tr	67.9	9.0	5.7	10.1
H_2SO_4	4	tr	42.9	5.4	3.5	43.1
Whole chick pea	0.7	tr	13.6	1.9	1.6	81.7

tr: trace.

DISCUSSION

Following the consumption of the retrograded starch gel, in addition to the polysaccharide derived from starch, the ileostomy effluent will also contain mucin of gastric, intestinal and other origins. In studies on the carbohydrate composition of intestinal mucins (Wesley *et al.*, 1983, 1985; Slomiany *et al.*, 1984; Podolsky, 1985a, b; Neutra & Forstner, 1987) it was found that the glycoprotein contained between 70 and 80% carbohydrate, consisting of a number of different oligosaccharides linked to a peptide core. The oligosaccharides contained core structures, although the structure and composition of the oligosaccharides is dependent on the blood group of the host (Slomiany *et al.*, 1984), and is also altered in certain disease states (Wesley *et al.*, 1983). Despite this structural variation it is possible to identify a typical composition of the mucin. For example, small intestinal mucins contain the amino sugars *N*-acetyl glucosamine, *N*-acetyl galactosamine and sialic acid, with fucose and

galactose comprising the bulk of the remaining carbohydrate. In the present study, the analytical procedures used do not permit the estimation of the amino sugars. However, the occurrence of fucose and galactose in the carbohydrate component of the ileostomy effluent indicates the occurrence of mucin. In studies on the composition of mucin from the small intestine (Forstner *et al.*, 1979; Gold *et al.*, 1981; Wesley *et al.*, 1983, 1985; Neutra & Forstner, 1987) it was found that fucose and galactose accounted for between 41 and 56% w/w of the mucin carbohydrate with an average of 50%. If an average value of 50% is assumed the calculated ratio of starch-derived carbohydrate to mucin carbohydrate in the effluent collected after the retrograded starch meal is 1 to 0.4.

It is predicted from *in vitro* experiments that a linear dextrin derived from retrograded amylose is the main resistant product obtained on the amylolysis of starch gels (Jane & Robyt, 1984; Ring *et al.*, 1988; Leloup *et al.*, 1992; Eerlingen *et al.*, 1993). The present *in vivo* experiments demonstrate the occurrence of branched starch fragments in the "resistant" starch fraction. If these branched fragments were small oligosaccharides the ratio of terminal to branched residues would be 2:1, whereas for the high molecular weight branched polymer amylopectin, the ratio would be 1:1 (Manners & Matheson, 1981). In the present experiments the average molar ratio of terminal to branched residues is 1:1.2, indicating the occurrence of polymeric fragments derived from amylopectin in the effluent. The average constituent chain length of the starch residue in the effluents after all three test meals, calculated from the ratio of terminal to 1-4 linked glucose was 14, taking into account any contribution to 1-4 linked glucose from any xyloglucan present.

From the neutral sugar and linkage analysis of peak effluent after consumption of the homogenized and whole chick pea, it is possible to identify polysaccharide components of cell wall, starch and mucin. Until recently it was thought that the non-starch polysaccharides of plant tissues were the main source of fermentable carbohydrate for the bacteria in the human colon. Mucin and oligosaccharides such as raffinose and stachyose were thought to make a relatively minor contribution. There is continuing interest and debate on the main carbon source for the bacterial biomass of the colon. As starch is the main polysaccharide in our diet it is important to estimate its potential contribution to the fermentable carbohydrate. Following consumption of the chick pea starch gel containing 50 g of starch, the average dry weight of the peak of effluent production in a two hour period was 5.4 g. The average neutral carbohydrate content of this material was 47% w/w. Of this carbohydrate, 79% was glucose originating from starch. The calculated starch content in the 5.4 g of effluent was 2.0 g, and the calculated amount of mucin carbohydrate, 0.8 g. In this two hour period 4.0% of the ingested starch had escaped digestion, and could be considered as resistant. This value is a minimum, as the excretion of starch-derived polysaccharide extends over a longer time than the two hour period examined. The average dry weight of the peak fraction is 57% of the dry weight of the six hour period extending from 2 hours before to 2 hours after the peak. If these effluents are assumed to have a similar composition, then an estimate of 7% w/w is obtained as an upper limit for the level of resistant starch.

In the effluents obtained after feeding the whole and homogenized chick pea, an estimate of the level of resistant starch may be obtained by comparing the ratio of arabinose of cell wall origin to glucose obtained from both cellulose and starch. From the neutral sugar composition of the feed and the neutral sugar composition of the ileostomy effluent (at the peak in production), it is calculated that, on average, 14% and 16% of the ingested starch following consumption of homogenized and whole chick peas, respectively had escaped digestion in the small intestine.

CONCLUSIONS

Using methylation analysis it was possible to obtain detailed structural information on the polysaccharides in ileostomist effluent obtained after feeding a starch-rich test meal. While *in vitro* studies predict that the resistant starch comprises a linear fragment of amylose, chemical characterization of the ileostomy effluent after the ingestion of retrograded starch gel and cooked, cooled whole and homogenized chick pea, showed the presence of branched fragments of amylopectin which had escaped digestion. After consumption of the cooked chick pea, the starch of which had been allowed to retrograde, on average 15% of the ingested starch had escaped digestion in the small intestine. Analysis of the effluents showed that carbohydrate deriving from starch and mucin can make a substantial contribution to the carbohydrate available for fermentation in the colon.

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