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A physicochemical characterization of chick pea starch resistant to digestion in the human small intestine

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The digestibility of a retrograded starch gel in the human small intestine was assessed in an ileostomist model. The resistant product was characterized by size-exclusion chromatography and X-ray diffraction. Carbohydrate from mucin and starch in the effluent was identified by monosaccharide analysis. The resistant starch contained molecules with a wide range of molecular weights. A major fraction consisted of fragments of amylose with an average degree of polymerization of 70–80. The resistant product was partially crystalline, giving a wide-angle powder diffraction pattern typical of the B-type crystalline polymorph of starch. The resistant product obtained *in vivo* was comparable with the product that was resistant *in vitro*.

INTRODUCTION

Starch is our main dietary polysaccharide and it can occur in foods in a number of different physical forms that have different susceptibilities to pancreatic α-amylase. As a consequence, the rate and extent of digestion of starch in the small intestine is affected, and there has been recent interest in defining the amount of starch that we eat that escapes digestion in the small intestine and is a substrate for fermentation in the colon.

Starch occurs in plants as native granules that are partially crystalline. The identity of the crystalline polymorph present depends on the structure of the starch polysaccharides, which is related to botanical origin. Most cereal starch granules give an A-type X-ray diffraction pattern while tuber starches such as potato give a B-type pattern (Gallant *et al.*, 1992). Recent interpretations of the X-ray intensity distribution of these polymorphs are based on the packing of double helices in hexagonal (B form) (Imberty & Perez, 1988) and monoclinic (A form) (Imberty *et al.*, 1987, 1988) unit cells. The amylolysis of native starch granules *in vitro* is affected by botanical source. Most cereal starches are hydrolysed relatively readily, while some starches such as that from potato are very resistant to hydrolysis but the molecular origin of this resistance is unclear. It is tempting to ascribe the resistance to the presence of the B-type crystalline polymorph (Gallant *et al.*, 1992), although other factors such as granule size and surface properties could be of importance. The extent of enzymic hydrolysis will also depend on the accessibility of the enzyme to starch. Granule and particle size, a compact or porous microstructure, and the presence of other constituents, such as cell walls and proteins, are all factors that can act as physical barriers to the enzyme. In a recent ileostomy study (Botham *et al.*, 1994), the effect of cellular material on the structure and the amount of starch appearing in the effluent was investigated. The presence of intact and disrupted cell walls markedly increased the level of resistant starch.

Most of the starch that we eat has been processed by a heat moisture treatment that results in disruption and gelatinization of the native granular structure. During gelatinization, the starch polysaccharide amylose is solubilized preferentially. On cooling a gelatinized starch dispersion to room temperature, the solubilized amylose reassociates as does the partially solubilized amylopectin, a process known as retrogradation. The retrogradation of amylose at room temperature is a rapid process that results in the formation of a network structure, the individual strands of which consist of many associated amylose chains. This process, monitored in terms of stiffness is complete within a few hours. Subsequently limited crystallization of the

amylose is observed to last several days (Miles *et al.*, 1985*b*). The amylose network can be melted by heating to above 140°C.

Retrogradation of amylopectin (Miles *et al.*, 1985*a*; Ring *et al.*, 1987) is a slower process, lasting days or weeks depending on sample concentration. In this case, crystallization occurs at the same rate as the development of stiffness and a partially crystalline amylopectin network is formed. The rate and extent of retrogradation of amylopectin is markedly influenced by the fine structure of the branched molecule (Kalichevsky *et al.*, 1990). In excess water, the retrogradation of amylopectin may be reversed by heating to 70°C. The B-type crystalline polymorph of starch is obtained on the crystallization of both amylose and amylopectin from water at room temperature.

There have been a number of studies (Colonna *et al.*, 1992; Leloup *et al.*, 1992; Jane & Robyt 1984; Ring *et al.*, 1988; Eerlingen *et al.*, 1993*a*) examining the digestibility by α-amylase of retrograded starch *in vitro*. A number of physicochemical factors are important. For amylose gels it was found that increasing the concentration of amylose in the gel reduced porosity and hence access of the enzyme, thus increasing the level of resistance (Leloup *et al.*, 1992). The structural organization of starch is an important determinant of digestibility. While retrograded amylopectin is hydrolysed extensively by α-amylase at 37°C (Ring *et al.*, 1988), retrograded amylose is substantially resistant, but the molecular origin of this difference is not known. Highly crystalline spherulitic material of the B-type crystalline polymorph is highly resistant to amylolysis, and it is suggested that the crystallization of amylose chains during retrogradation conveys resistance. Englyst and Cummings (1987) using ileostomy subjects, showed that freshly cooked potatoes were digested more completely than cooled cooked potatoes. When the cooled potatoes were reheated, the digestibility was greatly improved and it was suggested that retrograded amylopectin is partly responsible for the incomplete digestion of starch in cooled potato.

The main resistant product obtained from the amylolysis of starch and amylose gels *in vitro* is a linear dextrin with a relatively broad distribution of degree of polymerization (DP) with an average DP of 20–80, which depends on how the sample is prepared and on the type of average DP measured (Leloup *et al.*, 1992; Jane & Robyt, 1984; Ring *et al.*, 1988; Eerlingen *et al.*, 1993*b*).

There have been a number of studies *in vivo* demonstrating that a fraction of starch remains undigested at the end of the small intestine (Englyst & Cummings 1985; Schweizer *et al.*, 1990; Englyst & Cummings, 1986, 1987). More recently in an ileostomist study, and through the use of an intubation technique on normal subjects, the digestibility of starch *in vivo* after feeding bean and potato flakes has been assessed and the resistant product characterized (Faisant *et al.*, 1993). Three carbohydrate fractions derived from starch were obtained. The main resistant product was a linear dextrin with a weight average DP of 69, which melted at 150°C and gave a B-type X-ray diffraction pattern. In addition, there was an oligosaccharide fraction and a high molecular weight fraction.

In a recent related study (Botham *et al.*, 1994) the chemical composition of the polysaccharides in ileostomy effluent was examined using sugar and linkage analysis. Following consumption of a retrograded starch gel, carbohydrate from starch and mucin was found in the effluent, in the ratio of 1:0·7. After consumption of cooked, retrograded whole tissue, cell wall polysaccharides were additionally identified. The linkage analysis showed the resistant fraction to contain a small fraction of branched starch chains in addition to the main resistant linear glucan.

These studies demonstrate that a fraction of starch escapes digestion in the small intestine and we report on the physicochemical characterization of this material.

EXPERIMENTAL

Materials

Chick peas (*Cicer arietinum*) were obtained from a local supermarket. Chick pea starch was prepared by homogenizing chick peas that had been soaked for 16 h at 4°C. The homogenate was passed through a sieve and muslin to remove cell wall debris and the starch collected by sedimentation. Protein was removed from the sediment by washing successively with 0·1 M NaCl and water. The starch was allowed to dry in air at room temperature.

Meal preparation

The test meal was a chick pea starch gel, which was prepared by gelatinizing a 30% w/w starch dispersion for 5 min at 90°C with sufficient agitation before gelatinization to prevent sedimentation of the starch granules. The gelatinized dispersion was cooled to 4°C and kept at this temperature for 18 h. The retrograded starch gel was mashed and mixed with 5 ml lemon juice and 2 g sucrose immediately before consumption.

Diet and subjects

Four ileostomy subjects (all female, average age 70 (range 46–74)) took part in the study. All had had their large bowel removed for ulcerative colitis and were studied for, on average, 19 years (range 11–31 years) after surgery. None had evidence of small bowel disease, and as far as could be ascertained, none had more than 20 cm ileum resected. Volunteers were housed in the

metabolic unit of the Dunn Clinical Nutrition Centre in Cambridge and fed a plant polysaccharide-free diet throughout the study, including a 24 h period before the test breakfasts. Foods included prawns, mayonnaise, chicken, pork, salmon, beef, cheese, milk, cream, tea and coffee. The test breakfasts, in addition to a starch gel, comprised an omelette (made with one egg and milk), bacon, tea, milk and sugar. The diet provided 7 MJ and contained 123 g protein, 107 g fat, and 45 g carbohydrate.

Effluent collection

Ileostomy effluent was collected by the subjects either emptying or changing their bag every 2 h during the day from 7 am in the morning until retiring at 11 pm. Each sample of effluent was immediately frozen in solid CO_2. The effluent weight was recorded and the dry matter content was determined by drying alcohol rinsed aliquots over P_2O_5 under vacuo. The effluent production as a function of time was obtained. Before analysis, the ileostomy effluent was thawed and extracted with 3×5 ml of 70% aqueous methanol in a centrifuge to remove soluble small molecular weight carbohydrate. The residue was dried over P_2O_5 at room temperature.

Physicochemical characterization

The polysaccharide in the effluent was dissolved in 1 M KOH at room temperature, effectively dissolving the starch granules and retrograded starch. 0·1 M $NaBH_4$ was added to reduce the reducing end groups of the polysaccharide chain to the corresponding alditol, to arrest the progressive peeling of glycosyl units from the reducing end of the chain in alkaline conditions. After 16 h, the solution was centrifuged at 10 000 *g* for 10 min to remove cellular debris. The polysaccharide in the supernatant was separated by size-exclusion chromatography (Hizukuri & Takagi, 1984) on linked 0·75 cm × 30 cm columns of Anagel-TSK G 6000, 4000 and 3000 PW, with an Anagel TSK PW guard column. The column was calibrated with a series of pullulan fractions (Polymer Laboratories) of known molecular weight and limited polydispersity. The carbohydrate was eluted with 0·01 M NH_4OH at a flow rate of 1 ml/min. The eluent was monitored using a refractive index detector. Fractions (2 ml) of the eluent were collected for analysis.

The interaction of starch polysaccharide in the eluent with the polyiodide ion was examined, after neutralization of the eluent, by determining the absorbance of the starch/polyiodide ion complex between 500 and 700 nm. The λ_{max} of the complex was used to estimate the average chain length of the dextrin (John *et al.*, 1983).

The monosaccharide composition of the fractions from the size-exclusion chromatography was determined after hydrolysis of the polysaccharide with 2 M trifluoroacetic acid, TFA, for 2 h at 100°C (Selvendran *et al.*, 1979). TFA was removed by evaporation in an air stream at 50°C. The liberated monosaccharides were analysed by ion-exchange chromatography on a 0·4 cm × 25 cm Carbopac PA 1 column (DIONEX), fitted with a PA 1 guard column. The column was eluted with 0·15 M NaOH and the monosaccharides in the eluent were detected using a pulsed amperometric detector (Clarke *et al.*, 1991).

Characterization of crystalline form of starch

X-ray diffraction measurements were carried out using radiation of wavelength 0·154 nm. The diffractometer was a Philips Scientific PW 1820 vertical goniometer with attached camera. Data were collected using a proportional detector, stored and processed using Philips (Version 3.5) PC-APD software.

Samples were placed in the camera and scanned over the range 10·05–30° 2-theta (0·87943–0·29762 nm) at a speed of 0·02° 2-theta per second with a step size of 0·15. Identification of peak positions was accomplished using the peak search facility of PC-APD. This automatically locates peaks in a crystalline diffraction pattern by detecting minima in the second derivative of the diffractogram. The standard Peak Search settings were used, except that the minimum peak half-width was changed from 0·0 to 0·2° 2-theta (producing a slight smoothing of the data, which reduces the danger of detecting noise-induced peaks) and the maximum peak half-width was changed from 1·0 to 1·5° 2-theta (taking into account the general broadness of the starch peaks).

RESULTS

Physicochemical characterization

One subject was unable to consume the chick pea starch test meal, thus the effluent was not used for the analysis. The primary focus of this study was the characterization of the starch in the ileostomy effluent, and, therefore, a relatively mild dissolution in alkali was used to minimize depolymerization of the starch chain. A more vigorous alkaline treatment at elevated temperature is required to cleave quantitatively the oligosaccharides from mucin (Carlson 1968; Slomiany *et al.*, 1984; Podolsky 1985*a*, 1985*b*). Quantitative alkaline cleavage of the carbohydrate side-chains of mucins, which vary in length from 2–19 sugar residues, would result in the appearance of a muco-oligosaccharide fraction at small molecular weight. Figure 1 shows the chromatographic profiles of the three effluents examined following size-exclusion chromatography of the KOH-soluble polysaccharide. The profiles obtained are similar but complex, with material eluting from the void volume onwards, showing that the polymers in the effluent have a wide range of molecular size and hence molecular

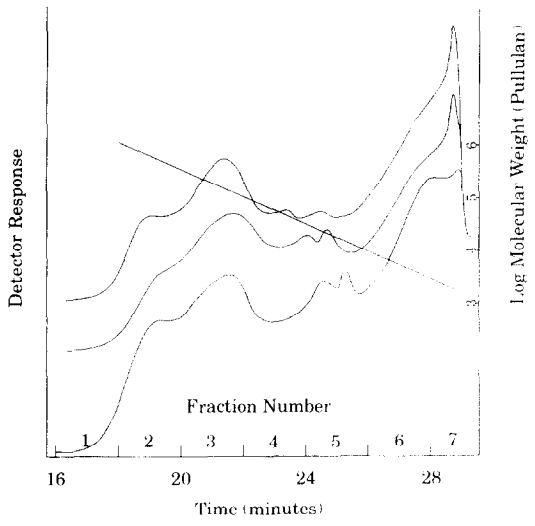


Fig. 1. Size-exclusion chromatograms of the alkali-soluble material in three ileostomy effluents obtained after feeding a retrograded chick pea starch gel.

weights. Fractions were collected and the monosaccharide composition determined after acid hydrolysis. Figure 2 shows the carbohydrate content of the fractions. Muco-oligosaccharides yield fucose, galactose, galactosamine and glucosamine upon hydrolysis (Slomiany *et al.*, 1984; Podolsky 1985*a*, 1985*b*; Wesley *et al.*, 1983; Allen, 1978), while hydrolysis of the starch yields glucose. The monosaccharides from mucin are eluted throughout the profile, indicating that fractions of mucin ranging in molecular size are present. Glucose from starch is present in the hydrolysates of all the fractions examined, indicating that the starch present is also polydisperse. There is a peak in the elution of glucose-containing polymers at a retention time corresponding to the elution of pullulans with a degree of polymerization (DP) ranging from 20 to 62. The average degree of polymerization was determined also by examining the starch/polyiodide interaction. Table 1 shows the λ_{max} of the starch/polyiodide ion complexes of each fraction and the calculated average DP of the starch chain.

Characterization of the crystallinity

Figure 3a shows the X-ray diffraction pattern obtained from the chick pea starch gel consumed as the test meal in this study. The positions of the diffraction peaks are shown by vertical lines along the abscissa and the ordinate shows the intensity of the diffracted X-rays. The position and relative intensity of these peaks closely match those of the B-type crystalline polymorph of starch (Imberty & Perez, 1988). The diffractograms obtained from the ileostomy effluents of three subjects after consumption of the starch gel test meal are shown in Fig. 3b. The positions of the diffraction peaks from these effluents are marked and compared with those from the original feed material (numbered). There is generally good correspondence in peak position between the diffraction peaks present in the gel and the

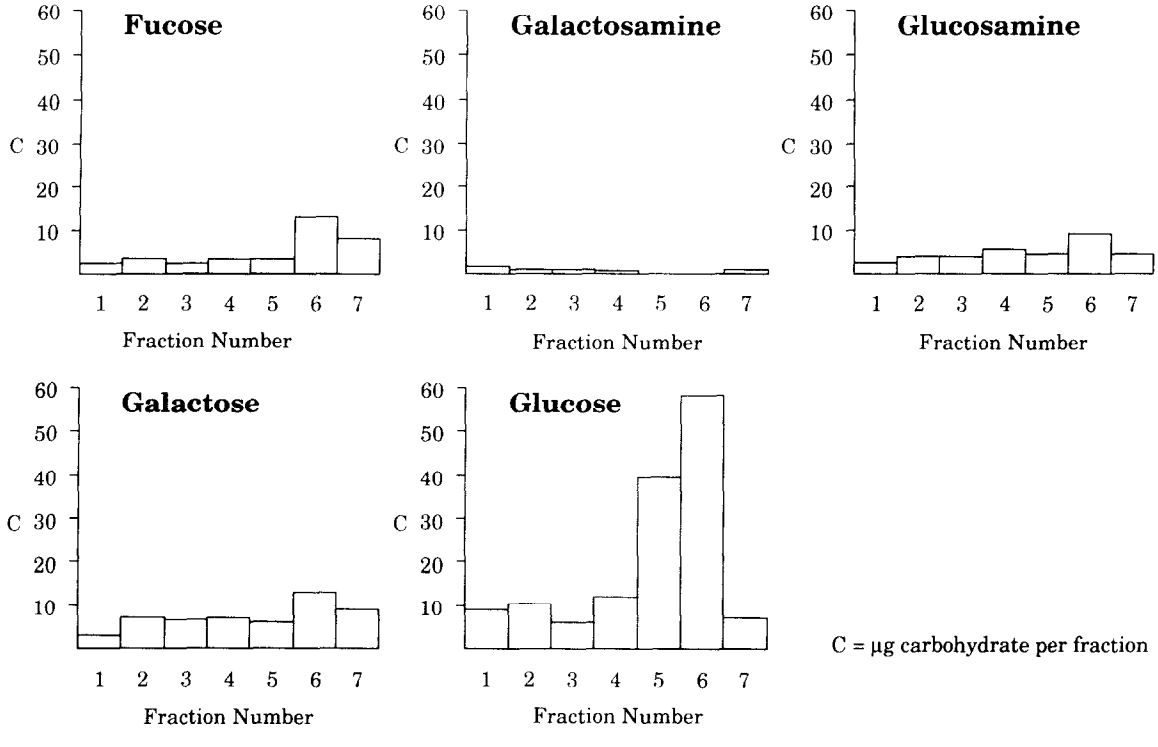


Fig. 2. Carbohydrate analysis of the fractions obtained by size-exclusion chromatography of the alkali-soluble material in ileostomy effluent.

Table 1. Iodine binding characteristics of the starch fractions obtained by size exclusion chromatography

Fraction	λ_{max} (nm)	DP
1	562	48
2	556	44
3	nd	
4	594	79
5	590	73
6	562	48
7	nd	

nd, not determined.

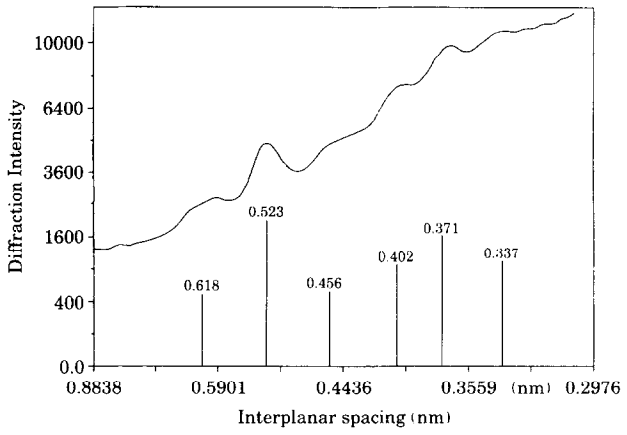
effluent obtained after feeding the gel, particularly with respect to the strongest peak at 0·52 nm and the medium-strength peaks at 0·61 and 0·37 nm. The B-type crystalline polymorph of starch is, therefore, present in the effluent. Figure 3c shows the X-ray diffraction patterns from two ileostomy effluents after consumption of the control meal containing no starch. Although there are three possible diffraction peaks which were detected, there is no evidence for the strong (0·52 nm) or medium-strength (0·61 nm, 0·37 nm) peaks characteristic of the B-type structure. This shows that the diffraction peaks detected in the effluents containing starch are not artefacts of the peak searching software.

DISCUSSION

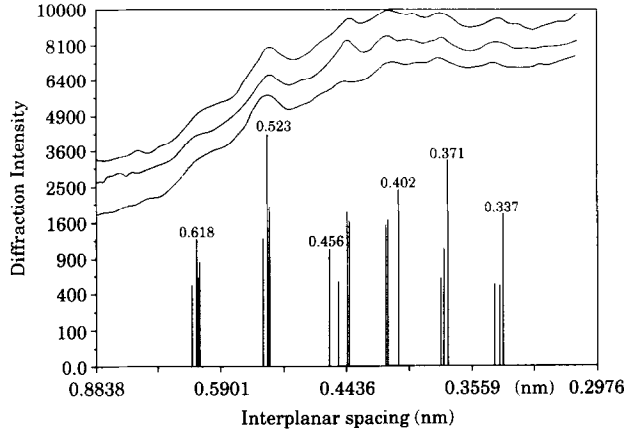
A peak in effluent production occurred between 5 and 7 h after consumption of the test meal indicating the time taken for the residue of the meal to reach the terminal ileum. The effluent collected by three subjects during 2 h spanning this peak in production was analysed. From the neutral sugar composition and the linkage analysis it was shown that the carbohydrate in the effluent was derived from mucin and starch (Botham *et al.*, 1994).

For glucose-containing polymers, there is a peak in the elution at a retention time corresponding to the elution of pullulans in the range DP 20–62. Linear amylose and pullulan chains of the same molecular weight differ in molecular size and hence elution time. In a comparison of the chromatographic behaviour of pullulan and amylose it was found that at the same retention time the molecular weight of the amylose was approximately 35% greater than that of the pullulan (Hizukuri & Takagi, 1984). To obtain more information on the chain lengths of the starch fragments eluted from the column, their interaction with the polyiodide ion in aqueous solution was examined.

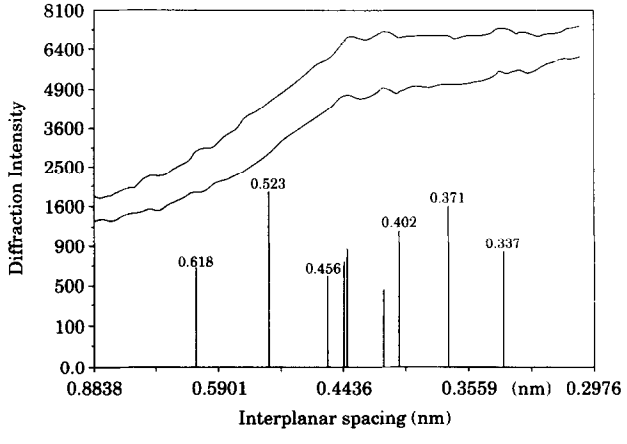
The blue amylose/polyiodide ion complex usually has a λ_{max} of 640 nm, but the λ_{max} decreases with decreasing chain length, and this dependence may be used to determine the length of an unsubstituted amylosic chain



(a)



(b)



(c)

Fig. 3. X-ray diffractograms of a (a) chick pea starch gel; (b) three ileostomy effluents containing resistant starch; and (c) two effluents containing no starch.

(John *et al.*, 1983). The λ_{max} of amylopectin usually ranges from 530 to 540 nm, indicating the presence of relatively short unsubstituted chains of DP 30–35. The λ_{max} of the major starch fraction was 590 nm and the calculated DP 70–75. In a study on the characterization of the α-amylase-resistant products of amylose and pea

starch gels *in vitro*, it was found that the λ_{max} of the polyiodide ion complex with the products was 590 nm. It was concluded (Ring *et al.*, 1988) that the resistant product from the starch gel consisted of fragments of amylose of DP ~70. Although retrograded amylopectin was hydrolysed by pancreatic amylases, the presence of the resistant amylose fraction hindered the conversion of amylopectin. The present study shows that the products resistant *in vitro* and *in vivo* are similar. The linkage analysis of the resistant starch showed the presence of fragments derived from amylopectin (Botham *et al.*, 1994). Separation of the starch by size-exclusion chromatography showed that fragments of amylose with a broad distribution of molecular weights with an average DP of 70–75 were a major resistant product.

In a study on the retrogradation of a pea starch gel it was found that the crystallinity detected after 24 h was primarily a result of the limited crystallization of amylose (Miles *et al.*, 1985*a*). These results show that a residue of the B-type crystalline polymorph of starch is found in the effluent of subjects who were fed the chick pea starch gel. The resistant residue of the starch gel includes the crystalline fraction of starch. This does not imply that the amorphous starch fraction plays no part in resistance to digestibility, but its contribution cannot be quantified using these results.

CONCLUSIONS

The main resistant product obtained at the end of the human small intestine after feeding a chick pea starch gel was a linear amylosic chain with a DP in the region of 70. The resistant fraction of starch is partially crystalline, with a B-type X-ray diffraction pattern. The resistant product obtained *in vitro* is comparable with that obtained from the amylolysis of starch and amylose gels *in vitro* (Ring *et al.*, 1988; Cairns *et al.*, 1990) and those products obtained *in vivo*, after feeding bean and potato flakes (Faisant *et al.*, 1993), although in the latter case a more obvious fraction of a partially crystalline high molecular weight starch was present. This indicates that the cellular structure of the plant food additionally influences the digestibility of starch in the small intestine as well as the intrinsic digestibility of a particular physical form of starch.

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