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Structural discrepancies in resistant starch obtained *in vivo* in humans and *in vitro*

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In-vivo resistant starch (RS) was collected at the end of the human ileum by an intubation technique after ingestion of two partly resistant starches (retrograded (R) or complexed (C) high amylose maize starch). In-vitro RS fractions were prepared enzymatically from R and C according to three methods of RS determination in food. Physicochemical characteristics of these two different fractions were compared. The ileal RS appeared to consist of three fractions: a first population of high molecular weight α-glucans attributed to amorphous potentially digestible material, a second made of B-type retrograded amylose crystallites and a third containing oligosaccharides. The in-vitro RS fractions showed no high molecular weight molecules, due to more extensive hydrolysis in the in-vitro procedure. Therefore, none of the in-vitro RS determinations allowed the isolation of a fraction qualitatively similar to in-vivo RS.

INTRODUCTION

Resistant starch (RS) has been defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals. In-vivo ileal digestibility of some starchy foods was studied using different techniques: ileostomy (Schweizer *et al.*, 1990), intubation of healthy volunteers (Flourié *et al.*, 1988; Molis *et al.*, 1992), hydrogen breath-test (Flourié *et al.*, 1988) and rats treated with antibiotics (Björck *et al.*, 1986). Several in-vitro methods have been proposed in order to quantify RS in food (Englyst *et al.*, 1982; Berry, 1986; Björck *et al.*, 1986; Englyst *et al.*, 1992). Some of them gave RS values in agreement with quantitative in-vivo measurements done either on rats (Björck *et al.*, 1986) or on ileostomized subjects (Englyst *et al.*, 1992) but none was assessed on structural similarity. The aim of this study was to compare physicochemical characteristics of RS obtained *in vivo* in humans and *in vitro* using three different procedures.

MATERIALS AND METHODS

Starchy products

Two starchy products with high content of RS were studied: retrograded starch (R) and complexed starch (C). Both were obtained from high amylose maize starch (70% amylose, Roquette, Lestrem, France) as described by Molis *et al.* (1992).

Obtention of in-vivo RS fractions

The ileal contents were collected by Molis *et al.* (1992) on healthy human volunteers by the intubation technique. After ingestion of either 33 g equivalent starch of R or C in a complex meal, ileal content was aspirated during 14 h. Fractions each of a 30-min period were frozen, freeze-dried and milled. RS content of each experimental starch was considered as all the starch that reached the terminal ileum. Each characterized ileal sample was obtained by making a pool of dry ileal contents collected for one subject in the time interval from 5 h 30 min to 7 h after the meal. This period corresponded to the maximum starch concentration in the ileal effluents.

In-vivo ileal samples were submitted to a partial purification: molecules soluble in pH 9·0 were removed by centrifugation and delipidation was performed on the sediments with a chloroform–methanol–water mix (Faisant *et al.*, 1992).

In-vitro RS determinations

Three methods were used to determine in-vitro RS content of R and C.

Method a was based on the method described by

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Berry (1986) and slightly modified (Champ, 1992). In this method, RS was starch not hydrolysed after 16 h of α-amylase hydrolysis.

Method b was elaborated by Asp and collaborators (Björck *et al.*, 1986). RS was defined as starch remaining in the fibre residue obtained by the AOAC method (Prosky *et al.*, 1988) and available for amyloglucosidase hydrolysis only after solubilization with 2N KOH.

Method c was elaborated by Englyst *et al.* (1992). RS corresponded to starch not hydrolysed after 120 min with pancreatin, amyloglucosidase and invertase.

Preparation of in-vitro RS fractions for physicochemical characterizations

In-vitro RS fractions were prepared from sample R and C according to methods *a*, *b* and *c* described previously.

RSa: samples were hydrolysed for 16 h by α-amylase at 37°C, then four volumes of absolute ethanol were added. After 1 h at room temperature, samples were centrifuged and supernatant discarded. Residues were then washed twice with 80% ethanol and once with acetone. RS residues were dried in a vacuum oven at 40°C and milled in a mortar.

RSb: the fibre residues were prepared according to the AOAC method (Prosky *et al.*, 1988) by hydrolysis with a thermostable amylase at 100°C. After cooling, proteolysis was performed at 60°C. Final hydrolysis with amyloglucosidase was performed after another cooling step. The total dietary fibre residues were obtained by successive centrifugations: three washings with 80% ethanol and two with 95% ethanol. Residues were then collected in acetone, dried and milled in the same conditions as for RSa. In that case, RS was considered to be all the starch contained in the AOAC solid residue.

RSc: after hydrolysis by pancreatin, amyloglucosidase and invertase during 120 min at 37°C, two volumes of absolute ethanol were added, samples were centrifuged, and the residues were washed with acetone and then dried and milled in the same conditions as for RSa. In that case, RSc would represent only the insoluble fraction of starch that escaped hydrolysis. Indeed, in their method, Englyst *et al.* (1992) considered RS as all the products of starch hydrolysis after 120 min except free glucose released before that time.

Preparation of lintnerized starchy products

Acid hydrolysis of retrograded starch and complexed starch was performed as described by Robin *et al.* (1974).

Physicochemical characterizations

Size exclusion chromatography (SEC) on Superose 12TM, determination of oligosaccharides by HPLC, differential scanning calorimetry (DSC) and X-ray diffraction were performed as described by Faisant *et al.* (1992).

RESULTS

Quantitative determinations of RS

Table 1 gives RS content of samples R and C determined by the in-vitro methods *a*, *b* and *c* compared to the in-vivo results obtained by Molis *et al.* (1992). RS content of R was 29·9, 13·5 and 26·1% of total starch (TS) with methods *a*, *b* and *c* respectively, while the in-vivo value was 49·4%. For C, RS content was 12·7, 9·7 and 13·9% with the respective in-vitro methods and 20·6% *in vivo*.

Physicochemical characteristics of in-vivo RS

SEC showed that starch that escaped digestion in the upper part of the intestine was composed of three populations of α-glucan molecules (Fig. 1). The first one (I), which represented a small proportion, was composed of oligosaccharides, mainly glucose, maltose, maltotriose and maltotetraose. The second population (II) exhibited a broad peak centred at $K_{av} \approx 0{\cdot}60$. Average number of degrees of polymerization (DP_n) were 35 and 38 for ileal samples after ingestion of C or R respectively. The third one (III) was constituted of high molecular weight α-glucans ($DP_n > 100$) excluded from the column. After soluble fraction removal, populations II and III were still observed, meaning that these molecules were involved in insoluble particles at least partially in crystalline constitutive of in-vivo resistant starch.

Both dry ileal contents exhibited a B-type pattern by X-ray diffraction analysis. Differential scanning calorimetry analysis showed an endotherm with a maximum temperature at 145°C in both cases while the starting

Table 1. RS determination (% of total starch) retrograded starch (R) and complexed starch (C) by different in-vitro methods compared to in-vivo RS measured by intubation[a]

	In vitro[b]			*In vivo*
Sample	Method *a*	Method *b*	Method *c*	Intubation technique
R	29·9	13·5	26·1	49·4
C	12·7	9·7	13·9	20·6

[a]Source: Molis *et al.* (1992).
[b]Method *a* derived from Berry (1986), *b* from Björck *et al.* (1986), *c* Englyst *et al.* (1992).

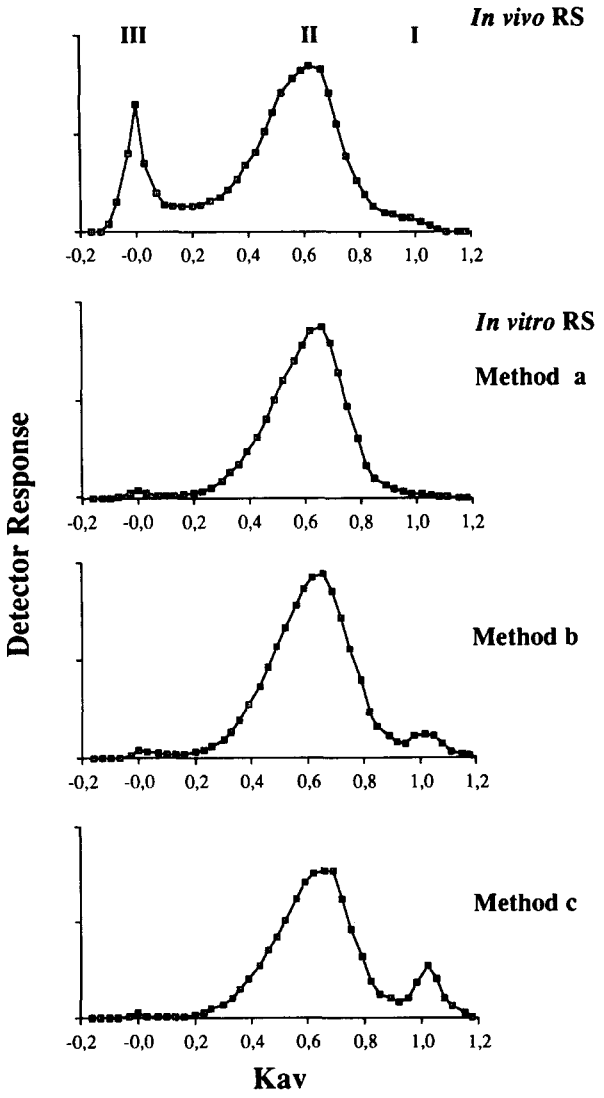

Fig. 1. Elution profiles on Superose 12TM of ileal content after ingestion of retrograded starch (in-vivo RS), and in-vitro residues obtained by methods *a*, *b* and *c* from retrograded starch R.

Table 2. Relative proportions (% of total starch) of constitutive α-glucan chains of in-vivo resistant starch from retrograded starch (R) and complexed starch (C). Comparison to the in-vitro RS fractions (RSa, RSb, RSc) from the three methods. I: oligosaccharides, II: middle molecular weight molecules, III: high molecular weight molecules

	I	II	III
In-vivo RS from R	8	79	13
In-vitro RS from R			
RSa	0	98	2
RSb	5	94	1
RSc	10	89	1
In-vivo RS from C	6	83	11
In-vitro RS from C			
RSa	2	97	1
RSb	6	92	2
RSc	18	81	1

products did not exhibit such an endotherm before ingestion.

Physicochemical characteristics of in-vitro RS

Figure 1 shows the elution profile on Superose 12TM of the RS fractions obtained from R by the different in-vitro methods. Results were very similar for sample C. In both cases, in-vitro residues from R and C showed one main population, characterized by one large peak centred at $K_{av} \approx 0{\cdot}65$. Average DP_n values of this population were 26, 40 and 23 for RSa, RSb and RSc from C, and 23, 22 and 22 for RSa, RSb and RSc from R respectively. The high molecular weight α-glucans (population III) disappeared almost totally in all six samples. However, some oligosaccharides could be observed in RSc and to a lesser extent in RSb from R and C. Table 2 shows the relative proportions of these three populations of α-glucans in in-vitro RS fractions estimated by the areas under the chromatograms.

RSa from R and C exhibited a sharp B-type diffraction pattern and an endothermic transition at 142°C for R, and at 93 and 147°C for C. X-ray diffraction of RSb fractions did not exhibit a clear pattern. However, an endothermic transition was observed at 142°C for R and 148°C for C. On the other hand, RSc did exhibit a sharp B-type pattern while no endotherm was observed at 145°C for the two samples. RSc from C exhibited an endotherm at 90°C.

The lintnerized starchy products showed a unique broad peak centred at $K_{av} \approx 0{\cdot}62$. DP_n values were 28 and 23 for lintner from C and R respectively. They exhibited a sharp B-type diffraction pattern. Lintner from R exhibited endothermic transitions at 118 and 148°C, and lintner from C a multiple stage transition at 43, 64, 113 and 149°C.

DISCUSSION

As in-vivo estimations were considered as references for RS content of experimental starches (49·4 and 20·6% for retrograded (R) and complexed (C) starch respectively), all the in-vitro methods seemed to underestimate RS values. Indeed, quantitative measurements of RS content of R and C were more than 35% higher with the in-vivo intubation technique than with all the in-vitro methods (Table 1). On the other hand, Champ *et al.* (1992) had used normal rats as a model for the estimation of ileal digestibility of both R and C starches. The figures appeared to be closer to in-vitro evaluation; 30·2% and 6·9% (on total starch basis) for R and C respectively. Such discrepancies between in-vitro and in-vivo determinations of RS raise the problem of the choice of the in-vivo technique which

has the highest significance for the human healthy subject. However, two in-vitro methods were validated on RS values obtained *in vivo* for several starchy products: the ileostomy model had been used to validate quantitatively method *c* (Englyst *et al.*, 1992), and Björck and collaborators (1986) used the rat model treated with antibiotics to validate method *b*. The present study proposed a qualitative comparison of the structural characteristics of RS obtained *in vivo* and *in vitro*.

Characterization of starch at the end of the ileum showed that oligosaccharides could reach the colon (population I). The high molecular weight α-glucans (population III) could not be totally crystalline because of their constitutive chain length over DP 100. This third population could then be described as semi-crystalline starch fragments with amorphous areas. Apart from this potentially digestible starch, in-vivo hydrolysis of starchy products led to isolation of a crystalline fraction constituted of retrograded amylose chains with $DP_n \approx 35$ (population II) and characterized by an endotherm at around 150°C and a B-type X-ray diffraction pattern (Faisant *et al.*, 1992; Leloup *et al.*, 1992).

Characterization of in-vitro resistant residues showed that in all cases, no high molecular weight molecules (population III) could be observed. Hydrolysis had been extensive and all 'potentially susceptible fraction' had disappeared. The presence of this potentially digestible starch *in vivo* is actually due to many factors related to the environment of starch during its progression in the upper intestinal tract: structures which could trap starch granules such as cell walls, time that enzymes would be in contact with their substrate, activity of enzymes, viscosity and other components of the meal.

However, the three methods could isolate a similar crystalline fraction than *in vivo* (population II), attributed to retrograded amylose crystallites. Average DP_n (about 23) were slightly lower than *in vivo* but were very close to those observed for lintnerized products. The fact that smaller molecules were obtained after in-vitro hydrolysis could be due to a more extended hydrolysis than *in vivo*: some potentially digestible fraction in population II *in vivo* could actually be hydrolysed *in vitro*, decreasing the average chain lengths. Thus, population II *in vivo* could be composed of retrograded amylose but also of a potentially digestible fraction, bound to the crystalline fraction.

Structural characterizations of RSb and RSc were somewhat difficult because of the high protein content and buffer mineral residues obtained after alcoholic precipitation. These proteins probably might lead to Maillard reactions within the caps during DSC analysis. Exothermic transitions around 135°C were observed, hiding any endothermic transition over this temperature. In-vitro residues exhibited the same characteristics as in-vivo residues and lintners: a B-type X-ray diffraction pattern and an endothermic transition around 150°C. These characteristics were consistent with the observations of Sievert and Pomeranz (1999) and Leloup *et al.* (1992) on RS residues obtained *in vitro* by enzymic hydrolysis.

The presence of oligosaccharides in in-vitro fractions was artificial since it was conditioned by the extent of washing of the residue. While RSa did not contain any oligosaccharides (washed twice with 80% ethanol and once with acetone), about 5% of oligosaccharides were observed in RSb (washed several times with alcohol). As RSc was washed only once with acetone, the highest proportion of oligosaccharides was observed. It is then difficult to discuss the proportion of this population I in in-vitro residues. Moreover, as products of starch hydrolysis, these oligosaccharides could be considered *in vivo* as 'digestible' starch, and their presence could also be related to external factors. Characterization of in-vivo ileal contents showed that oligosaccharides could represent more than 20% of the starch that reached the colon (Faisant *et al.*, 1992).

According to these results, none of the three in-vitro methods for RS determination (Berry, 1986; Björck *et al.*, 1986; Englyst *et al.*, 1992) gave satisfactory qualitative similarities with in-vivo RS fractions. The 'potentially digestible' fraction of starch observed *in vivo* was never taken into account by these tests. This gap could explain the quantitative underestimation of RS content of R and C by analytical methods compared to direct measurements on humans. Further work is in progress to characterize starch at the end of the human ileum after ingestion of several starchy foods. Such results should give a structural basis for any elaboration of an in-vitro test which will be able to determine RS in food, i.e. all the starch that escapes digestion in the small intestine.

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