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Resistant Starch: Its Chemical Form in Foodstuffs and Effect on Digestibility *in vitro*

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ABSTRACT

The chemical forms and resistance to hydrolysis in vitro *of raw and gelatinised starch from peas, maize, wheat and potatoes were measured. Raw granular starch proved very resistant to amylolysis. Only wheat starch was fully degraded after 24 hours' incubation with amylase (20 units/mg polysaccharide) at 37°C. In contrast, hydrolysis of freshly gelatinised starches was essentially complete within 1 h. To investigate the onset of resistance to hydrolysis after gelation, dispersions of amylose and amylopectin were stored at 20°C prior to amylolysis. Retrogradation of amylose was rapid, and the resulting material was highly resistant to amylolysis. In contrast, amylopectin underwent retrogradation more slowly and was almost completely degraded by amylase after incubation for 24 h. The onset of resistance to starch-hydrolysis in an amylose-rich food (cooked peas) was confirmed using a simulated digestion technique.*

INTRODUCTION

It has long been known that the physico-chemical form of starch affects both the rate and the extent of its hydrolysis by amylolytic enzymes, and that there are corresponding differences in the digestibility of starch in foods. A relatively slow rate of starch hydrolysis is a characteristic of foods which provoke a low glycaemic response in human subjects. Furthermore, starch

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which escapes digestion in the small intestine is likely to have physiological effects similar to some of the components of dietary fibre. There is a debate at present as to whether the resistant starch fraction should be included as dietary fibre, or deliberately excluded by the adoption of the appropriate analytical methods (Berry, 1986). This controversy cannot be resolved at present because the behaviour of the resistant starch fractions in the intestine is not known, and because the physiological effects of any starch which escapes digestion have not yet been fully investigated.

The physical form of the starch polysaccharides may undergo several transformations during processing and storage of a starch-based food material. Raw granular starch occurs as a semi-crystalline birefringent material which is usually processed by heating in the presence of water. If heated in an excess of water, at a characteristic temperature known as the gelatinization temperature, which for most starches is ~70°C, granular order is lost. The granules swell to many times their original size and the starch polysaccharide amylose is preferentially solubilized.

At temperatures below 100°C true molecular solution is not achieved, and swollen hydrated granules consisting of mainly amylopectin remain. If the water content of the suspension is reduced the dissolution temperature of the crystallites within the starch granule is raised. The melting temperature of the crystallites has been estimated at ~190°C (Biliaderis *et al.*, 1980). The extent of dissolution of the granular starch will therefore depend on the heat-moisture treatment received during processing. In foods a range of granular forms may be found immediately after processing, from granules which are still crystalline and birefringent to fully gelatinized granules.

Upon cooling, the dispersed starch polysaccharides reassociate or retrograde. Recent work has identified the roles played by amylose and amylopectin in starch retrogradation (Miles *et al.*, 1985*a*). Concentrated amylose solutions rapidly gel on cooling to room temperature; the gel arises as a result of a phase separation which produces a polymer-rich network (Miles *et al.*, 1985*b*). Subsequently some of the amylose slowly crystallizes as the double helical B-form. The gel can only be melted by heating to 160°C (Ring *et al.*, 1987*a*). Amylopectin within gelatinized granules can also recrystallize (Miles *et al.*, 1985*a*), in this case the association can be reversed by heating to ~70°C. The interactions involving amylose and amylopectin are time- and concentration-dependent (Miles *et al.*, 1985*a*,*b*). At low concentrations of solids, amylose gelation and crystallization is the dominant process. At high solids concentration amylopectin crystallization is much more significant (Orford *et al.*, 1987). Amylose chains can also form inclusion complexes with hydrophobic guest molecules.

The inclusion complex crystallizes as single helices in the V-form of amylose. The starch polysaccharides may therefore occur in quite diverse

physical forms. The objective of this study was to use *in vitro* techniques to determine how the physical form of starch polysaccharides affects their rate and extent of digestion.

MATERIALS AND METHODS

For the physico-chemical studies, analytical grade reagents were used throughout. A twice crystallized porcine pancreatic α-amylase and a bacterial pullulanase (*Enterobacter aerogenes*) were obtained from Sigma (Poole, Great Britain). For simulated digestion experiments, a porcine α-amylase (BDH, Poole, Great Britain) was dissolved in distilled water ($15\,mg\,ml^{-1}$), adjusted to pH 6·9 and stored at −20°C prior to use. A bacterial protease (Pronase, BDH, Poole, Great Britain) was also incorporated in these incubations.

Starches were prepared from potato (var. Desirée), smooth seeded pea (var. Filby), wheat and maize by an aqueous extraction procedure (Adkins & Greenwood, 1966). Waxy maize starch (Amioca) was a commercial sample obtained from Laing National. Amylose was prepared by aqueous leaching of pea starch granules at 70°C and purified as its 1-butanol complex as described elsewhere (Miles *et al.*, 1985*b*). Amylopectin was obtained from waxy maize starch after dissolution in dimethyl sulphoxide followed by precipitation with ethanol. The iodine binding capacity of the starches and starch polysaccharides was determined by a semi-micro differential potentiometric technique (Banks *et al.*, 1971).

Preparation of starch, amylose and amylopectin gels is described in detail elsewhere (Miles *et al.*, 1985*a*,*b*; Ring *et al.*, 1987*a*). Prior to enzyme hydrolysis, gel samples were disrupted in a tissue homogeniser consisting of a glass cup with a PTFE plunger, or an Ultraturax.

For the physico-chemical studies, hydrolysis was performed *in vitro* on raw starches and on gels, either freshly prepared or stored for up to 7 days. Incubations were carried out at 37°C in 0·05M phosphate buffer, pH 6·9, containing 0·04% NaCl using pancreatic amylase at concentrations of 2 and 25 units/mg polysaccharides. The extent of hydrolysis was assayed either by measuring the production of soluble carbohydrate using a phenol sulphuric acid colorimetric method (Dubois *et al.*, 1956) or by measuring the generation of reducing sugar using Nelson's colorimetric modification of the Somogyi method (Nelson, 1944).

For simulated digestion studies the procedure was similar to that previously described (Gee & Johnson, 1985). Dried mature peas (var. Filby) were pre-soaked in distilled water for 18 h at 5°C, ground in a pestle and mortar and homogenized in distilled water to give a final volume of 100 ml

containing 7 g of starch. Prior to digestion the homogenate was heated to 95°C, covered and maintained at that temperature for 10 min. In addition to α-amylase, the incubation procedure incorporated a non-specific protease ('pronase'; Sigma) to degrade proteins which could potentially impede the action of α-amylase. The amylose gel or whole-pea homogenate was cooled to 37°C for immediate use, or stored for 1 or 4 h at 20°C or 18 h at 5°C. Each sample was then diluted 1:1 with double-strength Krebs bicarbonate buffer and re-homogenized, giving a final starch concentration of 3·75 g/100 ml. To simulate the gastric phase, the pH of the homogenate was reduced to approximately 2·5 using 1 mM HCl, prior to incubation for 1 h at 37°C with occasional stirring.

This step was omitted, however, where the samples were to be studied immediately after preparation. After the gastric phase, the pH was re-adjusted to 7·2 using 2M NaOH, and sufficient α-amylase, and pronase were added to provide activities of 1·1 and 0·1 units per mg starch, respectively. The homogenate was then incubated for 2 h at 37°C with continuous stirring by means of an overhead rotary stirrer (180–200 rev/min). A single sample for determination of wet:dry weight ratio and similar duplicate samples for ethanol extraction were removed immediately prior to the addition of enzymes and at 2, 5, 10, 15, 20, 30, 60, 90 and 120 min afterwards. Samples for dry weight determination were dried at 85°C for 18 h. Hydrolysis of starch in weighed samples for extraction was arrested by addition of 20 ml ethanol:water (90:10 by vol). The samples were then extracted by boiling for 1 min and decanting the liquid through Whatman No. 1 filter paper. The procedure was repeated twice using 25 ml ethanol:water (20:20 v/v) and the extracts combined and made up to 100 ml prior to assay for free sugars by an automated version of Roe's anthrone method (Roe, 1955). The total starch content of the peas was determined as described previously (Gee & Johnson, 1985), and hydrolyses of all samples were expressed as a percentage of total starch present. Differential scanning calorimetry was performed as described by Miles *et al.* (1985*b*).

RESULTS

Physico-chemical characterization of starch samples

The gelatinization characteristics and amylose contents of the starches used in this study are shown in Table 1. Amylose is usually defined (Banks & Greenwood, 1975) as that starch polysaccharide which, under standard conditions, binds 19·5 w/w of iodine. Under the same conditions amylopectin binds less than 0·5% w/w iodine. The iodine binding capacities

TABLE 1
Characterization of Granular Starches, Amylose and Amylopectin

Starch	*Iodine binding capacities*	*% w/w amylose*	*Gelatinization temperature*	*% solubilized at 90°C*
Pea	5·8	29	62	21
Maize	4·6	23	71	13
Wheat	4·6	23	59	15
Potato	3·8	19	65	12
Amylose	19·5	100	—	—
Amylopectin	<0·4	<2	—	—

(IBC) of the amylose and amylopectin fractions used in this present study indicated that they were of high purity. The amylose contents of the starches ranged from 19% for potato up to 29% for smooth-seeded pea. The gelatinization temperatures of the starches ranged from 59 to 71°C while the amount of material solubilized by gelatinizing at 90°C ranged from 12% for potato starch to 21% for pea starch. The IBC's of the solubilized material indicated that it was >98% amylose.

The extent of association of amylopectin chains in crystallites can be conveniently measured by differential scanning calorimetry (DSC). Aqueous dispersions of starch and aged starch gels give an endothermic peak at ~70°C which can be attributed to the dissolution of amylopectin crystallites. Typical data for the starches and starch gels are shown in Table 2. The spherulitic amylose is very crystalline and gives a very sharp, intense X-ray diffraction pattern (Ring *et al.*, 1987*b*). The extent of chain association involving amylopectin in crystallites for the starches and 7 day-old starch gels is considerably less.

Enzyme studies

The extent of hydrolysis of raw granular starch by α-amylase after 24 h at 37°C is shown in Table 3. At enzyme levels of 2 units/mg polysaccharide, hydrolysis of the starch was very slow. Pea and potato starch were very resistant to hydrolysis while the cereal starches, wheat maize and waxy maize were slightly less so, with 16–25% of the material being converted to soluble carbohydrate. With higher levels of the enzyme (20 units/mg) the granular cereal starches were extensively hydrolysed. Pea starch was resistant to some extent, while potato starch was still very resistant to enzyme action. These results are in agreement with previous work on the hydrolysis of starches by α-amylase (Walker & Hope, 1963).

TABLE 2
Differential Scanning Calorimetry Data for Granular Starches and Starch Gels

Fraction	*Characteristics of endotherm*	
	Enthalpy change (mJ/mg polysaccharides)	*Transition temperature (°C)*
Pea starch	12·5	64
Maize starch	11·6	71
Wheat starch	9·7	60
Potato starch	16·1	65
Amylose gel	9·4	153
Amylopectin gel	15·0	54
Pea starch gel	6·0	61
Maize starch gel	1·7	59
Wheat starch gel	1·0	56
Potato starch gel	4·5	64
Spherulitic amylose	42·1	74

The α-amylolysis of the freshly gelatinized starches resulted in the production of clear solutions; no visible residue was present. At enzyme levels of 20 units/mg polysaccharide, hydrolysis, measured by the generation of reducing sugar, approached a plateau after only 5 min while for a smaller amount of enzyme (2 units/mg) the plateau value was approached after 40 min. High concentrations of α-amylase hydrolyse solutions of the starch polysaccharides in an essentially random manner producing glucose, maltose and a series of dextrins containing the α1–6 branch point (Marshall, 1974). If reducing sugar is measured and a conversion into maltose assumed then typical literature values for the extent of degradation of amylose are 110–120% and, for amylopectin, 85–95% (Marshall, 1974). In the present

TABLE 3
Extent of Hydrolysis of Raw Granular Starch by α-Amylase after 24 h at 37°C

Sample	*% Hydrolysis*	
	2 units/mg polysaccharide	*20 units/mg polysaccharide*
Pea starch	10	67
Maize starch	16	95
Wheat starch	25	100
Potato starch	<5	15

experiments conversion of the gelatinized starches was ~ 105%. Conversion of the gelatinized starches to low molecular weight oligosaccharides and glucose was therefore essentially quantitative.

To investigate the onset of enzyme resistance, gelatinized starch dispersions (30% w/w) were stored at 20°C prior to amylolysis. After 1 day the retrogradation of amylose was essentially complete (Miles *et al.*, 1985*a*) while the retrogradation of amylopectin was not detected. After 7 days the retrogradation of amylopectin was easily detected and quantifiable by DSC (Table 2). The extents of enzymolysis of 1 day-old and 7 day-old starch gels and an amylose and amylopectin gel are shown in Table 4. For the 1 day-old starch gels 22–33% w/w of the starch was resistant to hydrolysis, this figure rose to 30–42% for the 7-day-old gels.

TABLE 4
Extent of Hydrolysis of Starch Gels with α-Amylase

Sample		*% Hydrolysis*	
		2 units/mg polysaccharides	*20 units/mg polysaccharides*
Pea starch gel	1 day	67	91
	7 days	58	83
Potato starch gel	1 day	75	94
	7 days	68	92
Wheat starch gel	1 day	78	95
	7 days	70	95
Maize starch gel	1 day	71	
	7 days	65	90
Amylose gel		10	33
Amylopectin gel		91	100

The amylose gel was very resistant to hydrolysis while the amylopectin gel was almost completely degraded by the enzyme (>90%) with only a small amount of residue remaining after a 24 h incubation. The iodine binding behaviour of the residues was examined. The λ_{max} absorbance of the high molecular weight amylose iodine complex occurred at 642 nm. The α-amylase resistant residue representing 67% w/w of the original material had a maximum absorbance at 590 nm. The shift in λ_{max} indicated that the amylose had been substantially depolymerised. From the iodine binding characteristics of monodisperse synthetic amyloses (Banks & Greenwood, 1975) it is possible to estimate the average degree of polymerisation DP_w of the residue to be approx. 70. This is in agreement with previous observations

TABLE 5
Wavelength of Maximum Absorbance of Starch Iodine Complex

Residue	λ_{max}/I_2 *complex*
Amylose gel	590
Amylopectin gel	580
Wheat starch gel	570
Potato starch gel	585
Maize starch gel	560
Pea starch gel	590

on the α-amylase resistant residues from retrograded amylose (Jane & Robyt, 1984). The iodine complex of the starch gel residues shows a similar λ_{max} (Table 5). From these results it can be inferred that the starch gel residues are mainly amylose, although they must also include material derived from amylopectin, as the amylose content of the starch is insufficient to account for the amount of material in the residue. Thus, while retrograded amylopectin can be hydrolysed, the presence of retrograded amylose has an inhibiting effect on its conversion.

Studies on the extent of hydrolysis of purified starches were extended to a starch-rich food material using a simulated digestion procedure previously applied to a range of foods (Gee & Johnson, 1985). The hydrolysis of dissolved amylose, and amylose which had gelled after storage, is shown in Fig. 1.

The amylose in solution was hydrolysed very rapidly, reaching

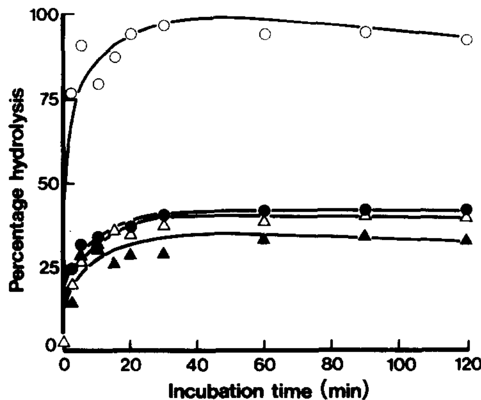

Fig. 1. Time-course for the hydrolysis of purified pea amylose immediately after gelatinisation (○) or after storage for 1 h (●) or 4 h (△) at 20°C, or for 18 h (▲) at 5°C. Each point is the mean of three separate determinations.

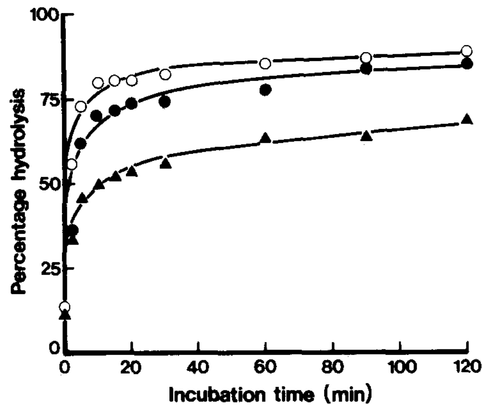

Fig. 2. Time course for the hydrolysis of starch in homogenised whole peas, immediately after cooking (○) or after storage for 1 h at 20°C (●) or for 18 h at 5°C (▲). Each point is the mean of three separate determinations.

approximately 75% of the eventual maximum of 95% within 2–3 min. This was true also of the gelled material, but the final extent of hydrolysis had fallen from close to 100% to approximately 40% in the material stored for 1 or 4 h at 20°C and there was a further reduction to 34% in the material stored for 18 h at 5°C. The starch in freshly cooked and homogenized whole peas was readily and almost completely hydrolysed by α-amylase. However, after storage at 5°C for 18 h, the extent of hydrolysis fell to 68% (Fig. 2). It is apparent that formation of resistant starch does occur during storage of cooked foodstuffs, and that the simulated digestion procedure has practical potential for the analysis of the physiological significance of resistant starch.

DISCUSSION

There is currently considerable interest in the nutritional significance of resistant forms of starch in foods. The passage of starch into the human large intestine presents nutritionists and regulatory bodies with an important dilemma because, although this material behaves as dietary fibre, it does not fit into any currently accepted definition, and the quantity present in foods will vary with cooking and storage. This situation will be difficult to resolve satisfactorily, and it raises a larger question concerning the usefulness of a single global figure for the many polysaccharides which comprise the dietary fibre complex. The accumulation of knowledge over the last decade has amply confirmed the physiological importance of these substances, but the diversity of their effects demands a much more sophisticated approach to their measurement and classification. There seems little doubt that resistant starch will need to be fitted into such a system, but many problems

concerning its chemical characteristics and physiological effect must first be resolved.

As with any emerging area of work, it is important to identify and evaluate differences in the technical procedures adopted by different laboratories. For example, in the present study the starch gels were homogenized prior to incubation with enzyme; they were not subjected to additional treatments which may modify the physical form and hence digestibility of the starch polysaccharides. In some studies starch gels have been taken through a drying step prior to analysis. It is well known that drying encourages chain associations which, because of the non-equilibrium nature of these systems, may be difficult to reverse. In addition, drying alters the crystallinity of starchy materials. The dried materials have also been submitted to vigorous milling to reduce particle size. This treatment is perhaps best avoided. The phenomenon of mechanical damage in starch granules, which destroys the native granular structure and renders the starch polysaccharides soluble in cold water, is well known and serves to illustrate how mechanical treatments can modify the physical form of the starch.

A crystalline pancreatic α-amylase was chosen for the *in vitro* hydrolysis of starch. A survey of the literature revealed that the levels of enzyme used in similar studies on resistant starch varied widely. In one study Björck *et al.* (1986) used 0·5 units/mg polysaccharide, whereas Berry used 50 units/mg. A method developed by Englyst *et al.* (1982) for dietary fibre analysis uses 5 units/mg polysaccharide. In the present study it was decided to perform experiments using 2 units/mg and 20 units/mg polysaccharide. The defined unit of enzyme activity is the amount of enzyme which will liberate 1 mg of maltose from soluble starch in 3 min at 20°C; therefore, even the lower levels of enzyme used represent a large excess.

One difficulty in assessing the nutritional significance of poorly digestible forms of starch is that the mechanisms and extent of resistance vary greatly between foods. Englyst has tackled this problem by proposing a classification system involving three forms of partially resistant starch and a single wholly resistant form (Englyst & MacFarlane, 1986). The present study confirms the poor digestibility of raw granular starch, which is classified as a partially resistant form in the Englyst system.

The reasons for this resistance are not known. The cereal starches give an A-type X-ray diffraction pattern, while potato starch gives a B-type pattern. The C-type pattern given by pea starch is thought to arise as a result of a mixture of A and B forms. It is tempting to speculate, therefore, that it is the B-type crystalline form which is resistant to hydrolysis. The granular starches are semi-crystalline; estimates for the percentage crystallinity in the starch granule range from 30–50%.

If it is assumed that the spherulitic amylose is $>90\%$ crystalline the DSC

results in the present study indicate levels of crystallinity in the granular starches ranging from 24 to 38% w/w. Therefore even the amorphous material within the potato starch granule is resistant to enzyme attack.

Another factor to be considered is particle size; potato starch granules are comparatively large with an average major axis of ~140 μm, pea, wheat and maize starch are smaller with an average major axis of 20–30 μm. Corresponding differences in surface area are ~20:1. If the major site of enzyme action is at the granule surface it is inevitable that the potato starch will be more slowly hydrolysed. The nature of the granule surface also needs to be considered; an adsorbed layer of non-starch material would effectively impede the action of the enzyme.

The present study confirms the very high resistance to enzymatic hydrolysis of retrograded amylose and the lesser resistance of amylopectin. These findings are in close accord with the classification of these materials suggested by Englyst & MacFarlane (1986). In most current literature on resistant starch the proposal is made that the crystalline nature of some forms of starch endows resistance. Matured gels of amylose and amylopectin give weak X-ray diffraction patterns of the B-type. The amylopectin gel has a higher level of crystallinity than the amylose gel yet it is the amylose gel which is the most resistant.

This apparent paradox warrants further study. It is important to determine whether α-amylolysis itself modifies the extent of interaction between the starch polysaccharides; for example, the enzyme may remove restrictions and hence encourage more extensive chain association.

The formation of resistant amylose in whole peas significantly reduced the susceptibility of the starch to amylolysis, though the rate of hydrolysis of the digestible fraction was not significantly slowed. Thus both the proportion of amylose present, and the treatment of a food after cooking may influence its availability for digestion in the small intestine. Goddard *et al.* (1984) showed that the glucose and insulin response to high amylose rice in human subjects was less than that to rice containing higher levels of amylopectin, and concluded that this might be due to slower hydrolysis of amylose to glucose. The present results suggest that the formation of resistant amylose after cooking such foods might reduce the proportion of starch available for uptake as glucose, and this should be borne in mind when designing or evaluating experiments concerned with the glycaemic index of foods (Jenkins *et al.*, 1982).

The present study confirms that a variety of poorly digestible starches may exist in foods, at levels which can be controlled by the food technologist. The unhydrolysed amylose and amylopectin residues passing into the large bowel behave in a similar way to the non-starch polysaccharides of unrefined plant foods.

An understanding of the physiological effects of these materials might therefore lead to the design of manufactured foods with improved nutritional characteristics.

Besides a reduction in the plasma-glucose response, the presence in foods of a significant quantity of resistant starch is likely to lead to a decline in total energy value and a modification of the intracolonic environment. Recent reports suggest that undegraded amylose residue is fermented by the colonic microflora of rats (Berry, 1986). The end-products of fermentation are volatile fatty acids which will be utilised by the mucosal cells or absorbed and metabolised at other systemic sites. However, the energy made available to the body will be less than would result from absorption as glucose. The influence of the various forms of undigested starch on colonic function in man, and their contribution to faecal bulk, whether as unfermented residue or bacterial cells, must be properly quantified before a correct classification of these materials in relation to dietary fibre can be achieved.

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