# Mechanisms of starch digestion by α-amylase–structural basis for kinetic properties

*Sushil Dhital[1], Frederick J. Warren[2], Peter J. Butterworth[3], Peter R. Ellis[3], Michael J. Gidley[1*]*

[1]ARC Centre of Excellence in Plant Cell Walls, Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Qld 4072, Australia.

[2]Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Qld 4072, Australia.

[3]King's College London, School of Medicine, Diabetes and Nutritional Sciences Division, Biopolymers Group, London, UK

* Corresponding author.

Phone: +61 7 3365 2145; Fax: +61 7 3365 1177. Email address: m.gidley@uq.edu.au (M. J. Gidley)

**Abstract**

Recent studies of the mechanisms determining the rate and extent of starch digestion by Ŭ-amylase are reviewed in the light of current widely-used classifications for (a) the proportions of rapidly-digestible (RDS), slowly-digestible (SDS), and resistant starch (RS) based on *in vitro* digestibility, and (b) the types of resistant starch (RS 1,2,3,4é ) based on physical and/or chemical form. Based on methodological advances and new mechanistic insights, it is proposed that both classification systems should be modified.

 

Kinetic analysis of digestion profiles provides a robust set of parameters that should replace the classification of starch as a combination of RDS, SDS, and RS from a single enzyme digestion experiment. This should involve determination of the minimum number of kinetic processes needed to describe the full digestion profile, together with the proportion of starch involved in each process, and the kinetic properties of each process.

The current classification of resistant starch types as RS1,2,3,4 should be replaced by one which recognises the essential kinetic nature of RS (enzyme digestion rate vs small intestinal passage rate), and that there are two fundamental origins for resistance based on (i) rate-determining access/binding of enzyme to substrate and (ii) rate-determining conversion of substrate to product once bound.

**Keywords**: Enzyme resistant starch, *in vitro* digestion, *in vivo* digestion, kinetic processes, digestion profiles

## 1. Introduction

Starch is one of the main sources of energy in the human diet. The release of glucose from starchy foods and its relevance to obesity, diabetes and other metabolic disorders has resulted in much interest in the dietary intake of starch necessary to maintain a state of good health of an individual. In humans, before being absorbed as glucose in the small intestine, starch is successively hydrolysed by salivary and pancreatic Ŭ-amylase in the mouth and small intestine, respectively, to smaller oligomers eventually leading to mainly maltose and maltotriose as end-products as well as Ŭ-limit dextrins, which contain branch points resistant to Ŭ-amylase. The Ŭ-amylase products are further hydrolysed to glucose by the combined action of two brush border exo-hydrolase double-headed enzymes, maltase-glucoamylase (MGAM) and sucrase-isomaltase (SIM) (Nichols et al. 2003). Recent work with recombinant enzymes has suggested that these mucosal enzymes not only have a synergistic effect on starch digestion by removing amylase hydrolysis products that may otherwise inhibit Ŭ-amylase activity if allowed to reach a sufficient concentration (Warren, Butterworth and Ellis 2012), but are also capable of hydrolysing starch by themselves (Ao et al. 2007a). A similar mechanism of starch digestion occurs in all monogastric animals, whereas in ruminants, starches are firstly hydrolysed by amylolytic enzymes of micro-organisms in the rumen and the remaining fraction is subjected to digestion in the small intestine by the hostôs amylolytic enzymes (Huntington, Harmon and Richards 2006). Similarly, the hydrolysis of starch granules to glucose is necessary for biological processes in plants, e.g. germination of grains, sprouting of tubers, and mobilisation of leaf starch during the night, as well as for technological processes, such as glucose syrup and bioethanol production

where starch is hydrolysed by different microbial amylases and amyloglucosidase to convert it to glucose and finally to ethanol by fermentation (Robertson et al. 2006, Smith et al. 2003).

As starch is the major source of glucose in the human diet, the postprandial rise in blood glucose concentrations and corresponding insulin response can be potentially manipulated by the selection of starches with varying degrees of susceptibility to amylolysis. Thus, from a nutritional perspective, starch can have diverse effects on postprandial metabolism, depending on its rate and extent of digestion in the gastrointestinal tract, and this has important implications for human health. Starch that escapes digestion in the small intestine is termed resistant starch (RS), and has been shown to have additional beneficial effects through fermentation in the large intestine to short chain fatty acids (Topping and Clifton 2001). Rapid digestion of starch can provide a large amount of glucose over a short period of time, resulting in substantial rises in blood glucose and exaggerated insulinaemic responses, which are a prime risk factor for type II diabetes, with insulin-resistance being an important marker of this risk. Slower digestion leads to a lower and more controlled rise in blood glucose, which avoids large excursions in insulin. Moreover, if digestion is sufficiently slow that the food passes to the large intestine before being digested, the residual RS becomes potentially of benefit to health through fermentation by colonic microflora, producing short chain fatty acids (SCFA). The major SCFA (acetate, propionate, and butyrate) are thought to generally contribute to optimal colonic function, but butyrate is of particular importance as an energy source for the colonocytes and is thought to be protective against colorectal cancer (Conlon et al. 2012). Thus the study of the rate and extent of hydrolysis of starch catalysed by amylases, often defined as hydrolysis kinetics, can in principle

help to identify food materials or to modify processing parameters for desired rates of release of glucose in the small intestine.

Hydrolysis kinetic studies in human subjects are challenging, given the complexity of the human digestive process with the involvement of several enzymes plus various hormonal controls operating on these enzymes. Furthermore, individual variation in response to the extent of starch digestion (Englyst et al. 1996a) and ethical issues in dealing with human subjects should not be underestimated. In fact, due to the inconvenience and high cost of using human subjects to study starch digestion, much work has been done to develop *in vitro* assays as biochemical mimics of *in vivo* conditions (Woolnough et al. 2008, Hur et al. 2011, Germaine et al. 2008). However, it should be noted at the outset that there are predictive limitations due to the fact that *in vitro* starch digestion methods rarely take into account several physiological parameters such as gastric residence time, small intestinal passage rate, or blood glucose clearance rates, which can all modulate a direct relationship between starch hydrolysis kinetics and blood glucose/insulin responses.

Gastric residence time and the ógrationingô of starch delivered from the stomach through the pylorus into the small intestine needs to be considered in predicting glucose uptake rates (Ranawana et al. 2011, Ranawana et al. 2010). Also, small intestinal passage rate can affect resistant starch levels as this determines the time available for pancreatic Ŭ-amylase to act in the small intestine before its starchy substrate is transported to the large intestine where microbial fermentation occurs. In relating glucose uptake to circulating plasma glucose and consequent insulin secretion, the rate of glucose clearance from the blood stream also needs to be considered. Recent evidence (Monro, Mishra and Venn 2010) suggests that individual variation

 

in this clearance rate may be an important contributor to the relationship between glucose uptake rate and insulin response.

As considerable advances have been made in understanding various health and nutritional aspects of starch with *invivo* and *invitro* studies, it is timely to review these advances with a view to a better understanding of the kinetics of starch digestion in relation to molecular and microstructural properties of starch systems. This review is focused on a critical evaluation of the current classifications of starch digestion rate and extent based on digestion kinetics, in order to understand the mechanisms controlling amylase hydrolysis of starch systems and to suggest mechanism-based classifications and potential predictive markers of metabolic responses.

## 2. Fundamental mechanisms controlling α-amylase action on starch

For any enzyme-catalysed reaction, there are a number of factors that may be rate-determining, reflecting the nature and concentration of the enzyme and substrate as well as environmental factors such as temperature and pH. As starch polymers are primarily Ŭ-1,4 ï glucans with only a small percentage of additional Ŭ-1,6 branches, almost all glycosidic linkages represent a potential substrate for pancreatic/salivary mammalian Ŭ-amylase, which is an endo-acting Ŭ-1,4ï glucan hydrolase. However, condensed forms of starch polymers as well as specific local conformations of Ŭ-glucan chains may prevent effective binding of amylase and/or catalysis. For example, native starches are semi-crystalline and molecules that are packed in tight crystalline structure can resist enzymic hydrolysis compared to loosely packed and more mobile molecules, or segments thereof. Commercial and domestic processing such as milling and cooking, depending upon the severity of processing parameters, can expose more available Ŭ-glucan chains or convert ordered structures of native semi-crystalline starch into more disordered conformations,

making them more vulnerable to amylolysis. In addition, in some cases, such as cooked and cooled starches, the leached starch polymers reorganise/recrystallise thus retarding the rate of enzyme catalysis (Bird et al. 2009, Lopez-Rubio et al. 2008)

The enzyme catalysed hydrolysis of solid starch (e.g. granules) is a heterogeneous reaction, occurring in the solid phase, involving the diffusion of enzyme to the solid surface, adsorption on the granule surface and finally initiation of degradation (Colonna, Leloup and Buleon 1992). The average starch granule of 25 µm size is almost 3000 times larger than the size of amylase, which has a hydrodynamic radius of 3-4 nm (Payan et al. 1980). Thus the granule is likely to provide many potential sites for adsorption of enzymes. Adsorption of enzyme to the granule surface, however, may be non-specific and will only result in a catalytic event when a run of contiguous glucose residues is accommodated in the enzyme active site (Quigley et al. 1998, Leloup, Colonna and Ring 1991, Gilles et al. 1996). The localised structure of the starch in a potential binding area is likely to affect the number of amylase subsites that can be filled with glucan residues and thus influence catalysis as a consequence. Human pancreatic Ŭ amylase has five binding subsites that span the active site with cleavage of the substrate occurring between subsites -1 and +1. Optimal substrate binding and cleavage occurs when a maltose residue serves as the leaving group (sites +1 and +2) and there are three sugars residues in the -1, -2, -3 sites (Brayer et al. 2000). Whether the enzyme acts in the liquid (solution) phase, as in the case of aqueous solutions of gelatinised starch, or the solid phase as in the case of granular starch, either *in vivo* or *in vitro*, the overall mechanisms involved in limiting amylase digestion rates of starches, can be broadly classified into two groups:

(a) Barriers that slow down or prevent access/binding of enzyme to starch; and

(b) Starch structural features that slow down or prevent amylase action (when access is not limiting).

This grouping of starch hydrolysis mechanism by substrate features can be equally relevant to other enzyme systems such as conversion of lignocellulosic bio-mass to bio-fuel, where the extensive covalent cross linking of lignin with other polysaccharides (primarily hemicelluloses) limits accessibility to carbohydrases (polysaccharide degrading enzymes) and prevents extraction of polysaccharides by neutral aqueous solvents (Harris and Stone 2008, Vidal et al. 2011). After de-lignifying, in an analogous manner to how starch structural features can slow down or prevent amylase action, the structural features of cellulose, such as crystallinity and fibre thickness, may affect the enzymic conversion of cellulose to glucose.

## 2.1 Barriers that slow down or prevent access/binding of enzyme to starch

### 2.1.1 Plant cell walls

Starches trapped in a plant or plant-based food matrix, irrespective of intrinsic enzyme resistance, are not readily accessible to endogenous enzymes of non-ruminant animals. For example, starches in the endosperm of whole grains are encapsulated in cells with the cell wall matrix providing a barrier for amylase to access and catalyse the hydrolysis of the starch as shown schematically in **Figure 1.** Intestinal enzymes in humans and non-ruminant animals cannot hydrolyse plant cell wall polysaccharides, potentially protecting the entrapped starch from amylase action in the small intestine. However, these wall components have little effect on the overall accessibility of starch for ruminants as they are degraded readily by rumenal microorganisms. Furthermore, the intact cell wall can prevent the complete swelling and

leaching of molecules from swollen starch granules thus impeding the action of pancreatic enzymes. During processing e.g. milling or cooking, the intactness of the cell wall is disrupted to varying degrees, making starches more readily available to amylases for hydrolysis. O'Dea et al. (1980, 1981) demonstrated greater postprandial blood glucose and insulin responses when ground rice was ingested instead of whole rice. Similarly, larger particle sizes of wheat, corn (maize) and oat grains have lower *in vitro* starch digestibly and lower glycaemic and insulinaemic responses when tested *in vivo* compared with milled fractions (Heaton et al. 1988), whereas no difference in blood glucose or insulin concentrations was observed in test subjects fed regular white and whole-wheat flour breads or pasta (Jenkins et al. 1981b, Jenkins et al. 1983). These findings suggest that the presence of an intact cell wall is an effective mechanism for slowing down starch digestibility by pancreatic Ŭ-amylase. For solid foods, individual differences in mastication habits can lead to differences in particle sizes produced by chewing, with consequent effects on inter-individual glycaemic responses (Ranawana et al. 2010). More generally however, the use of particle size disruption as part of *in vitro* digestion protocols, while a useful experimental tool, rarely reflects true mastication patterns (Hoebler et al. 1998).

### 2.1.2 Protein matrices

Starch granules in the endosperm of some cereals and legumes have protein bodies adjacent to, and sometimes indented into, starch surfaces as well as providing a more continuous protein matrix (Berg et al. 2012, Oria, Hamaker and Shull 1995) that may act as a further barrier for amylolysis of starch granules. In some grains such as sorghum, these protein matrices and bodies may form a contiguous layer around individual starch granules. The markedly slow amylase digestibility of sorghum compared to maize (Ezeogu, Duodu and Taylor 2005), in spite of having

similar starch granule structures and protein composition, has been mainly attributed to the disulphide cross linking of storage proteins in sorghum endosperm (Zhang and Hamaker 1998, Chandrashekar and Kirleis 1988), which on cooking forms indigestible aggregates providing barrier properties equivalent to the hull in wheat grain (Belton et al. 2006, Dona et al. 2010). Removal of proteins by protease or addition of reducing agents such as sodium sulphite or bisulphite, that prevent the formation of disulphide crosslinks, increased the rate and extent of amylase digestion of sorghum (Zhang and Hamaker 1998, Rooney and Pflugfelder 1986, Choi et al. 2008).

For milled grains, Al-Rabadi et al. (2009) (**Figure 2a and b**) and Mahasukhonthachat et al. (2010a) found an approximate doubling of amylase digestibility of ground barley and sorghum grains on decreasing the particle size from about 500 µm to about 250 µm. This increased amylase activity was inferred to be due to the destruction of both cell wall and protein networks during milling. Individual endosperm cells are typically 50 to 150 µm in diameter, so that starch granules in particles of ~500 µm will be predominantly located within intact cell walls, whereas particles of ~250 µm would be expected to have many broken cells with exposed intra-cellular starches for adsorption and catalytic action by amylases. In addition to specific effects of cell size on starch accessibility, smaller particles generally have more available surface area. Assuming that (a) the amount of enzyme is not a limiting factor, (b) adsorption of enzymes is a random phenomenon, and (c) the plant tissue particles are spherical; 500 µm diameter sorghum grain fragments (starch-containing endosperm cells) have an apparent specific surface area of 80 $cm^2$ per gram, which is 5 times lower than for 100 µm particles. Thus there is a 5-fold increase in the surface area available for enzyme adsorption. This increased probability of enzyme

adsorption, however, may or may not be associated with the attenuation of hydrolysis rate and extent, depending upon several factors such as exposure of starch granules at the outer surface, the degree of damage to starch granules, as well as the presence of intact cell wall or cell wall fragments and protein bodies or matrices.

### 2.1.3 Granular architecture of native starches

In several studies with size-fractionated native starches (Franco and Ciacco 1992, Franco, Ciacco and Tavares 1998, Kasemwong et al. 2008, Noda et al. 2005, Vasanthan and Bhatty 1996, Naguleswaran et al. 2012), larger granules have been reported to have lower amylolysis rates compared with smaller counterparts. Although compositional differences between the larger and smaller granules may partly affect the amylolysis (Dhital et al. 2011, Naguleswaran et al. 2012), the lower number of available Ŭ-glucan chains for initial adsorption/binding has been suggested to be the primary factor controlling the starch hydrolysis (Warren et al. 2011). In another study, Dhital et al. (2010b) reported the almost three times faster hydrolysis of small potato granules compared with the rate for larger counterparts **(Figure 3a),** but did not observe much difference between larger and smaller maize starch granules **(Figure 3b).** This was explained in terms of available surface area which in the case of maize starch, is much higher than the boundary surface area (calculation based on the diameter of granules) due to the presence of surface pores and channels, a common feature of A-polymorphic cereal starches (Fannon, Hauber and BeMiller 1992, 1993). Channels in A-polymorphic starches are reported to be lined with proteins and lipids (Benmoussa et al. 2010, Han et al. 2005, Naguleswaran et al. 2011) and have apparent diameters ranging from 0.007 to 0.1 µm, whereas pores (opening of channels) are larger with diameters varying from 0.1 to 0.3 µm, large enough for enzymes such as Ŭ-amylase (radius of ca

3-4 nm (Planchot and Colonna 1995, Payan et al. 1980)) to diffuse inside the granules. The easy access of enzymes to the less organised hilum area inside the granules leads to a specific digestion pattern described as óinside outô (**Figure 4a, b**). However for granules lacking pores and channels e.g., potato starch, the enzymes initiate the digestion from the surface towards the granule interior by óexo pittingô in an óoutside inô pattern (**Figure 4c, d**) (Gallant et al. 1992) as the surface of granules is impermeable to amylase owing to higher local concentration and tight packing of amylose (AM) and amylopectin (AP) (Oates 1997, Jane 2007).

Apart from cereal protein matrices and bodies such as in sorghum, granular starches contain varying amounts of non-starchy substances such as surface proteins (Baldwin 2001) and lipids (Baldwin, Davies and Melia 1997), that can reduce the surface accessibility by blocking the adsorption sites and therefore influence the enzyme binding (Oates 1997). In contrast, the presence of less ordered Ŭ-glucan (Warren et al. 2011, Tahir, Ellis and Butterworth 2010) and ólooseô material such as damaged surfaces facilitates binding of enzymes at the granule surface (Oates 1997).

### 2.1.4 Food composition and architecture

The microstructure of starch-containing foods and resultant properties such as viscosity may have major effects on amylase activity towards starches. In cereal-based products, arabinoxylans and (1-3,1-4)-ß-D-glucans have been given much attention as they are the primary water-soluble non-starch polysaccharides (soluble fibre), responsible for increasing the viscosity of the food bolus (Juntunen et al. 2002) and are considered as forms of dietary fibre fermented by bacteria in the human colon (Crittenden 2006). Similarly, pectins and other water-soluble cell wall polysaccharides such as galactomannans and xyloglucans found in starchy pulses and vegetables,

can also contribute to higher viscosity of food boli and digesta (Dikeman and Fahey 2006, Dikeman, Murphy and Fahey 2006, Judd and Ellis 2006). An increase in viscosity of digesta caused by the ingestion of water-soluble forms of dietary fibre is expected to slow down many of the processes associated with digestion of starch and other nutrients. This includes gastric function (sieving, emptying), small intestinal transit, mixing of enzymes with substrates, and transport from lumen to mucosal surface (Montagne, Pluske and Hampson 2003, Tharakan et al. 2010, Leeds 1979, Taylor 1979) all of which are mainly linked to the rheological behaviour of the digesta (Ellis et al. 2001).

Several studies have shown that the addition of soluble fibres in ingested food significantly reduces the postprandial rise in blood glucose and insulin concentrations in both human subjects (Jenkins et al. 1978, Ellis et al. 1981, Jarjis et al. 1984, Goni, Valdivieso and Garcia-Alonso 2000) and experimental animals (Ellis et al. 1995, Sambrook and Rainbird 1985). Similar to *in vivo* models, the incorporation of soluble fibre in the food or digestion medium has been shown to reduce the rate and extent of starch hydrolysis by amylases (Lee Wah et al. 2009, Chillo, Ranawana and Henry 2010, Aravind, Sissons and Fellows 2012, Regand et al. 2012, Dhital et al. 2014).

Also important, particularly in complex food matrices such as wheat bread, is that the soluble fibre can potentially form an enzyme-resistant ñbarrierò around the starch granules that inhibit the rate of digestion of starch by amylases (Ellis, Dawoud and Morris 1991, Brennan et al. 1996). This suggests that, in addition to rheological effects, the soluble fibres may also act as a physical barrier to enzymeï substrate interactions and the release of nutrients from the food matrix. Furthermore, soluble fibres such as galactomannans can have a direct noncompetitive inhibitory

effect on Ŭ-amylase (Slaughter et al. 2002). Noncompetitive inhibition could result from adsorption of Ŭ-amylase to galactomannan, with the resulting inactive galactomannanï amylase complex inhibiting the action of pancreatic amylase.

Accumulation of starch hydrolysis products in a food matrix during digestion, caused by hindered release from the matrix in the presence of soluble fibre or intact cell walls (see above), could also have an inhibitory effect on Ŭ-amylase activity (Colonna et al. 1992). However, it is known that maltose, the main product of amylolysis, is a relatively poor inhibitor because its binding to amylase is weak particularly in the presence of starch (Zhang et al. 2014 , Elödi, Móra and Krysteva 1972, Warren et al. 2012). Furthermore, soluble fibres may also limit water availability, restricting the swelling and gelatinisation of starch granules (Kaur et al. 2008, Slaughter et al. 2002), during food processing and thus decrease the susceptibility of starch to amylolysis (Slaughter et al. 2002).

The viscosity induced by the presence of soluble fibres is dependent on a number of factors including the rate and extent of hydration of the fibre, the molecular weight and concentration (dose) of the polymer. There is, however, very little information available on the effects of soluble fibre on the rheological behaviour of digesta in the human small intestine, partly because of the considerable problems of measuring viscosity directly *in vivo* and the difficulty of interpreting such complex rheological data. For a discussion of these issues, the reader should refer to more detailed reviews (Wang and Ellis 2013, Ellis et al. 2001).

## 2.2 Starch structural features that slow down or prevent amylase action

### 2.2.1 Granule organisation

The probability of direct contact between amylase and starch granules is hindered by various barriers. While the adsorption of enzymes onto granule surfaces is a prerequisite for catalytic action, adsorption per se is not the only determinant of catalytic rate and action. Depending upon the substrate conformation, either binding (e.g. granular starches) or catalysis (e.g. gelatinised/solubilised starches) can be a rate limiting step for enzyme catalysed hydrolysis of starches. The localised structure of starch granules such as amylose/amylopectin (AM/AP) ratio, amount of helical structure giving rise to crystallites, packing of crystallites, and interaction of starch molecules with non-starch components, individually or in combination can affect both binding and catalysis of starch granules. Catalysis involves formation of a covalent intermediate, which is dependent on substrate binding and may be subsequently attacked by water. Therefore water availability is another important parameter for successful catalytic breakdown of starch granules.

In non-waxy starch granules, AM and AP are packed in alternating layers of semi-crystalline and amorphous regions synthesized by apposition, where starch chains elongate towards the periphery of granules, i.e. radially (Yamaguchi, Kainuma and French 1979, Yoshida et al. 1958, French 1984). The low-density amorphous rings consist of AM and AP in less ordered conformations, whereas the dense semicrystalline rings are formed by a lamellar structure of alternating crystalline and amorphous regions with a repeat distance of 9 to 11 nm as measured by small angle scattering (Blazek and Gilbert 2010). The crystalline regions of the lamellae are mainly formed by double helices of AP side chains packed laterally into a crystalline lattice, whereas amorphous regions contain AM and clusters of AP branch points **(Figure 5).** The distribution of AM in starch granules still remains unresolved. However, it is now well accepted

 

that AM in most native starch granules is randomly distributed among the radial AP chains and is largely free of strong interactions with AP as judged by the ability of many swollen granules to leach amylose whilst retaining AP-based crystallinity. Double helices formed from AP branches are stabilised by hydrogen bonds and van der Waalsô forces to give either A- or B- type crystal structures with characteristic diffraction patterns in most cereal and tuber starches, respectively. The amorphous regions containing non-ordered structures contribute a featureless background in X-ray diffraction patterns (Jenkins and Donald 1995, Oostergetel and Vanbruggen 1989, Jenkins, Cameron and Donald 1993, Zobel 1988, French 1984).

At the molecular level, the crystalline structure and the packing of amorphous phases is considered to be an important factor in defining the rate and extent of enzymic hydrolysis of starches (Colonna et al. 1992). Processing such as hydrothermal treatment of starch granules involves the swelling of amorphous regions of granules with disruption and subsequent hydration-driven swelling of ordered structures making them more readily available for amylase-catalysed hydrolysis (Tahir et al. 2011). Although the Ŭ-glucan chains are largely complexed or crystallized into specific configurations, given time, amylase can digest even strongly entangled macromolecular chains (Oates 1997). A-type starches such as those from cereals (except for high amylose variants) are more readily hydrolysed than the B-type starches from high amylose cereals and potatoes. This difference in digestibility has been described as a function of crystallinity type, which is further dependent upon the branch chain length of AP (Sujka and Jamroz 2009, Planchot, Colonna and Buleon 1997, Jane et al. 2003, Gallant et al. 1992). In general, B-type starches consist of AP with relatively few short branch chains (A, B1 as defined by Hizukuri (1986)), but a greater number of long branch chains (B2, B3 and longer), which

 

extend through multiple crystals and stabilise the internal structure of granules. This makes B-type starches more resistant towards enzymic digestion compared with A-type polymorphic starches, which have more short branch chains that may destabilise the lamellar structure (McPherson and Jane 1999, Jane et al. 1999). In studies of model systems, Williamson et al. (1992) and Planchot et al (1997) made A- and B-type polymorphs from amylose spherocrystals (DP 15-20) and subjected these to enzymic digestion. The B-type polymorphs were found to be more resistant to Ŭ- and ɓ-amylase and amyloglucosidase, which concurs with the results discussed above for native granules. In contrast, Cai and co-workers reported greater enzyme resistant properties of A-type crystals made from debranched waxy starches compared with B-type crystals prepared from the same starch source by varying the method of preparation (Cai and Shi 2013, Cai and Shi 2010, Cai et al. 2010). Furthermore, with respect to helical structure, the double helices within two polymorphic forms are essentially identical (Gidley 1987, Imberty et al. 1991), but the packing of these double helices in A- type monoclinic unit cells is relatively more compact with a lower water content compared with B-type crystals packed in a hexagonal unit cell (Gallant et al. 1992, Tester, Karkalas and Qi 2004). Thus, it may be expected that A-type starches would be more resistant to enzymic hydrolysis. However, irrespective of the crystalline type, the distribution of crystallites within granules and their influence on the local granule organisation would be expected to impact on the overall behaviour of enzymes in starch hydrolysis (Gerard et al. 2001), and enzyme hydrolysis studies conducted on amylose or debranched amylopectin crystals may not be representative of the lamellar structure within native starch granules.

At the next level of starch structure, supramolecular organisation has been reported to favour digestion of less organised amorphous region compared with more ordered semi-crystalline regions based on microscopic (Gallant et al. 1992, Blazek and Gilbert 2010) and molecular structure analysis (Blazek and Gilbert 2010). These reports proposed that the presence of pores and channels in A-polymorphic starch allows enzymes to diffuse inside granules and preferentially hydrolyse amorphous regions prior to ordered crystallites. In contrast, the surface of B-type starches does not possess any pores that would enable the enzyme to access the more easily digestible core and thus digestion occurs from outer resistant surface as an ǿoutside-inô pattern. As a result, preferential hydrolysis of amorphous material is not as substantial in B-type starches compared to A-type starches **(Figure 4).** Zhang et al. (2006) and Shrestha et al. (2012), on the other hand, proposed that for both crystalline types, crystalline and amorphous regions are evenly digested. Although the amorphous regions may be more susceptible to hydrolysis, the glucan chains not involved in crystalline zones are not free and mobile, but are densely packed and arranged tightly adjacent to the crystalline regions, which inhibits hydrolysis (Zhang et al. 2006, Planchot et al. 1997). The entrapment of amorphous regions within imperfect crystals and the presence of double helical structures not necessarily part of crystallites have also been suggested as mechanisms of enzymic resistance of non-crystalline fractions (Shrestha et al. 2010). Similarly, some authors inferred that organisation of amylopectin lamellae into spherical blocklets in the surface regions of B-type starches, resulting in comparatively larger structures (200 to 500 nm) than in A-type starches (20-120 nm) (Gallant et al. 1992, Gallant, Bouchet and Baldwin 1997, Tang, Mitsunaga and Kawamura 2006), can hinder the accessibility of enzymes to the inside of granules. Additionally, for other starches such as high amylose maize starches

 

(HAMS), the presence of a higher concentration of AM may serve as a reinforcing and stabilising structural feature in the amorphous growth rings hindering amylase attack in comparison to amylopectin-rich starch granules such as normal maize starches (MS). In contrast, potato starch (PS), has an amylose content similar to MS, but a slow digestion rate and pattern similar to that of HAMS. It is more likely that granule architectural features with a size of at least 100 nm (such as pores and channels, damage to granule surface) are primary determinants of digestion rate and pattern among starches, with shorter scale features such as size of AM and AP, chain lengths of AP, amount and types of crystallinity having minor or secondary roles (Shrestha et al. 2012, Dhital, Shrestha and Gidley 2010a).

Several non-starch components associated with starch granules may also affect the enzymic hydrolysis of raw or gelatinised starch granules. As discussed previously, surface proteins and lipids may reduce the accessibility and binding of enzyme on the granule surface. Furthermore, cereal starches contain integral (internal) lipids, essentially monoacyl lipids (free fatty acids and lysophospholipids) (Morrison 1981) either independently or as amylose-lipid complexes (Morrison 1988). The independent lipids can also complex with amylose during heat processing of starch (Bjorck et al. 1984) restricting the swelling and solubility of granules. Amylose-lipid complexes, either native or induced by processing, are much less readily accessible to the active site of Ŭ-amylase compared with single helical amylose chains (Holm et al. 1983).

The enzyme susceptibility of amylose-lipid complexes is dependent on the structure of complexing lipids. More specifically, short-chain and some medium-chain fatty acids (<10 carbon units) are water soluble and are less effective complexation agents compared with water-insoluble long-chain fatty acids (>12 carbon units and above). Similarly, the long chain and

 

saturated fatty acids form more stable and enzyme resistant complexes compared with short chain and unsaturated fatty acids. Moreover, crystalline amylose-lipid complexes (so-called form II with a higher melting temperature) are more enzyme resistant than their amorphous counterparts (so-called form I with a lower melting temperature) (Tufvesson, Wahlgren and Eliasson 2003a, Tufvesson, Wahlgren and Eliasson 2003b, Yotsawimonwat et al. 2008, Kitahara, Sugnuma and Nagahama 1996, Gelders et al. 2005).

### 2.2.2 High density processed starches

Treatment of starch granules with various combinations of temperature, moisture, and time can affect the enzymic hydrolysis rate of starches (Thompson 2000, Roder et al. 2009). Hydrothermal treatment, such as annealing (performed at moisture levels greater or equal to 40%) and heat moisture treatment (performed at moisture levels below 35%) that includes heating of starch granules below the relevant gelatinisation temperature but above the effective glass transition temperature, can modify starch structure while retaining the granule form. Changes to structure (crystallite disruption, change in polymorphic form, crystallite reorientation, AMï lipid interaction and starch chain interaction) and morphology (formation of fissures and cracks on the granule surface) while retaining the granular structure (Varatharajan et al. 2011, Jacobs and Delcour 1998) can affect the enzymic digestibility of starches. Enzymic susceptibility of hydrothermally-treated starches varies with processing conditions (Zavareze and Dias 2011, Varatharajan et al. 2011, Kweon et al. 2000, Dias et al. 2010, Zavareze et al. 2010, Brumovsky and Thompson 2001), possibly reflecting the balance between re-crystallisation/densification (expected to retard amylolysis) and partial melting/swelling

 

(expected to enhance amylase action) achieved for different starch types under different processing conditions.

The hydrothermal treatment of starches in excess water beyond the gelatinisation temperature causes a more complete destruction of ordered structure. Dispersions of starch into paste or solution forms, plus swollen granular remnants, can gradually develop double helical structures and lose water holding capacity in a series of processes collectively termed as retrogradation. The tendency of Ŭ-glucan chains to retrograde depends on several factors including storage temperature, pH, and concentration, presence of surface active agents, and the molecular weight and type of starch (Jacobson and BeMiller 1997). AM molecules having a more linear structure are more prone to develop double helical crystallites compared with branched AP (Ring et al. 1987, Jane and Robyt 1984) that interferes with AM retrogradation (Berry 1986). The enzyme resistant property of retrograded starch has been described as the result of formation of stable B-type AM crystallites. However, as discussed previously, crystalline type or amount of crystallinity per se is not the sole determinant of enzyme resistant properties, rather it can be a route to achieve the dense packing of starch chains that hinders enzymic accessibility or catalytic action.

Non-crystalline dense packing of starch chains can also be achieved, for example by cooling starch with limited (plasticising) water from a high temperature ómoltenô state such as by extrusion cooking, which can result in a similar level of enzyme resistant properties as, for example, native HAMS granules (Lopez-Rubio et al. 2008, Htoon et al. 2009). In this context, retention of the natural packing of glucan chains within the native granular structure is, in principle, an effective and economic way (less processing required) of decreasing the enzyme

 

susceptibility of starches for improved health properties. As an example, considering retrograded starch as a potential source of ERS, retrograded HAMS produced from repeated autoclaving and cooling operations yields almost 30% ERS (Berry 1986), which is still lower than that obtained from native HAMS granules (35-70%) (Htoon et al. 2009, Bird et al. 2009, Evans and Thompson 2004, Berry 1986). However, the retention of natural granule packing (e.g., by avoiding gelatinisation/melting) depends upon the botanical source of the starch and processing conditions. HAMS incorporated into bakery products, is likely to retain its native structure (assessed by X-ray diffraction pattern) during baking, compared, for example, to normal wheat starch (Hoebler et al. 1999). The degree of swelling and gelatinisation in a baked cereal product is known to vary according to the amount of water in different parts of the food material during heat processing. Crust regions of wheat-based bread (Moss 1975) and the surface of short dough biscuits (Chevallier et al. 2000), which have much lower moisture contents, tend to contain birefringent starch granules that largely retain therefore their native characteristics. In addition, wheat starch granules were found to be in an almost native-like condition in sugar cookies but more than half of the starch in high ratio (water /flour ratio of 0.8) cake and bread was gelatinised (Lineback and Wongsrikasem 1980, Wootton and Chaidhry 1980). Apart from the moisture content, the restriction of swelling and gelatinisation of starch during baking can be affected by other constituents such as sugar, protein and lipids in the dough and also by manufacturing conditions (such as mixing, temperature and time).

The modification of a starch microenvironment during sheeting of pasta dough creates a protein network entrapping the starch granules. The protein network provides a physical barrier that alters or delays the access of starch to amylase (Fardet et al. 1998). Furthermore, it restricts the

diffusion of water into the granules limiting the swelling and gelatinisation of starch in cooked pasta (Colonna et al. 1990, Hermansen et al. 1986). However, increasing the sheeting cycles of pasta dough can ópull awayô the protein network, enhancing starch digestibility in cooked pasta (Kim et al. 2008a). Similarly, disintegration of the pasta into a coarse óporridgeô significantly increased the blood glucose response compared with intact pasta (Granfeldt and Björck 1991). This shows that macrostructural integrity is an important determinant of the glycaemic response of food material. However, even after disintegration, firm pasta particles remained, and the pasta porridge produced a lower glucose response than did bread made from the same raw material (Granfeldt and Björck 1991) signifying that the densely packed pasta particles limits enzymic hydrolysis by restricting access to the Ŭ-glucan chains of starch.

Apart from processing, enzyme-resistant structures can also be formed during the digestion process. Although it has not been demonstrated *in vivo*, re-crystallisation or re-organisation (retrogradation) of starch degraded polymers during the digestion process *in vitro* has been reported for extrusion-processed HAMS (Lopez-Rubio et al. 2008) thereby affecting the subsequent digestion rate. Consequently, the amylolysis of a specific starch variety in a longer time frame could depend on the competition between the kinetics of amylase digestion and the kinetics of molecular re-organisation (as these influence local molecular density) (Bird et al. 2009). Therefore the measurement of structural features of granular or processed starches may not necessarily predict the overall enzyme digestion rates, which can be related to the competition between dis-organisation and re-organisation of the polymers during the digestion process.

### 2.2.3 Clusters of branch points

The limit dextrins produced by Ŭ-amylase hydrolysis of starches include highly branched oligosaccharides, characteristic of the clustering of branch points within the AP structure (Zhu and Bertoft 1996). These branch points are sufficiently close together to prevent endo-hydrolysis between them, and so are resistant to further Ŭ-amylase action. Increasing the branching density of starches by, for example, ɓ-amylase treatment (which removes maltose units from the non-reducing end of AP chains but cannot bypass branch points) can therefore increase resistance to Ŭ-amylase action (Ao et al. 2007b). The importance of this mechanism was illustrated by the finding that digestion was most rapid for maize starches with an intermediate branching density. For starches with either longer or shorter branches, digestion was slowed, showing two mechanisms of digestion control (Zhang, Sofyan and Hamaker 2008) based on the potential of long branches to form crystallites and for short branches to be associated with increased clustering of branch points.

### 2.2.4 Chemical modification

Enzymic digestibility of starches can also be altered by chemical substitution (introduction of bulky side group to the Ŭ-glucan chains) and cross-linking (covalently linking two Ŭ-glucan chains together) of starches and has been recently reviewed (Maningat and Seib 2013). The substituent groups along the α-1,4 D-glucan chains hinder enzyme attack and also make neighbouring glycosidic bonds resistant to degradation. The presence of cross-linked polymer chains inhibits granular swelling and also provides steric hindrance to the approach of the active site of amylase enzymes. Moreover, cross-linking of starch may restrict the movement of α-amylase through the granule surface pores and channels. Unusual glycosidic bonds, such as α-1,2, α-1,3 or β-1,6 bonds, which form during pyrolysis reactions on starch, are not substrates for

amylases. It should be noted however, that the enzymic susceptibility of chemically-modified starches depends upon a variety of factors such as botanical origin of starches, types and amounts of modifying agents used, subsequent chemical bonds and derivatives formed, and the extent of granule gelatinisation.

## 3 Current classifications of starch digestion

There are two classification systems commonly used to describe starch digestion properties. Firstly, for nutritional purposes, Englyst et al. (1992) introduced a classification scheme that is based on a combination of rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). RDS and SDS were arbitrarily defined as the fraction of total starch hydrolysed after incubation with an excess of pancreatic amylase and amyloglucosidase at 37$^{o}$C for 20 minutes and a further 100 minutes, respectively. The non-hydrolysed residue remaining after 120 min of incubation was regarded as RS. For a starchy food product containing simple sugars, the content of each fraction was designated as rapidly available glucose (RAG), and slowly available glucose (SAG) rather than RDS and SDS (Englyst, Veenstra and Hudson 1996b). These classifications were used to reflect the rate at which glucose from starch becomes available for absorption in the human small intestine, based on *in vivo* data available at the time (Englyst et al. 1999). A number of other methods have, however been proposed for analytical quantification of RS (Champ et al. 2003, McCleary 2013, Champ et al. 1999) in food material. Each method varies in terms of the amount and type of enzymes, incubation time and temperature specified and thus generate different results for the amount of RS from the same foods. Some of the *in vitro* methods have been validated using *in vivo* data, which in most cases was obtained from ileostomates. Various other validation studies involved data from intubation

of healthy volunteers or from rats and pigs. In general, the analytical parameters of *in vitro* methods are optimised using the RS values from limited *in vivo* data. Parameters such as location of starch in food matrices, chewing and gastrointestinal transit time, and quantitative enzyme secretion are likely to vary from one subject to another. Thus the analytical techniques based on limited *in vivo* data may not represent the amount of starch reaching the terminal ileum in healthy human subjects having different digestive physiological environments. RS is a physiological term that describes the amount of starch that enters the large intestine. It is not just a property of starch but also a property of the food and the individual consuming it. Thus RS in one subject can be a slowly digestible starch in another subject, and *vice versa*. As the *in vitro* enzyme methods cannot completely simulate the complex physiological processes in the gastrointestinal tract of humans or test animals, this can consequently lead to a disparity between the molecular properties of RS produced *in vitro* (enzymic digestibility) and *in vivo* (physiological measurements) (Faisant et al. 1993, Hasjim et al. 2010a).

In a second scheme, RS has been sub-divided into (initially) four types according to the nature of the enzyme resistance and the structure of the starch (Englyst et al. 1992, Eerlingen and Delcour 1995). RS type 1 (RS1) is physically inaccessible starch, which is protected by a protein matrix or cell wall material, such as is found in whole grains, legumes, and pasta. RS type 2 (RS2) is native, uncooked semi-crystalline granular starch of B- and some C-type polymorphic structures, such as uncooked potato starch, green banana starch, and high-amylose maize starch. RS type 3 (RS3) is retrograded amylose formed in cooked starchy food. RS type 4 (RS4) is chemically modified or cross-linked starch, which is less accessible for enzyme hydrolysis. In addition to the four types of RS originally identified, resistant dextrins (Wang, Kozlowski and Delgado 2001)

and amylose-lipid complexes (Jane and Robyt 1984) can also be included as additional types of RS as they are resistant to enzyme hydrolysis. Indeed, it has been proposed that amylose-lipid complexes be designated as RS5 (Ai, Hasjim and Jane 2012, Hasjim et al. 2010b)

The early empirical classification of starch as RDS, SDS, and RS using time-based demarcation during amylase digestion is still widely used because of its simplicity, but is frequently linked to physiological significance with the assumption that RDS corresponds to rapidly absorbed glucose (and hence high glycaemic and insulinaemic responses) and that RS corresponds to starch that escapes digestion in the small intestine and enters the large intestine. There is now much more *in vitro* and *in vivo* information (as discussed above) showing that starch digestibility is highly variable and does not always follow a simple time-based classification into rapidly, slowly or resistant to digestion. For example, the 20 min cut-off point to separate RDS and SDS in the Englyst test was based on the observed deflection point of *in vitro* hydrolysis curves of starchy foods with the implicit assumption that this corresponded to a transition from rapid to slow digestion. In fact, Zhang et al., (2006) observed similar digestion rates from re-digestion of isolated undigested residues of maize starch recovered after either 20 or 120 min incubation with amylase to those found for native granules. This finding is also true for spherulites (Cai and Shi 2013), and the size and chain-length profile of starch polymers in enzyme resistant HAMS residues are similar to that of native granules (Evans and Thompson 2004). Thus, there is no clear biochemical distinction between slowly, rapidly or resistant portions of starches, because the deflection point on the digestogram of starchy material is not related to intrinsic enzyme resistance properties. The reason for the decrease in the rate of hydrolysis during a starch digestion experiment is in fact due to the exhaustion of available substrate since the digestion

process follows a first order kinetic curve (Goni et al, 1997, Butterworth, Warren and Ellis, 2011). The observed reduction in rate has nothing to do with the existence or otherwise of two physically different substrates being hydrolysed at different rates, as is implied by the RDS/SDS system. Essentially there is no fundamental physical basis for discriminating between RDS and SDS. Similarly, the classification of RS as the starch remaining after 120 min *in vitro* digestion has no physical meaning, as this too is often a reflection of the exhaustion of substrate under the particular digestion conditions used. Thus, the classification of digestibility as RDS, SDS or RS can be misleading if interpreted (as originally intended) to have physiological meaning; enzyme susceptibilities of starches are measurement- and method-dependent and are highly variable for different *in vitro* conditions and between individual human subjects.

RS is a physiological concept, i.e. it is defined as the fraction of starch that enters the large intestine. It is not a precise physical entity (Thompson 2000). The likelihood that all non-modified starches can be completely digested by amylases given sufficient time, enzyme activity and optimal environmental conditions demonstrates that true physiologically resistant starch depends on both digestion rate and gastrointestinal passage rate, which cannot be estimated from *in vitro* digestion data. While the activity/secretion of pancreatic enzymes is influenced by the nature of consumed foods (Buļko et al. 1982), their activity can be further affected by inhibition or non-catalytic binding on food components.

Assigning a numerical classification for RS can be ambiguous. For example, almost all amorphous extruded high-amylose starches provide a level of enzyme resistant properties similar to those seen in native HAMS granules (Lopez-Rubio, Htoon and Gilbert 2007, Htoon et al. 2009). The resistant properties of extruded HAMS do not arise from retrogradation of AM/AP

chains (RS3), nor from amylose lipid complexes (RS5), or the presence of intact granular structure (RS2). Rather, they are probably due to a close packing of amorphous chains providing a substrate with comparable polymer density to granular starch. Should this then be categorised as RS6? How many more types of RS could be categorised in this way? Would highly branched substrates be categorised as RS7? There is clearly a need for simplification of the nomenclature used to classify resistant starch.

Enzyme-based *in vitro* procedures that reasonably predict biochemical *in vivo* conditions for starch digestion have been discussed (Germaine et al. 2008, Woolnough et al. 2008, Sopade and Gidley 2009). *In vitro* processes commonly use fungal or bacterial amyloglucosidase as a final step to convert the α-limit dextrins and maltose produced by the action of α-amylase to glucose, which is in contrast to the *in vivo* system where mucosal enzymes hydrolyse products of amylase action to glucose. *In vitro* conditions typically use an excess of amyloglucosidase in order to ensure 100% conversion of Ŭ-amylase reaction products to glucose almost immediately, primarily for ease of measurement as glucose, although it has the added benefit of depleting potential product inhibition of Ŭ-amylase. Despite the multiple stages involved in reaction pathways, the kinetics of starch digestion, either with amylase or in combination with amyloglucosidase, often show simple decay curves with apparent first-order behaviour. This pattern can often be described by a single exponential decay equation $C = C_Đ (1 - e^{-kt})$ (Goñi, Garcia-Alonso and Saura-Calixto 1997). The decay equation suggests that the rate of reaction decreases with time due to substrate depletion, such that a semi logarithmic plot of starch digested against time shows a linear relation with a slope of ï *k*, where C represents the starch hydrolysis at time t and $C_{Đ,}$ the total concentration digested at the end point or the maximum

 

extent of hydrolysis. The *k* values reflect the susceptibility of starch or starchy foods towards hydrolysis by amylase, and typically range from $10^{-5}$ to $10^{-3}$ $min^{-1}$ but are dependent on enzyme concentration (Butterworth, Warren and Ellis 2011). The gradual decay of starch hydrolysis with time (as also seen in **Figure 2 and 3**) has been described as depletion of substrate (Butterworth et al. 2012, Dhital et al. 2010a). Although, the fraction of the starch remaining after the digestion plateau is reached has been described as enzyme resistant as previously discussed, provided the starch is not chemically modified, all starches can be hydrolysed completely by a combination of endo- and exo-enzymes, so that the presence of absolutely resistant starch without chemical modification may not be possible. Nonetheless, an appropriate description and mechanism to quantify the portion of starch that reaches the terminal ileum is lacking. The word ósresistantô used to describe and quantify those starches is ambiguous and is inappropriately used in many cases to describe the properties of starch. Secondly, there are kinetic parameters which determine digestion profiles related to structure, e.g. the early stage of granule digestion versus later stages. Furthermore, for conditions where a single kinetic process does not describe all of the digestion profile, there are structural differences which result in a more complex kinetic profile with evidence of more than one type of reaction that differ in the rate of catalysis. Thus the term ósresistant starchô does not reflect the physical condition of starch but is the kinetically determined value that reflects the susceptibility of enzyme towards the starch under given conditions and at a given time. This implies that the enzyme resistant starch from the first digestion course will generate an identical digestion curve with no apparent difference in potential kinetics parameters if treated with the fresh enzyme again. In fact, the repetition of the process can generate the complete hydrolysis of the starch no matter its source, for example native potato or HAMS (see

 

above). Thus expressing the digestion as a kinetic parameter can give a more straightforward description of relative rate of digestion of starch or starchy foods.

The initial stages (within the first 10-15 min) of a starch digestion experiment, in common with other enzyme catalysed reactions, show an approximately linear increase in product concentration with time. The rate of increase in product concentration can be related to the initial substrate concentration S using the Michaelis-Menten (M-M) equation:

$$v = \frac{V_{max} S}{K_m + S}$$

Where $V_{max}$ is the maximum reaction velocity and $K_m$ is the Michaelis-Menten constant.

$$V_{max} = k_{cat} E_0$$

Where $k_{cat}$ is the catalytic constant (or turnover number) for the enzyme and $E_0$ is the total enzyme concentration.

Reliable estimates for the kinetic parameters may be obtained during the initial stages of hydrolysis, before substrate exhaustion and product inhibition cause the reaction rate to decay (Butterworth et al. 2011, Slaughter, Ellis and Butterworth 2001).

The kinetic parameters obtained from the M-M equation may be used to compare the hydrolysis of different starch substrates. The $K_m$ value indicates the substrate concentration that will support a catalytic rate of $V_{max}/2$ (*i.e.* half the maximum reaction velocity), and has been suggested to be dependent on the available (or digestible) starch concentration. Available starch is that which is readily accessible for enzyme hydrolysis. This may be altered by processing the starch substrate by gelatinisation for example, which dramatically increases the amount of available starch, and markedly reduces the value of $K_m$. Following gelatinisation, a 5 to 20 fold reduction in $K_m$ occurs

as a result of increased substrate availability due to the loss of the semi-crystalline native starch granule structure (Tahir et al. 2011, Slaughter et al. 2001). For native starch granules, the value of $K_m$ has recently been shown to be dependent on the starch granule particle size (and more specifically, to surface area to volume ratio), as a result of the greater binding affinity for amylase to smaller granules (Warren et al. 2011, Tahir et al. 2010, Kim et al. 2008b).

The value of $k_{cat}$ also varies slightly depending on both the botanical origin of the starch, and on any processing of the starch substrate that alters its structure. Gelatinisation of starch dramatically increases the $k_{cat}$ value for amylase catalysed hydrolysis of a range of starches (Slaughter et al. 2001, Tahir et al. 2011). This presumably reflects the fact that a less structured substrate, following gelatinisation, is more accessible for the enzyme active site which requires a run of 5 contiguous Ŭ-1-4-linked anhydroglucose residues (Warren et al. 2012, Prodanov, Seigner and Marchis-Mouren 1984, Seigner, Prodanov and Marchis-Mouren 1987), allowing the enzyme to achieve a higher turnover number. Following gelatinisation, the value of $k_{cat}$ is very similar for starches from a range of botanical origins, as it is an intrinsic property of the enzyme.

The ratio $k_{cat}/K_m$, termed the catalytic efficiency (CE), or specificity constant, can be a useful tool for comparing the relative digestibility of different starches from different botanical origins, and/or starches which have been subjected to different processing conditions. CE, also termed the specificity constant, is a constant that describes the interaction between the enzyme and substrate, and may be used to indicate the preferred substrate of the enzyme (Cornish-Bowden 2004).

$K_m$ and $k_{cat}$ values are derived from M-M initial rate studies, so it could be asserted that values are not particularly useful for relating to biological endpoints. However, this appears not to be

the case for glycemic response where *in vivo* measurements in humans have consistently demonstrated that the peripheral blood glucose concentration rises within a relatively short time following ingestion of a starch meal (Ellis et al., 2005). Indeed, more direct measurements of glucose absorption in the portal blood show that glucose derived from starch enters the blood circulation in the first 10-15 min, reaching a peak between 30-60 min (Anderson, 1973; Ellis et al., 1995). This early rise in glucose originates from the initial stages of amylase action. Moreover, the physiological and clinical significance of the initial stage of starch digestion is amplified by the concomitant stimulation of insulin secretion and gut hormone signalling such as blood GIP and GLP-1 responses (Ellis et al., 1995).

From a mechanistic standpoint, measurements of $K_m$, $k_{cat}$ and $k_{cat}/K_m$ have revealed much about the susceptibility of starches from different botanic origins to amylolysis and how the susceptibility is affected by hydrothermal treatments (Slaughter et al., 2001; Tahir et al., 2010, 2011). In particular, changes in measured $K_m$ values accompanying hydrothermal processing can reveal how the availability of substrate increases as processing proceeds (Butterworth et al., 2011). Binding constants (measured by direct binding experiments) have been shown to correlate with values of $K_m$ values and absorption coefficients obtained from MM type experiments (Warren et al., 2013). Such findings add confidence to the usefulness of the MM approach.

One approach to extending MM kinetics beyond the initial phase of the enzyme reaction is to perform full progress curve analysis (Dona et al, 2010; Duggleby and Clarke, 1991) for starch

amylolysis, although this requires the assumption that the nature of the substrate does not change during the reaction, a debatable assumption when applied to starch digestion. Progress curve analysis of starch digestion has not been reported, but we believe that analysis of digestograms by extended pseudo-first order kinetics goes some way towards maximising the information available from progress curves. Log of Slope (LOS) plots enable digestibility constants and the total amount of digestible material to be estimated. In addition, changes in the ease of digestion as the reaction proceeds can also be identified (Butterworth et al., 2012).

Significant differences have been observed in the postprandial rise in blood glucose and insulin concentrations of test subjects fed with varying types of starch-rich foods containing the same amounts of starch (Crapo, Reaven and Olefsky 1977). This has led to starch-rich foods being ranked according to the extent of their postprandial blood glucose response, the so-called glycaemic index (GI) (Jenkins et al. 1981a). *In vitro* amylolysis kinetics have been used to predict the GI or equivalent biometrics (O'dea, Snow and Nestel 1981, Jenkins et al. 1982, Bornet et al. 1989, Granfeldt et al. 1992b). Goni et al. (1997) found a good correlation (r =0.909) between percentage of starch hydrolysed at 90 min ($H_{90}$) and metabolic glycaemic response *in vivo* expressed as GI= 39.21+0.803($H_{90}$). Similarly, the GI was related to hydrolysis index (HI) by the equation GI = 39.71+0.549 HI, where the HI was obtained by dividing the area under the hydrolysis curve (0-180 minutes) of the sample by the area of the standard material (white bread) over the same period of time. It needs to be stressed however, that both of these equations have been empirically derived. Several other studies have indicated a good correlation between the *in vitro* starch digestion and the glycaemic response of the foods and proposed different empirical equations. However, such studies (e.g. see Granfeldt et al. (1992a)) are dependent on the

methods used to prepare samples and measure the hydrolysis kinetics. Most of the methods use a limited time period (up to 180 min) to predict the GI, which may not well represent the slow digestion properties of starches (Zhang and Hamaker 2009, Lee et al. 2013). Thus, accumulated evidence presented above suggests that a full kinetic analysis can provide a more appropriate *in vitro* approach to investigating factors related to the rate (relevant to GI) and extent (relevant to RS) of starch digestion in the small intestine *in vivo.* However, it should be expected that there will be a limitation to the direct application of starch hydrolysis to physiological outcomes due to individual differences, such as in mastication, gastric residence time, small intestinal passage rate and digestive fluid secretion (i.e., variations in enzyme activity). All these factors need to be considered in addition to starch hydrolysis kinetics in predicting individual physiological parameters, such as GI and the peak blood glucose response (see DeVries (2007) for variability of GI measurement) and RS.

## 4.0 Can digestion kinetics be used to assign underlying starch digestion mechanisms?

As discussed above, reducing the enzymic digestibility of starch or starchy foods can be achieved either by (i) barriers that slow down or prevent access/binding of enzyme to starch or (ii) starch structural features that slow down or prevent subsequent amylase action. Where there are barriers preventing access of enzyme to starch, and which cause this to be the rate-limiting step, the kinetics of digestion will reflect the diffusion rate of enzyme across the barrier. Diffusion controlled digestion would typically follow first order kinetics (Fickôs Law). For smooth spherical particles, the surface area is proportional to the square of the radius or diameter of the particle. Moreover, if the rate-determining step is assumed to be the diffusion of enzyme onto the surfaces of spherical particles, the rate of digestion from first-order kinetics can be

related to the average particle diameter by $1/K = (X^2/6D)$, where K, X and D represent digestion rate coefficient ($min^{-1}$), particle diameter (m) and diffusivity coefficient ($m^2s^{-1}$), respectively, as a close approximation (Al-Rabadi et al. 2009). Thus, a plot of the reciprocal of K against the square of the particle diameter is linear and the inverse of the slope of the plot gives the apparent diffusivity of the enzyme within particles such as grain fragments or starch granules (Al-Rabadi et al. 2009, Dhital et al. 2010b, Mahasukhonthachat et al. 2010a).

The study of digestion rate as a function of the number/size of barriers (e.g. particle size) can be used to calculate an apparent enzyme diffusion coefficient, which can be compared with measured values in solution to determine the relative effectiveness of barriers. This approach has been used to study the rate of starch digestion by pancreatic Ŭ-amylase on size-fractionated samples of isolated granules from maize and potato (Dhital et al. 2010b), and granules embedded within milled sorghum and barley grains (Mahasukhonthachat, Sopade and Gidley 2010b, Al-Rabadi et al. 2009). For starch granules, controlled sedimentation was used to separate granules into narrow distributions with particle sizes (10-70 µm) as predicted by Stokesô law. For milled grains, fractions with average particle sizes ranging from 0.1 to 0.6 mm were obtained by variation in milling time (Mahasukhonthachat et al. 2010b), and size fractions of 0.1 to 3 mm were obtained by sieving of hammer-milled grains (Al-Rabadi et al. 2009). For all samples and size ranges, digestion followed first order kinetics and first order rate coefficients were shown to vary with the inverse square of the average particle size consistent with enzyme-substrate complex formation being rate limiting (**Figure 2**). For each granule/grain type, rate coefficients were inversely proportional to the square of particle size, interpreted as being due to effective surface area (granules) or diffusion within particles (milled grains) being the quantitative

determinants of digestion rate. When expressed as apparent amylase diffusion coefficients to eliminate size effects, milled grain digestion was found to be 100-1000 times slower than starch granule digestion, suggesting that cell walls and/or protein matrices have a controlling effect on starch digestion in uncooked grains. The apparent amylase diffusion coefficient within sorghum grain was ca 2.2 times less than within barley, suggesting that the more tightly packed cellular and protein matrix structures in sorghum provides a significantly greater barrier to amylase movement than in barley (Al-Rabadi et al, 2009).

In the context of generalised enzyme-substrate interactions (below), access barriers are likely to result in $k_1$ being rate-determining, provided that there are not additional molecular features that make $k_2$ very slow or $k_1$ / $k_{-1}$ very small.

$$E + S \underset{k_1}{\overset{k_{-1}}{\Leftarrow\Rightarrow}} ES \overset{k_2}{\rightarrow} E + P$$

Where there are molecular features of starch that control digestion rates, this is more likely to be due to inefficient formation of enzyme-substrate complex (making $k_1$ / $k_{-1}$ very small) or slow conversion of enzyme-substrate complex to products, making $k_2$ rate determining.

Thus, we can state that there are two possible limitations on the rate and extent of starch digestion: (i) the formation of an enzyme-substrate complex, hence enzyme accessibility to and/or binding to the starch substrate is rate-limiting; and (ii) catalysis, in the case of starch which is readily accessible to enzyme, but where the rate of hydrolysis is limited by the catalytic activity of the enzyme, as the enzyme substrate complex forms rapidly, with a high affinity.

We therefore suggest that the above observations lead to a natural division of the mechanisms for the rate determining aspect of starch digestion into two possibilities for the rate-determining step,

namely; the formation of an enzyme-substrate complex and the catalytic action. We propose that this classification offers a significant improvement over the existing system of RDS, SDS, RS1, RS2, RS3, RS4 etc., simplifying these many classes of starch to two only, namely binding limited or catalysis limited. A system based on intrinsic properties of the enzyme interaction with its substrate has the potential to be both more logical, and simpler to use, than the existing arbitrary systems. As an example, starch that is currently defined as RS1, i.e. starch that is physically inaccessible to enzyme due to cell wall barriers etc., may be re-classified as an example of starch for which enzyme binding is rate limiting. This is a more satisfying definition than RS1, as encapsulated starch is not intrinsically resistant to digestion, but its rate of digestion is limited by enzyme binding, rendering it a functionally resistant starch. The same may be said of RS2, where slow enzyme binding to those native starch granules which lack surface pores and channels limits the rate of digestion. RS3 may be a combination of both slow enzyme binding to condensed, retrograded structures, and resistance of the highly crystalline starch to catalysis by the enzyme. Whether slow binding predominates may vary depending on the nature of the retrograded material. RS4 may similarly be divided into chemical crosslinks, which slow the binding of the enzyme, and chemical modifications that slow down the catalysis of starch breakdown. Thus, we can begin to define the digestibility of starches not by arbitrary labels, but through a mechanistic understanding of how the enzyme interacts with its substrate. This will allow us to understand resistant starch, not as an absolute value, but as a functional property that is dependent on the rate and extent of starch digestion as it transits through the human digestive tract.

This may also explain other aspects of starch digestion that have been puzzling to researchers, such as the so called ñside by sideò digestion, the propensity for amorphous and crystalline parts of native granules to be digested at similar rates (Zhang et al. 2006; Shrestha et al, 2012). If the rate determining step for the hydrolysis of native starch is enzyme binding to the granule, then any differences in the rate with which amylase catalyses the hydrolysis of amorphous and crystalline regions of the granule will become negligible relative to the slow binding step.

## 5.0 Conclusions and prospects

It is more than 20 years since classifications were first proposed for (a) the proportions of rapidly-digestible, slowly-digestible, and resistant starch based on *in vitro* digestibility, and (b) the types of resistant starch based on physical and/or chemical form. While these empirical classifications have been widely used and have provided a useful shorthand nomenclature for researchers, it is becoming increasingly clear that they neither reflect *in vivo* behaviour accurately nor provide a basis for understanding the underlying mechanisms involved. Based on this review we propose the following:

1. Kinetic analysis of digestion profiles, which provides a robust set of parameters, should replace the classification of starch as a combination of rapidly-digestible, slowly-digestible, and resistant starch based on a single enzyme digestion experiment. The first step in such a kinetic analysis should be to determine the minimum number of distinct kinetic phases needed to describe the full digestion process and the proportion of starch involved in each kinetic phase using, for example, the log of slope plots (Butterworth et al. 2012). For each process so identified, the kinetic properties (e.g. digestion rate coefficients) should be obtained under

standardised conditions and compared with those for standard reference materials. These data can be used to assess the potential rate-determining steps involved and to provide a ranking of relative starch digestibility for empirical comparison with *in vivo* data, e.g. blood glucose response or resistant starch content. It is particularly important that the current *in vitro* classification of resistant starch should not be assumed to be equivalent to *in vivo* levels of starch entering the large intestine.

2. The current classification of resistant starch types as RS1/2/3/4/5 etc should be replaced by one which recognises the essential kinetic nature of RS (in combination with gastric residence and passage rate), and that there are two fundamental origins for resistance based on rate-determining access/binding of enzyme to substrate and rate-determining conversion of substrate to product once bound. The target should be to use kinetic analyses (see 1 above) to identify which of the two mechanisms (or a combination of both) are responsible for resistance in any specific starch/food.

3. It is important that *in vitro* assessments of starch digestion kinetics are carried out under controlled (preferably standardised) laboratory conditions in order to define underlying mechanisms and their structural basis, and that a large number of data points are collected throughout the time course of digestion (particularly for the early stages) in order to allow for robust analyses of digestive processes.

4. *In vitro* measurements will always only provide an approximate guide to *in vivo* properties such as glycaemic response and the amount of starch entering the colon, as these properties also depend on mastication processes, gastric processing and residence time, small intestinal enzyme concentrations and residence time, and hormonal responses. We should expect *in vivo* values,

 

notably the glycaemic response or resistant starch content, to vary both between and within individuals. Therefore, we should articulate more clearly that *in vitro* assessment can, at best, only provide indicative data unless the *in vitro* method is calibrated empirically against a substantial body of *in vivo* data on very similar substrates (e.g. foods not starches).

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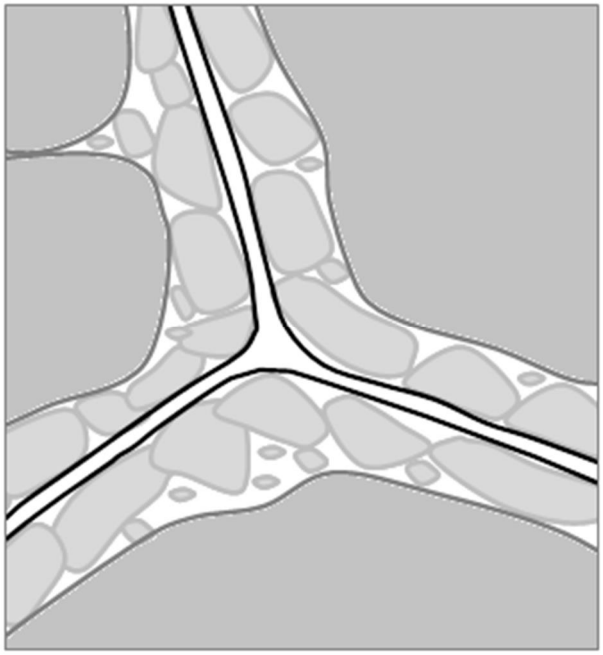

**Figure 1**: Model illustrating location of starch granules in plant cells. S, P and C refer to starch granules; protein bodies and cell wall matrices respectively. Starches in cereals, pulses and tubers are surrounded by varying proportions of proteins (bodies, matrices) and cell walls. These non-starch components provide a physical barrier towards the movement of amylases. Processing, such as by milling or cooking for example, can destroy the physical barrier that when intact, attenuates the rate and extent of amylolysis of starch.

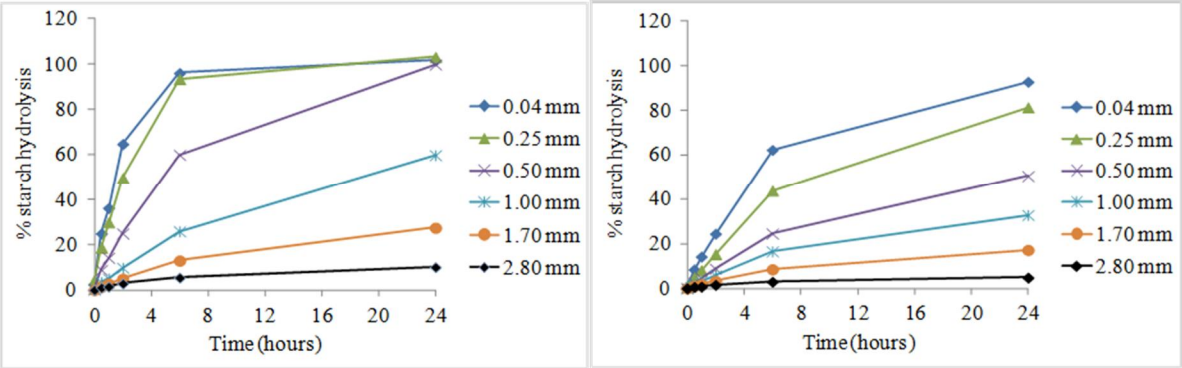


**Figure 2**: Starch hydrolysis kinetics of barley (a) and sorghum (b) grain milled to varying fragments sizes (Al-Rabadi et al. 2009). In similar grain fragment sizes, the higher hydrolysis extent of barley was assumed to reflect the presence of comparatively fewer protein bodies/matrices than in the sorghum grains that can hinder the amylase accessibility toward the starch granules. The hydrolysis extent is negatively correlated with particle size, as milling to smaller fragments can break down the cell wall and protein matrices increasing the accessibility of amylases towards the starch granules.

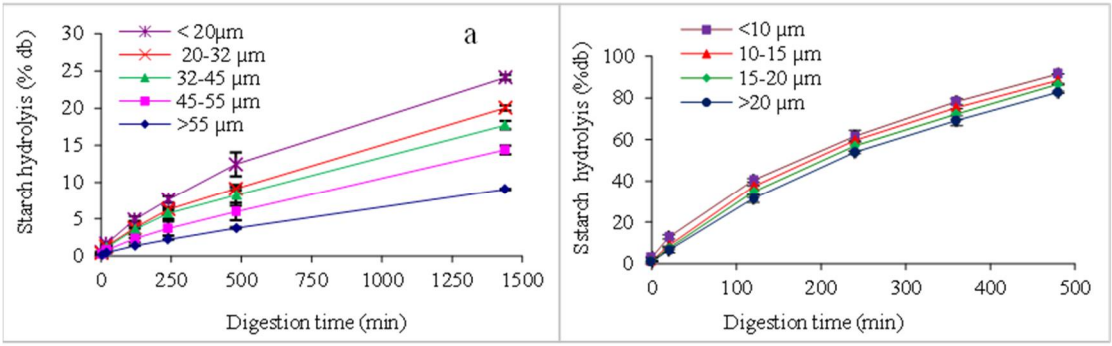


**Figure 3**. Starch hydrolysis kinetics of native potato starch (a) and maize starch (b) with varying granular sizes (Dhital et al. 2010b). The larger granules of potato starch (> 55 µm) were almost 3 times more resistant to amylolysis compared withsmaller granules (less than 20 µm diameter). This difference in hydrolysis rate was interpreted as resulting from differences in available surface area; smaller granules having more available surface area compared with that of larger granules, on the same weight basis. In contrast, the larger granules of maize (> 20 µm) were almost equally susceptible to amylolysis compared with smaller granules (less than 10 µm diameter). This similarity in hydrolysis extent was assumed to arise from surface pores and channels in maize starch granules that can dramatically increase the available surface area for amylase catalysed hydrolysis.

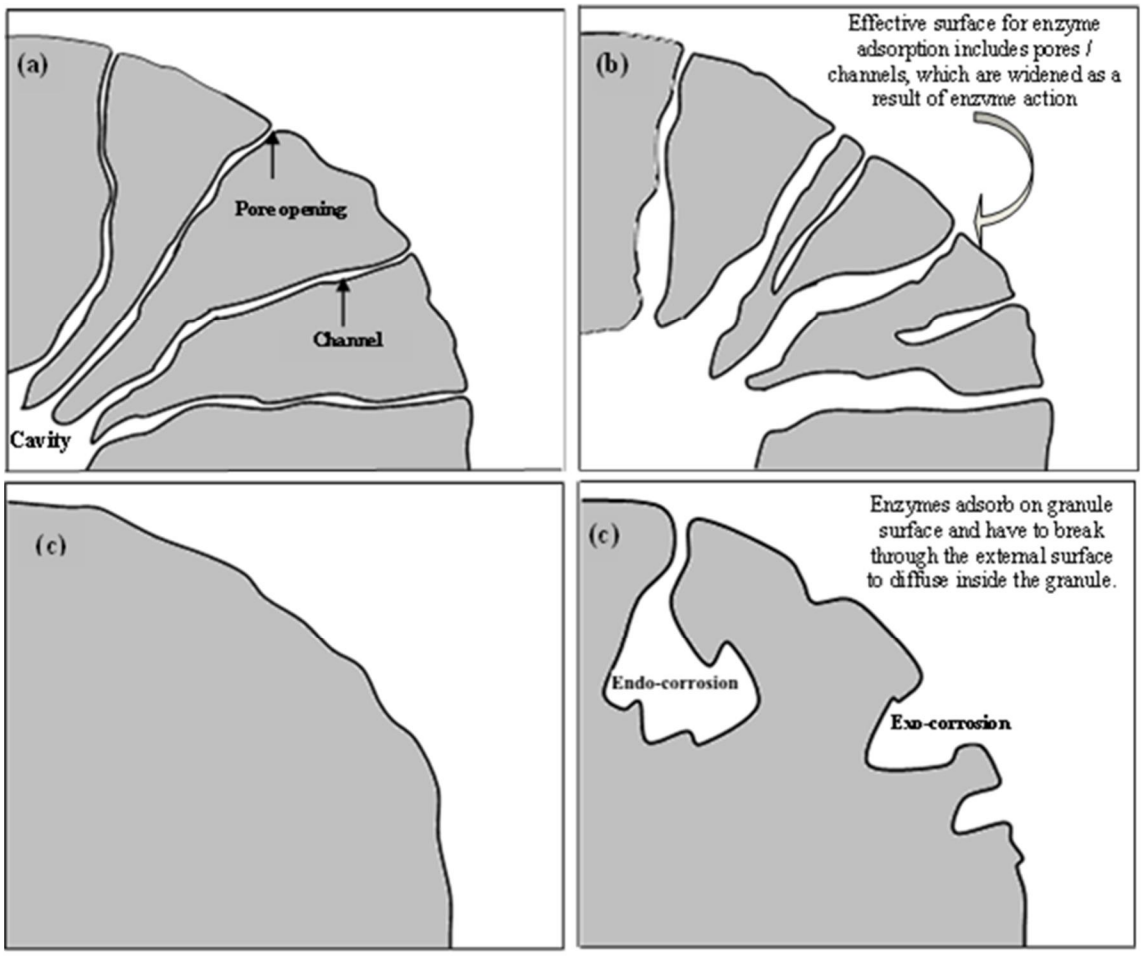


**Figure 4**. Model illustrating diffusion of amylase and its catalytic patterns in maize and potato starches (Dhital et al. 2010b): Maize starch showing pores, channels and cavity (a), maize starch hydrolysed by amylase with enlarged pores, channels and cavity (b), potato starch lacking pores, channels and cavity (c), and potato starch exo- and endo-corroded by amylase (d).

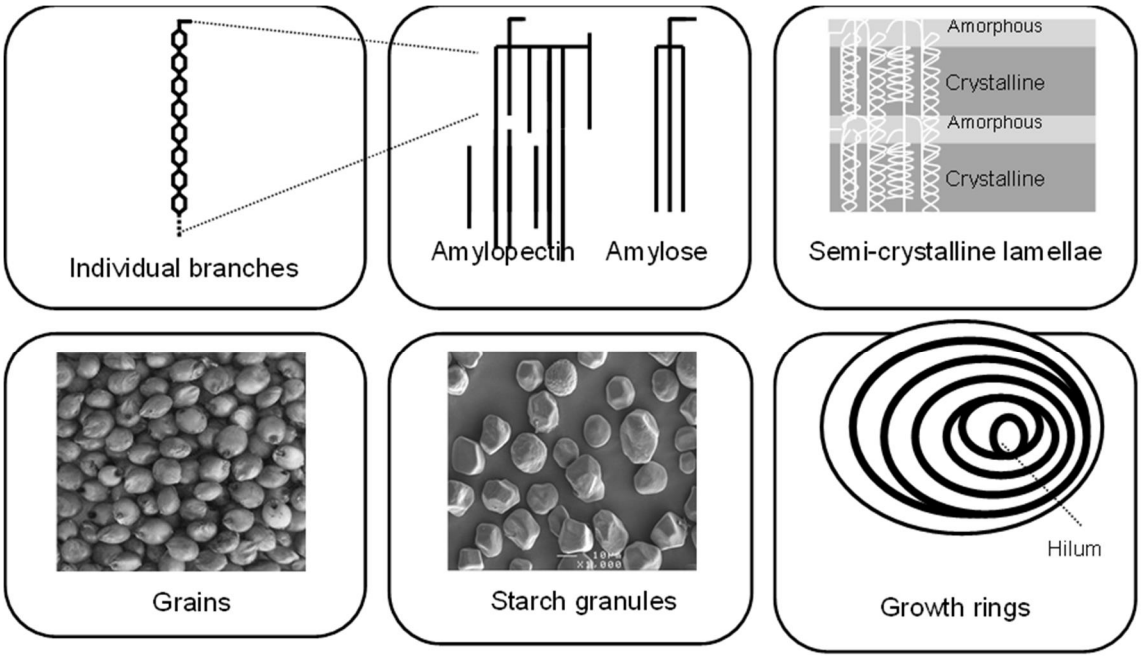


**Figure 5**. Different levels of starch structure that can control the rate and extent of amylolysis of starch granules. Figure modified from Tran et al. (2011).