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Review

Digestion of starch: *In vivo* and *in vitro* kinetic models used to characterise oligosaccharide or glucose release

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ABSTRACT

We give an overview of the kinetics of starch digestion, emphasizing *in vitro* studies and the various mathematical models used to analyse the data. The emphasis in this review is on ungelatinised starch (wherein granules are still intact and unswollen), applicable to domestic animal feed and to some human food. The mammalian digestive system uses a complex but well ordered series of processes to degrade and absorb nutrients from the diet of an individual. Mechanical action like mastication and churning of food throughout the various subsections of the gastrointestinal tract work together with biochemical components in secretions containing acids, buffers, and hydrolytic enzymes. Many attempts have been made to mimic digestion *in vitro* in an effort to model its complexities. Characterisation of enzyme-catalysed hydrolysis *in vitro* using purified enzymes, separately for simplification, has proven difficult, as the starch granule's complex structure causes enzyme action to follow unconventional kinetics. The susceptibility of starch granules to digestion by glucohydrolases depends on a set of factors that include the granular structure, the method of preparation and the nature of the starch, and those molecules bound to it. Within a starch granule, the branching structure, molecular size and molecular weight distributions, and crystallinity, may all affect its physical properties, thus controlling digestibility. Characteristics such as solubility and the presence of fibre, fat and protein all contribute to the rate of digestion.

1. Introduction

The digestion of starch is not a simple, single chemical process. The process can be partially quantified by several measures which differ from one another depending on the enzyme(s) and reaction conditions used to catalyse the hydrolysis. These measures include the rate of starch loss, rate of glucose appearance, and the rate of appearance of various oligosaccharides.

Understanding the factors influencing starch digestion is best done through a causal, mechanistically-based approach through the following paradigm: biosynthesis → growth and processing conditions → structure of starch and of starch-containing substances → digestion properties.

In other words, biosynthetic events control the formation and hence structure of the starch, which is dependent on growth conditions (Buleon, Colonna, Planchot, & Ball, 1998; Copeland, Blazek, Salman, & Tang, 2009; Smith, 2007) (e.g., diurnal temperature, water availability, etc.) and on any processing, such as cooking. It is important to be aware that causal relations follow the arrows given above. While there might be a *correlation* between, say, plant variety and rapid digestibility, this is not causal; observing that a particular plant variety gives rise to a structure which is rapidly digested is causal (Copeland et al., 2009).

As discussed in detail below, there are a number of different structural scales to be considered in starch-containing foods, ranging through the distribution of individual starch branches, through the overall branching structure of the starch molecules in a granule, through to the macroscopic structure of a grain. This structure includes not only the starch but also proteins, lipids and non-starch polysaccharides that may be present. Properties such as the kinetics of digestion are controlled by this structure at one or more of the various structural levels (Smith, 2007).

The starch consumed by domestic animals is largely uncooked (ungelatinised), whereas most human starch-containing food is

Abbreviations: ADP-glucose, adenosine5′-diphosphoglucose; AF^4, asymmetric flow-field flow fractionation; AUC, area under curve; CEST, chemical exchange saturation transfer; DMSO, dimethylsulfoxide; DP, degree of polymerisation; DSC, differential scanning calorimetry; FACE, Fluorescence-assisted capillary electrophoresis; GI, Glycemic Index; GL, Glycemic Load; GPC, gel-permeation chromatography; HPAEC, high-performance anion-exchange chromatography; HR MAS, high-resolution magic-angle spinning; NMR, nuclear magnetic resonance; NSP, non-starch polysaccharide; RDS, rapidly digested starch; RS, resistant starch; SAXS, small-angle X-ray scattering; SDC, slowly digested carbohydrate; SEC, size-exclusion chromatography; SDS, slowly digested starch; SEM, scanning electron microscopy; XRD, X-ray diffraction.

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cooked (gelatinised), with some exceptions such as muesli. Cooking greatly changes, and may destroy, granule structure. Unlike most enzymic reactions, the digestion of starch in its ungelatinised form is controlled, for the most part, by the granular architecture of the substrate. The focus of this review is on the characterisation of the functional property of digestibility in starches. Although structural and physical properties of starch are presented, the introduction is kept brief by using references to many literary sources covering this broad area.

2. Starch sources

Starch is abundant in the grains of all cereal crops including rice, wheat, maize, barley, and sorghum, as well as in pulses and tubers. The structure of starch and its packaging into granules varies widely between plant species, but the most important as foods are rice and wheat so these are emphasized here. The generalisation of concepts for studying digestion, to other sources of starch, can be readily made.

Native starches exist in many varieties. For example, rice can be waxy, non-waxy, and sticky; while maize can contain low or high amylose. In other words these types differ in their amylose, amylopectin, protein, and lipid contents together with other structural differences. About 15–30% of the rice grain is protein, lipid, and non-starch polysaccharides (Buleon et al., 1998; Chiou, Fellows, Gilbert, & Fitzgerald, 2005). Protein fractions in most other grains, such as wheat, are significantly higher.

The protein is also an important structural component in grain. We here consider only grains which have been hulled (or milled), i.e., from which the hard outer layer(s) has/have been removed. In wheat, much of the protein, predominantly friabilin, links the surface of starch granules to a complex matrix of proteins and lipids. Matrix proteins in wheat seeds create a need for special extraction methods to release the starch granules. Once released, the fraction of protein located in the starch granules themselves is relatively small (0.1–0.3%) (Csoti, Bako, Tamas, & Gardonyi, 2005). Similarly, in sorghum, the starch is tightly bound to a protein (kafirin), and this plays an equivalent role to the hull in wheat (Belton, Delgadillo, Halford, & Shewry, 2006).

3. Molecular architecture of starch

Regardless of their botanical origins, starch varieties contain primarily two different types of anhydroglucose polymers, amylopectin and amylose; both are linked by α (1,4) bonds in linear segments, with α (1,6) bonds at branching points. Amylopectin makes up ~70–80% of most starch varieties and so is the major component, strongly influencing the physiochemical and culinary characteristics of starch. Amylopectin contains many short branches with a non-random distribution of branching points (4–5%). On the other hand, amylose is primarily a linear macromolecule with less than 1% long-chain branches forming predominately single chain helices. Amylose is also known to exhibit a sixfold left-handed double helical conformation.

The structure of starch in a grain can be categorised (Ball et al., 1996) into six levels (Fig. 1), ranging in scale from nm to mm: i.e., 6 orders of magnitude.

Level 1: Individual branches (see Fig. 1) This is the distribution of the chain lengths (degrees of polymerisation, DP) of the branches in a sample, often termed the chain-length distribution. The length scale of chains is of the order of 1 nm.

Level 2: Whole starch molecules (see Fig. 1) This is the structure of the branched molecules, which is exceptionally hard to fully characterise both theoretically (Gray-Weale & Gilbert, 2009) and experimentally, but which can usefully be characterised by averages such as the number- and weight-average molecular weight, or the weight-average size distribution (Gidley et al., 2010). The branching properties of each amylopectin anhydroglucose chain

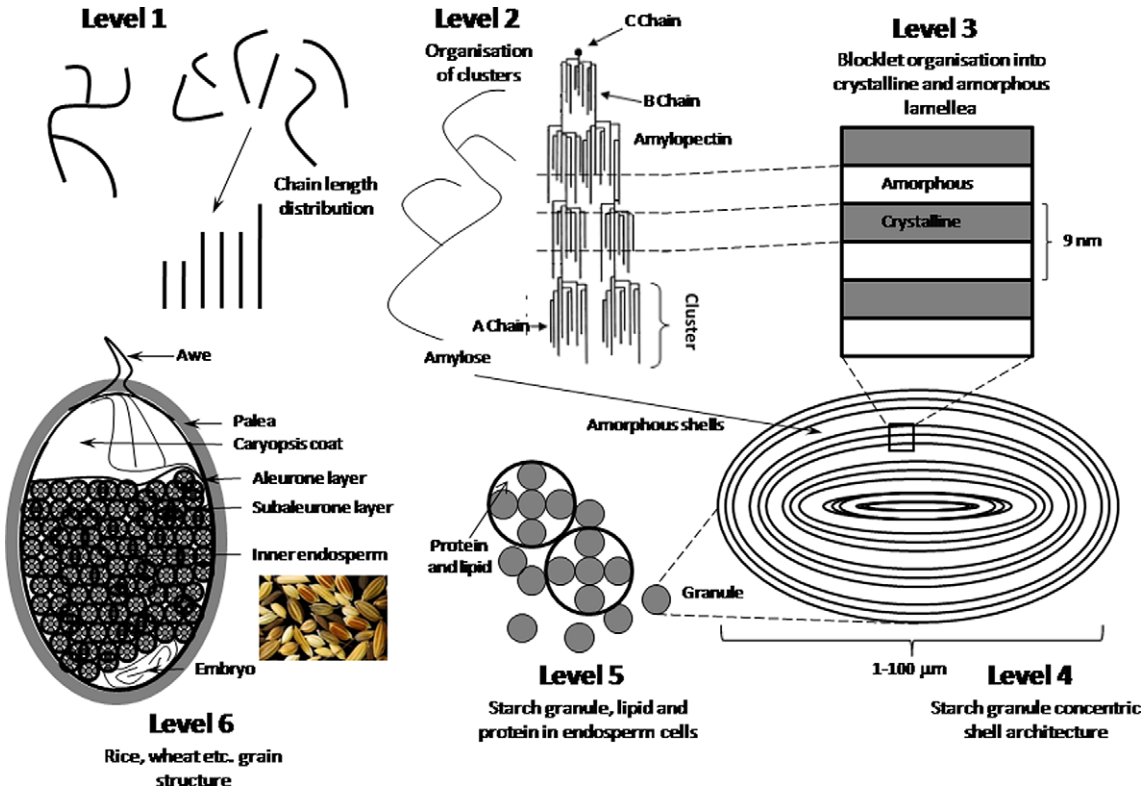

Fig. 1. Six supramolecular levels of the rice grain, highlighting the microscopic structural contribution of starch.

can be separated into three distinct groups (Peat, Whelan, & Thomas, 1952) (Fig. 2): (1) 'A chains' have no consequential branches and are joined to a B or C chain through an α (1,6) bond at their reducing end. (2) 'B chains' differ from A chains by possessing subsequent branches that are connected to them via an α (1,6) bond at the reducing end of branches. (3) 'C chains' are the backbone of the amylopectin molecule having a free reducing end. Numerous branches are attached to a C chain, thus laying the foundation for the complex structure of amylopectin.

Level 3: Lamellar structure (see Fig. 1) In native starch, the tight packing of glucan chains enables them to intertwine to form double helices, which in turn create regions of crystalline order, with the crystalline component largely comprising clusters of shorter portions (typically ~17 monomer units) of amylopectin branches; the amylose is thought largely to reside in the amorphous layers. This structure can be characterised by small-angle X-ray and neutron scattering, and by scanning electron microscopy (SEM). Isolated amylose molecules can form into a sixfold left-handed double helical structure (Fig. 3), which also can exist in whole starch after gelatinisation (Section 4).

Level 4: Granules (see Fig. 1) The lamellar structures occupy the inner architecture of native starch granules in concentric growth ring shells of thickness 100–400 nm; these are separated by regions of amorphous structure. The crystalline and amorphous lamellae alternate radially, creating concentric shells. The amorphous regions of the granule contain the branch points of amylopectin molecules, as they tend not to align, and amylose, as it is also does not form tightly packed crystalline regions (Donald, 2004). These semi-crystalline and amorphous 'growth rings' that make up the granules are ~15–30 μm in size. Characterisation is by SEM. This level of structure is greatly changed by the process of gelatinisation.

A conceptual model of starch granule structure called the 'hairy billiard ball' (Jane, Wong, & McPherson, 1997; Lineback, 1984, 1986; Robertson et al., 2006) illustrates both the single helical organisation of amylose, and the double helical organisation and clustering of amylopectin. The model describes the outside of the granule with protruding branches of amylopectin (hair) rather than a smooth exterior. The outside of the billiard ball, inside the hairy layer, defines a boundary between initially accessible, or surface-substrate sites, and initially inaccessible or interior substrate sites (Robertson et al., 2006). The hairy billiard ball model was developed to explain short range surface-order in X-ray diffraction (XRD) patterns. These patterns are unrelated to the X-ray patterns observed due to the semi-crystalline lamellae of granules (Sevenou, Hill, Farhat, & Mitchell, 2002). The digestion time courses of glucohydrolase action on starch granules are also best explained by variations in accessibility of the enzyme, seen in the hairy billiard ball model. Unlike synthetic polymers, such as polyethylene, starch amylopectin crystallites do not assemble into semi-crystalline rings with chain-folded lamellae. The repeat distance of starch crystallites upon annealing also does not appear to change as with

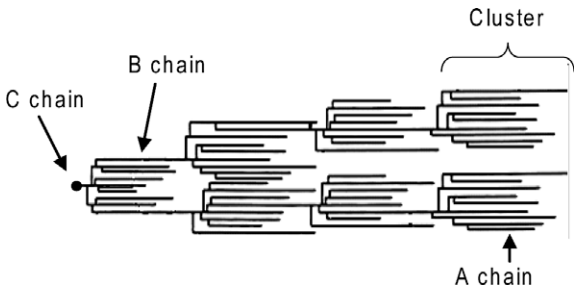

Fig. 2. Cluster model of amylopectin structure. The organisation of the chains is made evident. An amylopectin molecule contains only one reducing end (denoted by the black dot). (Inferred from Peat et al., 1952.)

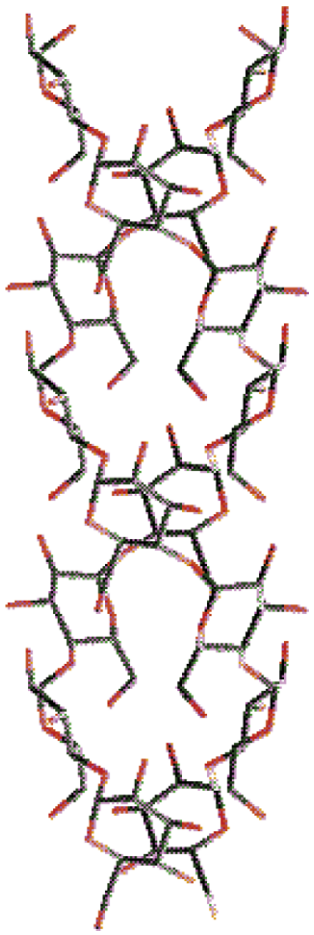

Fig. 3. Double helical conformation of amylose is dissimilar to other carbohydrates of glucose like cellulose and β (1,3) glucan that form single and triple helices, respectively. The helix is tightened upon binding with iodine and iodide ions, or short-chain alcohols.

synthetic polymers (Jenkins et al., 1994). Instead, the double helices appear to arrange side-by-side in a smectic or nematic-type structure (Waigh et al., 1997). These properties led to another conceptual model of the granular architecture of starch, based on side chain liquid–crystalline polymers. The rigid double helices of amylopectin branches provide the fundamental units of structural order in the liquid crystals, although they need to be adequately decoupled from the backbone for liquid crystalline packing to occur (Shibaev, 1994).

The alternating semi-crystalline shells, elliptical in shape, regularly alternate with a repeat distance of ~9 nm (Waigh, Donald, Heidelbach, Riekel, & Gidley, 1999; Waigh et al., 2000). Regardless of their botanical origins, amylopectin molecules exhibit the same repeat distance for crystalline and amorphous regions, as measured by XRD (Waigh et al., 1997, 1999). The molecular order of starch granules is also confirmed by their birefringent nature when viewed in polarised light, displaying a Maltese cross (BeMiller, 2007).

Level 5: Endosperm (see Fig. 1) This comprises the starch granules, together with protein and lipids. This level of structure is not usually important for digestion, with the exception of grains such as sorghum that lack a hull and instead have a dense protein–starch matrix which is not readily broken down.

Level 6: Whole grain (see Fig. 1) This final level, ~1 mm in size, includes the highest-level structures such as the hull, etc.

The role of the granular structure in nature is as an energy-storage medium for the germinating plant, to release glucose slowly

given the right external stimuli. The compact crystalline structure of amylopectin is the most important structural component for this purpose. Although amylose generally makes up a significant fraction (20–30%) of starch varieties, its role in the starch granule as a source of glucose for energising an embryo is yet to be completely understood. While amylopectin alone is sufficient for the formation of starch granules, amylose also plays a central role in the initial stages of granule crystallisation (Ziegler, Creek, & Runt, 2005). Interestingly, details such as the amylopectin–amylose ratio, size, morphology, and size distribution of the starch granule all determine the physicochemical characteristics of the granule (Ellis et al., 1998).

4. Functional properties

The addition of water and application of heat to raw grains is essential to transform them into a food with pleasing textural attributes. There are several stages that occur during cooking, including glass transition, gelatinisation (the non-equilibrium swelling of the crystalline regions (Slade & Levine, 1988)), swelling, pasting, and retrogradation (see below). The stages evolve in this order during the heating and subsequent cooling of suspended granules, often depending on one another (Slade & Levine, 1988). In its native form, a starch granule is insoluble in cold water, thus creating a suspension when mechanically dispersed in water. Heat treatment of these suspensions in excess water is generally used to make homogeneous starch–water mixtures. The hydration and rheological properties of carbohydrate polymers is well described (Matser & Steeneken, 1997; Steeneken, 1989).

The glass transition temperature is that at which the amorphous (glassy) regions of crystalline polymers become soft or rubbery. The semi-crystalline nature of starch granules mean that starch must lose its glassy, brittle properties before gelatinisation can occur (Biliaderis, Page, Maurice, & Juliano, 1986). The glass transition temperature is depressed by water, so at higher moisture contents the glass transition occurs at lower temperature (Biliaderis et al., 1986; Levine & Slade, 1989; Slade & Levine, 1988). Unlike most synthetic polymers, which return to their glassy state upon cooling below the glass transition temperature, cooling starch granules to below the glass transition temperature does not necessarily reverse the formation of soft, rubbery amorphous regions (Biliaderis et al., 1986; Levine & Slade, 1989).

The gelatinisation of starch occurs in the neighbourhood of 60 °C (see above) depending on its source (Shiotsubo, 1983). Above this temperature, the starch granule absorbs considerable amounts of water and loses its semi-crystalline nature while swelling to many times its original volume. Complete homogeneity of starch may only occur when swollen granules are heated well beyond the gelatinisation temperature, causing the disruption of the starch granules into smaller aggregates. At even higher temperatures and pressures, spontaneous hydrolysis of starch molecules increases the textural homogeneity of the mixture by producing smaller oligosaccharides (Miyazawa, Ohtsu, Nakagawa, & Funazukuri, 2006).

To characterise the size distribution of whole starch in mixtures requires dissolution of the starch without degradation of the molecules. Some starch varieties become completely solubilised after heating in water to 100 °C. Autoclaving starch suspensions is also used to create a homogenous solution, although the effectiveness of autoclaving varies with the starch variety; this process may well degrade the starch (Gidley et al., 2010; Ratnayake & Jackson, 2009). Starch may be completely dissolved in anhydrous dimethylsulfoxide (DMSO), either in the presence of a small amount of water (Bello-Pérez, Roger, Baud, & Colonna, 1998) or with the addition of LiBr as a hydrogen-bond disruptor (Dona et al., 2007; Schmitz, Dona, Castignolles, Gilbert, & Gaborieau, 2009); the latter can be carried out at ~80 °C with minimal shear and mild mechanical disruption of the granule structure, and it is a recommended technique for characterisation. Organic solvents such as DMSO are not useful as media (rather than for subsequent characterisation) for studies of digestion, as these environments may inhibit hydrolytic enzyme action.

Once starch has been gelatinised, the granules have swollen to many times their original volume. In the absence of shear, the granules maintain their molecular integrity (Parker & Ring, 2001). The swelling of starch granules is quantitatively related to the pasting temperature: this is the first temperature at which the viscosity of a starch suspension rapidly increases on heating. Swelling of granules is accompanied by leaching of amylose molecules and lipids, normally bound in the granule, into the continuous phase (Bhattacharya, Sowbhagya, & Swamy, 1972, 1978). The presence of amylose and lipids in the granule is known to retard the swelling because amylopectin contributes mainly to the uptake of water during swelling (Sasaki & Matsuki, 1998; Tester & Morrison, 1990). The proportion of long chains in amylopectin correlates with the rate of starch swelling. Starches with higher swelling potential tend to contain higher proportions of long chains (DP $\geqslant$ 35) in amylopectin (Bogracheva, Wang, & Hedley, 2001a; Sasaki & Matsuki, 1998). Amylose and lipid content, and amylopectin structure, are therefore all related to the swelling and gelatinisation characteristics of starch granules.

Gelatinised starch samples are far more susceptible to degradation by α-amylase than are native starch granules. Furthermore, characteristics such as the amylopectin/amylose ratio and the amylose complexes with lipids affect the rate of hydrolysis. The extent of digestibility of starches generally decreases as the amylose content increases (Vesterinen, Myllärinen, Forssell, Söderling, & Autio, 2002), although amylose content alone is not a sole predictor of digestibility (Htoon et al., 2009; Lopez-Rubio, Flanagan Bernadine, Shrestha Ashok, Gidley Michael, & Gilbert Elliot, 2008). Similarly, amylose complexed with lipid is more resistant to attack by hydrolytic enzymes than is free carbohydrate (Nebesny, Rosicka, & Tkaczyk, 2004).

The molecules in a gelatinised starch sample, when stored under certain conditions, can undergo inter- and intra-molecular association into an ordered structure; this is the process known as retrogradation. The association of amylose and amylopectin crystals as gelatinised samples are cooled can lead to the formation of a gel, or gelation, during the retrogradation process (Ring et al., 1987). To retrograde, amylose must first be heated to greater than 100 °C, destroying the granular architecture (Miles, Morris, Orford, & Ring, 1985). Amylopectin recrystallises at a rate which is much slower than that at which amylose forms its single or double helices, and the amylose-derived helices can thus aggregate (Baik, Kim, Cheon, Ha, & Kim, 1997). Consequently, the duration of the first stage of retrogradation depends on the amylose content of starch. High molecular weight amylose promotes retrogradation more than lower molecular weight polymers suggesting that the molecular weight distribution of amylose long-chain branches (and the main chain of the amylose) in the sample are one of the determinants of the temperature of the initial stage of retrogradation (Tsai & Lii, 2000). The later stages of retrogradation correlates with the chain-length distribution of the amylopectin, in particular the proportion of short A chains (Fredriksson, Silverio, Andersson, Eliasson, & Aman, 1998; Silverio, Fredriksson, Andersson, Eliasson, & Aman, 2000; Tsai & Lii, 2000). It is noted that the correlations cited here are not necessarily causal. The retrogradation of starch has been extensively studied by using several different techniques including differential scanning calorimetry (DSC) (Jane et al., 1999; Lai, Lu, & Lii, 2000; Tako & Hizukuri, 2000; Tsai & Lii, 2000), XRD (Jagannath, Jayaraman, Arya, & Somashekar, 1998;

Jouppila, Kansikas, & Roos, 1998), enzyme kinetics (Kim, Kim, & Shin, 1997; Tsuge, Tatsumi, Ohtani, & Nakazima, 1992), and dynamic viscoelastic measurements (Morikawa & Nishinari, 2000; Otobe, Yoshii, Sugiyama, & Kikuchi, 1995; Tako & Hizukuri, 2000).

Starch granules can display different diffraction patterns (via XRD) depending on their botanical origin; an A-type for normal starches and B-type for tuber starches (Katz, 1930) (Fig. 2). Differences between the two allomorphs relate to the relative amounts of water and the organisation of the double helices in the unit cell of the crystal (Bogracheva, Wang, & Hedley, 2001b; Bogracheva, Wang, Wang, & Hedley, 2002). Amylopectin molecules in A-type starches have a larger number of short-chain fractions (Hizukuri, 1985), but the crystallite is more susceptible to enzymic attack over longer times (Planchot, Colonna, & Buleon, 1997).

In native starch, the fraction of double helical order (Fig. 3) is significantly greater than crystalline order, suggesting not all helical formations are involved in starch crystallites (Cooke & Gidley, 1992). Even so, helical starch molecules resist amylase hydrolysis, explaining, at least in part, why high amylose starches resist digestion more than waxy varieties, even though they are often less crystalline (Tester, Qi, & Karkalas, 2006).

Crystallinity of the starch granule is not the only structural property affecting enzymic degradation. Amylose–lipid complexes also affect the rate of digestion. These complexes accumulate in the granule as starch granules are degraded (Gernat, Radosta, Anger, & Damaschun, 1993). Whether amylose–lipid complexes observed are always present or formed during digestion is still unknown, although they are resistant to enzymic attack.

5. Digestive enzymes

There are many hydrolytic enzymes within the digestive tract of animals that catalyse the breakdown of polymeric macromolecules. Rates of enzymic action are very dependent on conditions such as temperature and pH, although they occur generally at the optimal pH of ~5 and at temperatures around 37 °C. Two basic types of enzyme catalyse polysaccharide degradation. Although phosphorylases play a key role in the metabolism of starch in the plant kingdom, we concentrate here on the action of specific hydrolases in the digestion of carbohydrates in mammals (Bae et al., 2005; Schinzel & Nidetzky, 1999).

*Endo*hydrolases (Werner & Keilich, 1965) are generally excreted by cells and so operate outside them. Carbohydrate endohydrolases cleave large carbohydrates that are unable to diffuse into cells, to give smaller products that can traverse cell membranes. Endohydrolases randomly cleave a hydrated starch molecule into two smaller molecules; in the specific case of α-amylase this is done by cleaving any accessible α (1,4) bond. *Exo*hydrolases (Akerberg, Zacchi, Torto, & Gorton, 2000) release a monomer or dimer from the non-reducing end of the substrate molecule. Enzymes such as amyloglucosidase, and β-amylase, release glucose or maltose units, respectively, from the non-reducing end of starch and oligosaccharides.

The surfaces of starch granules are affected in different ways by enzyme action, as seen by light microscopy, confocal laser scanning microscopy (Apinan et al., 2007), and SEM (Tester, Morrison, Gidley, Kirkland, & Karkalas, 1994; Zhang, Ao, & Hamaker, 2006) (Fig. 4). Many different modes of enzyme attack have been identified, including pin-hole, sponge-like erosion, medium-sized holes, single holes in individual granules, and surface erosion (Sujka & Jamroz, 2007). Depending on the enzyme and variety of starch, enzymes can erode the entire granule surface (exo-corrosion) or digest channels at specific locations on the surface towards the centre (endo-corrosion). Starches from wheat, barley and rye have specific 'susceptible zones' that become pitted as a result of endo-corrosion (Fig. 4). Pits become enlarged and form numerous channels within the granule, thus weakening its structure. The granules eventually fragment, leaving behind residual starch (Fig. 4d). Although the physiochemical properties of the crystalline and

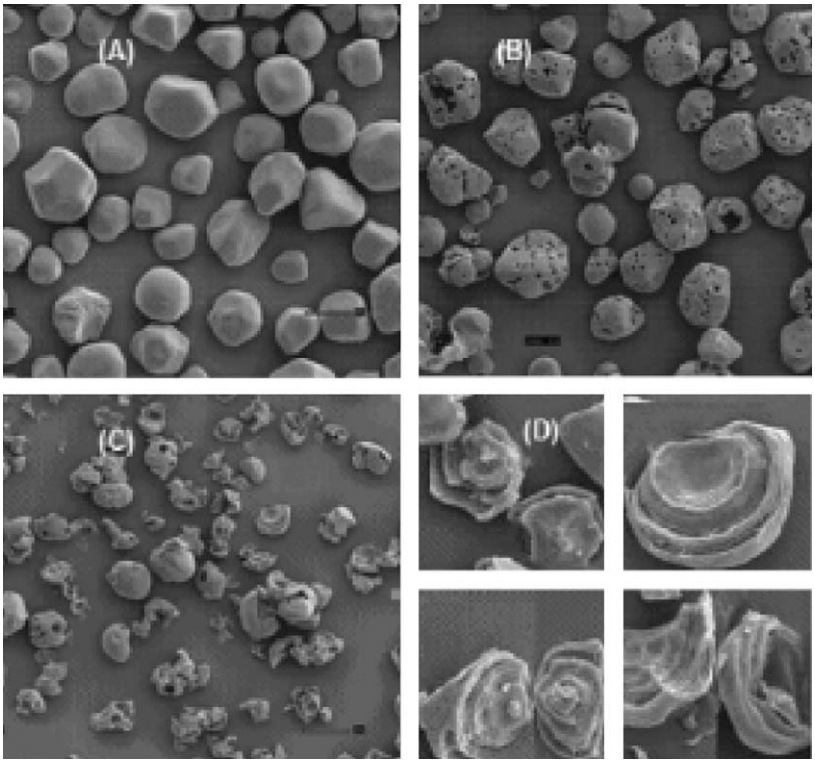

Fig. 4. SEM images (Zhang et al., 2006) of normal maize starch hydrolysed for: (A) 0 min (control); (B) 40 min (note the pit-hole action of α-amylase and amyloglucosidase); and (C) 120 min. The final image, D, shows residual pyramid shaped fragments that remain after digestion.

amorphous regions of the granule are different, the residual starch after digestion has a virtually unchanged debranched distribution, as measured by size-exclusion chromatography with multiple-angle laser light scattering and refractive index detection (Zhang et al., 2006). A similar profile after debranching implies a progression of digestion through the crystalline and amorphous lamellae; this is also seen with SEM (Zhang et al., 2006) (Fig. 4). Residues left after digestion also show comparable properties, such as gelatinisation temperature and enthalpy, with native granules as analysed by differential scanning calorimetry (Zhang et al., 2006).

High amylose starches characterised at different stages of *in vitro* digestion display properties that suggest that amorphous areas of the granule are hydrolysed by α-amylase, leaving the helical crystalline region intact. Analysis with SEM, small-angle X-ray scattering (SAXS), infrared spectroscopy, solid state ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and XRD show that an increase in the molecular order is favoured by the hydrolytic action of the enzyme (Lopez-Rubio et al., 2008). The changes suggest that resistant starch (RS) does not have a specific structure in pre-digested samples, but may be formed during digestion by a rearrangement of amylose chains into resistant structures of higher crystallinity (Leloup, Colonna, & Ring, 1991; Oates, 1997). Consequently, the resistance to enzyme digestion is a result of competition between the kinetics of enzyme hydrolysis, and the kinetics of amylose retrogradation. Furthermore, when hydrolysis is stopped after different times, the yield of high amylose RS fragments diminishes, but the size of the fragments stays constant (Jane & Robyt, 1984). This is attributed to the amorphous regions being digested more readily, leaving the chains in the helical crystalline regions (Fig. 3).

5.1. Human digestive system

Starch is met by salivary amylase in the mouth, which is the first enzyme to act on carbohydrates during digestion (Hoebler, Devaux, Karinthi, Belleville, & Barry, 2000). In a relatively short time, the bolus of food is carried by oesophageal peristalsis into the stomach. One of the key cells in the stomach for starch digestion is the parietal cell, which secretes HCl. The pH of the gastric juice is ~2.6, retarding the action of α-amylase but increasing the acid hydrolysis of starch. Also in the upper gastrointestinal tract, lipids bound to starch are hydrolysed by lipases secreted by various exocrine glands. A key step in lipid digestion is the creation of an emulsion that increases the area of the oil–water interface enabling more efficient action of the enzymes. The emulsion is produced first through the mechanical process of mastication and then peristaltic movements of the digestive tract. The emulsion is stabilised by its droplets being coated with membrane lipids, denatured proteins, and fatty acids, thus preventing them from coalescing (Boron & Boulpaep, 2009).

From the stomach, the ingested food proceeds to the duodenum where it encounters the pancreatic secretion whose rate of release is controlled by 'satiety' hormones. Pancreatic fluid contains two important components for starch digestion. Sodium hydrogencarbonate(bicarbonate) neutralises the acidity of the fluid arriving from the stomach to a pH of ~8. Pancreatic fluid also contains α-amylase that continues the hydrolysis of starch into glucose and oligosaccharides. The latter include linear and branched structures that are not absorbed into the bloodstream without further hydrolysis to glucose. Glucose is only a very minor product of endohydrolases, such as α-amylase which operates during the initial stages of digestion, so further enzymic processes are definitely required.

Substrates not digested by α-amylase, such as α-limit dextrins, and small linear oligomers, along with larger α-glucans, are later degraded into single glucose units. This conversion occurs via enzymes that are integral to the plasma membrane of enterocytes in the small intestine; these include mucosal maltase–glucoamylase, and sucrase–isomaltase. These are exo-glucosidases that act on the non-reducing end of glucose oligomers and catalyse not only the hydrolysis of α (1,4) bonds but also to a lesser extent that of α (1,6) branch bonds, ensuring further degradation of nonlinear oligosaccharides. The resulting monosaccharides, such as glucose and galactose, are absorbed by secondary active transport across the apical membrane of enterocytes and subsequently exit the gastrointestinal tract across the basolateral membrane, into the bloodstream (Boron & Boulpaep, 2009).

The large intestine (colon) contains a very large population of micro-organisms (showing considerable variation between individuals) that are beneficial to the digestion of polysaccharides in humans who might be enzyme-deficient, or in normal subjects with carbohydrates that have not been broken down in the jejunum and ileum. The bacteria inhabiting this segment of the digestive tract metabolise, through fermentation, undigested polysaccharides like RS and soluble fiber. Fermentation creates short-chain fatty acids, which stabilise blood glucose levels and suppress cholesterol synthesis in the liver (MacFarlane & MacFarlane, 2006; Wong, de Souza, Kendall, Emam, & Jenkins, 2006).

6. Digestibility indices

In the last 25 years the *in vivo* digestibility of foods has been classified by a number of metrics, the most popular of which is the Glycemic Index (GI). The GI was first defined by Jenkins et al. (1981) as the total glycemic response in the blood [area under the curve of glucose concentration versus time (Table 1)] in the 2 h period immediately subsequent to the consumption of a fixed amount of carbohydrate; it is expressed as a value relative to that of a standard food, normally white bread or glucose (Roberts, 2000). Many intrinsic and extrinsic factors affect the nature of starch and so affect the GI of a food, including starch structure at all six levels (Fig. 1); these factors are gastrointestinal motility, the method of cooking, and the presence of fibers, fats and proteins (Krezowski, Nuttall, Gannon, & Bartosh, 1986; Thorne, Thompson, & Jenkins, 1983). The extent of starch retrogradation, the granular morphology and the dietary fiber content are factors that have been examined *in vitro* (Urooj & Puttraj, 1999).

Through the process of *retrogradation*, gelatinized or solubilised starch can be transformed from an unstructured into a more ordered or crystalline state. This large physical change causes heat-processed starchy foods to harden or become stale as they spontaneously approach a metastable state of lower free energy. This has been reported to decrease the GI value, due to an increased resistance to amylase (Chung, Lim, & Lim, 2006).

Other systems used to rank the *in vivo* digestibility of carbohydrates are Glycemic Load (GL), Glycemic Response, and RS (Section 7.1). The GL is a metric used as a basis for weight loss, or for diabetes control. Also controversial (Das et al., 2007), the GL is calculated by multiplying the GI by the percentage of carbohydrate present within the food consumed. Like the GI, the GL has a scale used to collect food varieties into groups suitable for certain diets. The Glycemic Response is a measure of the increase in the glucose concentration in the blood at any given time after the ingestion of carbohydrate and RS, along with the other starch fractions (Section 7.1).

Conducting experiments on commercial foods to estimate their GI, or other *in vivo* measures, is an expensive process, so recent research has been aimed at developing *in vitro* methods to predict GI or an equivalent metric (Garsetti et al., 2005; Goni, Garcia-Alonso, & Saura-Calixto, 1997; Okuda, Aramaki, Koseki, Satoh, & Hashizume, 2005) (Section 7).

Table 1
Methods of kinetic analysis of the enzymic digestion of starch sorted by type of model.

	Source and treatment	Techniques used	Model used	Results	Commentary
In vivo monitoring of starch digestion					
Jenkins et al. (1981), Ross, Brand, Thorburn, and Truswell (1987)	Range of carbohydrate-based meals tested prepared using a range of cooking methods	Blood glucose concentrations of subjects measured in 30 min intervals after consumption of commercial, starch-based products	GI calculated by comparing area under the curve of blood glucose concentration for 2 h after ingestion relative to a white bread standard	Commercial products GI ranged from 30–130 AUC. Products with a GI below 55 AUC are generally considered healthier	– The GI measure of starch hydrolysis to glucose is dependent on many factors including the age, sex, race, protein ingestion (Krezowski et al., 1986), food processing (Brand, Nicholson, Thorburn, & Truswell, 1985), and health (Brand-Miller et al., 2002; Jenkins et al., 2002; Thorne et al., 1983) of the subject. Also GI only considers glucose absorption into the blood stream leaving other sugars unconsidered. The meaningfulness of this measure remains controversial (DeVries, 2007)
Exponential kinetic models					
Frei et al. (2003), Goni et al. (1997)	Range of carbohydrate-based meals tested prepared using a range of cooking methods	Digestions performed both *in vivo* and *in vitro* using α-amylase and amyloglucosidase. The areas under the curves are compared for various foods	A simple exponential model is used to calculate the area under *in vitro* digestion curves	The *in vitro* data show a correlation with the *in vivo* GI data	– No mechanistic basis for the exponential model used to analyse *in vitro* data – Time points only taken every 30 min both *in vivo* and *in vitro* leaving 'interesting' parts of the digestion unmonitored during *in vitro* analysis
Apar & Ozbek (2007)	No prior treatment, rice starch granules digested at 60 °C and pH 6.5	Digestion time courses of α-amylase monitored over the first 10 min of digestion with residual starch concentration measured by iodine binding	Simple exponential model fitted to a range of initial concentrations. Initial velocities analysed using Lineweaver–Burk plots	Both glucose and maltose cause uncompetitive product inhibition	– No mechanistic basis for the exponential model used to analyse data – Only the first 10 min of digestion was monitored – Remaining starch determined by iodine binding rather than sugar production measured by reducing power of solution, decreasing reliability
Wang et al. (2006)	No prior treatment, corn starch granules digested in an incubator at 50 °C	Digestion of starch using amyloglucosidase monitored by glucose assay and spectrophotometry	Simple exponential model used to describe the product inhibition of glucose	– Glucose found to inhibit amyloglucosidase after a "grace" period at low glucose concentrations – Optimal activity at 40 °C	– Mathematical model was created although no simulations of the model or fit to the data were shown – No mechanistic basis for the exponential model used to analyse *in vitro* data
Al-Rabadi et al. (2009)	Barley and sorghum starch digested at 37 °C in three stages with pH ranging from 2.0 to 7.0	Digestion performed mimicking conditions *in vivo*. Mechanical shaking of samples along with α-amylase and amyloglucosidase solutions subsequently followed by glucose assay and spectrophotometry	Simple exponential model fitted to fraction of digested starch using linear fits to logarithmic plots	– First order kinetics describes grain fragment digestion – Small starch particle sizes increase the rate of enzymic action	– Although model closely describes starch digestion, only the SDS stage was monitored as during the first 30 min of digestion no aliquots were taken
Starch fractions determined, no fitted model					
Benmoussa, Moldenhauer, and Hamaker (2007), Englyst and Hudson (1996), Pizzoferrato, Rotilio, and Paci (1999)	Englyst – Starch based meals prepared using various cooking techniques but digested at 37 °C and pH 5.2 Pizzoferrato – Chestnuts roasted and ground and incubated at 37 °C and pH 5.0 Benmoussa – Rice flour samples were boiled for 20 min and digested at 37 °C	*Englyst* test (Englyst et al., 1992) used to quantify hydrolysis	Fractions quantified for commercial foods and various starches of differing fine structure	Amylopectin fine structure correlated to digestibility values (RDS and SDS)	– Englyst and related methods only uses two time points during a digestion, leaving the mechanism of starch hydrolysis unconsidered

(*continued on next page*)

Table 1 (*continued*)

	Source and treatment	Techniques used	Model used	Results	Commentary
Chung et al. (2006)	Waxy rice starch was gelatinised at various temperatures for 5 min and digested at 37 °C	Englyst digestion values and time courses of α-amylase digestion plotted	Fractions quantified although no kinetic model fitted to time courses	Gelatinized and retrograded samples digestibility values compared	– Glucose concentration measured while other oligosaccharides ignored
Ao et al. (2007), Zhang et al. (2006)	Zhang – Maize, potato and wheat starch with no prior treatment were incubated at 37 °C and pH 5.2 Ao – Maize starch heated to 80 °C for 10 min, autoclaved for 20 min then incubated at 50 °C and pH 5.0 during digestion	Englyst digestion values and time courses of α-amylase and amyloglucosidase digestions of native and modified starches from different varieties were characterised	Fractions quantified although no kinetic model fitted to time courses	Modified starches with larger branch density and smaller chain length have slower digestion properties	– No attempt is made at characterising digestion by using a kinetic model
Manelius et al. (1997), Manelius et al. (2000), Shu et al. (2006)	Manelius – maize and wheat starch digestion performed at 25 °C and pH 6.5 with no prior hydrothermal treatment Shu – rice starch samples cooked at 50 °C and digested at 37 °C	Total reducing sugar after digestion with α-amylase determined for native and resistant starch varieties. Also different degrees of substitution and different sized granules were analysed using reducing sugar assay or gel-permeation chromatography	Time points taken every >30 min although no model used to fit data	Resistant starch demonstrated slow and incomplete hydrolysis. Furthermore, high degrees of substitution and large-granule sizes decrease the rate of enzymic attack	– No kinetic model used to analyse *in vitro* data – Time points only taken every >30 min leaving 'interesting' parts of the digestion unmonitored
Bertoft and Manelius (1992)	Waxy maize starch boiled for 60 min then digested at 23 °C and pH 5.5	α-Amylase digestion monitored by gel-permeation chromatography at various time points	No kinetic model used to fit to the data from chain-length distributions	The method described allows for the hydrolysis of granules to be monitored and the products collected at any stage during a digestion	– Recovery of sample from chromatography is not complete (Ward et al., 2006) – No attempt made to describe the digestion of starch using a kinetic model
Michaelis–Menten multiple stage model					
Dona et al. (2009)	Granular wheat and rice starch suspensions were digested at pH 5.2 and 25 °C without prior hydrothermal treatment	Digestion of wheat flour and rice starch by both α-amylase and amyloglucosidase monitored by ^{1}H NMR spectroscopy	RDS and SDS stages of starch digestion analysed separately, initially using Lineweaver–Burk plots	Both stages of starch digestion could be characterised separately using classical Michaelis–Menten kinetics	–Lineweaver–Burk plots used have large systematic errors
Michaelis–Menten without inhibition or inactivation					
Nitta et al. (1979)	Soybean starch and potato amylose suspensions were digested at a range of pHs at 25 °C without prior hydrothermal treatment	The kinetics of β-amylase action of soy bean starch was analysed over a range of pH values by testing the reducing end concentration over time	Data fitted to Hanes–Woolf plot to find Michaelis parameters at various pH values and a model developed to predict degradation at a given pH	The optimal pH of β-amylase was found to be 5.85	– An elaborate kinetic model was designed to consider enzymic action and pH dependence although no examples of the predictions of the model were compared to a digestive time course
Heitmann, Wenzig, and Mersmann (1997)	Potato starch digestion of samples incubated at 60 °C	α-Amylase digestion monitored by GPC	Reducing sugar liberated estimated by Michaelis–Menten analysis	Kinetics of three native starches fitted using Lineweaver–Burk analysis	– Very limited time points, whole time course not considered – Recovery of sample from chromatography is not complete (Ward et al., 2006)
Park and Rollings (1995)	Purified amylose and amylopectin from Sigma incubated for 30 min at 50 °C in H_2O/DMSO mixture	α-Amylase digestion monitored by SEC with low angle laser light scattering	Reducing sugar liberated estimated by Michaelis–Menten analysis	Kinetics of amylopectin and amylose compared so branching density affect on digestion characterised	– Same criticisms as above – Digestion halted by NaOH although starch is further degradated in alkaline environments – Very limited time points, whole time course not considered
Akerberg et al. (2000)	Wheat starch heated to 90 °C for 15 min and then incubated at 30 °C and pH 5.0 during digestion	Digestion with both α-amylase and amyloglucosidase was characterised by HPAEC	A model based on Michaelis–Menten kinetics considering hydrolysis by both enzymes and various sizes of substrate along with pH dependence was simulated	Various starch concentrations had their products during digestion simulated accurately	– Although digestions were run over 80 h normally only ~10 measurements were taken of products – The number of variable parameters in the kinetic model created allows the simulated model to fit experimental data accurately with many different parameter values

Table 1 (*continued*)

	Source and treatment	Techniques used	Model used	Results	Commentary
Amato et al. (2004)	Wheat flour suspensions with no prior hydrothermal treatment monitored at 27 °C and pH 6.8	Digestion of wheat flour by endogenous enzymes monitored by HR MAS NMR spectroscopy	Michaelis–Menten model without inhibition or inactivation	– No inhibition occurs during hydrolysis – Spectroscopy can be used to monitor hydrolysis	– HR MAS spectroscopy takes ~30 min to prepare the sample for analysis after enzyme addition, missing the initial stages of digestion
Considering the adsorption of enzyme onto substrate before enzymic hydrolysis					
Slaughter, Ellis, and Butterworth (2001)	Wheat, potato and rice starch suspensions were incubated at 37 °C and pH 7.4 during digestion	Monitoring α-amylase digestion by means of reducing-end assay	Freundlich plot considering adsorption used to analyse suspended samples and regular Michaelis–Menten used for gelled starch	A linear relationship found with Freundlich plot suggesting enzyme adsorption onto the surface of starch limits the rate of suspended starch digestion	– No fit to the integrated Michaelis–Menten equation was displayed for either gelled samples of suspended samples after considering adsorption step
Product or substrate inhibited Michaelis–Menten					
Fujii and Kawamura (1985)	Starch of unspecified source incubated at 30 °C and pH 5.25	Digestion with both α-amylase and amyloglucosidase were characterised by measuring the reducing power of aliquots	Product-inhibited Michaelis–Menten model used to estimate kinetic parameters by used of Lineweaver–Burk plots	The action of α-amylase can be neglected after substrate molecular weight decreases below 5000	– Digestion halted by strong alkaline solution although starch is further degraded in alkaline environments – Product inhibition equation used to fit data although other papers report no inhibition of glucosidases
Pastrana et al. (1998)	Starch of unspecified source incubated at 23 °C and pH 5.37	Digestion by glucoamylase was monitored by glucose assay and diffusional restriction in starch solutions of varying concentration	Kinetic model used to fit digestion data was classical Michaelis–Menten multiplied by an exponential describing restriction of the enzyme due to an increase in solution viscosity	Digestion is inhibited by the substrate only due to changing rheological properties of the reacting system, not by classical substrate inhibition effects	– The rheological properties of starch solutions depend greatly upon the preparation technique (autoclaved or heated or suspended only). This paper has not explained how starch solutions were prepared although rheological properties were used to explain inhibition

Acronyms: AUC, area under curve; HR MAS, high-resolution magic-angle spinning; RDS, rapidly digested starch; SDS, slowly digested starch.

6.1. Glycemic Index

The GI of food is a measure of the rate at which the contained carbohydrate is hydrolysed in the digestive system and absorbed via active and facilitated diffusion across enterocyte membranes into the bloodstream. The immediate effects of carbohydrates on an individual's blood glucose concentration are of interest not only for nutritional guidance but the glucose concentration has various health implications (Jenkins et al., 2002). The human subjects used to test varieties of food must be carefully chosen, as the rate of digestion of carbohydrates varies with health status, race and gender. Although widely used, both for research and commercially, the validity of the GI as a guide for dietary design is controversial. Skeptics question fundamental properties of this functional measure, including its reproducibility and therefore its meaning (Barclay et al., 2008; DeVries, 2007; Livesey, Taylor, Hulshof, & Howlett, 2008), as well as its relevance to diseases with a nutritional basis.

6.2. Health inferences of GI

Although controversial, the GI has proven useful as a numerical classification in the treatment of diabetes (Wolever et al., 2008). This health inference derives from blood glucose concentration and the insulin response of diabetics, relating directly to the rate of digestion of carbohydrates (Jenkins et al., 2002). Obesity, which eventually leads to a variety of circulatory and respiratory problems, is another major aspect of health that can be related to GI. Obesity was once thought to be best controlled by low-fat, high-carbohydrate diets, although commonly these are counterproductive to weight loss due to promotion of carbohydrate oxidation at the expense of fat oxidation, encouraging body fat gain (Brand-Miller, Holt, Pawlak, & McMillan, 2002). The consumption of high-GI foods can therefore be related to obesity for physiological reasons, but obesity is also thought to develop from an altered appetite (Thornley, McRobbie, Eyles, Walker, & Simmons, 2008). Foods that are hydrolysed relatively quickly in the digestive system leave subjects hungry in a relatively short time, increasing a tendency to eat between meals. This increased appetite created by high-GI foods is also thought to contribute to obesity (Roberts, 2000).

7. *In vitro* starch digestion

The digestibility of starch measured *in vivo* is a time-consuming, expensive process that requires many human subjects with specific attributes. Since there is no human subject dependence on the measurement of *in vitro* starch digestion, investigation of *in vitro* digestibility as a replacement for the GI is an increasingly researched topic (Table 1) (Frei, Siddhuraju, & Becker, 2003; Goni et al., 1997).

7.1. Starch fractions

For nutritional purposes, starch can be classified into three categories by the Englyst test (Englyst, Kingman, & Cummings, 1992), depending on their rate and extent of digestion; these include rapidly digested starch (RDS), slowly digested starch (SDS), and resistant starch (RS). The kinetic time course of the digestion of suspended starch samples reveals very different rates between stages, suggesting that a change in the physical nature of the substrate determines the kinetics of digestion (Table 1).

RDS is the fraction of starch granules that cause a rapid increase in blood glucose concentration after ingestion of carbohydrates. The fraction of starch that is said to be RDS *in vitro* is defined as the amount of starch digested in the first 20 min of a standard digestion reaction mixture (Englyst et al., 1992). In practice, the end of the RDS stage is considered to involve a change in the properties of the substrate, causing a transition in the rate of oligosaccharide or glucose production. The 'extent of reaction' at which this occurs can vary, depending on the conditions of digestion and of course will not always occur in the first 20 min after enzyme addition (Akerberg et al., 2000; Ao et al., 2007; Apar & Ozbek, 2007; Wang, Zeng, Liu, & Yuan, 2006); this time depends on how much enzyme is added. Although RDS is defined by experimental analysis of digestion *in vitro*, the rate of starch conversion to sugar follows similar kinetics in the human digestive system. Both free sugar and RDS in a meal must travel to the small intestine before the majority of the sugar is absorbed into the bloodstream. Coincidently, RDS is not to be confused with the total sugar content of a meal, which also contributes to the rate of increase in blood glucose concentration.

SDS is the fraction of starch that is digested slowly but completely in the human small intestine. From studies of *in vitro* digestion it is clear that there is a transition in the smoothness of the progress curves of reducing sugar production from RDS to SDS (Figs. 5 and 6). SDS is defined as the starch that is digested after the RDS but in no longer than 120 min under standard conditions of substrate and enzyme concentration (Englyst et al., 1992). The potential health benefits of SDS *in vivo* include stable glucose metabolism, diabetes management, mental performance, and satiety (Lehmann & Robin, 2007). Commercially, there are no SDS products available, although new slowly digestible carbohydrates (SDCs) such as isomaltulose (Palatinose, *Palatinit*) have been commercialised. These substances yield a slow and sustained blood glucose concentration after intake (Lina, Jonker, & Kozianowski, 2002).

The fraction of starch that escapes digestion in the small intestine, and may be subject to bacterial fermentation in the large intestine, is termed RS, derived from *in vitro* studies where starch undergoes limited enzymic hydrolysis. RS can have both beneficial and detrimental effects on a person's health; e.g., polysaccharides undigested until reaching the large intestine become metabolised to short-chain fatty acids by bacteria. Saturated fatty acids are commonly linked to cardiovascular disease including heart attack, heart failure, and stroke. On the other hand, the beneficial action of RS is similar to that of fibrous polysaccharides that resist endogenous human enzymes. Dietary fibre is composed mostly of non-starch polysaccharides (NSPs) such as arabinoxylan and β-glucan. By decreasing the plasma insulin and glucose response, NSP and RS are acknowledged as important contributors to the prevention and management of diabetes, obesity, and colon and rectal cancers (Topping et al., 2008). A range of physiological influences affect starch digestibility: e.g., people who chew their food sparingly swallow larger particle sizes. Larger particles allow less access for the digestive enzymes and have a faster oro-ileocaecal transit time, making the presence of RS more likely, therefore increasing the contribution to the various health benefits associated with RS.

8. Kinetic models of *in vitro* digestion

As discussed above, the trajectory of digestion time courses is changed dramatically by variations in the methods of starch preparation; starch digestibility depends on the many physical properties of the starch granule (Fig. 1). This section describes a range of kinetic models that are summarised in Table 1; these have been used to characterise the kinetics of starch digestion, in an attempt to correlate digestibility and the physical properties of starch granules.

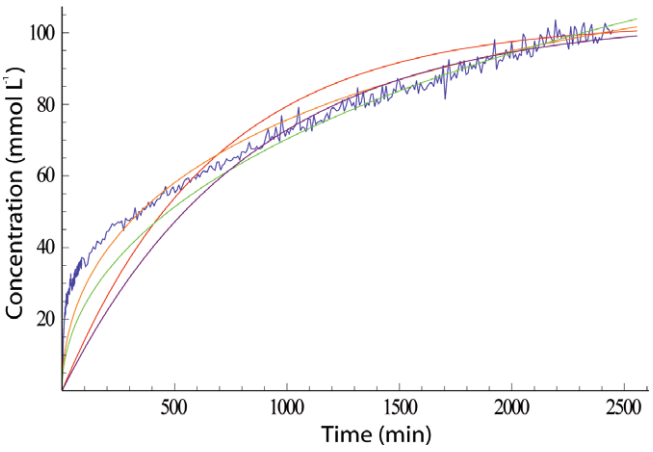

Fig. 5. Time courses of starch hydrolysis simulated with various enzyme kinetics models (real data in blue). The fitting of parameters used least squares regression analysis (also see Fig. 6). For all models: $C_0 = 130$ mmol L^{-1}. Red – Michaelis–Menten where $K_m = 8.9 \times 10^2$ mmol L^{-1} and $V_{max} = 1.3$ mmol L^{-1} min^{-1} (Eq. (2)); Orange – Michaelis–Menten with product inhibition where $K_m = 1.5$ mmol L^{-1}, $V_{max} = 86$ mmol L^{-1} min^{-1} and $K_i = 6.2 \times 10^{-4}$ mmol L^{-1} (Eq. (8)); Green – Michaelis–Menten with high-substrate inhibition where $K_m = 5.3 \times 10^2$ mmol L^{-1}, $V_{max} = 9.1 \times 10^{-1}$ mmol L^{-1} min^{-1} and $K_s = 8.4 \times 10^{-3}$ mmol L^{-1} (Eq. (9)); and Purple – Michaelis–Menten with diffusional restriction where $K_m = 1.3 \times 10^5$ mmol L^{-1}, $V_{max} = 5.7 \times 10^{-2}$ mmol L^{-1} min^{-1}, $R_m = 2.1$ and $\mu = 3.8 \times 10^3$ $mmol^{-1}$ L (Eq. (10)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

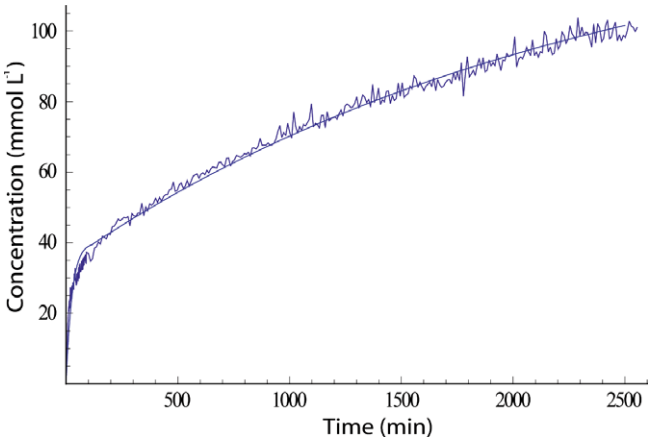

Fig. 6. Digestion of 6% (w/w) starch suspended in H_2O with 12 U mL^{-1} α-amylase at 25 °C. The RDS (<120 min) fitted with the integrated Michaelis–Menten equation (Eq. (2)) used parameters $K_m = 1.4 \times 10^2$ mmol L^{-1} and $V_{max} = 5.3$ mmol L^{-1} min^{-1} and the SDS (>120 min) had parameters $K_m = 3.6 \times 10^2$ mmol L^{-1} and $V_{max} = 1.9 \times 10^{-1}$ mmol L^{-1} min^{-1}.

8.1. Monitoring digestion

8.1.1. Reducing capacity

Digestion of starch is most commonly monitored by measuring the reducing capacity of the filtrate of aliquots taken at intervals during the reaction (Goni et al., 1997; Sayago-Ayerdi, Tovar, Osorio-Diaz, Paredes-Lopez, & Bello-Perez, 2005; Shu et al., 2006). For exohydrolases, such as amyloglucosidase, an exact concentration of product can be determined by this method, due to the specificity of the enzyme. When this method is used for α-amylase, the concentration of oligosaccharide can be estimated, although the

amount of starch left undigested must be determined by weighing the residual substrate, after filtration and drying. Measuring both oligosaccharide production and remaining substrate allows for a number-average weight of the products to be calculated, as α-amylase can produce a range of products from glucose to large-branched limit dextrins (Manelius, Qin, Avall, Andtfolk, & Bertoft, 1997). The oligosaccharide products can be converted to glucose via an exohydrolase and the glucose concentration measured using a glucometer, to estimate the amount of starch digested (Aarathi, Urooj, & Puttaraj, 2003).

Strong alkali which is generally used to terminate enzymic reactions is known to hydrolyse polysaccharides. This leads to a change in the reducing capacity of the solution after the digestion is presumed to have ceased. The estimated reducing capacity will therefore always be biased when hydroxides are used.

8.1.2. Chromatography

Digestion can be monitored by two different chromatography techniques (Gidley et al., 2010; Morell, Samuel, & O'Shea, 1998). Size-exclusion chromatography (SEC, often termed gel-permeation chromatography, GPC) can be used to measure the evolution in the molecular size distribution of the carbohydrate substrate during the course of digestion. It is essential to realise that SEC separates by size (as indicated by its name), not by molecular weight, and for a branched polymer such as starch there is normally no unique relationship between hydrodynamic radius and molecular weight (although there is a unique size/molecular weight relationship for linear polymers). Obtaining the full, quantitative size distribution of starch in a sample, without degradation, is not a simple matter (Cave, Seabrook, Gidley, & Gilbert, 2009; Gidley et al., 2010; Gray-Weale & Gilbert, 2009; Hoang et al., 2008; Schmitz et al., 2009). When it becomes possible to achieve routinely such undegraded distributions, it is anticipated that such data, as a function of starch source and of time and site of digestion, will be of considerable help in understanding digestive mechanisms.

Fluorescence-assisted capillary electrophoresis (FACE) (Morell, Samuel, & O'Shea, 1998; O'Shea et al., 1998), and high-performance anion-exchange chromatography (HPAEC) are used to obtain the distributions of oligosaccharide products, including the chain-length distribution of the amylopectin after treatment with a debranching enzyme (Manelius, Nurmi, & Bertoft, 2000). These techniques are restricted to relatively low degrees of polymerisation (less than ~80 anhydroglucose units) and so cannot characterise chain-lengths distributions of amylose. The latter can be determined by using SEC.

8.1.3. ^{1}H NMR spectroscopy

Techniques that can directly reflect the concentration of sugars in a solution, including NMR spectroscopy (Berthon & Kuchel, 1995; Dona, Pages, Gilbert, Gaborieau, & Kuchel, 2009; Kuchel, 1981, 1989), and high-resolution magic-angle spinning (HR MAS) ^{1}H NMR, have been implemented (Amato et al., 2004). The initial phase of the reaction is often overlooked when using HR MAS NMR, as there is an inability to acquire data at a sufficiently rapid rate, immediately after mixing the sample and enzyme.

Solution-state ^{1}H NMR spectroscopy is often used to monitor directly the kinetics of chemical processes in homogeneous, and heterogeneous, solutions such as starch mixtures (Dona et al., 2009; Minerath, Casale, & Elrod, 2008; Walker et al., 2007). Recently, NMR methodology has been developed to monitor progress of physical and chemical processes in heterogeneous starch suspensions (Dona et al., 2007, 2009) provided that: (1) the nucleus (nuclei) monitored is in solution during the entirety of the reaction; and (2) the heterogenous solution can be kept from settling (producing a vertical concentration gradient) during the time course. NMR spectroscopy only measures from a small portion of the sample (within the receiving coil), creating a bias in the measured concentration if a heterogeneous sample settles.

Introduction of ^{1}H NMR spectroscopy for the direct analysis of both α- and β-reducing ends of oligosaccharides produced during hydrolysis enables the accurate estimation of product formation during digestion of carbohydrate (Fig. 7). The method can be applied to many forms of starch including extracted, milled flour, and cooked, uncooked, macerated, and whole grain starch. Recording the progress of a reaction using NMR spectroscopy is less time-consuming than current assay techniques, and yields large data sets which include the initial RDS stage. Investigation of RDS is significant as the initial rate of absorption of sugar into the blood is determined by the RDS stage, and the total sugar content of a meal, thus directly affecting the *in vivo* measurement of starch digestion.

NMR spectroscopy, while able to monitor rigorously the kinetics of slow (minute time scale) reactions in solution, has some drawbacks. NMR equipment is expensive making it unsuitable for routine use as a large-scale industrial technique. Also, most applications of NMR require the solvents to be deuterated, adding to its expense.

8.2. Michaelis–Menten kinetics

Michaelis–Menten kinetics describes the action of many reactions where the enzyme concentration is small relative to the sub-

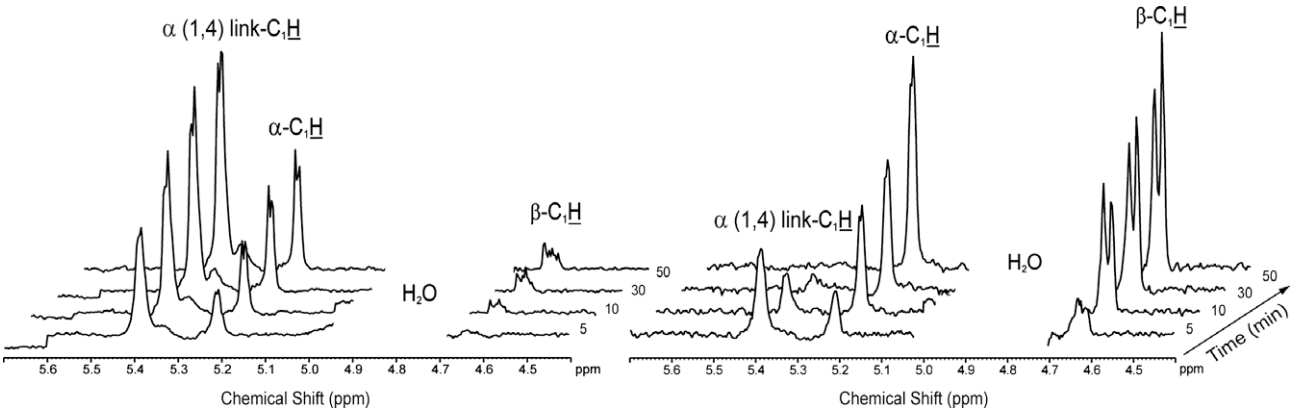

Fig. 7. ^{1}H NMR (400.13 MHz) spectra displaying the time dependence of digestion of starch (4% w/w) with: (A) α-amylase (3 U mL^{-1}); and (B) amyloglucosidase (0.3 U mL^{-1}). Adapted from Dona et al. (2009). The signal from both α and β-anomer reducing ends allow for measurement of the concentration of mono- (amyloglucosidase) or oligosaccharide (α-amylase). The α (1,4) bond signal at 5.4 ppm increases due to oligosaccharide production by α-amylase (A), and decreases as amyloglucosidase (B) hydrolyses all accessible α (1,4) bonds.

strate concentration. The derivation of the Michaelis–Menten equation assumes that a slow, product forming reaction follows the rapid, reversible formation of an enzyme–substrate complex (Eq. (1)),

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P, \quad (1)$$

where E is the enzyme, S is the substrate (starch) and P is the product. The Michaelis–Menten equation (Eq. (2)) is then derived by using the steady-state approximation for the ES complex: specifically the concentration of the enzyme–substrate complex is assumed to change much more slowly than the concentration of the substrate, so the rate equation takes the form,

$$\frac{d[P]}{dt} = \frac{V_{max}[S]}{K_m + [S]}, \quad (2)$$

where K_m is the Michaelis constant (Eq. (3)), and V_{max} is the maximum velocity of the reaction achieved when the enzyme active sites in the sample are all complexed with substrate all the time, and [P] is the concentration of product at any given time during the time course. The relationship between the K_m and the unitary rate constants in the reaction scheme is:

$$K_m = \frac{k_{-1} + k_2}{k_1}. \quad (3)$$

Taking the reciprocal of both sides of the Michaelis–Menten equation (Eq. (2)) gives the Lineweaver–Burk equation (Eq. (4)) that is often used to graphically analyse enzyme kinetic data. The equation is:

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}. \quad (4)$$

This relationship was used to estimate V_{max} and K_m values before the days of nonlinear least squares regression of Eq. (2) directly onto the data. The latter approach is now routinely used with readily available software packages (e.g., Castillo, Hadi, & Minguez, 2009).

Several time courses measured under identical conditions, except for the amount of enzyme used, should be superimposable when the time axis is scaled by the initial enzyme concentration. This is known as Selwyn's test (Selwyn, 1965); failure to pass this test means that the shape of the time course curve is not entirely governed by product formation or substrate consumption during the enzyme-catalysed reaction. Commonly, instability of the enzyme, or its aggregation, is responsible for this failure, although instability of substrate or product could also be factors. These problems are often rectified by changing the reaction conditions. Expressions have been derived to describe the initial reaction rate of a simple enzyme with a single substrate and unknown mechanism of inactivation (Schnell & Hanson, 2007). Unlike mechanism-based enzyme inactivation, product formation and enzyme inactivation are considered to occur independently of one another.

Hydrolysis of carbohydrates of similar properties to amylopectin, such as glycogen and many small soluble oligosaccharides, by glucohydrolases can be described by Michaelis–Menten kinetics (Table 1) (Inokuchi, Takahashi, & Irie, 1981; Miranda, Murado, Sanroman, & Lema, 1991). On the other hand, digestion of starch, and amylopectin samples digested with either α-amylase or amyloglucosidase, show clear deviation of time courses from those predicted by the Michaelis–Menten equation. This deviation cannot be a property of the enzyme, so it is concluded that the deviation is a consequence of either a chemical property of the substrate, such as an indigestible starch core, or a chemical property of the starch, such as its time-dependent interaction with water. Variation of starch hydration in solution during digestion affects the accessibility of the enzyme to the starch.

Substrate branching characteristics have been shown to influence depolymerisation reaction rates, as enzyme susceptibility decreases with increased branching density (Ao et al., 2007; French & Knapp, 1950; Kerr, Cleveland, & Katzbeck, 1951) (Section 9). In some cases, kinetic models that account for the multiple phases of starch degradation are used to give an explanation for the variations in starch hydrolysis rates with the changes in branch density of the starch sub-fractions (Dona et al., 2009; Park & Rollings, 1995) (Section 8.7). In an attempt to elucidate features of starch digestion, many other single-phase models have been proposed to describe the time courses, although they are generally found to be inadequate (Section 8.4–8.6).

8.3. Solutions of the Michaelis–Menten equation

As mentioned previously, the kinetic constants of the Michaelis–Menten equation can be estimated by using Lineweaver–Burk plots. Although this analysis avoids solving the Michaelis–Menten equation, the double reciprocal plot has a large systematic error. Fitting the transformation of an inherently nonlinear equation to experimental data distorts the errors in the measured variables, subsequently impacting on the veracity of estimates of the kinetic parameters. A more accurate graphical analysis of enzyme kinetics, the Hanes–Woolf plot, graphs the ratio of the initial substrate concentration to the reaction velocity plotted against substrate concentration (Nitta, Kunikata, & Watanabe, 1979). Even more statistically robust is the direct linear plot method of Eisenthal and Cornish-Bowden (1974). Direct linear plots are created by plotting experimental observations in parameter space and extrapolating lines through the observed points to a point of common intersection. Errors associated with experimental observations normally mean there is no unique intersection point; however, the most accurate estimates of the Michaelis parameters from direct linear plots are generally easily located (Eisenthal & Cornish-Bowden, 1974).

Integration of the Michaelis–Menten equation (Eq. (2)) yields an expression that relates time (t) to the concentration of substrate [S] at any given time. The solution therefore describes a reaction time course:

$$V_{max}t = ([S]_0 - [S]_t) + K_m \ln([S]_0/[S]_t), \quad (5)$$

where $[S]_0$ is the initial substrate concentration. The solution is nonlinear and clearly implicit with respect to the substrate concentration. An explicit form of the integrated Michaelis–Menten equation (Eq. (5)) is often approximated by introducing reasonable constraints, causing only one of the terms on the right hand side to dictate its value. If $[S]_0 \ll K_m$, then the first term dominates, restricting the value of the solution, indicating that the depletion of the substrate is approximated by a single exponential function. If the opposite occurs such that $K_m \ll [S]_0$, then the solution becomes a simple zero-order decay where the apparent rate constant that describes the reaction is independent of the extent of reaction.

The product-inhibition model for enzymic action (Eq. (9)) can also be integrated to produce, like the integrated Michaelis–Menten equation, a nonlinear expression which is implicit in the substrate concentration:

$$V_{max}t = ([S]_0 - [S]_t)\left(\frac{K_m}{K_i} - 1\right) + \left(K_m + \frac{K_m[S]_0}{K_i}\right)\ln([S]_0/[S]_t), \quad (6)$$

where K_i is the product inhibition constant. In the special case where K_i is equal to K_m, the integrated solution becomes a pure simple exponential decay (Kuchel, 1985; Kuchel & Ralston, 1997):

$$[S] = [S]_0 e^{\frac{-V_{max}t}{K_m + [S]_0}}, \quad (7)$$

The implicit nature of both Eqs. (5) and (6) explains the use of classical, graphical approaches to analyse experimental data, or more recently numerical approaches. Alternative techniques to compute the substrate concentration as a function of time, such as root-solving including the 'bisection method' or Newton–Raphson root approximation, have been applied to analyse enzyme time courses (Scheid, 1988; Tjalling, 1995). Other approximation methods such as the fourth order Runge–Kutta integration, and a low-order recursive method known as the 'decomposition method' (Sonnad & Goudar, 2009) have provided a simple alternative to numerical integration. However recently, using the Lambert W function solution (Goudar, Harris, McInerney, & Suflita, 2004), the Michaelis–Menten equation can be written in an explicit form in substrate concentration:

$$[S] = K_m W\left(\frac{[S]_0}{K_m} e^{\frac{[S]_0 - V_{max}t}{K_m}}\right), \quad (8)$$

where W is the Lambert omega function. This solution allows a systematic analysis of the errors associated with the different approximate methods of integrating the Michaelis–Menten equation (Sonnad & Goudar, 2009).

Since numerical integration has become a routine procedure, multiple iterations of numerical integration can be applied to the analysis of experimental data from enzymic reactions. Thus non-linear least squares regression analysis of the iterated solutions can be used to determine the 'best-fit' estimates of the constants in the Michaelis–Menten equation. This data fitting technique is constrained by the desirability of data being collected over the entire reaction time course. By comparison, the Lineweaver–Burk plot uses only the initial velocities of reactions over a range of substrate concentrations.

8.4. Product inhibition

Deviations from Michaelis–Menten kinetics in the early stages of hydrolysis of starch by both endohydrolases and exohydrolases have been reported (Akerberg et al., 2000; Ao et al., 2007; Apar & Ozbek, 2007; Wang et al., 2006; Zhang et al., 2006). After the initial stage of starch digestion, time courses predicted theoretically by integrating the Michaelis–Menten equation deviate from the experimental data. Various hypotheses have been proposed to explain this outcome. Based on these hypotheses, existing variations to the Michaelis–Menten model, or new kinetic models, have been developed in order to explain starch digestion (Table 1).

Competitive-inhibition occurs when the binding of a ligand to the enzyme prevents binding of the substrate to the active site, and *vice versa*. Used previously to explain the kinetics of many enzyme–substrate systems, the competitive-inhibition model has been used to describe product inhibition of glucoamylase by glucose (Fujii & Kawamura, 1985; Lim, Lee, Shin, & Lim, 1999; Wang et al., 2006) (Eq. (9)):

$$\frac{d[P]}{dt} = \frac{V_{max}[S]}{K_m\left(1 + \frac{[P]}{K_i}\right) + [S]}. \quad (9)$$

The parameter estimates obtained from fitting experimental data of carbohydrate digestion with a product-inhibition model are generally more precise. Whether the fitting is improved because product inhibition occurs, or because the number of floating variables in the theoretical model has increased, is an important consideration in the data analysis (Fig. 6). Studies that model starch digestion using product inhibition assume an effect irrespective of the concentration of the product (Fujii & Kawamura, 1985). However, studies on both glucoamylase and α-amylase reveal a "grace zone" where inhibition does not occur below a critical concentration of product (Lim et al., 1999; Wang et al., 2006). However, the critical glucose concentration is much greater than that of glucose generated during typical starch digestion. Furthermore, product inhibition, if occurring mechanistically, will take place regardless of the product concentration and does not correspond well with the notion of a "grace period". Studies on the inhibition of the main products of α-amylase, maltose and maltotriose, are inconclusive as to whether the inhibition by these products is competitive or non-competitive (Kazaz, Desseaux, Marchis-Mouren, Prodanov, & Santimone, 1998).

Experiments characterising inhibition of enzymic starch hydrolysis use various amounts of glucose added to a starch solution before adding glucoamylase. At the concentrations of glucose ($<100\ mmol\ L^{-1}$) produced during standard starch digestions, no significant change is observed in the initial velocity of the hydrolytic reaction (Wang et al., 2006). Similarly, the study of immobilised α-amylase inactivation during the hydrolysis of starch particles shows the same relative enzymic activity for long periods after the beginning of digestion (Lim et al., 1999). Furthermore, models of product-inhibited enzymic reactions still produce smooth time courses (of a different shape from that of Michaelis–Menten reactions (Eq. (2))) not explaining the sharper transitions that are seen during digestion of starch suspensions (Akerberg et al., 2000). Evidence is therefore against product inhibition as the explanation for deviations of Michaelis–Menten kinetics from real carbohydrate-polymer digestion curves.

8.5. Substrate inhibition

Inhibition of enzymic processes is normally either competitive, where the inhibitor binds to the enzyme competitively with the substrate, or non-competitive, in which case the inhibitor has identical affinities for the enzyme and the enzyme–substrate complex, decreasing the maximum velocity of the reaction. Another special case is uncompetitive inhibition where the inhibitor binds only with the enzyme–substrate complex (Kuchel & Ralston, 1997). The binding of the inhibitor is at a subsidiary site on the enzyme, causing the catalytic action of the enzyme to be altered, and slowing the formation of product.

High-substrate inhibition is a form of uncompetitive inhibition that is characterised by a decrease in the reaction rate at high substrate concentrations. The kinetic behaviour of high-substrate inhibited enzymic processes follows the Briggs–Haldane model (Briggs & Haldane, 1925):

$$\frac{d[P]}{dt} = \frac{V_{max}[S]}{K_m + [S] + K_s[S]^2}, \quad (10)$$

where K_s is the high-substrate inhibition constant. The quadratic term is not interpreted mechanistically; it is only justified phenomenologically and many studies have used this model to describe starch hydrolysis (Lopez, Torrado, Fucinos, Guerra, & Pastrana, 2006; Miranda et al., 1991; Pastrana, Gonzalez, Miron, & Murado, 1998).

Models of substrate inhibiting α-amylase action on starch have been based upon the uncompetitive mechanism in recent research (Table 1) (Lopez et al., 2006; Pastrana et al., 1998). However, the less commonly used competitive model of substrate inhibition is phenomenologically a more likely explanation for the effect (Fig. 8). Given the numerous sub-sites for glucose residues at the catalytic centre of α-amylase (Fig. 8), ineffective binding or partial occupancy of the sub-sites is more probably responsible for inhibition than an uncompetitive process.

Physical properties of the solutions, such as viscosity and diffusional restrictions, have also been used to explain high-substrate

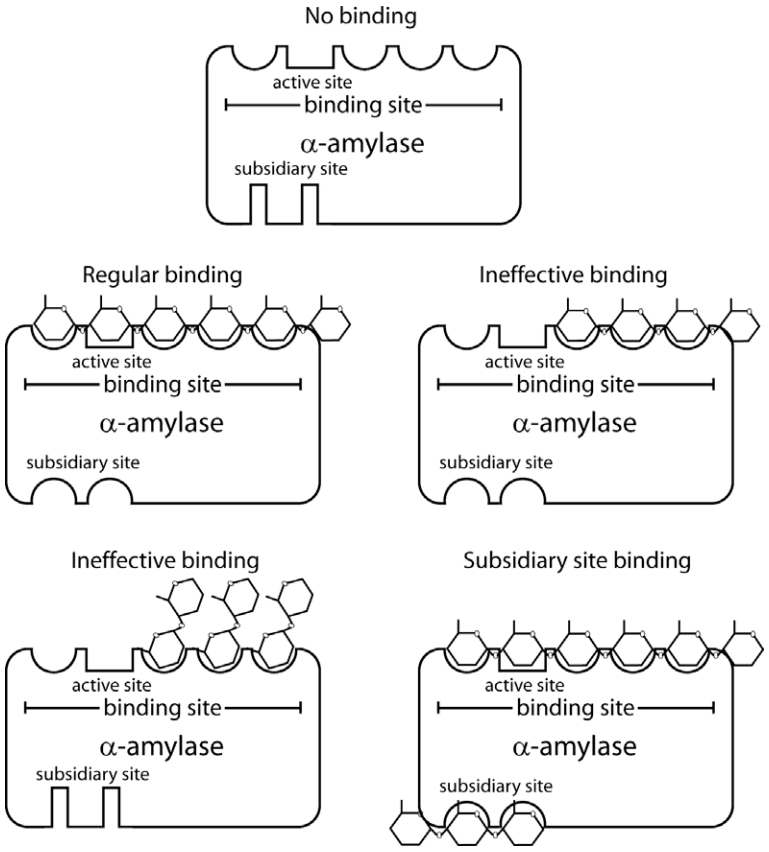

Fig. 8. Phenomenological interpretation of how the binding sites of α-amylase are bound during either product or substrate inhibition. The enzyme is first shown unbound, then regular binding without inhibition is presented. Ineffective binding shows how the enzyme might be bound in the instances of competitive-inhibition. Binding at the subsidiary site, that opens up only on occupation of the catalytic site by the substrate, brings about uncompetitive inhibition. (Based on Davies, Wilson, & Henrissat, 1997; Yoon & Robyt, 2003).

inhibition. In polysaccharide solutions, the concentration of solute strongly affects rheological properties. It is therefore reasonable to assume that particular kinetic profiles are at least partially conditioned by diffusional restrictions of the reaction mixture. The typical Michaelis–Menten differential equation is multiplied by a decaying exponential term to take diffusional restriction into account. Thus the reaction rate decreases asymptotically as the substrate concentration increases (Eq. (11)) (Pastrana et al., 1998),

$$\frac{d[\mathrm{P}]}{dt} = \frac{V_{\max}[\mathrm{S}]}{K_m + [\mathrm{S}]} e^{D_v R}, \tag{11}$$

where D_v is a first order coefficient that characterises the relationship between reaction velocity and diffusional restriction, and R expresses the diffusional restriction (Pastrana et al., 1998). Thus,

$$R = R_m + (R_0 - R_m)e^{-\mu[\mathrm{S}]}, \tag{12}$$

where R_0 is the diffusional restriction when [S] = 0, R_m is the largest value of diffusional restriction, and μ is a specific coefficient for diffusional restriction.

Combinations of product and substrate inhibition are used in models to fit digestion data from experiments where estimates are not satisfactory with single mechanisms (Lopez et al., 2006). Although both types of inhibition might well occur during starch digestion, as mentioned above, increasing the number of fitting parameters in models of catalysis will always lead to a more precise fit to the experimental data; this occurs at the expense of high coefficients of variation on parameter estimates.

Assignment of the reported inhibition by glucose of glucoamylase as either competitive or non-competitive has proven difficult (Hill, Macdonald, & Lang, 1997; Lopez et al., 2006). The Lineweaver–Burk plots obtained from inhibition experiments have not been able to distinguish between the two mechanisms. Research characterising the binding of substrate molecules to the enzyme sub-sites, followed by subsequent reorganisation of the enzyme–substrate complex, has not been able to shed light on this question either. This leads to multiple models still being used to describe the reaction mechanism of glucoamylase (Matsumura, Hirata, Ishii, & Kobayashi, 1988; Natarajan & Sierks, 1997).

8.6. Empirical exponential models

Given the above difficulties with characterising starch digestion using product/high-substrate-inhibition models, other empirical exponential models have been developed (Apar & Ozbek, 2007; Hill et al., 1997; Wang et al., 2006). Moreover, exponential models allow for easy manipulation, enabling different types of product or substrate inhibition to be added (Table 1). A "grace zone" (Section 8.4) where inhibition only occurs after a limiting value of product has been formed can also be added to an empirical exponential model; this is another reason for its use. Although easily controlled, variations of exponential models used to fit digestion time courses have no mechanistic basis.

Hill et al. (1997) proposed an exponential model which accounts for product inhibition as follows,

$$\frac{d[\mathrm{S}]}{dt} = \left(\frac{d[\mathrm{S}]}{dt}\right)_{[\mathrm{P}]=0} e^{-K_i[\mathrm{P}]}, \tag{13}$$

where K_i is the product inhibition constant.

This model was extended to incorporate a threshold concentration of product, below which product inhibition does not occur:

$$\frac{d[S]}{dt} = \left(\frac{d[S]}{dt}\right)_{[P]=0} e^{-K_i([P]-[P]_{th})}, \quad (14)$$

where $[P]_{th}$ is the threshold concentration of product below which product inhibition does not occur. Introduction of this constant enabled a closer fit of the equation to the multiple stages that are evident during *in vitro* starch digestion. Often, to make the model more able to fit the data a factor is included to account for the fact that 1 g of starch is converted into 1.11 g of glucose (the extra mass being from the added water) during digestion (Wang et al., 2006).

Another exponential model (Eq. (15)) introduced by Goni et al. (1997) has been used to described closely the digestion of both cooked (Frei et al., 2003) and raw (Al-Rabadi, Gilbert, & Gidley, 2009) grain starch samples. Specifically,

$$C = C_\infty(1 - e^{-kt}), \quad (15)$$

where C corresponds to the concentration of glucose at any time, C_∞ is the concentration of glucose at the completion of the reaction, and k is a first order kinetic constant. This equation is simply the solution of the relevant first order differential equation.

This same model has been used by Al-Rabadi et al. (2009) to describe the digestion by α-amylase of raw starch grain from both barley and sorghum. Results indicated that digestion of these raw grains was controlled by diffusion of enzymes within the porous grain (Al-Rabadi et al., 2009). The simple exponential models used to fit both the degradation of starch and the production of oligosaccharide closely simulates the digestion process over the initial stage (RDS stage) (Apar & Ozbek, 2007; Komolprasert & Ofoli, 1991) or over the entirety of the reaction (>24 h, or more correctly a large 'extent of reaction') where the RDS stage becomes comparatively insignificant (Al-Rabadi et al., 2009). Like the deviations from the Michaelis–Menten model, attempts to simulate digestion time courses, where both stages of starch digestion are carefully monitored, have proven difficult (Dona et al., 2009; Hill et al., 1997; Wang et al., 2006).

8.7. Multiple stage models

The obvious transition in the velocity of a reaction from being high to being much lower after the initial stage of a digestion time course has led to the implementation of two/multi-stage kinetic models (Table 1). One such model (Dona et al., 2009) uses Michaelis–Menten kinetics (Eq. (2)) to fit two stages of starch digestion by using Lineweaver–Burk analysis to estimate V_{max} and K_m values that are pertinent to each of two stages of the time course and later the whole time course is simulated by numerically integrating the Michaelis–Menten equation (Eq. (2)). The model successfully describes time courses from various starch samples prepared under different conditions, and digested with different hydrolytic enzymes. The time courses are measured with ^{1}H NMR spectroscopy (Dona et al., 2009).

Current models of the conformation of starch in solution, especially the hairy billiard ball, support the use of such a kinetic model to describe digestion time courses. Furthermore, the parameters estimated can be substituted back into the Michaelis–Menten differential equation to closely describe the whole time course of production of oligosaccharides during starch digestion (Fig. 6).

A similar approach was taken for fitting starch digestion (Park & Rollings, 1995) with multiple stages, although the rationale for using a multi-stage model was different from the previous case. The susceptibility of linear and branch points on a carbohydrate to enzymic hydrolysis is the mechanistic basis for fitting the reaction over multiple stages (Park & Rollings, 1995). The kinetic model supposes that enzyme attack is simultaneous for both linear and branch points, although it assumes different reaction rates of hydrolysis for the different domains of amylopectin.

The final model described here uses multiple applications of the Michaelis–Menten equation to account for the various rates of hydrolysis by α-amylase and amyloglucosidase (Akerberg et al., 2000). The model simulates digestion by using various rates and binding affinities of oligosaccharides of different lengths, produced by α-amylase and amyloglucosidase. Although this analysis considers both types of enzyme, and all lengths and complexities of substrate, the system of starch digestion is, perhaps, better analysed under simplified *in vitro* conditions, such as with single enzymes, or with well defined oligosaccharides.

9. Starch properties affecting digestion

The kinetics of enzymic starch digestion depends largely on two factors. The first is the molecular architecture and physicochemical characteristics of the starch granule, at all six levels of structure (Ao et al., 2007; French & Knapp, 1950; Kerr et al., 1951; Kruger & Marchylo, 1985) (Fig. 1; Section 3). Research correlating the botanical origin, largely responsible for the architecture of the starch granule, with both *in vivo* and *in vitro* digestibility is advanced; this is a correlation and does not follow the causal relationship (biosynthesis causes structural properties that determine digestibility) (Goni et al., 1997; Jenkins et al., 2002; Thompson, 2000). The second is the hydration or physical conformations that starch granules assume in aqueous solution (Section 4); these conformations control digestibility. Both heat and pressure during the preparation of starch suspensions alter the progression towards gelatinisation, which increases the availability of polysaccharide starch chains to digestive enzymes, thus affecting rates of hydrolysis (Chung, Yoo, & Lim, 2005; Chung et al., 2006; Eerlingen, Jacobs, & Delcour, 1994; Holm, Lundquist, Bjorck, Eliasson, & Asp, 1988).

Digestibility is strongly influenced by shearing and heating the sample (Farhat et al., 2001; Guraya, James, & Champagne, 2001; Shiotsubo, 1983). The relative amount of starch in each digestibility fraction can be altered by a range of various techniques for starch preparation (Fassler et al., 2006). The hydrolysis patterns of samples that are partially gelled after heating at different temperatures, behave very differently during the early stages of hydrolysis (Chung et al., 2006; Granfeldt, Eliasson, & Bjorck, 2000). The time required for oligosaccharide formation to reach a plateau decreases as the temperature of heat treatment is increased, indicating that starch in its native state is very resistant to digestion. This property is explained by the disruption of inter- and intra-molecular hydrogen bonds between starch chains, allowing the chains to separate and become more accessible to enzymes. Progressive gelatinisation then increases the availability of polysaccharide chains to the digestive enzymes (Chung et al., 2006). The extent of starch gelatinisation is positively correlated with its *in vitro* digestibility and the *in vivo* plasma glucose response in rats (Holm et al., 1988).

Starch retrogradation has also been correlated to rates of enzyme-catalysed hydrolysis; it depends on the time and temperature of sample storage (Eerlingen et al., 1994) and also on the amylose–amylopectin ratio (Fredriksson et al., 2000). Both storage conditions and the fraction of amylose which affect re-crystallisation are correlated with the digestion rates of retrograded starch samples. Retrogradation over periods of up to a week of gelatinisation, and partially gelled starch, show greater rates of hydrolysis than native starch (Chung et al., 2005, 2006).

The granular architecture established during starch synthesis in the grain also plays a role in determining the digestibility of starch. Amyloglucosidase and α-amylase show a decrease in the rate of

hydrolysis, as the digestion approaches the branch points of the polysaccharide (French & Knapp, 1950; Kerr et al., 1951). It has been proposed that partially shortening the length of exterior branched chains slows their rate of hydrolysis. Modification of normal maize starch, by treatment with β-amylase and maltogenic α-amylase, removes maltose residues and reduces the chain length on the exterior of the starch molecule. Digestion of this modified maize starch is slower than unmodified maize starch (Ao et al., 2007). Further modification by increasing the branch density on the exterior of the starch granule using transglucosidase slows the rate of hydrolysis (Ao et al., 2007). Aligned with this concept, altering the branch density by concentrating the number of α (1,6) bonds or changing the chain distributions in amylopectin molecules decreases the rate of hydrolysis of starch by α-amylase (Ao et al., 2007).

Although it appears that an increased branch density decreases the rate of glucosidases, in the other extreme where branch density is very low, the rate of digestion is also reduced. Comparison of the rate of hydrolysis of amylopectin with an equal weight-concentration of amylose shows that the highly linear amylose chains yield sugars more slowly than highly branched amylopectin (Kerr et al., 1951). This is postulated to occur because of the very low concentration of reducing ends on amylose that are able to be acted upon by glucosidases. Thus, it is concluded that both linear polymers of anhydroglucose and highly branched polymers of anhydroglucose inhibit the action of hydrolytic enzymes, slowing rates of digestion (Kerr et al., 1951).

Granules of starch that have been separated by sedimentation analysis into different sizes, and inspected by light microscopy, have also shown differences in rates of digestion by α-amylase (Fig. 4). Small granules were much more efficiently hydrolysed by digestive enzymes compared with their large-granule counterparts (Kruger & Marchylo, 1985). The rate of digestion is not only much greater in the case of small granules but also the total percentage of starch digested over the duration of the reaction is greater in the case of small granules (Manelius et al., 1997). The initial rates of hydrolysis of granules of various sizes are explained by the dependency on the amount of enzyme that is absorbed into the granule and thus the increased surface area of starch (McLaren, 1963).

10. Future directions

There is increasing evidence that the nutritional value of food is controlled by the microstructure of the contained carbohydrate. The structure of starch plays an important role in determining its accessibility to digestion, thus influencing the time course of the postprandial blood glucose response. The many structural features of starch granules have led to numerous models for estimating *in vitro* digestion rates, even under the simplest of experimental conditions. Progress towards correlating structural and physical properties of the starch granule to its digestibility, by use of more advanced kinetic models, is evident (Dona et al., 2009). However, quantitative mechanistic account for the rate limiting step of starch digestion, obtained with different preparation techniques, and at various stages during digestion, is still being sought.

Analysis of the time dependence on the structural and physical properties of starch granules during the course of digestion is of great importance (Gernat et al., 1993; Wang et al., 2006). Unveiling what physiological property controls the rate of starch granule digestion at various stages during, and under various conditions of preparation, is the next step towards completely understanding the processes of glucose release by hydrolytic enzymes. A better understanding of the rate limiting property of granular digestion by hydrolases will allow the development of kinetic models with a proper mechanistic basis, whether this involves a model with multiple stages or is explained by a continuous mathematical function (Chung et al., 2006; Cooke & Gidley, 1992; Dona et al., 2009).

In an effort to elucidate the finer details of the features of the digestion of starch, new analytical methodologies are being developed (Cave et al., 2009; Gidley et al., 2010; Hoang et al., 2008; Podzimek, Lebedat, & Johann, 2009; Roger, Baud, & Colonna, 2001; Rolland-Sabate, Colonna, Mendez-Montealvo, & Planchot, 2007). One such technique being improved upon is non-aqueous SEC of starch substrate samples (Cave et al., 2009; Hoang et al., 2008; Podzimek et al., 2009), as quantitative recovery of starch samples from SEC is contentious (Ward, Gao, De Bruyn, Gilbert, & Fitzgerald, 2006). Other chromatography techniques, specifically asymmetric flow-field flow fractionation (AF^4) (Roger et al., 2001; Rolland-Sabate et al., 2007), are becoming more generally applied to analyse hydrodynamic volume distributions of starch before and during digestion. It has been proved that SEC can cause significant shear degradation of the amylopectin component of starch (Cave et al., 2009). Problems arising from chromatography of starch such as incomplete recovery and shear scission of samples are expected to be solved by using a more gentle technique such as AF^4. Finally, NMR methods such as chemical exchange saturation transfer (CEST) (Ward, Aletras, & Balaban, 2000; Zhou & van Zijl, 2006), normally used in imaging sugars and carbohydrates, is currently being applied to the digestion of starch revealing before unseen features of oligosaccharides during digestion (Dona, Pages, & Kuchel, unpublished).

Overall the future is bright for the quantitative analysis of starch digestion, using advanced analytical methods, mathematical modeling of enzyme kinetics with complex substrates, and therefore prediction of nutritional outcomes. An *in vitro* counterpart of the GI seems plausible and is possibly imminent.

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