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The interplay of α-amylase and amyloglucosidase activities on the digestion of starch in *in vitro* enzymic systems

Frederick J. Warren, Bin Zhang, Gina Waltzer, Michael J. Gidley, Sushil Dhital*

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

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ABSTRACT

In vitro hydrolysis assays are a key tool in understanding differences in rate and extent of digestion of starchy foods. They offer a greater degree of simplicity and flexibility than dynamic *in vitro* models or *in vivo* experiments for quantifiable, mechanistic exploration of starch digestion. In the present work the influence of α-amylase and amyloglucosidase activities on the digestion of maize and potato starch granules was measured using both glucose and reducing sugar assays. Data were analysed through initial rates of digestion, and by 1st order kinetics, utilising logarithm of slope (LOS) plots. The rate and extent of starch digestion was dependent on the activities of both enzymes and the type of starch used. Potato required more enzyme than maize to achieve logarithmic reaction curves, and complete digestion. The results allow targeted design of starch digestion experiments through a thorough understanding of the contributions of α-amylase and amyloglucosidase to digestion rates.

1. Introduction

Complex carbohydrates have been recommended to make up over 50% of the energy intake in the human diet (Nishida, Uauy, Kumanyika, & Shetty, 2004). The main source of digestible carbohydrate in the human diet is starch, a complex carbohydrate comprised of two glucose polymers, amylose, an essentially linear polymer of α-(1 → 4) linked anhydroglucose resides, and amylopectin, a large branched molecule comprising chains of α-(1 → 4) linked anhydroglucose resides linked by α-(1 → 6) branch points (Gidley et al., 2010). Following ingestion, the α-(1 → 4) linkages are hydrolysed by α-amylase to produce predominantly maltose, maltotriose and branched α-limit dextrins, which are then hydrolysed to glucose by the brush border enzymes maltase-glucoamylase and sucrase-isomaltase, to be absorbed into the portal blood (Beeren, Petersen, Bøjstrup, Hindsgaul, & Meier, 2013; Butterworth, Warren, & Ellis, 2011; Diaz-Sotomayor et al., 2013; Nichols et al., 2003, 2009). Thus, ingestion of starchy foods may result in significant departures in blood glucose levels. It has been known for some time that different starchy foods elicit very different postprandial blood glucose responses (Crapo, Reaven, & Olefsky, 1977; Wolever & Jenkins, 1986), and this has been attributed to differences in the rate and extent of digestion between different starch containing foods (Butterworth et al., 2011; Butterworth, Warren, Grassby, Patel, & Ellis, 2012; Dona, Pages, Gilbert, & Kuchel, 2010; Holm, Lundquist, Björck, Eliasson, & Asp, 1988).

Due to the time and expense of carrying out human feeding trials, and the difficulty of elucidating mechanistic information regarding the differences in digestion rate between different starch containing foods from human studies, a great deal of research effort has been focused on developing *in vitro* models of starch digestion. These may use either pancreatic extracts or purified enzymes to digest starch to sugars (Butterworth et al., 2012; Dona et al., 2010; Englyst, Kingman, & Cummings, 1992; Hasjim, Lavau, Gidley, & Gilbert, 2010; Slaughter, Ellis, & Butterworth, 2001; Woolnough, Bird, Monro, & Brennan, 2010). From such experiments the rate and extent of starch digestion may be rapidly and conveniently assessed in the laboratory, and from there it may be possible to suggest mechanisms by which some starchy foods are more slowly digested than others, potentially allowing the rational design of foods with more favourable digestion profiles (Butterworth et al., 2012; Dhital, Shrestha, & Gidley, 2010; Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Goñi, Garcia-Diz, Mañas, & Saura-Calixto, 1996; Slaughter et al., 2001; Tahir, Ellis, & Butterworth, 2010; Zhang, Dhital, & Gidley, 2013).

Achieving these aims requires reliable and robust *in vitro* assay techniques, analysed in a logical manner that reflects the kinetics of the enzymes involved. Two main approaches have been taken to mimic the *in vivo* digestion process *in vitro*. The first alternative is to use purified pancreatic α-amylase in isolation at an enzyme

* Corresponding author. Tel.: +6133667373.
E-mail address: s.dhital@uq.edu.au (S. Dhital).

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activity representative of activities measured in the human small intestine (Slaughter et al., 2001). This approach has not been generally adopted, however, due to the paucity of available studies on enzyme activities in the human small intestine. This makes it hard to accurately determine the activity of α-amylase in the small intestine, and the surprisingly low α-amylase activities in the studies that do exist can pose technical problems for *in vitro* experiments due to the difficulty in measuring such low enzyme activities (Auricchio, Rubino, & Mürset, 1965; Borgström, Dahlqvist, Lundh, & Sjövall, 1957; Butterworth et al., 2011; Layer, Jansen, Cherian, Lamers, & Goebell, 1990; Slaughter et al., 2001). A second and more widely adopted alternative, is to use a combination of α-amylase (or pancreatin containing α-amylase activity) with a fungal amyloglucosidase under conditions which are determined to give results after a fixed time of digestion that are in line with the findings from ileostomy studies (Englyst & Cummings, 1985; Englyst et al., 1992; Hasjim et al., 2010; Muir & O'Dea, 1993). From a practical view point, this has advantages as it provides an assay where a significant proportion of digestion will be completed in an experimentally accessible timeframe, and amyloglucosidase will convert all the products from α-amylase to glucose, so that the glucose oxidase–peroxidase (GOPOD) assay can be used to quantify the products of digestion. The most popular implementation of this approach has been the Englyst assay, in which starch is digested by a combination of α-amylase and amyloglucosidase, and glucose release is determined after 20 min (termed rapidly digestible starch, or RDS), 120 min (termed slowly digestible starch, or SDS) and the remaining undigested starch (termed resistant starch, or RS). Although used extensively, the Englyst assay is a limited approach as it fails to take into account that starch digestion is a 1st order kinetic process, and may be analysed more succinctly with a 1st order kinetic model, as has been discussed elsewhere ((Butterworth et al., 2012; Dhital, Warren, Butterworth, Ellis, & Gidley, 2014; Goñi et al., 1997). As the conditions for the Englyst and related assays are calibrated against the results of ileostomy studies, it has been suggested by a number of workers that the results of *in vitro* experiments may be directly extrapolated to the *in vivo* situation (Englyst, Veenstra, & Hudson, 1996; Englyst, Englyst, Hudson, Cole, & Cummings, 1999; Englyst, Vinoy, Englyst, & Lang, 2003; Zhang & Hamaker, 2009). While it appears logical that the faster a starch is digested *in vitro*, the faster it is likely to be digested *in vivo*, great care should be taken when extrapolating from *in vitro* experiments, as the enzyme activities and conditions used are markedly different from those present in the human intestine (Ells, Seal, Kettlitz, Bal, & Mathers, 2005; Hasjim et al., 2010; Seal et al., 2003).

Amyloglucosidase has been assumed to act predominantly on the products of α-amylase digestion, rapidly converting them to glucose, which has the advantage of removing the inhibitory effects of maltose on amylase activity during long digests (although it should be remembered that maltose is not a very potent inhibitor of α-amylase; Alkazaz et al., 1996; Seigner, Prodanov, & Marchis-Mouren, 1985; Warren, Butterworth, & Ellis, 2012). Amyloglucosidase is also capable of hydrolysing α-(1 → 6) linkages, which α-amylase is unable to attack, removing limit dextrins, and allowing starch digestion to go to completion, as is the case *in vivo* where brush border enzymes undertake the same function (Diaz-Sotomayor et al., 2013; Nichols et al., 2003, 2009).

Recently, a number of workers have noted that there is an apparent synergism in the action of α-amylase and amyloglucosidase, particularly when attacking granular starches, as amyloglucosidase is capable of directly attacking starch granules, as well as hydrolysing α-amylase digestion products (Brewer, Cai, & Shi, 2012; Kimura & Robyt, 1995; Miao, Zhang, Mu, & Jiang, 2011; Ueda, 1981; Zhang et al., 2013). This has important consequences for interpreting the results of *in vitro* digestion studies, as varying the concentration of one, or both, enzymes may have unpredictable consequences on the rate and extent of starch digestion. In the present paper, we undertake a systematic study of the effects of varying concentrations of α-amylase and amyloglucosidase over a wide range on the rate and extent of the digestion of granular maize and potato starch. The products of digestion are measured using the GOPOD assay (specific to glucose) and the 4-hydroxybenzoic acid hydrazide (PAHBAH) reducing sugar assay, which is sensitive to not only glucose, but also maltose and maltotriose products of amylolysis (as well as, to a lesser extent, other products e.g. α-limit dextrins). The resultant digestion time courses are analysed by 1st order kinetics and log of slope (LOS) plots (Butterworth et al., 2012; Edwards, Warren, Milligan, Butterworth, & Ellis, 2014) to determine the rate and extent of digestion, and using initial rates, to allow comparison between experiments when the enzyme activity is too low to significantly deplete the substrate, and thus allow determination of a 1st order rate constant. The results obtained will allow targeted design of future starch digestion experiments through a thorough understanding of the contributions of α-amylase and amyloglucosidase to overall digestion rates.

2. Methods

2.1. Materials

Potato starch (S-4251) (PS) was purchased from Sigma-Aldrich Pty Ltd., Sydney, Australia and regular maize starch (MS) was purchased from Penford Australia Ltd., Sydney, Australia. The average apparent amylose contents of PS and MS, determined by an iodine colorimetric method (Hoover & Ratnayake, 2001), were 36.8% and 27.1%, respectively.

Porcine pancreatic α-amylase was obtained from Sigma-Aldrich® (Cat. no. A6255), and had an activity of 49,700 U/mL as defined by the manufacturer (confirmed by assay against soluble starch). One unit was defined by the manufacturer as the amount of enzyme required to liberate 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20 °C. Fungal amyloglucoside (*Aspergillus niger*) was obtained from Megazyme® (Megazyme E-AMGDF), and had an activity of 3260 U/mL as defined by the manufacturer (confirmed by assay against soluble starch). One unit was defined by the manufacturer as the amount of enzyme required to release one micromole of glucose from soluble starch per minute (10 mg/ml starch; pH 4.5; 40 °C). All other chemicals were obtained from Sigma-Aldrich® and were of the highest quality available.

2.2. Starch digestion

Starch (100 mg) was accurately weighed and added to a 15 mL polypropylene tube. To this was added 9.9 mL of acetate buffer (0.2 M, pH 6, containing 200 mM $CaCl_2$ and 0.5 mM $MgCl_2$). The pH value chosen is a compromise between the pH optima of the two enzymes, and would be expected to result in adequate activity from both enzymes. This was incubated in a water bath at 37 °C and 100 μL of a mixture of α-amylase and amyloglucosidase, diluted with buffer, was added to give the appropriate enzyme activities for each assay. Aliquots (200 μL) were taken at time intervals between 20 min and 4 h and immediately placed in boiling water for 5 min to inactivate the enzymes (Slaughter et al., 2001). These were then centrifuged (2000 × *g*, 5 min) to remove any unreacted starch residue and the supernatant analysed for glucose (GOPOD) and reducing sugar (PAHBAH). The GOPOD assay (Thermo Electron Noble Pk, Victoria, Australia. Cat no. TR 15104)) was carried out as per the manufactures instructions. The glucose value was multiplied by a factor of 0.9 to convert glucose concentration into starch with results presented as gram per 100 g dry starch. All the

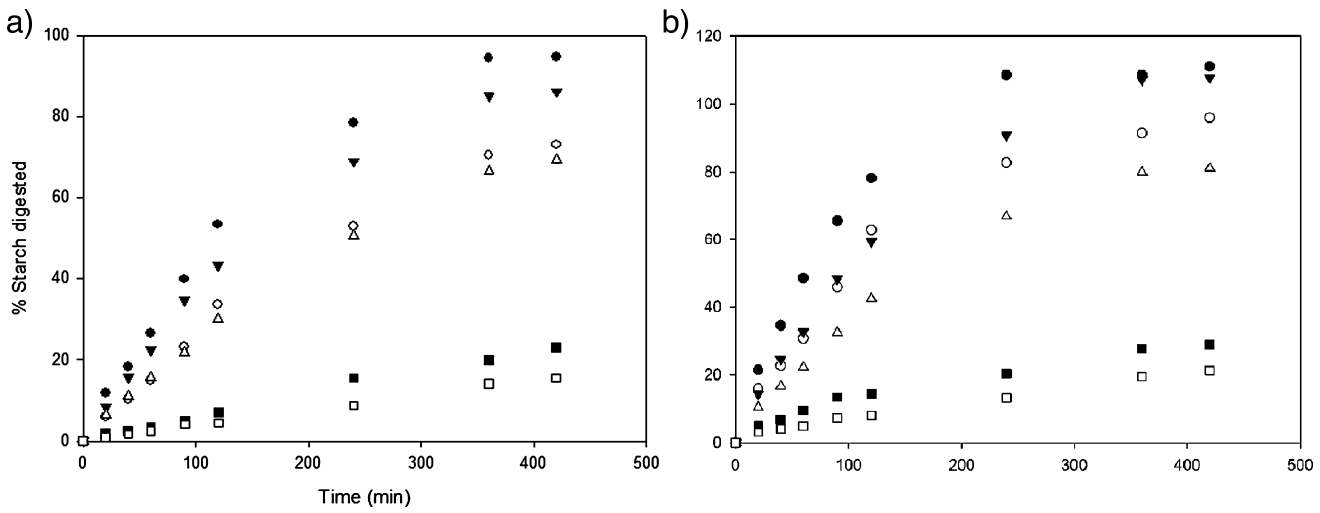

Fig. 1. Exemplar raw data for maize starch digestion at various enzyme activities. (A) Measured by glucose assay. (B) Measured by reducing sugar assay. Closed circles, 2 U α-amylase and 1.12 U amyloglucosidase per mL; open circles, 2 U α-amylase and 0.28 U amyloglucosidase per mL; closed triangles, 1 U α-amylase and 0.56 U amyloglucosidase per mL; open triangles, 0.5 U α-amylase and 0.56 U amyloglucosidase per mL; closed squares, 0 U α-amylase and 1.12 U amyloglucosidase per mL; open squares, 0 U α-amylase and 0.14 U amyloglucosidase per mL.

measurements were carried out in duplicate and results are expressed as means ± standard deviation of replicates. The PAH-BAH reducing assay was carried out as described by Moretti and Thorson (2008), using maltose standards. The reducing sugar values were also converted to starch equivalents and the results presented as gram per 100 g dry starch.

2.3. Data analysis

Enzymic starch digestion is a pseudo-first order kinetic process, producing a digestion curve that is initially linear with a constant rate at early time points as the substrate is not significantly depleted (Slaughter et al., 2001). As the reaction proceeds and the substrate is depleted the reaction rate shows an exponential decay that may be fitted using the familiar 1st order equation:

$$C = C_{\text{inf}}\left(1 - e^{-kt}\right)$$

where C is the amount of starch digested at time t, C_{inf} is the amount of starch digested at the reaction end point, and k is the pseudo-first order rate constant (Butterworth et al., 2012; Edwards et al., 2014). For the purposes of the present study, the data were analysed in two ways from both the reducing sugar and glucose analyses. Initial rates were obtained from the slope of the initial linear region of digestion curves. This allowed rates to be obtained for all enzyme concentrations, including when there was not enough enzyme activity to significantly deplete the substrate concentration during the time course of the reaction, and thus accurately determine a 1st order rate constant. First order rate constants were obtained using the log of slope (LOS) plot method (Butterworth et al., 2012), where the data permitted.

3. Results

Digestion rates for potato starch and maize starch were measured at a wide range of α-amylase and amyloglucosidase concentrations. Both starches showed systematic variation in the rate and extent of digestion at different enzyme concentrations. Both α-amylase and amyloglucosidase activities appear to independently enhance the rate of digestion of native starch when used alone or in combination. A simple visual inspection of the digestion curves for both maize and potato starch (Figs. 1 and 2) at a range of α-amylase and amyloglucosidase activities shows that there is an increase in both the rate and extent of starch digestion, when measured both through reducing sugar and glucose assay, with both increasing α-amylase activity at a fixed amyloglucosidase activity, and increasing amyloglucosidase activity at a fixed α-amylase activity. As would be expected, in the absence of amyloglucosidase, α-amylase releases very little glucose from potato or maize starch, the primary products of α-amylase being maltose and maltotriose (Prodanov, Seigner, & Marchis-Mouren, 1984; Seigner, Prodanov, & Marchis-Mouren, 1987). Thus, the GOPOD assay detects only a very small amount of product, while reductometry indicates significant breakdown of starch when α-amylase alone is present. The addition of even small amounts of amyloglucosidase activity leads to a dramatic increase in glucose release, as would be expected (McCleary, Gibson, & Mugford, 1997; Pazur & Ando, 1959; Tester, Qi, & Karkalas, 2006). It should be noted that in no case does the starch digestion rate in the absence of amyloglucosidase (measured by reductometry) equal the rate following the addition of amyloglucosidase, and increasing amyloglucosidase activity at a fixed α-amylase activity will always lead to an increasing rate of digestion- clearly indicating that the role for amyloglucosidase during *in vitro* digestion procedures is not simply to convert products of α-amylase digestion to glucose. As indicated in Figs. 1b and 2b, even in the absence of α-amylase, amyloglucosidase will directly attack starch granules to liberate glucose (Kimura & Robyt, 1995; Ueda, 1981). It should be noted in Figs. 1b and 2b that, because maltose was used as a standard for the reducing sugar assay, when a large amount of amyloglucosidase was present, and hence a significant amount of product was released in the form of glucose, the reducing sugar assay apparently over estimates the amount of starch digested, resulting in some values above 100%.

At low total enzyme activities, the observed digestion curves are not of a logarithmic form, for example the closed squares and open squares in Figs. 2a and 2b, i.e. insufficient substrate is converted to product during the time course of the reaction to result in a significant decay in the overall rate of reaction. Under such conditions, the digestion progress curves are essentially linear, and therefore unsuitable for first-order kinetic analysis, severely limiting the amount of information that may be obtained about the progress of the reaction. While a simple reaction velocity may be calculated, as has been done in the present study for comparative purposes, a rate coefficient and reaction end point may not be determined. The quantity of enzyme required to achieve a reaction rate adequate to consume a significant amount of substrate, and

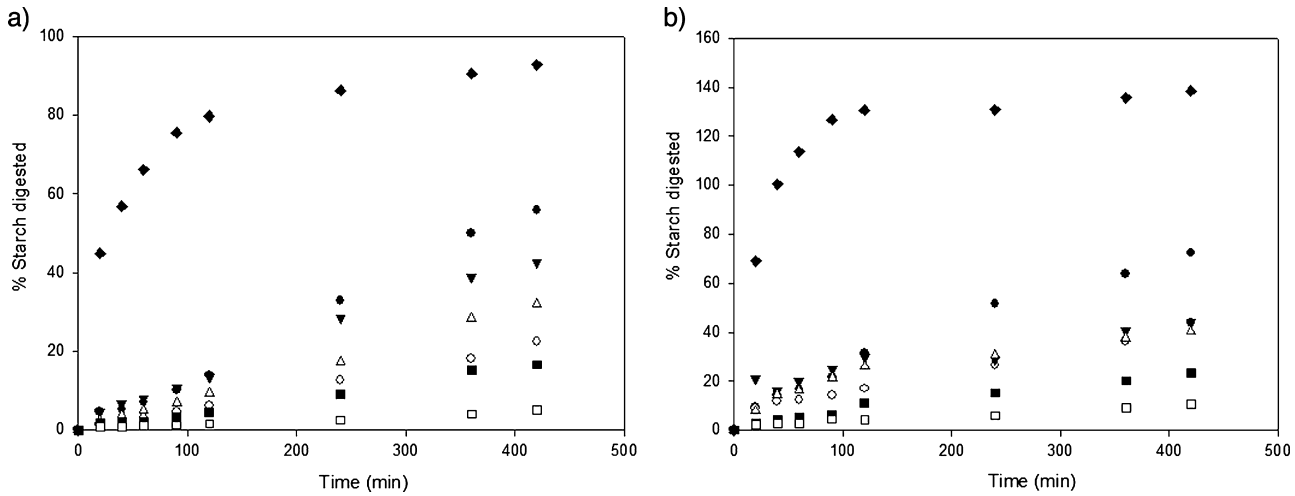

Fig. 2. Exemplar raw data for potato starch digestion at various enzyme activities. (A) Measured by glucose assay. (B) Measured by reducing sugar assay. Closed diamonds, 24 U α-amylase, 18 U amyloglucosidase; closed circles, 2 U α-amylase and 1.12 U amyloglucosidase per mL; closed triangles, 2 U α-amylase and 0.28 U amyloglucosidase per mL; open circles, 1 U α-amylase and 0.56 U amyloglucosidase per mL; open triangles, 0.5 U α-amylase and 0.56 U amyloglucosidase per mL; closed squares, 0 U α-amylase and 1.12 U amyloglucosidase per mL; open squares, 0 U α-amylase and 0.14 U amyloglucosidase per mL.

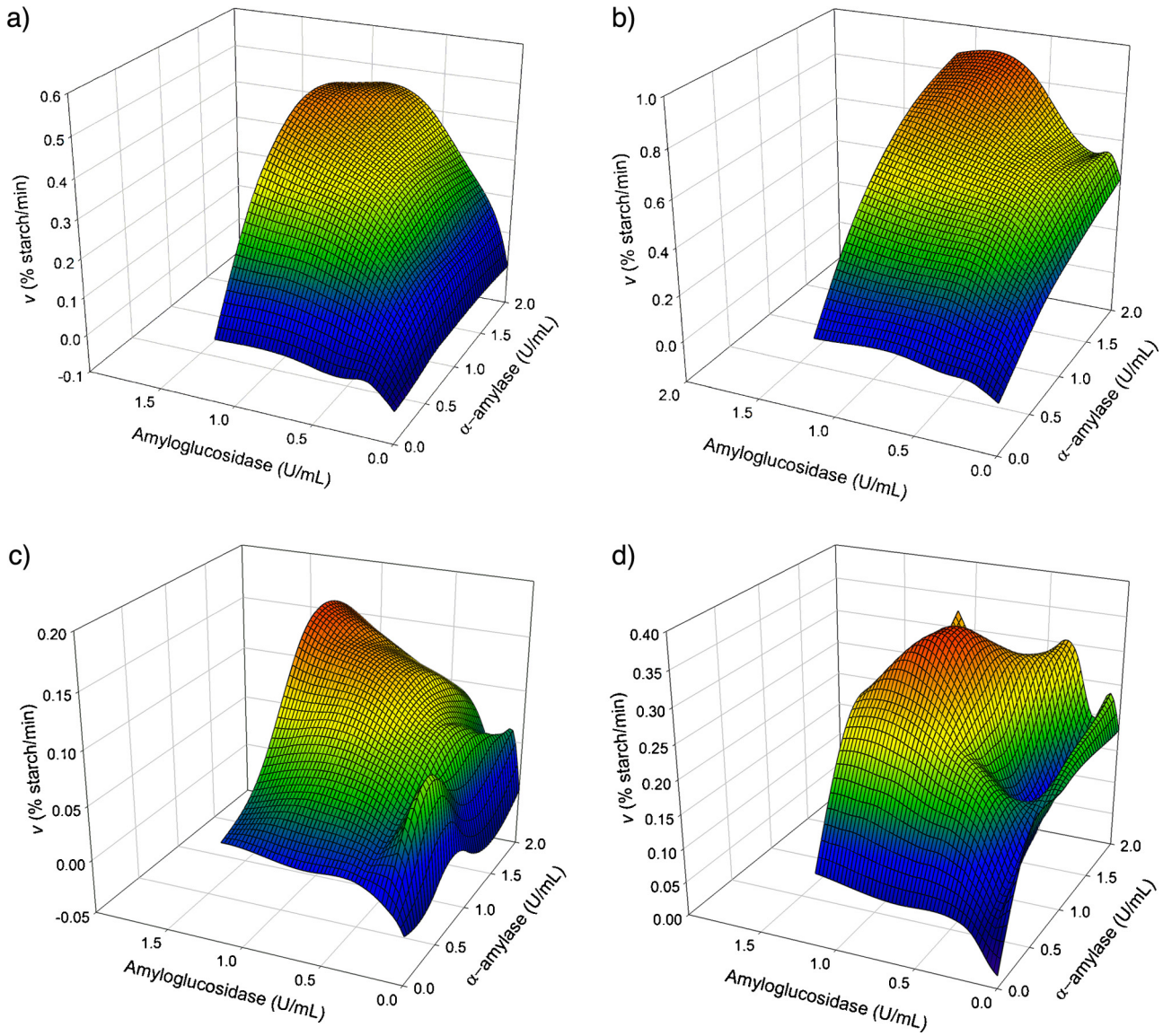

Fig. 3. Initial rates of starch digestion at various α-amylase and amyloglucosidase activities for starch shown as interpolated surface plots. (A) Maize starch measured by glucose assay; (B) Maize starch measured by reducing sugar assay; (C) Potato starch measured by glucose assay. (D) Potato starch measured by reducing sugar assay.

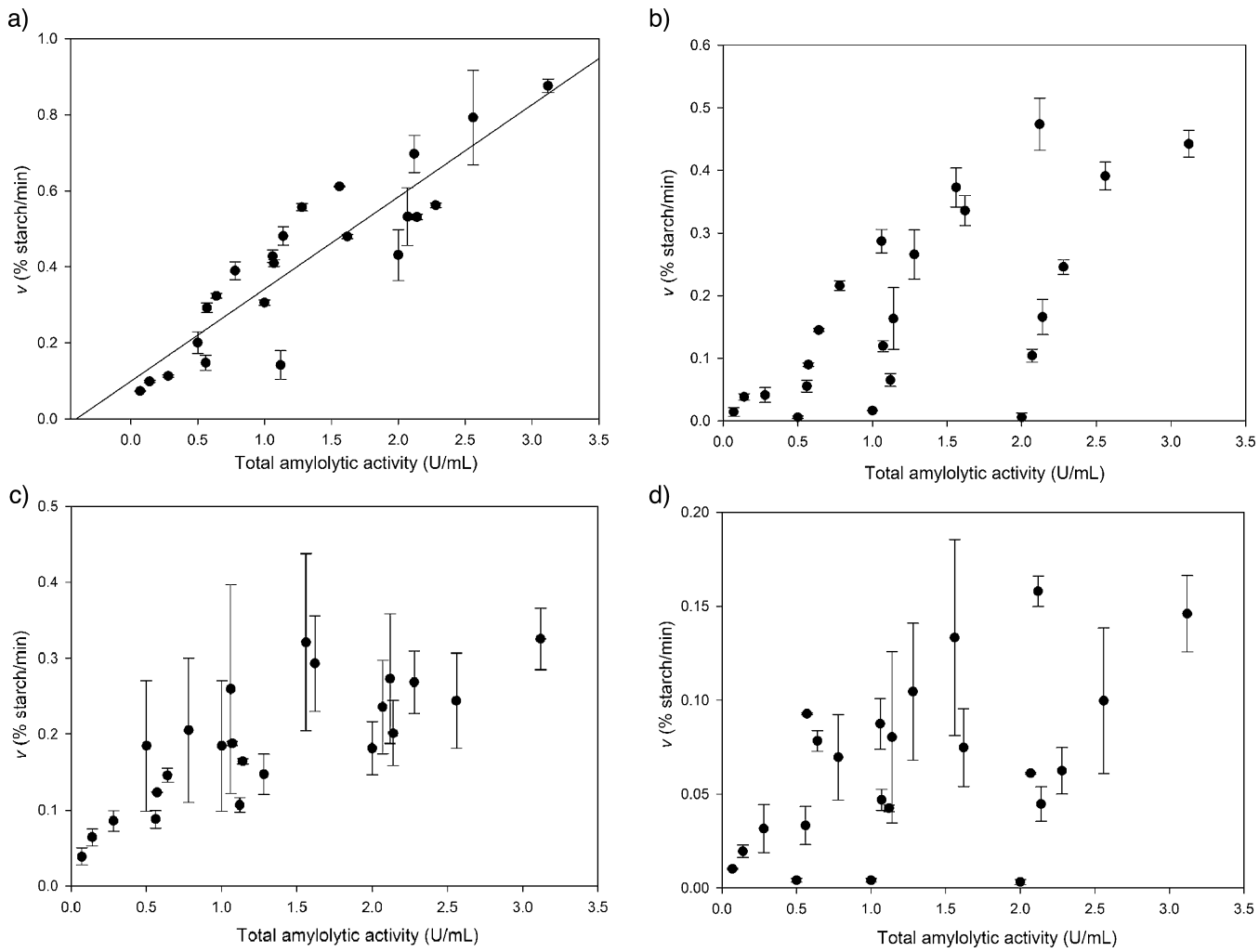

Fig. 4. Plots of total amylolytic activity (the sum of α-amylase and amyloglucosidase activity) against v. Values are shown ± S.D. (A) Maize starch measured by reducing sugar assay; (B) Maize starch measured by glucose assay; (C) Potato starch measured by reducing sugar assay; (D) Potato starch measured by glucose assay.

subsequently produce a logarithmic digestion curve, was dependant on the substrate used. Maize, a more rapidly digested starch granule, displayed a logarithmic digestion curve at far lower total enzyme concentrations than potato starch granules, a more slowly digested starch, showing a logarithmic curve in all the examples shown in Fig. 1, whereas in Fig. 2 the lower enzyme concentrations (closed squares and open squares), are essentially linear.

As the enzyme activity is increased, both the rate and extent of digestion is increased. A useful way to visualise this is through the use of surface plots, allowing the effects of both α-amylase and amyloglucosidase on the rate of starch digestion to be viewed simultaneously. Looking first at the data for maize starch (Fig. 3a and b), the initial rate (v) of digestion in the absence of amyloglucosidase is close to zero when sugar production is measured by glucose assay, as would be expected. Measured by reductometry, the initial rate is low at low α-amylase concentration, but then increases linearly with increasing α-amylase concentration. The initial rate for amyloglucosidase in the absence of α-amylase is uniformly low, measured by either method, indicating the importance of the combined action of α-amylase and amyloglucosidase on starch. There are some minor irregularities observed in the surface plots, but these are likely to be the result of small errors in the data being amplified through the interpolation procedure used to generate the plots.

The method used to measure the action of the two enzymes has some influence on the results. Measurement by reductometry results in rates that are dependent equally on the activity of both enzymes. Indeed a plot of v against the cumulative activity of both enzymes results in a linear plot ($R^2 = 0.79$) (Fig. 4a), indicating that both enzymes have nearly equal roles in the production of reducing sugar, a surprising result given the differences in enzyme activities between the two enzymes. A similar plot produced for v measured by glucose assay (Fig. 4b) reveals a far more complex relationship. Glucose release is dependent on the action of amyloglucosidase, and amyloglucosidase activity is much faster on the products of α-amylase than acting directly on starch, but this is contingent on having an adequate amyloglucosidase activity (relative to α-amylase) to generate significant amounts of glucose. Thus, a complex relationship results in which at each α-amylase concentration, there is a dramatic increase in rates with increasing amyloglucosidase activity, which saturates at high amyloglucosidase activities. It should be noted that during initial stages of the reaction, from which initial rates were obtained, maltose levels in the absence of amyloglucosidase were not sufficient to have a significant inhibitory action on α-amylase, so increases in initial rate on addition of amyloglucosidase cannot be ascribed to the removal of product inhibition by conversion of maltose to glucose (Warren et al., 2012).

First order rate constants (k) were also obtained from LOS plot analysis of the reaction progress curve (Butterworth et al., 2012). In this case a single rate constant was used to describe the total reaction curve. It should be noted that a faster rate may have been

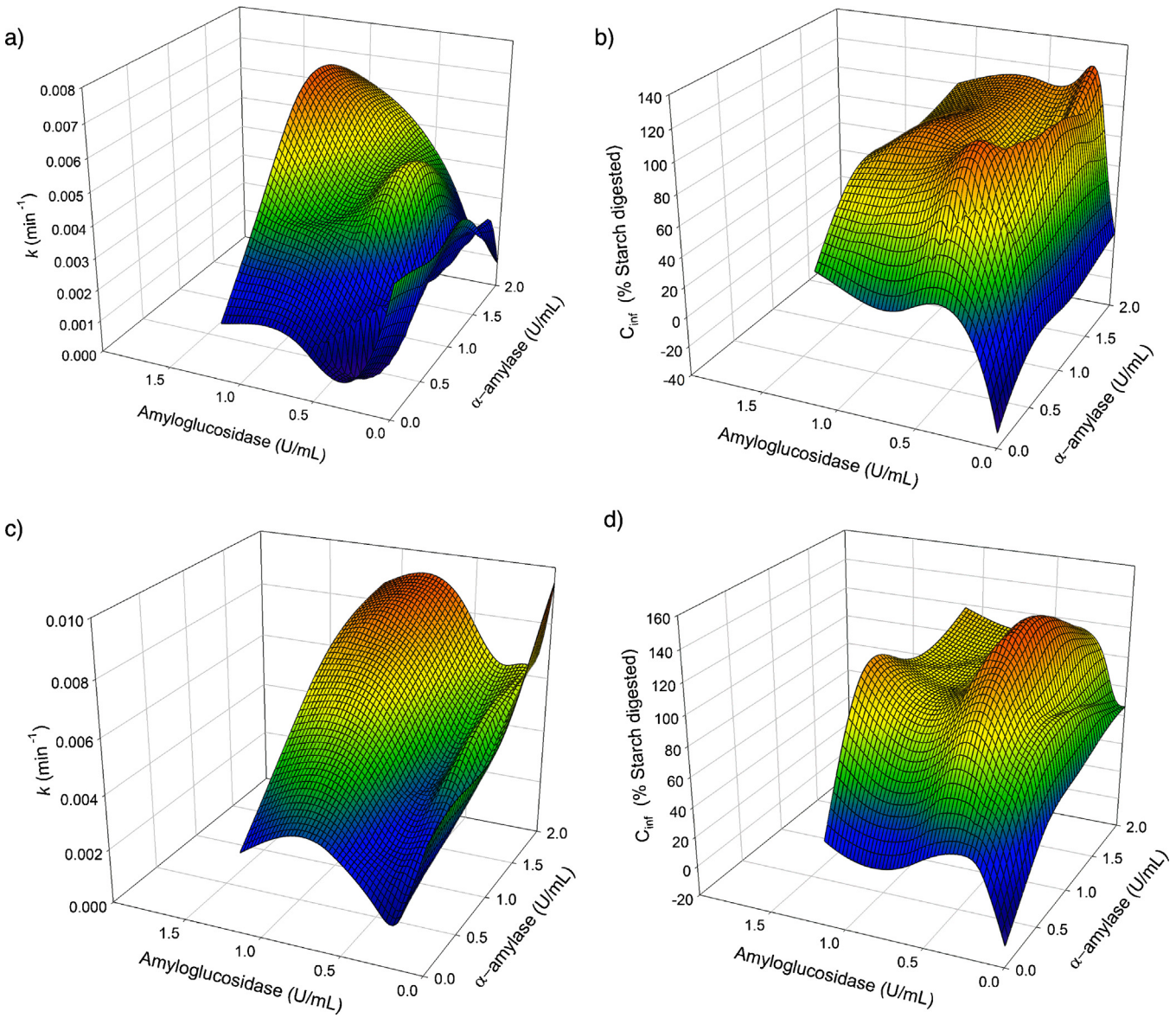

Fig. 5. LOS plot parameters for maize starch digestion at various α-amylase and amyloglucosidase activities shown as interpolated surface plots. (A) k measured by glucose assay. (B) C_{inf} measured by glucose assay. (C) k measured by reducing assay. (D) C_{inf} measured by reducing assay.

present, as observed by Butterworth et al. (2012), but for comparative purposes it was found that a single rate constant could adequately describe the reaction curves observed in the present study. For maize starch the reaction rate constants (k) were found to follow a very similar pattern, with relation to enzyme activity, as the initial rates (Fig. 5a and c). The values for the terminal extent of digestion (C_∞) also vary dependent on enzyme activity. Measured by both GOPOD assay and reductometry, complete digestion of the starch is dependent on adequate levels of activity of both enzymes (Fig. 5b and d). Potato starch is significantly more resistant to enzyme hydrolysis than maize starch, as has been well established in the literature (Dhital et al., 2010; Tahir et al., 2010), but what is less well appreciated is how this impacts upon the design of experiments to monitor the digestion of these slow to digest substrates. In the present study it was found that rate constant values could not be determined when the total enzyme activity (α-amylase and amyloglucosidase combined) was below 3 U per mg of starch (when product was measured by GOPOD assay), as there was insufficient enzyme activity present to adequately deplete the starch during the time course of the reaction, resulting in an essentially linear reaction curve (Fig. 2a and b). As a consequence, there was not enough data available to interpolate surface plots, similar to those produced for maize starch; complete data for all the values of k and C_{inf} that could be obtained are presented in Table S2.

Reaction curves approaching a logarithmic form can be obtained at lower enzyme concentrations when product is measured by reducing sugar assay (e.g. comparing the open squares and closed squares in Fig. 1a and b), presumably as with low amyloglucosidase activity significant proportions of the product released is in the form of maltose or longer oligosaccharides, and has not been converted to glucose. While at the same enzyme activity, the rate of hydrolysis of potato starch was always found to be slower than maize starch (Figs. 3 and 4), it was found that at high enzyme activities, potato starch could be completely hydrolysed, at a rate comparable to that which can be achieved for maize starch (Fig. 2a and b, and Table S2). Thus, there is no fraction of potato starch which is intrinsically resistant to hydrolysis, rather it has a structure which at comparable enzyme activities is more slowly digested

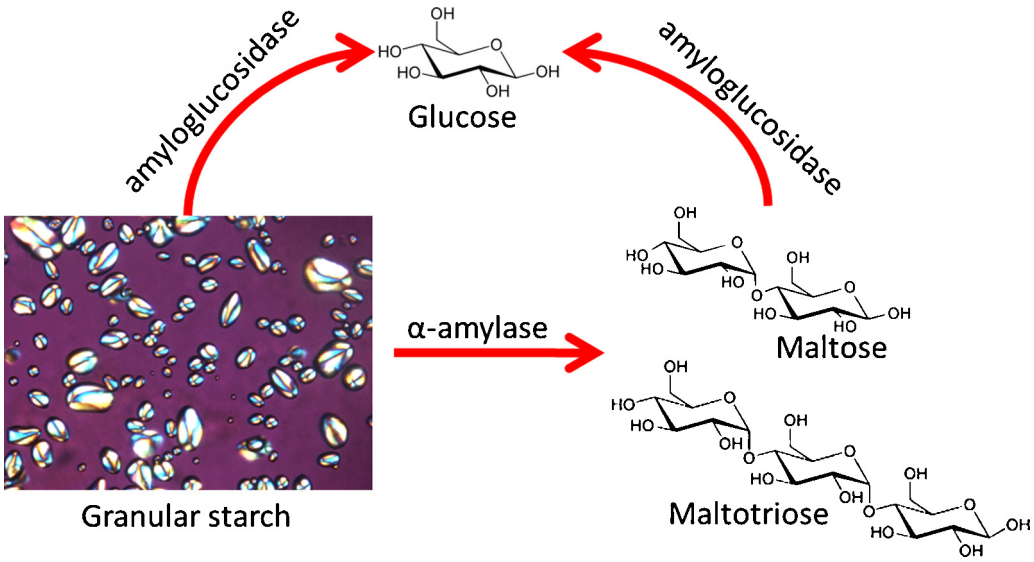

Fig. 6. Schematic showing a reaction scheme whereby α-amylase acts directly on starch, releasing mainly maltose and maltotriose, while amyloglucosidase acts both releases glucose directly from action on starch, and from action on the products of amylolysis.

than maize starch, but the rate and extent of digestion is simply a function of time and enzyme activity.

4. Discussion

The data presented in the current work represent a detailed exploration of the effect of varying activities of α-amylase and amyloglucosidase on the hydrolysis rates of two common granular starches, maize and potato. This work builds upon previous literature suggesting that unexpected effects occur when α-amylase and amyloglucosidase are used together, which cannot be explained through the actions of the individual enzymes (Zhang et al., 2013). Two methods of determining starch breakdown were used, glucose assay and reducing sugar assay, allowing the simultaneous determination of the total amount of sugar released through starch hydrolysis, and the amount of sugar converted all the way to glucose. Thus, in this study we were able to compare the overall rate of starch breakdown, with the rate of conversion of the starch fully to glucose.

An immediate observation is that the reaction rate (k or v) is far more dependent on the combined activity of both enzymes when that activity is measured by glucose release (Fig. 3a and c). Clearly, as amyloglucosidase is responsible for the production of the majority of glucose, in the absence of amyloglucosidase the reaction rate falls to nearly zero when the glucose assay is used, while the starch is being digested at a significant rate, as measured by reducing sugar assay. The addition of small amounts of amyloglucosidase does not immediately result in the hydrolysis rate measured by both methods becoming equal when the rates are measured by glucose assay, indicating that when the α-amylase activity is greatly in excess of the amyloglucosidase activity, the rate of product produced by α-amylase exceeds the rate at which amyloglucosidase can convert this product and granular starch to glucose (see reaction scheme in Fig. 6), especially considering that amyloglucosidase is relatively inefficient at converting shorter α-amylase products (maltose and maltotriose) to glucose (Sierks & Svensson, 2000; Zhang et al., 2013). The rates (k) of hydrolysis only approach similar values for the two measurement methods when the amount of α-amylase and amyloglucosidase were similar, i.e. adequate amyloglucosidase was present to convert all the α-amylase products to glucose (Tables S1 and S2). The initial rates (v) were always faster when measured by reducing sugar assay (Fig. 4), but this can be accounted for as the reducing sugar assay used maltose as a standard, and would therefore overestimate the amount of product produced if some of the product was in the form of glucose.

The amount of starch hydrolysed at the endpoint of the reaction, termed C_∞, also showed dependence on enzyme activity. For neither of the starches was a fraction observed which was fully resistant to enzyme digestion, as has often been suggested to exist, in particular for native potato starch (Åkerberg, Liljeberg, Granfeldt, Drews, & Björck, 1998; Planchot, Colonna, Gallant, & Bouchet, 1995; Tester et al., 2006). Fig. 5b and d clearly illustrate that once an adequate activity of both enzymes is used, the amount of maize starch digested at the reaction completion point plateaus at 100% starch digestion, while the digestion rate (Fig. 5a and c) continues to increase. A similar pattern was observed for potato starch (Table S2), although significantly more enzyme was required to achieve 100% digestion. It should be noted that when measured by reducing sugar assay the value of C_{inf} is overestimated somewhat as at high amyloglucosidase activities the majority of the maltose is converted to glucose, which was not taken into account in the present calculations for simplicity. These observations have important implications for the concept of resistant starch as measured *in vitro* and *in vivo*. Resistant starch may be most succinctly defined through a physiological description as "starch which avoids digestion in the small intestine, and may be fermented in the large intestine" (Åkerberg et al., 1998; Dhital et al., 2014; Zhang & Hamaker, 2009), but resistant starch often has a secondary *in vitro* definition as "starch which is not digested after a given time during *in vitro* digestion" (Englyst et al., 1992). The results presented here suggest that this simple *in vitro* definition is inadequate, as native potato granule resistant starch (often termed RS2 (Englyst et al., 1992; Sajilata, Singhal, & Kulkarni, 2006)) is not completely enzyme resistant, nor is any fraction of it completely enzyme resistant. The fraction of the starch that is resistant to digestion is simply a function of the amount of enzyme used and the digestion time. With analogy to the *in vivo* situation, the resistance of the starch (i.e. the proportion that reaches the large intestine), will be a function of the amylolytic enzyme activity which is present in the small intestine and the time exposed to that enzyme (i.e. small intestinal transit time). Thus, the key determinant from an *in vitro* assay of whether a proportion of a starch will be resistant to digestion *in vivo* is the rate at which the starch is digested under defined conditions of enzyme activity, rather than any reaction endpoint. The more

slowly digested a starch is *in vitro*, the higher the likelihood there is of a fraction of that starch reaching the large intestine *in vivo*, subject to individual variations in intestinal enzyme activities and transit rates. Consequently, the inter- and intra-individual variations in enzyme secretion levels and transit times means that the amount of any given starch that reaches the large intestine will vary for both an individual and populations *i.e.* physiologically resistant starch levels are intrinsically variable.

5. Conclusions

In the present work, the digestion rate and extent of two starches was measured for a wide range of enzyme activities. There was a large and systematic variation in digestion rate and extent, depending on both the relative activities of the enzymes and the measurement methods used. The data presented is expected to be of use in future studies of *in vitro* starch digestion, through informing the design of experiments to achieve adequate reaction rates necessary to allow first order reaction kinetic analysis. In the present study it was found that a minimum of 2 U/mL of α-amylase and 1.12 U/mL of amyloglucosidase was required to produce curves that were rapid enough to be analysed by 1st order kinetic methods, using both glucose and reducing sugar assay, and which resulted in a C_{inf} value of 100% of starch being digested.

Furthermore, the results show that native potato starch granules (an archetypal 'resistant' starch), although digested slowly, do not have a fraction which is completely resistant to digestion *in vitro*. Therefore, the endpoint of an *in vitro* enzymic digestion should not be used in isolation to predict an absolute value for resistant starch. The finding that potato starch granules can be completely digested *in vitro* given enough enzyme and time illustrates the likely dependence of *in vivo* resistant starch levels on endogenous enzyme activity and small intestinal passage rate, either or both of which may vary between meals and/or between individuals. *In vitro* assays can be a useful indicator but should not be expected to provide accurate quantitative prediction of *in vivo* resistance levels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.09.043.

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