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# The relation of physical properties of native starch granules to the kinetics of amylolysis catalysed by porcine pancreatic α-amylase

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ABSTRACT

Various native starch granules were tested as substrates for porcine pancreatic α-amylase and enzyme kinetic parameters obtained from initial rate studies. Surface area per kg weight values and gelatinisation enthalpies ($\Delta H_{gel}$) for the different granules were measured. $\Delta H_{gel}$ was used as an indication of the degree of disorder within the starch granules. $K_m$ values, that are indicative of starch availability, decreased with the particle size but not in a linear fashion. The catalytic efficiency of amylase and the initial rate of catalysed hydrolysis were both significantly correlated with the surface area. Also, $K_m$, catalytic efficiency ($k_{cat}/K_m$) and initial hydrolysis rate were all significantly correlated with $\Delta H_{gel}$. Thus the surface area of granules and the degree of order of starch have important influences on the initial rate at which native starch is digested by amylase. We believe that this is the first systematic report linking kinetic data with surface area and gelatinisation parameters of native starch.



## 1. Introduction

Hydrolysis of native starch granules by α-amylase involves a reaction between an enzyme in solution and a solid surface represented by the granule. Thus the surface area accessible to the enzyme and the efficiency of adsorption onto this surface are important kinetic parameters (Bertoft & Manelius, 1992). Adsorption of amylase on the granule is a pre-requisite for subsequent hydrolysis of starch (Slaughter, Ellis, & Butterworth, 2001). Upon adsorption, the specific forces between the enzyme binding sites and an accessible run of glycosidic linkages results in the formation of a productive enzyme–substrate complex that can be converted to products. The number of possible adsorption sites for the enzyme is likely to depend on a number of factors including the total surface area, pores/crevices in the structure that are wide enough to accommodate a molecule of α-amylase and the degree to which accessible glycosidic bonds in the starch (e.g., those particularly located in non-crystalline regions) are exposed at the surface (Colonna, Leloup, & Buleon, 1992).

The ease with which α-amylase can hydrolyse native starch granules has been related to the particle size (Franco, do Rio Preto, Ciacco, & Geraldo-Campinas, 1992; Franco, do Rio Preto, Ciacco, & Tavares, 1998; Kong, Kim, Kim, & Kim, 2003; Langworthy & Deuel, 1922; Noda et al., 2005; Riley et al., 2004; Tang, Mitsunaga, & Kawamura, 2004). Larger granules with relatively smaller surface area per unit weight are likely to possess lower affinity than smaller granules with respect to amylase binding/adsorption. In fact several studies made with native starches of a single botanical type that had been fractionated into different particle sizes, have shown that smaller granules are digested more rapidly than larger ones (Franco et al., 1992, 1998; Knutson, Khoo, Cluskey, & Inglett, 1982; Tang et al., 2004). It is well established that potato starch has the largest granule size of commercial preparations and that this is, in part, responsible for its reduced susceptibility to the action of amylase (Noda et al., 2005). Its B-type crystallinity is also a key factor in explaining the reduced rate of amylolysis (Planchot, Colonna, Buleon, & Gallant, 1997).

In contrast to the studies reported above however, several workers have shown that granule size has little influence on the susceptibility of the starch to amylolysis (Balls & Schwimmer, 1944; Leach & Schoch, 1961; Planchot, Colonna, Gallant, & Bouchet, 1995; Valetudie, Colonna, Bouchet, & Gallant, 1993; Zhang & Oates, 1999). The lack of uniformity in reports in the literature of amylolysis-size relationships remains unexplained and clearly warrants further study from an enzymological viewpoint.

It is widely believed that amylase attacks regions of starch polysaccharide that are non-crystalline and thus the degree of crystallinity will have an influence on the rate of digestion of the starch granule (Planchot et al., 1997). Zhang, Ao, and Hamaker (2006) have reported however, that both crystalline and amorphous regions of maize starch are digested with equal ease by α-amylase, thus leaving the question open of the susceptibility of crystalline regions to digestion. Differential scanning calorimetry (DSC) performed under quasi-equilibrium conditions in excess water can be used for structural studies including estimation of the proportion of ordered

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structures (Bogracheva, Wang, Wang, & Hedley, 2002). The gelatinisation enthalpy ($\Delta H_{gel}$) reflects the disruption of double helices and crystalline order, partial solubilisation of amylose and the development of glucan chain–water complexes and chain–chain interactions (Bogracheva et al., 2002). However, $\Delta H_{gel}$ determined under quasi-equilibrium conditions mainly reflects the loss of ordered structures (Bogracheva et al., 2002; Cooke & Gidley, 1992). Therefore it is of interest to compare the kinetics of amylase action on starches with gelatinisation enthalpies determined by DSC. Mechteldis, Wolters, and Cone (1992) reported an inverse correlation between $\Delta H_{gel}$ and the percentage of starch hydrolysed by amylase in 4 h, but relationships between $\Delta H_{gel}$ and enzyme kinetic parameters were not explored.

We have performed enzyme kinetic studies of amylase acting on a number of starch granules from several sources that differ in size, and have performed DSC studies in parallel with the enzyme work. The investigation contributes to our goal of seeking molecular explanations for the differences in susceptibility to amylolysis of starch granules of various botanical sources.

## 2. Materials and methods

### 2.1. Starches and biochemicals

Commercially extracted starch granules of high purity were obtained from various sources mainly as gifts. Wheat starch was provided by Prof. C. Hedley (formerly of the John Innes Centre, Norwich, UK), potato starch came from the National Starch Chemical Corporation (Manchester, UK) and rice starches (non-waxy-Remyline DR and waxy-Remyline-AX-DR) were gifts from Cairn Chemical Ltd. (Chesham, Bucks, UK).

Starches from wild type and mutant (*lam* and *r*) pea seeds were extracted by the method of Bogracheva et al. (1999). The pea mutants originated from the John Innes Centre (Norwich, UK). The *r* mutant is associated with decreased activity of starch branching enzyme and results in a decrease of the overall rate of starch synthesis, an increase in the proportion of amylose and a fall in the proportion of amylopectin (Lloyd, Hedley, Bull, & Ring, 1996). In addition, the morphology of starch granules is affected. The *lam* mutant lacks starch synthase 1 (Wang et al., 1997) and produces starch with very low amylase content, hence the designation as *lam*. The contamination by non-starch polysaccharides (NSP) was estimated from microscopy and chemical analysis (Roder et al., 2009) and found to be very low (<1%), and only fragments of cell walls from ruptured cells of pea seeds, rather than cell walls of whole cells, were present.

Phosphate buffered saline (PBS) tablets and porcine pancreatic α-amylase (type 1) were obtained from Sigma–Aldridge Co. Ltd. (Poole, Dorset, UK). The activity of this preparation is stated by the suppliers to be approximately 1000 units per mg protein, where 1 unit catalyses the release of 1 mg of maltose from starch in 3 min at 20 °C. This corresponds approximately to 0.97 μmol maltose/min/mg protein (~1 IU/mg protein at 20 °C). The purity of the preparation was checked by electrophoresis on denaturing polyacrylamide gels. Using the Mr of 56 kDa for amylase (Roder et al., 2009), a stock solution of 572 nM in PBS containing 1 mg/ml of bovine serum albumin was prepared fresh for each assay experiment. All other reagents, in the best grade available, were purchased from the same company (i.e. Sigma–Aldridge).

### 2.2. Light microscopy and particle sizing of native starches

For sizing, both digital image analysis coupled with light microscopy (DIALM) and a Beckman Coulter Counter Multisizer™ were used. Starch samples were mounted in distilled water before examination under a microscope with a video camera attachment connected to a PC equipped with ImageProPlus® 4.0 Software. The videos of microscopic images of particles were analysed for diameter, surface area and shape factors (Allen, 1997). The surface area per unit weight of particles was calculated assuming a specific gravity of 1.5 for all of the different starch granules (Donovan, 1979). In all cases, at least 1000 granules were examined to allow calculation of median diameters of the particles. The choice of DIALM for particle sizing allowed individual particles to be observed and measured. Many starch preparations contain granule clusters as well as individual grains. Calibration of the Counter was checked using Coulter Counter Standard L10 polystyrene latex (mode diameter 9.92 μm) and an aperture of 100 μm was used. The Coulter Counter may treat clusters as single particles and thus introduce anomalies in the data.

### 2.3. Differential scanning calorimetry

Gelatinisation parameters of native starches were measured and recorded on a Seteram Micro-DSC III (Seteram Instruments Ltd., Lyons, France) instrument, equipped with a thermal analysis data station and data recording software (Roder et al., 2009). Samples of starch suspensions (~3.5% in distilled–deionised water) were added to pre-weighed cuvettes and hermetically sealed. The sample was equilibrated at 20 °C for 2 h then scanned from 20 to 120 °C with a heating rate of 0.5 °C $min^{-1}$. The relatively slow heating rate in excess aqueous solution ensures that the gelatinisation process is 'quasi-equilibrium' (Davydova, Leont'ev, Genin, Sasov, & Bogracheva, 1995; Wang, Bogracheva, & Hedley, 1998). The sample and reference were cooled from 120 to 20 °C at a rate of 1.0 °C $min^{-1}$ and held at 20 °C for a further 60 min before scanning again from 20 to 120 °C, at a heating rate of 0.5 °C $min^{-1}$, followed by cooling to 20 °C. The DSC thermograms were analysed using Origin® software (MicroCal Europe, Milton Keynes, Bucks, UK) to obtain values for gelatinisation enthalpies ($\Delta H_{gel}$).

### 2.4. Chemical characterisation of the starches

A reagent kit (Megazyme Bray, Co. Wicklow, Ireland) was used for amylase/amylopectin assays. The method involves solubilisation of starch using dimethyl sulphoxide, followed by precipitation of amylopectin using concanavalin A and hydrolysis of the remaining amylose using amyloglucosidase/α-amylase. The glucose formed is then estimated colorimetrically. From the total starch content, the amylopectin fraction is determined by difference. Replicate samples ($n$ = 4–6) were analysed.

Starch moisture contents were determined in duplicate, by drying weighed amounts of starch in pre-dried aluminium dishes in an air oven at 103 °C to reach constant weight (Roder et al., 2009). Non-carbohydrate components of the samples were analysed by standard methods (Wang, Ellis, Ross-Murphy, & Reid, 1995). Protein contents of duplicate samples were estimated from total nitrogen measurements (N × 6.25) determined by a micro-Kjeldahl method. Samples for fat analysis were dispatched to Eclipse Scientific Group (Cambridgeshire, UK).

### 2.5. Processing of starches before use as enzyme substrates

Suspensions of starch granules (10–20 mg/ml) were prepared in 20–30 ml of freshly prepared PBS (pH 7.4). The mixture was agitated gently for 20 min in a 150 ml conical flask. This was conducted at room temperature (~25 °C). For preparation of 10–20 mg/ml suspensions of *r* pea starch, the starch–PBS mixtures were gently mixed (Ultra-Turrax homogenizer®, Janke & Kunkel, IKA-Werk, Staufen, Germany) at the lowest speed using 4–6 pulses

of 1–2 s duration, to break up any granule aggregates and produce an homogenous suspension, as viewed by light microscopy.

### 2.6. Assay of α-amylase and determination of kinetic parameters

The assay system has been described in detail elsewhere (Roder et al., 2009; Slaughter, Ellis, Jackson, & Butterworth, 2002; Slaughter et al., 2001). In brief, starch suspensions ranging in concentration from 1.25 to 10 mg/ml in PBS were reacted with 1.43 nM α-amylase at 37 °C under constant mixing in a total reaction volume of 4 ml. Samples were withdrawn at timed intervals up to 12 min and transferred to an ice cold 0.3 M $Na_2CO_3$ stop solution. After sedimentation of non-reacted starch by centrifugation, the supernatants were frozen and stored at −20 °C for up to 1 week before the concentration of reducing sugar in the supernatants was determined by a Prussian blue method. Reducing sugar was expressed as maltose equivalents by reference to a standard curve prepared for this sugar. Initial reaction rates were calculated from linear progress curves of the data obtained from the timed intervals up to 12 min and analysed by a weighted non-linear regression fit to the Michaelis–Menten equation using Enzfitter software (Biosoft® Great Shelford, Cambridgeshire, UK) to provide values for $K_m$ and $k_{cat}$ (the turnover number of amylase) and $k_{cat}/K_m$ (catalytic efficiency, CE) plus the standard errors of the estimated kinetic parameters. Where comparative rates of hydrolysis are reported in the Results, the data were obtained from the rate of release of maltose from 0.5% starch (5 mg/ml) during the initial 12 min of reaction. The enzyme concentration used for determining relative rates of hydrolysis was the same for each starch (1.43 nM or approximately $8 \times 10^{-3}$ IU). The amount of starch hydrolysed during the assay was less than 1% of the total added to the reaction mixture. The various kinetic parameters were obtained from the results of 3–5 separate experiments, each carried out in duplicate.

### 2.7. Statistical analysis

Pearson correlation and regression analyses were performed between the kinetic data and the values obtained for surface areas and gelatinisation enthalpies using standard statistical software (Excel 2003). Statistically significant correlations were accepted at $P < 0.05$.

## 3. Results and discussion

Some physical and chemical properties of the starches used in this investigation are shown in Table 1. Although it is known that the lipid content of starch granules can affect the amylolysis of starch (Svihus, Uhlen, & Harstad, 2005), such effects have not been considered further in this report given the close similarity of the amounts of measured lipid in the various starch granules used in our study. The protein content of starches from potato, non-waxy rice and waxy rice were also very similar (mean, 0.1 g/100 g of starch). Wheat plus *wt* and *lam* pea starches were somewhat higher in protein content (mean 0.26%), but the *r* pea starch was much higher at 0.67%. If a significant fraction of the protein associated with the granules is located at exposed surfaces (Han, Benmoussa, Gray, BeMiller, & Hamaker, 2005), then differences in the protein content of the various starches could influence the accessibility of surface located glucan chains to amylase. Although most starch granules possess certain characteristic proteins at their surface (Oda & Schofield, 1997), it is known that a considerable fraction of protein is comprised of granule-bound starch synthase located within the granule (Han & Hamaker, 2002; Rahman et al., 1995) and would not be expected to affect initial rates of hydrolysis of native starch. The *r* mutant is characterised by a relatively inactive starch branching enzyme II (Morell, Blennow, Kosar-Hashemi, & Samuel, 1997) but little information seems to be available of the extent to which the inactive enzyme protein is expressed. Whether the relatively high protein content of the *r* mutant granule is related to over expression of the impaired enzyme cannot be ascertained from our experiments. Investigation of the effects of granular protein on initial rate kinetics of amylolysis could be an important subject for future studies.

The water content of the granules ranged from 10.1% to 14.4% with a mean of 12.6%. In some preliminary treatments of the data to obtain $K_m$ and CE values, the starch absolute concentrations were calculated by allowing for the water content. For example, the $K_m$ value for wheat starch will change from 8.4 to 7.4 mg/ml if allowance is made for the weight of water in the weighed sample of starch. The plots shown below however, were obtained from calculations that ignored the differences in water content because the preliminary analyses revealed that allowing for the water had no significant effect on the conclusions reached for relationships of kinetic properties with particle size or gelatinisation enthalpy.

The surface areas shown in Table 1 were obtained from DIALM and Coulter Counter Multisizer™ data. The agreement between the two methods was reasonably good for most of the starches, but there were disparities in the respective values obtained for both wheat and potato and also particularly for the *r* pea mutant. The differences were not entirely unexpected given that wheat starch has a well known bimodal distribution and potato granules have a broad size range when viewed under the microscope. Apart from *r* pea starch, the other starches were essentially unimodal. The multilobular nature of the *r* granules plus the existence of aggregates revealed by microscopy, accounts for the disparity in estimated sizes by the two methods. Also the accuracy of estimates

**Table 1**
Some properties and characteristics of the starches used in the investigation.

| Starch | $\Delta H_{gel}$ (J/g) | Surface area/unit weight $m^2/kg$ Method | | Amylose ± SD (%) | Moisture (%) | Lipid (%) | Protein (%) |
|---|---|---|---|---|---|---|---|
| | | DIALM | Counter | | | | |
| Wheat | 9.7 | 421 | 571 | 28.3 ± 3.0 | 11.7 | 0.4 | 0.31 |
| Potato | 17.9 | 211 | 254 | 21.5 ± 2.2 | 14.7 | 0.2 | 0.09 |
| Non-waxy rice | 11.1 | 833 | 750 | 18.6 ± 1.1 | 10.1 | 0.2 | 0.11 |
| Waxy rice | 13.0 | 833 | 749 | 2.4 ± 0.7 | 10.2 | 0.3 | 0.1 |
| Wild type pea | 13.0 | 211 | 194 | 30.0 ± 2.0 | 14.0 | 0.25 | 0.25 |
| *lam* mutant | 14.9 | 296 | 253 | 11.3 ± 0.5 | 13.2 | 0.15 | 0.21 |
| *r* mutant | 6.2 | 296[a] | 458[a] | 71.8 ± 2.9 | 14.4 | 0.15 | 0.67 |

All entries in the table represent the mean of replicate measurements except for the particle surface areas which were calculated from median diameters of replicates. SD = the standard deviation.

[a] The starch from *r* pea mutant is multilobular.

of surface area parameters for *r* are likely to be compromised for the Coulter Counter method given that the shape of *r* granules is far from spherical (Bogracheva et al., 1999).

Table 2 shows a summary of the kinetic data obtained for all the native starches. $K_m$ values can be used as a guide to the substrate availability for amylase. If the quantity of available starch is low, a relatively large concentration of granules will be required to provide a sufficient concentration of substrate to allow a reaction rate of $V_{max}/2$. Therefore the calculated $K_m$ value based on the total starch added to the reaction mixture will be relatively high. Conversely, if the availability of starch is high, the measured $K_m$ will be relatively low. It might be expected therefore, that the measured $K_m$ value for the various starch granules would be related to their size. Fig. 1 shows that although $K_m$ increases with the size of the granule (decreases with surface area per unit weight), the relationship between $K_m$ and surface area is not linear. It is clear that the results for *r* starch do not follow the general trend exhibited by the other starches. The anomalous behaviour may be related, in part, to difficulties in obtaining reliable estimates of size and shape parameters (see above). In order for the *r* results to fit the general relationships shown for the other starches, the actual surface area of the *r* granules would need to be much greater than the value we obtained using our sizing methods. However, the very high amylose content of this starch is also likely to influence the kinetics of amylase action directly and contribute to the anomalous data. Studies of the effects of amylose-rich starches on the kinetics of amylolysis are currently under investigation in our laboratory.

The kinetic parameter $k_{cat}/K_m$ is known as the catalytic efficiency (CE) or specificity constant (Cornish-Bowden, 2004) and is useful for comparing the relative effectiveness of a number of starches as substrates for α-amylase (Slaughter et al., 2001, 2002). This parameter can be equated with the second order rate constant for enzyme and substrate collision leading to formation of an ES complex. The 'hit' rate is expected to be related to the surface area, but with starch granules, the availability of segments of susceptible α-glucan chains at the surface to which amylase may bind, to form an ES complex, have also to be considered.

Fig. 2a shows that CE is significantly correlated with the surface area of the granules provided that the data for pea mutant *r* starch are ignored; $r = 0.90$, $P < 0.02$ and $r = 0.922$, $P < 0.01$ (both with 4 degrees of freedom, df) for DIALM, and Coulter Counter data, respectively. Similarly, with the exception of *r*, the rate of hydrolysis determined during the first 12 min of reaction is linearly related to the surface area of the granules (Fig. 2b). Ignoring the hydrolysis rate data for pea mutant *r* starch, $r = 0.81$, $P < 0.05$ and $r = 0.856$, $P < 0.05$, (both with 4 df) for DIALM and counter data, respectively. Overall, the results again point to a clear difference in the behaviour of *r* relative to the other granules and also to the difficulties in obtaining reliable estimates of its surface area.

The literature contains several instances of similar findings relating particle size and hydrolysis rate but the earlier studies have rarely measured initial reaction rates and have usually involved long incubations with amylase, Kong et al. (2003) measured

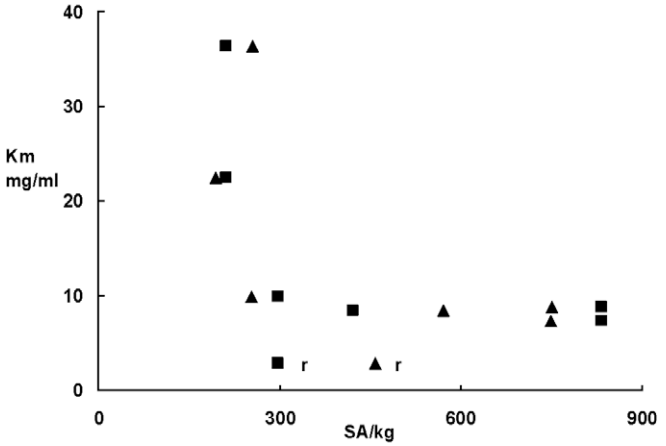


**Fig. 1.** Relationship between $K_m$ for α-amylase acting on native starch granules and the granule surface area. Individual data points represent areas calculated by microscopy (DIALM) (■) and Coulter Counter™ (▲). Data for the pea *r* mutant starch are identified with *r*. The units for surface area per unit weight are $m^2/kg$.

reaction rates in the initial 10 min of incubation, but they used much higher enzyme concentrations in their reaction mixtures than we did (>3 orders of magnitude) and consequently their reaction rates departed from linearity within 1 min. Under our conditions the rate of release of maltose was linear with time for at least 15–20 min.

The more complex relationship of $K_m$ with granule surface area than is seen for the CE and hydrolysis rate relationships is puzzling, but may be indicative of differences in the relative amounts of susceptible starch at the surface of the various granules. If the proportion of susceptible α-glucan chains distributed at the granule surface is not identical for different botanical starches, there is likely to be an impact on measured $K_m$ values particularly.

The relationship between $K_m$ and $\Delta H_{gel}$ is shown in Fig. 3. It is clear that the greater the gelatinisation enthalpy, the greater is the $K_m$ value. The relationship appears to be reasonably linear ($r = 0.78$, $P < 0.05$, 5 df) and suggests, predictably, that in granules with a high degree of ordered structures, the availability of α-glucan chain segments that interact favourably with amylase is limited. Linear relationships with significant correlations were also found for both CE ($r = 0.827$, $P < 0.05$, 5 df) and hydrolysis rate with $\Delta H_{gel}$ ($r = 0.873$, $P < 0.02$, 5 df) (Fig. 4a and b), but there was no obvious relationship between $K_m$ and/or CE values and the amylose content of the granules (data not shown).

These findings of a relationship between $\Delta H_{gel}$ and the catalytic properties do not support the conclusions reached by Zhang et al. (2006) for maize starch, that amorphous and crystalline fractions of starch are digested with equal ease by amylase. The methods used by Zhang were very different from ours in that they used much higher concentrations of amylase (by a factor of greater than $10^3$) and they determined digestion rates over time periods of up to

**Table 2**
Summary of the enzyme kinetic data obtained for α-amylase acting on native starch granules. Reaction rates were determined from the linear release of product with time over the initial 12 min of reaction. The hydrolysis rate was determined with a starch concentration of 5 mg/ml. The concentration of amylase was kept constant for all the rate assays. Kinetic parameters and their standard errors were determined from a computer fit to the Michaelis–Menten equation.

| Starch | $K_m$ (mg/ml) | $k_{cat}$ ($min^{-1} \times 10^{-5}$) | $k_{cat}/K_m$ ($\times 10^{-4}$) | Hydrolysis rate (μM/min) |
|---|---|---|---|---|
| Wheat | 8.4 ± 0.0 | 0.25 ± 0.0 | 0.30 ± 0.0 | 1.48 |
| Potato | 36.4 ± 8.3 | 0.30 ± 0.05 | 0.08 ± 0.02 | 0.49 |
| Non-waxy rice | 8.8 ± 1.1 | 0.38 ± 0.03 | 0.43 ± 0.09 | 2.25 |
| Waxy rice | 7.3 ± 1.5 | 0.39 ± 0.01 | 0.52 ± 0.12 | 2.18 |
| Pea *wt* | 22.4 ± 1.1 | 0.39 ± 0.01 | 0.17 ± 0.013 | 1.25 ± 0.35 |
| Pea *lam* | 9.9 ± 0.11 | 0.15 ± 0.0 | 0.15 ± 0.0 | 0.83 ± 0.21 |
| Pea *r* | 2.8 ± 0.02 | 0.36 ± 0.01 | 1.27 ± 0.13 | 3.44 ± 0.36 |

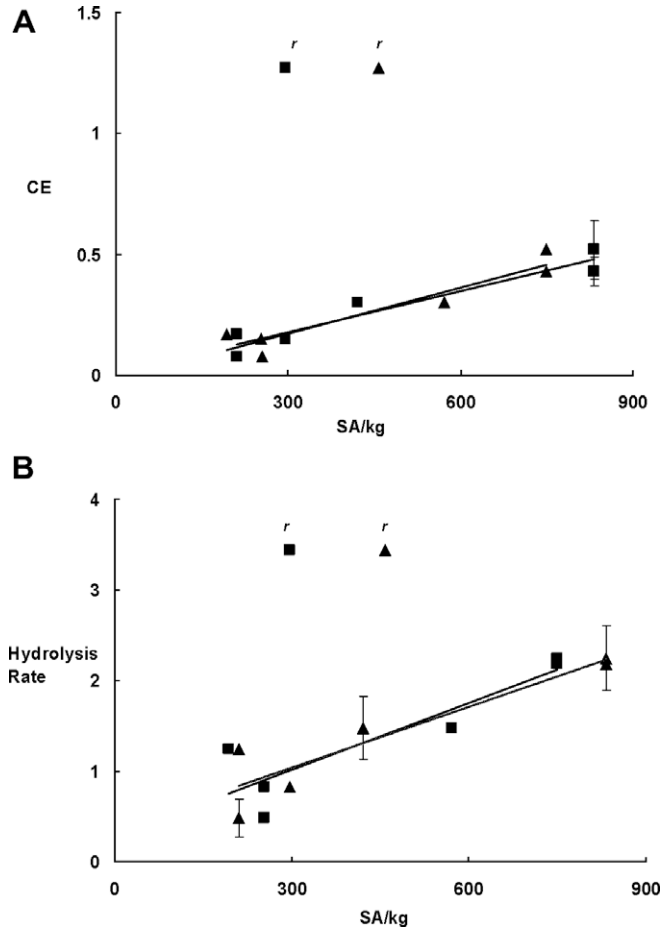


**Fig. 2.** (a) Relationship between the catalytic efficiency (CE) of α-amylase action on native starch granules and surface area. Individual data points represent areas calculated by DIALM (■) and Coulter Counter™ (▲). Data points for *r* starch are indicated within the figure. The units of CE are [μM/min (mg per ml)$^{-1}$ × 10$^4$]. The correlation data for DIALM and Coulter Counter, respectively, are $r = 0.9$, $P < 0.02$ and $r = 0.922$, $P < 0.01$ (both with 4 degrees of freedom, df). (b) Relationship between hydrolysis rate and surface area. Individual data points represent areas calculated by DIALM (■) and Coulter Counter™ (▲). The units for hydrolysis rate are μM/min and the starch concentration was 5 mg/ml in each case. Data points for *r* starch are indicated within the figure. The correlation data for DIALM and Coulter Counter, respectively, are $r = 0.81$, $P < 0.05$ and $r = 0.856$, $P < 0.05$ (both with 4 df).

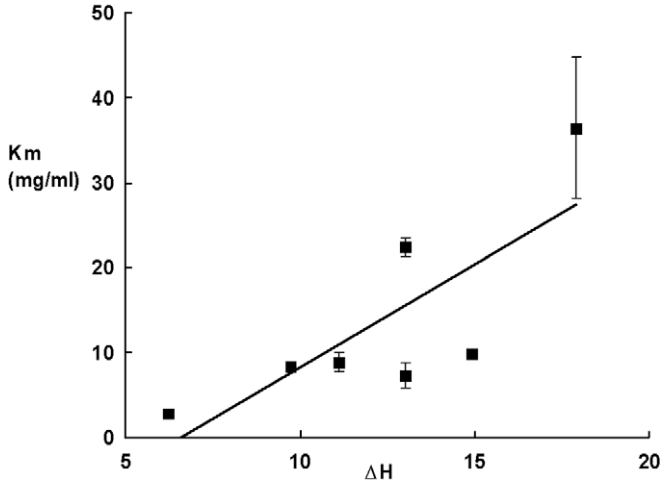


**Fig. 3.** Relationship between $K_m$ for α-amylase action and the gelatinisation enthalpies of the starches. The units for Δ*H* are joules/g. The *r* starch data points are included on the plot. The correlation data are $r = 0.78$, $P < 0.05$ (5 df).

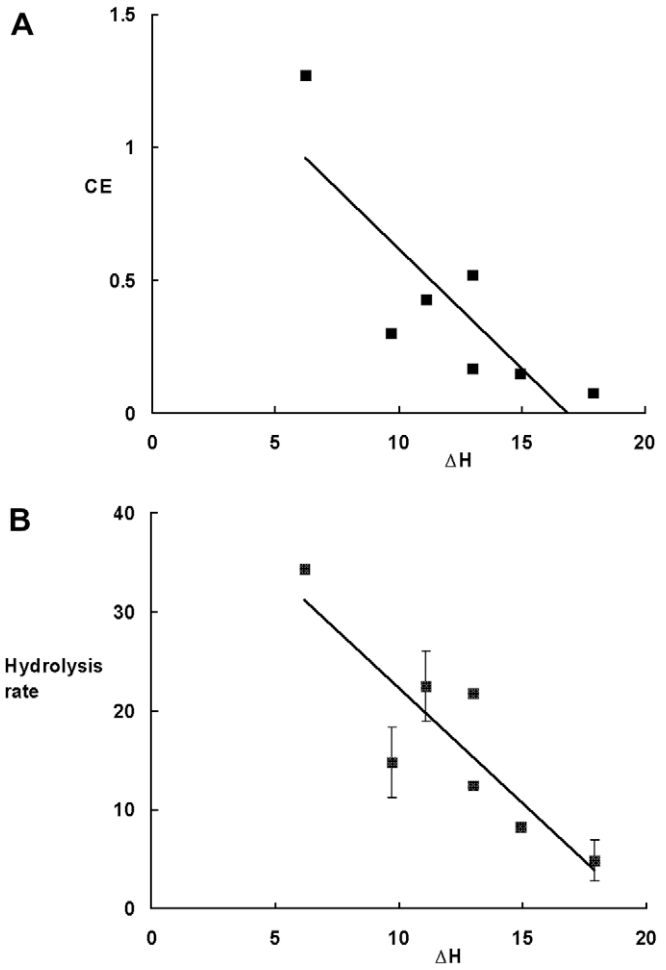


**Fig. 4.** (a) Relationship between the catalytic efficiency (CE) of α-amylase action and gelatinisation enthalpy of starch. CE has units of [μM/min (mg/ml)$^{-1}$] × 10$^4$. Δ*H* is in joules/kg. The correlation data are: $r = 0.827$, $P < 0.05$ (5 df). The *r* starch data are included in the plot. (b) Relationship between the hydrolysis rate for α-amylase acting on starch solutions (5 mg/ml) and gelatinisation enthalpy. The units for hydrolysis rates are μM/min. The correlation data are: $r = 0.873$, $P < 0.02$ (5 df). The *r* starch data are included in the plot.

3 h compared with the 12 min initial rates that we studied. Also they did not measure the proportions of amorphous and crystalline material in their digested samples to check whether amorphous and crystalline starch fractions are really digested at similar rates. It is quite possible that the 'side-by-side' digestion suggested by Zhang et al. (2006) can occur if the amylase concentration is high enough. Calculations of the likely activity of amylase in the small intestine (Slaughter et al., 2001) place the enzyme activity in our assays at approximately 10–20% of the amount seen *in vivo*. If Zhang and colleagues are correct and side-by side digestion of crystalline material occurs at relatively high amylase activities, we believe that it would not be realised *in vivo* where the activity is so much lower than that used in their studies.

## 4. Conclusions

It is clear that the relative surface area of the starch granule affects the kinetic properties of α-amylase action. Granules with larger diameters, and therefore smaller surface area per unit weight, are poorer substrates for amylase in respect of $K_m$, catalytic efficiency, and initial rate of hydrolysis. The CE and hydrolysis rate show a strong correlation with the area per unit weight of granules, but the relation with $K_m$ is more complex. This parameter increases with particle size, but not in a linear fashion. Differences

in the proportion of accessible (available) starch distributed on the surface of the various granules may contribute to the complexities seen in the $K_m$ responses, given that the constant is a useful guide to the fraction of starch that is a suitable substrate for amylase.

In addition, the comparisons of kinetic parameters and gelatinisation enthalpies reveal that the degree of disordered starch in granules is an important determinant of the kinetic responses; a large $\Delta H_{gel}$ and thus a greater degree of crystallinity, is associated with less favourable catalytic efficiencies and hydrolysis rates.

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