



Amylase binding to starch granules under hydrolysing and non-hydrolysing conditions

Sushil Dhital^{a,b}, Frederick J. Warren^b, Bin Zhang^{a,b}, Michael J. Gidley^{a,b,*}

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

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

ARTICLE INFO

Article history:

Received 24 December 2013

Received in revised form 13 June 2014

Accepted 16 June 2014

Available online 1 July 2014

Keywords:

Alpha-amylase

Starch granules

Confocal microscopy

Enzyme binding

Surface structure

ABSTRACT

Although considerable information is available about amylolysis rate, extent and pattern of granular starches, the underlying mechanisms of enzyme action and interactions are not fully understood, partly due to the lack of direct visualisation of enzyme binding and subsequent hydrolysis of starch granules. In the present study, α -amylase (AA) from porcine pancreas was labelled with either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) fluorescent dye with maintenance of significant enzyme activity. The binding of FITC/TRITC-AA conjugate to the surface and interior of granules was studied under both non-hydrolysing (0 °C) and hydrolysing (37 °C) conditions with confocal microscopy. It was observed that enzyme binding to maize starch granules under both conditions was more homogenous compared with potato starch. Enzyme molecules appear to preferentially bind to the granules or part of granules that are more susceptible to enzymic degradation. The specificity is such that fresh enzyme added after a certain time of incubation binds at the same location as previously bound enzyme. By visualising the enzyme location during binding and hydrolysis, detailed information is provided regarding the heterogeneity of granular starch digestion.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Starch is a major component in the human diet, as well as a feedstock for a range of industrial processes. The enzymic hydrolysis of starches to smaller oligomers either in living organisms or industrial processes involves the action of α -amylase (AA), an endo-acting enzyme that hydrolyses α -1 $\rightarrow$ 4 glycosidic bonds of amylose or amylopectin molecules. The amylolysis rate, extent and pattern of starch granules vary depending upon the barriers the enzyme encounters to access and then bind to the starch granules; or upon structural features of starch granules that prevent catalysis after initial binding. These mechanisms have been recently reviewed (Dhital, Warren, Butterworth, Ellis & Gidley, 2014).

Studies of starch hydrolysis either *in vivo* or *in vitro* inevitably provide an average value from a population of starch granules. Recent evidence, however, indicates that there is a great deal of heterogeneity in the internal architecture (Dhital, Shelat, Shrestha & Gidley, 2013) and physical and chemical structures (Liu et al., 2013) within individual granules. This could in principle affect enzyme binding and ultimately the catalytic process.

Studies of amylase binding to starch granules by solution depletion assay at 0 °C, found a dependence of enzyme affinity for starch on the surface area, and therefore particle size of starch granules (Schwimmer & Balls, 1949; Walker & Hope, 1963; Warren, Royall, Gaisford, Butterworth, & Ellis, 2011). Due to the lack of visualisation of enzyme bound to the granules, it could not be determined from these studies whether the enzyme was uniformly bound to all granules or preferentially bound to individual granules with special granular structures.

The morphological changes of starch granules during α -amylolysis have been investigated by analysis of remnant undigested granules by using various microscopic techniques such as light (bright or polarised field) (Leach & Schoch, 1961), scanning electron (Planchot, Colonna, Gallant, & Bouchet, 1995),

* Corresponding author at: ARC Centre of Excellence in Plant Cell Walls, Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia 4072, Qld., Australia.

Tel.: +61 7 3365 2145; fax: +61 7 3365 1177.

E-mail addresses: m.gidley@uq.edu.au, mike.gidley@uq.edu.au (M.J. Gidley).

transmission electron (Gallant, Bouchet, & Baldwin, 1997), atomic force (Sujka & Jamroz, 2009) and confocal laser scanning (Apinan et al., 2007; Lynn & Cochrane, 1997) microscopy. The α -amylolysis patterns of starches from different botanical origins have been described, for example, cereal starches are hydrolysed from the inside of granules towards the periphery (endo-corrosion, inside-out or centrifugal hydrolysis pattern); whereas high-amylose and tuber starches are hydrolysed from the surface towards the interior of granules (exo-corrosion, outside-in, or centripetal hydrolysis pattern). These differences in digestion pattern have been inferred to be related to the surface features of granular starch, possibly reflecting the presence of pores and channels within cereal starches that allow amylase to penetrate towards the less organised granule interior compared to the rigid and smooth surface and interior of tuber starches (Huber & BeMiller, 1997; Jane & Shen, 1993; Pan & Jane, 2000). Although these techniques provide general information regarding the hydrolysis pattern, they do not allow the visualisation of enzyme at the sites of hydrolysis.

Previous authors have attempted to visualise the location of enzyme molecules hydrolysing inside granules. Thomson, Miles, Ring, Shewry, & Tatham (1994) carried out real-time atomic force microscopic (AFM) imaging of wheat starch degradation by α -amylase. The AFM method is, however, limited to observations of the granule surface, and could not directly visualise the location of enzyme molecules. Similarly, Helbert, Schüle, & Henrissat (1996) studied the degradation of starch granules with direct localisation of the amylase by immunogold-labelling. The method, however, was unable to quantify the gold labelling efficiency of enzymes. Furthermore, the cross-sectioning of granules for electron microscopic observation may induce artefacts, for example cracks resembling the channels. Most recently, Tawil et al. (2010) used synchrotron ultraviolet fluorescence microscopy to visualise the adsorption and diffusion of amylase during starch degradation. The technique directly visualised the location of protein by imaging the auto-fluorescence from tryptophan present in AA. This method, while a powerful technique, can only visualise one granule at a time, rather than whole populations of granules. Furthermore, fluorescence from AA cannot be discriminated from other granule associated protein components.

Thus different aspects of the mechanism of amylase reaction with starch granules have been proposed as the outcome of observation using different techniques. However, there are a number of questions which remain unresolved:

1. Do enzymes bind uniformly to the granule surface?
2. Do the surface structure and botanical origin of starch granules affect amylase binding?
3. Why is there heterogeneity in starch granule digestion?
4. Is the heterogeneity of starch granules digestion related to enzyme binding?
5. Do surface features such as pores and channels enhance the diffusion of amylase inside the granules?

The present paper aims to address these questions based on the outcomes of direct localisation of fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) labelled AA during binding (under both non-hydrolysing (0 °C) and hydrolysing (37 °C) conditions) of starch granules from different botanical origins using confocal microscopy. The role of surface pores and channels towards amylase action was further studied through visualisation of the diffusion of fluorescent dextran probes followed by diffusion of labelled AA into starch granules.

2. Materials and methods

2.1. Materials

Potato starch (PS, Sigma S4251) was purchased from Sigma-Aldrich, Australia. Three types of maize starches: high amylose maize starch (Gelose 80) (HAMS, G80), regular maize starch (MS) and waxy maize starch (WMS) were purchased from Penford Australia Ltd., (Lane Cove, Sydney, Australia).

2.2. α -Amylase labelling with FITC and TRITC

α -Amylase from porcine pancreas (A6255, Sigma) was labelled with FITC (F7250, Sigma) and TRITC (87918, Sigma) at 10 $\times$ molar excess in carbonate buffer (0.1 M, pH 9) following the method of The and Feltkamp (1970). The unbound FITC from the conjugate was separated using a desalting column (Sephadex, PD-10) with phosphate buffered saline buffer (PBS, P4417, Sigma, pH 7.2). Following labelling, the enzyme solution was immediately aliquoted and frozen for storage. The enzyme was defrosted immediately prior to use. Freezing did not affect the enzyme activity. The dye:protein (F/P) molar ratio is defined as the ratio of moles of fluorescent moiety to moles of protein in the conjugate (The & Feltkamp, 1970), and was 2.36 and 4.67 for the FITC- and TRITC-AA conjugates, respectively. A unit of activity was defined as the enzyme required to liberate 1.0 mg of maltose from starch in 3 min at pH 6.9 and 37 °C, and activity was found to be 1078 and 1713 unit/mg of protein for FITC and TRITC conjugates, respectively, compared to 2485 unit/mg of protein for the unlabelled enzyme. The protein concentration of FITC, TRITC and unlabelled enzyme stock solutions was 1.39, 2.56 and 29 mg/mL, respectively.

Michaelis–Menten kinetic parameters for unlabelled and FITC labelled AA were determined using MS as a substrate, using a modification of the method of Tahir, Ellis, & Butterworth (2010). Briefly, 4 mL of various concentrations of starch (2.5–25 mg/mL) in PBS buffer were incubated at 37 °C in a water bath. At time 0, enzyme was added to a concentration of 1.5 nM. At 0, 4, 8 and 12 min, 300 μ L of starch suspension was removed and immediately added to 300 μ L of 0.3 M Na₂CO₃ in a microcentrifuge tube to stop the reaction. These samples were then centrifuged at 16,000 $\times$ g for 5 min to remove unreacted starch, and 300 μ L of supernatant removed to a fresh microfuge tube. The reducing sugar content was measured by the *para*-hydroxybenzoic acid hydrazide (PAHBAH) assay (H9882, Sigma) as described by Moretti and Thorson (2008) and expressed as maltose reducing sugar equivalents. Kinetic parameters were obtained from non-linear regression analysis using Sigmaplot® 12.5. All kinetic analysis was carried out in triplicate.

2.3. Confocal laser scanning microscopy

Unless otherwise stated, labelled α -amylase (FITC-AA and TRITC-AA) was observed using a confocal microscope (LSM 700, Carl Zeiss, Germany) with a Plan-Apochromat 20 $\times$ lens (with digital zoom of 2 $\times$ for maize, waxy maize), with and without differential interference contrast (DIC) using Zen Black 2011 software (Carl Zeiss Version 7.1). Starch images were taken using a frame size of 1024 $\times$ 1024 at a scan speed of 8 bit and a pixel dwell time of 1.58 μ s, from an optical slice of 2 μ m thickness. All imaging was performed with a 10 mW argon ion laser at 2% power with excitation of 488 and 555 nm for FITC and TRITC, respectively, either singly or in combination.

2.4. Enzyme binding to starch granules at 0 °C

The binding of FITC- and TRITC-AA conjugates to MS and PS granules was monitored at 0 °C. A 10 mg/mL starch granule dispersion

(2 mL) in sodium acetate buffer (0.2 M, pH 6.0) in 10 mL flat bottom tubes (97 × 16 mm) was immersed fully in an ice water bath placed above a stirrer plate. The dispersion was equilibrated for 10 min with continuous stirring at 200 rpm to ensure that the starch suspension obtained a temperature of 0 °C. The binding experiment was carried out in three different combinations. In the first set, 0.8 unit of FITC-AA conjugate per mg of starch was added and 100 µL aliquots were transferred to 1.5 mL microfuge tubes after 5, 10 and 20 min of incubation. Subsequently, 0.8 units TRITC-AA conjugate per mg of starch was added to the same incubation tube and aliquots were taken 5, 10 and 20 min after addition of the second enzyme. In a second set, TRITC-AA conjugate was added first followed by FITC-AA conjugate as described for the first set. In a third set, both FITC and TRITC-AA conjugates were added simultaneously.

Aliquots were immediately centrifuged at 2000 × g for 30 s, supernatants discarded, and the starch pellet observed using the confocal microscope as described in Section 2.3.

2.5. Enzyme binding to porous starch

In order to evaluate the roles of surface pores and channels in enzyme binding, porous starch granules were obtained by hydrolysing 3 mL of 1% maize starch suspension with 0.8 units/mg of AA (un-labelled) for 20 min at 37 °C. The reaction was halted by the addition of 10 mL of absolute ethanol. The tube was centrifuged at 2000 × g for 5 min. The pellet was washed 3 times with deionised water and the volume adjusted to 3 mL with acetate buffer (0.2 M, pH 6.0). The tube was then incubated at 0 °C for 10 min under the same mixing condition (200 rpm), and the binding experiment was carried out as described in Section 2.4.

2.6. Evaluation of the role of pores and channels during initial amyolysis

To evaluate the role of pores (and channels) in the initial stages of amyolysis, 250 µL (2 mg/mL in distilled water) of average molecular weight >65,000 Da TRITC dextran (Sigma, T1162) was mixed with 5 mL of 10 mg/mL MS in acetate buffer (0.2 M, pH 6.0) with 0.02% (w/v) sodium azide overnight at 37 °C under stirring (200 rpm). The FITC-AA conjugate (0.8 unit/mg of starch) was added to the solution and incubated for 1 h under the same condition. Aliquots (50 µL) were taken after 5, 30 and 60 min. The diffusion of dextran probes inside maize starch granules and the status of diffused probes following further amyolysis were assessed by observing the granules after centrifugation as described in Sections 2.3 and 2.4.

2.7. Enzymic digestion of granular starches

Enzymic digestion was carried out using 0.1, 0.4 and 0.8 unit of FITC-AA conjugate per mg of starch (WMS, MS, PS or HAMS). Starch suspension (5 mL, 10 mg/mL) in acetate buffer (0.2 M, pH 6, containing 0.02% (w/v) sodium azide) was incubated with FITC-AA conjugate and mixed at 37 °C. At set times between 5 and 1440 min of incubation, aliquots (100 µL) were transferred into 1.5 mL microfuge tubes and immediately centrifuged at 2000 × g for 30 s. The supernatant was used to determine the reducing sugar content using the PAHBAH assay as described by Moretti and Thorson (2008), and starch pellets from 0.1 and 0.8 unit of FITC-AA conjugate were used for confocal microscopic observation. Pellets from 0.8 unit of FITC-AA conjugate were also oven dried at 40 °C overnight for electron microscopic observation.

2.8. Scanning electron microscopy

The oven-dried samples were thinly spread onto circular metal stubs covered with double-sided adhesive carbon tape, and then platinum coated in a Sputter coater (Eiko IB3, Mito, Japan). Images of the granules were acquired with a JEOL 6300 scanning electron microscope (JEOL Ltd., Tokyo, Japan) under an accelerating voltage of 5 kV. Multiple micrographs of each sample were examined at multiple magnifications and typical representative images selected.

3. Results

3.1. Kinetic analysis of FITC labelled and unlabelled α -amylase

Michaelis–Menten kinetic parameters were obtained for both the FITC labelled and unlabelled enzymes. The $V_{\max}$ value was found to drop from 30.50 (± 3.10) to 15.27 (± 1.65) $\mu\text{M}/\text{min}$ following labelling of the enzyme, indicating that the addition of the FITC significantly reduced the catalytic activity of the enzyme. The K_m value, however, was relatively unchanged following labelling, with a value of 12.94 (± 2.67) and 17.30 (± 3.52) mg/mL for the unlabelled and labelled enzyme, respectively. Thus, while the labelling had a large effect on the enzyme's catalytic rate, substrate binding was far less affected. This indicated that the labelled enzyme was still able to bind to starch with an affinity similar to the unlabelled form.

3.2. α -Amylase binding to native starch granules

Representative confocal microscopic images of FITC and TRITC-AA conjugates bound to MS after 5 and 20 min of incubation under non-hydrolysing condition (0 °C) are shown in Fig. 1A, and clearly reflect the heterogeneity of AA (both FITC and TRITC) binding to MS granules.

Double labelling of maize starch, using FITC-AA conjugate followed by TRITC-AA conjugate, is shown in Fig. 1B. It can be observed that the TRITC-AA conjugate added after 20 min of incubation at 0 °C, binds to exactly the same granules at the same locations, as the FITC-AA conjugate was bound previously. In some granules (as marked), FITC-AA conjugate was observed in the core of granules as well as the surface, while TRITC-AA, which has had less incubation time, is only bound at the granule surface.

Double labelling of TRITC-AA followed by FITC-AA, as presented in Supplementary information Fig. S1, followed a similar pattern as shown in Fig. 1B. Labelling of FITC-AA simultaneously with TRITC-AA on maize starch granules is presented in Supplementary information Fig. S2. Similar to sequential labelling, one subsequent to the other, when used together the two conjugates also show the heterogeneous, but preferential binding towards specific maize granules.

Similar to MS, Fig. 1C shows the heterogeneity of FITC- and TRITC-AA conjugates binding to the surface of PS granules. The binding is more heterogeneous in PS compared to MS, with apparently a smaller fraction of the granules showing fluorescence after both 5 and 20 min incubation time. In contrast to MS, binding was mostly limited to the outer circumference of PS granules. Binding was not observed to be dependent on granule size.

Double labelling of PS, FITC-AA conjugate followed by TRITC-AA conjugate, is shown in Fig. 1D. Specificity of binding location to PS was also observed similar to that of MS. TRITC-AA conjugate added after 20 min of incubation at 0 °C, bound to the same granules/location where FITC-AA conjugate was bound previously. The double labelling of TRITC-AA followed by FITC-AA, as presented in Supplementary information Fig. S3, also followed a similar pattern

to that of individual labelling as shown in Fig. 1C. Similarly, double labelling of FITC-AA and TRITC-AA together on PS granules is presented in Supplementary information Fig. S4. In parallel to double labelling, one followed by the other, the two conjugates added simultaneously also showed heterogeneous, but preferential, binding to specific PS granules.

3.3. Amylase binding to enzyme treated (porous) starch granules

In order to study the effect of starch porosity on amylase binding, porous MS was obtained by partial hydrolysis with 0.8 unit of

unlabelled amylase per mg of starch for 20 min as described in Section 2.5. Numerous pores on the surface of maize starch granules were observed after 20 min of hydrolysis as seen in Fig. 2A. Though limited by magnification and resolution, channels extending towards the granule interior can be seen in the confocal microscopic picture (Fig. 2B). The confocal and differential interference contrast images after 5 min incubation of porous starch with the FITC-AA conjugate are shown in Fig. 2C. Compared to non-porous granules (Fig. 1A), amylase can freely diffuse inside the porous granules as observed by the higher intensity of the FITC-AA conjugate in the granule interior (marked by the solid arrow in

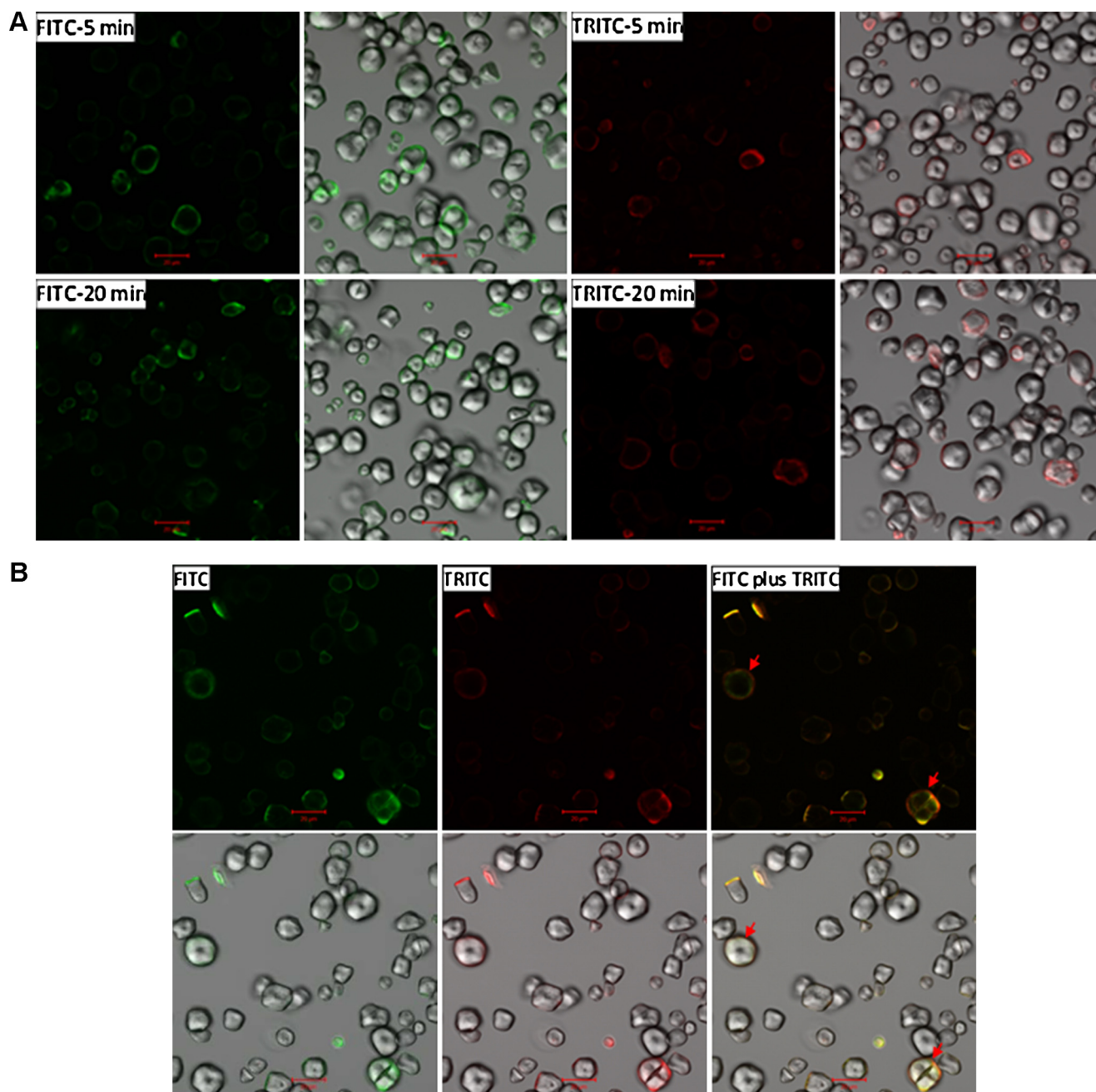


Fig. 1. (A) Confocal (first and third column) and differential interference contrast (second and fourth column) images of bound FITC- and TRITC-AA conjugate on maize starch granules incubated for 5 and 20 min at 0 °C. (B) Confocal (top panel) and differential interference contrast (bottom panel) images of bound FITC- and TRITC-AA conjugate on maize starch granules incubated for 25 min at 0 °C. TRITC-AA conjugate, 8 units/mg of starch, was added after 20 min incubation of FITC-AA conjugate. (C) Confocal (first and third column) and differential interference contrast (second and fourth column) images of bound FITC- and TRITC-AA conjugate on potato starch granules incubated for 5 and 20 min at 0 °C. (D) Confocal (top panel) and differential interference contrast (bottom panel) images of bound FITC- and TRITC-AA conjugate on potato granules incubated for 25 min at 0 °C. TRITC-AA conjugate, 8 unit/mg of starch, was added after 20 min incubation of FITC-AA conjugate.

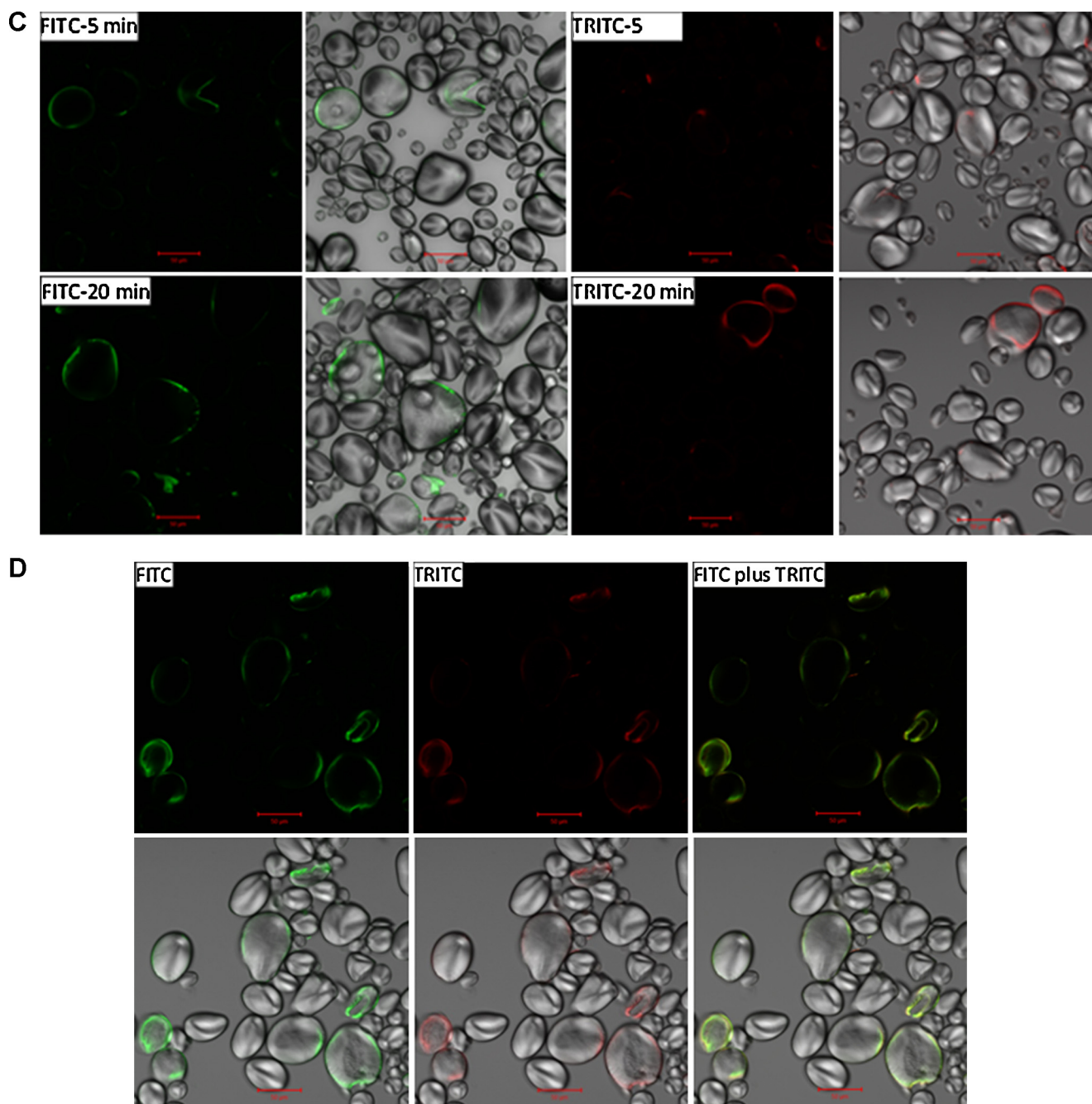


Fig. 1. (Continued).

Fig. 2D). For non-porous or less porous granules, enzyme was concentrated in the outer surfaces, as marked by the dotted arrow in Fig. 2D, similar to what was observed for untreated MS in Fig. 1A.

3.4. Diffusion of dextran probes and initial amylolysis of starch granules

As shown in Fig. 3B and C, following an overnight incubation with TRITC labelled dextran, a small number of granules have (red) dextran probes inside them (shown by arrows in Fig. 3B and C). After 5 min amylolysis, granules are observed with varying degrees of hydrolysis (damage) with the green fluorescence (FITC-AA conjugate) bound either to the interior or the peripheral regions of the granules (Fig. 3D–F). TRITC dextran was still observed in some granules, but there was not an obvious co-localisation between

TRITC dextran and FITC-AA (shown by arrows in Fig. 3E and F). After incubation for 30 and 60 min, however, the TRITC dextran was not observed, suggesting that starch hydrolysis by the amylase had resulted in release of the labelled dextran.

3.5. Amylolysis of granular starches

The digestion progress curves of MS and PS with 0.1, 0.4, and 0.8 unit of FITC-AA conjugate are shown in Fig. 4. As expected, the extent of hydrolysis is dependent upon the concentration of enzyme applied, as the substrate concentration is constant in all cases. The hydrolysis extent of starches followed the order of WMS > MS > PS > HAMS at all the enzyme concentrations used in the experiment.

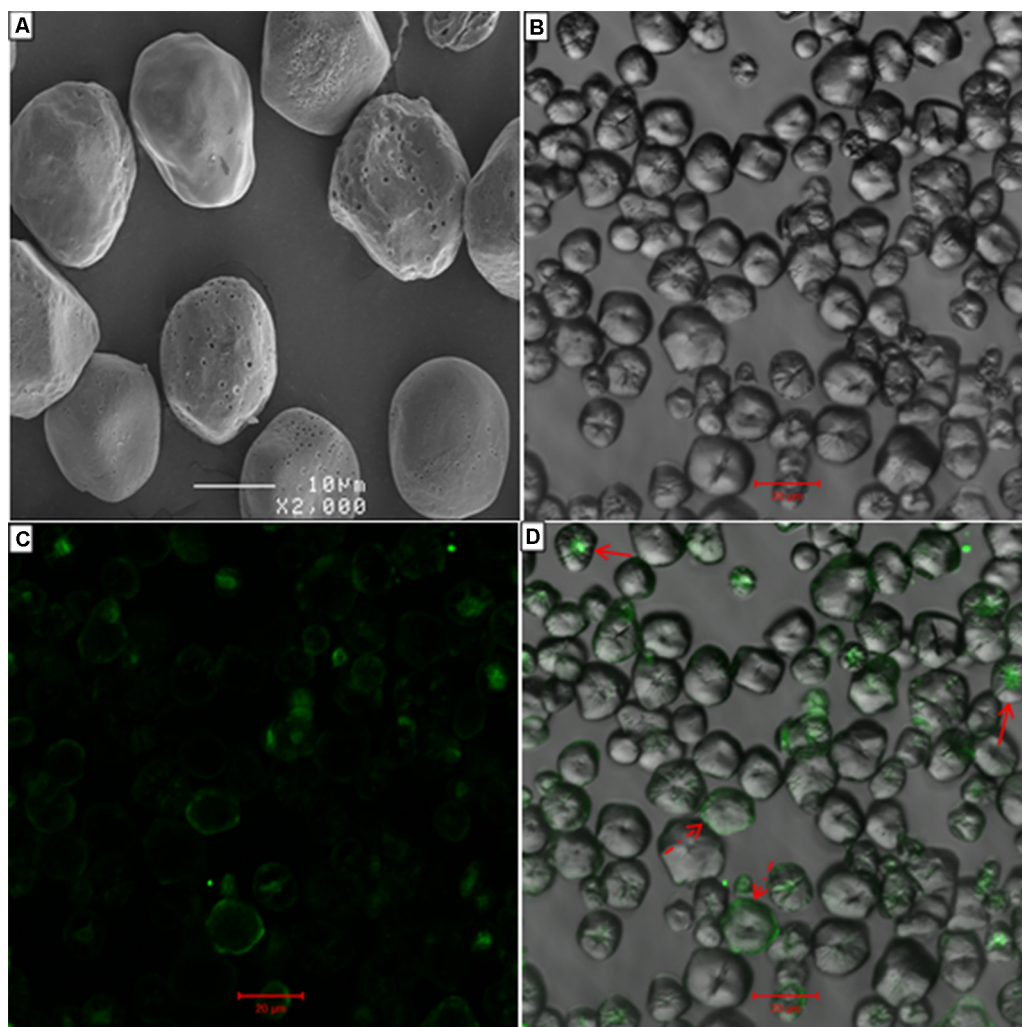


Fig. 2. Electron, confocal and differential interference contrast images of porous granules. (A) Electron microscopic picture of maize starch granules incubated with non-labelled AA for 20 min at 37 °C. (B–D) Confocal microscopic and differential interference contrast images of porous granules bound with FITC-AA conjugate for 5 min at 0 °C.

The hydrolysis pattern observed by electron microscopy is shown in Figs. 5 and 6, and Supplementary information Figs. S5, S6 for MS, PS, WMS and HAMS (Gelose 80), respectively. A-type polymorphic starches (WMS and MS) were hydrolysed by formation and enlargement of pores during the digestion time course, whereas B-polymorphic starches, PS and HAMS, were hydrolysed from the surface of the granules towards the interior.

Confocal and differential interference contrast images of hydrolysed MS and PS (0.8 units FITC-AA conjugate per mg of starch) are presented in Fig. 7. Similarly, confocal and differential interference contrast images of MS and PS incubated with 0.1 units FITC-AA conjugate per mg of starch, and WMS and HAMS at both enzyme concentrations are presented in Supplementary information Figs. S7–S9, respectively. The digestion pattern of MS with labelled enzymes was observed to be heterogeneous. In the initial 5 min of incubation, separate populations of high and low enzyme labelled granules were observed. On further incubation to 2 h, in contrast to the initial heterogeneous binding, almost all of the granules (Fig. 7) showed bound FITC-AA conjugate, with only a few exceptions. Electron microscopy also showed that almost all the MS granules after 2 h incubation were similarly porous. In contrast to MS, more selective enzyme binding of FITC-AA conjugate to digested residues of PS was observed (Fig. 7). This is in accordance with SEM observations, where only a few PS granules were eroded

during the digestion time course. Enzyme binding was found to be concentration dependent; at higher enzyme concentrations (0.8 units/mg of starch) comparatively more granules were observed with bound enzyme compared to a lower enzyme concentration (0.1 units/mg of starch). Binding was still observed to be preferential (heterogeneous) even at higher enzyme concentrations.

For MS and WMS, the enzyme which initially bound to the outer surface, subsequently diffused towards the granule interior with longer incubation times (Fig. 7, Supplementary information Figs. S7 and S8). For example, the intensity and number of granules with internal fluorescence after 2 h of incubation time was comparatively higher than that at 30 min incubation. In contrast, the diffusion of enzyme inside PS and HAMS granules was not observed. They were digested from the outer surface towards the interior. Even after 24 h incubation time, a few granules were highly eroded with enzyme bound at the erosion surfaces whereas the rest were intact without any substantial enzyme binding (Fig. 7).

4. Discussion

For the first time, we have been able to identify the location of bound amylase to starch granules under both non-hydrolysing and hydrolysing conditions. The results obtained lead us to propose that the heterogeneity of amylase action on starch granules

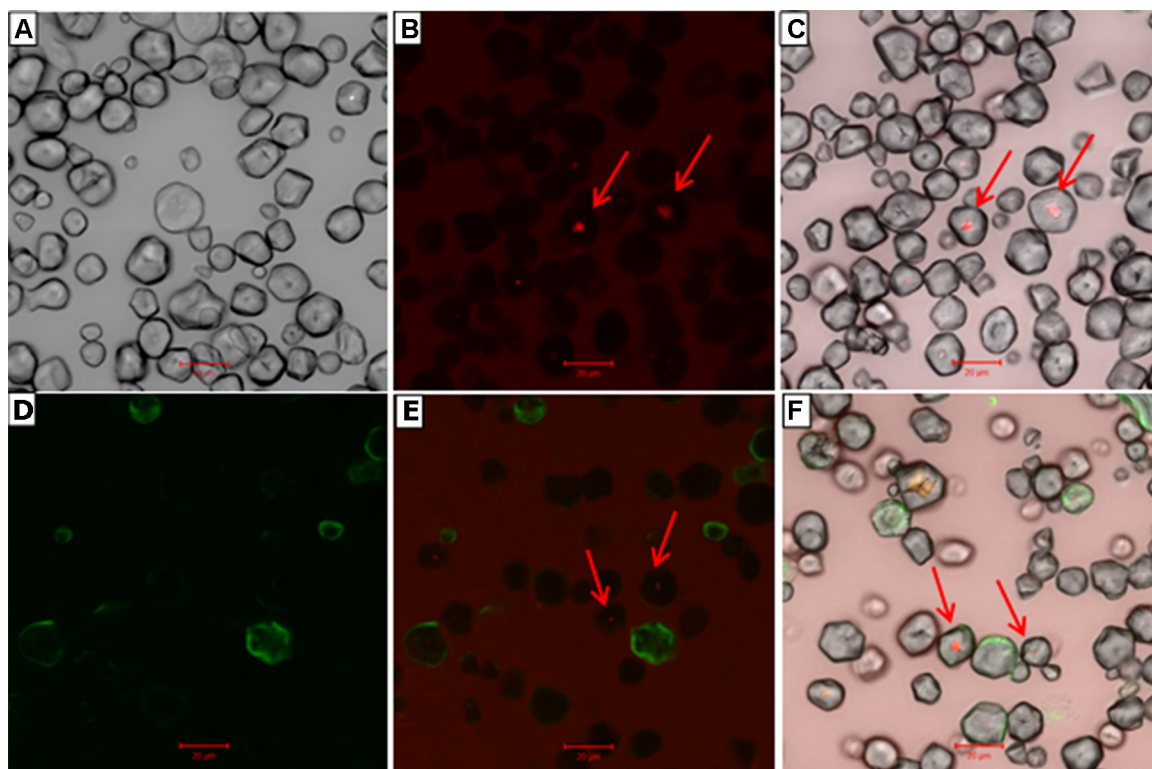


Fig. 3. Confocal and differential interference contrast images of diffused dextran probes and initial amylolysis of maize starch granules with diffused dextran probes. (A) Maize starch granules (differential interference contrast image), (B and C) confocal and differential interference contrast images of diffused dextran probes inside the maize starch granules after overnight incubation. (D and E) confocal image after 5 min of amylolysis by FITC-AA conjugate. (F) Differential interference contrast image after 5 min of amylolysis by FITC-AA conjugate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

during hydrolysis is due to preferential or selective binding of amylase to the granule surface. The possible reasons for the preferential binding are discussed below.

4.1. Binding of amylase to starch granules under non-hydrolysing conditions

Interactions between amylase and starch granules require transportation of the amylase by diffusion to the solid starch granules. The initial interaction (binding) of enzyme to starch surfaces may involve (1) non-catalytic binding *i.e.* adherence of enzyme to the granule surface by non-specific hydrogen bonding between OH groups of the starch moieties and enzyme (protein) molecule or by Van der Waals interactions; or (2) catalytic binding *i.e.* binding with at least 5 contiguous glucose residues in the active site of

the enzyme (Prodanov, Seigner, & Marchis-Mouren, 1984; Seigner, Prodanov, & Marchis-Mouren, 1987).

Initial binding can affect the subsequent catalytic events. If the binding occurs at the active site, catalysis can proceed. Alternatively, if the binding is non-catalytic in nature, the overall rate of enzyme action is decreased as enzyme molecules have to dissociate from the nonspecific sites and return to solution before they can rebound to the starch substrate (Henis, Yaron, Lamed, Rishpon, Sahar, & Katchalski-Katzir, 1988). Measuring the concentration of enzyme that is not bound to starch granules during the experiment conducted under non-hydrolysing condition (usually 0 °C) has been used to determine the binding rates of amylase to starch granules (Walker & Hope, 1963; Warren et al., 2011). These experiments, however, represent an average of both catalytic and non-catalytic binding over a population of granules.

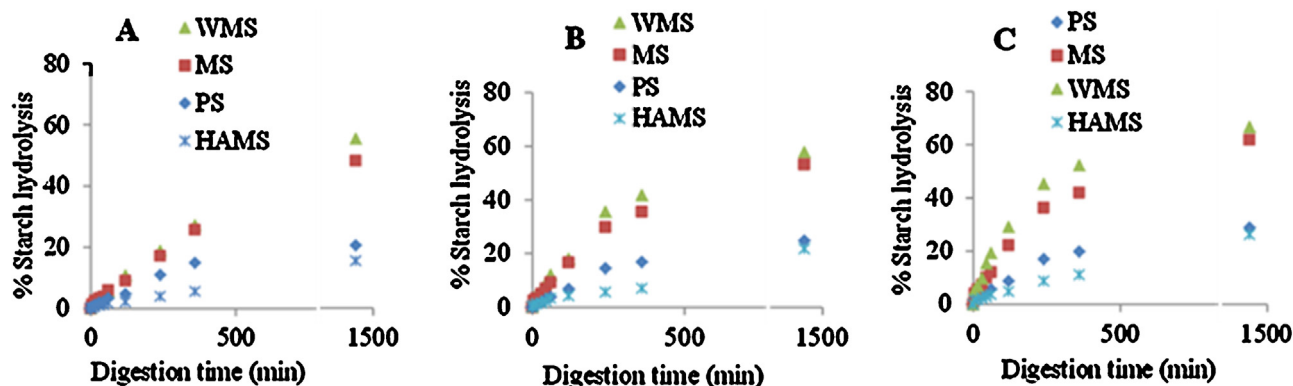


Fig. 4. FITC-AA conjugate catalysed hydrolysis rate of starches; waxy maize (WMS), maize (MS), potato (PS), high amylose maize-Gelose 80 (HAMS). (A–C) represents the digestogram at 0.1, 0.4 and 0.8 unit of FITC-AA conjugate per mg of starch.

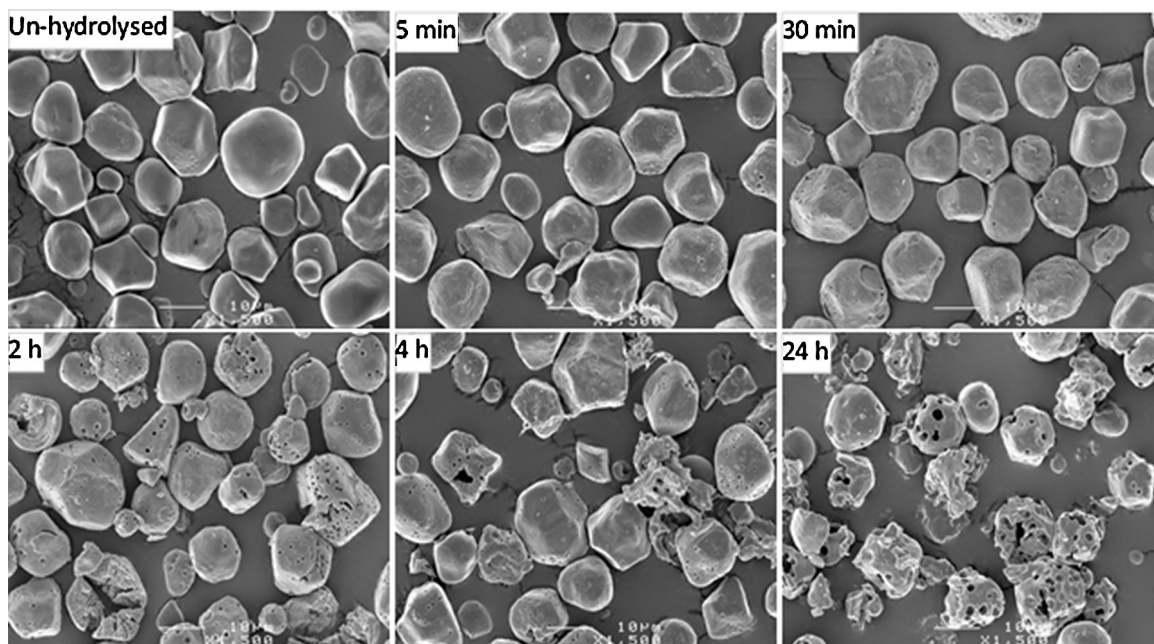


Fig. 5. Electron microscopic images of un-hydrolysed maize starch granules and granule remnant after hydrolysis for 5, 30 min, 2, 4 and 24 h with 0.8 unit FITC-AA conjugate per mg of starch.

The efficiency of enzyme adsorption has been previously reported to be inversely proportional to the granule size, or, more precisely, to the surface area of the granules (Schwimmer & Balls, 1949; Walker & Hope, 1963; Warren et al., 2011). The higher relative binding efficiency of smaller granules may be a factor contributing to higher digestion rate of smaller starch granules naturally occurring in bulk samples or obtained from fractionation of starches compared to larger granules (Dhital, Shrestha, & Gidley, 2010b; Tahir et al., 2010).

Roughness and porosity at the surface of MS granules (Dhital, Shrestha, & Gidley, 2010a), in addition to increasing the available surface area, can also elevate the probability of catalytic binding

due to the presence of more accessible (available) starch molecules on exposed, damaged, rough, and/or porous structures. The less organised regions are more accessible for initial enzyme binding compared to regions with greater molecular order (Warren et al., 2011).

The enzyme preference towards some specific granules in both MS and PS is not apparently related to granule size or surface area, and is therefore more likely to be governed by the 'available substrate' (starch chains that are sufficiently accessible as single chains to potentially lead to catalytic binding) than the 'available surface area'. Based on the data reported here, we propose that there can be localised variation in the amount of 'available substrate' within

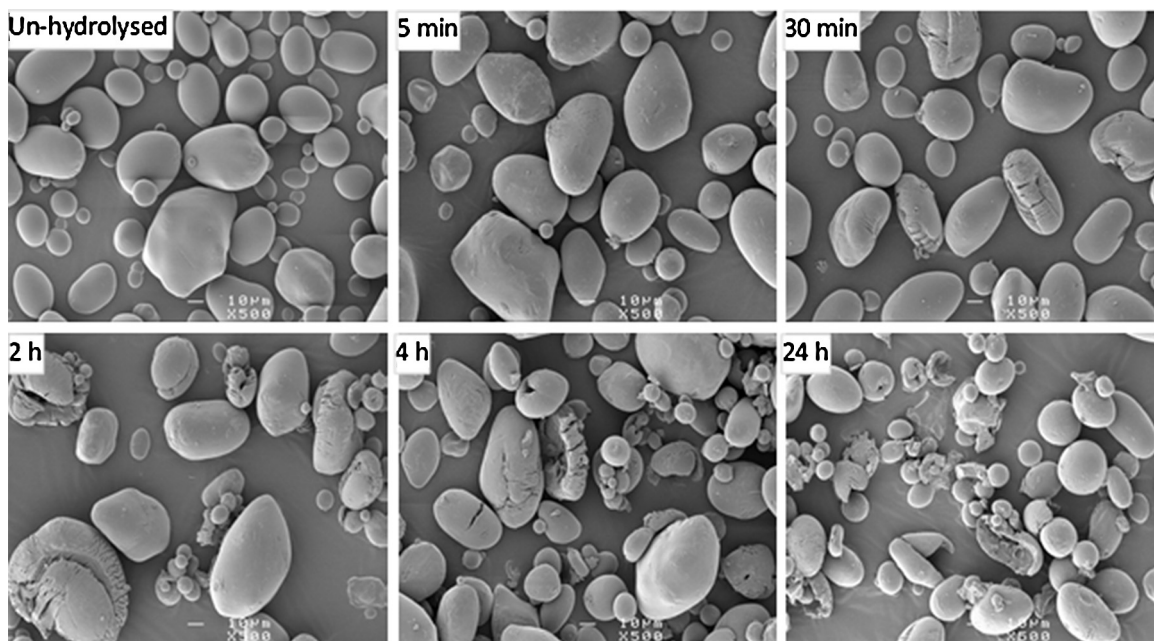


Fig. 6. Electron microscopic images of un-hydrolysed potato starch granules and granule remnant after hydrolysis for 5, 30 min, 2, 4 and 24 h with 0.8 unit FITC-AA conjugate per mg of starch.

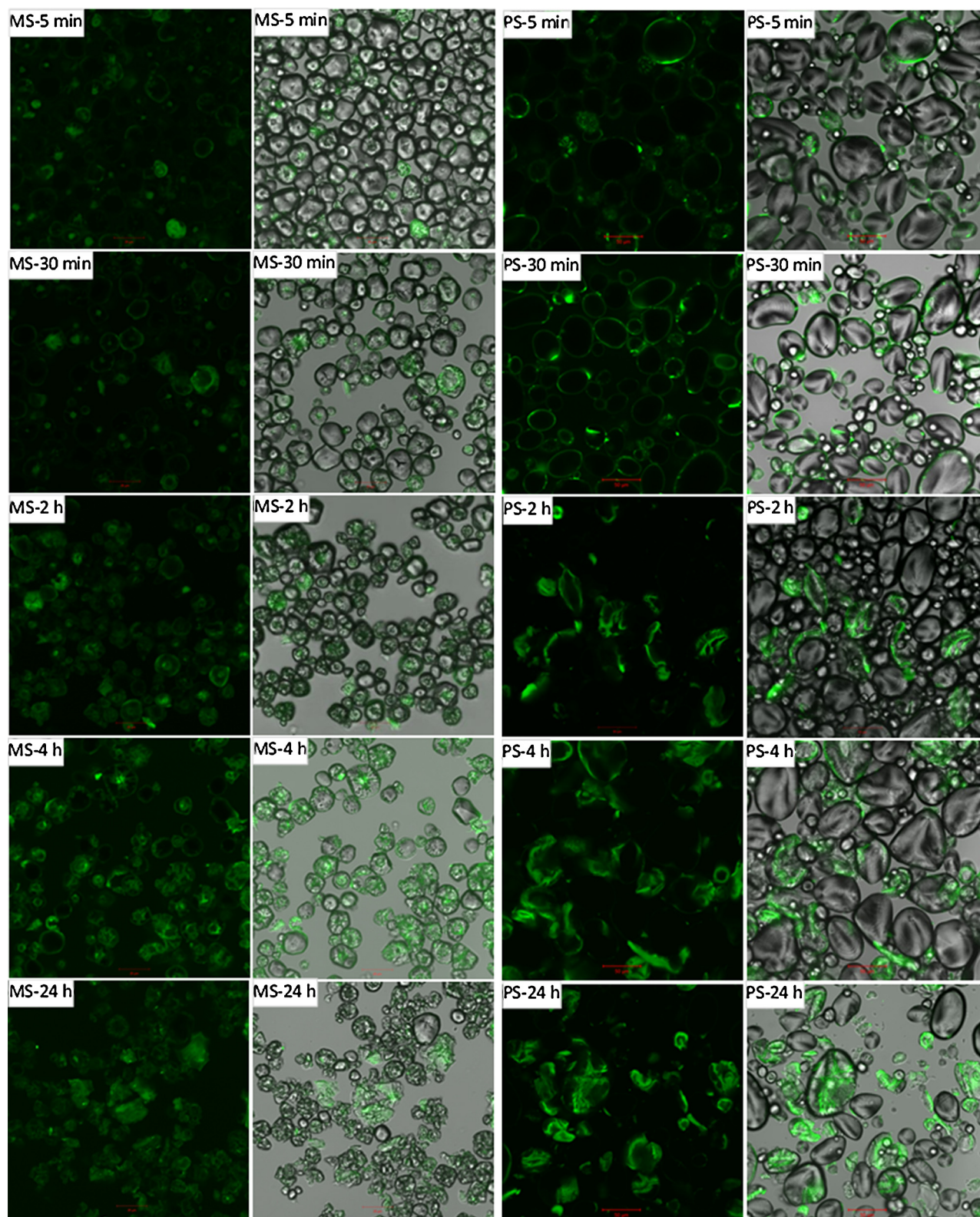


Fig. 7. Confocal (first and third column) and differential interference contrast (second and fourth column) images of maize (MS) and potato (PS) starch granule remnants after hydrolysis for 5, 30 min, 2, 4 and 24 h with 0.8 unit FITC-AA conjugate per mg of starch.

or at the granule surface due to local polymer organisation factors, and that enzyme binds preferentially to these specific regions of the granule. This is also evident in Fig. 2, where the preferential binding of enzyme to porous regions was observed. In contrast, for granules without pores, enzyme was concentrated at the outer

periphery similar to non-treated starch (Fig. 1A). The hilum (Fig. 2, bold arrow) appeared to be the least organised part of the granules since a relatively high proportion of enzyme was bound in the hilum area within 5 min of incubation under non-hydrolysing condition.

The role of local surface structures in controlling the specificity of enzyme binding was evident during double (consecutive) labelling experiments as shown in Fig. 1B and Supplementary information Fig. S1 for MS, and Fig. 1D and Supplementary information Fig. S3 for PS. The fresh enzyme bound at exactly the same granule sites that had previously bound enzyme. The structural features associated with granules which bind amylase compared to those which do not is the subject of current investigations.

4.2. Amylolysis of starches

The FITC-AA conjugate at 0.1, 0.4 and 0.8 units/mg of starch granules was used to study the hydrolysis of starches with both A- (WMS, MS) and B- (PS, HAMS) type polymorphism. The rate and extent of starch digestion were proportional to the concentration of enzyme (Fig. 4). As expected, the hydrolysis extent, at all enzyme concentrations, was highest in WMS, followed by MS, PS and HAMS. The role of molecular, supra-molecular and granular structures that affect the hydrolysis rate and extent of starch granules after initial binding has been recently reviewed (Dhital et al., 2014). The electron microscopic images of granule remnants after amyloysis with 0.8 unit FITC-AA conjugate per mg of starch (Figs. 5 and 6, Supplementary information Figs. S5 and S6) were in agreement with several previous reports (Dhital et al., 2010a, 2010b; Planchot et al., 1995; Zhang, Dhital, & Gidley, 2013).

4.3. Binding of amylase to starch granules at hydrolysing conditions

For the first time, we have been able to localise amylase on starch granules during binding and hydrolysis. Recently, Tawil et al. (2010) studied the location of bacterial amylase in maize and waxy maize starches using light and synchrotron UV fluorescence microscopy (measuring the auto-fluorescence of tryptophan in the enzyme). Starch samples were incubated with enzyme under a microscope, and the changes in the granule morphology were observed at different times. The experimental methodology employed by Tawil et al. (2010) while highly innovative, was in some ways limited, as the authors were only able to visualise one granule at a time, and the enzyme-starch interaction was observed under a microscope coverslip, meaning that no mixing or temperature control could be employed. The present study builds upon the findings of Tawil et al. (2010) by extending the study of the localisation of enzyme during binding and hydrolysis of starch to starch samples from multiple botanical origins, under a range of conditions, and with whole populations of granules.

The adsorption of enzyme to starch granules during hydrolysis was found to be a highly selective process. This selectivity is reflected in confocal microscopic images taken at different digestion times (Fig. 7 and Supplementary information Figs. S7–S9). Similar to enzyme binding under non-hydrolysing conditions, after 5 min incubation under hydrolysing conditions, the binding of FITC-AA conjugate to MS is more homogenous compared to that of PS (Fig. 7 and Supplementary information Fig. S7). Confocal microscopy observations of amylase binding to MS and WMS at different incubation times (Fig. 7, Supplementary information Figs. S7 and S8) appear to confirm the usually accepted ‘inside-out’ digestion pattern for A-polymorphic starches ascribed to the presence of pores and channels that allow the easy diffusion of enzymes inside the granule to access the less organised interior. However, the mere presence of surface pores and channels does not necessarily mean that enzymes diffuse through them to the granule interior. In the present study, it was observed that very few of the maize starch granules for which labelled dextran was able to diffuse to their hilum, also showed diffusion of labelled enzyme to their hilum (Fig. 3).

In contrast to A-polymorphic WMS and MS (Fig. 7, Supplementary information Figs. S7 and S8), the enzyme was bound only to selective granules in B-polymorphic starches (PS and HAMS, Fig. 7 and Supplementary information Figs. S7 and S9) during incubation under hydrolysing conditions. This selectivity between granules and within granules would suggest that the enzyme binding is restricted to sites on the starch granule surface that are suitable for enzyme catalytic actions, as it is these regions that are subsequently degraded by enzyme, while granules without enzyme bound are left untouched. Thus, the comparatively homogenous binding of amylase under both non-hydrolysing and hydrolysing conditions in MS suggests that the surface of maize starch contains more readily available substrates possibly at the periphery of the pores. The enzyme initially catalytically binds at these substrates and keeps hydrolysing with enlargement of pores (channels) until the enzyme can access the less organised hilum region. After that, the enzyme starts hydrolysing from the hilum towards the granule surface. In contrast, due to the absence of pores and channels in PS and HAMS, amylase catalytically binds the granules that have a damaged surface or exposed substrate and keep hydrolysing externally, so called ‘exo-corrosion’ (Dhital et al., 2010b). The inaccessibility of enzyme to the granule interior further suggests that the surface structure of PS and HAMS is rate limiting to the hydrolysis of these starches.

5. Conclusion

This study shows that amylase binds to starch granules in selected local regions under both hydrolysing and non-hydrolysing conditions. It is proposed that binding occurs to those regions which have less local molecular order and therefore contain abundant potential binding sites for α -amylase. Once bound, subsequent catalytic action exposes more potential binding sites, thus granule digestion becomes comparatively easier during digestion, resulting in extensive digestion of some granules in the presence of limited if any digestion of other granules. The different behaviour of α -amylase to dextran probes of similar size suggests that physical accessibility is not the determinant for enzyme localisation, and that therefore binding interactions are more likely to be the most important factor in determining the specificity of enzyme location.

Acknowledgements

This work was supported in part by the Australian Research Council (Discovery Grant DP130102461) and a University of Queensland Postdoctoral Fellowship awarded to FW. We acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy and Microanalysis, The University of Queensland.

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.2014.06.063>.

References

- Apinan, S., Yujiro, I., Hidefumi, Y., Takeshi, F., Myllärinen, P., Forssell, P., et al. (2007). Visual observation of hydrolyzed potato starch granules by α -amylase with confocal laser scanning microscopy. *Starch/Stärke*, 59(11), 543–548.
- Dhital, S., Shelat, K., Shrestha, A. K., & Gidley, M. J. (2013). Heterogeneity in maize starch granule internal architecture deduced from diffusion of fluorescent dextran probes. *Carbohydrate Polymers*, 93, 365–373.
- Dhital, S., Shrestha, A. K., & Gidley, M. J. (2010a). Effect of cryo-milling on starches: Functionality and digestibility. *Food Hydrocolloids*, 24(2), 152–163.

- Dhital, S., Shrestha, A. K., & Gidley, M. J. (2010b). Relationship between granule size and in vitro digestibility of maize and potato starches. *Carbohydrate Polymers*, 82(2), 480–488.
- Dhital, S., Warren, F. J., Butterworth, P. J., Ellis, P. R., & Gidley, M. J. (2014). Mechanisms of starch digestion by α -amylase—structural basis for kinetic properties. *Critical Reviews in Food Science and Nutrition* (in press).
- Gallant, D. J., Bouchet, B., & Baldwin, P. M. (1997). Microscopy of starch: Evidence of a new level of granule organization. *Carbohydrate Polymers*, 32(3), 177–191.
- Helbert, W., Schüle, M., & Henrissat, B. (1996). Electron microscopic investigation of the diffusion of *Bacillus licheniformis* α -amylase into corn starch granules. *International Journal of Biological Macromolecules*, 19(3), 165–169.
- Henis, Y. I., Yaron, T., Lamed, R., Rishpon, J., Sahar, E., & Katchalski-Katzir, E. (1988). Mobility of enzymes on insoluble substrates: The β amylase-starch gel system. *Biopolymers*, 27(1), 123–138.
- Huber, K. C., & BeMiller, J. N. (1997). Visualization of channels and cavities of corn and sorghum starch granules. *Cereal Chemistry*, 74(5), 537–541.
- Jane, J. L., & Shen, J. J. (1993). Internal structure of the potato starch granule revealed by chemical gelatinization. *Carbohydrate Research*, 247, 279–290.
- Liu, D., Parker, M. L., Wellner, N., Kirby, A. R., Cross, K., Morris, V. J., et al. (2013). Structural variability between starch granules in wild type and in *ae* high-amylase mutant maize kernels. *Carbohydrate Polymers*, 97(2), 458–468.
- Leach, H. W., & Schoch, T. J. (1961). Structure of the starch granule. II. Action of various amylases on granular starches. *Cereal Chemistry*, 38(1), 34–36.
- Lynn, A., & Cochrane, M. P. (1997). An evaluation of confocal microscopy for the study of starch granule enzymic digestion. *Starch/Stärke*, 49(3), 106–110.
- Moretti, R., & Thorson, J. S. (2008). A comparison of sugar indicators enables a universal high-throughput sugar-1-phosphate nucleotidyltransferase assay. *Analytical Biochemistry*, 377(2), 251–258.
- Pan, D. D., & Jane, J. L. (2000). Internal structure of normal maize starch granules revealed by chemical surface gelatinization. *Biomacromolecules*, 1(1), 126–132.
- Planchot, V., Colonna, P., Gallant, D. J., & Bouchet, B. (1995). Extensive degradation of native starch granules by α -amylase from *Aspergillus fumigatus*. *Journal of Cereal Science*, 21(2), 163–171.
- Prodanov, E., Seigner, C., & Marchis-Mouren, G. (1984). Subsite profile of the active center of porcine pancreatic α -amylase. Kinetic studies using maltooligosaccharides as substrates. *Biochemical and Biophysical Research Communications*, 122(1), 75–81.
- Schwimmer, S., & Balls, A. (1949). Starches and their derivatives as adsorbents for malt α -amylase. *Journal of Biological Chemistry*, 180(2), 883–894.
- Seigner, C., Prodanov, E., & Marchis-Mouren, G. (1987). The determination of subsite binding energies of porcine pancreatic α -amylase by comparing hydrolytic activity towards substrates. *Biochimica et Biophysica Acta (BBA)—Protein Structure and Molecular Enzymology*, 913(2), 200–209.
- Sujka, M., & Jamroz, J. (2009). α -Amylolysis of native potato and corn starches—SEM, AFM, nitrogen and iodine sorption investigations. *LWT—Food Science and Technology*, 42(7), 1219–1224.
- Tahir, R., Ellis, P. R., & Butterworth, P. J. (2010). The relation of physical properties of native starch granules to the kinetics of amylolysis catalysed by porcine pancreatic α -amylase. *Carbohydrate Polymers*, 81(1), 57–62.
- Tawil, G., Jamme, F., Réfrégiers, M., Viksø-Nielsen, A., Colonna, P., & Buléon, A. (2010). In situ tracking of enzymatic breakdown of starch granules by synchrotron UV fluorescence microscopy. *Analytical Chemistry*, 83(3), 989–993.
- The, T. H., & Feltkamp, T. E. W. (1970). Conjugation of fluorescein isothiocyanate to antibodies: I. Experiments on the conditions of conjugation. *Immunology*, 18(6), 865–873.
- Thomson, N., Miles, M., Ring, S., Shewry, P., & Tatham, A. (1994). Real-time imaging of enzymatic degradation of starch granules by atomic force microscopy. *Journal of Vacuum Science and Technology B: Microelectronics and Nanometer Structures*, 12(3), 1565–1568.
- Walker, G. J., & Hope, P. M. (1963). The action of some α -amylases on starch granules. *Biochemical Journal*, 86(3), 452–462.
- Warren, F. J., Royall, P. G., Gaisford, S., Butterworth, P. J., & Ellis, P. R. (2011). Binding interactions of α -amylase with starch granules: The influence of supramolecular structure and surface area. *Carbohydrate Polymers*, 86(2), 1038–1047.
- Zhang, B., Dhital, S., & Gidley, M. J. (2013). Synergistic and antagonistic effects of α -amylase and amyloglucosidase on starch digestion. *Biomacromolecules*, 14(6), 1945–1954.