Carbohydrate Polymers 93 (2013) 365–373

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Heterogeneity in maize starch granule internal architecture deduced from diffusion of fluorescent dextran probes

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ARTICLE INFO

Article history:
Received 20 August 2012
Received in revised form 5 December 2012
Accepted 8 December 2012
Available online 7 January 2013

Keywords:
Starch
Diffusion
Fluorescence recovery after photobleaching
Granule heterogeneity
Surface pores and channels

ABSTRACT

Heterogeneity in maize starch granules was investigated by studying the diffusion of fluorescent dextran probes (20, 70 and 150 kDa) inside granules using fluorescence recovery after photobleaching combined with confocal microscopy. Access of probes to the interior of granules was greatly enhanced by limited (2.4%) amylolysis. The diffusion of probes within granules was found to be either 'fast' with diffusion coefficients in the order of 10^{-6} cm^2 s^{-1} or 'slow' with diffusion coefficients in the order of 10^{-7} cm^2 s^{-1}, independent of the size of dextran probes or prior treatment of the granules by α-amylase. Results were compared with observations of pores and channels in granules by electron microscopy and by confocal microscopy after labelling with 8-amino-1,3,6-pyrenetrisulfonic acid. It is proposed that there is an inherent heterogeneity of internal architecture in maize starch granules due to the presence or absence in individual granules of (a) pores leading to a central cavity, resulting in 'fast' diffusion of dextran probes and (b) accessibility of the starch polymer matrix to dextran probes, leading to 'slow' diffusion behaviour. The observed heterogeneity of maize starch granule porosity has implications for chemical modification reactions and the kinetics of digestion with amylases.

1. Introduction

Starch granules are heterogeneous with difference in molecular structures, amount and type of molecular organisation, shape, size and surface features. Even structure and morphology of granules from a single source e.g. kernel, tuber or root are not homogenous (Fannon, Gray, Gunawan, Huber, & BeMiller, 2004). Most of the A-polymorphic cereal starches contain pores on the granule surface (Fannon, Hauber, & BeMiller, 1992) that lead to serpentine channels, varying in number, size and depth, penetrating towards the centre of granules which in some starches e.g. maize can be hollow cavities (Huber & BeMiller, 1997; Huber & BeMiller, 2000). These channelled microstructural features in starch granules are thought to influence the reactivity of starch with enzymes, chemical agents and dyes (Huber & BeMiller, 2000; Huber & BeMiller, 2001). Though the reason behind the absence of pores and channels in B-polymorphic starches such as potato and high amylose maize starches is still not understood, their presence in A-polymorphic starches may be potentially beneficial for production of animal feeds, ethanol (Benmoussa et al., 2010; Benmoussa, Suhendra, Aboubacar, & Hamaker, 2006) and chemical modification process (BeMiller, 1997; Fannon, Gray, Gunawan, Huber, & BeMiller, 2003) utilising either raw or partially cooked starches.

During enzymatic hydrolysis, the presence of pores and channels increases the available surface area compared to the boundary surface area fostering initial enzyme adsorption and diffusion inside the granules (BeMiller, 1997; Oates, 1997). This allows enzymes an easy and speedy access to the less organised 'hilum' region, hydrolysing the starches from the hilum towards the granule surface in an 'inside-out' (Gallant, Bouchet, Buleon, & Perez, 1992) or 'centrifugal' (Helbert, Schulein, & Henrissat, 1996) digestion pattern. Starch granules with high porosity are more susceptible to enzymes compared to granules with low porosity (Gunawan, 2002). B-polymorphic starches, apparently lacking surface pores and channels, are slowly digested compared to A-polymorphic starches as enzymes need to 'dig' holes in the granule surface to diffuse inside the granules or alternatively digest the granules creating erosion on surfaces (Gallant et al., 1992). However, in both polymorphic forms, the rate limiting step in enzymatic hydrolysis is considered to be the diffusion of enzymes into the granules (Colonna, Leloup, & Buleon, 1992) by successive formation of pits and/or larger pores (Dhital, Shrestha, & Gidley, 2009).

The morphology and composition of pores and channels in cereal starch granules have been well studied (Benmoussa et al., 2010; Chen et al., 2009; Han, Benmoussa, Gray, BeMiller, & Hamaker, 2005; Huber & BeMiller, 1997; Huber & BeMiller,

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http://dx.doi.org/10.1016/j.carbpol.2012.12.017

2000; Juszczak, Fortuna, & Wodnicka, 2002; Kim & Huber, 2008; Naguleswaran, Li, Vasanthan, & Bressler, 2011). These studies suggest that the channels contain proteins and phospholipids and have an apparent diameter ranging from 0.007 to 0.1 μm, whereas pores (opening of channels) are larger with diameters varying between 0.1 and 0.3 μm. These pores and channels in starch granules are large enough for enzymes such as alpha amylase (radius of ca 3–4 nm (Payan et al., 1980; Planchot & Colonna, 1995)) to diffuse inside granules. However, the direct measurement of amylase diffusivity in native starches (e.g. maize starch) is rather complex as diffusion occurs concurrently with starch degradation which increases the granule porosity. As an alternative approach, the apparent diffusivity of amylase in milled sorghum (Al-Rabadi, Gilbert, & Gidley, 2009; Mahasukhonthachat, Sopade, & Gidley, 2010), barley grains (Al-Rabadi et al., 2009), potato and maize starches (Dhital, Shrestha, & Gidley, 2010) was determined by plotting the reciprocal of amylase digestion rate against the square of the particle size, the linear nature of which suggests that the rate limiting step in enzymatic digestion of granular starch is access of enzymes to starch molecules. In the case of milled grain fragments, enzyme diffusion is hindered by a tightly packed cellular structure and a possible protein matrix, whereas a molecularly dense surface without pores and channels provides an effective barrier in e.g. potato starch granules. However, these studies are averaged across a population of granules or grain fragments with no information on the behaviour of individual particles within the population. In the present study, the heterogeneity within individual maize starch granules was studied in terms of the rate of diffusion of fluorescently labelled dextran probes using the fluorescence recovery after photobleaching (FRAP) technique. FRAP has been used to investigate the diffusion of labelled enzymes (Cuyvers et al., 2011; Henis et al., 1988; Jervis, Haynes, & Kilburn, 1997) or probes in polymer solutions and gels (Axelrod, Koppel, Schlessinger, Elson, & Webb, 1976; Burke, Park, Srinivasarao, & Khan, 2000; Perry, Fitzgerald, & Gilbert, 2006; Shelat et al., 2010). In the FRAP technique, a small region of a sample containing mobile fluorescent molecules is exposed to a brief intense pulse of light, thereby causing irreversible photochemical bleaching of the fluorophore in that region. Diffusion coefficients are then determined by measuring the rate of recovery of fluorescence which results from transport of fluorophore into the bleached region from un-irradiated parts of the system. The FRAP method allows an assessment of heterogeneity within a population of granules, as individual granules can be selectively irradiated. Additional information on surface pores and channels was obtained by observing the starches by electron and confocal microscopy using 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) as a fluorescent dye that binds to the reducing ends of starch molecules. The study further sheds light on the mechanisms of polymer diffusion operating inside individual maize starch granules, with implications for the control of enzymatic hydrolysis and modification of starch granules.

2. Materials and methods

2.1. Materials

Maize starch (MS) was purchased from Penford Australia Ltd., Lane Cove, NSW, Australia. Fluorescein isothiocyanate conjugate tagged dextrans (FITC-dextran) of weight-average molecular weight 2.0×10^4, 7.0×10^4, and 15.0×10^4 were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia) and used as received. These probes, as reported in supplier product information sheets, contain 0.003–0.025 mol of FITC per mol of glucose and have approximate Stokes' radii of 3.3, 6.0 and 8.5 nm for 20, 70 and 150 kDa dextran probes, respectively. The size of these probes is similar to the size of α-amylases that are reported to have crystallographic and hydrodynamic radii of ca 3 and 4 nm respectively (Payan et al., 1980; Planchot & Colonna, 1995).

2.2. Methods

2.2.1. Pre-treatment of starch granules with enzymes

To introduce further porosity in granules, raw maize starch was hydrolysed by pancreatin (after saliva mimic pre-treatment) according to the method of Htoon et al. (2009) with slight modification. About 500 mg of the starch was treated with 1 mL of artificial saliva containing porcine α-amylase (Sigma A-3176; 250 U/mL of carbonate buffer, pH 7) for 15–20 s before 5 mL of pepsin (Sigma P-6887) solution (1 mL per mL of 0.02 M HCl, pH 2) was added and incubated at 37 °C for 30 min in a reciprocating (85 rpm) water bath (SWB20; Ratek Instruments Pty. Ltd. Boronia VIC 3155, Australia). The carbonate buffer consisted of 14.4 mM sodium hydrogen carbonate, 21.1 mM potassium chloride, 1.59 mM calcium chloride, and 0.2 mM magnesium chloride which was neutralised (5 mL, 0.02 M NaOH) before adjusting the pH with 25 mL of 0.2 M sodium acetate buffer (pH 6) prior to adding 5 mL of a mixture of pancreatin (Sigma P1750, 2 mg/mL of acetate buffer) and amyloglucosidase (Sigma A-7420, 28 U/mL of acetate buffer) in acetate buffer. Immediately after addition of the enzyme mixture, the flask was placed in an ice water bath to stop enzymatic activity and later frozen. The thawed digestion mixture was centrifuged at $2000 \times g$ for 10 min and the residues were washed twice with distilled water and freeze dried for succeeding experiments. The percentage starch hydrolysis during the enzyme treatment, as determined from the amount of glucose in the supernatant (Dhital et al., 2009), was 2.4%.

2.2.2. Electron microscopic observation of native and enzyme-treated starch

Native and porous (enzyme treated) starches were observed by scanning electron microscopy (JSM 6300, JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 5 kV following the method used by Dhital et al. (2009).

2.2.3. Labelling of starches with APTS

Native and partially digested (porous) starch samples were labelled with APTS before observing by confocal microscopy as previously described (Blennow et al., 2003). Starch granules (10 mg) were dispersed in 15 μL of freshly made APTS (A 7222, Sigma–Aldrich) solution (10 mM APTS dissolved in 15% acetic acid), and 15 μL of 1 M sodium cyanoborohydride (S 8628, Sigma) was added. The reaction mixture was incubated at 30 °C overnight, after which the granules were washed five times with 1 mL of distilled water and finally suspended in 20 μL of 1:1 (v/v) glycerol–water mixture. A drop of the mixture was then mounted on a microscope slide for observation.

2.2.4. Confocal and fluorescence microscopic observation of dyed starches

The APTS labelled samples were observed with a ZEISS LSM 510 META Confocal Microscope (Carl Zeiss, Inc. Thornwod, NY) using a Plan-Neofluar 40 × 1.3 oil DIC objective lens. Starch images were taken using frame size 512 × 512 with scan speed of 8 bit, pixel time of 3.20 μs, and an optical slice of less than 5 μm thickness. All imaging was performed with a 10 mW argon ion laser at 5% power with excitation and emission at 488 nm and 505 nm respectively. The fluorescence microscopic images of APTS-labelled samples were taken using an Olympus BX61 fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

2.2.5. FRAP experiments

Starch (~1 mg) was dispersed in 500 μL of FITC dextran (2 mg/mL (w/v) in deionised water) solution in a microcentrifuge tube and mixed at 300 rpm overnight at ambient temperature in a thermomixer (Eppendorf AG 22331, Hamburg, Germany). The sample (2 drops ~40 μL) was placed on a microscopic slide, covered with a cover slip, and sealed using liquid nail polish. FRAP experiments were carried out with a ZEISS LSM 510 META Confocal Microscope using a Plan-Neofluar 40×/1.3 oil DIC objective lens.

All photobleaching was performed at 488 nm emission at 100% laser power with confocal pinhole fully opened. FRAP image acquisition was performed using stack size 256 × 256 × 1 with scan mode of 8 bit and pixel time of 3.20 μs. All imaging was performed with a 10 mW argon ion laser at 5% power with excitation at 488 nm and emission at 505 nm. The region of interest (ROI) were kept constant at 7.5 μm (radius) and granules containing FITC-dextran were bleached at the ROI for 18.8 s, with recovery of fluorescence intensity measured every 2.5 s, for at least 3 min depending on the recovery rate. Experiments with constant fluorescence intensity in an unbleached portion (reference region) over the course of the experiment were the only ones considered for data processing. Due to the movement of granules, drifting of the bleached region was often observed. Care was taken to avoid possible drift e.g. by vibration, and experiments showing drift of the ROI were not taken into account.

The diffusion coefficient (D) was calculated from a range of FRAP experiments using Axelrod's equation (1976) (Eq. (1)). Leddy and Guilak (2003) also studied molecular diffusion in cartilage using the same equation. In this equation, the mobility of the probe due to Brownian motion is expressed as the diffusion coefficient D, which is related to the diffusion time t as:

$$D = \left(\frac{\omega^2}{4t_{1/2}}\right)\gamma d \quad (1)$$

where ω is the bleach spot radius which is kept constant at 7.5 μm, $t_{1/2}$ is the half recovery time, defined as the time when normalised fractional intensity (f) is half of $f(\infty)$. γd is the bleaching parameter which is kept constant at 0.88 for a circular laser beam (Axelrod et al., 1976). The mean fluorescence in the bleached region over time was converted to normalised fractional fluorescence intensity (f) as

$$f = \frac{F(t) - F(0)}{F(\infty) - F(0)} \quad (2)$$

where $F(t)$ is the fluorescence at time t, $F(0)$ is the fluorescence immediately after bleaching, and $F(\infty)$ is the maximum fluorescence obtained during the recovery phase.

Eq. (1) assumes unrestricted two-dimensional diffusion in a circular bleached area, with no recovery from above and below the focal plane. Thus, the present study measures apparent two-dimensional diffusion into and within starch granules. Factors such as incomplete bleaching of granules or drift in ROI and/or reference regions may affect the reproducibility of FRAP experiments. Diffusion coefficients in the present experiments were calculated as the average of at least 2 consistent experiments that avoided these factors with variation expressed as standard deviations (Table 1).

Analysis of variance (ANOVA) was used to determine the significant differences at $P < 0.05$ using Genstat-1995 (Genstat Release 5 (3.2), VSN International, Hemel Hempstead).

3. Results and discussion

3.1. Surface morphologies of granules using SEM and confocal microscopy

Maize starch granules imaged by electron microscopy were polyhedral or near-spherical in shape with varying numbers of pores clustered on the surfaces of some granules. Though the pores are not distinct at lower magnification e.g. 1000× (Fig. 1A), they are clearly visible at higher magnification e.g. 2000× and over (Fig. 1A1 and A2). Presumably granules might also contain other micro-pores either too small to be seen with the magnification and resolution used in this experiment or covered by native proteins or with platinum during sputter coating. These surface pores, as the opening of channels into starch granules, have been explored extensively (Fannon et al., 1992; Fannon, Shull, & BeMiller, 1993; Huber & BeMiller, 1997; Huber & BeMiller, 2000). A characteristic central cavity and extended channels varying in size were observed in APTS-labelled maize starch granules (Fig. 2A), but were difficult to observe by conventional fluorescence microscopy (Fig. 2A1) without optical sectioning. After limited pre-treatment of granules with amylases (2.4% hydrolysis) and pepsin, the granule surface appeared to be roughened, with an increase in the number and size of pores. Digestion occurred in a heterogeneous fashion, some granules having a highly porous surface with surface pores of typically ca 300 nm in diameter whilst others only had roughened surfaces without obvious pores (Fig. 1B and B1). Pre-treated granules labelled with APTS were observed to have an enlarged central hilum and channels extending to the granule surface in both confocal and conventional fluorescence microscopy images (Fig. 2B and B1), consistent with the inside-out or centrifugal hydrolysis pattern (Gallant et al., 1992; Helbert et al., 1996).

Granule surfaces are relatively impermeable to large molecules such as amylases owing to tight packing of amylopectin and a higher concentration of amylose chains (Oates, 1997) acting as the

Table 1
Diffusion coefficients of FITC dextran probes in maize starch granules.

		Diffusion coefficient ($cm^2\ s^{-1}$)[a,b]	
Dextran probe	Diffusion type	Raw granules	Pre-treated granules
20 kDa	Fast	2.76E−06 (1.41E−06)[a]	2.18E−06 (9.85E−07)[a]
	Slow	3.17E−07 (1.04E−07)[b]	1.71E−07 (1.08E−07)[b]
70 kDa	Fast	1.60E−06 (4.45E−07)[a]	3.12E−06 (7.75E−07)[a]
	Slow	1.87E−07 (6.01E−08)[b]	4.12E−07 (1.23E−07)[b]
150 kDa	Fast	1.68E−06 (6.29E−07)[a]	3.76E−06 (2.10E−06)[a]
	Slow	2.37E−07 (1.06E−08)[b]	5.21E−07 (3.17E−07)[b]

Dextran probe	20 kDa	70 kDa
Solution diffusion coefficient	8.26E−06 (1.41E−07)	8.10E−06 (8.14E−08)

[a] Results are expressed as means (standard deviation) from at least duplicate determinations.
[b] Values with similar superscript are not significantly different ($p > 0.05$).

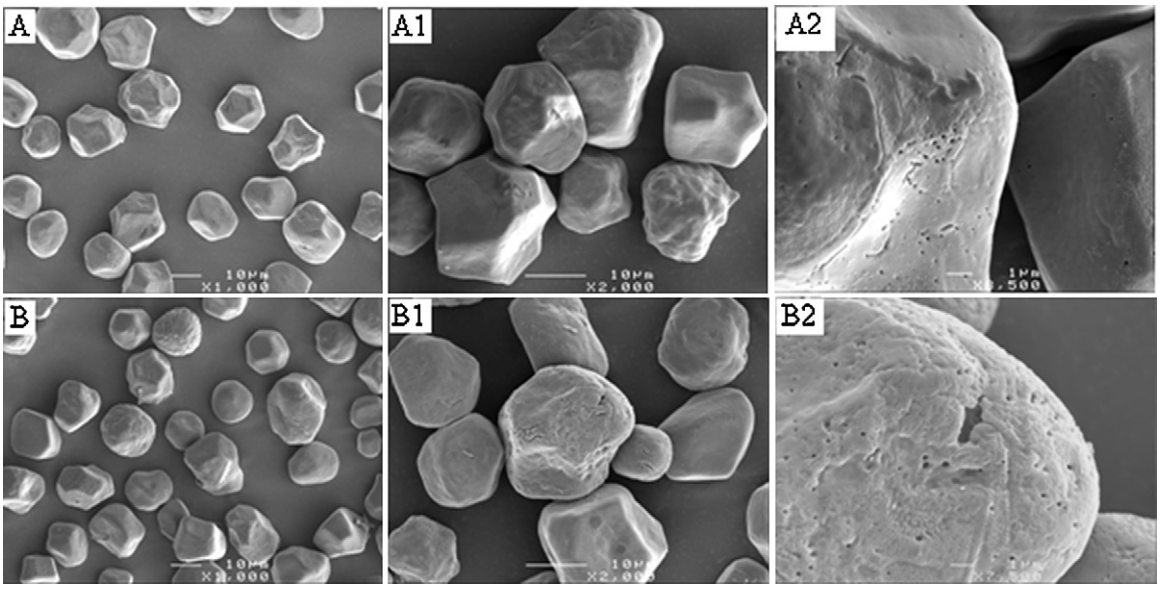

Fig. 1. Electron micrographs of raw maize starch granules (A) and after pre-treatment with pepsin and amylase (B).

first hurdle in starch granule hydrolysis. However, enzyme action is not exclusively an external surface phenomenon; as soon as the attack develops via weak areas e.g. surface pores, hydrolysis proceeds very rapidly in a radial direction with either enlargement of existing channels or the formation of new channels (Gallant et al., 1992) subsequently proceeding outwards over a broader front until almost complete digestion or fragmentation of starch granules occurs (Zhang, Ao, & Hamaker, 2006). The hydrolysis is expected to be even faster for granules with existing channels. This leads to the potential for heterogeneity in granule digestion patterns. Although enzyme adsorption on the granule surface is expected to be a random phenomenon, the movement of the enzyme into the granule is not random, but is likely to be a diffusion-controlled process through the densely packed polymer chains from the granule surface to granule interior. Thus depending on the granule architecture, differences in the rate of enzyme diffusion inside the granules can be anticipated.

3.2. Diffusion of dextran probes

In order to characterise the heterogeneity within granule population, the diffusion of inert enzyme-sized FITC-dextran probes into maize starch granules was studied with confocal microscopy. Though pores, channels and cavities were observed in SEM and

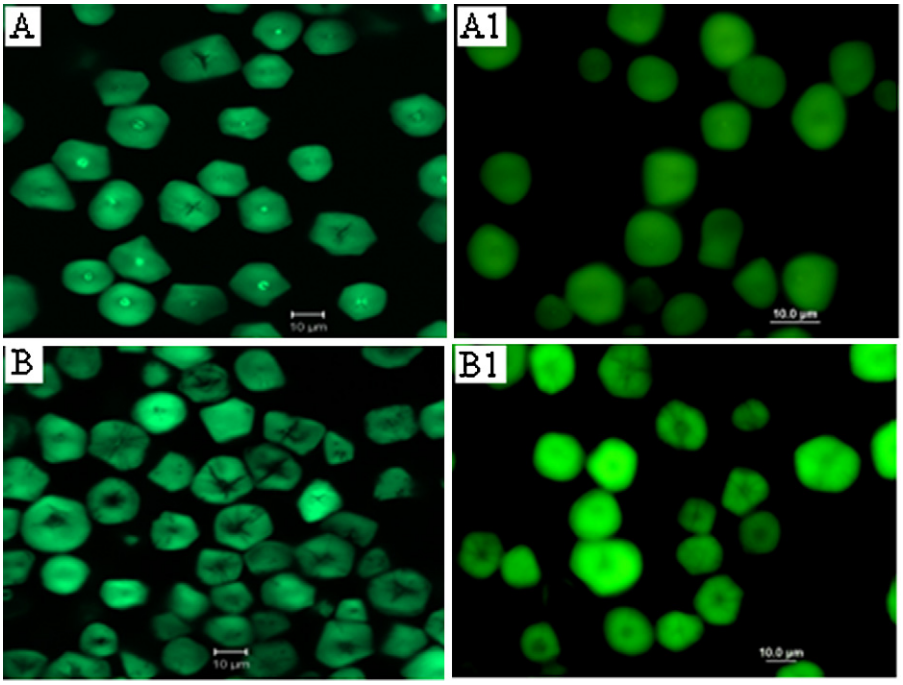

Fig. 2. Confocal micrographs of RMS granules (A) and enzyme pre-treated RMS (B) after labelling with APTS. Fluorescence light micrographs of RMS granules (A1), and enzyme pre-treated RMS (B1) after labelling with APTS.

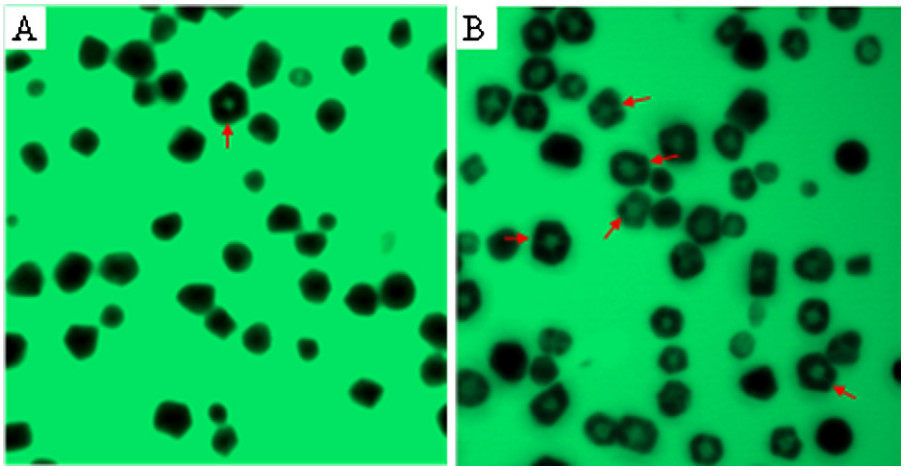

Fig. 3. Confocal micrographs of MS granules suspended overnight in 20 kDa FITC dextran solution before (A) and after (B) enzyme pre-treatment. Only a few untreated granules (A) have a light fluorescent centre (example arrowed) due to diffusion of FITC dextran, whereas most of the pre-treated granules (B) have a fluorescent central cavity and channels (examples arrowed).

APTS-labelled confocal images of raw maize starch granules (Figs. 1A1 and 2A1), only a few granules (marked in Fig. 3A) showed detectable internal fluorescence when exposed to FITC dextran overnight (Fig. 3A). However, most of the enzyme pre-treated granules (Fig. 3B) showed fluorescent central cores and channels extending from the core to the granule surface (which appeared as dark areas in APTS-labelled granules) after overnight treatment (Figs. 2B and B1).

The observation of at least some fluorescent granules after soaking in FITC dextrans allows FRAP experiments to be performed to characterise diffusion of dextran probes before and after enzyme pre-treatment. A typical diffusion curve, of normalised fractional fluorescence intensity obtained from Eq. (1) plotted vs. time (t), is shown in Fig. 4. Since the granules have different shapes and sizes as well as numbers and sizes of pores and channels, FRAP parameters were kept constant during the whole experiment. The difference between the initial (pre-bleaching) fractional intensity (which is defined as 1.0) and maximum fractional intensity after bleaching is termed the immobile fraction. In this case, the immobile fractions could be due to physical adsorption e.g. trapping of dextran probes within channels, cavity and matrix of starch granules during the recovery time series. To reduce any instrumental effects on the loss of fluorophore from the ROI, intensity from a reference region in the same focal plane but away from any granules is collected concurrently with the intensity in the ROI, and only those experiments without significant difference in intensity of the reference region during pre- and post-bleaching are considered for diffusion calculations. Assuming no loss of fluorescence intensity, there is a linear relation between FITC dextran concentration inside the granule and fluorescence intensity.

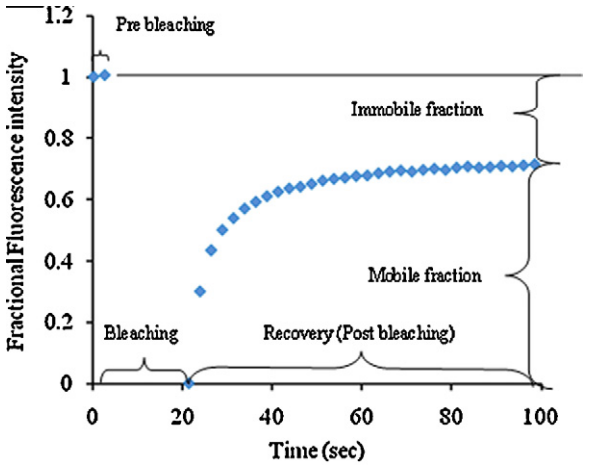

Fig. 4. Description of a typical FRAP recovery curve (20 kDa FITC dextran in enzyme pre-treated MS).

Irrespective of the dextran size, almost 100% recovery was obtained for the blank FRAP experiments (experiments conducted on FITC dextran solution without MS granules, figures not shown) whereas in starch granules, the recovery ranged from 55 to 75% (Figs. 5 and 6), suggesting that granule architecture restricted movement of a substantial minority (25–45%) of FITC dextran molecules during the recovery phase.

The calculated diffusion coefficients of FITC dextran of 20, 70 and 150 kDa inside raw and enzyme pre-treated maize starch granules are shown in Table 1. Irrespective of dextran molecular weight (size) and level of granule porosity, either slow-type or fast-type diffusion was observed and individual granules within a single sample demonstrated one or other of these distinct behaviours. Typical FRAP recovery curves for 20 kDa FITC dextran in MS and pre-treated MS are shown in Figs. 5 and 6 respectively.

As presented in Table 1, the fast-type diffusion coefficients ranged from 1.6 to 3.7×10^{-6} cm^2 s^{-1}, whereas slow-type diffusion was of the order of 10^{-7}, at least 4 times slower than that of fast diffusion and 8 times slower than the solution (blank) diffusion coefficient. Diffusion coefficients between fast- and slow-type for different molecular sizes of dextran probes and granular treatments were significantly different ($p < 0.05$) whereas within fast- and slow types there was no significant difference based on dextran size or granular treatment (ANOVA, $p > 0.05$). The number of slow-type or fast-type diffusing granules for each dextran size and granular treatment were comparable but not quantified, as the FRAP experiment was conducted on only those selected granules showing fluorescence inside them. The values presented in Table 1 are an average of at least 2 good experiments without loss of fluorescence in either the ROI or reference region during the recovery phase. There are therefore three types of granules within any of the preparations i.e. those which did not take up the fluorescent probe after mixing overnight (lack of internal fluorescence), and if there was internal fluorescence, those with either fast-type diffusion characterised by a recovery curve that levelled off within 1 minute after bleaching or slow-type diffusion that took more than 6–7 min for recovery. For example, the 'fast' diffusion

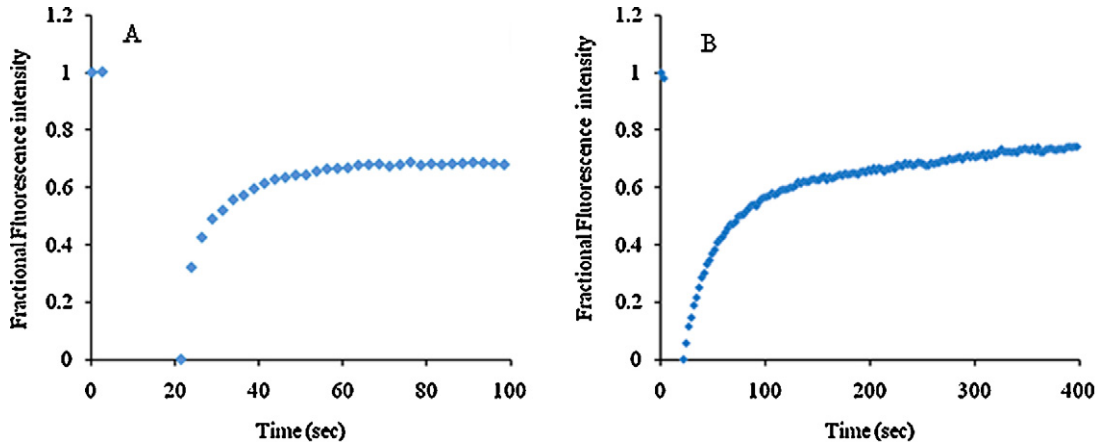

Fig. 5. Example FRAP recovery curves of 20 kDa FITC dextran in MS. (A) Fast diffusion type and (B) slow diffusion type.

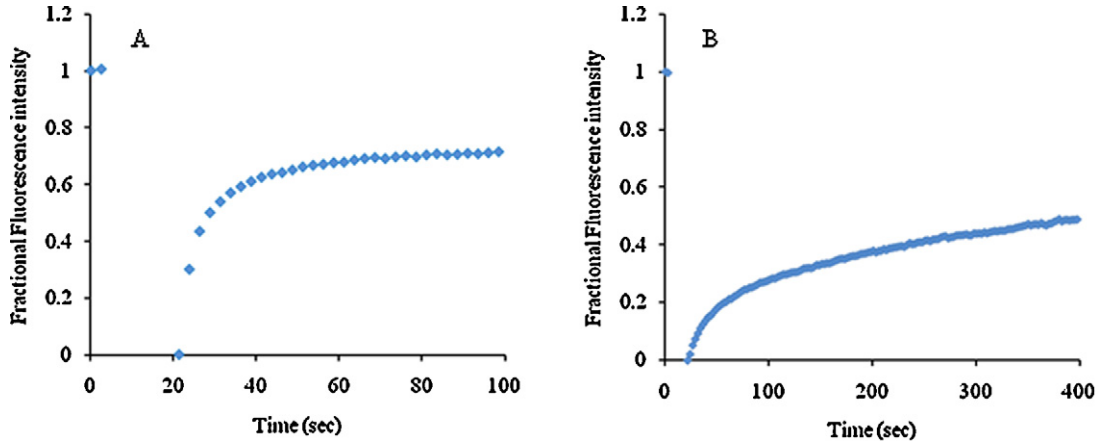

Fig. 6. Example FRAP recovery curve of 20 kDa FITC dextran in enzyme pre-treated MS. (A) Fast diffusion type and (B) slow diffusion type.

coefficients of FITC dextran in MS and pre-treated MS as shown in Figs. 5A and 6A were 4.9×10^{-6} and 3.2×10^{-6} cm^2 s^{-1}, respectively with $t_{1/2}$ values of 3.0 and 3.8 s, respectively. The 'slow' type diffusion coefficients of FITC dextran in MS and pre-treated MS as shown in Figs. 5B and 6B were 4.13×10^{-7} and 2.91×10^{-7} cm^2 s^{-1} with corresponding $t_{1/2}$ values of 30 and 42 s respectively, whilst the $t_{1/2}$ of the solution blank experiment irrespective of dextran size was ca 1 s with an average diffusion coefficient of 8.0×10^{-6} cm^2 s^{-1}. This value is almost 20 times higher than that reported by Perry et al. (2006). This variation together with the insignificant molecular size dependence of dextran diffusion in solution further suggests the possibility of 'flow' enhancing diffusion in the current system. However, as the main purpose of dextran solution measurement is to compare with much slower in-granule diffusion, limited flow effects are acceptable.

The immobile fraction of dextran probes in Figs. 5 and 6 ranged from 25 to 55% whereas it was close to zero in solution blank experiments. The percentage of immobile fraction was apparently independent of diffusion coefficient, size of dextran probes or granular treatments. This suggests that the diffusion of dextran inside MS granules (irrespective of pore sizes) is not solely free Brownian diffusion but that the dextran probes are subjected to varying levels of physical barriers or interactions which are essentially absent in solution blank experiments. Similarly, the bleached dextran probes occupying the diffusive volume may hinder the diffusion of fresh un-bleached probes further increasing the immobilised fraction. DeSmedt et al. (1997) also reported the incomplete diffusion of dextran probes in dextran methacrylate gels, whilst complete fluorescence recovery was observed in dextran methacrylate solutions. It is to be noted that the granules were conditioned with FITC dextran solution overnight before performing FRAP experiments. Thus it is possible that longer (but experimentally impractical) FRAP experiment times would allow dextran probes sufficient time to overcome those barriers responsible for immobile fractions. For example, Fig. 6B has more than 50% immobile fraction, but if the Axelrod fit is extrapolated to 2.5 h, almost 100% fluorescence recovery is predicted (extrapolated graph not shown).

The distinct slow- and fast-type diffusion mechanisms of dextran probes, irrespective of granule treatment and dextran size, suggest heterogeneity within the granules and that the underlying mechanism is a feature of granule architecture that controls the passage of dextran probes inside the granule matrix. The probes inside the granule can move through channels towards the central cavity (radial movement) or can diffuse within the granule matrix (lateral movement) either from the surface of channels or from the edges of the cavity. We propose that the former radial movement represents the fast-type diffusion mechanism, whereas lateral diffusion of dextran-probes is the origin for the slow-type diffusion mechanism. However, it is to be noted that even for the slow-type diffusion, the initial inward diffusion is always the fast-type i.e. diffusion from the surface to the granule interior via channels. As the dextran probes were allowed to diffuse into granules overnight prior to measurement, the observation of fast diffusion suggests that these granules do not allow passage into a dense diffusion-restraining polymer environment ('lateral' diffusion) but instead are contained within pores and channels. Although limited enzyme

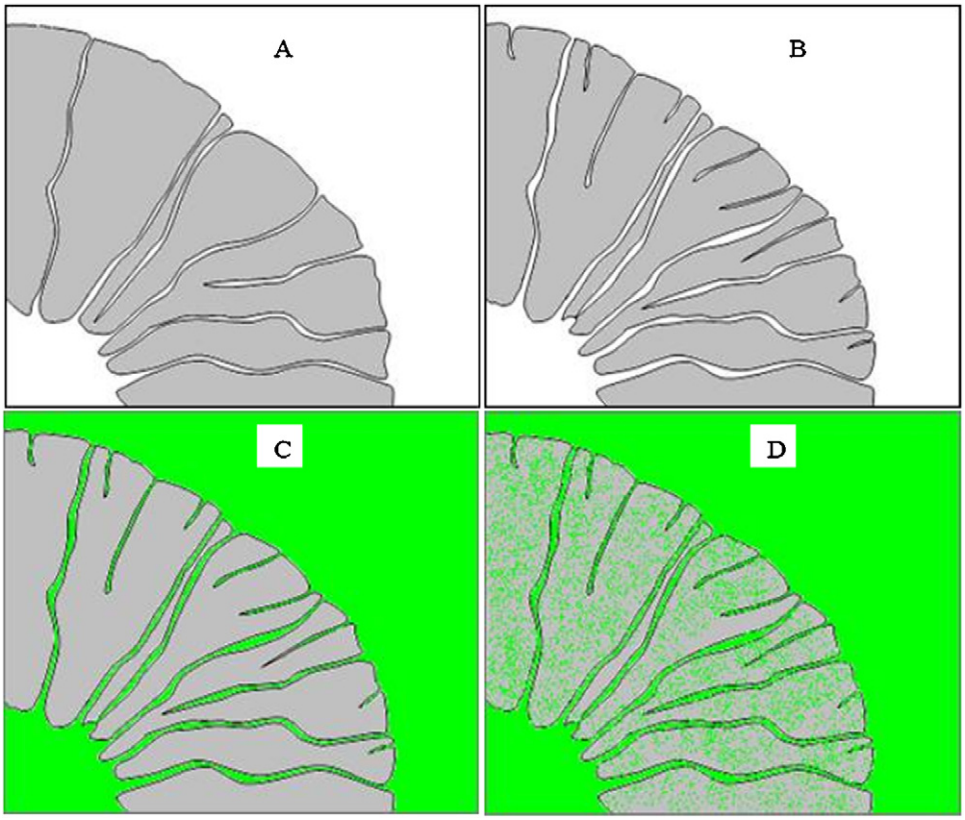

Fig. 7. Model illustrating the diffusion mechanisms of dextran probe in maize starch granules. Pores and channels in native maize starch granules (A). Opening of new pores and channels and enlargement of existing pores and channels by limited hydrolysis (B). Diffusion of FITC-Dextran (represented by bright green colour) via pores and channels towards the central cavity (fast diffusion) (C). Diffusion of FITC dextran within the granule matrix (slow diffusion) (D). (For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of this article.)

pre-treatment (2.4% hydrolysis) allows many more granules to take up dextran probes after overnight soaking (Fig. 3) consistent with limited enzyme action opening up surface pores, slow-type diffusion is still observed after enzyme pre-treatment consistent with a bulk polymer network origin for the observed slow diffusion.

It is apparent that the pores and channels with diameter of almost 100 nm (Fannon et al., 1992, 1993) can easily accommodate the labelled dextran probes. However, if the slow-type diffusion is due to the lateral diffusion of probes within the bulk of the granule matrix, then the matrix should have voids to contain dextran probes with hydrodynamic radius of 4–7 nm. This apparently contradicts previous reports from Brown and French (1977) and Planchot, Roger, and Colonna (2000), who reported that only molecules no larger than 800–1000 Da (equivalent to about a 6 glucose unit oligosaccharide) or with a hydrodynamic radius <0.6 nm can penetrate between radially aligned amylopectin chains in a starch granule matrix. However, based on the super-helical model proposed by Oostergetel and Vanbruggen (1993), Oates (1997) speculated that the voids inside an amylopectin-superhelix are empty and about 8 nm wide, larger enough for dextran probes to diffuse inside them. Once, the probes diffuse into cavities or other small voids via pores and channels or from the hilum, it is possible that probes could further migrate into the granule matrix in an inside-out fashion (Huber & BeMiller, 1997; Villwock, 1999). Polysaccharides with molecular weights as large as 2.000 kDa have been found to be able to penetrate granule matrices (Villwock, 1999) suggesting that the starch matrix is capable of capturing larger molecules than previously thought possible from size exclusion studies (Brown & French, 1977; Planchot et al., 2000). The slow-type diffusion coefficient of dextran probes in maize starches (ca 2×10^{-7} cm^2 s^{-1}) is quite similar to that inferred by Al-Rabadi et al. (2009) for diffusion of amylases in milled sorghum/barley grains. In those grains, the diffusion of enzymes was suggested to be controlled by dense packing of cellular and/or protein matrices analogous to the proposal that slow-type diffusion is controlled by the porosity of a densely packed starch matrix. The observed slow- or fast-type diffusion of dextran probes inside maize starch granules provides some insights into the heterogeneity of maize starch granules and their digestion by amylases. The existence of sub-populations of granules with differing degrees of crystallinity and reactivity within waxy maize starch (Jia, Ao, Han, Jane, & BeMiller, 2004) may be another manifestation of the heterogeneity observed in this study. In general, within a starch preparation, it has been observed that granules are not equally susceptible to enzymatic degradation (Colonna et al., 1992; Gallant et al., 1992; Oates, 1997; Tester, Qi, & Karkalas, 2006). For maize starch, the heterogeneity was assumed to be due to variation in the presence and numbers of pores and channels (Fannon et al., 2004). The marked increase in accessibility of granule interiors to APTS (Fig. 2) and dextran probes (Fig. 3) after modest (2.4%) amylolysis suggests both the formation of new pores by the pitting action of amylase and removal of starch or protein coatings over surface pores by the action of amylase or pepsin. Once entrance to the granule interior has been secured, we propose that for those granules characterised by fast-type diffusion of dextran probes, amylases quickly diffuse into the granule interior hydrolysing and thereby widening pores and channels, followed by slow-type diffusion of amylases, hydrolysing the granule matrix with an inside-out pattern. Thus, granules having larger size and number of pores and channels are assumed to be hydrolysed faster than granules having fewer pores and channels. However, extrapolation of the current dextran probe diffusion behaviour to amylase action is difficult due to the concurrent catalytic action of

amylase leading to differences in the local diffusion environment, so further experiments e.g. diffusion study of labelled enzymes are needed to further explore the granular heterogeneity of amylolysis.

The clear distinction of granules into fast- or slow-diffusion types is intriguing and suggests that there are two categories of internal structure within maize starch granules. One type of architecture allows rapid entry and exit of polymeric probes, consistent with not only the presence of pores and channels but also an inability of the polymeric probes to access the more densely packed regions of the granule that could lead to slower diffusion properties. The second type of architecture is proposed to allow access of polymer probes to the densely packed bulk of the granule where voids are smaller than pores and channels and from which there is a tortuous exit path leading to slow-type diffusion. The model representing this mechanism is presented in Fig. 7. Although the present data cannot distinguish unambiguously between direct access to the granule bulk from the exterior and access via internal and channels and pores, the latter seems a more likely mechanism based on the much greater access of probes to granule interiors after very limited amylolysis (Figs. 2 and 3). The inability of granules showing fast-type diffusion to allow penetration of probes into the bulk of the granule even after overnight mixing, suggests that either pores and channels are effectively coated with non-carbohydrate components (Han et al., 2005) or that starch polymers adjacent to pores and channels are densely packed.

4. Conclusion

In the present study, we have demonstrated that the local diffusion of dextran probes inside maize starch granules are either fast-type with apparent diffusion coefficients in the order of 10^{-6} cm^2 s^{-1} or slow-type with apparent diffusion coefficients in the order of 10^{-7} cm^2 s^{-1}. This suggests that granule architecture primarily controls the diffusion of dextran probes and is independent of probe molecular weight from 20 to 150 kDa.

The two types of diffusion behaviour are proposed to be due to (a) probe access limited to pores, channels and central cavity ('fast-type') and (b) probe access to the more densely packed bulk of the granule ('slow-type'). Access to granule interiors was greatly increased following 2.4% amylolysis, suggesting that starch polymers restricting access to pores and channels are an important initial site of enzyme action. These findings have shed light on the mechanism underlying the heterogeneity of maize starch granules and their digestion with amylases. The approach of using FRAP to study the diffusion of labelled probes within individual starch granules has the potential to further define the heterogeneity and mechanisms of enzymatic hydrolysis and chemical modification of maize and other starch granules.

Acknowledgements

We thank Dr Steven Mason (School of Chemistry and Molecular Biosciences, The University of Queensland) for expert advice and assistance.

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