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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

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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.

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 → 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).

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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, Schülein, & 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× 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 μL of starch suspension was removed and immediately added to 300 μL of 0.3 M Na_2CO_3 in a microcentrifuge tube to stop the reaction. These samples were then centrifuged at 16,000 × *g* for 5 min to remove unreacted starch, and 300 μL 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, Carls Zeiss, Germany) with a Plan-Apochromat 20× lens (with digital zoom of 2× 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 × 1024 at a scan speed of 8 bit and a pixel dwell time of 1.58 μs, from an optical slice of 2 μm 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