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Effect of short-chain fatty acids on the formation of amylose microparticles by amylosucrase

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ABSTRACT

Amylose microparticles can be produced by self-assembly of amylose molecules through an amylosucrase-mediated synthesis. Here we investigated the role of short-chain fatty acids in the formation of amylose microparticles and the fate of these fatty acids at the end of the reaction. The rate of self-assembly and production yields of amylose microparticles were significantly enhanced in the presence of fatty acids. The effect was dependent on the length of the fatty acid carbon tail; butanoic acid (C4) was the most effective, followed by hexanoic acid (C6) and octanoic acid (C8). The amylose microparticles were investigated by carrying out SEM, XRD, Raman, NMR, FT-IR and DSC analysis. The size, morphology and crystal structure of the resulting amylose microparticles were comparable with those of amylose microparticles produced without fatty acids. The results indicated the carboxyl group of the fatty acid to be responsible for promoting the self-assembly of amylose chains to form microparticles. The fatty acids were eventually removed from the microstructure through the tight association of amylose double helices to form the amylose microparticles.

1. Introduction

Amylose is a mostly linear polysaccharide consisting of α-1,4-D-glucose units, and makes up around 20–30% of the starch granules in general cereal starches. The amylose content can be as low as 0% for waxy starch while that of high-amylose starches is higher than 50%. This polysaccharide is also known as a safe natural material and is widely used in the food, medical, and chemical industries because of its non-toxicity, biocompatibility, and biodegradability (Aburto et al., 1999; Dumoulin, Cartilier, & Mateescu, 1999; Fu, Meng, Lu, Zhang, & Gao, 2007; Gao et al., 2011; García, Martino, & Zaritzky, 1998; Siew, Man, Newton, & Basit, 2004). It is well established that amylose can form complexes with various guest molecules such as alcohols (Brisson, Chanzy, & Winter, 1991; Buléon, Delage, Brisson, & Chanzy, 1990; Nishiyama et al., 2010; Rappenecker & Zugenmaier, 1981), polymers (Kaneko, Saito, Nakaya, Kadokawa, & Tagaya, 2008), aroma compounds (Biais, Le Bail, Robert, Pontoire, & Buleon, 2006), iodine (Stein & Rundle, 1948), carbon nanotubes (Lim, Seo, Jung, Park, & Kim, 2014), and iron oxide nanoparticles (Lim et al., 2015) *via* hydrophobic interactions. Amylose-based helical inclusion complexes with various fatty acids have also been extensively studied (Biliaderis, Page, Slade, & Sirett, 1985; Cao et al., 2013; Godet, Buleon, Tran, & Colonna, 1993; Zabar, Lesmes, Katz, Shimoni, & Bianco-Peled, 2009). The aliphatic part of the fatty acid is presumed to be held within the cavity of the helical amylose chain by hydrophobic interactions while the carboxyl group remains outside due to steric effect and its hydrophilic nature (Godet, Tran, Colonna, Buleon, & Pezolet, 1995).

Enzymatic synthesis of amylose has attracted much attention because it can produce pure amylose with a controllable molecular weight. Having a material with a defined and controlled molecular weight is beneficial in terms of controlling the properties of final product. The most extensively studied enzymes for the synthesis of amylose are phosphorylase and amylosucrase. Phosphorylase synthesizes amylose in the presence of maltotetraose (G_4) as a primer and α-D-glucose 1-phosphate (Glc-1-P) as a glycosyl donor (Fujii et al., 2010; Kadokawa, 2012; Ziegast & Pfannemüller, 1987). Amylosucrase is another enzyme that synthesizes amylose from a single substrate, in this case sucrose (Cambon, Barbe, Pizzut-Serin, Remaud-Simeon, & André, 2014; Guérin et al., 2012; Roblin et al.,

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2012). The synthesis is initiated by the hydrolysis of sucrose, and the released D-glucose molecule is used as an acceptor of free D-glucose to produce maltose. The elongation of maltose to the longer amylose is achieved via a repeated polymerization cycle. Recently, an amylosucrase-mediated production of an amylose nanocomposite embedding carbon nanotubes and iron oxide nanoparticles as guest molecules was reported (Lim et al., 2015, 2014). Phosphorylase has also been reported to produce an amylose-fatty acid complex using maltohexaose and Glc-1-P as substrates and fatty acids with chain lengths greater than C14 as guest molecules (Gelders, Goesaert, & Delcour, 2005).

There have been several reported studies of the complexation of amylose molecules with long-chain fatty acids, but few with short-chain fatty acids. In this study, we investigated the role of short-chain fatty acids in the formation of amylose microstructures by amylosucrase and the fate of these fatty acids at the end of reaction. We introduced water-miscible short-chain fatty acids such as butanoic acid (C4), hexanoic acid (C6), and octanoic acid (C8) to the synthesis. The associations of the rate of self-assembly to form amylose microparticles and of the production yield with the presence or absence of fatty acids were thoroughly investigated. The effects of the fatty acid tail length on the synthesis and the physical characteristics of resulting amylose microparticles were also examined.

2. Materials and methods

2.1. Chemicals and bacterial strain

Sucrose, maltose, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), sodium chloride (NaCl), monosodium phosphate (NaH_2PO_4), sodium dodecyl sulfate (SDS), imidazole, ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), butanoic acid, hexanoic acid, octanoic acid, and butanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luria-Bertani (LB) broth was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Distilled water was used in all experiments. *Escherichia coli* MC1061 was used as the host microorganism for the expression vector pHCDGAS, encoding recombinant amylosucrase from *Deinococcus geothermalis* (DGAS). DGAS was prepared as described earlier (Lim et al., 2014). Briefly, recombinant *E. coli* MC1061 harboring pHCDGAS was grown in 500 mL LB broth (0.1 mg/mL ampicillin) at 37 °C for 24 h at 250 rpm. The cells were harvested by centrifugation (7000*g* for 20 min at 4 °C) and washed with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0). The bacterial pellet was resuspended in lysis buffer and disrupted by sonication in an ice bath and the cellular debris was spun down by centrifugation at 10,000*g* for 30 min at 4 °C. The supernatant was passed through a Ni-NTA affinity column (Qiagen Inc, Valencia, CA, USA), and the column was washed with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0) and the recombinant DGAS was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0). The activity of the purified enzyme was measured by applying the dinitrosalicylic acid (DNS) method, using fructose as a standard (Seo et al., 2008; Sumner & Howell, 1935).

2.2. Synthesis of amylose microparticles with fatty acids

To produce the amylose microparticles, an aqueous solution containing 500 mM sucrose and 5000 U DGAS in 10 mL of 50 mM Tris-HCl buffer (pH 7.0) was incubated at 40° C for 24 h (Fig. 1). The formation of amylose microbeads in the presence of fatty acids was carried out by adding 10 mM of each fatty acid to 10 mL of the aqueous reaction solution. After the enzymatic reaction, the produced amylose microparticles were harvested by centrifugation. The collected beads were washed with filtered DI water three times and dried using a vacuum dryer for further analysis.

2.3. Determination of the average degree of polymerization ($\overline{DP}$)

During the enzyme reaction, an aliquot of the reaction mixture was collected every hour over the course of 24 h reaction, followed by ethanol precipitation and vacuum drying. The reaction solution was homogenized by vortexing before collecting the aliquot. To determine the $\overline{DP}$ of amyloses from the collected reaction mixture, the dried samples were solubilized in 1 M KOH to a final concentration of 10 mg/mL. Then, 10.5 vols of 0.1 M HCl, 111.5 vols of DI water, and 2 vols of an iodine solution (2% KI and 0.2% I_2) were mixed with one volume of solubilized amylose solution. The maximum absorption wavelength (λ_{max}) value of the solution (200 μL) was measured in a 96-well plate. The λ_{max} of the amylose-iodine complexes was determined by recording the adsorption spectra ranging from 450 to 750 nm with 2 nm steps using a microplate spectrophotometer (Infinite M200, Tecan, Durham, NC, USA). The $\overline{DP}$ of amylose synthesized with and without fatty acids was calculated by using the equation (John, Schmidt, & Kneifel, 1983)

$$10^3/\lambda_{max} = 11.6/\overline{DP} + 1.537$$

2.4. Turbidimetry

The rate of self-assembly for the formation of amylose microparticles was monitored by measuring the turbidity of the reaction. The absorbance of the reaction solution was measured at 600 nm as a function of time over 24 h using a spectrophotometer (Optizen POP, Mecasys Co. Ltd., Daejeon, Korea). A volume of 1 mL of reaction solution was transferred into a cuvette with a 1 cm path length and sealed with a customized cap to prevent the evaporation of reaction solution and fatty acids. Then, the reaction was carried out at the same condition as described above. The reaction solution of the cuvette were homogenized by gentle vortexing just prior to each measurement.

2.5. Scanning electron microscopy (SEM)

Amylose microparticles generated from each reaction were dispersed in filtered DI water to a final concentration of 1 mg/mL and were dropped onto a plasma-cleaned silicon substrate. The water was allowed to evaporate, and then the morphologies of the amylose microparticles were visualized by using a field emission-scanning electron microscope (FE-SEM, Leo Supra 55, Genesis 2000, Carl Zeiss, Oberkochen, Germany) with an accelerating voltage of 5 kV. The average size of the amylose microparticles was determined by measuring the diameter of 100 beads from the images.

2.6. X-ray diffraction (XRD)

Powder X-ray diffraction patterns of the amylose microparticles were analyzed from 3 to 30° (2θ) using Cu Kα radiation on a Bruker D8 Advance diffractometer (Bruker, Karlsruhe, Germany). All of the samples were dehydrated in a vacuum desiccator before XRD analysis.

2.7. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectra were recorded on KBr pellets on a Perkin-Elmer Spectrum One System spectrometer (Foster City, CA, USA) from 4000 to 500 cm^{-1}. Vacuum-dried samples were mixed with KBr