ACS Catalysis

Research Article

pubs.acs.org/acscatalysis

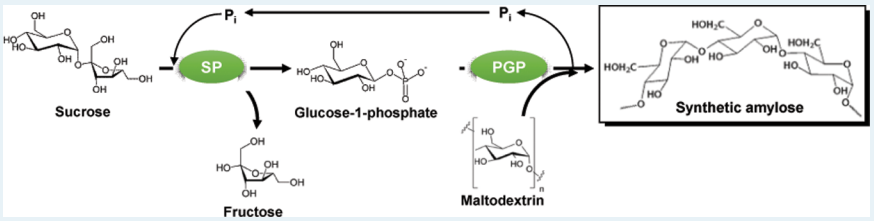
One-Pot Enzymatic Conversion of Sucrose to Synthetic Amylose by using Enzyme Cascades

Peng Qi,[†] Chun You,[†] and Y.-H. Percival Zhang*[,†,‡]

[†]Biological Systems Engineering Department, Virginia Tech, 304 Seitz Hall, Blacksburg, Virginia 24061, United States

[‡]Cell Free Bioinnovations, Inc., Blacksburg, Virginia 24060, United States

Supporting Information



ABSTRACT: Synthetic amylose could be a very important compound as a precursor of low-oxygen diffusion biodegradable plastic films, a healthy food additive, and a potential high-density hydrogen carrier. In this study, one-pot reactions composed of sucrose phosphorylase and potato alpha-glucan phosphorylase or supplemented with the three other enzymes (i.e., glucose isomerase, glucose oxidase, and catalase) were carried out to convert cheap sucrose to synthetic amylose, whereas one glucose unit from sucrose was added into the nonreducing end of the primer—maltodextrin. A thermostable sucrose phosphorylase was cloned from a thermophilic bacterium *Thermoanaerobacterium thermosaccharolyticum* JW/SL-YS485. The values of k_{cat} and K_m on sucrose were 15.1 s^{-1} and 20.2 mM, respectively, at 37 °C. The half-life time of this enzyme was 3.1 h at 70 °C. The yield of synthetic amylose was not significantly improved when glucose isomerase, glucose oxidase, and catalase were used to remove fructose, which was an inhibitor to sucrose phosphorylase. This result suggested that the two-enzyme system equipped with the sucrose phosphorylase with a high value of fructose dissociation constant (34.4 mM) did not require the three other enzymes to mitigate product inhibition. The number-average degree of polymerization of synthetic amylose was controlled from 33 to 262 by adjusting primer maltodextrin concentration and reaction time.

KEYWORDS: *cascade enzymatic reaction, potato alpha-glucan phosphorylase, sucrose phosphorylase, synthetic amylose, thermostable enzyme*

INTRODUCTION

Enzyme-based biocatalysis has become an attractive alternative to chemical catalysis because of its higher reaction selectivity as well as milder and greener reaction conditions.[1,2] Multiple enzymes in one-pot reactions and even in vitro biosystems comprising dozens of enzymes have been developed to implement complicated biological reactions, such as the synthesis of chiral alcohols with NAD(P)H regeneration,[3,4] the synthesis of carbohydrates,[5,6] polymers,[7] and proteins,[8] as well as the production of hydrogen,[9,10] alcohols,[11,12] and electricity.[13−15] The consolidation of cascade enzymatic reactions in one vessel has numerous benefits: fewer unit operations, smaller reactor volume, higher volumetric and space-time yields, shorter cycle times, and less waste generation. Also, by coupling steps together, unfavorable equilibria may be driven toward the formation of desired products.[1,7,16]

Sucrose is a disaccharide composed of glucose linked to fructose via an ether bond between C1 on the glucosyl subunit and C2 on the fructosyl unit. It is the most abundant disaccharide in nature, and approximately 168 million metric tonnes was produced from sugar cane, sugar beet, and sorghum in 2011.[17] Although its price varied greatly by several fold in the past 10 years,[18] sucrose is among the cheapest fermentable sugars. Sucrose in Brazil is usually cheaper than starch in the United States, allowing Brazil to produce the most cost-competitive ethanol in the world.

Sucrose phosphorylase (SP, EC 2.4.1.7) can catalyze the reversible phosphorolysis of sucrose into glucose 1-phosphate (G-1-P) and fructose, shown in eq 1[19] with an equilibrium constant of 5.3 at pH 7.0[20,21]

$$\text{sucrose} + \text{phosphate} = \text{fructose} + \text{glucose 1-phosphate} \quad (1)$$

Numerous SPs have been isolated and characterized from *Bifidobacterium longum* SJ32,[22] *Streptooccus mutans* UA159,[23] *Leuconostoc mesenteroides* no. 165,[24] *Leuconostoc mesenteroides* NRRL B-742,[25] and *Pseudomonas saccharophila*.[26] SP is classified as a glycosyl transferase, but it belongs to the glycoside hydrolase family 13[27] with a characteristic double displacement mechanism of retaining glycosidases.[28] SP can be

Received: October 22, 2013
Revised: March 16, 2014
Published: March 18, 2014

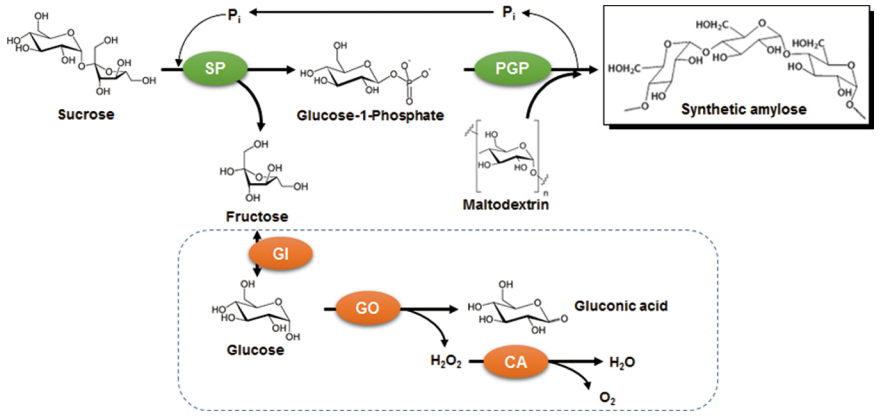


Figure 1. Scheme of the synthetic enzymatic pathway from sucrose to amylose. SP, sucrose phosphorylase (EC 2.4.1.7); PGP, potato alpha-glucan phosphorylase (EC 2.4.1.1); GI, glucose isomerase (EC 5.3.1.5); GO, glucose oxidase (EC 1.1.3.4); and CA, catalase (EC 1.11.1.6).

used to produce G-1-P, which is an efficient donor for chemical and enzymatic glycosylation reaction.[29] In addition, it can be used to produce a number of glycosylated compounds because it has a broad substrate specificity.[30,31] For example, Goedl et al.[32] developed an efficient and selective process for the production of glyceryl α-D-glucoside, which is a moisturizing agent for cosmetics. For the production of glycosylated compounds, these reactions may be preferably run at high temperature to avoid possible microbial contamination.[33] A mesophilic SP has been engineered for enhanced thermostability, but its half lifetime remains short.[34] To our limited knowledge, no SP enzyme was cloned and characterized from thermophilic organisms.

Amylose is a linear polysaccharide made up of anhydroglucose units linked by alpha-1,4-glycosidic bonds, accounting for 20−30% weight of natural plant starch. The other component is branched amylopectin. Amylose is more valuable than amylopectin because of its promising applications: a precursor for making high-quality clear, transparent and flexible low-oxygen diffusion plastic sheets and films,[35,36] a functional food additive for lowering the risk of serious noninfectious diseases (e.g., diabetes and obesity),[37,38] and a potential high-density hydrogen carrier.[10,39,40] A large amount of high-purity linear amylose without branches is not available because the complete separation of amylose from amylopectin from plant starch is difficult and very costly. Recently, we demonstrated the conversion of nonfood cellulose to amylose by using four enzymes in one pot, including endoglucanase, cellobiohydrolase, cellobiose phosphorylase, and potato alpha-glucan phosphorylase (PGP).[16] However, it is relatively difficult to produce a large amount of synthetic amylose from cellulose because commercial cellulase mixtures cannot produce cellobiose as a major product from biomass. In this study, we redesigned the synthetic enzymatic pathway that can convert sucrose to synthetic amylose in one vessel (Figure 1). Also, the first thermophilic SP was cloned and characterized from *Thermoanaerobacterium thermosaccharolyticum* JW/SL-YS485.

EXPERIMENTAL SECTION

Chemicals and Materials. All chemicals were reagent-grade or higher and purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. The PCR enzyme was the Phusion DNA polymerase for New England BioLabs (Ipswich, MA). Maltodextrin (dextrose equivalent 13.0−17.0) used as a primer for the synthesis of amylose had molecular weights in the range of 1162−1564. The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). Insoluble regenerate amorphous cellulose (RAC) was prepared from Avicel PH105 (FMC) via cellulose dissolution followed by water precipitation.[41] Glucose isomerase, glucose oxidase, and catalase were purchased from Sigma-Aldrich. The genomic DNA sample of *T. thermosaccharolyticum* JW/SL-YS485 was a gift from Lee Lynd at Dartmouth College.[42]

Strains and Media. *Escherichia coli* Top 10 was used as a host cell for DNA manipulation, and *E. coli* BL21 Star (DE3) (Invitrogen) was used as a host cell for recombinant protein expression. Luria−Bertani (LB) medium was used for *E. coli* Top 10 cell culture, and autoinducing medium ZYM-5052 was used for recombinant protein expression.[43] The final concentration of antibiotic for *E. coli* was 100 mg/L ampicillin.

Plasmid Construction and Protein Expression/Purification. The full length DNA fragment encoding the putative sucrose phosphorylase gene from *T. thermosaccharolyticum* JW/SL-YS485 was amplified with a pair of primers (IF-SP 5′- TTAAC TTTAA GAAGG AGATA TACAT ATGGC ACTTA AAAAT AAGGT ACAAC −3′ and IR-SP5′-AGTGG TGGTG GTGGT GGTGC TCGAG TACTA AATAC TTTAC TTCTT CACCG-3′). The pET20b vector backbone was amplified with a pair of primers (VF-SP 5′-CGGTG AAGAA GTAAA GTATTTAGTACTCGAGCAC-CACCACCACCACCACT-3′ and VR-SP 5′- GTTGT ACCTT ATTTT TAAGT GCCAT ATGTA TATCT CCTTC TTAAA GTTAA −3′). The plasmid pET20b-SP was assembled on the basis of the two PCR products by prolonged overlap extension PCR—called the restriction enzyme-free and ligase-free Simple Cloning.[44] The strain *E. coli* BL21 Star (DE3) containing the pET20b-SP was cultivated in the MDG medium at 37 °C overnight.[43] Seed culture (0.5 mL) was added into 100 mL of the autoinducing medium ZYM-5052.[43] The cell culture was grown at 37 °C and 300 rpm. When the absorbency reached approximately 8, the temperature of the cell culture was decreased to 20 °C overnight to promote the correct folding of recombinant SP. After centrifugation, the cell pellets were lysed in an ice bath by ultrasonication twice with the Fisher Scientific Sonic Dismembrator Model 500 (3 s pulse, total 120 s, at 50% amplitude). The His-tagged SP was purified by using Ni-nitrilotriacetic acid resin as described elsewhere.[45] The expression and purification of the recombinant enzyme PGP-Ctdoc-LL-PGP and scaffoldin, which helped purify the dockerin-containing PGP through the simple adsorption on RAC, was described elsewhere.[16] Protein mass concentration was measured by the Thermo Scientific Pierce Bradford method with bovine serum albumin as a reference protein.[46] The specific activities of SP and PGP were 18 and 3.3 U/mg at 37 °C, respectively.

Enzyme Activity Assay. The activity of SP was determined at 37 °C. The product G-1-P was measured by coupling phosphoglucomutase (PGM) and the liquid glucose assay kit (Pointe Scientific, Canton, MI) that reduced NAD^+ to NADH. The recombinant PGM from *Clostridium thermocellum* was produced and expressed as described

dx.doi.org/10.1021/cs400961a | *ACS Catal.* 2014, 4, 1311−1317

previously.[47] The SP activity assay was performed in 450 μL of the mixture containing 400 μL of the liquid glucose assay reagent and 50 μL of the other reagents with the final concentrations of 50 mM phosphate, 10−200 mM sucrose, 20 U/mL PGM, and 0.2 U/mL SP. The formation of NADH with time was monitored spectrophotometrically at 340 nm for 10 min. To test this enzyme's substrate specificity, its activity assay was performed on 100 mM maltose or lactose instead of sucrose.

Enzymatic Conversion of Sucrose to Amylose. The one-pot reactions were conducted in 1 mL of the100 mM TrisHCl buffer (pH 7.4) containing 50 mM phosphate, 100 mM sucrose, 5 U of SP, 5 U PGP, and 10−1000 μM maltodextrin (dextrose equivalent 13.0−17.0) at 37 °C. The three supplementary enzymes: 20 U of glucose isomerase, 20 U of glucose oxidase, and 30 U of catalase were added into the reaction solution if needed. The reaction was terminated by 20 min in boiling water. After centrifugation to remove deactivated enzymes, the supernatants were used for the quantification of sucrose, fructose, G-1-P, and amylose.

Quantification of G-1-P, Sucrose, and Fructose. The G-1-P concentration in the supernatant was measured by the liquid glucose assay kit supplemented with PGM. The 450 μL of reaction mixture contains 400 μL of the liquid glucose (hexokinase) kit reagent and 50 μL of the other reagents, including the sample with final concentrations of 50 mM phosphate and 20 U/mL PGM. The mixture was incubated at 37 °C for 20 min. The formation of NADH was monitored at 340 nm. The lumped concentration of sucrose and G-1-P in the supernatant was measured by the liquid glucose (hexokinase) kit reagent supplemented with 2 U/mL SP and 20 U/mL PGM. The formation of NADH was monitored at 340 nm. The remaining sucrose concentration was calculated by the lumped concentration of sucrose and G-1-P minus the measured G-1-P concentration. In the two-enzyme system, fructose concentration in the supernatant was measured by the liquid glucose (hexokinase) kit reagent supplemented with 2 U/mL glucose isomerase and 10 mM $MgCl_2$ at 37 °C for 10 min.

Amylose Calculation and Characterization. In the DP-controlled amylose reaction system, the optimal concentrations of sucrose, inorganic phosphate, and enzymes were 100 mM sucrose, 50 mM sodium phosphate, 10 to 1000 μM maltodextrin, 5 U of SP, and 5 U of PGP at 37 °C. After 25 h of incubation, the reactions were stopped by 20 min in boiling water. After centrifugation that removed deactivated proteins, an equal volume of 100% ethanol was added to precipitate amylose. After centrifugation, the amylose pellets were washed three times with 50% ethanol and then freeze-dried. Amylose pellets were examined by using Coomassie brilliant blue dye to make sure that there was no detectable protein in the dehydrated amylose samples. The cross-polarization magic-angle spinning ^{13}C NMR spectra of synthetic amylose and natural starch were characterized on a Bruker II Avance-300 spectrometer.[16] The FTIR spectroscopy was conducted using a Thermo Nicolet 6700 ATR/FT/IR spectrometer (Fisher Scientific). Two hundred fifty-six scans at a resolution of 6 cm^{-1} were averaged for each sample. All FTIR spectra were subjeted to Saviszky-Golay smoothing.[16] The absorbance of the bands obtained was solved using the Voigt distribution function by PeakFit 4.12 software. The yield of amylose (Y_a) was calculated on the basis of eq 2

$$Y_a = \frac{\text{Amylose}}{S_i - S_r} 100\% \quad (2)$$

in which Amylose was the weight concentration of the precipitated amylose, S_i was the initially added sucrose weight concentration, and S_r was the residual sucrose weight concentration.

The number-average degree of polymerization (DP_n) of synthetic amylose was calculated as

$$DP_n = \frac{GE}{RE} \quad (3)$$

where GE was the concentration of amylose in terms of glucose equivalent measured by the phenol−sulfuric acid method,[48] and RE was the concentration of reducing ends of amylose measured by the modified bicinchoninic acid method.[48] The molecular weight of synthetic amylose can be calculated as eq 4

$$M_w = DP_n \times 162.2 + 18.0 \quad (4)$$

where 162.2 and 18.0 are the molecular weights of anhydroglucose and water, respectively.

RESULTS AND DISCUSSION

Cloning, Expression, and Purification of a Thermophilic SP. Although the genomic sequences of numerous thermophilic microorganisms have been sequenced and annotated elsewhere (e.g., Kyoto Encyclopedia of Genes and Genomes), the genes encoding thermophilic sucrose phosphorylases is not as popular as other phosphorylases. A 1467 kb open reading frame of a thermophilic bacterium *T. thermosaccharolyticum* JW/SL-YS485 was hypothesized to encode a putative SP because the deduced 488-amino acid SP has an identity of 96.9% with the annotated SP gene from *T. thermosaccharolyticum* DSM 517 reported in the KEGG database. This SP also is highly homologous compared with the validated sucrose phosphorylases from *L. mesenteroides* no. 165,[24] *L. mesenteroides* NRRL B-742,[25] *S. mutans* UA159,[23] and *P. saccharophila*[26] (Figure S1). A 1467 kb open reading frame was amplified on the basis of the genomic DNA of *T. thermosaccharolyticum* JW/SL-YS485 and cloned into plasmid pET20b. The *E. coli* BL21 (DE3) strain harboring the T7 promoter based plasmid pET20b-SP was cultivated in the autoinducing high cell density medium for the production of recombinant SP. The soluble SP accounted for about 5% of total *E. coli* cellular protein according to SDS-PAGE analysis (data not shown). The protein was purified via nickel-nitrilotriacetic acid resin, appearing homogeneous (Figure 2a, Lane 1). Approximately 10 mg of SP was purified from 1 L of the cell culture. The values of k_{cat} and K_m on sucrose were 15.1 ± 0.4 s^{-1} and 20.2 ± 3.9 mM at 37 °C, respectively. The value of K_m on phosphate was 6.3 ± 0.6 mM at 37 °C (Figure S2c). The optimal temperature of this SP was 80 °C, and it lost its activity rapidly at 90 °C (Figure S3). The half-lifetimes of this enzyme were 0.5 h at 80 °C and 3.1 h at 70 °C, much longer than other reported mesophilic SPs in the literature. This SP did not exhibit activity on either maltose or lactose (data not shown).

Synthesis of Amylose. One-pot cascade reaction composed of up to five enzymes was designed to convert sucrose to synthetic amylose (Figure 1). This cascade reaction had three modules: (1) the phosphorylation of sucrose to G-1-P and fructose by sucrose phosphorylase (EC 2.4.1.7); (2) the synthesis of amylose from nonreducing ends of maltodextrin added by a glycosyl transferase family 35 potato α-glucan phosphorylase (PGP, EC 2.4.1.1); and (3) the mitigation of fructose inhibition to SP via glucose isomerase (GI, EC 5.3.1.5), glucose oxidase (GO, EC 1.1.3.4), and catalase (CA, EC 1.11.1.6). In this system, the phosphate ions were internally recycled to maintain phosphate levels. This designed pathway is completely different from the natural starch biosynthesis pathway in plants mediated by two enzymes: ADP-glucose pyrophosphorylase, which synthesizes sugar nucleotide precursor, and starch synthase, which extends the alpha-1,4-linked glucan chains using ADP-glucose.

The proof-of-concept experiment catalyzed by SP and PGP was carried out under the following conditions: 100 μL of 100 mM TrisHCl buffer (pH 7.4) containing 50 mM sucrose, 10

dx.doi.org/10.1021/cs400961a | ACS Catal. 2014, 4, 1311−1317

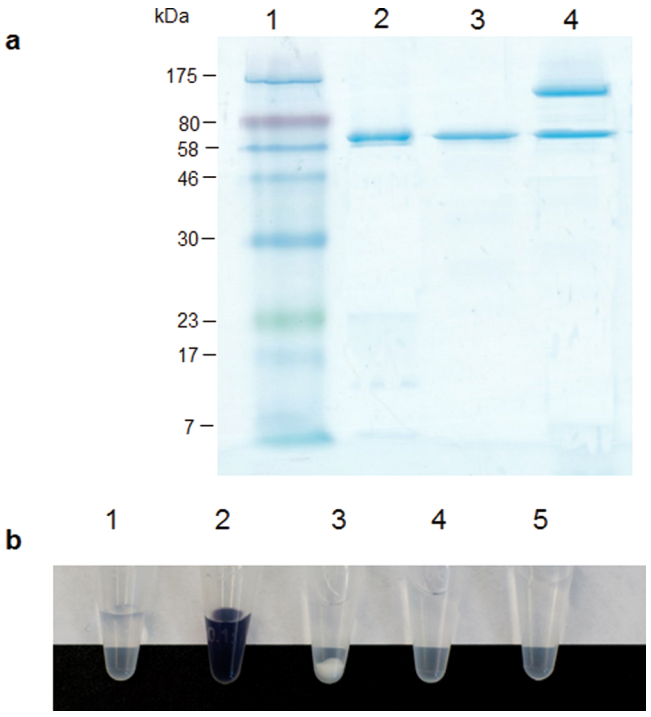


Figure 2. (a) SDS-PAGE analysis of the purified recombinant enzymes (Lane 1, protein markers; Lane 2, sucrose phosphorylase; Lane 3, the scaffoldin purified with RAC; and Lane 4, purified PGP with scaffoldin bound on RAC). (b) Pictures of the proof-of-concept for the synthesis of amylose from sucrose. Tube 1, the sucrose reaction mixture mixed with iodine/potassium iodide at hour 0; Tube 2, the enzymatic reaction mixture mixed with iodine/potassium iodide after 12 h incubation at 37 °C; Tube 3, the precipitated synthetic amylose by the addition of ethanol; Tube 4, the synthetic amylose solution hydrolyzed by alpha-amylase; and Tube 5, the synthetic amylose solution hydrolyzed by alpha-amylase was mixed with iodine/potassium iodide.

mM sodium phosphate, 100 μM maltodextrin, 1 mg/mL SP, and 1 mg/mL PGP at 37 °C (Figure 2). The temperature was chosen as 37 °C because PGP, along with GI, GO, and CA, is not thermostable. TrisHCl buffer was used due to its low cost compared to the HEPES buffer used before.[16] The recombinant PGP was purified through its dockerin that can bind to the cohesin of the mini-scaffoldin, which was pulled down through the high-affinity adsorption of regenerated amorphous cellulose, as described previously.[16] The recombinant SP and PGP along with mini-scaffoldin were purified to be homogeneous in SDS-PAGE (Figure 2a, Lanes 1 and 3). At the beginning, the mixture containing sucrose and short-chain maltodextrin as a primer was colorless in the presence of iodine (Figure 2a, Tube 1). After 12 h, the solution exhibited a deep blue color in the presence of iodine, suggesting the formation of synthetic amylose (Tube 2). This synthetic amylose was precipitated by the addition of ethanol (Tube 3). The freeze-dried amylose was also confirmed by using ^{13}C NMR and FTIR (Figure S4). When the synthetic amylose solution was treated with 10 U of alpha-amylase for several hours, the solution was colorless in the presence of iodine (Tube 4). This result suggested that synthetic amylose linked by alpha-1,4-glycosidic bonds was hydrolyzed by alpha-amylase. The hydrolyzed amylose by alpha-amylase, that is, short chain maltodextrin, cannot be precipitated by the addition of ethanol (Tube 5).

In the two-enzyme (i.e., SP and PGP) system on 100 mM sucrose (i.e., 200 mM glucose equivalent), the concentrations of phosphate and two-enzyme loadings were optimized. The unit of SP used was chosen to equal that of PGP, ensuring the matching reaction rates for the sucrose phoshorylation and amylose synthesis. When 5 units of SP and PGP each was used, too low phosphate levels resulted in slow initial sucrose cleavage rates because phosphate is a substrate of SP, whereas excessive phosphate levels resulted in slow amylose synthesis rates because phosphate is an inhibitor of PGP for the amylose synthesis direction (data not shown). The optimal phosphate concentration was 50 mM. To ensure the completion of the reaction within 24 h, it was found that the enzyme loadings of SP and PGP were both 5 U/mL, and the doubled SP and PGP loading did not enhance the product yield significantly. The optimal reaction conditions were 50 mM sodium phosphate, 200 μM maltodextrin, 5 U/mL SP, and 5 U/mL PGP at 37 °C. The amylose concentration in terms of glucose equivalent in the reaction mixture reached 32.3 mM at hour 25 (Figure 3a). Because the sucrose hydrolysis utilization efficiency was 72.9% at hour 25, the amylose synthesis yield was 0.221 g of amylose per gram of sucrose. Also, 71.8 mM fructose and 41.1 mM G-1-P were coproduced in the end of the reaction.

To decrease fructose inhibition to SP, the three other enzymes (i.e., 20 U/mL of GI, 20 U/mL of GO, and 30 U/mL of CA) were added, yielding the five-enzyme system. In this system, fructose was catalyzed into glucose by GI, glucose was oxidized into gluconic acid and hydrogen peroxide by GO, and hydrogen peroxide was degraded to water and oxygen by CA. When the concentration of sucrose in the five-enzyme system decrease from 100 mM to 20.8 mM at hour 25, amylose concentration in terms of glucose equivalent reached 34.7 mM (Figure 3b). Compared to the two-enzyme system, the sucrose hydrolysis efficiency in the five-enzyme system increased from 72.9% to 79.2%, but the amylose yield was nearly unchanged,

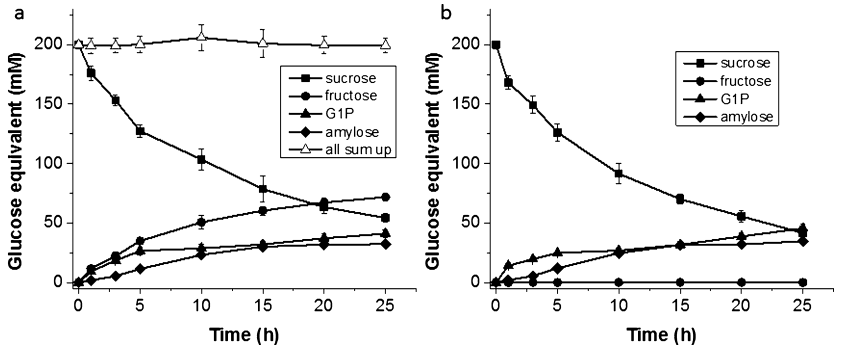


Figure 3. Concentration profiles of substrate and products by the two-enzyme mixture (a) and by the five-enzyme mixture (b).

dx.doi.org/10.1021/cs400961a | ACS Catal. 2014, 4, 1311−1317

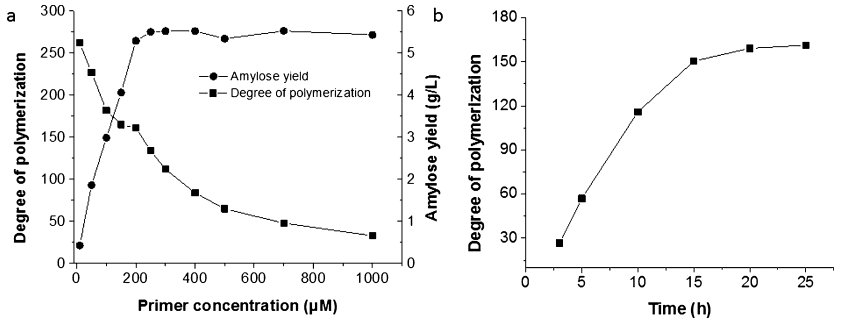


Figure 4. Number-average DP of synthetic amylose in the two-enzyme system. (a) The DP and yield of synthetic amylose in terms of maltodextrin concentration. (b) Profile of the DP of synthetic amylose under conditions (100 mM TrisHCl (pH 7.4), 100 mM sucrose, 50 mM sodium phosphate, 200 μM maltodextrin, 5 U/mL SP, and 5 U/mL PGP at 37 °C).

being 0.219 g of amylose per gram of sucrose. Although the addition of the other three enzymes increased sucrose conversion slightly, it decreased the G-1-P utilization efficiency for the synthesis of amylose (i.e., 45.5 mM G-1-P remained) possibly due to a slight negative influence from H_2O_2, a free radical, which could decrease activities and stability of the other enzymes,[50] although CA was added to remove H_2O. This result was completely different from the result that the addition of GI, GO, and CA nearly doubled amylose yield from cellobiose.[16,49] Furthermore, this SP was competitively inhibited by fructose, and the K_I value was up to 34.4 mM (Figure S2). Due to its high-value K_I, the removal of fructose was not so effective to increase yield of synthetic amylose. Therefore, the two-enzyme system could show multiple advantages over the five-enzyme system, such as fewer enzymes used and potentially better enzyme stability for a long time.

In the end of the two-enzyme system (Figure 3a), the amylose yield was 0.221 g of amylose per gram of sucrose, lower than the theoretical yield (i.e., 0.474 g of amylose per gram of sucrose) because a large amount of G-1-P was not utilized. The incomplete of the reaction was not due to the equilibrium achieved in the coupled SP/PGP reaction. The equilibrium constants for the coupled SP/PGP reaction, the sucrose cleavage catalyzed by SP only, and the amylose synthesis catalyzed by PGP only were calculated to be 120, 5.3, and 23, respectively, at pH 7.0.[20,21] We also found out that the use of tag-free PGP purified by anion-exchange chromatography resulted in a much higher amylose yield (0.346 g of amylose per gram of sucrose) than that of immobilized PGP on the surface of RAC. Therefore, low amylose yields were mainly due to the immobilized PGP, which was partially supported by the previous sucrose-to-amylose study featuring a high amylose yield.[51] The above study implied that it was important to further improve PGP stability by protein engineering and develop an easy purification approach of PGP without chromatography.

Synthesis of Different Chain Length Amylose. The effect of the initial amount of primer (maltodextrin) added in the enzyme systems on the DP of synthetic amylose was further investigated. Because the five-enzyme system did not exhibit significant advantage over the two-enzyme system in terms of the yield of amylose, the simple two-enzyme system was used. When primer concentration was changed from 10 to 1000 μM maltodextrin (Figure 4a), the number-average DP of synthetic amylose decreased from 262 to 33 (Figure 4a). When primer concentration was lower than 200 μM, the weight yield of synthetic amylose was lower, suggesting the primer was limited for the synthesis of amylose under these conditions. Also, the degree of polymerization of synthetic amylose can be controlled by the reaction time of the two-enzyme system (Figure 4b). At the primer concentration of 200 μM, the DP of synthetic amylose were 26.7 at hour 3, 57 at hour 10, 150 at hour 150, and 161 at hour 25, respectively.

The number-averaged DP of synthetic amylose from sucrose mediated by PGP was controlled by experimental conditions, such as the concentration of the primer used and reaction time (Figure 4). According to the literature,[49,52,53] synthetic amylose made from G-1-P catalyzed by PGP had a low degree of dispersity (e.g., almost one), a ratio of the weight-average molecular weight to the number-average molecular weight. Therefore, the molecular weights of synthetic amylose in this study were calculated to vary from 5370 to 42 514, with respective to the number-average degree of polymerization from 33 to 262. Here the molecular weight calculated from the number-average DP that can be measured without the polysaccharide standards could be more precise than data measured by using size-exclusion chromatography or NMR analysis, both of which heavily relied on the standard samples.

Synthetic amylose could have a variety of applications from high-value to low-value products, depending on its purity and DP. A lack of a large amount of synthetic amylose with well-controlled DPs from short to long is slowing the development of the application of this new polysaccharide. Top-quality high-DP amylose could be used as a chromatographic column matrix and a drug capsule material in the pharmaceutical industry. Amylose is also known as a high-performance material because it acts as a host molecule and forms amylose supramolecules by the inclusion of various guest molecules owing to its helical conformation.[54] High-DP amylose could be used as starting materials to make biodegradable films and sheets, which already account for half of the billion-dollar Bioplastic market.[35,36] Food-grade amylose could be mixed with cereals to make high-amylose tailored foods. This kind of food could have a low glycemic load, which may improve human health and decrease the risk of some serious diseases, such as diabetes and obesity.[37,38]

Sucrose phosphorylase is a promising biocatalyst for the glycosylation of many substrates. Previously studied mesophilic SPs lacking thermostability limited their applications. To increase their stability, the stability of mesophilic SPs can be extended by the immobilization on the solid support[29] or by protein engineering.[34] For example, the most stable SP mutant retained less than 5% of its original activity after 1 h of incubation at 70 °C.[34] The discovery of new SPs from

dx.doi.org/10.1021/cs400961a | ACS Catal. 2014, 4, 1311−1317

thermophiles in nature could be straightforward and time-saving. To our limited knowledge, this enzyme from *T. thermosaccharolyticum* JW/SL-YS485 was the first thermostable SP reported and had a half lifetime of 3 h at 70 °C.

To scale up the production of synthetic amylose, cheap sucrose is a more attractive substrate than costly cellobiose[49] or a hydrolytic product of cellulose through partial enzymatic hydrolysis by special and costly cellulase cocktails.[16] In addition to amylose, the coproduct of fructose is a useful sweetener. Because this two-enzyme system did not involve costly and labile coenzymes (e.g., NAD(P) and CoA) and ATP, this conversion might have potential industrial applications—the coproduction of synthetic amylose and fructose when both SP and PGP were stable enough.

CONCLUSIONS

Cascade enzyme reactions were designed to convert sucrose to amylose, whose DP was controllable by changing the concentration of primer and reaction time. Although the addition of the three other enzymes can mitigate fructose inhibition, the two-enzyme system had a comparative amylose yield with the five-enzyme system. This result was mainly attributed to this thermophilic enzyme featuring a high fructose dissociation constant.

ASSOCIATED CONTENT

Supporting Information

Sequence alignment of sucrose phosphorylase, reaction rate versus concentration plots, relative SP activity, and characterization of starch by ^{13}C NMR and FITR. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ypzhang@vt.edu. Fax: 001-540-231-3199. Tel.: 001-540-231-7414.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was funded by the Biological Systems Engineering Department of Virginia Tech. This study was partially supported by the DOE SBIR/STTR I award (DE-SC0009659TDD) and NSF STTR I award (IIP-1321528) to Y.P.Z.

REFERENCES

(1) Rollin, J. A.; Tam, W.; Zhang, Y.-H. P. *Green Chem.* **2013**, *15*, 1708−1719.
(2) Bornscheuer, U. T.; Huisman, G. W.; Kazlauskas, R. J.; Lutz, S.; Moore, J. C.; Robins, K. *Nature* **2012**, *485*, 185−194.
(3) Hummel, W. *Trends Biotechnol.* **1999**, *17*, 487−492.
(4) Wichmann, R.; Vasic-Racki, D. *Adv. Biochem. Eng. Biotechnol.* **2005**, *92*, 225−260.
(5) Shaeri, J.; Wright, I.; Rathbone, E. B.; Wohlgemuth, R.; Woodley, J. M. *Biotechnol. Bioeng.* **2008**, *101*, 761−767.
(6) Meyer, A.; Pellaux, R.; Panke, S. *Curr. Opin. Microbiol.* **2007**, *10*, 246−253.
(7) Chi, Y. G.; Scroggins, S. T.; Frechet, J. M. J. *J. Am. Chem. Soc.* **2008**, *130*, 6322−6323.
(8) Carlson, E. D.; Gan, R.; Hodgman, C. E.; Jewett, M. C. *Biotechnol. Adv.* **2012**, *30*, 1185−1194.
(9) Martín del Campo, J. S.; Rollin, J.; Myung, S.; Chun, Y.; Chandrayan, S.; Patiño, R.; Adams, M. W. W.; Zhang, Y.-H. P. *Angew. Chem., Int. Ed.* **2013**, *52*, 4587−4590.
(10) Zhang, Y.-H. P.; Evans, B. R.; Mielenz, J. R.; Hopkins, R. C.; Adams, M. W. W. *PLoS One* **2007**, *2*, e456.
(11) Guterl, J.; Garbe, D.; Carsten, J.; Steffler, F.; Sommer, B.; Reiße, S.; Philipp, A.; Haack, M.; Rühmann, B.; Kettling, U.; Brück, T.; Sieber, V. *ChemSusChem.* **2012**, *5*, 2165−2172.
(12) Krutsakorn, B.; Honda, K.; Ye, X. T.; Imagawa, T.; Bei, X. Y.; Okano, K.; Ohtake, H. *Metab. Eng.* **2013**, *20*, 84−91.
(13) Xu, S.; Minteer, S. D. *ACS Catal.* **2011**, *1*, 91−94.
(14) Zhu, Z. G.; Sun, F. F.; Zhang, X. Z.; Zhang, Y.-H. P. *Biosens. Bioelectron.* **2012**, *36*, 110−115.
(15) Zhu, Z.; Tam, T. K.; Sun, F.; You, C.; Zhang, Y.-H. P. *Nat. Commun.* **2014**, *5*, 3026.
(16) You, C.; Chen, H.; Myung, S.; Sathitsuksanoh, N.; Ma, H.; Zhang, X. Z.; Li, J. Y.; Zhang, Y.-H. P. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 7182−7187.
(17) United States Department of Agriculture: Foreign Agricultural Service. http://usda01.library.cornell.edu/usda/fas/sugar//2010s/2011/sugar-11-17-2011.pdf 2011.
(18) IndexMundi—Country Facts. http://www.indexmundi.com/commodities/?commodity=sugar&months=120 (2013).
(19) Luley-Goedl, C.; Nidetzky, B. *Biotechnol. J.* **2010**, *5*, 1324−1338.
(20) eQuilibrator—Biochemical Thermodynamics Calculator. http://equilibrator.weizmann.ac.il/ (2013).
(21) Alberty, R. A. *Biochemical Thermodynamics: Applications of Mathematica*; Wiley: Hoboken, NJ, 2006.
(22) Kim, M.; Kwon, T.; Lee, H. J.; Kim, K. H.; Chung, D. K.; Ji, G. E.; Byeon, E. S.; Lee, J. H. *Biotechnol. Lett.* **2003**, *25*, 1211−1217.
(23) Russell, R. R.; Mukasa, H.; Shimamura, A.; Ferretti, J. J. *Infect. Immun.* **1988**, *56*, 2763−2765.
(24) Kawasaki, H.; Nakamura, N.; Ohmori, M.; Sakai, T. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 322−324.
(25) Lee, J. H.; Moon, Y. H.; Kim, N.; Kim, Y. M.; Kang, H. K.; Jung, J. Y.; Abada, E.; Kang, S. S.; Kim, D. *Biotechnol. Lett.* **2008**, *30*, 749−54.
(26) Trethewey, R. N.; Fernie, A. R.; Bachmann, A.; Fleischer-Notter, H.; Geigenberger, P.; Willmitzer, L. *Plant, Cell Environ.* **2001**, *24*, 357−365.
(27) Henrissat, B. *Biochem. J.* **1991**, *280*, 309−316.
(28) Goedl, C.; Nidetzky, B. *ChemBioChem* **2009**, *10*, 2333−2337.
(29) Goedl, C.; Schwarz, A.; Minani, A.; Nidetzky, B. *J. Biotechnol.* **2007**, *129*, 77−86.
(30) Kitao, S.; Matsudo, T.; Sasaki, T.; Koga, T.; Kawamura, M. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 134−141.
(31) Sawangwan, T.; Goedl, C.; Nidetzky, B. *Org. Biomol. Chem.* **2009**, *7*, 4267−4270.
(32) Goedl, C.; Sawangwan, T.; Mueller, M.; Schwarz, A.; Nidetzky, B. *Angew. Chem., Int. Ed.* **2008**, *47*, 10086−10089.
(33) Haki, G. D.; Rakshit, S. K. *Bioresour. Technol.* **2003**, *89*, 17−34.
(34) Cerdobbel, A.; De Winter, K.; Aerts, D.; Kuipers, R.; Joosten, H. J.; Soetaert, W.; Desmet, T. *Protein Eng., Des. Sel.* **2011**, *24*, 829−834.
(35) van Soest, J. J. G.; Vliegenthart, J. F. G. *Trends Biotechnol.* **1997**, *15*, 208−213.
(36) Frische, R.; Wollmann, K.; Gross-Lannert, R.; Schneider, J.; Best, B. Special Amyloses and Their Use for Producing Biodegradable Plastics. U.S. Patent No.5,374,304, December 20, 1994.
(37) Regina, A.; Bird, A.; Topping, D.; Bowden, S.; Freeman, J.; Barsby, T.; Kosar-Hashemi, B.; Li, Z. Y.; Rahman, S.; Morell, M. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3546−3551.
(38) Maki, K. C.; Pelkman, C. L.; Finocchiaro, E. T.; Kelley, K. M.; Lawless, A. L.; Schild, A. L.; Rains, T. M. *J. Nutr.* **2012**, *142*, 717−723.
(39) Zhang, Y.-H. P.; Huang, W. D. *Trends Biotechnol.* **2012**, *30*, 301−306.
(40) Zhang, Y.-H. P. *Energy Environ. Sci.* **2009**, *2*, 272−282.
(41) Ye, X.; Zhu, Z.; Zhang, C.; Zhang, Y.-H. P. *Appl. Microbiol. Biotechnol.* **2011**, *92*, 551−560.
(42) Currie, D.; Herring, C.; Guss, A.; Olson, D.; Hogsett, D.; Lynd, L. R. *Biotechnol. Biofuels* **2013**, *6*, 32.

(43) Studier, F. W. *Protein Expression Purif.* **2005**, *41*, 207−234.
(44) You, C.; Zhang, X. Z.; Zhang, Y.-H. P. *Appl. Environ. Microbiol.* **2012**, *78*, 1593−1595.
(45) Wang, Y.; Huang, W.; Sathitsuksanoh, N.; Zhu, Z.; Zhang, Y.-H. P. *Chem. Biol.* **2011**, *18*, 372−380.
(46) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−254.
(47) Wang, Y.; Zhang, Y.-H. P. *J. Appl. Microbiol.* **2010**, *108*, 39−46.
(48) Zhang, Y.-H. P.; Lynd, L. R. *Biomacromolecules* **2005**, *6*, 1510−1515.
(49) Ohdan, K.; Fujii, K.; Yanase, M.; Takaha, T.; Kuriki, T. *J. Biotechnol.* **2007**, *127*, 496−502.
(50) Mishra, S.; Imlay, J. *Arch. Biochem. Biophys.* **2012**, *525*, 145−160.
(51) Waldmann, H.; Gygax, D.; Bednarski, M. D.; Shangraw, W. R.; Whitesides, G. M. *Carbohydr. Res.* **1986**, *157*, c4−c7.
(52) Kadokawa, J.-I. *Chem. Rev.* **2011**, *111*, 4308−4345.
(53) Ohdan, K.; Fujii, K.; Yanase, M.; Takaha, T.; Kuriki, T. *Biocatal. Biotransform.* **2006**, *24*, 77−81.
(54) Putseys, J. A.; Lamberts, L.; Delcour, J. A. *J. Cereal Sci.* **2010**, *51*, 238−247.