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Enzymatic Synthesis and Properties of Highly Branched Rice Starch Amylose and Amylopectin Cluster

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We enzymatically modified rice starch to produce highly branched amylopectin and amylose and analyzed the resulting structural changes. To prepare the highly branched amylopectin cluster (HBAPC), we first treated waxy rice starch with *Thermus scotoductus* α-glucanotransferase (TSαGT), followed by treatment with *Bacillus stearothermophilus* maltogenic amylase (BSMA). Highly branched amylose (HBA) was prepared by incubating amylose with *Bacillus subtilis* 168 branching enzyme (BBE) and subsequently treating it with BSMA. The molecular weight of TSαGT-treated waxy rice starch was reduced from 8.9×10^8 to 1.2×10^5 Da, indicating that the α-1,4 glucosidic linkage of the segment between amylopectin clusters was hydrolyzed. Analysis of the amylopectin cluster side chains revealed that a rearrangement in the side-chain length distribution occurred. Furthermore, HBAPC and HBA were found to contain significant numbers of branched maltooligosaccharide side chains. In short, amylopectin molecules of waxy rice starch were hydrolyzed into amylopectin clusters by TSαGT in the enzymatic modification process, and then further branched by transglycosylation using BSMA. HBAPC and HBA showed higher water solubility and stability against retrogradation than amylopectin clusters or branched amylose. The hydrolysis rates of HBAPC and HBA by glucoamylase and α-amylase greatly decreased. The k_{cat}/K_m value of glucoamylase acting on the amylopectin cluster was 45.94 $s^{-1}(mg/mL)^{-1}$ and that for glucoamylase acting on HBAPC was 11.10 $s^{-1}(mg/mL)^{-1}$, indicating that HBAPC was 4-fold less susceptible to glucoamylase. The k_{cat}/K_m value for HBA was 15.90 $s^{-1}(mg/mL)^{-1}$, or about three times less than that for branched amylose. The k_{cat}/K_m values of porcine pancreatic α-amylase for HBAPC and HBA were 496 and 588 $s^{-1}(mg/mL)^{-1}$, respectively, indicating that HBA and HBAPC are less susceptible to hydrolysis by glucoamylase and α-amylase. HBAPC and HBA show potential as novel glucan polymers with low digestibility and high water solubility.

KEYWORDS: Amylopectin cluster (APC); *Bacillus stearothermophilus* maltogenic amylase (BSMA); *Bacillus subtilis* 168 branching enzyme (BBE); branched amylose (BA); highly branched amylopectin cluster (HBAPC); highly branched amylose (HBA); *Thermus scotoductus* α-glucanotransferase (TSαGT)

INTRODUCTION

Although carbohydrates are the major energy source for living organisms, excessive carbohydrate intake may result in lifestyle diseases such as obesity and diabetes (*1, 2*). This problem may be ameliorated by changing the structure of starch to delay its digestion in the small intestine and prevent a surge in the blood glucose level. A number of physical, chemical, and enzymatic methods have been used to modify the structure of amylose and amylopectin in starch. Physical and chemical methods may produce unexpected byproducts in the process; in contrast, the enzymatic method offers high substrate selectivity and product specificity and better control of the biochemical reactions than chemical or physical methods.

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Enzymatic modification of starch usually involves hydrolysis and transglycosylation by carbohydrate enzymes. Several enzymes are highly useful for starch enzymatic modification. For example, 4-α-glucanotransferase catalyzes the transfer of an α-glucan chain from one α-glucan molecule to another (*3–5*). This intermolecular glucan transfer reaction, called disproportionation, is readily reversible. Such enzymes can catalyze intramolecular glucan transfer reactions within a single linear glucan molecule, as well as intermolecular disproportionation transfers (*6–9*). 4-α-Glucanotransferase from *Thermus scotoductus* (TSαGT), used in this study, shows maximum activity at pH 7.0 and 75 °C. It also exhibits high thermal stability, retaining 90% of its activity after 120 min of incubation at 80 °C (*10*). Branching enzyme (BE, 1,4-α-glucan, 1,4-α-glucan 6-glucosyltransferase; EC 2.4.1.18), which is also widely used to enzymatically modify starch, catalyzes the formation of α-1,6 branching points. Incubation with BE converts amylose into branched glucans containing cyclic forms (*11*). A third enzyme is maltogenic amylase, a glycoside hydrolase present in various Gram-positive bacteria, which hydrolyzes cyclodextrins (CDs) and starch mainly to maltose, and pullulan to panose, by the cleavage of α-1,4-glycosidic bonds. It also exhibits high transglycosylation activity via the formation of various glycosidic linkages, such as α-1,6- and α-1,3-linkages, which produce branched oligosaccharides from liquefied starch (*12*). Recent research on maltogenic amylase suggests that it produces branched side chains when reacted with amylopectin clusters (*13*).

We isolated the gene-encoding branching enzyme (BBE) from *Bacillus subtilis* 168 to investigate the action of BBE on amylose. Then, we produced the modified starches amylopectin cluster (APC), highly branching amylopectin cluster (HBAPC), branched amylose (BA), and highly branched amylose (HBA) using BBE, TSαGT, and *Bacillus stearothermophilus* maltogenic amylase (BSMA), and characterized their structural and functional properties.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *Bacillus subtilis* ISW1214 [hsrM1, leuA8, metB5, tets] (*14*), purchased from Takara Shuzo (Tokyo, Japan), was used as a recipient strain for the transformation. *B. subtilis* transformants were cultivated aerobically at 37 °C in A medium (pH 7.5), which contained 3.3% Bacto tryptone, 2% Bacto yeast extract, 0.74% NaCl, 0.8% Na_2HPO_4, 0.4% KH_2PO_4, 2% casamino acids, and 0.06 mM $MnCl_2$. The pLIP DNA vector used for *Bacillus* expression and secretion was purchased from BioLeaders Co. (Daejeon, Korea).

Enzymes and Reagents. α-Amylase from porcine pancreas (A-4268) and glucoamylase from *Aspergillus niger* (10115) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Isoamylase isolated from *Pseudomonas amyloderamosa* was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan). Amylopectin from waxy rice starch (Japonica) was prepared following the alkali method reported by Yamamoto et al. (*15*). Amylose was extracted from rice starch (Sigma) using the Montgomery and Senti method (*16*). All other chemicals were of analytical grade.

Overexpression and Purification of Enzymes. Transformation of *Bacillus subtilis* was carried out using a slight modification of the procedure of Dubnau (*17*) as follows. A single colony of *Bacillus subtilis* ISW1214 was precultured in 5 mL of LB medium at 37 °C for 12 h, transferred into fresh SPI medium, and cultured at 37 °C for 90 min with shaking. Then, 50 μL of the culture solution and 50 μL of the plasmid solution (10–100 ng) were mixed and shaken at 37 °C. After 30 min, 100 mL of LB medium were added, and the mixture was shaken at 37 °C for 60 min. The transformed cells were spread on LB agar medium containing kanamycin (100 μg/mL). Each *B. subtilis* cell carrying the BSMA (*18*), TSαGT (*10*), or BBE gene was cultivated in A medium containing kanamycin (100 μg/mL) at 37 °C for 22 h. Because the recombinant proteins each contained a six-histidine tag, they were purified using nickel−nitrilotriacetic acid (Ni−NTA) columns (1 × 4 cm, Qiagen, Hilden, Germany) (*19*). The molecular masses of the purified proteins were estimated by SDS-PAGE analysis (*20*), and each showed a single band.

BBE Activity Assay. BBE activity was analyzed following the procedure of Takata et al. (*21*). The enzyme (50 μL) and 0.05% amylose solution (50 μL) were mixed and incubated at 30 °C for 20 min. The reaction was terminated by addition of 1 mL of iodine reagent, which was made fresh daily by dilution of 0.5 mL of iodine stock solution (0.26 g I_2 and 2.6 g KI in 10 mL water) in 130 mL with distilled water. The absorbance at 660 nm was measured immediately with a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). One BE unit was arbitrarily defined as the amount of enzyme that degraded 1 μg/mL amylose per min under the above conditions.

TSαGT Activity Assay. TSαGT activity was determined by measuring the change in blue color absorbance resulting from iodine staining during the conversion of amylose by the enzyme (*22*). Each enzyme reaction mixture contained 250 μL of 0.2% amylose, 50 μL of 1% maltose, 600 μL of 50 mM Tris−HCl buffer (pH 7.5), and 100 μL of enzyme solution. The mixture was incubated at 75 °C for 10 min. The reaction was stopped by boiling for 10 min. Aliquots (0.1 mL) were mixed with 1 mL of iodine solution (0.02% I_2 and 0.2% KI), and the absorbance of the reaction mixture was measured immediately at 620 nm using a spectrophotometer. One unit of TSαGT was arbitrarily defined as the amount of enzyme that degraded 1 mg/mL of amylose per min under the conditions described above.

BSMA Activity Assay. BSMA activity was assayed according to the dinitrosalicylic acid (DNS) method (*23*), by determination of the amount of reducing sugars produced by the enzyme. Each enzyme reaction mixture was composed of 150 μL of 1% soluble starch in 50 mM sodium citrate buffer (pH 6.0), 120 μL of reaction buffer (50 mM sodium citrate buffer, pH 6.0), and 30 μL of enzyme solution. The reaction mixture was preincubated at 50 °C for 5 min before the diluted enzyme solution was added and incubation was continued for another 10 min. The reaction was terminated by addition of 750 μL of DNS solution and boiling for 5 min. Absorbance was measured at 575 nm using a spectrophotometer. One unit of BSMA activity was defined as the amount of enzyme that produced 1 mmol of reducing sugars in 1 min under the reaction conditions.

Kinetic Studies of Glucoamylase and α-Amylase in HBAPC and HBA Hydrolysis. Glucoamylase activity was assayed using the glucose oxidase/peroxidase (GOD-POD) method (*24*). The substrate solutions, composed of 200 μL of various concentrations of APC, HBAPC, BA, or HBA substrates in 50 mM sodium acetate buffer (pH 4.5), were prewarmed at 50 °C for 5 min. The enzyme solution was then added to the substrate solution, and aliquots (20 μl) of the reaction mixture were collected every 30 s for 2 min. The reaction was stopped by addition of an equal volume of 0.1 N NaOH and 360 μL of glucose determination reagent (Glucose-E; Asian Pharmaceutical, Seoul, Korea).

α-Amylase activity was measured using the copper-bicinchoninate reducing-value method with a microplate reader (*25*). Solutions of various concentrations of waxy rice starch, BA, HBA, APC, or HBAPC were prepared in 20 mM sodium phosphate buffer containing 6 mM NaCl (pH 6.9). The reaction mixture was prewarmed at 30 °C for 5 min; enzyme solution was added to the substrate solution, and 100-μL aliquots of the enzyme digest were collected at various time intervals. The reaction was stopped by addition of 100 μL of 0.1 N NaOH. After the addition of the copper-bicinchoninate reagent (200 μL) to each reactant, the solutions were heated at 85 °C for 35 min and then cooled at 4 °C for 10 min. Aliquots (0.3 mL) were transferred to microplate wells, and the absorbances were measured using a microplate reader (EL340, Biokinetics Reader, BioTek Instruments, Inc.). The kinetic parameters were analyzed using a Lineweaver–Burk plot.

HBA and HBAPC Production. A reaction mixture (400 mL) containing 0.5% amylose extracted from rice starch (Sigma), 50 mM Tris−HCl (pH 7.5), and 22.5% dimethyl sulfoxide was heated to 100 °C for 30 min. After the mixture was cooled to 30 °C, BE (5000 U/g substrate) was added to the amylose paste. The reaction was carried out at 30 °C for 6 h and stopped by heating in boiling water for 30

min, producing BA. After the insoluble materials were removed by centrifugation, the solution was precipitated by addition of two volumes of ethanol. The precipitate was redissolved in 40 mL of 50 mM sodium citrate (pH 6.0) and heated to 100 °C with mixing for 30 min. The solution was cooled to 55 °C, and BSMA (500 units/g substrate) was added to the paste. The reaction was carried out at 55 °C for 12 h and stopped by heating in boiling water for 30 min, producing HBA.

Next, 200 mL of 5% waxy rice starch solution containing 50 mM sodium phosphate buffer (pH 6.5) was gelatinized at 100 °C for 1 h. After the paste cooled to 75 °C, 1000 units of TSαGT (100 units/g substrate) were added. The reaction was carried out at 75 °C for 30 min and was stopped by heating in boiling water for 1 h, producing APC. After the paste had cooled to 55 °C, 1000 units of BSMA (100 units/g substrate) was added. This reaction was performed at 55 °C for 4 h and was stopped by heating in boiling water for 30 min, producing HBAPC. Insoluble materials were removed by centrifugation.

Each product was precipitated by addition of two volumes of ethanol, and the pellet was washed in 66% ethanol two more times. The precipitates were frozen at –70 °C for 5 h and freeze-dried (FD-5508, Freeze-Dryer, Ilsin Engineering Co., Seoul, Korea) for 12 h.

Thin-Layer Chromatography Analysis. A silica-gel K5F thin-layer chromatography (TLC) plate (Whatman, Brentford, UK) was activated by placing it in a 110 °C oven for 1 h. Samples were then spotted on the plate and developed twice in a TLC chamber containing a solvent mixture of isopropyl alcohol/ethyl acetate/water (3:1:1 v/v/v) at room temperature.

The plate was dried thoroughly and developed by dipping it rapidly into a methanol solution containing 3 g of *N*-(1-naphthyl)-ethylenediamine and 50 mL of concentrated H_2SO_4 per liter. The plate was dried and placed in an oven at 110 °C for 10 min until blue-black spots appeared on the white background.

To detect maltooligosaccharides labeled with ^{14}C at the reducing end, the plate was placed in a cassette and covered with an imaging plate for 10 h. The radioactivity detected by the imaging plate was measured using a Fuji Film BAS2500 image analyzer (Tokyo, Japan).

High-Performance Anion-Exchange Chromatography Analysis. To analyze the branched chain length distribution of the HBA and HBAPC starch samples, the samples were debranched by incubation with isoamylase in 25 mM sodium acetate (pH 4.3) at 60 °C for 60 h. The reaction was stopped by boiling for 5 min, and the branched chain length distribution was analyzed using a high-performance anion-exchange chromatography (HPAEC) system (Dionex-300, Dionex, Sunnyvale, CA,) coupled with an electrochemical detector (ED40, Dionex). A CarboPacTM PA-1 anion-exchange column (250 × 4 mm, Dionex) and a guard column were used to separate the debranched samples. After the column was equilibrated with 150 mM NaOH, the sample was eluted with multiple gradients of 600 mM sodium acetate in 150 mM NaOH at a flow rate of 1 mL/min. The linear sodium acetate gradients were as follows: 10–30% for 0–10 min, 30–40% for 10–16 min, 40–50% for 16–27 min, 50–60% for 27–44 min, and 60–64% for 44–60 min.

Size-Exclusion Chromatography-Multiangle Laser Light Scattering-Refractive Index Analysis. The molecular-weight distribution of the modified rice starch was analyzed using multiangle laser light scattering (MALLS; Dawn DSP, Wyatt Technology, Santa Barbara, CA) and a refractive index (RI) detector (Waters 410) coupled with size-exclusion chromatography (SEC). SUGAR KS-804 and KS-806 columns (8 × 300 mm; Shodex, Kawasaki, Japan) were connected in tandem and equilibrated at room temperature. The flow rate of the mobile phase (0.15 M $NaNO_3$ containing 0.02% NaN3) was 0.4 mL/min. The powdered sample was redissolved in the mobile phase, and the solution was autoclaved (121 °C) for 20 min. The sample was filtered through a 5-μm disposable syringe filter and injected into the SEC-MALLS-RI system. The weight-average molecular weight (M_w) of the sample was calculated using ASTRA, version 4.90.07, software (Wyatt Technology) with the Berry extrapolation method for curve fitting and a d*n*/d*c* value of 0.146 mL/g.

Differential Scanning Calorimetry (DSC) Analysis. The reference was 10 mg of distilled water. The samples (2.5 mg of product in 7.5 mg of DDW) were weighed and hermetically sealed in aluminum pans, which were then heated from 10 to 150 °C at the rate of 5 °C/min. The degree of retrogradation was expressed as the enthalpy calculated from the area of the endothermic peak between 30 and 80 °C (*26, 27*).

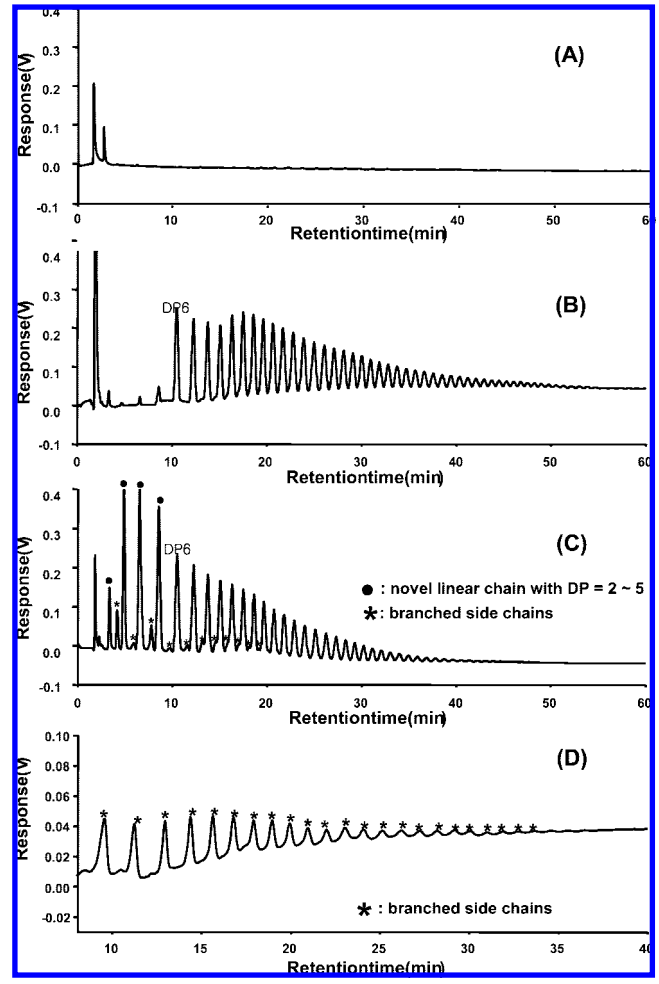


Figure 1. HPAEC analysis of side-chain distributions of highly branched amylose formed from amylose (**A**) using BBE (**B**), BSMA (**C**), or HBA after β-amylase treatment (**D**).

Solubility Measurement. BA, HBA, APC, and HBAPC were mixed with 250 μL of distilled water in Eppendorf tubes at 25 °C. After they were vortexed at room temperature for 2 h, the samples were saturated. The samples were centrifuged at 12 000 rpm for 1 h, after which the supernatants were diluted with 25 mM sodium acetate buffer (pH 4.5). Amyloglucosidase (0.5 U/mg) was added, and the mixtures were incubated at 60 °C for 16 h. The amyloglucosidase was inactivated by incubation of the tubes in a boiling water bath for 15 min. The amount of dissolved glucans was measured following the procedure of Brunt et al. (*28*).

RESULTS AND DISCUSSION

Preparation of Highly Branched Rice Amylose. Rice amylose was incubated with BBE, and the product was hydrolyzed using isoamylase. Side-chain analysis of the HPAEC product revealed that various glucans with side chains from DP6 to DP30 were produced, suggesting that the rice amylose (**Figure 1A**) was successfully transformed into branched glucan mixtures (**Figure 1B**). To obtain highly branched amylose, the branched amylose was further incubated with BSMA. Analysis of the side chains of the product by HPAEC revealed that the number of short chains ranging from DP2 to DP5 increased, whereas that of long chains longer than DP9 decreased. BSMA preferentially hydrolyzed longer branched side chains of branched amylose, yielding, as expected, predominantly carbohydrates

Table 1. Physicochemical Properties of Branched Amylose (BA), Highly Branched Amylose (HBA), Amylopectin Cluster (APC), and Highly Branched Amylopectin Cluster (HBAPC)

product	solubility in water (mg/mL)	av molecular weight (g/mol)	av chain length (DP)	branched side chain (%)
waxy rice starch	[a]	8.9×10^8	18.6	[a]
BA	414 (±5.2)	9.9×10^6	16.2	[a]
HBA	435 (±4.5)	5.9×10^6	10.2	7
APC	455 (±10.2)	1.2×10^5	18.6	[a]
HBAPC	565 (±12.1)	5.8×10^4	9.5	36

[a] Not determined.

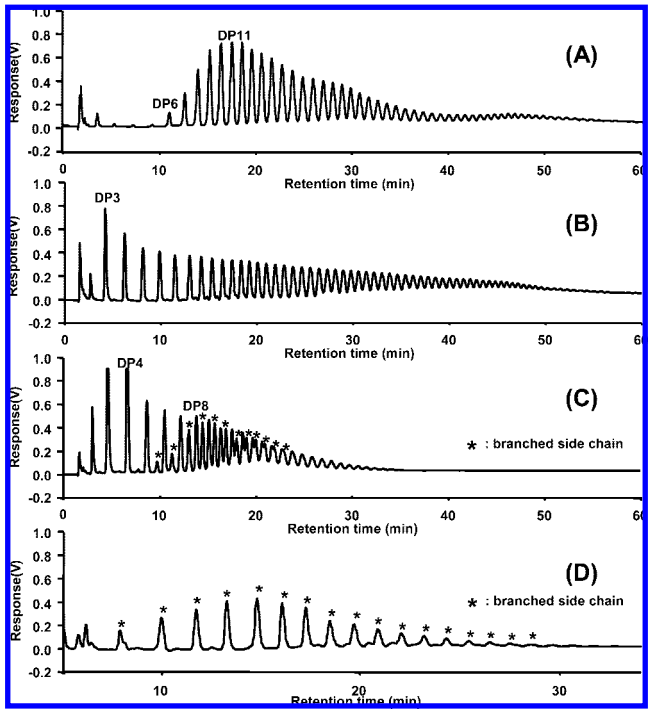


Figure 2. HPAEC analysis of side-chain distributions of highly branched amylopectin cluster formed from waxy rice starch (**A**) with TSαGT (**B**), BSMA (**C**), or HBAPC after α-amylase treatment (**D**).

with shorter side chains (**Figure 1C**). Interestingly, some novel peaks lacking branched amylose appeared after the BSMA treatment. The liquid chromatography analysis also revealed several amylose products that contained no branched sugar moiety. To identify the characteristics of the novel compounds, the debranched side chains (**Figure 1C**) were incubated with β-amylase. The HPAEC chromatogram showed that the peaks remained after β-amylase treatment (**Figure 1D**), indicating that the compounds were branched oligosaccharides. Thus, branched side-chain formation can be explained by the transfer by BSMA of small oligosaccharide moieties, such as glucose and maltose, from long side chains to the glycosyl residue of the nonreducing side chain ends in branched amylose, via the formation of α-1,6-glycosidic linkages, thereby producing highly branched amylose.

The production yield, estimated on the basis of HBA weight, was approximately 55%. The branched side chains in HBA comprised about 7%, estimated from the peak area produced in the HBAEC analysis.

Molecular weights were monitored during enzyme treatment using the SEC-MALLS-RI system. The average molecular weights of BA and HBA were approximately 9.9×10^6 Da and 5.9×10^6 Da, respectively, both greater than the molecular weight of amylose (**Table 1**).

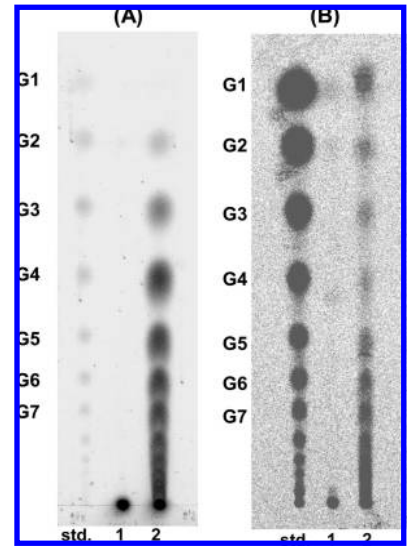


Figure 3. TLC analysis of hydrolysis products generated by isoamylase from HBAPC containing a ^{14}C-labeled glucosyl moiety. The chromatogram (**A**) was visualized using the naphtol−H_2SO_4 method and (**B**) autoradiography: lane 1, HBAPC; lane 2, HBAPC after isoamylase treatment.

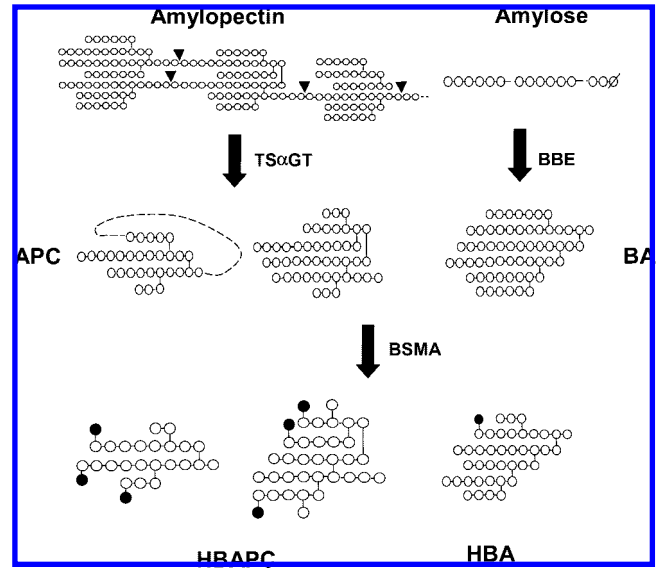


Figure 4. Schematic diagram of structural changes in amylopectin and amylose during enzyme treatment: APC, amylopectin cluster; BA, branched amylose; HBAPC, highly branched amylopectin cluster; HBA, highly branched amylose.

Preparation of Highly Branched Rice Amylopectin Cluster. To obtain amylopectin clusters, we reacted waxy rice starch with TSαGT and determined the molecular weight of the product using SEC-MALLS-RI. As shown in **Table 1**, the average molecular weight of the reaction products decreased from 8.9×10^8 to 1.2×10^5 Da, indicating that amylopectin was degraded into cluster units by the cleavage of the interchain segment between clusters by TSαGT. As shown **Figure 2**, BSMA treatment increased the number of branched side chains in modified amylopectin clusters. HPAEC analysis suggested that a 36% increase in branched side chains occurred. BSMA treatment decreased the molecular weight slightly, to 5.8×10^4 Da (**Table 1**). The yield of HBAPC formed from APC with BSMA was estimated at approximately 39%.

Mode of Action of BSMA in the Formation of Branched Side Chains. BSMA transglycosylation activity can also

Table 2. Kinetic Parameters of Hydrolysis by Glucoamylase and Porcine Pancreatic α-Amylase

enzyme	substrate	K_m (mg/mL)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot(mg/mL)^{-1}$)
glucoamylase (*Aspergillus niger*)	waxy rice starch	1.48 (±0.13)	74.85 (±1.88)	50.02 (±4.80)
	APC	1.14 (±0.31)	52.42 (±3.82)	45.94 (±3.34)
	HBAPC	4.15 (±0.19)	45.93 (±2.42)	11.10 (±1.11)
	BA	1.18 (±0.05)	50.85 (±0.86)	43.04 (±1.39)
	HBA	2.54 (±0.08)	40.41 (±0.95)	15.90 (±0.16)
α-amylase (porcine pancreas)	waxy rice starch	0.07 (±0.02)	264.7 (±22.5)	3775 (±324)
	APC	0.08 (±0.02)	242.5 (±15.4)	2964 (±254)
	HBAPC	0.24 (±0.04)	119.5 (±6.4)	496 (±96)
	BA	0.16 (±0.03)	376.3 (±23.0)	2343 (±264)
	HBA	0.40 (±0.01)	235.0 (±12.8)	588 (±14)

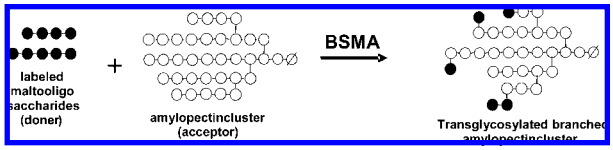
Table 3. Endothermic Characteristics of Products Stored at 4 °C for 20 Days

	To	Tp	Tc	Tc−To	Δ*H* (J/g)
waxy rice starch	36.69	46.94	56.74	20.05	1.6
APC	39.05	57.91	69.10	30.05	1.3
HBAPC	[a]	[a]	[a]	[a]	[a]
BA	40.50	59.82	68.31	27.81	0.6
HBA	[a]	[a]	[a]	[a]	[a]

[a] Not detected.

produce branched side chains, with BSMA catalyzing maltooligosaccharides as donors and amylopectin clusters as acceptors.

To elucidate the mode of action for the formation of branched side chains, we used maltooligosaccharides labeled with ^{14}C as donors and amylopectin clusters as acceptors. After the reaction, two volumes of ethanol were added; the mixture was centrifuged, and the resulting pellet was washed two times with 66% ethanol to remove small oligosaccharides. The precipitate was incubated with an excess of isoamylase to debranch the side chains containing ^{14}C-labeled glucose. As shown in the TLC radiogram (**Figure 3B**, lane 2), labeled glucose was detected in the fraction of debranched HBAPC side chains. The formation of ^{14}C-labeled glucose provided evidence that BSMA transferred the sugar moiety of ^{14}C-labeled maltooligosaccharides to amylopectin cluster *via* a transglycosylation reaction, as shown in eq 1.



(1)

On the basis of the conclusion drawn from the experiments, we proposed a model for modification of amylopectin and amylose by BBE, TSαGT, and BSMA as shown in **Figure 4**.

Hydrolysis Kinetics of Highly Branched Products Using Glucoamylase. The hydrolysis kinetics of the transfer products were studied using *Aspergillus niger* glucoamylase to investigate the digestibility of highly branched glucans. **Table 2** lists the K_m and k_{cat} values for HBA and HBAPC. The K_m values for APC and HBAPC were 1.14 and 4.15 mg/mL, respectively, indicating that the binding affinity of highly branched products was greatly decreased. The HBAPC k_{cat}/K_m value was 11.2 mL/s·mg, whereas that of APC was 45.04 mL/s·mg, indicating that highly branched glucans are less susceptible to glucoamylase hydrolysis.

Hydrolysis Kinetics of Highly Branched Products Using α-Amylase. To evaluate the digestibility of highly branched products, we studied branched product hydrolysis kinetics using porcine pancreatic α-amylase (PPA). The k_{cat}/K_m value of APC was 2964 mL/s·mg, slightly lower than that of waxy rice starch (3775 mL/s·mg). The K_m values for HBAPC and HBA were three times higher than the APC and BA values. The k_{cat}/K_m value of HBAPC was approximately one-sixth that of APC and one-eighth that of waxy rice starch, perhaps because of the increased K_m value. The kinetic data indicate that the highly branched glucans HBAPC and HBA were less susceptible to PPA hydrolysis. After BSMA treatment, small side chains (DP1−DP5) became the major compounds (**Figures 1** and **2**). Since PPA has a substrate preference for side chains greater than DP4 (*29, 30*), highly branched glucans were not easily hydrolyzed by PPA.

The modified starch products are expected to be poorly hydrolyzed by the digestive enzymes present in the small intestine. However, the biochemical data in vitro can be different from reality depending on many other factors that should be taken into consideration in vivo. Furthermore, the examination of the potential toxicity and undesirable effects of the branched amylose and amylopectin product would be necessary for specified health uses.

Retrogradation Rate of Highly Branched Glucans. The degradation rate of branched products was monitored using DSC, with the increase in the area of the first endothermic peak (*31*) representing the degree of retrogradation.

BE and BSMA treatment significantly retarded rice starch retrogradation (**Table 3**). During the 20-day storage period at 4 °C, no endothermic enthalpy, indicating the retrogradation of the highly branched glucans HBAPC and HBA, was detected by DSC. This suggests that highly branched glucans are only slightly retrogradable. Retrogradation properties are governed by the amylopectin side chain length (*32*). The average HBAPC chain length decreased from 18.6 to 9.5 (**Table 1**), possibly affecting the retrogradation rate of the highly branched product. Thus, the molecular weight and distribution of side chains of different lengths may be related to the physicochemical properties of starch.

Solubility of Highly Branched Products. The solubility of each sample was measured by determination of the total soluble glucose at saturation. The glucose content was analyzed using the GOD−POD method after the products were hydrolyzed to glucose units by amyloglucosidase and isoamylase. As shown in **Table 1**, HBA, APC, and BA did not differ in water solubility. However, the solubility of HBAPC significantly increased, compared to that of HBA, APC, and BA. Thus, highly branched glucans may have highly desirable applications in the beverage industry.

The low digestibility and high solubility of HBAPC and HBA mark them as potential candidates for new functionalities with low digestibility and ergogenic effects.

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