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Amylolytically-resistant tapioca starch modified by combined treatment of branching enzyme and maltogenic amylase

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ABSTRACT

Tapioca starch was modified using branching enzyme (BE) isolated from *Bacillus subtilis* 168 and *Bacillus stearothermophilus* maltogenic amylase (BSMA), and their molecular fine structure and susceptibility to amylolytic enzymes were investigated. By BE treatment, the molecular weight decreased from 3.1×10^8 to 1.7×10^6, the number of shorter branch chains (DP 6–12) increased, the number of longer branch chains (DP >25) decreased, and amylose content decreased from 18.9% to 0.75%. This indicated that α–1,4 linkages of amylose and amylopectin were cleaved, and moiety of glycosyl residues were transferred to another amylose and amylopectin to produce branched glucan and BE-treated tapioca starch by forming α–1,6 branch linkages. The product was further modified with BSMA to produce highly-branched tapioca starch with 9.7% of extra branch points. When subject to digestion with human pancreatic α-amylase (HPA), porcine pancreatic α-amylase (PPA) and glucoamylase, highly-branched tapioca starch gave significantly lowered α-amylase susceptibility (7.5 times, 14.4 times and 3.9 times, respectively), compared to native tapioca starch.

1. Introduction

Tapioca starch extracted from the cassava tuber is one of the most important carbohydrate food sources in many regions of the tropics. Due to the abundance and ease of extraction, tapioca starch has been one of a few commercially-exploited starches such as maize starch (Moorthy, 2004). Native starches are structurally too weak and functionally too restricted for vast range of applications. Therefore, the modification of starch in various ways such as physical, chemical or enzymatic method may be required to enhance the functionality of native starch. Among these, the enzymatic method has many advantages over other methods as it is safer for both the environment and consumers and the reaction can be more specifically controlled under the mild conditions thus consequently may result in fewer by-products (Butler, Van der Maarel, & Steeneken, 2004).

Branching enzyme (BE, 1,4-α-D-glucan: 1,4-α-D-glucan, 6-α-D-(1,4-α-D-glucano)-transferase, EC 2.4.1.18) is known to catalyze the formation of α-1,6-glucosidic linkages by transglycosylation in amylopectin, thereby creating branched glucan molecules. Furthermore, BE cleaves the α-1,4 glucosidic bond of the segment between clusters to produce amylopectin cluster from amylopectin. When BE from microorganism acts on starch containing amylose and amylopectin, both transglycosylation and cleaving reactions lead to form branched glucan and amylopectin cluster (Lee et al., 2007).

Maltogenic amylase (MAase), a glycoside hydrolase cloned from various Gram-positive bacteria, hydrolyzes cyclomaltodextrins and starch mainly to maltose, and pullulan to panose, by cleavage of α-1,4 glycosidic bonds. It also exhibits high transglycosylation activity via formation of various glycosidic linkages such as α-1,6 and α-1,3 linkages producing branched oligosaccharides from liquefied starch (Park et al., 2000). From the recent research of maltogenic amylase, it was evidenced that maltogenic amylase produced branched side chains by reaction with amylopectin cluster (Kim, 2006).

Recently, Takii, Ishihara, Kometani, Okada, and Fushiki (1999) developed a new type dextrin, highly-branched cyclic dextrin

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(HBCD) from waxy corn starch by the cyclization reaction of branching enzyme. They claimed that the product showed a sustained blood glucose availability in mice because of slower hydrolysis by α-amylase and α-glucosidase in small intestine.

In the previous study, we modified rice starch using combined treatment of *Thermus scotoductus* 4-α-glucanotransferase (TSαGT) and *Bacillus stearothermophilus* maltogenic amylase (BSMA) to produce highly-branched amylopectin clusters (Lee et al., 2007). It was evidenced that the product demonstrated significantly-reduced susceptibility to α-amylase and glucoamylase.

In this study, tapioca starch was first modified using BE isolated from *Bacillus subtilis* 168, and the resulting products were further branched by transglycosylation reaction of BSMA to produce highly branched tapioca starch. The molecular fine structures of BE-treated and highly-branched tapioca starch were analyzed and their susceptibilities to digestive enzymes, such as porcine pancreatic amylase (PPA), human pancreatic amylase (HPA), and glucoamylase were kinetically investigated.

2. Materials and methods

2.1. Materials

Commercial tapioca starch was kindly provided by Tay Ninh Tapioca Starch Company (Tay Ninh province, Viet Nam). Debranching enzyme, isoamylase, was purchased from Hayashibara Shoji Inc. (Okayama, Japan). Porcine pancreas α-amylase (A-4268) and *Aspergillus niger* glucoamylase (10115) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human pancreatic α-amylase was prepared according to the method of Brayer et al. (2000). Branching enzyme (BE) was isolated from *Bacillus subtilis* 168 according to Lee et al. (2007). *Bacillus stearothermophilus* maltogenic amylase (BSMA) was isolated from *E.coli* MC 1061 according to Cha et al (1998). Isoamylase isolated from *Pseudomanas amyloderamosa* was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan).

2.2. Assay for enzyme activity

Branching enzyme activity was analyzed according to the procedure of Takata, Takaha, Okada, Takagi, and Imanaka (1996). The 50 μL of enzyme solution and 50 μL of 0.05% (w/v) amylose solution were mixed and incubated at 30 °C for 20 min. The reaction was terminated by adding 1 mL of iodine reagent which was made by diluting 0.5 mL iodine stock solution (0.26 g I_2 and 2.6 g KI in 10 mL water) to 130 mL with distilled water. The absorbance at 620 nm was measured immediately with a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). One BE unit was defined as the amount of enzyme that degraded 1 μg/mL amylose per min under the above conditions. BSMA activity was assayed according to the dinitrosalicylic acid (DNS) method, by determining the amount of reducing sugars produced by the enzyme (Cha et al., 1998). Each enzyme reaction mixture was composed of 150 μL of 1% (w/v) β-cyclodextrin 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 continued the incubation for another 10 min. The reaction was terminated by adding 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 μmol of reducing sugars in 1 min under the reaction conditions. The activity was determined by dinitrosalicylic acid (DNS) method. Each enzyme reaction mixture was composed of 150 μL of 1% (w/v) pullulan in 50 mM sodium acetate buffer (pH 4.5), 120 μL of reaction buffer (50 mM sodium acetate buffer, pH 4.5), and 30 μL of diluted enzyme solution. The reaction mixture was preincubated at 60 °C for 5 min before the diluted enzyme solution was added and continued the incubation for another 10 min. The reaction was terminated by adding 750 μL of DNS solution and boiling for 5 min. Absorbance was measured at 575 nm using a spectrophotometer. One unit of isoamylase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars in 1 min under the reaction conditions.

2.3. Preparation of BE-treated and highly-branched tapioca starch

Tapioca starch slurry (5%, w/v) was prepared by mixing 4 g starch and 50 mM sodium phosphate buffer (pH 6.5) to be a final volume of 80 ml. The slurry was gelatinized in a boiling water bath for 30 min while stirring. The gelatinized tapioca starch paste was incubated with added BE (1000 U/g starch) in a water bath at 30 °C for 6 h for modification. The reaction was stopped by heating in boiling water for 30 min. After removal of insoluble materials by centrifugation (15,000g for 20 min), 2-volumes of ethanol was added to the solution. The precipitant recovered after centrifugation (15,000g for 10 min) and decantation was dried in a convectional air-oven (50 °C) overnight to finally produce BE-treated tapioca starch. To produce highly-branched tapioca starch, the BE reaction product was further treated with BSMA (1000 U/g starch) at 50 °C for 36 h. After it was precipitated with 2-volumes of ethanol and washed 4 times with distilled water to remove small maltooligosaccharides, highly-branched tapioca starch was recovered and dried at 50 °C overnight.

2.4. Branched chain length distribution of BE-treated and highly-branched tapioca starch

To analyze the branched chain length distribution of BE-treated and highly-branched tapioca starch, first, 1% (w/v) of each starch sample was dissolved in 50 mM sodium acetate buffer (pH 4.5), followed by incubation with isoamylase (360 U/g substrate) at 60 °C for 48 h for complete debranching. The reaction was stopped by boiling for 10 min. After centrifuging (15,000g for 10 min) and filtering (0.45 μm), the branched chain length distribution was analyzed using a high-performance anion-exchange chromatography (HPAEC) system (Dionex-300, Dionex Co., Sunnyvale, CA, USA) coupled with an electrochemical detector (ED40, Dionex Co.). The system was equipped with a CarboPac PA-100 column (4 × 250 mm, Dionex Co.) and eluted with a gradient of 0–0.4 M sodium acetated in 0.15 M sodium hydroxide at a flow rate 1.0 ml/min.

2.5. Molecular weight distribution of BE-treated and highly-branched tapioca starch

The molecular weight distributions of the modified tapioca starch samples were analyzed by using size exclusion chromatography (SEC), multi-angle laser light scattering (MALLS) (Dawn DSP, Wyatt Technology, St. Barbara, CA, USA), and refractive index (RI) detector (Waters 410, Waters Co., Milford, MA, USA). SUGAR KS-804 and KS-806 columns (8 × 300 mm, Showa Denko K.K., 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% NaN_3) was 0.4 ml/min. Each sample (0.5%, w/v) was dissolved in distilled water at boiling temperature for 1 h and then filtered through 5 μm disposable syringe filter before injecting to SEC-MALLS-RI system. The weight average molecular weight (M_w) of the sample was calculated using ASTRA V4.90.07 software (Wyatt Technology) with the Berry extrapolation method for curve fitting and the dn/dc value of 0.146 mL/g.

2.6. Kinetic study of α-amylases and glucoamylase for digestion of BE-treated and highly-branched tapioca starch

Porcine and human pancreatic α-amylase activities were measured using the copper-bicinchoninate reducing-value method with a microsample plate reader (Fox & Robyt, 1991). Varied concentrations of tapioca starch, BE-treated, and highly-branched tapioca starch were prepared in 20 mM sodium phosphate buffer containing 6 mM NaCl (pH 6.9), respectively. The reaction mixture was pre-warmed at 30 °C for 5 min, and then each enzyme was added to substrate solutions, respectively. One hundred microliter aliquots of each reaction mixture were taken at various time intervals and the reaction was stopped by adding 100 μl of 0.1 N NaOH. After 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 the wells of a microsample plate, and the absorbance was measured using a microsample plate reader (EL340, Biokinetics Reader, Bio-Tech Instruments, Inc., UK) at the wavelength 570 nm.

Assay of glucoamylase activity was carried out according to the glucose oxidase/peroxidase (GOD-POD) method (Mitchell, 1990). Varied concentrations of tapioca starch, BE-treated, and highly-branched tapioca starch were dissolved in 50 mM sodium acetate buffer (pH 4.5) The reaction mixture was pre-warmed at 50 °C for 5 min, and then glucoamylase from *A. niger* was added to the substrate solution, and aliquots (20 μl) of the reaction mixture were taken at every 30 s for 2 min. The reaction was stopped by adding the same volume of 0.1 N NaOH solution and 360 μl of glucose determination reagent (Glucose-E kit, Young-Dong Pharm., Seoul, Korea) was added. The analysis with a microsample plate reader was same as above at the wavelength 505 nm.

The kinetic data were transformed to Lineweaver–Burk plots, and the kinetic parameters (K_m, k_{cat}) were calculated.

3. Results and discussion

3.1. Effects of BE treatment on molecular fine structure of tapioca starch

During the period of BE treatment on tapioca starch, the reaction mixture was sampled at time intervals to monitor the change of the molecular weight distribution of the starch using SEC-MALLS-RI system (Fig. 1). The first peak in the elution profile for native starch, which corresponded to native amylopectin of tapioca starch, completely disappeared in 20 min. This probably indicated that internal chains of amylopectin spanning over clusters were readily cleaved by the reaction of BE to convert amylopectin macromolecules into relatively smaller clusters with narrow size distribution as proposed by Takata et al. (1997). None of intact amylopectin molecules remained intact after 20 min of

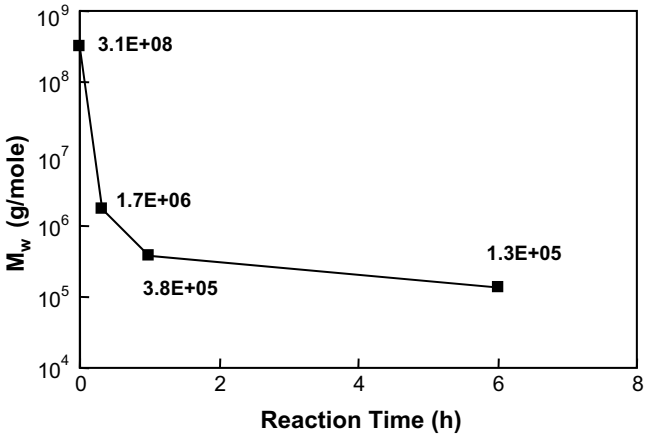

Fig. 2. Changes in weight average molecular weight of tapioca starch during branching enzyme (BE) treatment.

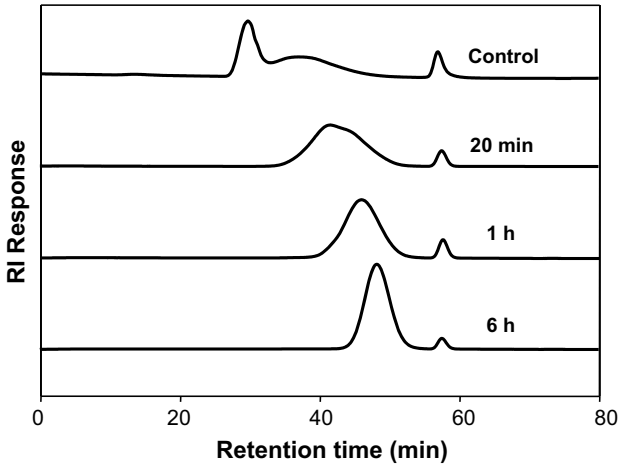

Fig. 1. Changes in molecular weight distribution of tapioca starch during branching enzyme (BE) treatment.

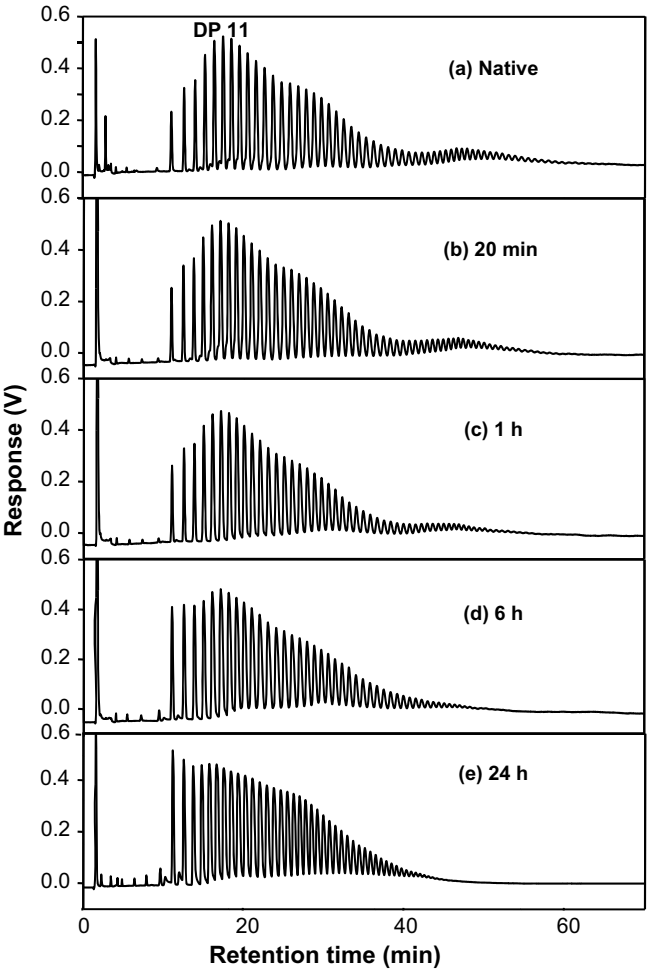

Fig. 3. HPAEC analysis of branch chain length distribution of tapioca starch during branching enzyme (BE) treatment.

Table 1
Changes in branch chain length distribution and amylose content of tapioca starch during branching enzyme (BE) treatment

Sample	Branch chain length distribution				Amylose content (%)
	A	B1-short	B1-long	B2	
	(DP <12)	(DP 13–24)	(DP 25–36)	(DP >36)	
Native	24.1	47.7	19.3	7.0	18.64
20 min	26.2	50.7	18.9	6.7	
1 h	29.8	51.9	13.4	4.5	
6 h	33.5	48.6	14.9	2.4	
24 h	43.6	39.4	14.5	2.5	0.74

BE reaction. After 20 min, the elution profile slowly shifted forward. The onset of the elution peak obviously delayed with reaction time, whereas endset of the peak hardly changed, resulting in a sharper peak thus more narrow molecular weight distribution after 6 h of BE treatment. The weight average molecular weight (M_w) was calculated for each profile and plotted as a function of BE reaction time (Fig. 2). The M_w rapidly decreased during 1 h of BE reaction (approximately 1000-fold reduction), and then very slowly decreased thereafter, leveling off at around 100,000.

The branch chain distributions of BE-treated tapioca starch at varied reaction times were shown in Fig. 3 and Table 1. The number of the short branch chains from DP 6–12 increased from 24.1% to 33.5% whereas the number of the longer branch chains, B1-long (DP 25–36) and B2 (DP >36) decreased from 19.3% to 14.9% and from 7% to 2.4%, respectively. The B1-short (DP 13–24) chains hardly changed or even slightly increased. These results may indicate that the longer branch chains are partially cleaved by BE to become shorter chains. The resulting linear chains could act as donors for transglycosylation to the branch chains of BE-treated tapioca starch by formation of α-1,6 linkages, which may increase the number of A chains or maintain the B1-chain quantity.

At the end of 6 h of BE treatment on tapioca starch, amylose content, measured by iodine affinity method, decreased from 18.9% to 0.75% (Table 1). This dramatic reduction of amylose content indicated that most of long chain amylose molecules in tapioca starch were cleaved. The cleaved linear glucans from amylose also could be transferred to either other chains of amylose or amylopectin by the formation of α-1,6-glucosidic linkage, as evidenced by previous studies (Lee et al., 2007), thus contributed to the branch chain length distribution in Fig. 3. The disproportionation action of BE led to the progressive disappearance of amylose and formation of amylopectin with a modified branch chain length distribution.

3.2. Production of highly-branched tapioca starch

To prepare highly-branched tapioca starch, BE-treated tapioca starch was further treated with BSMA. The final product was subject to HPAEC analysis for the determination of the branch chain length distribution. As shown in Fig. 4(c) and Table 2, the number of short side chains with DP 3–5 greatly increased whereas that of DP >6 significantly decreased. Branch chains longer than approximately DP 30 completely disappeared. In addition, a series of small peaks newly appeared between linear maltooligosaccharides (asterisked in Fig. 4c). As an effort to identify those peaks, highly-branched tapioca starch was digested with β-amylase, and the completely digested solution was analyzed as presented in Fig. 4(d). It was observed that those new peaks still remained after the β-amylase treatment. This finding could indicate that those new peaks correspond to extra-branched maltooligosaccharides.

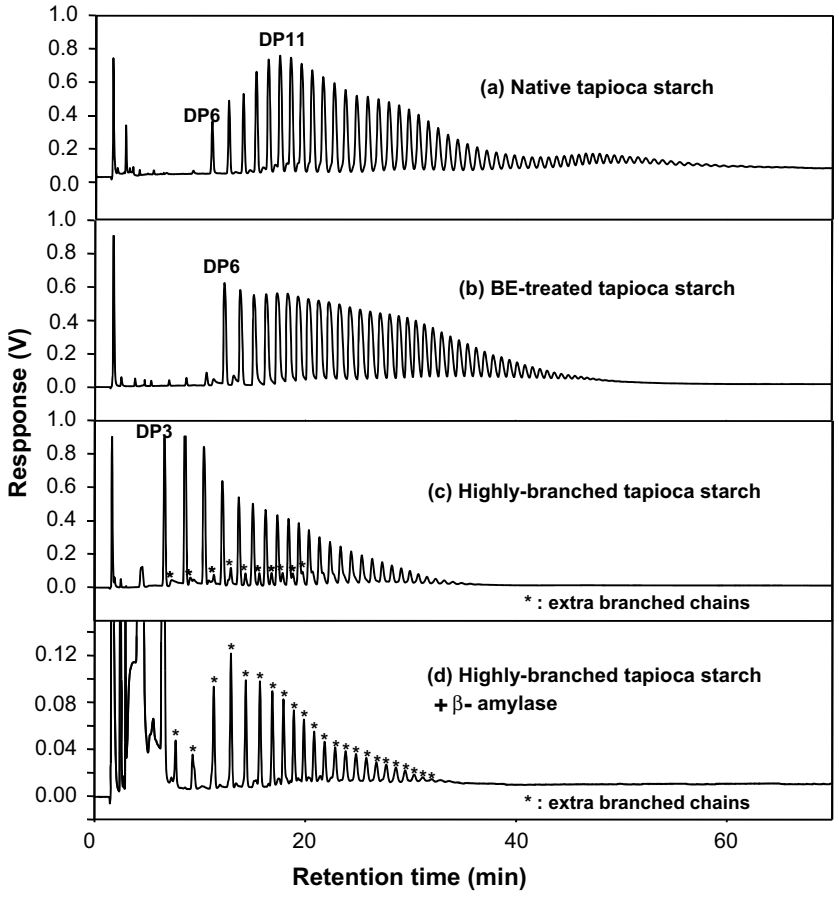

Fig. 4. Comparison of branch chain length distribution of native tapioca starch, BE-treated tapioca starch and highly-branched tapioca starch.

Table 2
Comparison of branch chain length distribution and amylose content for native tapioca starch, BE-treated tapioca starch and highly-branched tapioca starch

Sample	Amylose content (%)	Branch chain length distribution			
		A (DP <12)	B1-short (DP 13–24)	B1-long (DP 25–36)	B2 (DP >36)
Native	18.64	24.1	47.7	19.3	7.0
BE-treated	0.74	43.6	39.4	14.5	2.5
Highly-branched	0.16	75.1	24.4	0.5	0.0

BSMA preferentially hydrolyzed longer branch chains, releasing maltose and glucose from the non-reducing end of the branch chains, and transferred the resulting maltooligosaccharides to the non-reducing ends of the shorter branch chains by forming α-1,6-glucosidic linkages.

Table 3
Kinetic parameters of native tapioca starch, BE-treated tapioca starch and highly-branched tapioca starch for enzymatic digestion

Digestive enzyme	Sample	Kinetic parameters		
		K_m (mg/ml)	k_{cat} (1/s)	k_{cat}/Km (ml/mg-s)
HPA (human pancreatic α-amylase)	Native	0.10 ± 0.01	478.5 ± 43.5	3729 ± 321.0
	BE-treated	0.14 ± 0.01	436.2 ± 14.1	3133 ± 83.0
	Highly-branched	0.37 ± 0.02	196.2 ± 21.2	497 ± 85.0
PPA (porcine pancreatic α-amylase)	Native	0.07 ± 0.003	263.5 ± 33.3	4030.4 ± 599.9
	BE-treated	0.06 ± 0.006	208.7 ± 30.1	3651.5 ± 372.9
	Highly-branched	0.38 ± 0.08	100.5 ± 16.4	278.1 ± 25.8
Glucoamylase (*Aspergillus niger*)	Native	0.71 ± 0.02	44.1 ± 2.1	61.2 ± 2.7
	BE-treated	0.63 ± 0.08	39.7 ± 2.5	63.3 ± 4.9
	Highly-branched	1.6 ± 0.1	25.1 ± 2.2	15.5 ± 0.5

3.3. Kinetic parameters for enzymatic digestion of BE-treated tapioca starch and highly-branched tapioca starch

Susceptibility to enzymatic digestion of BE-treated tapioca starch and highly-branched tapioca starch was determined using human pancreatic α-amylase (HPA), porcine pancreatic α-amylase (PPA) and glucoamylase from *A. niger*, and the kinetic parameters were summarized in Table 3. In the case of PPA digestion, the K_m value of highly-branched tapioca starch was 0.38 mg/ml, which is more than 6× higher than that of native tapioca starch (0.07 mg/ml) or BE-treated tapioca starch (0.06 mg/ml). The k_{cat} value of highly-branched tapioca starch was 100.5 s^{-1} that was more than 2 times lower than that of native tapioca starch (263.5 s^{-1}) or BE-treated tapioca starch(208.7 s^{-1}). As a result, the k_{cat}/K_m value of highly-branched tapioca starch was 14 times lower, compared with that of tapioca starch and BE-treated tapioca starch. Similarly, kinetic parameters of native tapioca starch, BE-treated tapioca starch, and highly-branched tapioca starch for glucoamylase digestion were determined as summarized in Table 3. Km, k_{cat} and k_{cat}/K_m values were not significantly different between native tapioca starch and BE-treated tapioca starch, as similar as the case of PPA. However, highly-branched tapioca starch showed significantly higher K_m (>2 times) and lower k_{cat} and thus k_{cat}/K_m (<4 times) values compared to those of native tapioca starch and BE-treated tapioca starch. Compact molecular structure of highly-branched tapioca starch induced by the abundant short chains and the absence of long chains, and the addition of extra short

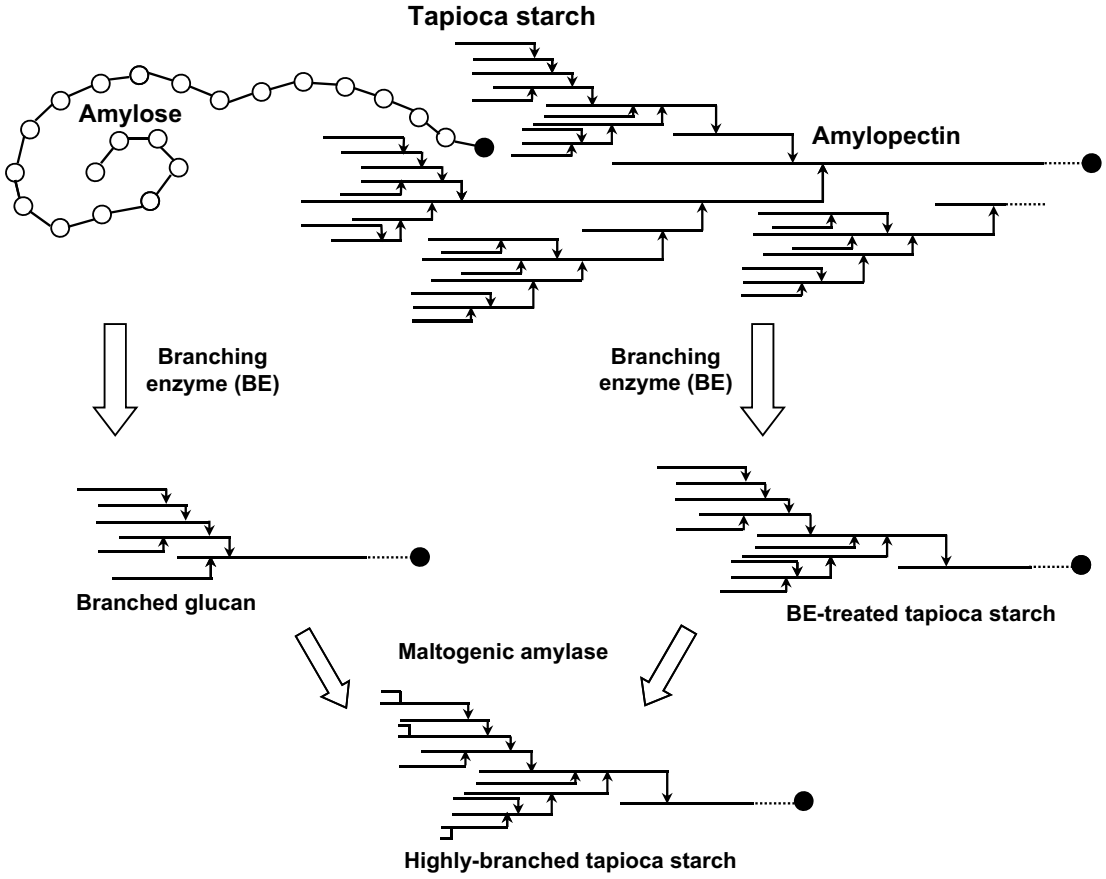

Fig. 5. Schematic diagram of enzymatic modification of tapioca starch with branching enzyme and maltogenic amylase. Arrows are α-1,6 glucosidic linkages. The reducing end of a glucan chain is shown by closed circle.

branches may be responsible for the resistance to enzymatic digestion. The lowered enzymatic susceptibility of highly-branched tapioca starch suggested that the BE modified tapioca starch and highly-branched tapioca starch are potentially useful to be applied in food industries as functional food materials especially for the diabetic, the obese and also for the athletic, because their slow digestion in small intestine would be helpful for continuous and prolonged glucose supply as reported by Takii et al. (1999).

4. Conclusions

The molecular structure of highly-branched tapioca starch produced by a combined treatment of BE and BSMA was found to contain amylopectin clusters with highly-branched side chains as proposed in Fig. 5. The low hydrolysis rate of highly-branched tapioca starch by α-amylases and glucoamylase suggested that it has a potential to maintain blood glucose level after intake and during prolonged exercise. The BE-treated tapioca starch is a product from both amylose and amylopectin with the molecular weight ranging from 10^6 to 10^8 Da depending on the degree of modification. This may not contribute to retard the enzymatic digestion, but the mixture of BE-treated and highly-branched tapioca starch at different ratios may be useful for the control of blood glucose level, which requires further study.

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