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Modification of Rice Starch by Selective Degradation of Amylose Using Alkalophilic *Bacillus* Cyclomaltodextrinase

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A cyclomaltodextrinase (CDase) isolated from alkalophilic *Bacillus* sp. I-5 (CDase I-5) exists in a dodecameric form, an assembly of six dimers, each catalytic site of which is located in a narrow groove at the interface of the dimeric unit. Because of the unique geometric shape of the catalytic site, the enzyme has the ability to discriminate the molecular size of substrates. An analysis of the hydrolysis reaction of the enzyme revealed that its k_{cat}/K_m value on amylose was 14.6 $s^{-1}(mg/mL)^{-1}$, whereas that for amylopectin was 0.92 $s^{-1}(mg/mL)^{-1}$, showing an exceptionally high preference toward amylose. CDase I-5 was applied to modify the starch structure to produce low-amylose starch products by incubating rice starch with this enzyme. We found that the amylose content of rice starch decreased from 28.5 to 9%, while the amylopectin content remained almost constant with no significant change in the side chain length distribution. When the CDase I-5-treated rice starch was stored at 4 °C for 7 days, the retrogradation rate was significantly retarded as compared to that in the control sample.

KEYWORDS: Alkalophilic *Bacillus* I-5; cyclomaltodextrinase (cDase); low-amylose starch; retrogradation

INTRODUCTION

Starch is the major carbohydrate reservoir in plants, where it is found in granular form. Starch consists mainly of two structurally different polymers, amylopectin and amylose. Amylopectin (AP) is an α-(1,4)-linked D-glucose polymer with about 4−5% α-(1,6)-linked branches, whereas amylose (AM) is an essentially α-(1,4)-linked linear D-glucan with very few branches. The proportion of AM and AP in starch is variable, depending largely on the plant source. For example, high amylose corn starch displays 50−70% apparent AM content, whereas waxy-type starch contains no AM. The various AM/AP ratios of plant starches result in different physical, functional, and nutritional properties.

Cyclodextrin-degrading enzymes known as cyclomaltodextrinases (CDases; E.C. 3.2.1.54) efficiently hydrolyze cyclomaltodextrins (CDs), which are cyclic glucose oligomers linked by α-D-(1,4)-glycosidic bonds (*1*). The gene encoding CDase has been cloned from alkalophilic *Bacillus* sp. I-5, and the gene structure, biochemical properties, and 3-D protein structure of CDase I-5 have been characterized in our laboratory (*2*). The CDase I-5 gene has an open reading frame of 559 amino acids with a relative molecular weight of 65 kDa. The 3-D structure of the CDase I-5 indicates that the enzyme exists in a dodecameric form, composed of six copies of dimers (*3*). The purified CDase preferably hydrolyzes cyclomaltoheptaose (*β*-CD) over soluble starch and pullulan and also displays transglycosylation activity. These enzymatic properties of the CDase are distinguished from those of a typical α-amylase (e.g., TAKA-amylase A (TAA; E.C. 3.2.1.1) from *Aspergillus oryzae* (*4*, *5*)). It has been reported that the TAA does not hydrolyze CDs, and only a few types of α-amylases are able to hydrolyze CDs at a very slow rate (*1*, *3*). The active site cleft of CD-degrading enzymes in dimeric forms is known to form a narrow and deep groove at the top of the $(\beta/\alpha)_8$ barrel, whereas a wide and shallow active site is observed in α-amylases (*1*, *3*). We have proposed that the substrate preference of CDase toward flat and thin CDs can be explained by this unique active site conformation of CDase (*1*, *3*). Similarly, Kamasaka et al. (*6*) obtained higher selectivity on relatively small, disordered amylose (AM) molecules in starch than on large amylopectin (AP) molecules by neopullulanase (*6*) with a qualitative comparison of various amylolytic enzymes on potato starch. The distinguishable action pattern of CDase can be utilized to produce specially made low-amylose starch by specifically degrading AM molecules.

In this study, the distinct action pattern of alkalophilic *Bacillus* I-5 CDase (CDase I-5) on the starch structure was investigated quantitatively, and the applicability was also examined for

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starchy food processing by evaluating the anti-retrograding effect of CDase treatment on rice starch. The enzyme modification of starch could be used to decrease viscosity and improve gel stability, feel, appearance, texture, and resistance to heat. Moreover, CDase I-5 could be used to produce amylose-free starch, which is of great interest in many industries.

MATERIALS AND METHODS

Materials. Amylose, amylopectin, and starches were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). All other chemicals used were of reagent grade unless otherwise stated.

Protein Purification, Bacterial Strains, and Plasmids. Gene cloning, transformation into *Escherichia coli*, and CDase overexpression were performed as described previously (*2*, *3*, *7*). The CDase of alkalophilic *Bacillus* I-5 was purified from the culture of *E. coli* MC1061[F^-, *araD*139, *araABC-leu*] 7696, *gal*U, *kal*K, *lac*X74, *rpsL*, *t6hi*, *hsdR2*, *mcrB*] harboring the CDase gene on plasmid pUC18. To characterize the enzymatic properties of CDase I-5, the protein was produced from recombinant *E. coli* MC1061 and purified using anion exchange chromatography. CDase I-5 was precipitated mainly in the presence of 50% (w/v) ammonium sulfate. Following ammonium sulfate fractionation, the protein was purified further by FPLC using a Q-sepharose column and a DEAE-8HR column (*3*).

Enzyme Activity. The activity of CDase I-5 was assayed using the 3,5-dinitrosalicylic acid (DNS) method of reducing sugar determination (*8*). An assay mixture containing 0.5% (w/v) β-CD and 0.1% (v/v) CDase I-5 in 50 mM sodium phosphate buffer (pH 7.0) was incubated at 50 °C for 10 min. The reaction was stopped by adding 3-fold DNS solution (v/v), followed by boiling for 5 min. The absorbance of the reaction mixture was measured at 575 nm. The equivalent of hydrolyzed glycosidic bonds was used as the equivalent of maltose based on a maltose calibration curve. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of reducing end per minute.

Kinetic Studies on the Hydrolysis of Various Substrates. The copper bicinchoninate method was applied to measure the concentration of reducing sugars produced from enzymatic hydrolysis (*3*, *9*), and the kinetic parameters of CDase I-5 toward three different substrates (β-CD, amylase, and amylopectin) were determined to compare substrates specificities.

Amylose and Amylopectin Content. The amylose content of starch was measured colorimetrically using the iodine method (*10*). One hundred milligrams of sample were weighed accurately and placed into a 50 mL Erlenmeyer flask, to which 1 mL of 95% ethanol and 9 mL of 1 N NaOH were added. The sample was heated for 10 min in boiling water to gelatinize the starch. After cooling the gelatinized sample to room temperature, it was transferred to a 100 mL volumetric flask; then the total volume was increased to 100 mL by adding distilled water. Five milliliters of the starch solution was pipetted into a 100 mL volumetric flask, and 1 mL of 1 N acetic acid and 2 mL of iodine solution (0.2 g of iodine and 2.0 g of potassium iodide in 100 mL of aqueous solution) were added. The solution was diluted to 100 mL with distilled water, shaken, and allowed to stand for 20 min. The absorbance was then measured at 620 nm using a spectrophotometer.

To determine the changes in the proportion of individual components during the CDase I-5 reaction, normal rice starch was solubilized in 90% DMSO by heating and used as a substrate for the reaction (*6*, *11*). The reaction mixture consisted of 0.5% rice starch and CDase I-5 (1 U/mg substrate) in 50 mM sodium phosphate buffer, and the contents of amylose, amylopectin, and reducing sugar were analyzed at 10, 30, and 60 min. The amylose content was measured using the iodine absorption method (*10*), and the reducing sugar content was measured using the DNS method (*8*) as the amount of maltose. The approximate content of amylopectin was estimated by subtracting the sum of amylose and reducing sugar content from the total percentage.

Protein Concentration. The amount of protein was measured using the method described in Bradford (*12*). The reaction mixture consisted of 100 mL of diluted enzyme solution and 900 mL of Bradford solution (100 mg of Coomassie Brilliant Blue G-250 in 50 mL of ethanol, 100 mL of 85% phosphoric acid, and 850 mL of distilled water). The reaction mixture was held at room temperature for 5 min while vortexing it once every minute, and then its absorbance was measured at 595 nm using a spectrophotometer. The blank was prepared by replacing the enzyme solution with buffer solution. A standard curve was prepared using bovine serum albumin (BSA; Sigma-Aldrich). The amount of protein was determined by extrapolating the standard curve.

SDS−PAGE. Protein samples were electrophoresed on a discontinuous polyacrylamide gel containing SDS according to Laemmli (*13*). The separating gel was typically prepared as a 10% (w/v) acrylamide gel using a stock solution of 33.5% (w/v) acrylamide containing 0.3% (w/v) *N*,*N*′-methylene bisacrylamide. The separating gel contained 12.0 mL of acrylamide/bisacrylamide solution, 15.2 mL of 1 M Tris-HCl buffer (pH 9.0), 0.4 mL of 10% (w/v) SDS solution, 11.2 mL of water, 1.0 mL of 3% (w/v) ammonium persulfate, and 20 mL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). The stacking gel was prepared from a stock solution of 30% (w/v) acrylamide and 0.44% (w/v) *N*,*N*′-methylene bisacrylamide to achieve a final concentration of 4% (w/v) acrylamide, 1.3 mL of acrylamide/bisacrylamide solution, 2.5 mL of 1 M Tris-HCl buffer (pH 6.8), 0.1 mL of 10% (w/v) SDS solution, 6.1 mL of water, 0.1 mL of 3% (w/v) ammonium persulfate, and 20 μL of TEMED. The electrode buffer was composed of 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS (pH 8.3). Samples were mixed with an equal volume of 2x loading buffer [0.25 M Tris-HCl buffer (pH 6.8), 10% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol, and 0.004% (w/v) bromophenol blue] and boiled for 3 min before loading onto the gel.

Thin Layer Chromatography. A thin-layer chromatography (TLC) was done according to Kim et al. (*14*). A TLC plate (K5F Silica Gel 150 Å; Whatman Filtration, Kent, UK) was activated for 30 min in a 110 °C oven. Prepared samples were spotted on the plate that was then developed in a TLC chamber with a solvent of *n*-butanol/acetic acid/water = 5:3:1 (v/v/v) at room temperature. The plate was dried and developed by dipping it rapidly into the solvent with 3 g of *N*-(1-naphthyl)-ethylenediamine and 50 mL of concentrated H_2SO_4 per 1 L of methanol. The plate was dried and placed in a 110 °C oven for 10 min

Gel Permeation Chromatography. The degradation of amylose and amylopectin was analyzed by gel permeation chromatography using connected Superdex 200 (10 × 300 mm; Amersham Pharmacia Biotech, Uppsala, Sweden) and Superdex 30 (10 × 300 mm; Amersham Pharmacia Biotech) columns. Elution was performed at room temperature with 100 mM NaCl at a flow rate of 0.7 mL/min. Eluted carbohydrates were detected with a refractive index (RI) detector (Waters R401; Millipore Co., Billerica, MA)

HPAEC Analysis. Hydrolysis products of rice starch and waxy starch were analyzed using high-performance anion exchange chromatography (HPAEC) equipped with an electrochemical detector (ED 40; Dionex Co., Sunnyvale, CA.). The column (CarboPac PA1; Dionex Co.) was able to separate the monosaccharides and oligosaccharides. Filtered samples were eluted along with a linear gradient of the solvent containing 600 mM sodium acetate and 150 mM NaOH.

Analysis of Side Chain Length Distribution of Amylopectin. A reaction mixture consisting of 0.5% amylopectin and CDase I-5 (1 U/mg substrate) was incubated at 50 °C for 3 h, and then the side chain lengths were analyzed. Control and CDase I-5-treated amylopectin samples were debranched with pullulanase (Promozyme 200L, NOVO Nordisk A/S, Bagsvaerd, Denmark) at 60 °C for 12 h. The products were analyzed by HPAEC with a CarboPac PA1 column and an electrochemical detector. The elution was performed with a 0−70% (v/v) gradient of 600 mM sodium acetate in 150 mM NaOH at a flow rate of 1.0 mL/min.

Differential Scanning Calorimetry. The retrogradation of cooked rice samples was determined using differential scanning calorimetry (DSC; Seiko Co., Ltd., Ohkuma, Japan) during 7 days of storage at 4 °C. DSC was calibrated with indium (156.6 °C, 28.591 J/g) and tin (232.2 °C, 60.62 J/g). Distilled water was used as a reference. Cooked rice grains (12 mg) were weighed and hermetically sealed in aluminum pans. The pans were heated from 20 to 120 °C at a rate of 5 °C/min. The degree of retrogradation was expressed as the enthalpy calculated from the area of the endothermic peak between 40 and 80 °C.

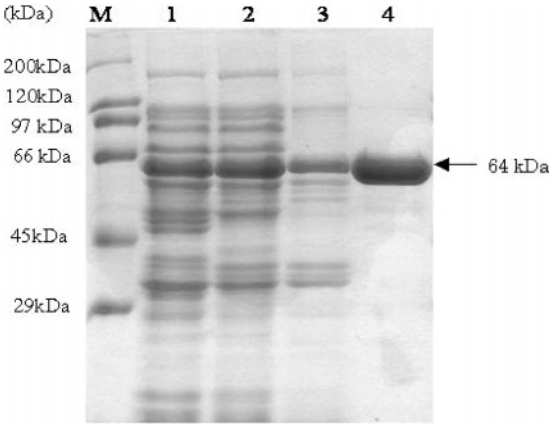

Figure 1. SDS–PAGE analysis of purified CDase I-5: lane M, size marker; lane 1, cell extract; lane 2, ammonium sulfate (50%); lane 3, Q-sepharose chromatography; and lane 4, DEAE-8HR chromatography.

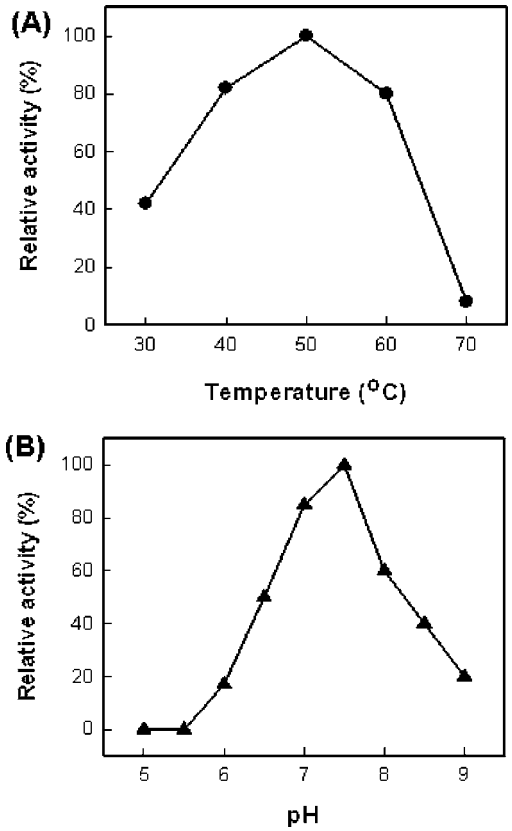

Figure 2. Effect of temperature and pH on CDase I-5 activity: the optimum temperature was 50 °C on β-CD as the substrate, and the optimum pH was 7.5 in a sodium-phosphate buffer.

RESULTS AND DISCUSSION

Purification of Cyclomaltodextrinase (CDase I-5). The molecular mass of purified CDase I-5 was estimated to be about 64 kDa as determined by SDS−PAGE (**Figure 1**). The molecular weight was similar to that of maltogenic amylases.

Effect of Temperature and pH on CDase I-5 Activity. The enzyme reaction was performed in a pH 7.0 sodium-phosphate buffer. The optimum reaction temperature of CDase I-5 was 50 °C with β-CD as a substrate (**Figure 2**). The optimum temperatures of other maltogenic amylase are as follows: 50 °C for BLMA (*15*), 45 °C for BBMA (*16*), 55 °C for BSMA (*17*), 55 °C for NPase from *Bacillus stearothermophilus* IMA6503 (*18*), 70 °C for BTMA (*19*), 60 °C for ThMA (*20*), and 45 °C for EFMA (*21*).

Table 1. Kinetic Parameters of CDase I-5 toward Various Substrates[a]

parameters	substrates		
	β-CD	amylose	amylopectin
K_m (mg/mL)	0.515 (±0.0080)	1.52 (±0.18)	55.15 (±9.91)
k_{cat} (s^{-1})	78.18 (±0.38)	22.11 (±0.91)	50.36 (±3.28)
k_{cat}/K_m (s^{-1}(mg/mL)$^{-1}$)	151.67 (±2.91)	14.60 (±1.27)	0.92 (±0.097)

[a] The copper bicinchoninate method was applied to measure the reducing sugars produced from enzymatic hydrolysis.

The CDase I-5 activity at various pHs was determined in sodium-phosphate buffer (pH 6−8) and showed the highest enzyme activity at pH 7.5 on β-CD (**Figure 2**). Sodium phosphate buffer (50 mM, pH 7.5) was also chosen for further characterization of the enzyme.

Kinetic Studies on the Hydrolysis of Various Substrates. The hydrolysis rate of CDase I-5 toward β-CD, amylose (AM), and amylopectin (AP) was determined and summarized in **Table 1**. Amylose exhibited a ~16 times higher k_{cat}/K_m value than amylopectin, indicating a unique specificity to amylose over amylopectin. This feature originates from its unusual quaternary structure, a dodecameric form, in which a hydrolyzed product released from one active site on the assembly would be readily accepted into the other active sites of a cluster (*3*). Through the spatial arrangement of the active site in the supramolecular assembly, CDase I-5 of the dodecameric form would be more advantageous to discriminate the molecules in terms of their sizes, as compared to previously reported CD-degrading enzymes exhibiting this selectivity (*6*). Therefore, a more linear shape of amylose molecules can be easily accessible to active site than amylopectin, resulting in a higher specificity on CDase I-5.

Thin Layer and Gel Permeation Chromatography Analysis. Commercial amylose and amylopectin were used as substrates to investigate the action pattern of CDase I-5 on starch components. The enzymatic reaction was initiated by adding 1.0 U/mg substrate of CDase I-5 to either 0.25% (w/v) amylose or 0.25% amylopectin in 50 mM sodium phosphate buffer (pH 7.5). Potato amylose and amylopectin dispersions (2% each, w/v) in 90% DMSO were prepared and diluted with deionized water, then immediately used for the enzyme treatment (*6*, *11*). After incubating at 50 °C for 0.5, 1, 5, and 24 h, an aliquot of the reaction mixture was removed, and the reaction was immediately stopped by boiling for 5 min. The hydrolyzed products of amylose and amylopectin by CDase I-5 were characterized by TLC analysis (**Figure 3**). Maltose and glucose were the major hydrolyzed products of amylose using CDase I-5 during the 24 h reaction period. However, degraded products were seldom detected from amylopectin hydrolysis. These results indicated that CDase I-5 preferentially degrades amylose, which clearly distinguishes CDase I-5 from other amylolytic enzymes.

Changes in molecular mass distribution of AM/AP caused by the CDase I-5 reaction were also analyzed using gel permeation chromatography (**Figure 3**). Samples were collected at designated incubation time intervals as described previously and loaded onto a Superdex 200 (10 × 300 mm, Amersham Pharmacia Biotech) column. Within the first 0.5 h, amylose started to degrade rapidly, and the peak of amylose gradually shifted to a longer retention time. As the reaction time reached 1 h, most of the original amylose molecules disappeared. However, in the CDase I-5 treatment, the amylopectin peak was

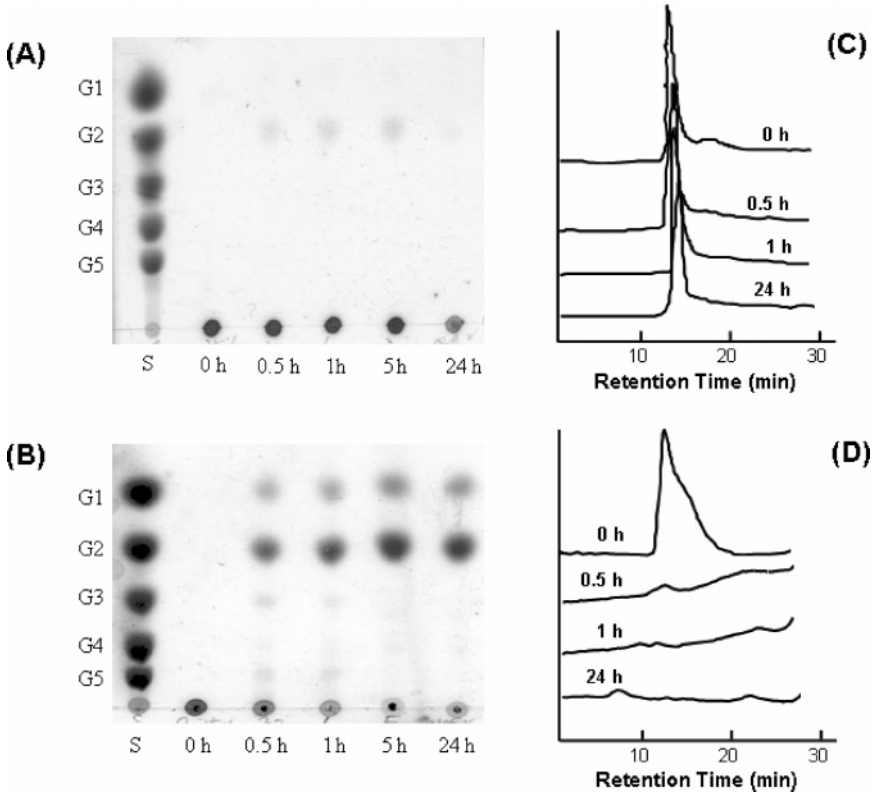

Figure 3. Thin layer (TLC) and gel permeation (GPC) chromatography analyses of hydrolyzed products from amylose and amylopectin with CDase I-5: (**A**) TLC analysis of 0.25% amylopectin reaction mixture, S represents the maltooligosaccharides standard (glucose–maltopentaose); (**B**) TLC analysis of 0.25% amylose reaction mixture; (**C**) GPC analysis of 0.25% amylopectin reaction mixture; and (**D**) GPC analysis of 0.25% amylose reaction mixture. All reactions were carried out with CDase I-5 (1 U/mg substrate) in 50 mM sodium phosphate buffer.

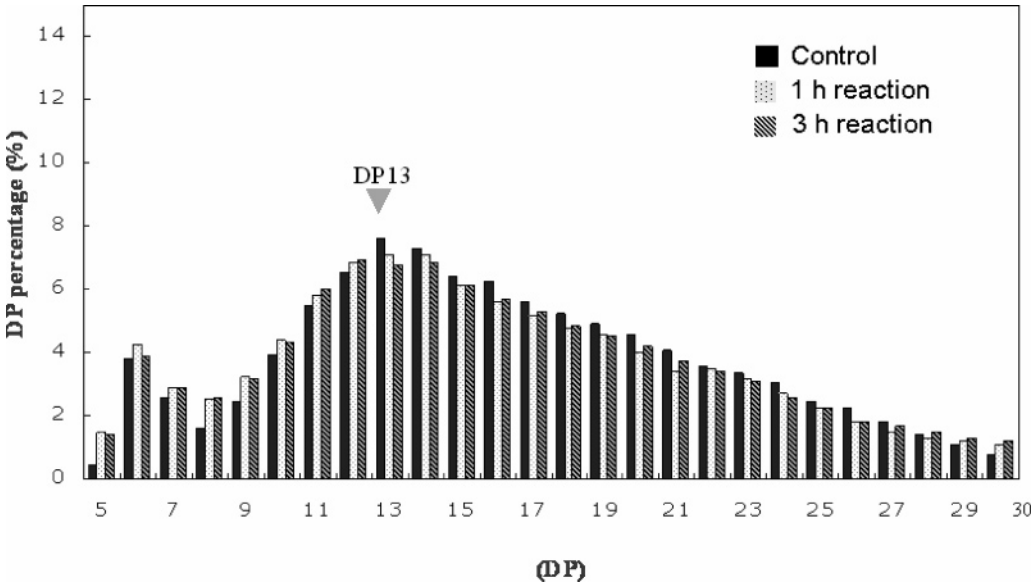

Figure 4. Area percentage (%) of the side chain of amylopectin from DP 5 to DP 30 (DP: degree of polymerization): a reaction mixture consisting of 0.5% amylopectin and CDase I-5 (1 U/mg substrate) was incubated at 50 °C and then debranched with pullulanase at 60 °C for 12 h. The products were analyzed by HPAEC with a CarboPac PA1 column, and the area percentages of peaks from DP 5 to DP 30 were calculated.

not affected, even after a reaction time of up to 25 h (**Figure 3**). Changes in molecular mass distribution caused by enzyme reactions clearly demonstrated that CDase I-5 had a selective degrading ability toward amylose over amylopectin. The same phenomenon was observed with rice and waxy starch, which supported the higher specificity of CDase I-5 on amylose even in the presence of amylopectin (data not shown). The previous results suggest that CDase I-5 can be a potential candidate to generate low-amylose starch by selectively removing or degrading amylose from starch.

Determination of the Contents of Starch Components. From the results mentioned previously, it could be positively concluded that amylose was selectively hydrolyzed by CDase I-5, even in the presence of amylopectin. The changes of starch components by CDase I-5 reaction were summarized in **Table 2**. As the reaction time proceeded, the amylose fraction gradually

Table 2. Determination of the Contents of Starch Components during the CDase I-5 Reaction[a]

Time (%)	Amylopectin	Amylose	Reducing sugar
0 min	71	28.5	0.5
10 min	72	12	16
30 min	72	10	18
60 min	72	9	19

[a] The reaction mixture consisted of 0.5% rice starch and CDase I-5 (1 U/mg substrate) in 50 mM sodium phosphate buffer.

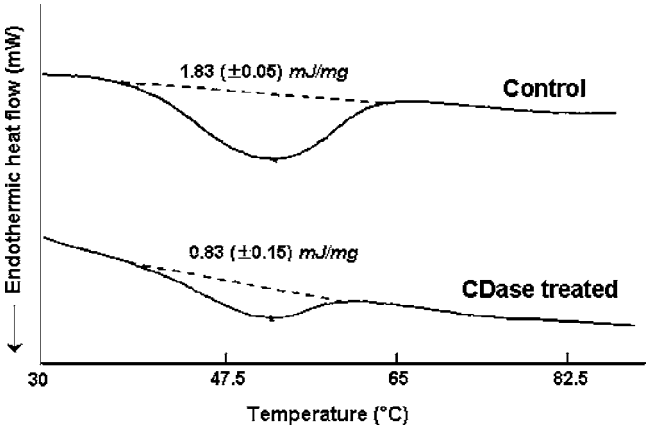

Figure 5. DSC thermogram of cooked rice after 7 days of storage at 4 °C. Endothermic peaks represent retrogradation of starch, and enthalpy can be calculated by the peak area.

decreased with the enzyme treatment, while the reducing sugar fraction increased in response to the amylose fraction decrease. Both independently measured amounts of amylose decrease and the increase in reducing sugar were well-matched, indicating that reducing sugars were produced almost solely from amylose and that the amylopectin fraction was not affected during the reaction period.

Effect of CDase I-5 on the Side Chain of Amylopectin. The change in the side chain length distribution of amylopectin was investigated using HPAEC after treatment with pullulanase, and the area percentages of peaks from DP 5 to DP 30 were calculated during the reaction period (**Figure 4**). There was little difference in the side chain length distribution between the control and CDase I-5-treated amylopectin, indicating that the enzyme scarcely affected the side chain length of amylopectin. We observed only a slight increase in the relative proportion of short chains (less than DP13) during the CDase I-5 treatment.

Effect of CDase I-5 Treatment on the Retrogradation of Rice Starch. In the DSC thermogram, the first endothermic peak of gelatinized and stored starch at around 40−60 °C, the so-called staling endotherm, originates from the melting of recrystallized amylopectin. The amylose component in starch retrogrades rapidly, followed by the slow recrystallization of the amylopectin component over time (*22*, *23*). Amylose is responsible for the short-term retrogradation and is also involved in aggregating double helices of amylose and amylopectin in the later stage, contributing synergetic effects on retrogradation (*22*). Thus, amylose contributes extensively to starch retrogradation, and the retrogradation rate depends greatly upon the amylose content as well as amylopectin (*24*−*28*).

When the gelatinized starch was rescanned after 7 days of storage at 4 °C, the control rice starch showed an endothermic peak of 1.83 mJ/mg, while the CDase I-five-treated sample exhibited 0.83 mJ/mg, which was 45.4% of the control (**Figure 5**). Therefore, the CDase I-5 treatment significantly retarded the retrogradation of cooked rice, possibly because a substantial amount of amylose was degraded by the enzymatic reaction. The reduced amylose content could lower the intermolecular rearrangement between amylose and amylopectin, resulting a retardation of starch retrogradation overall.

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