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Effect of Amylose Content on Corn Starch Modification by *Thermus aquaticus* 4-α-Glucanotransferase

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Corn starches with different amylose contents were enzymatically modified using *Thermus aquaticus* 4-α-glucanotransferase (TAαGTase). Upon the enzyme treatment, the chain-length distributions of isoamylolytically debranched products became broader [degree of polymerization (DP): 3–40] than those of untreated corn starches. In addition, a variety of cycloamyloses (CAs) with different sizes were formed by the glucanotransfer activity of TAαGTase. CAs with DP 5–40 were detectable in all of the TAαGTase-treated corn starches. From the results of high-performance anion-exchange chromatography and high-performance size-exclusion chromatography analyses, it was suggested that the amount of CAs produced by the enzyme treatment increased as the amylose content of the starches increased. Thus, we concluded that the extent of modification of starch molecules was enhanced in proportion to amylose content by the transfer activity of TAαGTase. This finding could be useful for developing an efficient process of CA production using this enzyme.

Keywords: *Thermus aquaticus* α-glucanotransferase (TAαGTase), corn starch, amylose, cycloamylose (CA)

Numerous studies have been conducted on starch to determine the structure–function relationships and to develop food and biotechnological applications [11]. Amylose and amylopectin are two major components of starch. Amylose molecules appear to exist as single helices within the starch granule where they are interspersed with semi-crystalline amylopectin molecules [3], yet their precise location within the granule awaits elucidation.

Starch-metabolizing enzymes have been classified depending on their reaction mechanism and substrate specificity [2] into glycosyl hydrolases (E.C. 3.2.1.x) and glycosyl transferases (E.C. 2.4.x.y). Recent research has attempted to develop tailor-made starchy materials using glycosyltransferase, and Park [9] proposed that current progress in protein engineering and molecular biology may lead us to a new way of creating "structured and modified starch." The 4-α-glucanotransferases (4-α-GTases; E.C. 2.4.1.25) belong to the α-amylase superfamily [5, 12, 15] and are known to catalyze the transfer of an α-glucan chain from one α-glucan molecule to another by the so-called disproportionating activity [16]. In addition, this type of enzyme can catalyze a reversible intramolecular glucan transfer reaction, which creates a cyclic form of α-glucan [1, 13, 15, 16]. If the donor molecule in the reverse direction is a cyclic glucan, the reaction may be called a coupling reaction. Because of this diversity of enzymatic reaction patterns, the products formed by the action of 4-α-GTases are widely heterogeneous. Cycloamylose (CA), a well-defined cyclic α-1,4-glucan, is one type of product generated by the intramolecular transfer activity of this enzyme. This product is itself highly soluble in water, which is a characteristic that can improve the solubility, adjust the reactivity, or increase the stability of various guest molecules by forming inclusion complexes. CA has various potential applications in the food and non-food industry sectors [16]. Therefore, in this study, we treated native corn starches having different amylose contents with TAαGTase and compared the differences in degree of structural modification and CA formation between them.

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The TAαGTase gene was cloned and prepared as described by Seo *et al.* [10]. *Thermus aquaticus* YT-1 (ATCC 25104) was cultured in *Thermus* B-P medium (0.4% beef extract, 0.4% polypeptone, 0.3% K_2HPO_4, 0.1% KH_2PO_4, and 2.5% agar) at 70°C for 7 days with shaking. The TAαGTase gene was cloned and expressed in *E. coli* MC1061 [F^- *araD139 recA13 Δ(araABC-leu)7696 galU galK ΔlacX74 rpsL thi hsdR2 mcrB*] according to Park *et al.* [8]. TAαGTase was purified using Ni–NTA affinity chromatography; the optimum reaction temperature and pH of the enzyme were 75°C and 7.5, respectively. TAαGTase activity was determined by measuring the optical change in iodine staining during the conversion of amylose by the enzyme [6]. One unit of enzyme activity was defined as the amount of enzyme that resulted in a change in absorbance of one unit per minute at 620 nm.

Three different types of corn starch varying in amylose content were chosen for comparison. Normal corn starch (NCS, 25–30% amylose) and waxy corn starch (WCS, 0–2% amylose) were acquired from Daesang Corporation (Seoul, Korea). Amylomaize V (55% amylose) was purchased from National Starch & Chemical Co. (Berkeley, CA, U.S.A.). Two hundred mg of each sample (NCS, WCS, and amylomaize V) was dispersed in 20 ml of 90% dimethyl sulfoxide (DMSO) and boiled in a water bath for 1 h. After heating, the sample was stirred overnight to obtain a transparent solution. Five ml of this solution was mixed with 30 ml of ethyl alcohol, and then centrifuged at 6,000 ×*g* for 15 min. Precipitated starch (50 mg) was dissolved in 5 ml of distilled water and treated with TAαGTase (40 U/g starch) at 75°C for 1 h. The reaction was terminated by heating for 30 min in boiling water.

The change in relative molecular weight of each of the TAαGTase-modified corn starches was determined. Molecular weight (MW) distributions were measured using high-performance size-exclusion chromatography (HPSEC; Summit HPLC System, Dionex, Sunnyvale, CA, U.S.A.) with a refractive index (RI) detector (Shodex RI-101, Showa Denko, Tokyo, Japan) and elution at 0.6 ml/min using distilled water [19]. Shodex OHpak SB-806 HQ and SB-804 HQ (8.0×300 mm; Showa Denko) columns were connected in tandem and equilibrated at 50°C, and 200 μl of the sample was injected for analysis. As shown in Fig. 1, untreated NCS contained amylopectin with an M_p (peak MW) of 1.0×10^8 g/mol (retention time, R_t=15.4 min) and amylose molecules with an M_p of 2.4×10^5 g/mol (R_t=26.0 min). Treatment with TAαGTase shifted the distribution profiles toward the lower molecular weight range. Amylopectin molecules from the three different starches were almost completely degraded to smaller molecules under the above reaction conditions. The number of molecules with an M_p around 2.4×10^4 g/mol (R_t=30.0 min) increased as a result of the TAαGTase treatment and were especially obvious in the WCS sample. These product peaks seem to indicate the

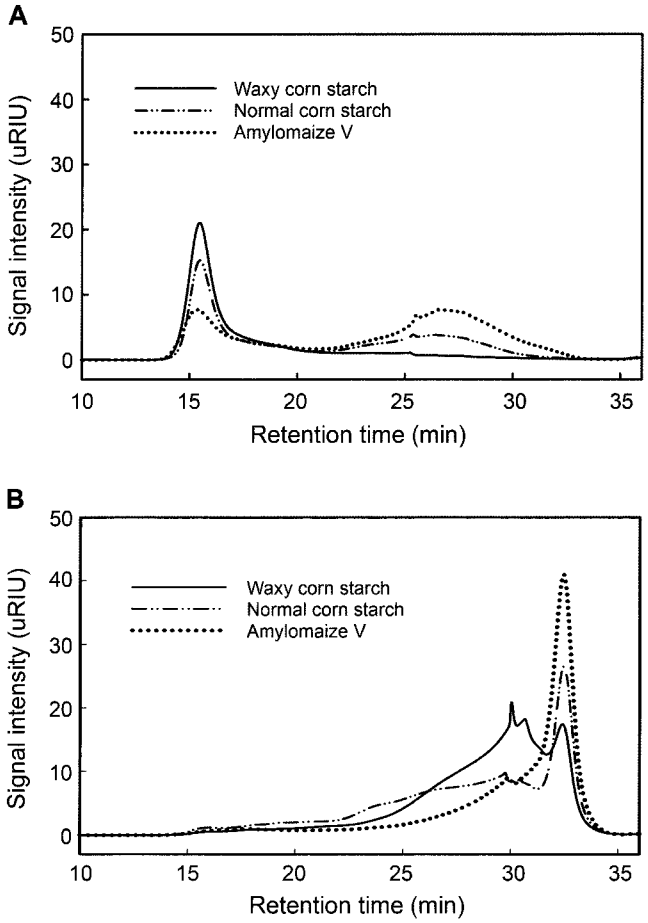

Fig. 1. Changes in molecular weight distributions of corn starches by enzymatic degradation of TAαGTase.
A. Molecular weight distributions of gelatinized native corn starches. **B**. Molecular weight distributions of corn starches reacted with TAαGTase (40 U/g starch) at 75°C for 1 h.

presence of amylose, small amylopectin clusters, and associations of modified amylopectin clusters as previously reported [7, 8, 15]. Another noteworthy observation is that the relative proportion of molecules with an M_p around 0.6×10^4 g/mol (R_t=32.4 min) increased significantly as the amylose content of the corn starch increased. The respective fractions may have consisted of CAs of various sizes.

Changes in the chain length-distributions of TAαGTase-treated corn starches were analyzed using a high-performance anion-exchange chromatography (HPAEC) system (Dionex-300, Dionex) coupled with a pulsed amperometric detector (ED40, Dionex) after debranching with isoamylase. Samples were treated with isoamylase (295 U/g substrate; Sigma-Aldrich Chemical Co., St. Louis, MO, U.S.A.) at 40°C for 48 h and applied to a CarboPac PA-1 column (4×250 mm; Dionex), and then eluted with a linear gradient of 0–0.4 M sodium acetate in 150 mM sodium hydroxide at a flow rate of 1.0 ml/min [10]. As can be seen in Figs. 2A–2C, untreated corn starches showed branch-chain-length distributions

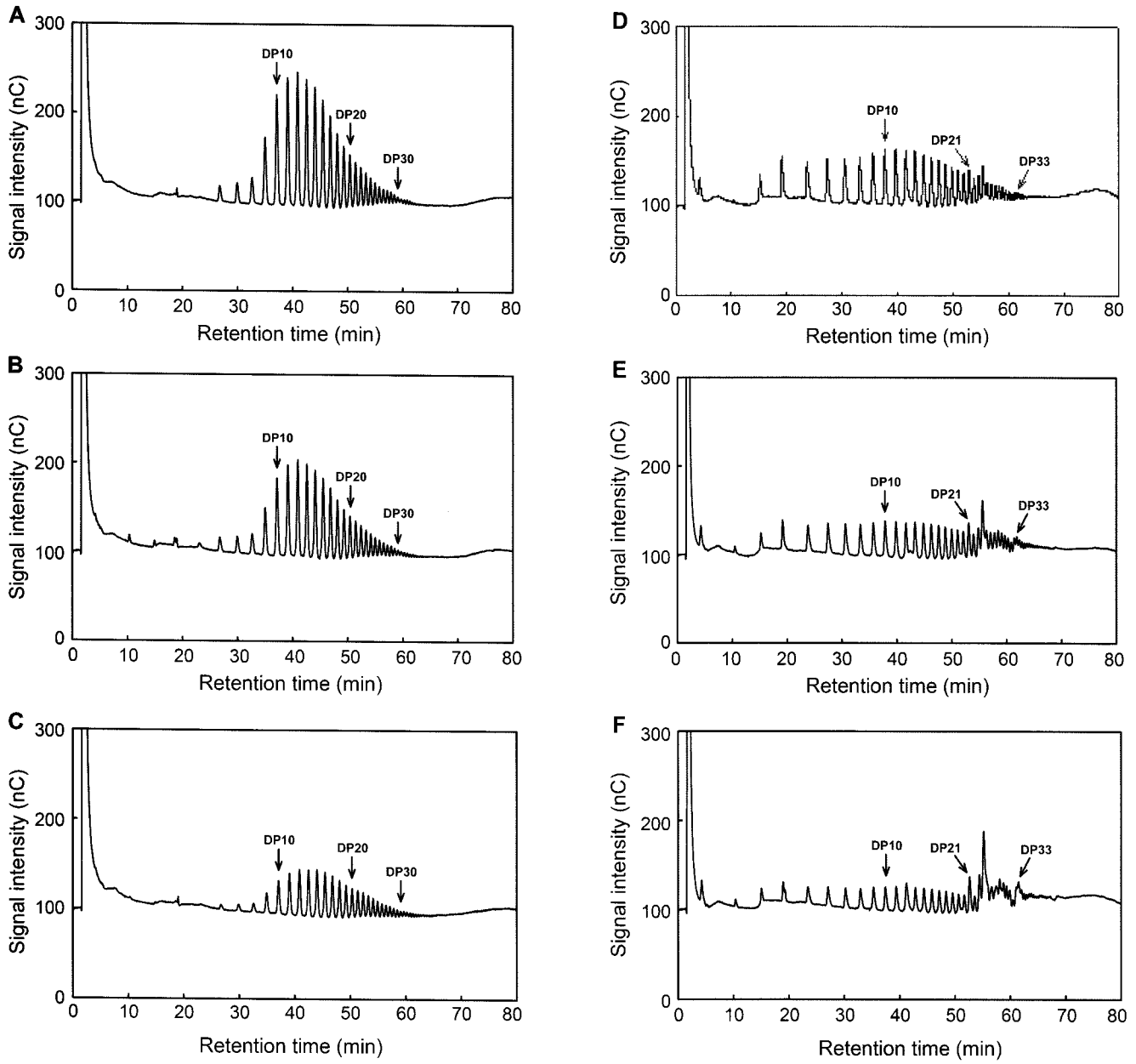

Fig. 2. HPAEC analysis of side-chain distributions of TAαGTase-treated corn starches.
A. Control waxy corn starch; **B**. control normal corn starch; **C**. control amylomaize V; **D**. TAαGTase-treated waxy corn starch; **E**. TAαGTase-treated normal corn starch; **F**. TAαGTase-treated amylomaize V. Samples were debranched with isoamylase (295 U/g substrate) at 40°C for 48 h and analyzed using a CarboPac PA-1 column (4×250 mm; Dionex).

from degree of polymerization (DP) 6 to around 37–40. On treating with TAαGTase, the relative proportions of both short and long branch chains increased when compared with those in untreated controls. It has been previously reported that *Thermus thermophilus* αGTase treatment of normal potato starch resulted in a broadened chain-length distribution from DP 2 to around DP 55 [18]. In the present study, an unusual pattern of chain length distributions was observed at around DP 21–35 following enzyme treatment, suggesting that CAs of various sizes were produced. Within this range of chain lengths, the peak intensity significantly increased in proportion to the amylose content of the corn starches. It should be noted that the DP range mentioned above may not be matched to the number of glucose moieties in cyclic glucan. Park *et al.* [8] showed an HPAEC chromatogram of a cycloamylose standard having an elution pattern and time that were very similar to those of the TAαGTase-treated amylose. This same elution pattern was obtained for the products of TAαGTase-treated corn starches in our study.

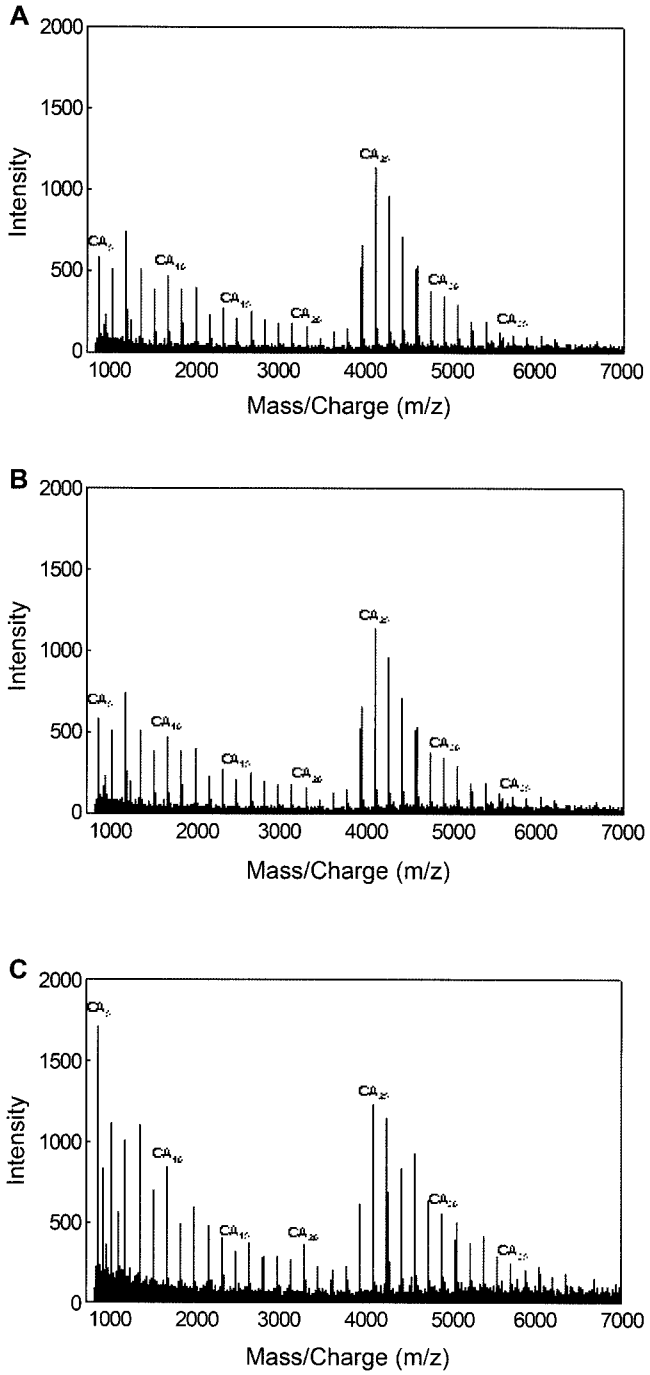

Fig. 3. MALDI-TOF-MS analysis of cycloamylose (CA) produced by TAαGTase.
A. Waxy corn starch; **B**. normal corn starch; **C**. amylomaize V. The theoretical *m/z* of $(CA)_n+Na^+$ is 162_n+23.

Formation of CAs by TAαGTase treatment was investigated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Voyager DE-STR Biospectrometry Workstation; Applied Biosystems Inc., Foster City, CA, U.S.A.). One μl of sample (0.1-0.001 mg/μl) was mixed with 1 μl of matrix (0.1-5 pmol/ml 2,5-dihydroxybenzoic acid or α-cyano-4-hydroxycinnamic acid) and then analyzed [12]. As shown in Fig. 3, the glucan profile was significantly affected by TAαGTase treatment. No signals were observed for untreated control starches, whereas cyclic glucans with DP 5-40 were found in all of the enzyme-treated samples. Thus, TAαGTase catalyzed the conversion of linear glucan chains into cyclic glucans of various sizes, from very small cyclodextrins to large ring structures. The highest signal intensity was displayed at CA_{25} regardless of starch type. However, the MALDI-TOF-MS analysis is not a quantitative method for determining the absolute amount of a product represented by an individual peak. Thus, we could not directly compare differences in the amount of CAs among the starch samples.

In conclusion, CAs are highly water soluble, are able to form inclusion complexes with inorganic and organic molecules [4, 14, 16], and can be produced by 4-α-glucanotransferase. CAs have various potential uses, as in food and drink preparations, food additives, infusion solutions, adhesives, and starch substitutes for biodegradable plastics [14, 17]. The results of the presented investigation suggest that corn starches with high amylose content can be used for the development of an efficient process for cycloamylose production with 4-α-glucanotransferase.

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