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Heterologous Expression and Characterization of Glycogen Branching Enzyme from *Synechocystis* sp. PCC6803

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A gene (*sll*0158) putatively encoding a glycogen branching enzyme (GBE, E.C. 2.4.1.18) was cloned from *Synechocystis* sp. PCC6803, and the recombinant protein expressed and characterized. The PCR-amplified putative GBE gene was ligated into a pET-21a plasmid vector harboring a T7 promoter, and the recombinant DNA transformed into a host cell, *E. coli* BL21(DE3). The IPTG-induced enzymes were then extracted and purified using Ni-NTA affinity chromatography. The putative GBE gene was found to be composed of 2,310 nucleotides and encoded 770 amino acids, corresponding to approx. 90.7 kDa, as confirmed by SDS-PAGE and MALDI-TOF-MS analyses. The optimal conditions for GBE activity were investigated by measuring the absorbance change in iodine affinity, and shown to be pH 8.0 and 30°C in a 50 mM glycine-NaOH buffer. The action pattern of the GBE on amylose, an α-(1,4)-linked linear glucan, was analyzed using high-performance anion-exchange chromatography (HPAEC) after isoamylolysis. As a result, the GBE displayed α-glucosyl transferring activity by cleaving the α-(1,4)-linkages and transferring the cleaved maltoglycosyl moiety to form new α-(1,6)-branch linkages. A time-course study of the GBE reaction was carried out with biosynthetic amylose (BSAM; $M_p \cong$ 8,000), and the changes in the branch-chain length distribution were evaluated. When increasing the reaction time up to 48 h, the weight- and number-average DP (DP_w and DP_n) decreased from 19.6 to 8.7 and from 17.6 to 7.8, respectively. The molecular size (M_p, peak $M_w \cong 2.45$–2.75×10^5) of the GBE-reacted product from BSAM reached the size of amylose (AM) in botanical starch, yet the product was highly soluble and stable in water, unlike AM molecules. Thus, GBE-generated products can provide new food and non-food applications, owing to their unique physical properties.

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Branching enzymes (BEs, 1,4-α-D-glucan: 1,4-α-D-glucan 6-α-D-(1,4-glucano)-transferase, E.C. 2.4.1.18) are known to play an important role in the biosynthesis of glycogen and amylopectin (AP) by transferring α-(1,4)-linkages into new α-(1,6)-linkages [3, 9]. Widely distributed in microorganisms, plants, and animal tissues, BEs are classified into two groups, starch BEs (SBEs) and glycogen BEs (GBEs). Although there is no essential difference in the basic biochemical activity of GBEs and SBEs, these two BE groups are separated based on the different structure of starch (semicrystalline granules) and glycogen (amorphous viscous dispersion), which is likely due to different α-(1,6)-branching frequencies (5% *vs.* 10%). In general, bacteria only contain one gene encoding a GBE, whereas plants express at least two SBE isoforms encoded by different genes [8, 25]. Several *in vitro* biochemical studies have already shown that different SBE isoforms in plants have different substrate specificities and branching manners. For example, in maize, SBE I is believed to transfer longer chains and preferentially acts on amylose (AM), in contrast to SBE IIa and SBE IIb that preferentially act on AP and transfer shorter chains, giving rise to the polymodel distribution of the branch-chain length of AP [7, 11]. In addition to the branching activity of GBEs, it has also been demonstrated that the GBE from *Bacillus stearothermophilus* catalyzes the cyclization reaction of AM and AP [26]. However, whether or not this activity has a role in glycogen or starch metabolism has yet to be elucidated.

Glycogen, which is composed of α-(1,4)-linked glucose residues with about 10% α-(1,6) branch linkages, is produced by many bacterial sources, as well as other diverse species [22]. Despite being a macromolecule with a similar chemical structure to AP, glycogen has a greater

proportion (10%) of α-D-(1,6)-glycosidic linkages and more random distribution of branch linkages [13]. The branch structures of glycogens have already been characterized using various analytical tools, such as the absorption spectra of glycogen-iodine complexes [5], molecular size distributions of debranched glycogen [28], and chemical analysis of the average chain length of glycogen [14]. Moreover, an advanced analytical technique using high-performance anion-exchange chromatography (HPAEC) enabled the proportion of individual branch chains in glycogen [15, 16, 21] and AP [29] to be determined, including a complete separation of the individual α-(1,4)-linked linear glucan chains released by isoamylolysis.

Cyanobacteria, previously known as blue-green algae, are photosynthetic prokaryotes that contain chlorophyll *a*, the same photosynthetic pigment used by plants. From an evolutionary point of view, cyanobacteria represent a link between bacteria and green plants, as they have been proposed to represent the endosymbiotic progenitors of plastids in plants [17]. Cyanobacteria and many other bacteria are known to produce glycogen as their carbohydrate reserve [6]. An analysis of the genomic DNA of *Synechocystis* sp. PCC6803 on the basis of sequence similarity with other organisms indicated that this cyanobacterium included one gene encoding a GBE [10, 31]. However, the biological function of this putative GBE gene has not yet been proven. Previously, the current authors generated a GBE-deficient mutant in *Synechocystis* sp. PCC6803 to determine whether this putative gene, *sll*0158, encodes a functional GBE [31]. As a result, it was proven that the mutant did not exhibit any GBE activity and accumulated insoluble α-(1,4)-linked linear glucan chains in the cells.

Food and non-food industries require starch with various types of structure [19], and until now physical and chemical treatments have been the predominant methods used to modify the structure of starch. However, with the advent of environmentally friendly technology, so-called green technology, and heightened consumer awareness, the biocatalytic modification of starch is attracting more attention. In addition to the sustainability of this processing technology, specifically tailor-made products can also be manipulated using biocatalytic processes. However, retrogradation is one of the detrimental phenomena when applying biocatalytic modification to starch, which is caused by the reassociation and recrystallization of α-(1,4)-linked linear glucan chains. Thus, the application of GBEs may provide an excellent solution based on the introduction of new branching points to the structure, resulting in shorter linear chain lengths, which in turn reduces the chances of glucan chain reassociation and inhibits recrystallization. Accordingly, in this study, a new *Synechocystis* GBE was heterologously expressed and biochemically characterized. Additionally, possible applications of the GBE reaction are discussed in relation to starch-related glucan substrates.

MATERIALS AND METHODS

Materials

The chemical components for the BG-11 medium were obtained from Fisher Scientific Co. (Pittsburgh, PA, U.S.A.), the molecular biology reagents obtained from Promega Biotech (Madison, WI, U.S.A.), and the potato amylose type 3 (PAM) and other chemicals purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.).

DNA Isolation, Expression Host, and Plasmids

The *Synechocystis* sp. PCC6803 (ATCC 27184) strain was grown at 25°C under continuous fluorescent light (40 μE m^2 s^{-1}) in a BG-11 medium supplemented with 5 mM glucose. The cell growth was monitored by measuring the absorbance of the cell cultures at 730 nm, and the cells were grown for 7–8 days (A_{730}=1.6–1.8) before genomic DNA extraction. The genomic DNA of *Synechocystis* sp. PCC6803 was isolated following the procedure of Ausubel *et al.* [1] with some modifications [31].

The isolated genomic DNA was used as a template for cloning the GBE gene. *E. coli* TOP10 [F^- *mrcA Δ(mrr-hdsRMS-Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 D(ara-leu)7697 galU galK rpsL endA1 nupG)*] (Invitrogen, CA, U.S.A.) was used for the gene manipulation and amplification. The *E. coli* BL21 (DE3) strain [F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)] (Novagen, Darmstadt, Germany) was used as the expression host, and the T-cloning vector (Takara, Tokyo, Japan) and pET-21a(+) (Novagen, WI, U.S.A.) were used as the DNA cloning and expression vectors, respectively.

Cloning and Overexpression of Putative GBE

The putative GBE gene, *sll*0158, was amplified by a PCR from the genomic DNA of *Synechocytis* sp. PCC6803 using Ex-*Taq* DNA polymerase (Takara, Tokyo, Japan) and pre-designed primers. The GBE gene-specific oligonucleotide primers were designed from the gene sequence of *sll*0158 and its 5'- and 3'-flanking regions [10]. The forward (5'-TT*CATATG*ACCTACACCATCAACGCTG-3') and reverse (5'-TT*CTCGAG*AGCTATGTTGCTAGCCTC-3') primers contained NdeI and XhoI restriction sites (*italics*), respectively. The PCR amplification was performed using the following heating cycle: an initial denaturation step at 98°C for 30 sec, denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 90 sec. This heating cycle was repeated 30 times and a last elongation was conducted at 72°C for 5 min. The amplified DNA fragments were purified using a PCR product purification kit (Promega, WI, U.S.A.) and digested with both NdeI and XhoI restriction enzymes (NEB, Ipswich, U.K.). The purified DNA fragment was ligated with T4 ligase (Roche, Mannheim, Germany) into a linearized T-cloning vector, and then subcloned into pET-21a to create the final pET21a-GBE. In this final construction, the C-terminus of the *sll*0158 gene product was extended with six additional histidine residues (His-tag). The other genetic manipulations were performed as described by Sambrook and Russell [23].

Enzyme Purification from Recombinant *E. coli*

The *E. coli* BL21 (DE3) transformant harboring pET21a-GBE was grown in an LB (Luria-Bertani) broth supplemented with 100 μg of ampicillin per ml at 37°C until the absorbance change at 600 nm reached 0.6. Expression of the recombinant GBE was then induced by IPTG (isopropyl-β-D-thiogalactoside; 0.1 mM) during 12 h of incubation at 16°C. The IPTG-induced cells were harvested by

centrifugation (5,000 ×*g* at 4°C for 20 min) and resuspended in 50 mM Tris-HCl (pH 7.0). A crude cell extract of the *E. coli* transformant was prepared using a cell disruptor (Sonic Dismembrator Model 100; Fisher Scientific, PA, U.S.A.). After centrifugation (10,000 ×*g* at 4°C for 20 min) of the crude extract, the supernatant was loaded on a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatographic column (Qiagen, Hilden, Germany) and the pure recombinant GBE obtained by eluting with a 250 mM imidazole buffer (50 mM Tris-HCl containing 300 mM NaCl). The purified GBE was concentrated using an Amicon Ultra-15 (MWCO 30,000; Millipore, CA, U.S.A.), and the protein concentration determined by the Bradford method [4].

Molecular Mass Determination of Recombinant GBE

The molecular mass of the GBE was measured by SDS-PAGE [12] and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Applied Biosystems Inc., CA, U.S.A.). The separating gel was prepared as 12% (w/v) acrylamide gel using a stock solution of 30% (w/v) acrylamide containing 1% (w/v) *N,N'*-methylene bisacrylamide. The electrophoresis was carried out at 80 V for stacking or 120 V for a separating gel, and a PageRuler Prestained Protein Ladder (Fermentas, St. Leon-Rot, Germany) was used as the molecular weight marker. The MALDI-TOF-MS analysis was performed in a positive mode using 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix.

Enzyme Assay Using Lugol's Method

The change in the iodine-binding capacity of PAM by converting α-(1,4)-linkages to α-(1,6)-linkages was used to quantify the GBE activity. The assay mixture containing 0.05% PAM, a 50 mM glycine-NaOH buffer (pH 8.0), and the GBE was incubated at 30°C for 10 min. The reacted samples (0.1 ml) were mixed with a 0.02% iodine/potassium iodide solution (Lugol's solution, 1.0 ml), and the absorbance at 620 nm was measured using a spectrophotometer (Beckman DU 730; CA, U.S.A.). One unit of GBE activity was arbitrarily defined as the amount of enzyme that caused a change in the absorbance of 1 unit during a 10-min reaction under the above conditions [7].

Analysis of Side Chain Distribution Using High-Performance Anion-Exchange Chromatography (HPAEC)

A PAM solution (1.0%, 1 ml, w/v) was reacted with 0.1 U (specific activity, 0.68 U/mg) of the GBE for 24 h at the optimal conditions described above. The resulting product was reacted with isoamylase (E.C. 3.2.1.68; Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) for the specific hydrolysis of α-(1,6)-linkages in a 10 mM sodium acetate buffer (pH 3.5). The side-chain distribution of the debranched product was measured using an HPAEC (DX-300; Dionex Corp., CA, U.S.A.) equipped with a CarboPac PA-1 pellicular anion-exchange column (Dionex Corp., CA, U.S.A.) and pulsed amperometric detector (PAD; Dionex Corp., CA, U.S.A.). The separation of the linear glucan chains was achieved using a linear gradient mode from 150 mM NaOH to 600 mM sodium acetate (in 150 mM NaOH) for up to 100 min [30].

Analysis of Molecular Size Distribution Using High-Performance Size-Exclusion Chromatography (HPSEC)

Biosynthetic amylose (BSAM; M_p, peak $M_w \cong 8,000$) obtained from an amylosucrase (AS; E.C. 2.4.1.4) reaction using 1 M sucrose as the substrate [20] was used as the substrate for the GBE reaction. The change in the molecular size distributions of the GBE-treated BSAM was determined using an HPSEC (Summit HPLC system; Dionex Corp., CA, U.S.A.). The GBE-treated samples were separated using sequentially connected Shodex SB-806 HQ and Shodex SB-804 HQ columns (Showa Denko Co. Ltd., Tokyo, Japan), and detected by a refractive index (RI) detector. The mobile phase was deionized water (18.2 MΩ) at a flow rate of 0.6 ml/min. Shodex pullulan standards (P-20, P-50, P-100, P-200, P-400, and P-800; Showa Denko Co. Ltd., Tokyo, Japan) were used to generate a calibration curve from which the relative molecular size of the GBE-treated BSAM was calculated with no further correction.

RESULTS AND DISSCUSSION

Cloning and Expression of *Synechocytis* GBE in *E. coli*

Based on the whole genome sequence of *Synechocystis* sp. PCC6803, a putative GBE gene (*sll*0158) was identified. The *sll*0158 gene was cloned in a pET-21a vector and expressed in *E. coli* as a recombinant protein tagged with His-tagged residues at the C-terminus. The nucleotide sequence of the putative GBE gene and its deduced amino acid sequence had an open reading frame encoding 770 amino acid residues, initiating from an ATG and ending with a TAG. The DNA sequence of this putative GBE gene was analyzed using an ABI 3730xl sequencer (Applied Biosystems, CA, U.S.A.) and found to match completely with the previously published *sll*0158 sequence of *Synechocystis* sp. PCC6803 (data not shown) [10].

Purification and Characterization of Recombinant GBE

The recombinant putative GBE was expressed by IPTG induction in *E. coli* BL21 (DE3), as described in Materials and Methods. The cells harboring the overexpressed His-tagged putative GBE were then disrupted, and the supernatant after centrifugation was used as the crude enzyme extract. A pure GBE preparation was achieved using Ni-NTA agarose affinity chromatography. The GBE was purified 3.4-fold with a yield of 38.4% from the cell extract (Table 1). SDS-PAGE of the final GBE preparation revealed a single band when compared with the cell extract (Fig. 1A), and the band was approximately 90.3 kDa when compared with the commercial protein marker. To confirm the exact molecular weight, the purified GBE molecular mass was

Table 1. Purification table for GBE expressed in *E. coli.*

Steps	Total volume (ml)	Total activity (unit)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell extract	50	87.42	300.55	0.29	100	1
Ni-NTA	10	33.59	33.68	1.00	38.42	3.45

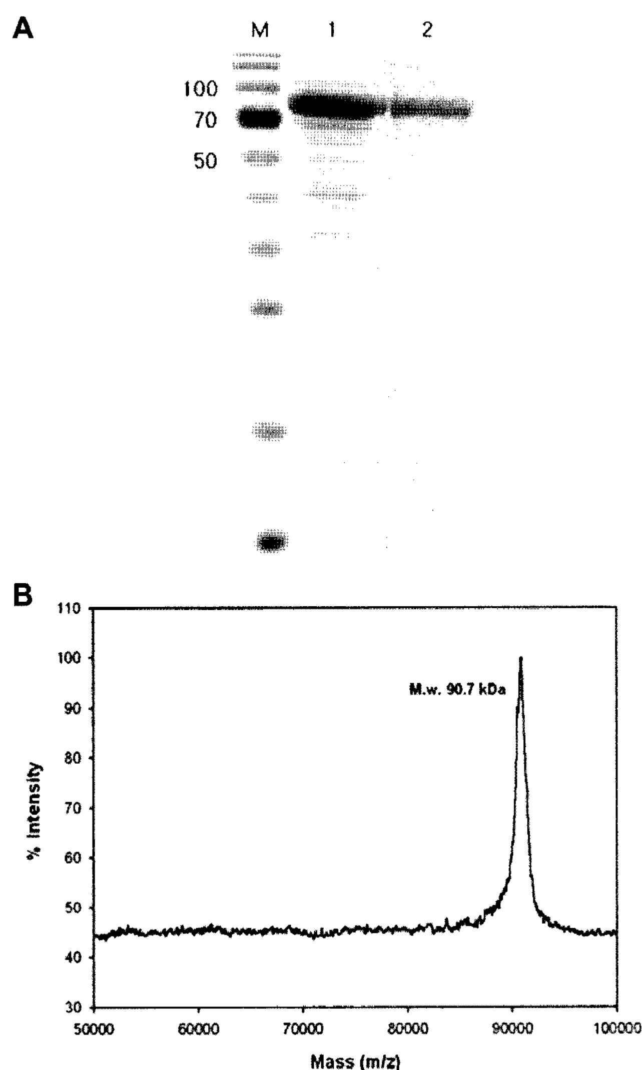

Fig. 1. SDS and MALDI-TOF-MS analyses of GBE expressed in *E. coli* BL21 (DE3).

A. The overexpressed protein was analyzed using SDS-PAGE. Lane M, commercial standard maker (Fermentas, St. Leon-Rot, Germany); lane 1, Cell extract of *E. coli* BL21 (DE3) harboring pET21a-GBE; lane 2, purified histidine-tagged GBE using Ni-NTA affinity column chromatography. **B.** MALDI-TOF-MS analysis of purified GBE using Ni-NTA chromatography (90,739 Da).

confirmed by a MALDI-TOF-MS analysis (Fig. 1B) to be about 90.7 kDa, which agreed well the molecular mass estimated using the pI/M_w tool (90.3 kDa) from Swiss-Prot [2].

Optimal Reaction Conditions for GBE Activity

The optimal temperature for the recombinant GBE was 30°C (Fig. 2A) with the greatest enzyme activity at pH 8.0 when using a 50 mM glycine-NaOH buffer (Fig. 2B). As the GBE activity was found to depend on the type of buffer, as well as the pH, the subsequent enzyme reactions were carried out in a 50 mM glycine-NaOH buffer (pH 8.0). However, when not considering the buffer effect, the GBE displayed activity across a fairly wide pH range, maintaining more than 80% of its maximum activity within pH 6–9.

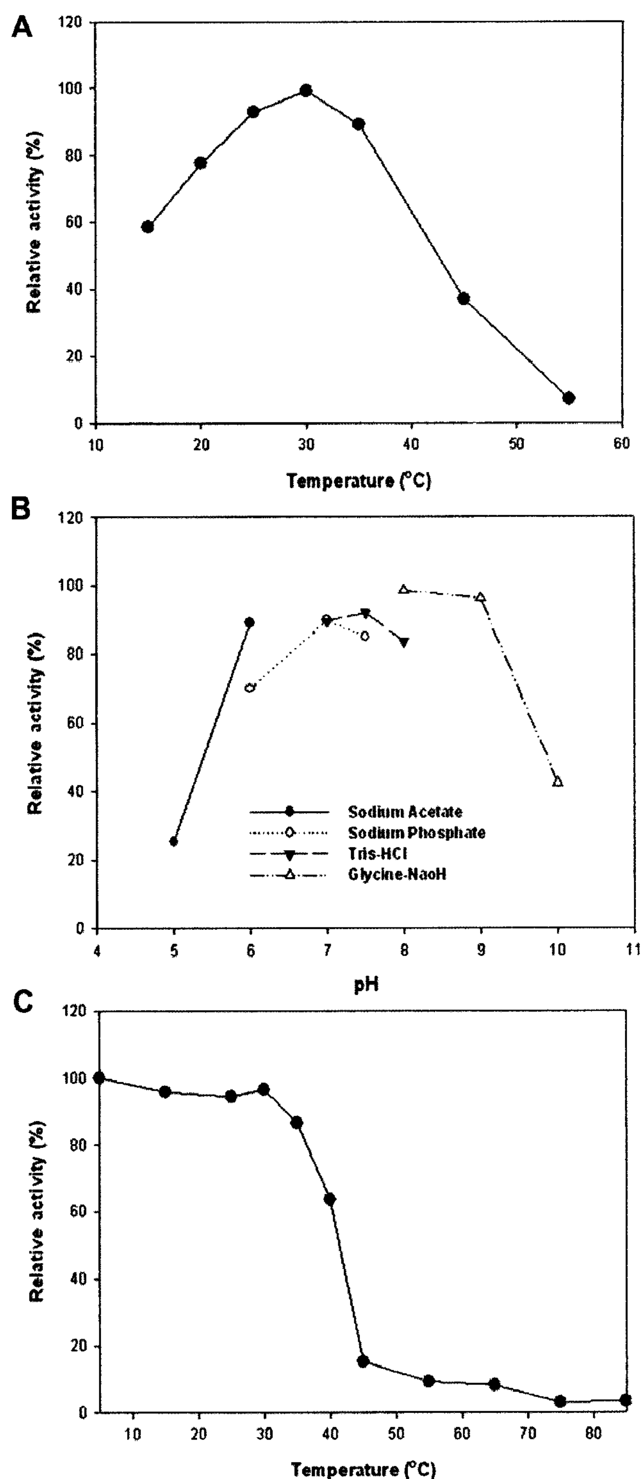

Fig. 2. Optimal temperature and pH conditions for reaction of purified GBE expressed in *E. coli*.

A. Effect of temperature on enzyme activity of purified GBE. **B.** Effect of pH on enzyme activity of GBE. **C.** Thermostability of GBE at various temperatures.

Meanwhile, the thermal stability of the GBE was examined across a temperature range of 5–85°C (Fig. 2C). After heating for 30 min, the GBE did not display any apparent inactivation up to 30°C, yet began to lose its activity

at 35°C. When the temperature reached 50°C, the enzyme displayed less than 10% of its maximum activity. Therefore, these results indicated that the GBE from *Synechocystis* sp. PCC6803 has a relatively low thermal stability.

Reaction Pattern of Recombinant GBE on Potato Amylose (PAM) and Biosynthetic Amylose (BSAM)

The glucan chain-length distributions were analyzed by HPAEC using a commercial PAM substrate essentially composed of α-(1,4)-linkages. The new branch chains generated by the reaction of the GBE with PAM were then released by the hydrolyzing activity of isoamylase that is specific for α-(1,6)-linkages. Without isoamylolysis, only a negligible amount of short- and intermediate-size chains was detected in the GBE-treated PAM [18], whereas no peak was detected with the native PAM (Fig. 3A), suggesting that the apparent hydrolyzing activity of GBE would only seem to occur on the donor molecule side [24, 26, 27]. The intermolecular transferring activity of GBE allows a certain length of glucan chain to be moved to another chain, meaning the residual glucan chain cannot be used anymore as a donor molecule and thus remains unchanged. Thus, after isoamylolysis, the GBE-treated PAM displayed a substantial amount of linearized glucan chains released from the newly generated α-(1,6)-linked product, with a maximum peak at DP6 (Fig. 3B). Meanwhile, the native PAM showed a small yet detectable amount of maltooligosaccharide chains after isoamylolysis, indicating that the commercial PAM was not pure and partially contaminated with the branched structure of amylopectin. The reaction pattern of the recombinant GBE was further investigated using biosynthetic amylase (BSAM) as the substrate. The BSAM used in this study essentially contained no α-(1,6)-linkages, as isoamylolysis did not produce any change in the chain distribution profile of the BSAM (data not shown). A time-course study of the GBE reaction was carried out, and the changes in the branch-chain length distribution were evaluated. When increasing the reaction time up to 48 h, the weight- and number-average DPs (DP_w and DP_n) decreased from 19.6 to 8.7 and from 17.6 to 7.8, respectively (Fig. 4), indicating that the average side-chain length on the branched structure became smaller and then unchanged when the side chains could no longer be used as donors. However, since this analysis did not provide information about the molecular weight change during the GBE-derived transferring reaction, the molecular size change was monitored during the GBE reaction using an HPSEC analysis.

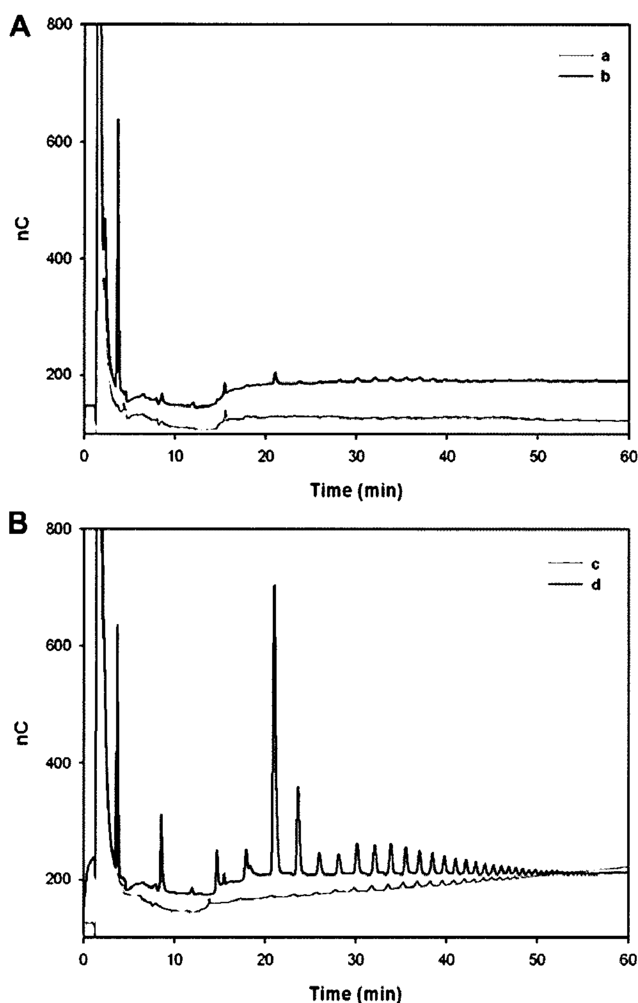

Fig. 3. Change in side-chain distributions after GBE reaction with potato amylose.

A. Without isoamylolysis data: amylose (a) and GBE-treated amylose (b). **B.** With isoamylolysis data: amylose (c) and GBE-treated amylose (d).

Molecular Size Distribution of GBE Reaction Products

To investigate the changes in the molecular size distribution of the GBE-treated products as regards the linear α-(1,4)-linked glucan chains, BSAM (M_p, peak $M_w \cong 8{,}000$) was used as the substrate under the optimal GBE reaction conditions. The HPSE chromatogram of the GBE-treated

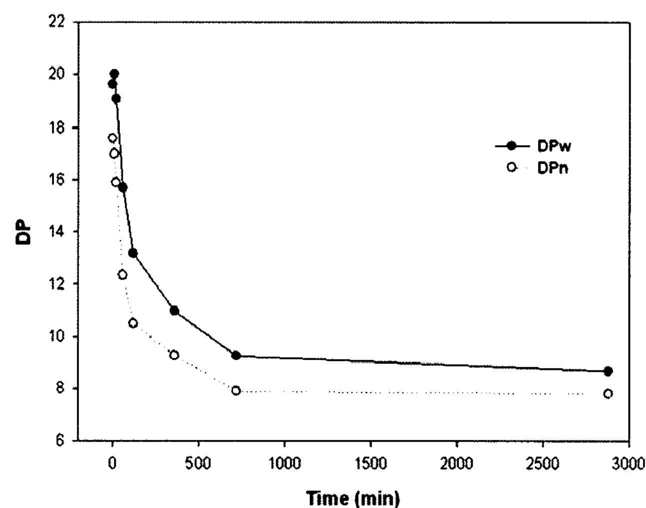

Fig. 4. Change in weight- and number-average DPs (DP_w and DP_n) for BSAM during GBE treatment.

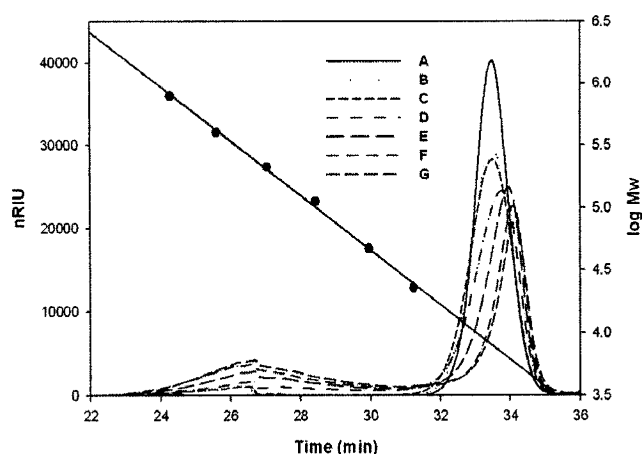

Fig. 5. Estimated molecular weight of GBE-treated BSAM using high-performance size-exclusion chromatography.
A, 0 min. B, 10 min. C, 20 min. D, 1 h. E, 2 h. F, 24 h. G, 48 h.

BSAM clearly showed a bimodal size distribution (Fig. 5). The first peak with a shorter elution time was newly produced by the inter-chain transglucosylating reaction of the GBE. The size distribution of this peak was much broader (M_r relative M_w=3.0×10^4–3.0×10^6) than that of the original BSAM, and its Mp value about 30 times greater. The peak area corresponding to the newly produced macro-size glucans (M_p≅2.45–2.75×10^5) continued to increase as the GBE incubation proceeded. However, whereas the molecular size of the GBE treatment product reached the size of amylose in general botanical starch, the physical properties of the product were totally different, as it was highly soluble and stable in water, and gave a clear solution [24]. These results correspond with those for amylopectin, which is more soluble in water than amylose owing to its branched α-(1,6)-linkages. In contrast, the second peak with a longer elution time shifted when further increasing the elution time, while the peak area decreased. The relative M_p decreased gradually from 8.0×10^3 to 5.8×10^3 during the 48-h enzyme reaction. Theoretically, if the GBE reaction had occurred within the same molecule, there would have been no change in the M_w of the substrate.

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