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Overexpression of the *gct* Gene Encoding 4-α-Glucanotransferase of a Hyperthermophilic Archaeon, *Thermococcus litoralis*

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The gene encoding a extremely thermostable 4-α-glucanotransferase from a hyperthermophilic archaeon, *Thermococcus litoralis,* was cloned, sequenced and expressed in *Escherichia coli*. The amino acid sequence of the enzyme was distantly related to other functionally-related ones, such as D-enzymes. The enzyme is of industrial interest because of a novel activity of producing cycloamylose and is also important for fundamental studies of protein, sugar-metabolizing enzymes. In this paper, the overexpression of 4-α-glucanotransferase in *E. coli* was carried out expression vector system with *lac* and T7 promoters. The enzyme was successfully overexpressed, and purified by the heat treatment of a cell-free extract, successive Butyl-Toyopearl and Mono Q chromatographies. The purified recombinant enzyme showed the same specific activity and the same mobility in SDS-PAGE as natural enzyme.

Key words – Overexpression, 4-α-Glucanotransferase, Hyperthermophilic archaeon, *Thermococcus litoralis,* Thermostable enzyme

Enzymes from organisms inhabiting extremely hot environments represent fascinating objects for basic as well as applied research. On one hand, the industry is interested in thermostable biocatalysts for biotechnological processes demanding elevated temperature. On the other hand, considerable efforts have been made over the last years to understand how the proteins of extreme thermophiles maintain their structures and functions at high temperatures [3].

4-α-Glucanotransferase (EC 2.4.1.25) transfers a segment of α-1,4-glucan to a new position of an acceptor, which is glucose or another α-1,4-glucan. And it catalyzes the intramolecular transglycosylation (cyclization) of amylose, yielding cyclic α-1,4-glucan (cycloamylose) [4]. Transglycosylation catalyzed by amylases and their related enzymes has been utilized in the industry for the production of various oligosaccharides. A similar enzyme is also present in plants and is called disproportionating enzyme (D-enzyme) (EC 2.4.1.25). Analyses of the activity of *E. coli* [9], *Thermus aquaticus* ATCC 33923 [14], *Clostridium butyricum* [1], *Chlamydia psittaci* [2] and the activities of the potato [5] and barley [16] enzymes indicated that amylomaltase and D-enzyme catalyze similar reactions.

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Cycloamylose is higher polymerized than cyclodextrins, and therfore shows improved features to form inclusion complexes, and to dissolve in water [8]. For these properties, cycloamylose is expected to be used in future industry. However, the molecular mechanism for the formation of such a large cyclic glucan remains unclear because structural information of the enzyme has never been reported.

The heterologous expression in *E. coli* is one of the most frequently used techniques in the laboratory and industry. When a strong promoter is used to express recombinant proteins, a yield of more than 10% of the total *E. coli* proteins can often be obtained. The gene encoding 4-α-glucanotransferase was cloned, sequenced and expressed in *E. coli* from *Thermococcus litoralis,* a hyperthermophilic archaeon [4]. The enzyme is of industrial interest because of a novel activity of producing cycloamylose and is also important for fundamental studies of protein such as X-ray crystallography. Therefore, a large scale preparation of the enzyme is required. If the 4-α-glucanotransferase gene could be overexpressed in *E. coli,* the enzyme could be easily purified from the cells, in particular, by heat treatment, because of its hyper thermostability. Here we report overexpression of 4-α-glucanotransferase by site-directed mutagenesis and its purification. The level of purified recombinant protein reached approximately 8.8 mg/L.

Material and Methods

Materials

Butyl-Toyopearl 650M was purchased from Tosoh Corp (Tokyo, Japan). Mono Q was purchased from Pharmacia LKB Biotechnology. Protein standards for the molecular weight determination on SDS-PAGE were purchased from Pharmacia LKB Biotechnology and Roche Applied Science, respectively. Malto-oligosaccharides were purchased from Wako Chemical Industries Ltd (Tokyo, Japan). Glucose oxidase, peroxidase, 2,2′-azino-di-(3-ethylbenzthiazoline)-6-sulfonate (ABTS), restriction endonucleases and polynucleotide kinase were purchased from Roche Applied Science. Agarose powder and T4 DNA ligase were from Takara Shuzo (Kyoto, Japan). The ingredients of *E. coli* media were purchased from Difco. Isopropyl-β-D-thiogalactopyranoside (IPTG) and ampicillin were purchased from Sigma.

Bacterial strains and DNA manipulation

The *E. coli* strains used in this study are listed in Table 1. The plasmid pUC19 and pUC119 were purchased from Takara Shuzo and pET-15b was purchased from Novagen. The recombinants DNA techniques used were essentially based on the methods described by Sambrook *et al.* [10]. Plasmid DNA isolated by the Wizard Plus Minipreps DNA Purification System (Promega).

Construction of plasmids

The plasmid pJGT1 contained a 3.6 kb *Hin*dIII fragment was digested with *Hin*dIII/*Sma*I. A 2.6 kb *Hin*dIII/*Sma*I fragment containing the *T. litoralis* 4-α-glucanotransferase gene was subcloned into pUC119, and pJGT2 was obtained (Fig. 1). The plasmid pJGT3, in which the correct reading frame was placed at an appropriate distance downstream of the vector promoter, *i.e.* the initiations codon adjacent to the Shine-Dalgarno sequence of the *lac* promoter, was constructed from the plasmid pJGT2 containing a 2.6 kb *Hin*dIII/*Sma*I insert, through site-directed mutagenesis. Site-directed mutagenesis of pJGT2 was done to delete 5′-flanking region of the gene by Kunkel's method [6] using an oligonucleotide having a sequence 5′-GATGCCA-AATATGAAGTTTATTCTTTCCATAGCTGTTTCCTGTGTG AAATGTT-3′ (Fig. 1). Uracil-containing single-strand DNA was prepared from *E. coli* MV1184 (pJGT2) and helper phage M13KO7 [14].

On the other hand, the expression vector for the T7 promoter was construct. *Nco*I/*Xho*I sites were introduced by site-directed mutagenesis into the plasmid pJGT2 containing the 2.6 kb *Hin*dIII/*Sma*I fragment. The resulting plasmid was designated as pJGT4 (Fig. 2). The expression plasmid pUT7 was constructed as follows; the plasmids pET15b and pUC19 were digested using *Bgl*II/*Agt*II and *Pvu*II/*Aat*II, respectively. The *Bgl*II site of 0.6 kb *Bgl*II/

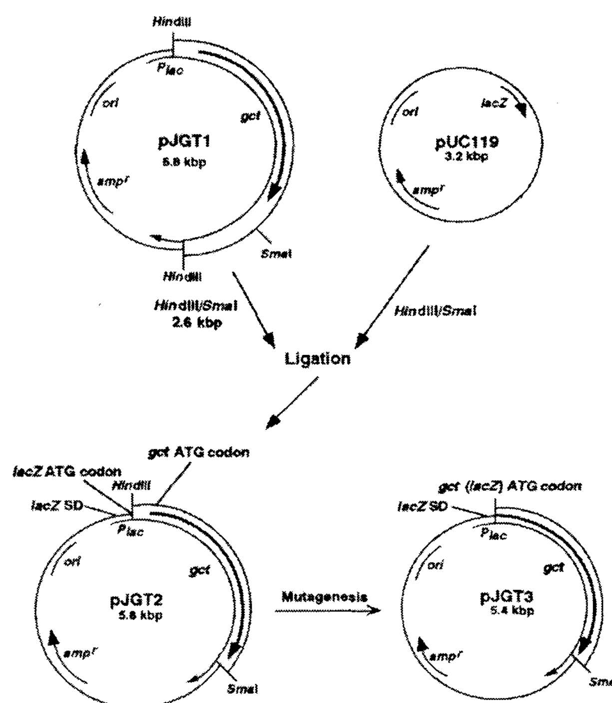

Fig. 1. Construction of the overexpression plasmid pJGT3 for the 4-α-glucanotransferase gene.
The position and direction of the 4-α-glucanotransferase gene are indicated by thick arrows. *amp*^{r}, ampicillin resistance gene; *ori*, replication origin of the plasmid; *gct*, the gene for 4-α-glucanotransferase.

Table 1. Bacterial strains used for this study

Strains	Relevant genotype
MV1184	*ara*, Δ(*lac-proAB*), *rspL*, *thi*(Φ80d*lacZ*ΔM15), Δ(*stl-recA*) 306::Tn10(*tet*^{r})/F′:*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15
JM109	*recA1*, *endA1*, *gyrA96*, *thi1*, *hsdR17*, *supE44*, *relA1*, Δ(*lac-proAB*)/F′ *traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15
BL21(DE3)	*E. coli* B, F$^-$, *dcm*, *ompT*, *hsdS*($r_B^- m_B^-$) *gal*(DE3)
BL21(DE3)pLysS	*E. coli* B, F$^-$, *dcm*, *ompT*, *hsdS*($r_B^- m_B^-$) *gal*(DE3) (pLysS Cam^r)

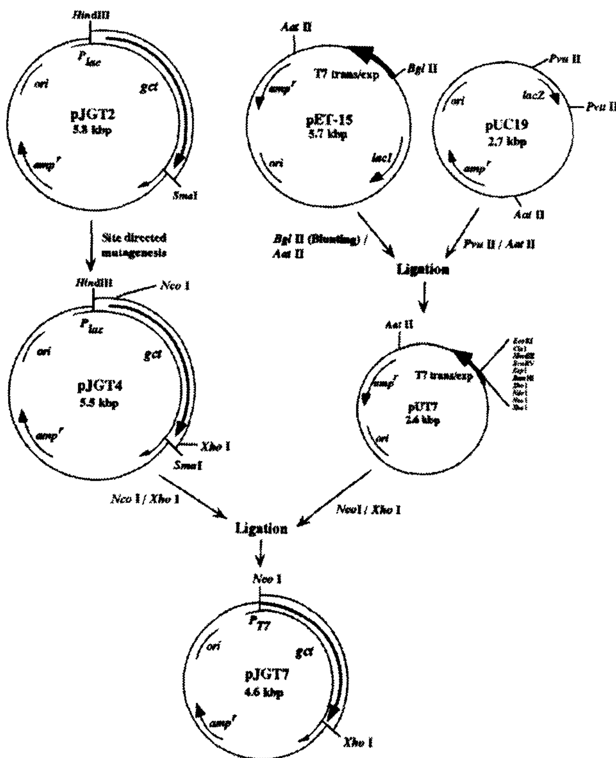

Fig. 2. Construction of the overexpression plasmid pJGT7 for the 4-α-glucanotransferase gene.
The position and direction of the 4-α-glucanotransferase gene are indicated by thick arrows. *amp*^{r}, ampicillin resistance gene; *ori*, replication origin of the plasmid; *gct*, the gene for 4-α-glucanotransferase.

*Agt*II fragment containing plasmid pET15b was made by blunt T4 DNA polymerase and blunted *Bgl*II site containing 0.6 kb *Bgl*II/*Agt*II fragment and 2.0 kb *Pvu*II/*Aat*II fragment containing plasmid pUC19 were ligated by T4 ligase. The resulting plasmid was designated as pUT7. And then, the plasmids pJGT4 and pUT7 were digested using *Nco*I/*Xho*I. A 2.6 kb *Nco*I/*Xho*I fragment of pJGT4 was ligated into pUT7 *Nco*I/*Xho*I sites. The resulting plasmid was designated as pJGT7 (Fig. 2). The confirmation of the recombinant plasmids were done by the dideoxy chain termination method of Sanger et al. [11] with ABI PRISM Dye Terminator Cycle Sequencing Core Kit using an Applied Biosystems model 377 DNA sequencer.

Assay of enzyme activity

The 4-α-glucanotransferase activity was determined by the GOD-POD method [12] as reported previously [4].

Overexpression and purification of the enzyme

E. coli MV1184 and JM109 cells harboring a recombinant plasmid (pJGT2 or pJGT3) carrying the 4-α-glucanotransferase gene were grown for 3 h at 37℃ in LB medium containing 100 μg/ml ampicillin. The *lac* promoter was subsequently induced by the addition of 0.5 mM IPTG. The cells were allowed to grow for an additional 12 h, with vigorous shaking, then cellected by centrifugation (7,000×g for 10 min), and resuspended in 20 mM sodium phosphate buffer, pH 6.0 (buffer A). The suspension was sonicated and centrifuged (20,000×g for 30 min). The supernatant was incubated for 1 h at 80℃ and centrifuged to remove denaturated proteins. This supernatant was used as the crude cell extract. The crude cell extract was applied to a Butyl-Toyopearl 650 M column (3 cm×27 cm) equilibrated with buffer A, 30% ammonium sulfate. The enzyme was eluted with a decreasing linear gradient of ammonium sulfate from 30% to 0 in buffer A. The active fractions were combined, dialyzed against buffer A, and applied to a Mono Q column (0.5 cm×5 cm) equilibrated with buffer A. After the column had been washed with buffer A, the enzyme was eluted with a linear gradient of NaCl from 0 to 0.5 M in buffer A. The active fractions were pooled and dialyzed against buffer A, and stored at -80℃. The chromatography columns were controlled with an FPLC system (Pharmacia).

E. coli BL21(DE3) and BL21[(DE3)pLysS] cells harboring a recombinant plasmid (pJGT7) carrying the 4-α-glucanotransferase gene were grown at 37℃ in LB medium containing 100 μg/ml ampicillin for 3 h. The T7 promoter was subsequently induced by the addition of 1 mM IPTG. The cells were allowed to grow for an additional 3 h, with vigorous shaking, then cellected by centrifugation (7,000×g for 10 min), resuspended in 20 mM sodium phosphate buffer (pH 6.0). Purification of the enzyme was performed by the same procedures as described above.

SDS-PAGE

SDS-PAGE was using method of Laemmli [7], a separating gel (10% acrylamide in 0.38 M Tris-HCl, pH 8.8) and stacking gel (4% acrylamide in 0.38 M Tris-HCl, pH 6.8) was performed at 25 mA and 25℃ in 25 mM Tris-HCl containing 192 mM glycine, and 0.1% SDS. The sample protein (15 μl) were mixed with 2×SDS sample buffer and thermally denaturated at 98℃ for 5 min prior to electrophoresis. The gels were stained with a solution containing 0.1% Coomassie brilliant blue R-250, 40% methanol, and 10% acetic acid and destained with a solution containing

10% methanol and 10% acetic acid.

Protein determination

Protein concentrations were determined with bicinchoninic acid protein-assay kit (Pierce Chemical Co.) with bovine serum albumin as a standard protein.

Thin Layer Chromatography

Reaction products derived from oligosaccharides by the 4-α-glucanotransferase were identified by thin layer chromatography (TLC) on precoated silica gel plates (Kiesel gel $60F_{254}$, Merck). The products were developed by means of multiple ascents with a solvent system of 1-butanol/ethanol/water (5:5:3), and were detected by spraying 3% sulfuric acid in methanol and then baking at 140℃ for 10 min.

Results and Discussion

Overexpression of 4-α-glucanotransferase gene from *T. litoralis* in *E. coli* and purification of the enzyme

For overexpression of 4-α-glucanotransferase in *E. coli*, the residual sequence not possibly related to the enzyme gene expression in pJGT1 was removed to obtain an expression vector pJGT2. The plasmid pJGT2 contained a 2.6 kb *Hin*dIII/*Sma*I insert (Fig. 1), which was subjected to site-directed mutagenesis to construct an overexpression plasmid pJGT3 (Fig. 1). The resulting overexpression plasmid pJGT3 placed the gene in the correct reading frame at an appropriate distance downstream of the vector promoter. The resultings construct positions the initiation codon of the gene adjacent to the Shine-Dalgarno sequence of the *lac* promoter.

As expected, the *T. litoralis* 4-α-glucanotransferase could be easily purified from *E. coli* harboring the plasmid (pJGT3) by heat treatment of cell-free extract (80℃ for 1 h) as previously reported [13], and by successive Butyl-Toyopearl and Mono Q chromatographies. The purification of recombinant enzymes is summarized in Table 2. The purified recombinant enzyme showed the same mobility in SDS-PAGE as the natural enzyme (Fig. 3). These results confirm that the cloned gene actually encodes *T. litoralis* 4-α-glucanotransferase, and that the recombinant enzyme retains catalytic activity (Fig. 4). The 4-α-glucanotransferase from *T. litoralis* was overexpressed in *E. coli*. The recombinant enzyme from *E. coli* MV1184 (pJGT3) was purified

Table 2. Purification of 4-α-glucanotransferase from *E. coli* MV1184 harboring pJGT3

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)
Cell-free extract	108	63.2	0.56	100
Heat treatment	32.8	61.8	1.88	97.8
Butyl-Toyopearl	8.72	35.2	4.04	55.7
Mono Q	1.76	25.2	14.32	39.9

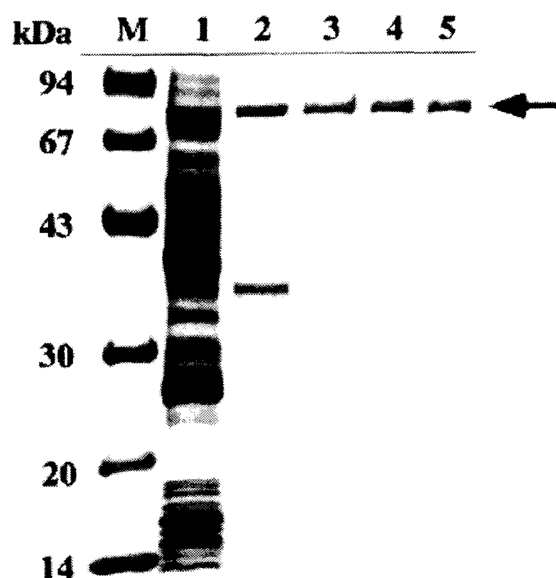

Fig. 3. SDS-PAGE of 4-α-glucanotransferase produced in *E. coli* harboring pJGT3.
Lanes M, size standard markers; lane 1, cell-free extracts obtained by centrifugation of disrupted cells by sonication; lane 2, soluble fraction obtained by centrifugation of heat-treated cell-free extracts at 80℃ for 1 h; lane 3, sample after Butyl-Toyopearl chromatography; lane 4, sample after Mono Q chromatography; lane 5, purified 4-α-glucanotransferase from *T. litoralis*. The arrow indicates the position of 4-α-glucanotransferase. The numbers on the left are the estimated molecular masses of the marker proteins.

through heat treatment with higher yield of 4.7-fold than does that from pJGT2 (Table 3).

The 4-α-glucanotransferase from *T. litoralis* was expressed in *E. coli* JM109. The recombinant enzyme was purified through heat treatment from *E. coli* JM109 (pJGT3) with higher yield than from *T. litoralis*. However, 4-α-glucanotransferase from JM109 (pJGT3) was not expressed at a high level (Table 3). On the other hand, the *gct* gene on the expression vector pJGT7 was expressed by T7 promoter in *E. coli*. But, the yield of the recombinant en-

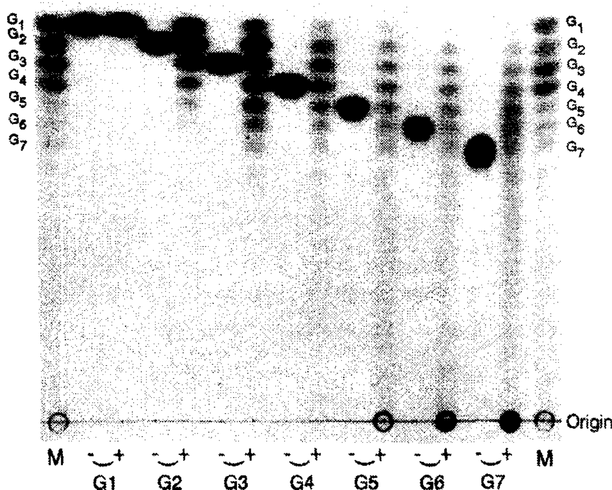

Fig. 4. Thin-layer chromatogram of reaction products from various substrates by the 4-α-glucanotransferase which was purified from *E. coli* MV1184 harboring pJGT3. The reaction mixtures, containing 20 mM sodium phosphate buffer (pH 6.0). 1% malto-oligosaccharides (G_1-G_7) and 0.1 μg of the purified recombinant enzyme, were incubated with(+) or without(-) the enzyme at 90 ℃ for 2 h. M, malto-oligosaccharides (G_1-G_7). Substrates: G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose; G_6, maltohexaose; G_7, maltoheptaose.

Table 3. Comparison of 4-α-glucanotransferase activity produced by *E. coli* strains harboring various plasmids and *T. litoralis* cells

Strains	Activity (U/g of wet cell)
MV1184 (pUC119)	0
T. litoralis	0.19
MV1184 (pJGT2)	0.55
MV1184 (pJGT3)	8.40
JM109 (pJGT3)	5.44
BL21[(DE3)pLysS] (pJGT7)	7.50
BL21(DE3) (pJGT7)	2.31

zyme produced was lower than that produced from pJGT3 (Table 3).

In summary, a procedure has been developed for the overexpression and purification of large quantities of 4-α-glucanotransferase. The expression of the *gct* gene in *E. coli* MV1184 (pJGT3) was the greatest, while the expression of the gene in *E. coli* JM109 (pJGT3) was approximately 65% of the highest one. The expression of the *gct* gene in *E. coli* BL21[(DE3)pLysS] (pJGT7) proceeded by the T7 promoter was 88%, but the *gct* gene in *E. coli* BL21(DE3) (pJGT7) was 27%, as compared in Table 3. The use of the *E. coli* strain MV1184 harboring the plasmid pJGT3, or any subsequent mutant derivatives, will enable the rapid production of the quantities of enzyme required for ongoing and future structural studies.

Substrate and reaction specificity of 4-α-glucanotransferase

To confirm the identity of the recombinant enzyme with 4-α-glucanotransferase and to investigate the specificity of the reaction, the activity of the enzyme toward a range of malto-oligosaccharides was investigated. Substrates were incubated with or without the enzyme at 90℃ for 2 h and then applied to TLC plate (Fig. 4). It showed that no transglycosylation reaction could be detected with glucose. In contrast, maltose, maltotriose, maltotetraose, malatopentaose, maltohexaose and maltoheptaose were shown to be effective substrates, producing other malto-oligosaccharides and glucose. These results confirm that the recombinant enzyme has the same catalytic activity as the natural enzyme [4]. The D-enzyme of potato tubers [13] and amylomaltase of *Thermus aquaticus* ATCC 33923 [14] were reported to exhibit similar activities toward malto-oligosaccharides. However, in the case of D-enzyme, no transglycosylation reaction of maltose was detected, and after the enzyme reaction, maltose was not produced, while maltose was detected with the amylomaltase. It will be interesting to determine what reaction mechanisms cause the differences between the two enzymes.

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초록 : 초호열성 고세균 *Thermococcus litoralis*로부터 4-α-glucanotransferase의 대량발현

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초호열성 고세균 *Thermococcus litoralis* 유래의 4-α-glucanotransferase는 클로닝되어 염기배열이 밝혀졌으며, 대장균에서 발현되었다. 발현된 이 효소는 기능적인 면에서는 D-enzyme과 유사하지만 아미노산 배열에서는 큰 차이점을 나타내었다. 이 효소는 cycloamylose를 생산하는 새로운 기능적인 특성을 가지고 있어 당대사 관련 효소 단백질에 관한 연구의 중요성 때문에 산업적으로 많은 각광을 받고 있다. 본 연구는 초호열성 고세균 *T. litoralis* 유래 4-α-glucanotransferase 유전자를 부위 특이적 변이 방법으로 재조합하여 lac와 T7 프로모터를 이용해서 대장균 발현 벡터 시스템에서 대량발현 시켰다. 대장균에서 대량 발현된 재조합 효소 단백질은 열처리, Butyl-Toyopearl, Mono Q 크로마티그래피 방법에 의하여 간단히 정제되었다. 정제된 재조합 효소 단백질은 본래의 효소 단백질과 같은 기능을 가지고 있는 것으로 확인되었다.