Cloning and Sequence Analysis of the Gene for Glucodextranase from \textit{Arthrobacter globiformis} T-3044 and Expression in \textit{Escherichia coli} Cells

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The \textit{gld} gene for glucodextranase from \textit{Arthrobacter globiformis} T-3044 was cloned by using a combination of gene walking and probe methods and expressed on the recombinant plasmid pGD8, which was constructed with pUC118, in \textit{Escherichia coli} cells. The enzyme gene consisted of a unique open reading frame of 3,153 bp. The comparison of the DNA sequence data with the N-terminal and 6 internal amino acid sequences of the purified enzyme secreted from \textit{A. globiformis} T-3044 suggested that the enzyme was translated from mRNA as a secretory precursor with a signal peptide of 28 amino acids residues. The deduced amino acid sequence of the mature enzyme contained 1,023 residues, resulting in a polypeptide with a molecular mass of 107,475 daltons. The deduced sequence showed about 38% identity to that of the glucanamylase from \textit{Clostridium sp}. G0005. The glucodextranase activity of transformant harboring pGD8 was about 40 mU/ml at 30°C for a 16-h culture. Although the GDase that was produced from the transformant was shorter than authentic GDase by 2 amino acid residues at the N-terminal end side, its enzymatic properties were almost same as the authentic one.

Two kinds of genes, \textit{dex1} and \textit{dex2}, for endo-dextranases from \textit{A. globiformis} T-3044 were also cloned into \textit{Escherichia coli} cells. The N-terminal of the purified endo-dextranase from \textit{A. globiformis} T-3044 agreed with the deduced amino acid sequence, after the 33rd alanine residue, of only the \textit{dex1} gene for endo-dextranase. This result suggests that the endo-dextranase is translated from mRNA as a secretory precursor with a signal peptide of 32 amino acids residues. The deduced sequence of endo-dextranase 1 and endo-dextranase 2 showed about 93% and 65% identity with that of known endo-dextranase from \textit{Arthrobacter} sp. CB-8, respectively.

Key words: glucodextranase; cloning; \textit{Arthrobacter}; glucanamylase; \textit{Clostridium}

Glucodextranase (EC 3.2.1.70; GDase) is an exo-splitting enzyme that consecutively removes the glucose units from the non-reducing end of dextran, an \(\alpha\)-glucan that is mainly composed of \(\alpha-1,6\)-glucosidic linkages, and its only product is \(\beta\)-d-glucose. The enzyme has been prepared from only two strains, \textit{Arthrobacter globiformis} I42 and T-3044, although dextran glucosidases that produce \(\alpha\)-d-glucose from dextran were prepared from \textit{Streptococcus mitis} and \textit{mutans}. GDase is a useful tool for the purification of cycloextrans (cyclosomaltooligosaccharides; CIs) from the reaction mixture for CI production, because it hydrolyzes only dextran and linear isomaltooligosaccharides that are contained as residues and byproducts in the mixture and does not hydrolyze CIs, which have no non-reducing ends. However, commercially available GDase, which is prepared from \textit{A. globiformis} strain I42 is too expensive to use on an industrial scale. On the other hand, the T-3044 strain produced not only GDase but also endo-dextranase (EC 3.2.1.11; dextranase; DXase) when it was cultured in a medium containing dextran. DXase must be removed from crude GDase enzyme solution for CI production because the enzyme hydrolyzes CIs the same as linear isomaltooligosaccharides. We had to carry out several procedures for the separation of DXase and GDase, although we have attempted the separation by using simple column chromatography. Therefore, we tried cloning of the gene for GDase from \textit{A. globiformis} T-3044 to obtain the DXase-free GDase sample by a simple procedure.

Many studies of molecular cloning on dextran hydrolyses have been reported and their amino acid sequences have been compared for investigation of their enzymatic properties. However, such studies of GDase have never been reported.

In this report, we describe the cloning and DNA sequencing of the gene coding for GDase from \textit{A. globiformis} T-3044 into an \textit{Escherichia coli} plasmid vector (pUC118), and the expression of GDase (\textit{gld}) gene in an \textit{E. coli} strain.

Materials and Methods

\textbf{Strains and growth conditions.} \textit{A. globiformis} T-3044, isolated from soil, was grown as described previously. \textit{E. coli} XL1-Blue MRF' \(\Delta\text{(merA) }I83 \Delta\text{(merCB)}\)
hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac (F' proAB lacZΔM15 Tn10 (Tet')) was used as a host strain for routine transformation. L-Broth and the L-broth plates contained 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-Gal) and 47.2 μg/ml of isopropyl-β-d-thiogalactopyranoside (IPTG) (X-Gal plates). X-Gal plates were used for screening of E. coli. Ampicillin (Sigma Chemical Co.) was routinely added up to the final concentration of 50 μg/ml, if necessary.

Enzyme assay and general analytical methods. GDase activity was assayed as described in our previous paper.\textsuperscript{3} DXase activity was assayed as follows. Reaction conditions were the same as the GDase assay and reducing power in the reaction mixture was measured by the method of Somogyi and Nelson.\textsuperscript{15} One unit of DXase activity was defined as the activity that increased the reducing power equivalent to 1 μmole of glucose in 1 min under these conditions. The protein concentration was estimated by the Coomassie blue method of Bradford with γ-globulin as a standard.\textsuperscript{16} SDS-PAGE was done by the method of Laemmli.\textsuperscript{17}

Optical rotation study. To identify the configuration of the anomeric carbon atom of the products, the change in the optical rotation were observed with time by the method of Kato \textit{et al.} on the reaction mixture of the GDase with dextran.\textsuperscript{18}

Purification of GDase and DXase from \textit{A. globiformis} T-3044. The GDase was purified as in our previous report.\textsuperscript{3} The DXase was also purified as follows. Four liters of the culture broth was centrifuged at 12,000 × g for 10 min. Ammonium sulfate, (NH₄)₂SO₄, was added to the supernatant up to 70% saturation. The precipitate was collected by centrifugation and suspended in 160 ml of 100 mM phosphate buffer (pH 7.0) as a crude extract. The crude extract was dialyzed against 100 mM phosphate buffer (pH 7.0) at 4°C for 16 h and the dialysate was centrifuged to remove insoluble materials. Ammonium sulfate was again added up to a final concentration of 1.0 M. After insoluble materials were removed by centrifugation, the enzyme was run on an HPLC (Toyo Co., Japan) using a preparative TSKgel Phenyl 5PW column (21.5 mm I.D. × 150 mm; Tosoh Co.). Elution conditions were as follows: solvent, 100 mM phosphate buffer (pH 7.0); elution, a linear gradient of (NH₄)₂SO₄ (1.0–0 M); flow rate, 4 ml/min; injection volume, 160 ml; detection 280 nm; fraction volume, 8 ml/each fraction. The enzyme was eluted at around 0.1 M of (NH₄)₂SO₄. The active fractions (total 88 ml) were concentrated to 1.0 ml by ultrafiltration using the PM 10 Amicon membrane. The concentrate was diluted 20 times with 10 mM phosphate buffer (pH 7.0) and was put on an HPLC using a TSKgel DEAE 5PW column (7.5 mm I.D. × 75 mm, Tosoh Co.) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl (0–0.4 M) and 1 ml/min flow rate. The active fractions (total 9 ml) were concentrated to 0.9 ml by ultrafiltration using the Centrprep (Amicon Co.). A portion (0.3 ml) of the concentrate was put on a molecular sieve HPLC using a TSKgel G3000 SW column (7.6 mm I.D. × 600 mm × 2, Tosoh Co.) and eluted with 200 mM NaCl-100 mM phosphate buffer (pH 7.0) at 1 ml/min flow rate. The enzyme was eluted as a single peak. The active fractions were combined (total 21 ml).

Identification of N-terminal and several internal amino acid sequences of the GDase and DXase. The purified GDase from \textit{A. globiformis} was digested completely with lysyl endopeptidase (Wako Pure Chemicals, Japan). The purified GDase (1.6 nmole/1.0 ml of 100 mM phosphate buffer (pH 7.0)) was allowed to react at 37°C with 0.016 nmole of the lysyl endopeptidases for 6 h. After addition of 50 mM acetic acid to adjust the pH to 5.0, the endopeptidase digestion sample was put on the 1 ml of an anhydrotrypsin agarose gel (Takara Shuzo Co. Ltd., Japan) column equilibrated at pH 5.0 with 50 mM acetic buffer (pH 5.0). The peptide fragments were eluted with 50 mM acetic acid buffer and then separated by HPLC with Cosmosil-C18 reversed phase column as in our previous report.\textsuperscript{19}

N-terminal sequence analyses of the native GDase and DXase, and GDase digestion peptides by the endopeptidase were done by repetitive Edman degradation in a pulse liquid protein sequencer (model 473A, Applied Biosystems).

DNA manipulation. Unless otherwise indicated, DNA manipulations were done essentially as described by Maniatis \textit{et al.}\textsuperscript{20} DNA sequences were analyzed on an automated DNA sequencer (model 370A, Applied Biosystems) with the DNA sequencing system/ Taq polymerase kit (Applied Biosystems).

Polymerase Chain Reaction (PCR) technique. A specific DNA fragment was amplified from \textit{A. globiformis} chromosomal DNA using sense and anti-sense primers that were designed using the DNA sequence information from upstream of DXase 2 (dex2) gene or the information from N-terminal and several internal amino acid sequences of the GDase. A Gene Amp PCR System 9600 model (Perkin Elmer Cetus) was used as the equipment for PCR. Each 50-μl reaction mixture was prepared by the protocol supplied with the Takara LA PCR kit (Takara Shuzo Co. Ltd., Japan). The temperature program for each PCR cycle was 98°C for 20 s, 68°C for 8 min. After 98°C for 1 min of heat treatment, thirty cycles were run.

Sequence similarity searches. The sequence similarity searches were used to search the current databases (Swiss prot) for amino acid sequence similarities between the GDase or DXase and other proteins.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have appeared in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers D88362 for \textit{gld} and \textit{dex2} genes, and D88361 for \textit{DXase 1 (dex1)} gene.
Results and Discussion

Cloning and DNA sequencing of dex and gld genes

We failed not only in obtaining a positive clone by the shotgun method using an X-Gal plate containing dextran 40 and glucose assay reagent (Glucose B test Wako; Wako pure chemicals) but also in making any available probes by PCR technique with the uncertain mix primers designed from the protein sequence information of the GDase.

In general, the genes coding for the enzymes that relate to same metabolic pathway exist close together on the chromosome as an operon. Therefore, we thought that the gld gene might make an operon with the dex gene, and changed the strategy of the cloning into the gene walking method. The cloning of the dex gene was done at first and then we analyzed the sequence up and downstream of the dex gene.

To support the cloning of the dex and gld genes, DXase was purified up to homogeneity with a 25.5% yield. The enzyme was finally eluted as a single symmetrical single peak by a molecular sieve HPLC and it migrated as a single band on SDS-PAGE. Its $M_r$ was estimated at about 70,000, the same as that of the DXase from Arthrobothacter sp. CB-8. Specific activity of the purified enzyme was 65 U/mg protein. Up to twenty-fifth of the N-terminal sequence of the purified DXase was identified.

Cloning for the dex gene was done by the shotgun method using X-Gal plates containing 0.5% blue dextran (Blue dextran plate) as previously reported. The Sau3AI-partial digested chromosomal DNA fragments were ligated to the BamHI site of pUC118 and introduced into the E. coli strain XL1-Blue MRF'. The transformants were cultured on a Blue dextran plate at 37°C for 24 h.

Eight of about 10,000 transformants showed positive halo signals. The plasmids were isolated from these clones and designated as pDX1–pDX8, which had from about 3 kb to 5 kb-insert of Sau3AI fragments. The dex genes were divided into two kinds of genes based on the restriction map and tentatively named as dex1 and dex2 (Fig. 1). The dex1 gene contained HindIII site in its sequence. On the other hand, the dex2 gene did not contain the site. The comparison of the deduced amino acid sequences among the known DXase from Arthrobothacter sp. CB-8 and the two DXases from A. globiformis T-3044 are shown in Fig. 2. DXase 1 and DXase 2 showed about 93% and 65% identity to the known DXase, respectively. Therefore these DXases seem to be typical DXases.

The N-terminal sequence of the purified DXase was found in the deduced amino acid sequence of only DXase 1 at 33rd after residues, although DXase 1 and DXase 2 also had about 63% identities with each other. These results suggest that the dex2 gene is not expressed under these culture conditions. The sequence analyses of the up and downstream of these two dex genes were done. There was a partial open reading frame (ORF) upstream from the dex2 gene. We could find several sequences coinciding with the internal peptide amino acid sequences of the GDase on the deduced amino acid sequence translated from this frame. This result suggested that the partial ORF was a part of a structural gene coding for the C-terminal end of the GDase. On the other hand, we could not obtain available sequence information from the up and downstream of the dex1 gene. It is very strange that the gld gene is around not the dex1 but the dex2 gene, because the two expressed dextran hydrolys, GDase and DXase 1, seem to be independently regulated. If gld gene is near the dex1 gene, the two genes could be regulated by a single regulator gene. This thing seems to be biologically more efficient for the bacterium. We have no ideas or data about this question.

We designed two primers from this sequence and then did PCR by using the primers and chromosome DNA from A. globiformis T-3044. The amplified DNA fragment was labeled with digoxigenin and then used as a probe. We started cloning for the gld gene by the probe method. We did colony hybridization using the probe and gene library described before. Five positive clones were obtained from about 10,000 transformants. The plasmids were extracted from transformants and then sequenced. Only one clone contained a complete ORF and was named pGD8.

Examination of the nucleotide sequence in Fig. 3 shows an ORF starting at position 1 and terminating at position 3,153. The N-terminal amino acid sequence of the purified GDase and six of lysyl endo-peptidase digested peptides were compared with the deduced amino acid sequences translated from this ORF. We found that the amino acid sequences of all peptides were consistent with the deduced amino acid sequence. These results strongly suggest that the ORF corresponds to the gld gene. The N-terminal amino acid sequence of the GDase was found in the deduced sequence at 29th and after. Therefore, this enzyme is a typical extracellular enzyme with a signal peptide consisted of 28 amino acid residues. The mature enzyme consisted of 1,023 residues resulting in a polypeptide with a molecular mass of 107,475 daltons. The molecular mass almost agreed with those from SDS-PAGE.

A potential ribosome-binding site begins at

![Fig. 1. The Restriction Maps of the DNA Fragments from A. globiformis T-3044 Inserted in the pDX1–pDX8. Closed arrows indicate the location, size, and direction of the dex genes. Open arrows indicate the direction of the vector-located promoter (P_lac).](image-url)
Cloning of Glucodextranase Gene from *Arthrobacter*

**Arthrobacter globiformis** T-3044 DXase 1
**Arthrobacter** sp. CB-8 DXase 7
**Arthrobacter globiformis** T-3044 DXase 2

\[
\text{MGVGLRLRAWVTAATAAVFLSTSGTVLAQATAPGGTSSAPAAVSVE} 49 \\
\text{MGVGLRLRATAMAAVFLISVTLAQATAPGGPVAALKA} 29 \\
\text{MGMHRLAQASSAFLPLLPPAAQGPEAPAT} 129 \\
\text{EVSLRTQITISSADQVIRIPSYFNEQKRDVTTVRXPSDAGYFSEFIGFQYLATANOMDSGSKLTLTSEAG} 248 \\
\text{EVSLRTQITISSADQVIRIPSYFNEQKRDVTTVRXPSDAGYFSEFIGFQYLATANOMDSGSKLTLTSEAG} 248 \\
\text{DVNLILITQITISSADQVIRIPSYFNEQKRDVTTVRXPSDAGYFSEFIGFQYLATANOMDSGSKLTLTSEAG} 248 \\
\text{SYNLHSGSNCHILDASVMKLQFASA-DAAEKLQDLQVTAVAEPPYFSFYVG--NEQFTHMNYEQVSGYQWTDIGEYGSTMKNTFFNDVLKMY} 445 \\
\text{SYNLHSGSNCHILDASVMKLQFASA-DAAEKLQDLQVTAVAEPPYFSFYVG--NEQFTHMNYEQVSGYQWTDIGEYGSTMKNTFFNDVLKMY} 445 \\
\text{SYPNHDSTHSTGANCHTVCMLFESSSMTQHVEGTTSEPPYFSFYVGPQAYQYDEMVRDNYQVSGYQWTDIGEYGSTKGMNRNTFFHSDVLKLY} 429 \\
\text{HSVDSTDVTNVKNEQPGVPQWQTPMNIDVNTTVHINRMWYDKVYNTCININSSHHWDEGSTDSADPNTPVVMRFNTSEVGMTCNAIRVYAL} 545 \\
\text{HSVDSTDVTNVKNEQPGVPQWQTPMNIDVNTTVHINRMWYDKVYNTCININSSHHWDEGSTDSADPNTPVVMRFNTSEVGMTCNAIRVYAL} 545 \\
\text{SDTENIKHNQGNGWCLTSQHSLKRLYNTSAEGVITNENPDPGNGLALEYSVGQIQEISGNWSDYQLRLGFDPGDNWDSNWNKAKSAP} 640 \\
\text{SDTENIKHNQGNGWCLTSQHSLKRLYNTSAEGVITNENPDPGNGLALEYSVGQIQEISGNWSDYQLRLGFDPGDNWDSNWNKAKSAP} 640 \\
\text{SNTENIKHNQGNGWCLTSQHSLKRLYNTSAEGVITNENPDPGNGLALEYSVGQIQEISGNWSDYQLRLGFDPGDNWDSNWNKAKSAP} 640 \\
\text{SNTENIKHNQGNGWCLTSQHSLKRLYNTSAEGVITNENPDPGNGLALEYSVGQIQEISGNWSDYQLRLGFDPGDNWDSNWNKAKSAP} 640 \\
\text{A. globiformis T-3044 and the other protein showing identity to this sequence is presented in Fig. 4. The N-terminal end side of the GDase sequence showed about 38% identity to that of the glycoamylase from *Clostridium* sp. G9005, although the amino acid sequence of the GDase was about 300 amino acids longer than that of the glycoamylase. There were no obvious similarities between the GDase and any other dextran hydroxases like as dextran glucosidase, isomaltodextranase, DXases, or CI glucanotransferase.}

In general the usual glycoamylase hydrolyzes α-1,4-glucan far faster than α-1,6-glucan. However, the glycoamylase from *Clostridium* sp. G9005 also shows high activity on isomaltooligosaccharides. On the other hand, although the glycoamylase activity of strain 142 GDase has about 1.0% of GDase activity, the activity of strain T-3044 GDase was about 15 times higher than that of strain 142. Therefore, the T-3044 GDase is characterized by a high activity toward starch, and these two enzymes, the T-3044 GDase and the *Clostridium* glycoamylase, seem to have similar substrate specificity, although the main substrate is obviously different.
previously described, the product of GDase is only β-D-glucose, the as same as that of glucoamylase. Moreover GDase also conserved the four amino acid sequences that were thought to be the catalytic domain of glucoamylase, as shown in Fig. 5. These data suggest that the GDase from *A. globiformis* T-3044 could be classified as a member of glucoamylase family. Recently, Morimoto et al. and Tomita et al. independently presented the deduced amino acid sequence of the GDase from *A. globiformis* 142 strain. They also reported that its sequence had high similarity to that of our GDase. Therefore, GDase and glucoamylase basically may have a common structure, and such a difference of substrate specificity may be caused by a difference of the sequence length or a minor difference of the amino acid sequence relating to substrate binding.

Expression of the *gld* gene in *E. coli* and some properties of the *Gld* enzyme from transforant

The strain XL1-Blue MRF' carrying pGD8 was incu-
Fig. 3. Nucleotide Sequence of a Sau3A1-DNA Fragment Inserted in pGD8 and Its Deduced Amino Acid Sequence.

Amino acids are described by single letters. SD sequence is represented by an under line. The boxed amino acid sequences represent analyzed mature enzyme (N-terminal sequence) and the lysyl endopeptidase fragments (internal sequences) by protein sequencer as described in Materials and Methods. The horizontal arrows show the palindromic sequence and vertical arrow shows the cleavage site by the signal peptidase.

hated aerobically in L-broth at 30°C for 16 h. The GDase activity in the sonicated crude lysate was examined. The activity of GDase in the strain harboring pGD8 was about 40 mU/ml when the culture was done on test tubes. Production of GDase in XL1-Blue (pGD8) was not stimulated by the addition of IPTG. This suggested that expression was not dependent on the lacZ promoter located on the vector strain, and

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A. globiformis T-3044 GDase
Clostridium sp. G0005 Glucoamylase 22)

Table 1. Comparisons of Properties of Dase from E. coli Transformant (pGD8) and A. globiformis T-3044 GDase

<table>
<thead>
<tr>
<th>Property</th>
<th>pGD8</th>
<th>A. globiformis T-3044 GDase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>E. coli transformant (pGD8)</td>
<td>A. globiformis T-3044 GDase</td>
</tr>
<tr>
<td>M.W. (SDS-PAGE)</td>
<td>120,000</td>
<td>120,000</td>
</tr>
<tr>
<td>N-terminal end sequence</td>
<td>E-P-A-P-G-S-P-G-A-S-β-D-glucose</td>
<td>Q-A-E-P-P-G-S-P-G-β-D-glucose</td>
</tr>
<tr>
<td>Product</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimum pH</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Heat stability</td>
<td>≤40°C</td>
<td>≤40°C</td>
</tr>
<tr>
<td>Hydrolysis ratio of dextran 40</td>
<td>35%</td>
<td>35%</td>
</tr>
<tr>
<td>GAase/GDase</td>
<td>14%</td>
<td>15%</td>
</tr>
</tbody>
</table>

The asterisks represent matched amino acid residues between the GDase from A. globiformis T-3044 and the glucoamylase from Clostridium sp. G0005. The vertical arrows show the cleavage sites by the signal peptidases.

Fig. 4. A Comparison of the Deduced Amino Acid Sequences between the GDase from A. globiformis T-3044 and the Glucoamylase from Clostridium sp. G0005.

that the gld gene, together with its promoter region, might be contained entirely within this plasmid. The GDase activity of the transformant is about 15% of that (261 mU/ml) from A. globiformis T-3044 and not sufficient yet. Furthermore, the activity was only about 6 mU/ml, when the culture was done in a mini jar fermenter. We have no idea why the activity was reduced by a scale-up of cultivation.

As reported by Okada et al., GDase can be simply purified by affinity chromatography using Sephadex resin and methyl α-glucoside. However, the crude extract prepared from A. globiformis T-3044 culture, which was dominantly contaminated with DXase, could not be purified by this method, because DXase hydrolyzed Sephadex resin. We easily prepared DXase free GDase sample from a cell-free extract of the transformant cells. SDS-PAGE analysis of the prepared sample showed one almost pure band with an M, of 120,000, although a minor band with an M, of about 100,000 was also detected. We consider that this minor band might be partially degraded GDase, because this protein also absorbed to Sephadex resin and was eluted by methyl α-glucoside.
Moreover a similar M₆ band was also detected in long stored purified GDase sample from *A. globiformis* T-3044. As shown in Table 1, the N-terminal analysis of the purified GDase showed that the GDase from the transformant was hydrolyzed for maturation at a different site from authentic GDase. This result suggests that the signal peptidase of *E. coli* recognizes a different site and produces a GDase that is shorter than authentic GDase by 2 amino acid residues at the N-terminal end side. Some properties of the partially purified transformant GDase were almost the same as those of the T-3044 GDase. These results suggest that the difference of the N-terminal end is not very important, although a slight difference of properties may be caused by this reason. We are trying to express the gd1 gene by using another host vector system in order to over-express the gd1 gene and in the future we will obtain a high GDase activity sample from the new system.

References

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