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Cloning and characterization of the glycogen branching enzyme gene existing in tandem with the glycogen debranching enzyme from *Pectobacterium chrysanthemi* PY35

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Abstract

The glycogen branching enzyme gene (*glg*B) from *Pectobacterium chrysanthemi* PY35 was cloned, sequenced, and expressed in *Escherichia coli*. The *glg*B gene consisted of an open reading frame of 2196 bp encoding a protein of 731 amino acids (calculated molecular weight of 83,859 Da). The *glg*B gene is upstream of *glg*X and the ORF starts the ATG initiation codon and ends with the TGA stop codon at 2 bp upstream of *glg*X. The enzyme was 43–69% sequence identical with other glycogen branching enzymes. The enzyme is the most similar to GlgB of *E. coli* and contained the four regions conserved among the α-amylase family. The glycogen branching enzyme (GlgB) was purified and the molecular weight of the enzyme was estimated to be 84 kDa by SDS–PAGE. The glycogen branching enzyme was optimally active at pH 7 and 30 °C.
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Keywords: *Pectobacterium chrysanthemi* PY35; Glycogen branching enzyme; Glycogen debranching enzyme

The branching enzyme [l,4-α-D-glucan:1,4-α-D-glucan 6-D-(1,4-glucano)-transferase; EC 2.4.1.18] catalyzes transglycosylation to form α-1,6-glycosidic linkages of starch or glycogen. The enzymes are widely distributed in plants, microorganisms, and animal tissues. The genes for the enzymes have been cloned and sequenced from various sources [1]. The branching enzyme from *Pectobacterium* sp. has not been reported. *Pectobacterium chrysanthemi* is one of the phytopathogenic enterobacteria, which causes soft rot disease in various plants. Its pathogenicity is due to its ability to secrete several extracellular enzymes including pectate lyases, cellulases, and proteases. Many researchers have reported that some of these enzymes such as pectinases, proteases, and cellulases have multiple enzymatic forms [2]. Recently a gene for the amylolytic enzyme from *P. chrysanthemi* was reported [3]. The metabolism of glycogen is not known to be involved in the pathogenicity of soft rot.

Structural similarities were found among starch hydrolases and related enzymes including branching enzymes, α-amylases (EC 3.2.1.1), cyclodextrin glucanotransferases (EC 2.4.1.19), pullulanases (EC 3.2.1.41), and isoamylases (EC 3.2.1.68). The existence of four highly conserved regions in their primary sequences has been demonstrated [4–6], and similarities in their secondary structures have been proposed [7]. The four regions are most likely to include the catalytic residues and the substrate-binding residues of the enzymes.

Some of the *P. chrysanthemi* genes, such as genes producing the pectinases and proteases, exist in tandem.

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As many as eight *P. chrysanthemi* 3937 endo-Pels have been characterized, PelA, PelB, PelC, PelD, PelE, PelI, PelL, and PelZ [2]. The corresponding genes are organized in tandem as four clusters of bacterial chromosome (*pel*A–*pel*E–*pel*D, *pel*B–*pel*C–*pel*Z, *pel*I, and *pel*L) [8–10]. Also, other genes in other bacteria have cluster structure, too [11–14]. Meanwhile, the genetic organization of the glycogen (*glg*) operon was determined in *Escherichia coli* [15,16]. A tandem structure of *glg*B and *glg*X of *E. coli* was observed [17]. Other tandem structures of *glg*B and *glg*X in bacteria have not been reported yet. It is possible that the arrangement of *glg*B and *glg*X is different to the organism.

In this article we report the cloning and sequencing of the glycogen branching enzyme (*glg*B) gene existing in tandem with the glycogen debranching enzyme (*glg*X; *amy*X in the previous paper [3] is renamed *glg*X in this paper) gene from *P. chrysanthemi* PY35, and the biochemical characterization of the recombinant enzyme expressed in *E. coli*.

Materials and methods

Bacterial strains and culture conditions. The *P. chrysanthemi* PY35 was grown at 28 °C in a tryptone–yeast extract (TY) medium. *E. coli* DH5α and recombinant *E. coli* cells were grown at 37 °C in Luria–Bertani (LB) medium containing the appropriate concentration of antibiotic: 50 μg ml^{-1} ampicillin.

Recombinant DNA techniques. Standard procedures for restriction of endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning related techniques were followed as described by Sambrook and Russell [18].

Chemicals and reagents. Restriction enzymes and DNA modifying enzymes were purchased from Gibco-BRL (Gaithersburg, MD, USA) and Boehringer–Mannheim (Indianapolis, IN, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA).

Cloning and DNA sequencing of glgB. To amplify *glg*B homologues from the *P. chrysanthemi* PY35 chromosome, degenerate oligonucleotide primers were designed based on conserved amino acid sequences adjacent to the high conservation regions II and IV available in the database. The sense and antisense degenerate oligonucleotide primers are 5′-CGSGTSGAYGCBGTKGCBTC-3′ (sense) and 5′-TGDACCACTTCRTCRTGVGA-3′ (antisense), respectively. After PCR amplification using *P. chrysanthemi* PY35 genomic DNA, SuperTherm DNA polymerase (JMR, Side Cup, Kent, UK), 1.5 mM $MgCl_2$, and 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. The anticipated product of approximately 400 bp was isolated from an agarose gel using a gel extraction kit (NucleoGen, Seoul, Korea). PCR product was confirmed sequenced by BLAST search. From these initially sequenced DNA regions we then amplified upstream 'outside' DNA by primer walking using only one specific primer 5′-CGATCGCCTCCAGATTCTCCTT GCC-3′. Downstream 'outside' DNA by using the specific oligonucleotide primers 5′-AATACCACC ACGACCTGCTGA-3′ based on known sequences and 5′-TCATCCACCACCCAACCGT-3′ based on the known *glg*X sequence [3]. The amplified fragments were isolated for further nucleotide sequencing. The complete open reading frame (*glg*B) was amplified from genomic DNA using 5′-CCAGGCG GAAACCAACAAG-3′ (sense), 5′-CCGGATGAGAACAGGGC GAG-3′ (antisense), and cloned into pGEM-T (Promega, Madison, WI, USA). The two complete open reading frames (*glg*B and *glg*X) were amplified from genomic DNA using 5′-AATGGATCCGCCA GGCGGAAACCAACAAG-3′ (sense) and 5′-GCTGGATCCAGTG TGCGACTTGA TACTGG-3′ (antisense) (*Bam*HI sites are indicated by underline) by LA *Taq* (Takara Shuzo, Otsu, Shiga, Japan). The PCR products were digested with *Bam*HI and cloned into pGEM3Zf(+) (Promega) digested with the same restriction enzyme. Nucleotide sequences were determined by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin–Elmer, Norwalk, CT, USA). The samples were analyzed with an automated DNA sequencer (Model 310; Applied Biosystems, Foster City, CA, USA). Additional oligonucleotide primers were designed based on available sequence information to extend and confirm the existing sequence. The BLAST program was used to find the protein coding regions.

Purification of GlgB. The glycogen branching enzyme of *P. chrysanthemi* PY35 was purified from cell extracts of *E. coli* harboring pLYPY340 as described by Guan et al. [19]. The cells were grown at 37 °C to an OD 600 nm of 0.6 before the expression of GlgB was induced by added 0.5 mM isopropyl-D-thiogalactoside. Following growth at room temperature for 4 h, the cells were harvested in a refrigerated centrifuge. Cell pellet was resuspended in 150 ml buffer A (50 mM Tris–acetate buffer, pH 7.5, 10 mM EDTA, and 2.5 mM DTT) and lysed by sonication. The homogenate was centrifuged at 12,000*g* for 20 min and the resulting supernatant was saturated to 40% by addition of ammonium sulfate. The precipitate was collected by centrifugation, then dissolved in 30 ml buffer A, and dialyzed overnight in buffer A. The dialyzed sample was loaded onto Q-Sepharose fast-flow column (Amersham Pharmacia Biotech, Buckinghamshire, UK) and eluted with a gradient of 0–0.4 M KCl in buffer A. The active fractions were pooled and precipitated with 40% ammonium sulfate. The precipitate was redissolved in buffer B (pH 8.0, 10 mM triethanolamine, 1 mM EDTA, and 2.5 mM DTT). Fractions with branching activity were pooled and concentrated with a Centricon concentrator (Amicon).

SDS–PAGE. SDS–PAGE was carried out as described previously [20].

Assay of GlgB activity. The enzyme solution (50 μl) was mixed with the substrate solution (50 μl) and incubated for 30 min at 30 °C. The substrate solution was 0.1% type III amylose (Sigma) dissolved in 100 mM Tris–HCl (pH 7.5). Reactions were terminated by the addition of 2 ml iodine reagent. Iodine reagent was made daily from 0.5 ml stock solution (0.26 g I_2 and 2.6 g KI in 10 ml water) mixed with 0.5 ml of 1 N HCl and diluted to 130 ml. One unit of enzyme activity was defined as the amount of GlgB that can decrease the A_{660} of the amylose–iodine complex by 1% min^{-1} [1].

Assay of GlgX activity. To detect glycogen debranching enzyme activities from *E. coli* cells harboring the cloned glycogen debranching enzyme genes, bacterial colonies were first grown on LB broth supplemented with 50 μg ml^{-1} ampicillin. After incubation overnight, cells of 3 ml culture harvested by centrifugation at 5000*g* for 5 min were resuspended in 200 μl of 10 mM Tris–HCl buffer (pH 7.5) and sonicated four times at 4 °C for 60 s at 3 min intervals. Supernatants obtained after centrifugation for 20 min at 17,000*g* were used as crude enzymes. Crude enzymes were applied to LB agar supplemented with 0.1% (W/V) glycogen (Sigma). After incubation for 2 h, the plates were stained with staining solution [0.15% (W/V) iodine and 1.5% (W/V) potassium iodine].

Characteristics of GlgB enzyme. The effects of pH and temperature on the glycogen branching enzyme activity were examined with the purified recombinant enzyme. The effect of pH on the glycogen branching enzyme activity was determined by using the protocol described above, to obtain values from pH 3 to 10; all of the assays were performed at 30 °C. To determine the effect of temperature on the enzymatic activity, samples were incubated at temperatures from 4 to 70 °C for 30 min. In all cases, the treatments were carried out in microcentrifuge tubes. After various time intervals, samples were withdrawn and clarified by centrifugation, and the enzyme activities were measured as previously described.

Nucleotide sequence accession number. The nucleotide sequence and the deduced amino acid sequence encoded by *glg*B have been submitted to GenBank under Accession No. AF434710.

Results

Isolation of the gene encoding GlgB

To isolate the *glg*B gene, degenerate primers based on amino acids from two high conserved regions of the four conserved regions in bacterial glycogen branching enzymes [D-(AG)-(ILFM)-R-V-D-A-V-A-(SN) and N-(FY)-(IVM)-(LF)-P-L-S-H-D-E-V-V-H-(GL)-K] allowed amplification of approximately 400 bp DNA fragment. Both strands of this fragment were sequenced and BLAST search showed that this DNA was in high homology with other bacterial *glg*B genes. From these initially sequenced DNA regions we then amplified upstream 'outside' DNA by primer walking using only one specific primer 5′-CGATCGCCTCCAGATTCTCCTT GCC-3′. Downstream 'outside' DNA by using the specific oligonucleotide primers 5′-AATACCACCACGAC CTGCTGA-3′ based on known sequences and 5′-TCA TCCACCACCCAACCGT-3′ based on known *glg*X sequence [3]. The 2.4 kb fragment was cloned into pGEM-T and the 4.5 kb PCR products were digested with *Bam*HI and cloned into pGEM3Zf(+) digested with *Bam*HI. The pGEM-T containing 2.4 kb insert was designated pLYPY200. The pGEM3Zf(+) containing 4.5 kb insert was designated pLYPY300 (Fig. 2).

To further localize the glycogen branching enzyme gene in the insert *Bam*HI, *Eco*RI, *Pst*I, and *Bst*XI-digested fragments of pLYPY300 were subcloned into pGEM3Zf(+) vector. The ligation mixture was used to transform *E. coli* DH5α and the transformants showing glycogen branching enzyme activity were screened. pLYPY310, pLYPY330, and pLYPY350 clones did not show glycogen branching enzyme activity (Fig. 2). But pLYPY300, pLYPY310, pLYPY330, and pLYPY350 clones showed glycogen debranching enzyme activity (Figs. 1 and 2). pLYPY340 was selected for further characterization. The glycogen branching enzyme gene of pLYPY340 clone was designated *glg*B gene and the restriction map of *glg*B and *glg*X is shown in Fig. 2.

Sequencing of the 2.7 kb insert encompassing glgB

Both strands of the entire 2.7 kb insert in pLYPY340 were sequenced and an ORF was identified (Fig. 3). *glg*B is 2196 bp in size and encodes a protein of 731 amino acids with a predicted molecular mass of 83,859 Da. The *glg*B gene is upstream of *glg*X (Accession No. AY044255) and the coding regions of the *glg*B and *glg*X open reading frames (ORFs) overlap by 1 bp. The ORF

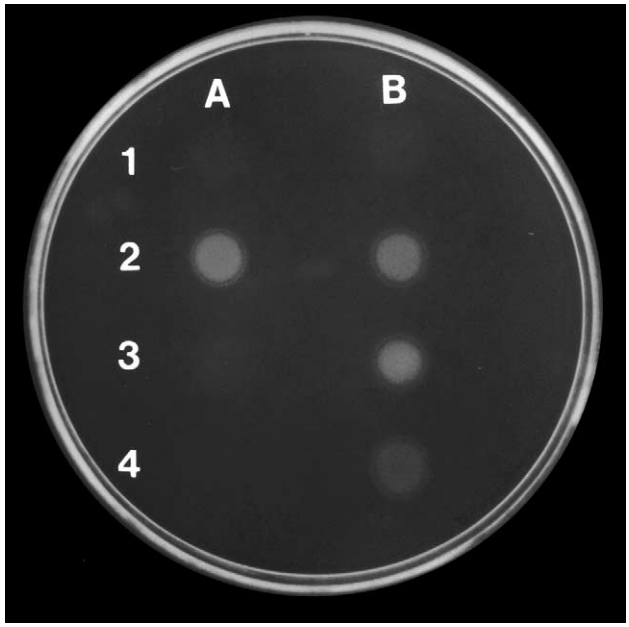

Fig. 1. Detection of a glycogen debranching enzyme positive clone by glycogen agar diffusion method. The cell extracts were incubated at 37 °C for 2 h. Lane 1A, *E. coli* as a negative control; lane 1B, pGEM3Zf(+) in *E. coli*; lane 2A, *E. coli* harboring pLYPY300; lane 2B, *E. coli* harboring pLYPY310; lane 3A, *E. coli* harboring pLYPY320; lane 3B, *E. coli* harboring pLYPY330; lane 4A, *E. coli* harboring pLYPY340; and lane 4B, *E. coli* harboring pLYPY350.

*glg*B starts the ATG initiation codon and the reading frame ends with the opal stop codon TGA at position 2301 (Fig. 3). The *glg*X gene was previously sequenced as *amy*X [3]. The N-terminal amino acid sequences of the protein eluted from SDS–PAGE gel closely matched the amino acid residues 1–6 of the translated sequences (data not shown). The calculated p*I* of GlgB is 5.84.

Comparison of amino acid sequence with those of GlgB from other sources

The amino acid sequence of the glycogen branching enzyme from *P. chrysanthemi* PY35 was compared with the sequences of other glycogen branching enzymes. Protein sequence databases (SWISS-PROT, PIR, and GenBank) were searched using the standard search algorithms (programs FASTA and BLASTP) to find polypeptides related to GlgB. GlgB of *P. chrysanthemi* PY35 shared significant sequence similarity with previously reported GlgB (P07762) of *E. coli* [21], GlgB (P52979) of *Agrobacterium tumefaciens* [22], and GlgB (P16954) of *Synechococcus* sp. PCC 7942 [23] (Fig. 4). GlgB of *P. chrysanthemi* PY35 shared 69% amino acid identity with GlgX (P07762) of *E. coli*, 62% with GlgB (P45177) of *Haemophilus influenzae*, 51% with GlgB (P52980) of *Streptomyces aureofaciens* [24], 50% with GlgB (P52979) of *A. tumefaciens*, 49% with GlgB (Q10625) of *Mycobacterium tuberculosis*, 48% with GlgB

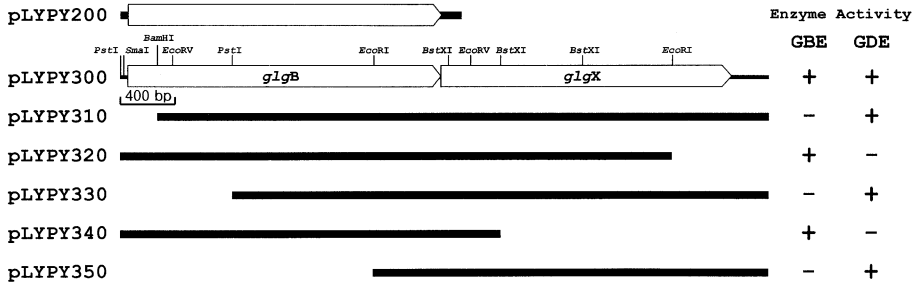

Fig. 2. Physical map of recombinant DNA pLYPY300 carrying *glg*B and *glg*X genes of *P. chrysanthemi* PY35. The cleavage sites of restriction enzymes *Pst*I, *Sma*I, *Bam*HI, *Eco*RV, *Eco*RI, and *Bst*XI are shown. pLYPY300 was constructed by cloning a 4.5 kb fragment of *P. chrysanthemi* PY35 into pGEM3Zf(+) vector.

```
   1 GTTTATGCGGCAGGCGGATGGTGACCTGCGTCAGCGGAATAAACAGCGGAGTTTTGCTCAACAGATAGGCAATGACCAGATAGACGCATG
   1                                                                                        M

  91 TTTGTTGCAGCAATGACAGAATCAGATCAGAACATTATTAATCTCCTTTTTTCCGGTCATTACGCCGATCCTTTTGCCGTGCTGGGTATG
   2  F  V  A  A  M  T  E  S  D  Q  N  I  I  N  L  L  F  S  G  H  Y  A  D  P  F  A  V  L  G  M

 181 CATGACACGGCATCGGGGCTGGAAGTACGTGCTCTGCTTCCCGATGCCATCGACGTCTGGGTCGTGGACGCACACAGCGGACGTAAAGTG
  32  H  D  T  A  S  G  L  E  V  R  A  L  L  P  D  A  I  D  V  W  V  V  D  A  H  S  G  R  K  V

 271 GCGAACCTGCAATGCCGGGATCCGCGCGGTTTTTTCGCCTCGGCGATTCCCCGTCGCAAAAAGCCGTTCAGCTATCGTCTGGCGGTGACC
  62  A  N  L  Q  C  R  D  P  R  G  F  F  A  S  A  I  P  R  R  K  K  P  F  S  Y  R  L  A  V  T

 361 TGGCCGCAGGATACGCAGGTCATTGATGACCCTTATCGTTTTGGCACGCTGTTGCAGGAGCTGGATATCTGGCTGCTGGCGGAAGGGCGC
  92  W  P  Q  D  T  Q  V  I  D  D  P  Y  R  F  G  T  L  L  Q  E  L  D  I  W  L  L  A  E  G  R

 451 CACTTGCGGCCGTTTGAAACGCTGGGGGCGCACCCTTCCACGCTGGATGGCGTCGTCGGCACCTGTTTTGCCGTCTGGGCGCCGAATGCT
 122  H  L  R  P  F  E  T  L  G  A  H  P  S  T  L  D  G  V  V  G  T  C  F  A  V  W  A  P  N  A

 541 CAGCGGGTGTCGGTGGTGGGCGATTTCAATTTTTGGGACGGCCGCCGCCATCCGATGCGGCGGCGGCGCGAAAACGGCGTATGGGAACTG
 152  Q  R  V  S  V  V  G  D  F  N  F  W  D  G  R  R  H  P  M  R  R  R  R  E  N  G  V  W  E  L

 631 TTCGTGCCGGGCGTCGGGCCGGGCCAATTGTACAAATTCGAAATCATCGACTGTTACGGCAACGTGCTGGTGAAGTCCGACCCTTATGCC
 182  F  V  P  G  V  G  P  G  Q  L  Y  K  F  E  I  I  D  C  Y  G  N  V  L  V  K  S  D  P  Y  A

 721 TTCGAATCCCAGATGCGGCCGGACACCGCGTCGGTGGTCAGCCGTCTGCCGCCCGCGCTGCCGGTGGATGAAGCGCGGCAGCACGCCAAC
 212  F  E  S  Q  M  R  P  D  T  A  S  V  V  S  R  L  P  P  A  L  P  V  D  E  A  R  Q  H  A  N

 811 GAGCTGCAGTCGCCGATTTCCATTTACGAAGTGCACCTGGGGTCATGGCGGCGTCACACCCACAATAATTTCTGGCTCAGTTACCGCGAA
 242  E  L  Q  S  P  I  S  I  Y  E  V  H  L  G  S  W  R  R  H  T  H  N  N  F  W  L  S  Y  R  E

 901 CTGGCGGACCAACTGGTGCCGTACGTGAAAGAAATGGGGTTTACCCATGTTGAGCTGATGCCGGTGCACAAACACCCGTTTGACGGCAGT
 272  L  A  D  Q  L  V  P  Y  V  K  E  M  G  F  T  H  V  E  L  M  P  V  H  K  H  P  F  D  G  S

 991 TGGGGCTATCAGCCGCTGGGGTTATACGCGCCGACCCGCCGTTTCGGTTCGCCGGACGACTTCCGCTATCTGGTGTCGGCTTTTCACGAG
 302  W  G  Y  Q  P  L  G  L  Y  A  P  T  R  R  F  G  S  P  D  D  F  R  Y  L  V  S  A  F  H  E

1081 GCGGGCATCAATGTGCTGCTGGACTGGGTTTCCGGCCATTTCCCGGCGGATAGCTACGGGCTGGCGCGTTTCGACGGCCCCGCGCTGTAC
 332  A  G  I  N  V  L  L  D  W  V  S  G  H  F  P  A  D  S  Y  G  L  A  R  F  D  G  P  A  L  Y

1171 GAATACGCCGACCCGAAAGAGGGCTACCACCAGGACTGGAACACGCTGATTTATAACTTTGACCGCCACGAAGTCCGTAACTATCTGGCG
 362  E  Y  A  D  P  K  E  G  Y  H  Q  D  W  N  T  L  I  Y  N  F  D  R  H  E  V  R  N  Y  L  A

1261 GGTAACGCACTGTACTGGACTGAGCGGTTTGGCGTCGATGGGCTGCGGGTGGACGCGGTGGCGTCGATGATTACCGCGATTACAGCCGC
 392  G  N  A  L  Y  W  T  E  R  F  G  V  D  G  L  R  V  D  A  V  A  S  M  I  Y  R  D  Y  S  R

1351 CGCGACGGGGAATGGGTGCCGAACTACTTCGGCGGCAAGGAGAATCTGGAGGCGATCGGCTTCCTGCGTTACACCAACCAGATGCTGGGG
 422  R  D  G  E  W  V  P  N  Y  F  G  G  K  E  N  L  E  A  I  G  F  L  R  Y  T  N  Q  M  L  G

1441 CAGCACCACGCCGGCGCGGTCACCATCGCCGAAGAGTCCACCGATTACGCCGGGGTGACGCTGCCGCCGGAGCATGGCGGGCTGGGCTTT
 452  Q  H  H  A  G  A  V  T  I  A  E  E  S  T  D  Y  A  G  V  T  L  P  P  E  H  G  G  L  G  F

1531 CACTACAAGTGGAACATGGGCTGGATGCATGACTCGCTGGCCTACATGCAACTGGACCCGGTGCACCGCAAATACCACCACGACCTGCTG
 482  H  Y  K  W  N  M  G  W  M  H  D  S  L  A  Y  M  Q  L  D  P  V  H  R  K  Y  H  H  D  L  L

1621 ACCTTCGGCATGCTGTACGCCTATAGCGAAAACTTGTGTTGCCGCTGTCGCATGACGAGGTGGTGCACGGCAAACGCTCGTTGCTGGAT
 512  T  F  G  M  L  Y  A  Y  S  E  N  F  V  L  P  L  S  H  D  E  V  V  H  G  K  R  S  L  L  D

1711 CGGATGCCCGGCGACGTCTGGCAGAAGTTCGCCAACCTGCGCGCCTATTACGGCTTCATGTGGGCCTACCCCGGCAAGAAGCTGCTGTTC
 542  R  M  P  G  D  V  W  Q  K  F  A  N  L  R  A  Y  Y  G  F  M  W  A  Y  P  G  K  K  L  L  F

1801 ATGGGCGGCGAATTCGCGCAGGGGCGGGAGTGGAACCACGACACCAGTCTGGACTGGCACCTGCTGGACGAGCCGGAGGGCTGGCATGCC
 572  M  G  G  E  F  A  Q  G  R  E  W  N  H  D  T  S  L  D  W  H  L  L  D  E  P  E  G  W  H  A

1891 GGGGTGCAGCAACTGGTGCGCGATCTCAACCACTGCTATCGCCAGCACCCGCCGCTCTATCAGTGCGACTACCTGCATCAGGGCTTTGAG
 602  G  V  Q  Q  L  V  R  D  L  N  H  C  Y  R  Q  H  P  P  L  Y  Q  C  D  Y  L  H  Q  G  F  E

1981 TGGGTGGTGGTGGACGATCGGGAAAACTCGGTGTTCGCCTTTATCCGGCGCGATGCCGACGGCAACGAAATGCTGATTATCAGCAACTTT
 632  W  V  V  V  D  D  R  E  N  S  V  F  A  F  I  R  R  D  A  D  G  N  E  M  L  I  I  S  N  F

2071 ACGCCGGTGCCGCGCGACAGTTATCGCGTCGGCATCAATCAGCCGGGGGGCCTGGCGCGAGGTACTCAATACCGATTCCTGGCATTACCAC
 662  T  P  V  P  R  D  S  Y  R  V  G  I  N  Q  P  G  A  W  R  E  V  L  N  T  D  S  W  H  Y  H

2161 GGCGGCAACCTGGGCAATCAGGGGCTGGTGTATAGCGAAACGGTGGGCAGCCATTCCCGGCCGCAGTCGCTGGTGCTGGCATTGCCGCCG
 692  G  G  N  L  G  N  Q  G  L  V  Y  S  E  T  V  G  S  H  S  R  P  Q  S  L  V  L  A  L  P  P

2251 CTCGCCACCCTGTATCTGGTGAAGGAGGCGTGATGGGTGAACTGTTGGCGGGTCGCCCGCGACCATTAGGCTCCCATTTCGATGGCGAAG
 722  L  A  T  L  Y  L  V  K  E  A  -

2341 GCGTGAACTTCGCCCTGTTCTCATCCGGGGCGTCGCGGGTGGAATTGTGCATTTTTGACGGCCTGCGGGAGCAGCGTTTGCCGCTGACGG
2431 CCCGCACCGGCGATATCTGGCACGGCTACCTGCCGGACGCGCAGCCGGGGTTGTGTTACGGCTACCGCGTCGATGGCGCGTTTGACCCGT
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Fig. 3. Nucleotide sequence and deduced amino acid sequence of the *glg*B gene. The nucleotide sequence is numbered from the first. The stop codon is indicated by bar. Initiation and termination codons are shaded. The consensus sequences among all type of GBE are boxed and designated regions I, II, II, and IV from start codon in order.

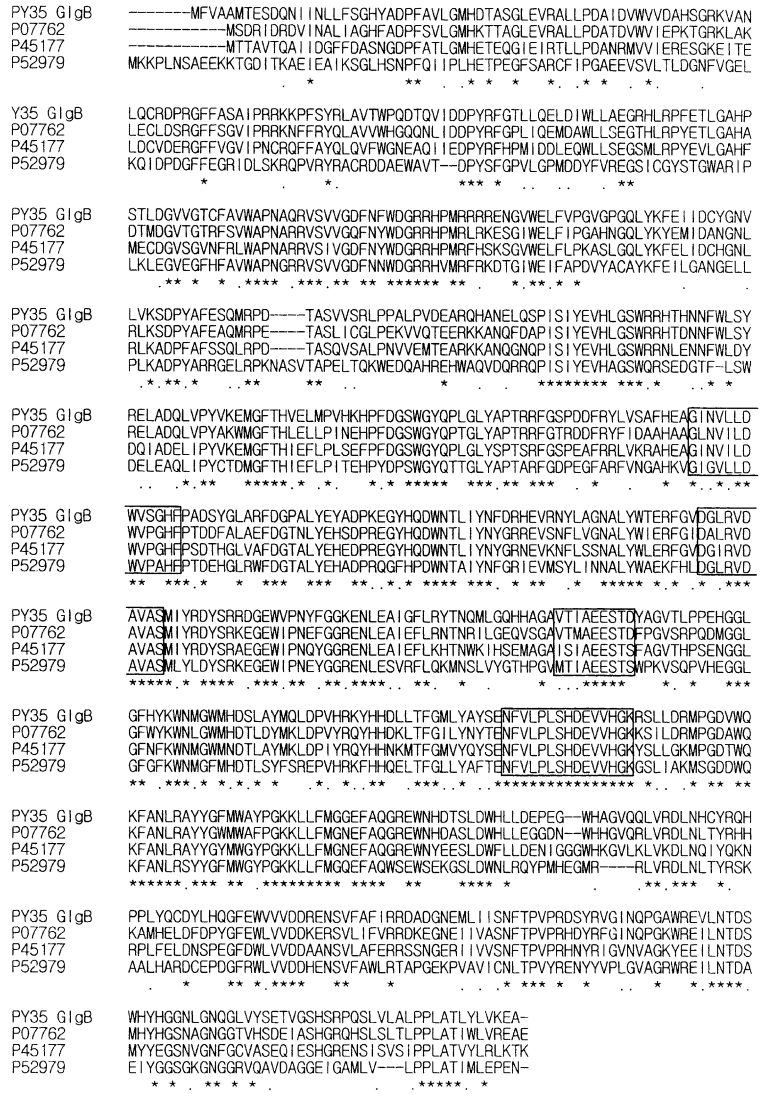

Fig. 4. Alignment of primary sequences of bacterial branching enzymes. The aligned enzymes are from *P. chrysanthemi* PY35 (AF434710), *E. coli* (P07762), *H. influenzae* (P45177), and *A. tumefaciens* (P52979). Asterisks indicate identical amino acid and dots indicate similar amino acids.

(P16954) of *Synechococcus* sp. PCC 7942, 47% with GlgB (P52981) of *Synechocystis* sp. PCC 6803, 47% with GlgB (P30539) of *Butyrivibrio fibrisolvens* [25], 45% with GlgB (P30537) of *Bacillus caldolyticus* [26], 45% with GlgB (P30538) of *Bacillus stearothermophilus* [27], and 43% with GlgB (P39118) of *Bacillus subtilis* [28].

The four regions conserved among the glycogen branching enzymes and glycogen debranching enzymes are shown in Table 1. These conserved regions have been reported in the α-amylase family previously. Svensson classified glycogen branching enzyme and glycogen debranching enzyme in the α-amylase family [29]. The conserved sequences of glycogen branching enzymes were XXXVXXDWVXXHF, DXXRXDAVAX, XXXAEXSTX, and XXXLXXSHDEVVXXK. Glycogen debranching enzymes had conserved sequences, GIEVILDXVXNHX, DGFRFDLXXX, KLIAEPWDX, and XX NXXTXHDGFTLXD. All of these enzymes, glycogen branching enzymes and glycogen debranching enzymes, had XXXVXX**D**XVXX**H**X, **D**XX**R**X**D**XXXX, XXX**A**E**X**XXX, and XXXXXXX **HD**XXXXX.

We have constructed a phylogenetic tree of the glycogen branching enzyme protein by the DNAMAN analysis system using above sequences, as shown in Fig. 5. The phylogenetic tree showed that the GlgB of *P. chrysanthemi* PY35 is very close to the GlgB of *E. coli*.

Purification of GlgB enzyme

For the purification of the enzyme, *E. coli* harboring pLYPY340 was grown at 37 °C. After centrifugation of the culture at 10,000*g* for 10 min at 4 °C, cell pellet was resuspended in buffer and lysed by sonication. The crude extract was used for further enzyme purification.

Table 1
The four conserved regions found in the glycogen branching enzymes and glycogen debranching enzymes

Source of enzyme	Region I	Region II	Region II	Region IV	Accession No.[a]
Glycogen branching enzyme					
Pectobacterium chrysanthemi PY35	GINVLLDWVSGHF	DGLRVDAVAS	VTIAEESTD	NFVLPLSHDEVVHGK	Pch PY35
Escherichia coli	GLNVILDWVPGHF	DALRVDAVAS	VTMAEESTD	NFVLPLSHDEVVHGK	P07762
Haemophilus influenzae	GINVILDWVPGHF	DGIRVDAVAS	ISIAEESTS	NFVLPLSHDEVVHGK	P45177
Synechococcus sp. PCC 7942	GIGVILDWVPGHF	DGIRVDAVAS	LSIAEESTS	NFMLALSHDEVVHGK	P16954
Bacillus caldolyticus	GLGVIIDWVPGHF	DGFRVDAVAN	WMIAEDSTD	NFILPFSHDEVVHGK	P30537
Bacillus stearothermophilus	GIGVILDWVPGHF	DGFRVDAVAN	LMIAEDSTD	NFILPFSHDEVVHGK	P30538
Butyrivibrio fibrisolvens	GIGVILDWVPAHF	DGLRVDAVAS	LTIAEESTA	NYILPLSHDEVVHLK	P30539
Agrobacterium tumefaciens	GIGVLLDWVPAHF	DGLRVDAVAS	MTIAEESTS	NFVLPLSHDEVVHGK	P52979
Streptomyces aureofaciens	GIGVIVDWVPAHF	DGLRADAVAS	VTIAEESTA	NFVLPISHDEVVHGK	P52980
Synechocystis sp. PCC 6803	GIGVIIDWVPGHF	DGMRVDAVAS	LSIAEESTS	NYMLALSHDEVVHGK	P52981
Mycobacterium tuberculosis	GIGVIVDWVPAHF	DGLRVDAVAS	VTIAEESTP	NYVLPLSHDEVVHGK	Q10625
Bacillus subtilis	NIGVILDWVPGHF	DGFRVDAVAN	MMIAEDSTE	HFVLPFSHDEVVYGK	P39118
Glycogen debranching enzyme					
Pectobacterium chrysanthemi PY35	GIEVILDVVFNHS	DGFRFDLATI	KLIAEPWDI	SVNMLTSHDGFTLRD	AY044255
Escherichia coli	GIEVILDIVLNHS	DGFRFDLAAV	KLIAEPWDC	AINLVTAHDGFTLRD	P15067
Pseudomonas aeruginosa PAO1	GLELILDVVYNHT	DGFRFDLATI	KLIAEPWDI	SVNFVTAHDGFTLRD	C83375
Haemophilus influenzae	GIEVILDVVFNHS	DGFRFDLATV	KLIAEPWDI	TLNFITAHDGFTLKD	P45178
Mycobacterium tuberculosis	GIEVILDVVYNHT	DGFRFDLAST	KLIAEPWDV	SINFVTAHDGFTLND	Q10767
Pasteurella multocida	GIEVILDVVFNHT	DGFRFDLGSV	KLIAEPWDI	I-NFITAHDGFTLRD	AAK02626
Conserved	* * *	* * *	* *	* *	

[a] Accession number from the SWISS-PROT protein (all start with 'P') and GenBank DNA sequence databases.

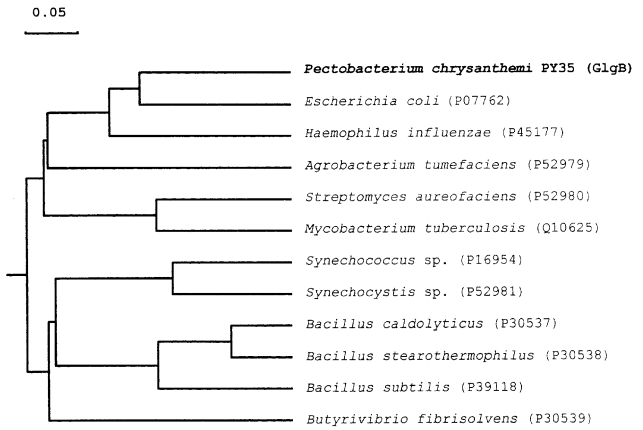

Fig. 5. Phylogenetic tree of prokaryotic glycogen branching enzymes. The aligned enzymes are from *P. chrysanthemi* PY35 (AF434710), *E. coli* (P07762), *H. influenzae* (P45177), *A. tumefaciens* (P52979), *Synechococcus* sp. PCC 7942 (P16954), *Synechocystis* sp. PCC 6803 (P52981), *B. fibrisolvens* (P30539), *B. caldolyticus* (P30537), *B. stearothermophilus* (P30538), *B. subtilis* (P39118), *S. aureofaciens* (P52980), and *M. tuberculosis* (Q10625).

After dialysis, a Q-Sepharose column was used to purify the branching enzyme (Fig. 6). The predicted *glg*B gene product consists of 731 amino acids with a calculated molecular weight of 83,859 Da, which corresponds well with the size of GlgB protein determined by SDS–PAGE. The molecular weight of the enzyme was estimated to be 84 kDa by SDS–PAGE (Fig. 6).

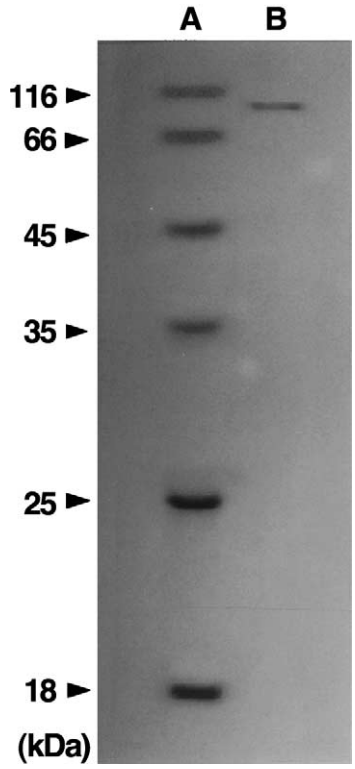

Fig. 6. Determination of molecular weight of glycogen branching enzyme by SDS–PAGE. Lane A, the molecular weight standard was stained with 0.025% Coomassie brilliant blue R-250 after electrophoresis. Molecular weight markers used were β-galactosidase (116,000), bovine serum albumin (66,200), ovalbumin (45,000), lactate dehydrogenase (35,000), restriction endonuclease *Bsp* 981 (25,000), β-lactoglobulin (18,400), and lysozyme (14,400). Lane B, the band is *E. coli* harboring pLYPY200 expressing purified glycogen branching enzyme.

Characterization of the glgB gene product

The effect of pH on the activity of the GlgB was determined at 30 °C in various buffers ranging from pH 3 to 10 (Fig. 7A). Maximal activity was observed at the pH 7. The temperature dependence of GlgB activity was determined by measuring activity at various temperatures at pH 7. As with pH dependence, GlgB was also dependent on temperature (Fig. 7B). Maximal activity was observed at 30 °C.

Discussion

Branching enzymes have been considered members of the α-amylase family [30], but little is known about this enzyme. Glycogen branching enzyme from *Pectobacterium* sp. has not been reported so far. We cloned and analyzed the structural gene for the glycogen branching enzyme from *P. chrysanthemi* PY35. We purified this

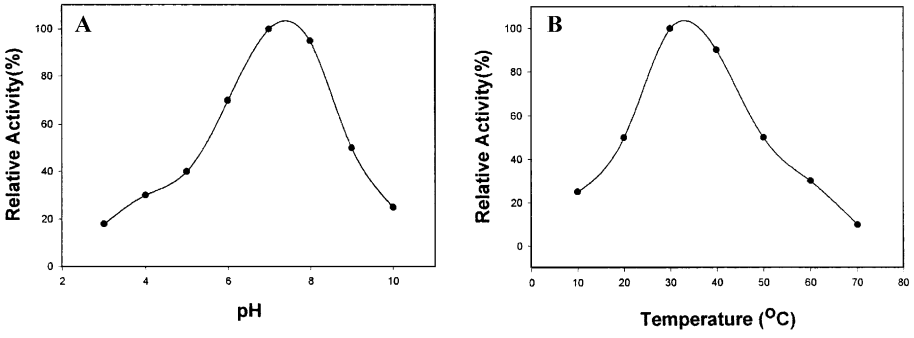

Fig. 7. Characteristics of the glycogen branching enzyme activity of GlgB enzyme expressed in *E. coli*. (A) The GlgB enzyme was assayed for its glycogen branching enzyme activity using amylose as a substrate at different pH values at 30 °C for 30 min. (B) The GlgB enzyme was assayed for its glycogen branching enzyme activity using amylose as a substrate at different temperature values at pH 7 for 30 min.

branching enzyme and analyzed the enzymatic properties of the enzyme. The ORF of *glg*B is 2196 bp in size, thus encoding a protein of 731 amino acids. The *glg*B gene is downstream of *glg*X. The coding regions of the *glg*B and *glg*X ORFs overlap by 1 bp. This tandem structure of glycogen branching and debranching enzyme in *P. chrysanthemi* PY35 may allow glycogen synthesis and disintegration to be controlled organically.

The amino acid sequence of GlgB is very similar to that of glycogen branching enzymes of other bacterium. The amino acid sequence of GlgB of *P. chrysanthemi* PY35 is the most similar to that of GlgB of *E. coli*, with 69% identity. GlgB of *A. tumefaciens* and GlgB of *Synechococcus* sp. PCC 7942 are of high similarity at the protein level to GlgB of *P. chrysanthemi* PY35. These amino acids have four conserved regions commonly even in the conserved regions reported in the α-amylase family previously. Glycogen branching enzymes belong to the α-amylase family. In the sequences of various bacteria, existing glycogen branching enzymes, proposed regions 1, 2, 3, and 4 matched each other quite well. In four conserved regions the sequences D (late) of region II, E of region III, and D of region IV are at the catalytic site. H is shared commonly with regions I and IV at the substrate binding domain [29,30].

The genetic organization of the glycogen operon (*glg*) was determined in *E. coli* [15,16], *Bst* [31], *Bsu* [28], and *Atu* [32]. The *glg* operon is located at approximately 75 min on the *E. coli* K-12 chromosome map [17]. The arrangement and nucleotide sequence of the entire *glg* cluster revealed that a continuous DNA fragment of over 15 kb flanked by the genes *asd* [33] and *glp* D [34] contains the genes encoding the branching enzyme (*glg*B), ADP-glucose pyrophosphorylase (*glg*C), and glycogen synthetase (*glg*A) and two genes, *glg*X (homologous to genes encoding α-amylases) and *glg* P (homologous to the rabbit glycogen phosphorylase gene) [15,17]. None of the latter genes are required for glycogen synthesis, but they are needed for glycogen metabolism. Detailed inspection of the organization of the *E. coli glg* cluster suggests that *glg* genes may be transcribed as two operons, *glg*BX and *glg* CAP [17]. The coding regions of the *glg*B and *glg*X open reading frames (ORFs) of *E. coli* overlap by 1 bp. In *P. chrysanthemi* PY35 GlgB and GlgX are the most similar to GlgB and GlgX of *E. coli*, respectively. The coding regions of the *glg*B and *glg*X of *P. chrysanthemi* PY35 ORFs overlap by 1 bp, as well. The organization of the *glg*BX operon in *P. chrysanthemi* PY35 is similar to that in *E. coli* [17]. Thus, it is possible that *glg*B and *glg*X genes of *P. chrysanthemi* PY35 are partial glycogen operon.

Microorganisms in nature are often faced with a "feast-or-famine" type of existence, and many bacteria have evolved biochemical systems for the production of storage compounds that serve material. These storage compounds become especially important under conditions of limited nutrient supply [35]. Our results provide that the genes of *P. chrysanthemi* responsible for uptake and utilization of glycogen are most likely to constitute an operon. Taken together, it is plausible that *Pectobacterium* sp. attack host plants resulting in degrading of the plant's cell walls and then pathogens absorb the metabolizable sugars into the cell. These sugars can be converted to glycogen by a series of enzyme systems for the food reservation of *Pectobacterium* sp. The conversion of monomer sugars into glycogen is likely to occur during periods of excess carbon availability. The lack of genetic studies on glycogen metabolism had prevented further insights into the physiological and evolutionary aspects of this pathway. Also, so far the metabolism of glycogen has not been studied whether it is related to the pathogenicity of soft rot caused by *Pectobacterium* sp. Further work is needed to search neighboring genes and to determine the function and regulatory system of GlgB in *P. chrysanthemi* PY35. This is possible to accomplish by cloning and characterizing the gene upstream *glg*B.

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