Cloning of Isoamylase Gene of *Pectobacterium carotovorum* subsp. *carotovorum* LY34 and Identification of Essential Residues of Enzyme

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The gene encoding for isoamylase of the *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) LY34 was cloned and expressed into *Escherichia coli* DH5 α . Isoamylase catalyzes the hydrolysis of α -1,6-glycosidic linkages specifically in amylopectin, glycogen, and derived oligosaccharides, while the enzyme did not hydrolyze α -1,4-glycosidic linkages of amylose. The isoamylase gene (*glgX*) had an open reading frame of 1,977 bp encoding 658 amino acid residues with a calculated molecular weight of 74,188 Da. The molecular weight of the enzyme was also estimated to be 74 kDa by activity staining of a SDS-PAGE gel. The mature GlgX had a calculated pI of 4.91. Isoamylase from *Pcc* LY34 had 70% amino acid identity with isoamylase from *Pectobacterium chrysanthemi* and contained the four regions conserved among all amylolytic enzymes. The isoamylase was optimally active at pH 7.0 and 40°C. GlgX was Ca²⁺-dependent. The changes of Asp-335, Glu-370, and Asp-442 into Ala, respectively, using site-directed mutagenesis techniques showed that three residues are essential to isoamylase (GlgX) activity. The sequences around those residues were highly conserved in isoamylase of different origins and GlgX of the *glg* operon in glycogen biosynthesis.

Key words – *Pectobacterium carotovorum* subsp. *carotovorum* LY34, isoamylase, essential residue, site-directed mutagenesis

Introduction

Pectobacterium carotovorum subsp. *carotovorum* is the most commercially important soft rotting pathogens in various plants. The phytopathogenicity of the *Pectobacterium* sp. is mainly due to their capacity to synthesize and secrete depolymerizing enzyme which macerates the major component of plant cell walls. *Pectobacterium* sp. produces several extracellular enzymes including pectate lyases, polygalacturonases, cellulases, and proteases [2].

Isoamylase (EC 3.2.1.68, glycogen 6-glucanohydrolase) hydrolyzed α -1,6-glucosidic linkage in α -glucans such as amylopectin and glycogen. Some bacteria are able to produce isoamylase: *Bacillus amyloliquefaciens* [32], *Flavobacterium* sp. [13], *Streptococcus bovis* [27], *Pseudomonas amyloclavata* [8], and *Escherichia coli* K-12 (as GlgX protein) [34]. Also, the nucleotide sequences of isoamylase genes have been de-

termined from some organisms mentioned above [13,24]. In the amino acid sequence of isoamylase, four regions conserved in α -amylases are also found [18]. Many bacterial species excrete amylolytic enzymes into their environment to digest exogenous amylopectin and amylose. Since some of them are of commercial importance, they have been studied extensively [35]. In contrast to extracellular enzymes, few genes encoding intracellular amylolytic enzymes have been reported [6,22].

The strain *Pcc* LY34, used in the present study, was originally isolated from Chinese cabbage tissue with soft-rot symptoms. Three cellulases, referred to as CelA (accession number AF025768), CelB (AF025769), and CelC (AY188753) and two β -glucosidases, referred to as AscB (AY622309), BglB (AY54253) of *Pcc* LY34 were previously isolated to analyze their importance in phytopathogenicity in our laboratory [16,20].

The gene of isoamylase from *Pcc* LY34 have been cloned and expressed. As our aim of this study is to understand the relationship between the function and structure of isoamylase, we first sequenced the entire isolated DNA frag-

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ment and deduced the primary sequence of isoamylase of *Pcc* LY34. In this article, we describe the cloning and sequencing of the isoamylase gene from *Pcc* LY34 and the biochemical characterization of the recombinant enzyme expressed in *E. coli* DH5 α and the catalytic residues of isoamylase clarified by site-directed mutagenesis.

Materials and methods

Bacterial strains and growth conditions

Pectobacterium carotovorum subsp. *carotovorum* LY34 was grown at 28°C in tryptone-yeast extract medium. *Escherichia coli* DH5 α and recombinant *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium containing the appropriate concentration of antibiotics: 50 μ g/ml 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 [26]. 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 Chemical Co. (St. Louis, MO, USA). Nucleotide sequences were determined by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CT, USA). The samples were analyzed with an automated DNA sequencer (Model 3100 Applied Biosystems, Foster City, CA, USA). The BLAST program was used to find the protein coding regions.

Cloning of the isoamylase gene

Chromosomal DNA was isolated from *Pcc* LY34 according to the method of Park *et al.* [21]. The genomic library was constructed by ligating partially *Sau*3AI-digested genomic DNA fragments with pBluescript II SK+ cut with *Bam*HI. To detect isoamylase activities from *E. coli* cells harboring the cloned isoamylase genes, bacterial colonies were first grown on LB broth supplemented with ampicillin (50 μ g/ml). After incubation overnight, cells of 3 ml culture harvested by centrifugation at 5,000 $\times$ g for 5 min were resuspended in 200 μ l of 10 mM Tris-HCl buffer (pH 7.5) and sonicated four times at 4°C for 60 sec in 3 min in-

tervals (Bandelin Sonopuls UW2070, Berlin, Germany). Supernatants obtained after centrifugation for 20 min at 17,000 $\times$ g were used as crude enzymes. Crude enzymes were applied to LB agar supplemented with 0.1% (w/v) amylopectin (Sigma Chemical Co., St. Louis, MO, USA). After incubation for 2 hr, the plates were stained with staining solution [0.15% (w/v) iodine and 1.5% (w/v) potassium iodine].

For high expression of GlgX, the PCR product generated with primers, 5'-GGATCCATGGCAGAATTGCAGACAGG-3' (sense, containing a *Bam*HI site as underlined) and 5'-AAGCTTGGCGGTCTGTTTATCAAGA-3' (antisense, containing a *Hind*III site as underlined) was cloned into expression vector of pET-21a(+) using *Bam*HI and *Hind*III sites, resulting in the addition of a C-terminal (His)₆tag. The resulting plasmid was designated pET-21a(+)/GlgX. The absence of mutations within the coding region of *glgX* was verified by DNA sequencing.

Expression and purification of enzyme

E. coli strain BL21 (DE3) carrying pET-21a(+)/GlgX was grown at 37°C in LB medium containing 50 μ g/ml ampicillin. The cells were harvested by centrifugation (6,000 $\times$ g, 10 min) and washed twice with 10 mM Tris-HCl buffer (pH 7.0). The frozen cells were mixed with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mg of bovine DNase I (Sigma Chemical Co., USA) and incubated at 37°C for 30 min. Triton X-100 was added to the suspension to a final concentration of 2.5%. The solubilized recombinant GlgX with His-tag (GlgX-His) was applied on a HisTrap kit (Amersham Pharmacia Biotech., Pennsylvania, USA). GlgX was eluted with 100 mM imidazole with 0.1% Triton X-100. The active fractions were combined and purified by ion exchange chromatography on a Q-Sepharose column (Amersham Pharmacia Biotech., Pennsylvania, USA) [23]. For the final purification step, the dialyzed sample was loaded onto an anion exchange Mono Q HR (Amersham Pharmacia Biotech., Pennsylvania, USA) column pre-equilibrated with 20 mM MOPS buffer (pH 6.5). The fractions with enzyme activity eluted as a single protein peak and the purity of the enzyme were assessed by SDS-PAGE. The protein concentration was determined by the method of Bradford [5].

Enzyme assay

The isoamylase activities in the recombinant *E. coli*

clones were determined by measuring the amount of reducing sugars released during incubation with amylopectin. To 250 μ l of 0.5 % (w/v) amylopectin dissolved in a 50 mM sodium acetate buffer (pH 7.0), 200 μ l of the enzyme solution was added, and the samples were incubated at different temperatures for 20 to 60 min. The amounts of reducing sugars released were determined by the dinitrosalicylic acid method [3,7]. Blank samples were used to correct the non-enzymatic release of reducing sugars. The effects of pH and temperature on the isoamylase activity were examined with the crude recombinant enzyme. The effect of pH on the isoamylase activity was determined by using the protocol described above, to obtain values from pH 3.0 to 11.0; all of the assays were performed at 40°C. To determine the effect of temperature on the enzymatic activity, samples were incubated at temperatures from 0 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 described above. The effects of various substances on the isoamylase activity were examined after co-incubation of the enzyme with metal ions and other reagents in various concentrations at 40°C for 30 min.

Isoamylase activity staining after SDS-PAGE

Identification of an *glgX* gene product was performed in 0.75 mm gels in a vertical slab unit by a modification described by Lim *et al.* [14,15]. The separating gel containing 10% acrylamide, 0.33% bisacrylamide, and 0.1% amylopectin was to detect activity of electrophoresed GlgX. The protein samples were separated by SDS-PAGE (Mini Protean 3, BioRad). Subsequently the protein was renatured by incubation in three changes of 250 ml of 10 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100 with shaking for 10 hr. Finally the gel was incubated in 10 mM Tris-HCl (pH 7.5) at 40°C for 8 hr. The gel was then immersed in staining solution for 5 min. Active band appeared as a white halo on a dark background.

Site-directed mutagenesis

The *glgX* gene (2.6 kb), encoding the isoamylase from *Pcc* LY34 was used for site-directed *in vitro* mutations. Site-directed *in vitro* mutations were used for D335A with 30 mer synthetic oligonucleotide primers 5'-GGCTTCCGTTTCGCACTGGCAACGGTGCTG-3' (forward) and 5'-CAGC

ACCGTTGCCAGTGC~~G~~AAACGGAAGCC-3' (reverse), for D370A with 30 mer primers 5'-CAAGCTGATTGCCGCACCGTGGGACATCGG-3' (forward) and 5'-CCGATGTCCCAACGGTGC~~G~~GCAATCAGCTTG-3' (reverse), and for D442A with 27 mer 5'-CTGACGGCACATGCCGGCTTTACGCTG-3' (forward) and 5'-CAGCGTAAAGCCGGGCATGTGCCGTCAG-3' (reverse). The 50 μ l of reaction mixtures were contained 1 μ l of the pET-21a(+)/GlgX DNA (80 ng/ μ l), 10 pmol of each primer, 1 μ l of 2 mM dNTP mixture, 5 μ l of 10 X *Pfu* DNA polymerase buffer containing 20 mM MgSO₄ and 2.5 U of cloned *Pfu* DNA polymerase purchased from Stratagene (La Jolla, CA, USA). After predenaturation at 95°C for 5 min, twelve cycles of treatments at 95°C for 30 sec, 55°C for 1 min, and 68°C for 5min followed by at 72°C for 10 min were applied to the reaction mixture with PCR cyclor. Negative control (without DNA) was included in every PCR procedure. PCR products were incubated on ice for 5 min, and added 1 μ l of *DpnI* restriction enzyme (10 U/ μ l), gently and thoroughly mix reaction mixture and incubated at 37°C for 1 hr. One micro-liter of incubated PCR mixture was used to transform into *E. coli* DH5 α according to the manufacturer's specifications of site-directed mutagenesis kit (Stratagene, La Jolla, CA) [9].

Nucleotide sequence accession number

The nucleotide sequence and the deduced amino acid sequence encoded by *glgX* have been submitted to GenBank under the accession number EU022588.

Results

Detection of isoamylase, positive clone, and subcloning

A clone showing amylopectin digesting activity was isolated from amylopectin containing agar plates. One clone showed the white ring that indicated isoamylase activity. The clone was found to contain an insert of 2.6 kb. The pBluescript II SK+ containing 2.6 kb insert was designated pKY200 (Fig. 1). To further localize isoamylase gene in the insert, *Bst*XI, *Sph*I, and *Eco*RI-digested fragments of pKY200 were subcloned into pBluescript II SK+ vector. The ligation mixture was used for transforming into *E. coli* DH5 α and the transformants showing isoamylase activity were screened on LB agar plates containing 0.1% amylopectin by staining of the plates. pKY210, pKY220, and pKY230 clones did not show any isoamylase activity (Fig. 1 and Fig. 2).

Fig. 1. Physical map of recombinant DNA pKY100 carrying *glgX* gene of *Pcc* LY34. The cleavage sites of restriction enzymes *Sac*II, *Eco*RV, *Eco*RI, *Cla*I, *Bst*XI, and *Nco*I are shown. pKY100 was constructed by cloning a 2.6 kb fragment of *Pcc* LY34 into pBluescript II SK+ vector.

Fig. 2. Detection of an isoamylase positive clone by amylopectin agar diffusion method. The cell extracts were incubated at 37°C for 2 hr. Lane 1A, *E. coli* DH5α as a negative control; lane 1B, *E. coli* DH5α harboring pBluescript II SK+ as a negative control; lane 2A, *E. coli* DH5α harboring pKY200; lane 2B, *E. coli* DH5α harboring pKY210; lane 3A, *E. coli* DH5α harboring pKY220; lane 3B, *E. coli* DH5α harboring pKY230.

pKY200 was selected for further characterization. The isoamylase gene of pKY200 clone was designated *glgX* and restriction map of *glgX* is shown in Fig. 1.

Sequencing of the 2.6 kb insert encompassing *glgX*

The entire 2.6 kb insert in pKY200 was sequenced in both strands, and an ORF was identified (Fig. 3). *glgX* is 1,977 bp in size and encodes a protein of 658 amino acids with a predicted molecular mass of 74.2 kDa. A Shine-Dalgarno sequence of GAAGG is present at positions -9 to -13 in relation to the ATG site. The reading frame ends with the ochre stop codon TAA at position 1,975.

Amino acid sequence homology of *glgX* with other amylolytic enzymes

Protein sequence databases (SWISS-PROT, PIR, and GenBank) were searched using the standard search algorithms (programs FASTA and BLASTP) to find poly-

peptides related to GlgX. GlgX of *Pcc* LY34 shared significant sequence similarity with many previously reported AmyX of *P. chrysanthemi* [14], GlgX of *E. coli* [24], GlgX of *Pasteurella multocida* [17], and GlgX of *Haemophilus influenzae*. GlgX of *Pcc* LY34 shared 70% amino acid identity with AmyX of *P. chrysanthemi*, 65% with GlgX of *Yersinia pestis*, 63% with GlgX of *E. coli*, 61% with GlgX of *S. typhimurium* and 51% with GlgX of *H. influenzae* (Table 1). Also, GlgX of *Pcc* LY34 has partial homology with isoamylase, pullulanase, α-dextran endo-1,6-α-glucosidase, alkaline amylopullulanase, α-amylase, and branching enzymes (data not shown). The four regions conserved among the amylolytic enzymes are shown in Table 2. These conserved regions have been reported in the α-amylase family previously. Specifically, Nakajima and his colleagues identified that region III was the most conserved in the four regions [18].

Characterization and identification of the *glgX* gene product.

To characterize the isoamylase in the recombinant *E. coli* DH5α cells, direct activity staining technique that allows rapid and specific detection of isoamylase on the polyacrylamide slab gels was employed. The predicted *glgX* gene product consists of 658 amino acids with an estimated molecular mass of 74,188 Da, which corresponds well with the size of GlgX protein determined by SDS-PAGE (Fig. 4). The technique takes advantage of the ability of isoamylase to degrade amylopectin even in very small amounts. After electrophoresis the gel was rinsed, re-natured, and incubated. The gel was then stained with staining solution. A white halo appeared around a band where amylopectin was degraded. The pattern was reproducible. A protein band with the apparent molecular weight of about 74,000 Da, produced a halo (Fig. 4).

A number of different α-glucans were incubated with GlgX in order to determine the substrate specificity. As shown in the Table 2, GlgX preferentially hydrolyzed amylopectin. Glycogen was hydrolyzed efficiently, while amylose and dextran were not cleaved. The effect of pH on the activity of GlgX against amylopectin was determined at 40°C in various buffers ranging from pH 3.0 to 11.0 (Fig. 5A). Maximal activity was observed at the pH 7.0, and 60% activity was observed at pH 5.0 and 9.0. The temperature dependence of GlgX activity toward amylopectin was determined by measuring activity at various temperatures

1 TCTGGACCTGGCATCTTCTGGATGAGCCGGAAGGTTGGCACCGCGCGTGCAACAGCTGGTGCGCGATCTCAACCACTTACTATCGCCAGCACCGCTCGCTGTATCAGCTTAGATTTTCAGCC
121 GCAGGGCTTTGAATGGCTGGTGGTGGACGATCGGGAATAATCAGTTTTCGCTTTTGTCCGGCTGATGACGAGGGCAACGAATGCTGGTGGTCAGTAACCTTACGCCGGTGCCACGTTA
241 TGGCTATCGGATTGGCATTAACTCGCCCGCGGCTGGCGAGAAGTCATCAACACCGATTCTGTTCACTACAACGGTAGCGATTTGGGCAATGTTGGGACGATTTACAGTGAGGAATGGG
361 CGCGATCAGCGCCAGCATCTCTGTTCTTGACTATTCGCGCGCTTGCTACGCTGTATCTGGTAGAGGAGGGCTAAATGGCAGAATTGCAGACAGCAAGCCGACGCCCTGGGAGCCAG
1 MAELQKTPTLGAS
481 TTTTGCAGGACAGGGCGTGAAATTCGCGCTGTTTTCAGCGGATGCTGAGCGGGTGGAGCTGTGCATATTTGATGAACGCCAGCAGGAGCAGCGCTGAACTGACCGCGCAGCGGCGA
16 F D G G G V N F A L F S S A D A E R V E L C I F D E R Q Q E Q R L E L T A R S G D
601 TATCTGGACGGCTACCTTCTGCTGGCAGCCCGGCTACGTTACGGTTTCGGGGTGTATGGCGCTTCGAGCGCTCACAAGGCTTGGCGTTCAACCCACATAAGCTGCTGTAGATG
56 I W H G Y L P A A Q P G L R Y G F R V D G P F E P S Q G L R F N P H K L L L D P
721 GTGGCTGTGACGCTTACGGCTGGTGGTGGATGATGATGCTGCAAGCGGGCATGACCAGCGGATGAACGGCATAGCGCTGACATCATGGGCAATGCGTAGTCACGGCAGAGGA
96 C A R Q L L D G W V V D D D C L Q G G I D Q R D E R D S A D I M A K C V V T A E D
841 TTACGATTGGCAGATGACCACTCGGCACACGCTCTGGCATCAGACGGTGATTTATAGGCAACAGCTTGCTGGGTTGACGCAACTGCACCCCTGATATTCTCGAAGATTTCGGCGTAG
136 Y D W Q D D Q H P H T L W H Q T V I Y E A H V R G L T Q L H P D I P E D I R G S
961 CTATCGGCGCTGGGCGACCCGGTGATGATTGACTATCTGACGTGCTGGGCGTGACGGCGCTGGAACCTGCTTCGGTGACAGCAGATGCCGATGAGCGCGGCTTCAGCAACTGGGCGT
176 Y A A L G H P V M I D Y L T S L G V T A L E L L P V Q H A D E P R L Q Q L G L
1081 GCGTAATTACTGGGCTATAAGCTATGTTGCCGTTGACGTTACAGTTTGGCTGTGGGTGATGACGCGCTGAATGAATTCGATATGCGGTGAAGCGCTGCATCGTGGGGGAT
216 R N Y W G Y N V L L P F A V D N S L A V G D D A L N E F R Y A V K A L H R A G I
1201 CGAGGTATTCTGGATGTGGTGTAAACCAAGCGCAGAACTGGATGTTGAAGGTCACCGCTGTGCCAGCGCGCATCGATAACCGCAGTTATTACTGGCTGGATGAAACGGTGAATA
256 E V I L D V V F N H S A E L D V E G P T L C Q R G I D N R S Y Y W L D E N G E Y
region I
1321 CCATAACTGGACCGGCTGCGGCAATGTGTTGCCGCTGAACCATCGGCGGTCATTGATTGGTGATGGACTGTCTGCGCTTCTGGCGGAGGCTGCCACGTTGATGGCTTCGTTTCGA
296 H N W T G C G N V L R L N H P A V I D W V M D C L R F W R E V C H V D G F R F D
region II
1441 TCTGGCAACGGTGCTGGGGCAACCCGGATTTTACGCCGCTGCGCGGCTGTTGTCCGCATGAAACAGATAGCCGCTTACAGGGCTGCAAGCTGATTGCCGACCGTGGGACATCGG
336 L A T V L G R T P D F T A A A P L L S A M K N D S R L L Q G C K L I A E P W D I G
region III
1561 GCATGGCGGTTATCAGTTAGGTCAGTTCGCGACCGGCTTCGGGAGTGAGTAGACCGCTACCGTGACGATATGCGCGGTTTTTGGCTGCATGGCGATTTCCCTCGGCCGCTTCGCTCG
376 H G G Y Q L G Q F P T P F A E W S D R Y R D M R F W L H G D I S L G A F A R
1681 CCGCTTTCGCCGCTCCAGCGATATTTCCAGCAGCAGCAGCGCTGCTTTCGCTCTATTAAACAACTAGCGGCACATGACGGCTTACGCTGCGGATCTGGTGAGTTTCAACCAAA
416 R F A A S S D I F Q Q H D R L P F A S I N K L T A H D G F T L R D L V S F N H K
region IV
1801 GCACAACGAGCCCAACGGCAGGGTAACCGCAGCGTACAGACAGTAACCTCAGCAATAACCGGTAACGGAAGGCTGGAAGCGGGCAGCAGCATCTCAGCGTTCGGCTGGCGAGCCA
456 H N D A N G E G N R D G T D S N F S N N H G T E G L E A G D D I L Q R R L A S Q
1921 GAAGCGGCTGCTGACGACGCTGATCTGTGCGAGGGCAGCCATGCTGCTGGCGGGCGATGAATCGGGCAGCAGCAGCAGGGAACAACAATGCCTACTGTGAGGATACGAAATGAC
496 C K A L L T T L I L S Q T P T M L L A G D E L G H S Q G N N A Y C D N E L T
2041 CTGGCTGATTTGGGAAACGCAATAGTGACCTGCGCGAGTTTGTGCGGGATGATCAACTGCGCGCGACAGATCCCGGCAATTACAGCAGAGAGCGTGGTGGCAAGAGGGGAGCGTGGC
536 W L H W E N A N S A L R E F V A G I L Q L R R T I P A L Q Q E T W W Q E G D G A
2161 AGTGACGTGGCTCAACAGAGAAGGCCAGCGGTTGAGCGCCGACAGATGGGAACAAGGGGAGCATCAGCTACAGATTTTACTGTGTCGTGGCTGTGGCTGTTTAAAGCCAGCGCTACA
576 V Q W L N R E G Q P L T P Q Q W E Q G E H Q L Q L I L S G R W L V L F N S A L H
2281 CGCGGTGATTTGATGTCGAGAGGCCACTGAGCAATTTACCGCGGTTTGTATGAGACGACCCGCGGAGGGCGGAATCTGGCAGCAGACAGCCGAGCGGATGTGTCTTGATATAA
616 A G E F M L P E G H W Q V S P P F D E T N P P E G G I W H G Q A Q A V C V L I K
2401 ACAGACCGGCTAAGCGCAAGCCATTAAAGAAATAATCAGGAGATAGCCATGGTGAATAACGACAAGCATGACCTCTGATGTTGGCAAGCAGCTCCCTCTTAAATCCGTGGCATTAAAT
656 Q T A *
2521 TTAGCGGAGGCGTGAAGCAGCGGCTGAAAGGGCTGACGGCGCTGCGTGCCAAGCCGCGGCTCCACTTTGGCGCAAAATTCGCAATTATGATTTCGCGCTCTCTAAGTGCCTGAATTT

Fig. 3. Nucleotide sequence and deduced amino acid sequence of *glgX* 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 types of amylolytic enzymes are gray boxed and designate region I, II, III, and IV.

at pH 7.0. As with pH dependence, GlgX was also dependent on temperature (Fig. 5B). Moreover, activity was observed at 40°C. GlgX was dependent on concentration of Ca^{2+} (Table 3).

Identification of essential residues for isoamylase activity

Site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships, identifying intramolecular regions of amino acids, which may mediate these functions, gene expression, and vector modification. In this study, we replaced all three possible candidates (one Glu residues and two Asp residues) with Ala. All the mutant plasmids were sequenced on both

strands, and we confirmed that no error other than the intended mutation was introduced. Enzyme activity assays were done for GlgX and the single-point mutants D335A, E370A and D442A. Every mutation of D335, E370, or D442 to Ala abolished enzyme activity, respectively (Table 4).

Discussion

Various extracellular enzymes such as cellulases and pectate lyases have been isolated from *P. chrysanthemi* and *Pcc*, which attack cell walls and membranes of plants [12,16,19-21,25,28,35]. But, the amylolytic enzymes from *Pcc* have not been reported so far. A gene encoding the isoamylase from *Pcc* LY34 was cloned and sequenced in this

Table 1. Pairwise similarity between isoamylase amino acid sequences¹⁾

Enzyme source ²⁾	Similarity(%) with debranching enzyme sequence											
	1	2	3	4	5	6	7	8	9	10	11	12
<i>Pcc</i> LY34	100.0	72.2	64.4	44.3	50.4	62.1	62.5	53.5	42.5	42.9	46.7	47.3
<i>Pch</i> PY35		100.0	62.1	43.8	48.3	61.2	61.6	50.9	41.9	43.4	47.5	47.3
<i>Eco</i>			100.0	44.5	50.6	85.4	85.3	51.0	42.0	43.1	45.5	47.9
<i>Asp</i>				100.0	40.8	44.7	44.7	43.4	48.0	59.4	52.2	53.4
<i>Hin</i>					100.0	51.2	51.2	68.7	39.4	42.4	42.2	45.0
<i>Sty</i>						100.0	99.7	52.0	42.7	43.5	45.7	47.4
<i>Sen</i>							100.0	52.1	42.5	43.6	46.1	47.5
<i>Pmu</i>								100.0	42.0	43.0	44.5	46.1
<i>Sme</i>									100.0	46.4	50.7	51.2
<i>Dra</i>										100.0	52.6	51.2
<i>Pae</i>											100.0	67.1
<i>Xca</i>												100.0

¹⁾Calculated with CLUSTAL W and PAM250 residue weight table.

²⁾The sequences are from the following sources: *Pcc* LY34, *Pectobacterium carotovorum* subsp. *carotovorum* LY34; *Pch* PY35, *Pectobacterium chrysanthemi* PY35; *Eco*, *Escherichia coli*; *Asp*, *Arthrobacter* sp.; *Hin*, *H. influenzae*; *Sty*, *S. typhimurium*; *Sen*, *S. enteritica*; *Pmu*, *P. multocida*; *Sme*, *S. meliloti*; *Dra*, *D. radiodurans*; *Pae*, *P. aeruginosa*; *Xca*, *X. campestris*.

Table 2. Regions Conserved among amylolytic enzymes¹⁾

Enzyme source	Amino acid sequence of conserved regions			
	I	II	III	IV
<i>Pcc</i> LY34	GIEVILDVVFNHS	DGFRFDLATV	KLIAEPWDI	PFASINKSTAHDGFTLRD
<i>Pch</i> PY35	GIEVILDVVFNHS	DGFRFDLATI	KLIAEPWDI	PWASVNMLTSHDGFTRLR
<i>Eco</i>	GIEVILDIVLNHS	DGFRFDLAHV	KLIAEPWDI	PSAAILNLVTAHDGFTLRD
<i>Hin</i>	GIEVILDVVFNHS	DGFRFDLATV	KLIAEPWDI	PHTTLNFITAHDGFTLRD
<i>Sme</i>	GIEVLMDVVYNHT	DGFRFDLAST	KLIAEPWDV	ATSSINLVSAHDGFTLRD
<i>Pae</i>	GLELILDVVYNHT	DGFRFDLATI	KLIAEPWDC	PYASVNFVTAHDGFTLRD
<i>Dra</i>	GIEVILDVVYNHT	DGFRFDLAST	KLIAEPWDV	PYASINLVTAHDGFTLRD
Conserved	* * * * *	*****	*****	* ***** *

¹⁾The underlined amino acids are conserved among all type of amylolytic enzymes, while those high lighted with an asterisk are conserved among all the enzymes listed here region I, II, III, and IV have been previously defined. *Pcc* LY34, *Pectobacterium carotovorum* subsp. *carotovorum* LY34; *Pch* PY35, *Pectobacterium chrysanthemi* PY35; *Eco*, *Escherichia coli*; *Hin*, *H. influenzae*; *Sme*, *S. meliloti*; *Pae*, *P. aeruginosa*; *Dra*, *D. radiodurans*.

study. Sequencing of the entire 2.6 kb insert in pKY200 confirmed the presence of an open reading frame. The ORF is 1,977 bp in size, thus encoding a protein of 658 amino acids.

Evaluation of amino acid sequences of GlgX from *Pcc* LY34, led to the prediction that it would be an intracellular enzyme because of devoid of any specific kind of prokaryotic signal peptide. The amino acid sequences of GlgX are very similar to that of AmyX of *P. chrysanthemi*, with 70% identity to that of GlgX of *Y. pestis*, with 65%. Isoamylase belongs to the α -amylase family. In the sequences of various bacteria, existing isoamylase, proposed regions 1, 2, 3, and 4 matched each other quite well. In four conserved re-

gions 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 [18,29,31]. From these results, it was suggested that the GlgX of *Pcc* LY34 belong to one of the subfamilies of isoamylase.

Mutation of one Glu residues (E370) and two Asp residues (D335 and D442) to Ala, resulted in the activity determination of the mutant enzymes clearly indicated that D335, E370, and D442 were essential for *Pcc* LY34 isoamylase (Table 4), and those residues were well conserved in all the sequences listed in Fig. 3. Recently, the three-dimensional structure of isoamylase from *Pseudomonas* has

Fig. 4. Detection of isoamylase activity band by Amylopectin-SDS-PAGE method. Lane S, the molecular weight standard was cut off after electrophoresis and stained with 0.025% Coomassie blue R-250. Molecular weight markers used were phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Lane A and B, extract of *E. coli* DH5 α harboring pKY200 expressing GlgX.

Fig. 5. Characteristics of the relative activity of isoamylase expressed in *E. coli* DH5 α . (A) The GlgX was assayed for the isoamylase activity using amylopectin as a substrate at different pH values at 40°C for 30 min. (B) The GlgX was assayed for the activity of isoamylase using amylopectin as a substrate at different temperature values at pH 7.0 for 30 min.

Table 3. Effect of calcium ion concentration on isoamylase activity¹⁾

Concentration of CaCl ₂	Relative activity (%)
None	100
1 mM	100
5 mM	130
10 mM	150
20 mM	150
30 mM	150
40 mM	150
50 mM	150
100 mM	150
200 mM	150

¹⁾Enzyme activity was assayed at pH 7.0 and 40°C for 30 min

Table 4. Specific enzyme activities of GlgX and mutant enzymes of *P. carotovorum* subsp. *carotovorum* LY34¹⁾

Enzyme	Specific activity (U/mg)
GlgX	6.2×10^4
D335A	<10
E370A	<15
D442A	<10

¹⁾Specific enzyme activities were assayed at pH 7.0 and 40°C for 30 min

been solved by Katsuya et al. [11]. According to their model, D375, E435, and D510 of *Pseudomonas* isoamylase are located at the bottom of a deep cleft, and their three-dimensional location resembles those of the catalytic residues of amylase. These residues corresponded to D335, E370, and D442 of *Pcc* LY34 isoamylase, respectively. All mutants were lost their enzymatic activity. This implies that the dissociation rate of the enzyme-substrate complex is faster for the GlgX enzyme than for the mutants. In previous, Takase [30] reported that substitution of any of these residues in *Bacillus subtilis* α -amylase (BSUA) causes an almost complete loss of catalytic activity, with substrate-binding ability being retained by site-directed mutagenesis. Isoamylase contains a bound calcium ion that has been reported in other α -amylase family enzymes [1].

GlgX was expressed in *E. coli*, and the action properties of the protein were found to be similar to those of bacterial glycogen debranching enzyme and isoamylases, although its primary sequence is rather similar to that of amylase proteins. One debranching enzyme has been isolated from *E. coli* [10], and it was reported that phosphorylase-limit dextrins of glycogens, which have a branch degree of polymerization of 4, are good substrates, but native

starches or glycogens are inert. Yang *et al.* [34] reported that this enzyme corresponds to GlgX. GlgX from *Pcc* LY34 hydrolyzed amylopectin and glycogen, but the enzyme was specific on amylopectin.

Glycogen is proved to be a rather poor substrate for GlgX. In spite of this result, we consider it the most likely natural substrate, since it is the only polysaccharide present in appreciable amounts in the intracellular of bacteria. Some bacteria accumulate glucoses intracellularly as glycogen. Light and electron microscopy studies of the bacterial glycogen revealed that the bacteria were embedded in an intracellular matrix [4]. Since the sequence of GlgX is very similar to those of glycogen debranching enzymes of glycogen operon in *P. chrysanthemi*, *Y. pestis*, *E. coli*, *S. typhimurium*, and *H. influenzae*, it is possible that the *glgX* of *Pcc* LY34 is a unit of glycogen operon. Further work is needed to search neighboring genes and to determine the function and regulatory system of GlgX in *Pcc* LY34. This is possible to accomplish by cloning and characterizing the gene flanking *glgX*.

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초록 : *Pectobacterium carotovorum* subsp. *carotovorum* LY34에서 Lsoamylase 유전자 클로닝 및 효소 활성의 필수 잔기 확인

조계만 · 김은주 · 레누카라디아 마스 · 샤 모하마드 아스라 폴 · 홍선주 · 신기재 · 김종욱 · 이영한² · 김훈³ · 윤한대^{1*}
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연부균인 *Pectobacterium carotovorum* subsp. *carotovorum* LY34로부터 이소아밀라제 유전자 (*glgX*)를 클로닝한 후 대장균 숙주에서 발현시켰다. 이 효소는 α -1,6-글루코시드 결합을 가수분해하였으나 α -1,4-글루코시드 결합은 가수분해 하지 못하였다. 유전자는 658개의 아미노산을 암호화하는 1,977개의 DNA 염기서열로 이루어져 있었고 이 유전자에 의해 암호화되는 아미노산 서열을 다른 아밀라제 효소들과 비교한 결과 이소아밀라제 유전자와 유사하였으며 4개의 보존 지역을 확인하였다. SDS-PAGE에 의해 확인된 단백질의 크기는 약 74 kDa 이었다. 효소 활성은 pH 7.0, 40°C에서 가장 높은 활성을 나타냈으며 Ca^{2+} 첨가로 활성이 증가되었다. 이 효소의 보존되어 있는 아미노산 중에 글루탐산 370번, 아스파르트산 335번 및 442번 잔기를 알라닌으로 치환시킨 결과 활성이 약해졌다. 이 결과로부터 이들 잔기들이 효소활성에 중요한 역할을 하는 것으로 추정된다.