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# Effect of C- or D-Domain Deletion on Enzymatic Properties of Cyclodextrin Glucanotransferase from *Bacillus stearothermophilus* NO2

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**Abstract** To analyze the role of the C and D domains in the cyclization activity of cyclodextrin glucanotransferase (CGTase), two plasmids, pKB1△C300 and pKB1△D96, were constructed in which DNA regions encoding 100 and 32 amino acids, respectively, from the C and D domains of *B. stearothermophilus* NO2 CGTase were deleted. The mutated CGTase from the pKB1△C300 produced much lower amounts of α-, β- and γ-cyclodextrin (CD) than the parental CGTase. However, the mutated CGTase from the pKB1△D96 showed a similar production pattern of CDs to wild-type CGTase. The production ratios of the α-, β- and γ-CDs were not affected by the deletions, when compared to those of parental CGTase. The optimum temperature of the mutated CGTase from the pKB1△C300 was decreased from 60°C to 55°C. The optimum pH of the mutated CGTase from the pKB1D96 was shifted from 6.0 to 7.0. The thermostability of the two mutant CGTases were not changed. From these results, it is suggested that the C and D domains are not related to cyclization activity directly because mutant-enzymes deleted C or D domains still possessed their activity. However, they are important for other enzymatic properties such as productivity and pH optimum as a partition of CGTase tertiary structure.

**Key words:** Cyclodextrin glucanotransferase, *B. stearothermophilus*, C/D domain, deletion mutants

Cyclodextrins (CDs) are cyclic oligosaccharides in which 6, 7, or 8 glucose units are linked by α-1,4 glucosidic bonds, and are named α-CD, β-CD, and γ-CD according to the number of glucose units [19]. They are able to form inclusion complexes with many organic and inorganic molecules, thereby changing the physical and chemical properties of the included compounds. CDs are produced from starch by intramolecular transglycosylation reactions of cyclodextrin glucanotransferase (CGTase; EC 2.4.1. 19), which is produced mainly by *Bacillus* strains [13]. CGTase is an important enzyme for the food and pharmaceutical industries. Although CGTase and α-amylase catalize the cleavage of α-1,4 glucosidic bonds in amylose and amylopectin, their products are quite different. Most α-amylases are composed of approximately 500 amino acids, and most CGTases of approximately 700 amino acids. The COOH-terminal region of CGTase contains an extra 200 amino acids, in addition to the $NH_2$-terminal region of α-amylase. It is well known that there are at least four highly conserved regions within 400 amino acids residues in the $NH_2$-terminal, in many amylolytic enzymes including CGTase [11, 17]. Recently, the tertiary structure of CGTase from *Bacillus stearothermophilus* TC-91 was determined [11] and found to consist of four globular domains, A, B, C, and D. Structural analysis showed that the $NH_2$-terminal domain (A and B domains) was similar to the structure of Taka-amylase and that the COOH-terminal domain (C and D domains) was unique to the CGTase.

By the site-directed mutagenesis of CGTase genes of *B. stearothermophilus* [5] and alkalophilic *Bacillus* sp. [18], it was known that the A domain in the $NH_2$-terminal region contained the catalytic site for cyclization function. The role of the extra C and D domains of the CGTase was also reported. The function of the COOH-terminal region of CGTases in enzymatic properties were different according to the origin of the enzymes. The optimum pH of alkalophilic *Bacillus* sp. #1011 [9] was dependent on the COOH-terminal segment. Deletion of 10 or 13 amino residues of the COOH-terminal of the CGTase from alkalophilic *Bacillus* sp. [10] reduced the pH stability of the enzymes in the alkaline pH range (pH 9-11). It was also known that the D domain of *B. stearothermophilus* NO2 played a role for the substrate

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binding site, and substrate binding at the D domain enhanced the thermostability of the enzyme. Furthermore, mutant enzyme deleted a part of the D domain (74 amino acid residues) was not secreted into the culture medium [4]. From these reports, it was supposed that the $NH_2$-terminal region was important for cyclization function and the COOH-terminal region was related to enzymatic properties such as pH stability, thermostability, secretion, and products ratio. However, the function of the COOH-terminal region was not obvious because the enzymatic characteristics of the COOH-terminal mutant enzymes were variable.

In order to analyze the functions of the COOH-terminal region of the CGTase from *B. stearothermophilus* NO2, we constructed two deletion mutants in which DNA regions encoding part of the C or D domains of the enzyme were deleted, and examined the characteristics of the mutant enzymes. In this paper, we demonstrated that the deletion of the C domain reduced the cyclization activity of the CGTase and the deletion of the D domain shifted the optimum reaction pH from 6.0 to 7.0.

## MATERIALS AND METHODS

### Bacterial Strains and Plasmids

*Escherichia coli* JM109 [*rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 *sup*E44 *rel*A1 $\lambda^-$ △(*lac-pro*AB)/F': *pro*AB, *lacI*$^q$Z△M15 *tra*D36] [21] and *Bacillus subtilis* NA1 (arg-15 *hsm*M *hsr*M Amy$^-$ Npr$^-$) [12] were used as hosts for recombinant DNA manipulations. Plasmid pTB523 [7] was used as a subcloning vector for *B. subtilis* transformation. Plasmid pKBR1, into which a 6.2 kb-DNA fragment containing the CGTase gene from *B. stearothermophilus* NO2 [4] was cloned in plasmid pBR322 [2], was used as a parental plasmid for construction of deletion plasmids. The deletion plasmids pKBR1△C300 and pKBR1△D96 were constructed from pKBR1 according to the strategy illustrated in Fig. 2.

### Media

CS1 medium containing 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.05% $K_2HPO_4$, and 0.01% $MgSO_4 \cdot 7H_2O$ was used for production of the CGTase. For agar plates (CS1 azure plate), soluble starch was replaced by 0.2% starch azure (Sigma, St. Louis, MO, U.S.A.) and 1.5% agar was added. Starch azure, in which potato starch is covalently linked with Remazol brilliant blue R., was used as the substrate for the colorimetric assay for α-amlase [13]. Amylase-producing bacteria can form clear halos around colonies on CS1 azure plates.

### DNA Manipulation

Plasmid DNA was prepared by either the rapid alkaline extraction methods [1] or CsCl-ethidium bromide equilibrium density gradient centrifugation [6]. Restriction endonucleases, S1 nuclease, alkaline phosphatase, and T4 DNA ligase were purchased from Promega (Promega, Madison, WI, U.S.A.) and used as recommended by the manufacturer. For the analysis of DNA, agarose gel electrophoresis was performed under standard conditions [16]. A Gene Clean Kit (Bio 101, La Jolla, CA, U.S.A.) was used to recover DNA from the agarose.

### Transformation

Transformation of *E. coli* and *B. subtilis* was carried out by the methods of Cohen [3] and Anagnostopoulous [8], respectively. Transformants were selected on CS1 azure plates supplemented with 15 μg/ml tetracycline.

### Assay of CGTase Activity

α-CD specific CGTase activity was assayed by the methyl orange method as described previously [15]. The reaction mixture (3,000 μl), containing 600 μl of 5% soluble starch in 50 mM phosphate buffer (pH 6.0), 105 μl of 1 mM methyl orange in 50 mM phosphate buffer (pH 6.0), 2,845 μl of 50 mM phosphate buffer, and 50 μl of enzyme solution, was incubated for 10 min at 60°C. α-, β-, and γ-CD were identified by HPLC. The reaction mixture (pH 6.0) containing 5% soluble starch (pH 6.0) was incubated with 200 U of the enzyme in a total volumn of 3 ml. The CDs formed were analyzed by HPLC with an Amide column (TSK-GEL Amide-80; 4.6×250 mm; Tosoh, Japan) and a refractive index detector (Waters, model 410, Milford, MA, U.S.A.). The temperature of the column was kept at 70°C. The mobile phase was acetonitrile/water (60/40) which flowed at a rate of 1 ml/min [9].

### Other Procedures

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at a concentration of 7.5% (w/v) polyacrylamide as described by Laemmli [14]. Protein concentration was measured by the method of Smith *et al.* [20] with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.).

## RESULTS AND DISCUSSION

### Construction of Deletion Mutants of CGTase

Based on the nucleotide sequence of CGTase from *B. stearothermophilus* NO2 [4], the deletion plasmid pKBR 1△C300 and pKBR1△D96 were constructed from pKBR1, according to the cloning strategy illustrated in Fig. 2. As shown in Fig. 1, restriction sites (*Nsi*I-*Sca*I, *Sca*I-*Sac*I) not disturbing the open reading frame after deletion were selected. The cleaved site, composed of cohesive ends, was then blunt-ended using S1 nuclease. For the construction of pKBR1△C300, in which a DNA

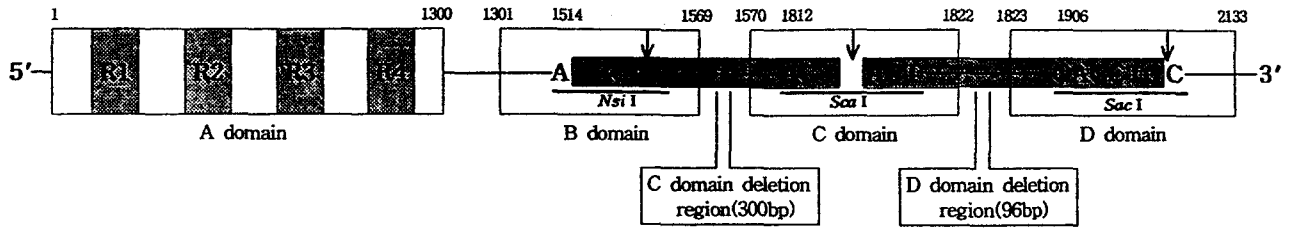


**Fig. 1.** Deletion region of the C or D domain of the CGTase gene of *B. stearothermophilus* NO2.
The number of the nucleotide sequence starts at the ATG start codon. The conserved regions of the amylolytic enzymes are indicated as R1-R4 and the deletion region is indicated by a gray box.

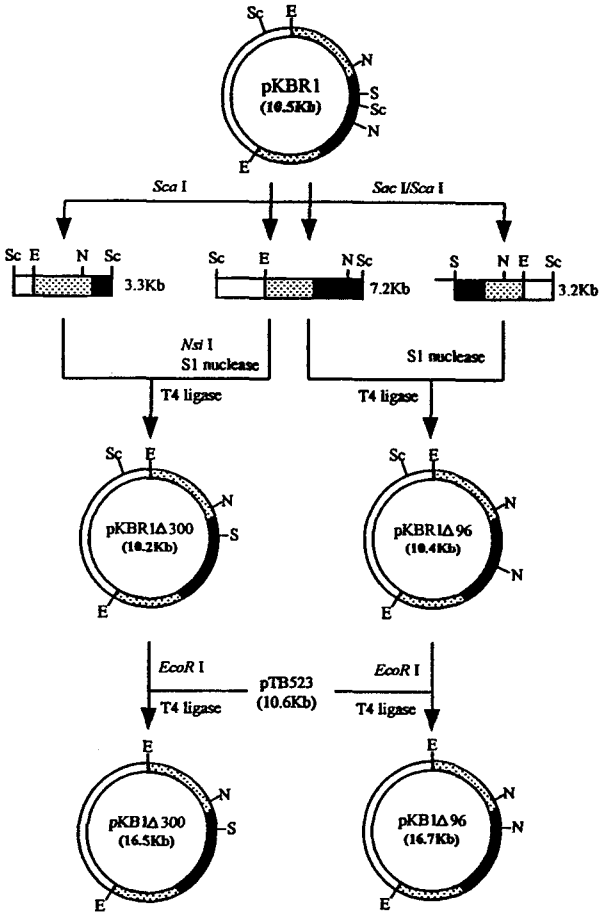


**Fig. 2.** Scheme for the construction of deletion plasmids.
Open reading frame of CGTase is indicated by a black box and the flanking region is indicated by a dotted box. Abbreviations: E, *EcoR*I; N, *Nsi*I; S, *Sac*I; Sc, *Sca*I.

region encoding 100 amino acids of the CGTase C domain was deleted, 7.2- and 3.3-kb fragments of pKBR1 resulting from *Sca*I digestion were recovered. The 7.2-kb fragment was digested with *Nsi*I and then ligated with the 3.3-kb fragment pretreated with S1 nuclease. For the construction of pKBR1△D96, in which a DNA region encoding 32 amino acids of the CGTase D domain was deleted, the pKBR1 was double digested with *Sca*I and *Sac*I, and three fragments of 7.2 kb, 3.2 kb, and 0.1 kb were recovered. The 3.2-kb fragment was digested with S1 nuclease, and ligated with the 7.2-kb fragment. *E. coli* JM109 cells were transformed with pKBR1△C300 or pKBR1△D96, and the transformants were selected on CS1 azure plates containing 15 μg/ml tetracycline. The structures of the constructed deletion plasmids were further confirmed by identifying the size reduction of *EcoR*I fragments and the loss of *Nsi*I and *Sca*I sites in the CGTase region of pKBR1△C300 or *Sac*I and *Sca*I sites in pKBR1△D96. The deletion plasmids pKBR1△C300 and pKBR1△D96 were stably maintained and efficiently expressed in *E. coli* (data not shown). However, the majority of mutated CGTases expressed in *E. coli* were localized in the periplasmic space of the cell.

## Subcloning of Deletion Mutant CGTase Genes in *B. subtilis* Vector

The deleted CGTase genes of pKBR1△C300 and pKBR 1△D96 were subcloned into the pTB523 vector [4] for efficient secretion in *Bacillus* due to the problem of secretion in *E. coli*. The *EcoR*I-*EcoR*I fragments of pKBR1△C300 and pKBR1△D96 were inserted into the *EcoR*I site of pTB523 treated with calf intestinal alkaline phosphatase, resulting in the recombinant plasmids pKB 1△C300 and pKB1△D96. These were transformed into *B. subtilis* NA-1.

## Expression of Deletion Mutant CGTases in *B. subtilis*

To determine the expression efficiency of the mutant CGTases, *B. subtilis* cells harboring each of the recombinant plasmid were cultivated on CS1 medium containing 25 μg/ml tetracycline at 37°C until equal cell concentrations ($OD_{660}$, about 2.0) were reached. The enzyme activities in the culture supernatants were then assayed. The CGTase activities of the *B. subtilis* containing pKB1△D96 or pKB1△C300 were 106.6 mU/ml (specific activity, 380 mU/mg-protein) and 5.5 mU/ml (sp. activity, 12 mU/mg-protein), respectively. On the other hand, the control cells containing pKB1, in which the whole CGTase gene was subcloned into the pTB523, produced 86.6 mU/ml (sp. activity, 340 mU/mg-protein) under the same culture conditions (Fig. 3). These results indicated that deletion of the D domain led to the

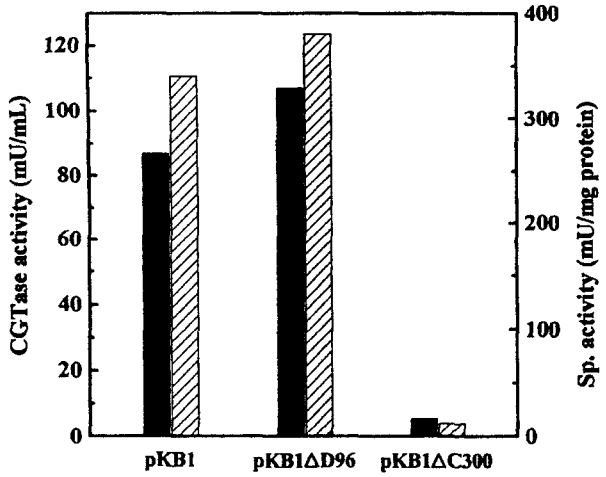


**Fig. 3.** Production of extracellular CGTases in the cultures of *B. subtilis* NA-1 containing plasmid pKB1, pKB1△C300, and pKB1△D96 on CS1 medium.
Total CGTase activity (black bar), specific activity (dashed bar).

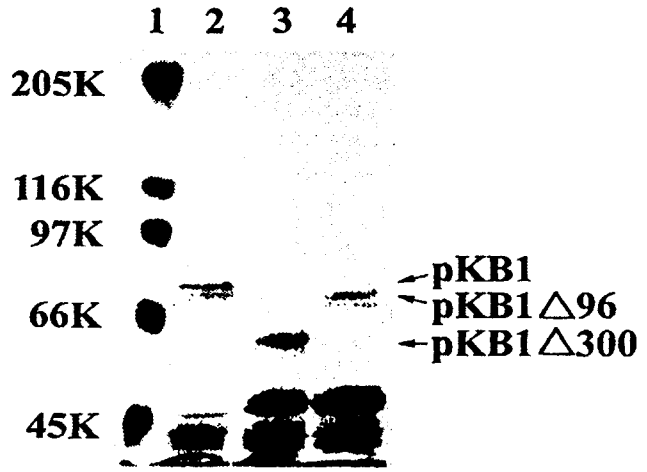


**Fig. 4.** SDS-polyacrylamide gel electrophoresis of partially purified CGTases.
*B. subtilis* NA-1 carrying wild-type and mutant CGTase genes was grown in CS1 medium at 37°C for 24 h. The culture supernatant was precipitated by 60% ammonium sulfate. The precipitate was dissolved in 50 mM phosphate buffer (pH 6.0) and dialyzed against the same buffer. The dialyzed solution was applied to a Sephadex G-150 column equilibrated with 50 mM phosphate buffer (pH 6.0) and eluted with the same buffer. Proteins were stained with Coomasie Brillant Blue G. Lane 1, protein molecular weight marker; lane 2, pKB1; lane 3, pKB 1△C300; lane 4, pKB1△D96.

enhanced expression of CGTase and that deletion of the C domain caused a very low level production of the enzyme. The C domain deletion was assumed to cause the low productivity of pKB1△C300 not only by affecting the efficiency of expression, but also the enzyme activity by conformational change, as the ratio of enzyme productivity to specific activity of the mutant CGTase toward wild-type CGTase was 1.8 (5.5/86.6 vs 12/340). To confirm this fact, the enzymes were partially purified by 60% ammonium sulfate precipitation, Sephadex G-150 gel filtration chromatography. Equal amounts of the enzymes (300 mU) were analyzed by SDS-PAGE. As shown in Fig. 4, the molecular weights of the mutant enzymes from pKB1△C300 and pKB1△D96 were estimated to be 64 kDa and 72 kDa, respectively, while that of the wild-type enzyme from pKB1 was 75 kDa. In the case of pKB1△C300, the protein band was thicker than the others in spite of the same amount of enzyme loaded. This result indicated that the deletion of the C domain resulted in a decrease of enzyme activity due to a conformational change.

It was reported that deletion of a part of the D-domain (74 amino acids) from the $NH_2$-terminal end of CGTase, which was the same enzyme with our work, was not secreted into the culture medium [4]. To investigate and compare the distribution of the enzymes, the localization of the intra- and extracellular enzymes were determined (Table 1). The wild-type and mutant enzymes of pKB1△C300 and pKB1△D96 were secreted at a 50~56% level into the culture medium. This fact reveals that the deletion of the C or D domains do not influence the secretion of the enzymes. This observation is contrary to the previous work by Fujiwara *et al.* [4]. It is interesting that the deletion of 74 amino acids from the $NH_2$-terminal end (637th-711th) did not result in secretion, and the deletion mutant of the other part of the D domain (605th-636th) still retained secretion ability. From these results, it is suggested that the C domain is involved indirectly in cyclization activity through a conformational stabilization, and the $NH_2$-terminal end of the D domain is partly necessary for secretion of the enzyme.

**Table 1.** Localization of CGTase activities in the cultures of *B. subtilis* NA-1 containing each plasmid on CS1 medium.

| Plasmid | CGTase Activity (mU/ml) | | | Secretion (%) |
|---|---|---|---|---|
| | Extracellular | Intracellular | Total | |
| pKB1 | 86.6 | 83.4 | 170.0 | 51 |
| pKB1△D96 | 106.6 | 105.5 | 212.1 | 50 |
| pKB1△C300 | 5.5 | 4.3 | 9.8 | 56 |

### Analysis of Reaction Products

To examine the function of the C and D domains in the CGTase reaction, the partially purified enzymes were reacted with 5% soluble starch at 55°C for 24 h. The reaction products were analyzed by HPLC and the ratios of α-, β-, and γ-CD were calculated. The ratios of α-:β-:γ-CD produced by the mutant CGTases from pKB 1△D96 and pKB1△C300 were found to be 39:45:16 and 43:39:18, respectively. These values are not significantly different from the 40:44:16 values of wild -type CGTase.

The function of the COOH-terminal region of β-CGTase of alkalophilic *Bacillus* sp. #1011 was previously examined using mutated enzymes in which DNA regions encoding 10 or 13 amino acids from the COOH-terminus were deleted [10]. Kimura *et al.* [10] reported that the reaction products produced from starch by the mutated enzymes were glucose, maltooligosaccharides, α-CD, and β-CD. Therefore, they suggested that the $NH_2$-terminal region of CGTase, which is common to other amylolytic enzymes, possesses starch degrading activity and that the COOH-terminal region is important for cyclization function. However, such an inference was not obvious because very small regions (only 13 or 14 amino acid residues) of the COOH-terminal had been deleted and the mutant enzymes still possessed the cyclization activity in spite of variation of the products. In this work, the enzymes with deleted C or D domains (100 or 32 amino acid residues) exhibited the cyclization activity and produced equal amounts of CDs when compared to parental CGTase. Hence, the C and D domains in the COOH-terminal region of *B. stearothermophilus* NO2 CGTase seem not to be involved directly in the cyclization function. The $NH_2$-terminal domains, A and B, may be important for the cyclization function.

## Effect of pH and Temperature on the Enzyme Activity

To examine the effect of C or D domain deletion on the enzymatic properties of CGTase, the pH, temperature optima, and thermal stability of two mutant enzymes were determined and compared with those of wild-type CGTase. The optimum pH of the mutant CGTases from pKB1△C300 and pKB1△D96 were found to be 6.0 and 7.0, respectively, compared to 6.0 of wild-type CGTase (Fig. 5). The CGTase activity of pKB1△D96 was maximal at 60°C, a temperature optimum identical to that of wild-type CGTase. However, the optimum temperature of the mutant CGTase from pKB1△C300 was decreased to 55°C (Fig. 6). The optimum pH shift of

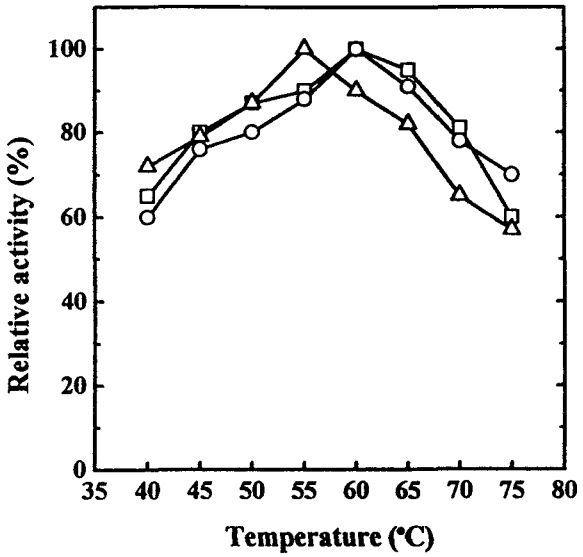


**Fig. 6.** Effects of temperature on the activity of CGTases.
The enzymes were assayed with 5% soluble starch (pH 6.0) for 30 min at various temperatures. Symbols: pKB1 (◇), pKB1△D96 (○), pKB 1△300 (△).

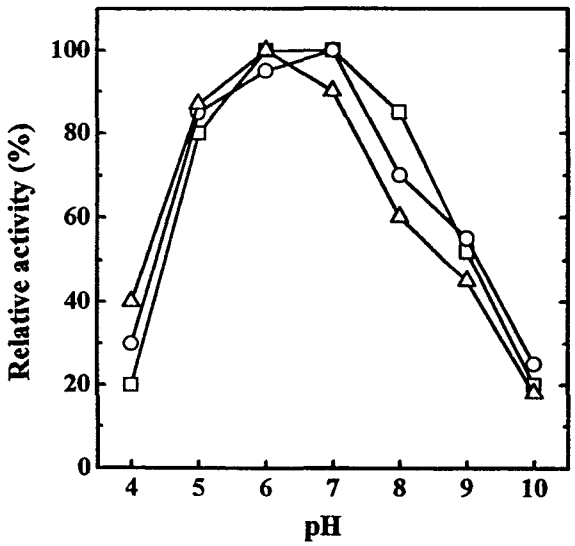


**Fig. 5.** Effects of pH on the activity of CGTases.
The enzymes were assayed with 5% soluble starch in the pH range indicated for 30 min at 60°C. The following buffers were used; pH 4-5, sodium acetate, pH 6-7, potassium phosphate, pH 8-10, glycine/NaOH. Symbols: pKB1 (◇), pKB1△D96 (○), pKB1△300 (△).

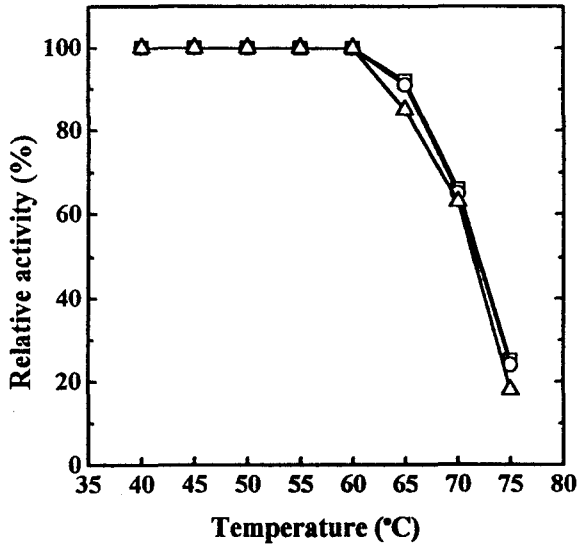


**Fig. 7.** Thermal stability of the CGTases.
Enzyme solutions were preincubated at the indicated temperature for 1 h in the absence of substrate, and then the relative activities were assayed under the conditions described in the Materials and Methods. Symbols: pKB1 (◇), pKB1△D96 (○), pKB1△300 (△).

CGTase from pKB1△D96 and optimum temperature reduction of CGTase from pKB1△C300 may have been induced by some modifications of the secondary and tertiary structure of the wild-type enzyme by deletion. After the enzymes were incubated at various temperatures for 1 h, the remaining CGTase activities were measured. All three enzymes (two mutant enzymes and wild-type enzyme) retained approximately 65% of the enzyme activity even after heat treatment at 70°C for 1 h (Fig. 7). Fujiwara *et al.* reported that substrate bound at the cleft between the A and D domains (30 Å) and this substrate binding stabilized thermostability of the enzyme [5]. In this work, C or D domain deletion might lead to an altering of the distance between the A and D domains by conformational change. However, the thermostability was not affected. More precise studies will be conducted to clarify the substrate binding ability at the D domain.

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