Journal of Biochemistry and Molecular Biology, Vol. 37, No. 4, July 2004, pp. 408-415

JOURNAL OF
Biochemistry and Molecular Biology
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Expression of Cyclodextrinase Gene from *Paenibacillus* sp. A11 in *Escherichia coli* and Characterization of the Purified Cyclodextrinase

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Received 9 September 2003, Accepted 30 September 2003

The expression of the *Paenibacillus* sp. A11 cyclodextrinase (CDase) gene using the pUC 18 vector in *Escherichia coli* JM 109 resulted in the formation of an insoluble CDase protein in the cell debris in addition to a soluble CDase protein in the cytoplasm. Unlike the expression in *Paenibacillus* sp. A11, CDase was primarily observed in cytoplasm. However, by adding 0.5 M sorbitol as an osmolyte, the formation of insoluble CDase was prevented while a three-fold increase in cytoplasmic CDase activity was achieved after a 24 h-induction. The recombinant CDase protein was purified to approximately 14-fold with a 31% recovery to a specific activity of 141 units/mg protein by 40-60% ammonium sulfate precipitation, DEAE-Toyopearl 650 M, and Phenyl Sepharose CL-4B chromatography. It was homogeneous by non-denaturing and SDS-PAGE. The enzyme was a single polypeptide with a molecular weight of 80 kDa, as determined by gel filtration and SDS-PAGE. It showed the highest activity at pH 7.0 and 40°C. The catalytic efficiency (k_{cat}/K_m) values for α-, β-, and γ-CD were 3.0×10^5, 8.8×10^5, and 5.5×10^5 M^{-1} min^{-1}, respectively. The enzyme hydrolyzed CDs and linear maltooligosaccharides to yield maltose and glucose with less amounts of maltotriose and maltotetraose. The rates of hydrolysis for polysaccharides, soluble starch, and pullulan were very low. The cloned CDase was strongly inactivated by *N*-bromosuccinimide and diethylpyrocarbonate, but activated by dithiothreitol. A comparison of the biochemical properties of the CDases from *Paenibacillus* sp. A11 and *E. coli* transformant (pJK 555) indicates that they were almost identical.

Keywords: Cyclodextrin, Cyclodextrinase, *Paenibacillus* sp. A11, Sorbitol

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Introduction

Cyclodextrinase (CDase; EC 3.2.1.54) catalyzes the hydrolysis of cyclodextrins (CDs) to form linear oligosaccharides of α-1,4-linkages (Depinto and Campbell, 1968). Its substrates, CDs, are cyclic oligomers of glucose that are formed by the action of cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) on starch. Three forms of CDs, the α, β, and γ (6-8 glucose units, respectively), are commonly produced by several CGTases (Bender, 1986; Rojtinnakorn *et al*., 2001; Martins and Rajni, 2002; Kaulpiboon and Pongsawasdi, 2003). CDs offer a variety of industrial applications in the foods, cosmetics, and pharmaceuticals fields as stabilizers, solubilizers, and control-released substances (Schmid *et al.,* 1988). More attention was thus given to CGTase studies, because of its CD-forming activity. Recently, the interest in CDase was intensified in order to gain a better understanding of the CD metabolism. The mode of CD metabolism was proposed in *Klebsiella oxytoca* M5a1, *Thermococcus* sp. B1001, and alkalophilic *Bacillus* sp. A2-5a (Fiedler *et al*., 1996; Ohdan *et al*., 2000; Hashimoto *et al*., 2001).

The CDase gene from *Clostridium thermohydrosulfuricum* 39E was first cloned and characterized (Podkovyrov and Zeikus, 1992) and studies in other strains followed (Oguma *et al*., 1993; Fiedler *et al*., 1996; Kim *et al*., 1998). The overproduction of CDase from alkalophilic *Bacillus* sp. I-5 in *Escherichia coli* MC1061 was reported by Kim *et al*. (1998). There are several reports on the overproduction of target proteins or enzymes in *Escherichia coli*, where the products were frequently observed to be insoluble aggregates (Lee and Tao, 1994; Hanning and Makrides, 1998; Kim *et al*., 1999). Attempts were reported on the successful prevention of the insoluble aggregation of proteins. These were made by changing the culture temperature (Chalmers *et al*., 1990), adjusting the inducer concentration (Donovan *et al*., 1996), adding an osmotic stabilizer such as sorbitol, glycyl betain and mannitol (Blackwell and Horgan, 1991; Kim *et al*., 1999), or non-metabolizing sugars, such as sucrose, and raffinose to the

growth medium (Bowden and Georgiou, 1988). Lowering the induction temperature to 30°C and/or adding mannitol osmolyte were reported to be effective in increasing the expression of the soluble active CGTase from *Brevibacillus brevis* in *Escherichia coli* (Kim *et al.*, 1999); however, no such study on CDase has ever been reported.

We previously reported the cloning and sequencing of the gene coding for *Paenibacillus* sp. A11 CDase (GenBank accession No. AY205309) (Kaulpiboon *et al.*, to be published). In this study, we attempt to overproduce the A11 CDase in *E. coli* in the presence of some polyols that were added to the culturing medium. The purification and characterization of the recombinant enzyme and comparison with *Paenibacillus* sp. A11 CDase will be performed.

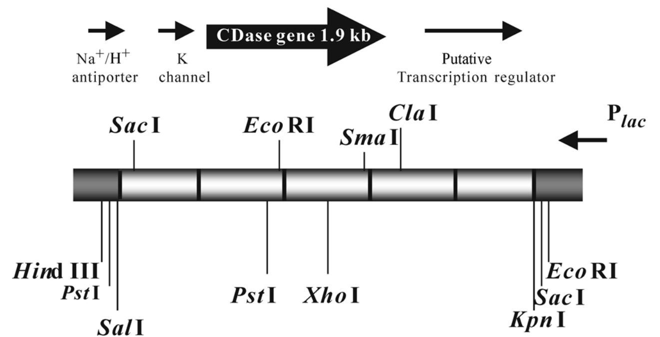

Fig. 1. Restriction map of inserted fragment containing CDase gene in pUC 18. The top arrow indicates orientation and size of CDase gene in pUC 18 including a partial Na^+/H^+ antiporter, a K channel and a putative transcription regulator.

Materials and Methods

Materials Cyclodextrins (α-, β- and γ-CDs), potato soluble starch, glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, D-sorbitol, and bovine serum albumin were purchased from Sigma (St. Louis, USA). Yeast extract and tryptone were obtained from Difco (Detroit, USA). Phenyl-Sepharose CL-4B and Sephadex G-200 were products of Amersham Pharmacia Biotech (Uppsala, Sweden). DEAE-Toyopearl 650M was a product of Tosoh (Tokyo, Japan). All of the other chemicals of analytical grade were obtained from Merck (Darmstadt, Germany) and Bayer (Leverkusen, Germany).

Bacterial strain, plasmid and media The *Escherichia coli* strain JM109 [F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}*lacZ ΔM15/recA1 endA1 gyr96 thi hsdR17 supE44 relA1 Δ(lac-proAB)*], harboring the plasmid pJK 555, was used for the CDase overproduction. *E. coli* cells were grown in a Luria-Bertani (LB) medium at 37°C (Maniatis *et al.*, 1982). Ampicillin (100 μg ml^{-1}) was added to the medium to allow the growth of the pJK 555 plasmid-carrying strain. The restriction map of the pJK 555 plasmid was constructed (Fig. 1). The CDase gene was located by DNA sequencing to the left of the insert, away from the *lac* promoter, and its orientation was opposite that of the *lac* Z′ gene in pUC 18.

Effect of polyols on CDase production *E. coli* JM 109 containing the pJK 555 plasmid was cultured in 100 ml of a LB medium with or without 0.5 M of each polyol. The culture was shaken at 250 rpm, 37°C for 24 h. The polyol that resulted in the highest CDase expression was chosen. The concentration of that polyol was then varied for the best CDase production. A suitable concentration was used to follow cell growth and CDase activity. A comparison of the CDase protein in the medium with and without polyol was performed on SDS-PAGE (Weber and Osborn, 1975).

Induction and extraction of CDase *E. coli* JM 109 (pJK 555) was grown in a LB medium containing 0.5 M sorbitol at 37°C for 24 h. The cells were harvested by centrifugation at 8,000 × *g* for 15 min at 4°C and washed twice in a 10 mM phosphate buffer, pH 6.5. The cells were then resuspended in a cold extraction buffer (10 mM phosphate buffer, pH 6.5 containing 1 mM DTT, 1 mM EDTA and 1 mM PMSF) and sonicated with a SONOPULS ultrasonic homogenizer (3 mm diameter-stepped microtip, 15% amplitude, Bandelin, Germany) for 5 cycles of 5 min sonicate and 5 min pulse. The cell debris was separated from the supernatant by centrifugation at 100,000 × *g* for 1 h. Both the supernatant and debris fractions were checked for the CDase protein on SDS-PAGE. The supernatant fraction was used as a crude CDase preparation.

Purification of CDase All operations were performed at 4°C. Buffer A that was used in all steps was a 10 mM phosphate buffer, pH 7.5, containing 1 mM DTT, 1 mM EDTA, and 10% glycerol. The crude CDase was precipitated overnight with 40-60% ammonium sulfate. The precipitate formed was collected by centrifugation at 27,000 × *g* for 30 min, dissolved, and dialyzed in buffer A. The dialyzed enzyme solution was applied to a DEAE Toyopearl 650 M column (1.7 × 17 cm), equilibrated with buffer A. The column was eluted with a linear 200 ml gradient of 0 to 0.5 M NaCl in buffer A at a flow rate of 30 ml/h. The active fractions from this column were pooled and loaded onto a Phenyl Sepharose CL-4B column (1.7 × 10 cm), equilibrated in buffer A containing 1 M ammonium sulfate. The column was washed with 160 ml gradient of a decreasing ammonium sulfate concentration from 1 to 0 M in buffer A. The fractions having CDase activity were collected as a purified pool and concentrated for subsequent studies.

CDase assay and protein determination CDase activity was assayed in a 0.5 ml reaction mixture that contained 1% β-CD (w/v) in a 0.1 M phosphate buffer, pH 7.0, and appropriately diluted enzyme. After incubation at 40°C for 30 min, the reducing sugar that was formed was measured by using the 3,5 dinitrosalicylic acid method (Bernfeld, 1955). One unit (U) of CDase was defined as the enzyme amount that produced 1 μmol of reducing sugar as glucose in 1 min per ml of the reaction at 40°C.

The amount of protein was determined by the Coomassie blue method (Bradford, 1976), using bovine serum albumin (BSA) as the standard.

CDase purity and amylolytic activity staining CDase in each step of purification was electrophoresed on a 7.5% native polyacrylamide gel (Weber and Osborn, 1975) and stained for both protein and activity. For activity staining, the gel was soaked in 10

ml of a 2.0% (w/v) potato soluble starch in a 0.1 M phosphate buffer, pH 7.0, at 40ºC for 30 min. It was then quickly rinsed several times with distilled water. Ten ml of the I_2 staining reagent (0.2% I_2 in 2% KI) was added for color development at room temperature. The clear band on the blue background indicated starch-degrading activity.

Molecular weight determination The molecular weight of the pure protein was estimated by SDS-PAGE on a 7.5% polyacrylamide gel using the Mini protein II electrophoresis apparatus (BioRad Laboratories, Richmond, USA). Protein bands were visualized by Coomassie blue staining (Weber and Osborn, 1975). Broad range molecular weight markers (BioRad) were used as the standards. Gel filtration on the Sephadex G-200 column (2.4 × 80 cm) was also performed to determine the molecular weight of the protein in the native state. The gel was equilibrated with buffer A containing 0.1 M NaCl at the flow rate of 20 ml/h. Catalase (232 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and cytochrome *c* (12.5 kDa) were used as the standard proteins.

Effect of pH and temperature on CDase activity The purified CDase was incubated with β-CD at different pHs or temperatures. The enzyme was assayed by using the 3,5 dinitrosalicylic acid method (Bernfeld, 1955). Then 0.1 M of acetate, phosphate, tris-HCl, and glycine-NaOH were used as the reaction buffers for pH 3.0-5.0, 5.0-7.0, 7.0-9.0, and 9.0-11.0, respectively. For temperature effect, the reactions were measured at 0 to 100ºC.

Substrate specificity of CDase Various substrates at 1% (w/v) concentration were incubated with CDase (10 µl, 0.3 mg/ml) in a 0.1 M phosphate buffer, pH 7.0 at 40ºC for 30 min. The total reaction volume was 0.5 ml. The enzyme was assayed by using the 3,5 dinitrosalicylic acid method (Bernfeld, 1955). The result was expressed as a percentage of the relative activity. The maximum activity was set as 100%.

High performance liquid chromatography The purified CDase (0.5 ml, 0.3 mg/ml) was incubated with 2.5 ml of various substrates at 2% w/v in a 0.1 M phosphate buffer, pH 7.0, at 40ºC for 24 h. Hydrolytic products of each substrate from the action of CDase were analyzed by HPLC with a Spherisorb 10 NH_2 column (4.6 × 250 mm) (Phenomenex, Torrance, USA) using the refractive index detector (Shimadzu model RID-10A). The column was eluted by 70% acetonitrile as the mobile phase using a flow rate of 1.0 ml min^{-1} at room temperature.

Thin layer chromatography TLC was performed on a silica gel plate (Kiesel Gel 60, Merck), and developed at ambient temperature for 5 h. with the solvent system of 1-propanol : ethylacetate : water (7 : 1 : 2). The spots were visualized by dipping in a solution of ethanol : sulfuric acid (9 : 1), drying, and heating at 110ºC for 15 min.

Kinetic studies of CDase Initial velocity studies for the hydrolysis reaction were carried out under the standard reaction condition (described in the previous section). The concentrations of CD substrates varied from 0.5-16 mM. The Lineweaver-Burk of the initial velocity against the CD concentration was plotted and the kinetic parameters were then determined using the EnzFitter program, version 2.0.14.0 (Biosoft, Ferguson, USA).

Effect of metal ions and protective chemicals on CDase activity The purified CDase (30 µl, 0.3 mg/ml) was incubated in a 10 mM phosphate buffer, pH 7.5, at 40ºC for 30 min in the presence of various metal ions and protective chemicals at a final concentration of 10 mM. The total incubation volume was 60 µl; 30 µl was withdrawn for the assay of the residual activity by using the 3,5 dinitrosalicylic acid method, as described. The residual activity was compared with the control condition and reported as a percentage of the relative activity.

Effect of group-specific reagents on CDase activity To investigate the active site residue of this enzyme, various group-specific reagents were tested for their ability to inactivate CDase activity. CDase in a 10 mM phosphate buffer, pH 7.5, was incubated with reagents at 40ºC for 30 min (Mattsson *et al*., 1992). The final concentration of the enzyme and reagents was 0.15 mg/ml and 5 mM, respectively. Only for the reagents, *N*-bromosuccinimide (NBS) and diethylpyrocarbonate (DEP), were 0.1 and 1 mM final concentrations used. The total volume of the reaction mixture was 60 µl. After incubation, samples (30 µl) were withdrawn and the residual activities were measured.

Results

Effect of polyols on the CDase expression Several polyols were studied for their effects on the expression of *Paenibacillus* sp. A11 CDase in *E. coli*. The result showed that glycerol, mannitol, and inositol did not change the level of enzyme production. Sorbitol, an osmotic stabilizer, was the only polyol that could increase CDase production. Its optimum effective concentration was evaluated in cells that were grown at 37ºC. As shown in Table 1, sorbitol gave a positive effect on the production of soluble CDase in *E. coli* harboring pJK 555. The maximum induction of soluble CDase, the three-fold increase in activity, could be achieved with 0.5 M sorbitol.

We further investigated the effect of sorbitol on the production of soluble CDase enzymes in cells. Sorbitol enforced the production of soluble CDase concurrently with

Table 1. Effect of sorbitol concentration on CDase overproduction

Sorbitol (M)	CDase activity (U/ml)
0	14.0
0.1	27.3
0.2	33.5
0.3	36.4
0.4	40.7
0.5	45.6
0.6	39.1
0.7	32.3

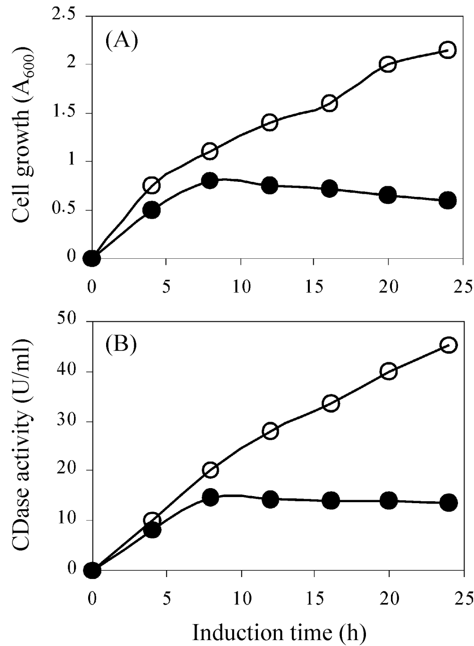

Fig. 2. Effect of sorbitol on cell growth and soluble CDase production. *E. coli* (pJK 555) cells were cultured in the LB medium with (○) and without (●) 0.5 M sorbitol. (A) Cell growth (B) CDase activity.

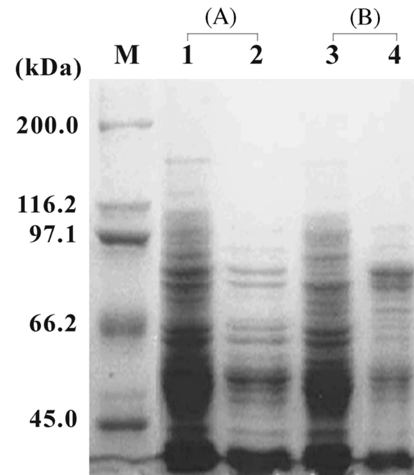

Fig. 3. SDS-PAGE analysis of sorbitol effect. (A) and (B) show induction results at 37°C in LB medium without and with 0.5 M sorbitol, respectively. Lanes 1 and 3, insoluble fraction; lanes 2 and 4, soluble fraction; lane M, molecular weight marker proteins. Arrow indicates induced CDase.

cell growth. As shown in Fig. 2A and 2B, after 8 h culturing, the cell growth and CDase activity were significantly higher when sorbitol was present in the culture medium.

The effect of sorbitol on the prevention of the insoluble CDase was demonstrated by SDS-PAGE. After a 24 h-culture in a LB medium under different conditions, the amount of CDase protein (80 kDa) was compared. When the cells were cultured at 37°C in the presence of sorbitol, a small amount of insoluble CDase protein was observed, while the soluble CDase from cytoplasmic fraction was mainly detected (Fig. 3B). The opposite result was observed when the cells were grown in a medium without sorbitol (Fig. 3A).

Purification of the CDase Recombinant CDase in *E. coli* (55 mg protein per litre culture) was highly purified after the following steps: cell disruption by ultrasonication, precipitation with ammonium sulfate, column chromatography on DEAE-Toyopearl 650 M, and chromatography on Phenyl-Sepharose CL-4B. The enzyme was purified about 14-fold with a 31% yield. The specific activity was 141 U/mg protein. The purified enzyme showed a single band on the native gel upon both protein and activity staining (Fig. 4).

Molecular weight determination The molecular weight of the enzyme was 80 kDa, as determined by SDS-PAGE. Gel filtration on a Sephadex G-200 column of the native CDase resulted in the estimation of the same molecular weight. These results indicate that the enzyme is a monomer with a molecular mass of 80 kDa.

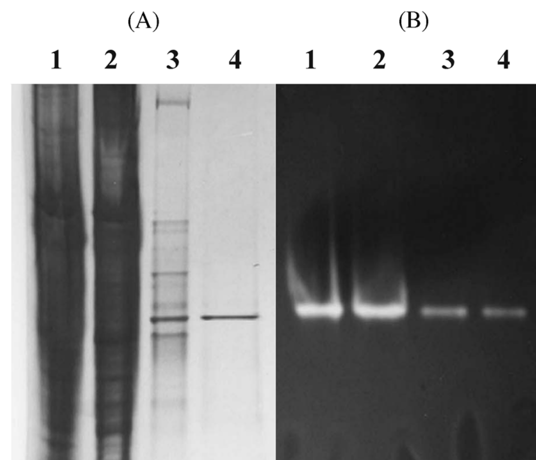

Fig. 4. Non-denaturing PAGE analysis of *E. coli* (pJK 555) CDase in each step of purification on a 7.5% acrylamide gel. (A) Coomassie blue staining (B) Amylolytic activity staining. Lane 1, crude enzyme; lane 2, enzyme from 40-60% ammonium sulfate precipitation; lane 3, enzyme from DEAE-Toyopearl 650 M; lane 4, enzyme from Phenyl-Sepharose CL-4B.

Effect of pH and temperature on CDase activity The effects of pH and temperature on the enzyme activity were examined (Fig. 5). The optimum pH and temperature were 7.0 and 40°C, respectively. The enzyme showed 20-60% of the activity at pH 5.0-6.0 and 8.0-9.0, while very low or no activity was observed at pH below 4.0 or above 11.0. Upon incubation of the enzyme at 50°C for 30 min, 80% of the activity remained.

Substrate specificity and hydrolysis products The recombinant CDase had high specificity towards β-CD, followed by γ-CD and α-CD, respectively. Hydrolytic

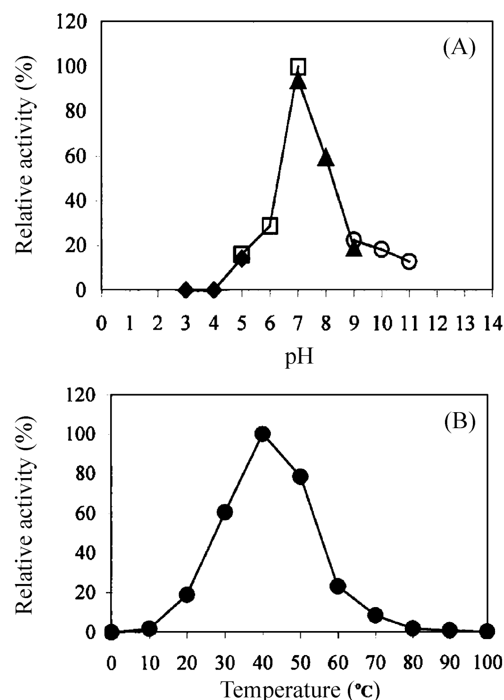

Fig. 5. Effects of (A) pH and (B) temperature on enzyme activity. Buffers used: (◆) 0.1 M acetate buffer pH 3.0-5.0; (□) 0.1 M K-phosphate buffer, pH 5.0-7.0; (▲) 0.1 M Tris-HCl buffer, pH 7.0-9.0; (○) 0.1 M glycine-NaOH buffer, pH 9.0-11.0.

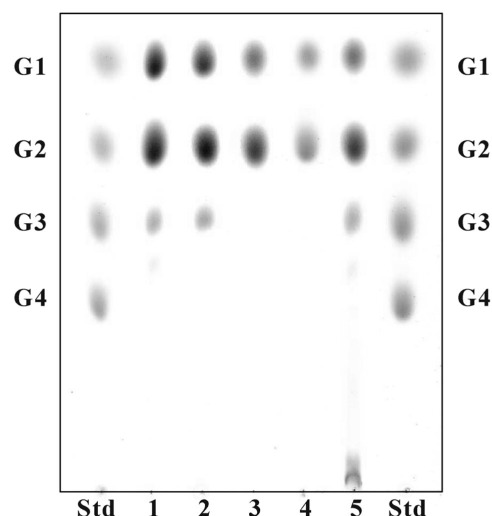

Fig. 6. Thin layer chromatogram of the hydrolysis products produced by *E. coli* (pJK 555) CDase. Reactions were performed as described under experimental procedures. Lane 1, α-CD; lane 2, β-CD; lane 3, γ-CD; lane 4, maltoheptaose; lane 5, soluble starch. G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose.

activities of 20-45% relative to that of β-CD were observed when the substrates maltopentaose, maltoheptaose, and maltohexaose were used. Lower activity (10-14%) was observed with maltotriose, maltotetraose, soluble starch, and amylose, while trace hydrolysis (3% hydrolytic activity) of pullulan could be detected (Table 2). In addition, this enzyme could not hydrolyze glycogen as well as other branch polysaccharides.

The end-products after a 24 h-incubation of the enzyme with various substrates were investigated and analyzed by TLC (Fig. 6) and HPLC (Table 2). Each substrate was degraded to give reducing oligosaccharides with maltose and glucose as the dominant products. It should be noted that hydrolysis of α- and β-CDs yielded more maltotriose than hydrolysis of γ-CD and maltoheptaose (G7).

Kinetic studies of CDase Kinetic parameters of the recombinant CDase with α-, β-, and γ-CDs as substrates are shown in Table 3. The K_m, k_{cat}, and k_{cat}/K_m values showed that the strongest binder, β-CD, was the best substrate with the highest turnover number and catalytic efficiency. Whereas the bigger ring, γ-CD, was significantly better than the small ring of α-CD when catalytic efficiency, k_{cat}/K_m, was compared.

Table 2. Substrate specificity and end-products of cyclodextrinase

Substrate (1%, w/v)	Relative activity (%)	End-product* (relative molar ratio) G1 : G2 : G3 : G4 : G5
β-Cyclodextrin	100	0.7 : 1.0 : 0.3 : 0.1 : 0.0
γ-Cyclodextrin	72	0.7 : 1.0 : 0.1 : 0.1 : 0.1
α-Cyclodextrin	57	0.6 : 1.0 : 0.3 : 0.1 : 0.0
Maltotriose (G3)	11	0.7 : 1.0 : 0.2 : 0.0 : 0.0
Maltotetraose (G4)	14	0.6 : 1.0 : 0.1 : 0.1 : 0.0
Maltopentaose (G5)	22	0.6 : 1.0 : 0.3 : 0.1 : 0.0
Maltohexaose (G6)	35	0.7 : 1.0 : 0.1 : 0.1 : 0.0
Maltoheptaose (G7)	43	0.6 : 1.0 : 0.1 : 0.1 : 0.1
Soluble starch	9	0.4 : 1.0 : 0.1 : 0.1 : 0.0
Pullulan	3	0.7 : 1.0 : 1.0 : 0.1 : 0.0
Glycogen	0	-

*after 24 h incubation

Table 3. Kinetic parameters of the recombinant CDase with cyclodextrins substrate

Substrate	K_m (mM)	V_{max} (μmoles/min)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
α-CD	5.0	0.17	1500	3.0×10^5
β-CD	2.5	0.25	2200	8.8×10^5
γ-CD	2.9	0.18	1600	5.5×10^5

Table 4. Effects of metal ions and protective chemicals on CDase activity

Compound*	Final concentration	Relative activity (%)
None	-	100
Metal ions		
$CaCl_2$	10 mM	100
KCl	10 mM	94
LiCl	10 mM	90
NaCl	10 mM	92
$BaCl_2$	10 mM	88
$MgCl_2$	10 mM	80
$ZnCl_2$	10 mM	37
$AgNO_3$	10 mM	0
$HgCl_2$	10 mM	0
Protective chemicals		
β-mercaptoethanol	10 mM	107
EDTA	10 mM	101

*Incubation with 0.15 mg/ml enzyme at 40ºC, pH 7.5 for 30 min.

Effects of metal ions and protective chemicals on CDase activity The effects of selected metal ions and protective chemicals on CDase activity are summarized in Table 4. The enzyme was completely inhibited by the 10 mM concentration of Hg^{2+} and Ag^{+} while moderately inhibited by Zn^{2+}. Chemicals that were often used as a protective substance for the enzyme, such as β-mercaptoethanol and EDTA, had no effect on the activity of this enzyme.

Effect of group-specific reagents on CDase activity The result in Table 5 shows that the CDase activity was totally inactivated by 1 mM *N*-bromosuccinimide (NBS) and diethylpyrocarbonate (DEP). There was a partial inactivation observed when incubated with 5 mM iodoacetamide (IAM), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), and phenylglyoxal (PG) at pH 7.5, 40ºC for 30 min. Also, 2,4,6-Trinitrobenzenesulfonic acid (TNBS), phenylmethylsulfonyl fluoride (PMSF), and *N*-acetylimidazole (NAI) at concentrations of 5 mM had no effect on CDase activity. Some activation was exhibited by a reducing agent like dithiothreitol (DTT), which suggests that the purified CDase was not in a fully reduced state, and CDase activity was increased upon reduction of the disulfide bond. These findings gave evidence of the involvement of Trp, His, Cys, Carboxylic amino acids, and Arg on CDase activity.

Discussion

When CDase was extracted from *E. coli* JM 109 harboring pJK 555, we found that the CDase protein was primarily localized in the cell debris fraction, while a less amount was found in the soluble cytoplasmic fraction (Fig. 3A). This was different from the observation in *Paenibacillus* sp. A11 where the CDase activity was only found in the cytoplasm. Similar

Table 5. Effect of various group-specific reagents on CDase activity

Reagents*	Concentration (mM)	Amino acid involved	Residual activity (%)
None	-	-	100
Iodoacetamide (IAM)	5	Cysteine	71
Dithiothreitol (DTT)	5	Cystine	115
2,4,6-Trinitrobenzenesulfonic acid (TNBS)	5	Lysine	97
Phenylmethylsulfonyl fluoride (PMSF)	5	Serine	94
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)	5	Carboxylic amino acids	76
	5		0
Diethylpyrocarbonate (DEP)	1	Histidine	0
	0.1		63
	5		0
N-bromosuccinimide (NBS)	1	Tryptophan	0
	0.1		5
N-acetylimidazole (NAI)	5	Tyrosine	99
Phenylglyoxal (PG)	5	Arginine	62

*Incubation with 0.15 mg/ml enzyme at 40ºC, pH 7.5 for 30 min.

Table 6. Comparison of biochemical properties of the CDases from *Paenibacillus* sp. A11 and the *E. coli* transformant

Parameter	*Paenibacillus* sp. A11*	*E. coli* JM 109 (pJK 555)
Relative molecular weight (kDa)		
Sephadex G-200	80	80
SDS-PAGE	80	80
Specific activity (U/mg)	133	141
Optimum pH	7.0	7.0
Optimum temperature (ºC)	40	40
k_{cat}/K_m (M^{-1} min^{-1})	8.2×10^5	8.8×10^5
Dominant product	G2, G1	G2, G1
Activator	DTT	DTT
Inactivator	NBS, DEP, IAM, EDC, PG	NBS, DEP, IAM, EDC, PG
Inhibitor	Ag^+, Hg^{2+}, Zn^{2+}	Ag^+, Hg^{2+}, Zn^{2+}

*(Kaulpiboon and Pongsawasdi, accepted to be published in enzyme Microb. Technol.)

results were frequently observed in the expression of several target proteins in the *E. coli* system (Donovan *et al*., 1996; Hanning and Makrides, 1998; Kim *et al*., 1999). It is possible that these differences were caused by the expression of protein in different types of bacteria. *Paenibacillus* sp. A11 is a gram-positive bacterium while *E. coli* JM 109 is gram-negative. In this study, adding 0.5 M sorbitol as an osmotic stabilizer into the *E. coli* culturing LB medium minimized the insoluble CDase problem. We demonstrated that sorbitol had a positive effect on the CDase overproduction of the *E. coli* transformant in parallel with the cell growth increase. It was apparent that the addition of sorbitol could prevent the formation of the insoluble CDase aggregate, enforcing the correct folding to the active soluble enzyme. Polyols, osmolytes, and some non-metabolizing sugars have already been reported as important for enzyme activity as well as for the secondary/tertiary structures of some proteins, including the prevention of protein aggregation (Lee and Lee, 1981). Bowden and Georgiou (1988) demonstrated that the addition of non-metabolizing sugars, such as sucrose and raffinose, to the growth medium prevented the aggregation of β-lactamase. In another study, some compatible osmolytes were effective in producing soluble dimethylallyl pyrophosphate: 5’AMP transferase in the transformed *E. coli.* Large amounts of soluble and active protein were obtained by growing and inducing the cells under osmotic stress that were built up by sorbitol and glycyl betain (Blackwell and Horgan, 1991). Recently, Kim *et al*. (1999) found that mannitol, sorbitol, glycerol, erythritol, xylitol, and arabitol could increase the expression of the soluble active CGTase from *Brevibacillus brevis* in *E. coli*.

The recombinant CDase was purified to a specific activity of 141 unit/mg protein, representing a 14-fold purification. It was a single polypeptide with M_r of 80 kDa. The optimum pH and temperature were 7.0 and 40ºC. All of the determined biochemical properties of the cloned enzyme were almost identical to those of the authentic one (Table 6). The substrate specificity of *E. coli* (pJK 555) CDase was quite similar to that of *Paenibacillus* sp. A11 and other strains (Podkovyrov and Zeikus, 1992; Oguma *et al*., 1993; Kim *et al*., 1998). The best substrate for the recombinant CDase was β-CD. Very low hydrolysis was observed with polysaccharides, such as soluble starch and pullulan, while highly branched saccharide, such as glycogen, could not be hydrolyzed by this enzyme. Usually, CDases had high specificity with cyclic oligosaccharides and appropriate size linear oligosaccharides, comprised of glucose units linked by the α-1,4 glycosidic bond (Depinto and Campbell, 1968; Podkovyrov and Zeikus, 1992; Oguma *et al.*, 1993; Kim *et al*., 1998). The final products from CDs and maltooligosaccharides that were hydrolyzed by the enzyme were maltose, glucose, and small amounts of maltotriose and maltotetraose. Maltose was the main product of both CDases. This pattern of hydrolysis products that were obtained was similar to the action of some other CDases (Podkovyrov and Zeikus, 1992; Oguma *et al.*, 1993; Kim *et al*., 1998; Hashimoto *et al.*, 2001).

In conclusion, this study demonstrated that sorbitol, a polyol osmolyte, had an important role in increasing the soluble CDase activity in parallel with an increase of transformant cell growth. In this context, our work might be useful to other systems of recombinant protein expression when the prevention of the insoluble protein formation is required. We also demonstrated that the recombinant and authentic CDases are identical in most of the biochemical properties that were determined.

Acknowledgments We would like to thank Dr. M. Iizuka from Osaka City U., Japan for his help in TLC. Also thanks to Dr. V. Rimphanitchayakit for adivice in DNA cloning. This work was supported in part by research grants from the Ministry of University Affairs through C. U.

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