Purification and refolding of the recombinant subunit B protein of the Aggregatibacter actinomycetemcomitans cytolethal distending toxin

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ABSTRACT

Purpose: *Aggregatibacter actinomycetemcomitans* is associated with localized aggressive periodontitis. It produces cytolethal distending toxin (CDT), which induces cell cycle arrest in the G2/M phase. The CDT holotoxin is composed of CdtA, CdtB, and CdtC. CdtB has structural homology to human DNase I and is an active component of the CDT complex acting as a DNase. In particular, the pattern homology seen in the CdtB subunit has been associated with specific DNase I residues involved in enzyme catalysis, DNA binding, and metal ion binding. So, to study the functions and regulation of recombinant CdtB, we made up a quantity of functional recombinant CdtB and tested it in relation to the metal ion effect.

Materials and Methods: We constructed the pET28a-cdtB plasmid from *A. actinomycetemcomitans* Y4 by genomic DNA PCR and expressed it in the BL21 (DE3) *Escherichia coli* system. We obtained the functional recombinant CdtB by the refolding system using the dialysis method and then analyzed the DNase activity and investigated the metal ion effect from plasmid digestion.

Results: The recombinant CdtB subunit was expressed as the inclusion bodies. We were able to obtain functional recombinant CdtB subunit using refolding system. We confirmed that our refolded recombinant CdtB had DNase activity and was influenced by the metal ions Mg^{2+} and Ca^{2+}.

Conclusion: We suggest that the factors influencing recombinant CdtB may contribute to CDT associated diseases, such as periodontitis, endocarditic, meningitis, and osteomyelitis. ***(J Korean Acad Periodontol 2008;38:343-354)***

KEY WORDS: *Aggregatibacter actinomycetemcomitans*; cytolethal distending toxin.

Introduction

Aggregatibacter actinomycetemcomitans is a small nonmotile gram-negative coccobacillus. It belongs to the family *Pasteurellaceae* and grows singly, in pairs, or in small clumps and is described as facultatively anaerobic. These organisms, which are associated with localized aggressive periodontitis, endocarditis, meningitis, and osteomyelitis, produce a variety of virulence factors, including cytotoxic factors, chemotactic inhibitor, collagenases, lipopolysaccharide, and cytolethal distending toxin (CDT)[1-8]. In particular, the interaction between *A. actinomycetemcomitans* and oral gingival cells seems to be important, since *A. actinomycetemcomitans* are found in the affected gingival pocket[7]. These organisms adhere to human cells and some of them invade the attached cells *in vitro*[8,9]. Also, a crude cell extract and a growth medium of them have been found to inhibit the growth of human fibroblasts and keratinocytes and to block the G2/M cycle of human gingival fibroblast[3,10]. Recently, CDT has been identified as a cell cycle specific factor[8,11]. CDT was first discovered as a new heat-labile toxin from *Escherichia coli* and *Campylobacter spp.*, where it has been associated with diarrheal disease. It is a unique bacterial toxin that induces cell distension and the cell cycle arrest of cultured cells in the G2 phase[12]. As with other members of the CDT family, *A. actinomycetemcomitans* CDT induces cell cycle arrest in the G2/M phase[8].

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The amino acid sequence of *A. actinomycetemcomitans* CDT is very similar to the *Hemophilus ducreyi* CDT, the structure of which has demonstrated that CDT is an AB_2 type toxin, with the catalytically active A subunit of the AB_2 toxin corresponding to the CdtB subunit[13]. Among the subunits composing the CDT holotoxin, CdtA, CdtB, and CdtC, CdtB has structural homology to human DNase I and it has been suggested that CdtB is an active component of the CDT complex acting as a DNase[14]. The CdtB subunit of *A. actinomycetemcomitans* presented highly conserved domains with the CdtB of other pathogenic bacteria, including *E. coli*, C. *jejuni*, and *H. ducreyi*[15]. The conserved domains observed in CdtB were found at specific DNase I residues involved in enzyme catalysis, DNA binding, and metal ion binding. In support of this, *E. coli* CdtB has been demonstrated to possess nicking activity toward purified plasmid *in vitro*.

The best characterized CdtB subunit shares conserved motif and residues of typical DNase. Especially, the pattern homology investigated in the CdtB subunit was associated with specific DNase I residues involved in enzyme catalysis, DNA binding, and metal ion binding. Among them, the metal ion binding sites, Glu66, Asp189, and Asp263 which are also conserved in Bovine pancreatic deoxyribonuclease (DNase) were considered to be critical regulators of DNase activity. His150, Asp228, and His264 are catalytic residues and Arg125 and Asn191 are DNA contact residues. Moreover, a penta peptide sequence (Ser-Asp-His-Tyr-Pro) containing the C-terminus of a CdtB subunit conserved the motif found in every DNase I C-terminus[16].

Many previous studies have investigated the correlation between metal ions, such as Mg^{2+}, Ca^{2+}, and Mn^{2+}, and DNase activity. These have shown that several other metal ions can also activate DNase, although the highest enzyme activity is reached with Mg^{2+} and Ca^{2+} or with Mn^{2+}. Kunitz showed that the concentration of Mg^{2+} needed for activity is proportional to the DNA concentration and also, when Ca^{2+} was added, the DNase activity increased to a greater extent[17,18]. Accordingly, we suggest that the metal ions, Ca2+ and Mg^{2}, may have pivotal roles in the *A. actinomycetemcomitans* CdtB subunit.

In this study, we reconstructed the CdtB subunit of *A. actinomycetemcomitans* Y4 and expressed it in the *E. coli* system. Although the CdtB subunit was expressed by inclusion bodies, we made the functional recombinant CdtB subunit using the refolding system. Further, we tested the significance of the effect of metal ions on plasmid digestion with the refolded CdtB protein.

Materials and Methods

1. Bacterial strains and culture condition

A. actinomycetemcomitans Y4 (serotype b, ATCC 43718) was cultured in Brain-Heart Infusion broth at 37°C in anaerobic chamber containing 85% N_2, 10% H_2 and 5% CO_2[18]. *E. coli* strains, Top 10F and BL21 (DE3), were grown aerobically in Luria-Bertari (LB) medium or on LB plates. Ampicillin (100ug/ml) and kanamycin (50ug/ml) was used when appropriate.

2. Plasmid construction

A. actinomycetemcomitans Y4 cultured in BHI broth for 48 h was harvested and genomic DNA was isolated by using the Promega genomic DNA kit according to the manufacturer's recommendations. Briefly, 5ml of *A. actinomycetemcomitans* was collected and resuspended in 600ul lysis solution. After 5-min incubation at 80°C, RNase solution was added into the bacterial suspension and further incubated at 37°C for 40min. Proteins in the lysates were precipitated and then genomic DNA was precipitated by isopropanol. Finally the genomic DNA was rehydration in 100ul of TE buffer for 1h at 65°C[19].

Using *A. actinomycetemcomitans* genomic DNA as a template, the cdtB gene was amplified by PCR using primers with restriction site; cdtBF: Cggga tccTCA AGT

TAT GCT AAC TTG AGT (BamHI), cdtBR: Ccgctc gag TTA GCG ATC ACG AAC AAA ACT (XhoI). PCR was carried out under the following amplification condition: initial denaturation at 94°C for 5min, followed by 30 cycles of denaturation at 94°C for 1min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10min.

Purified PCR products of cdtB gene were ligated into the pGEM-T vector and the ligation mixtures were transformed into the competent Top10F cells. In order to express cloned genes in *E. coli* system, selected plasmids containing the cdtB PCR product and pET28(a) expression vector were digested by *Bam*HI and *Xho*I restriction endonucleases and digested PCR products of CdtB gene was directly ligated with T4 DNA ligase into the multiple cloning site of pET28(a) expression vector. After selection of the transformants resisting the kanamycin, sequence of clone is conformed using T7 promoter and terminator.

3. Expression of recombinant CdtB

Single colonies of *E. coli* BL-21 (DE3) carrying the pET28a-cdtB gene plasmid were inoculated separately into 5 ml of LB broth containing 50ug/ml kanamycine and incubated at 37°C for overnight. After overnight culture, it was added to 500ml LB broth containing 50ug/ml kanamycine and grown at 37°C with vigorous shaking until an optical density of 0.8 was reached, and then the expression of 6xHis protein was induced by addition of 1mM IPTG (isopropyl-β-D-thio-galactopyranoside). After 6h of incubation, cultured bacteria were harvested by centrifugation, resuspended in 20mM Tris-HCl containing 0.5M NaCl and 5mM imidazole (pH 8.0), and disrupted with an Ultrasonic disruptor[14]. After centrifugation at 13000 rpm for 20min, the pellet was washed with Phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH2PO4, pH 7.3) containing 0.1% Triton X-100 twice. Cells were resuspended in PBS with 0.1% Triton X-100 and broken again by an ultrasonic disrupter and centrifuged at 13000rpm for 20min. The inclusion body which contained 6xHis protein[20-21] was obtained as the resulting pellet. Some of the inclusion body were mixed with gel loading dye and heated in a boiling water bath for 3min. Samples were applied to a 12% SDS-PAGE gel and the electrophoresis was carried out. The protein bands were transferred into a nitrocellulose membrane. The membrane was blocked for 1 h with 3% BSA in TBS at room temperature. Then the membrane was washed 3 times with TBS-Tween buffer, incubated for 1h at room temperature in TBS-Tween buffer containing a 1:3000 dilution of Ni-NTA conjugate stock solution, and stained with AP staining solution until the signal is clearly visible.

4. Solubilization and Refolding of CdtB

The expression protein that was contained in inclusion bodies were isolated, solubilized, and refolded using a modification of the previously procedure[14,22,23]. Briefly, after removing the soluble protein and cell membrane, the inclusion bodies were isolated by centrifugation. The pellet of the inclusion body was washed with 50mM Tri (pH 8.0) containing 2M Urea. For reconstruction of the expressed protein, the inclusion bodies were solubilized in 50mM Tris (pH 8.0) containing 8M urea and 100mM 2-ME; Solubilization was allowed to proceed for 2 h at 37°C. Following centrifugation, the solubilized protein was then refolded by sequential dialysis in 4, 2, 1 and 0.5M urea in PBS (pH 7.4) at 4°C. Resulting supernatants were added into the nickel-chelated agarose (Ni-NTA, Qiagen), washed with 20mM Tris-HCl containing 0.5M NaCl and 5mM imidazole and eluted with 20mM Tris-HCl containing 0.5M NaCl and 200mM imidazole. The supernatant containing the purified CdtB proteins were dialysis against PBS. Quantification of the purified and refolded recombinant proteins was carried out by Coomassie blue-staining of the SDS-PAGE gel using known quantities of bovine serum albumin as standards.

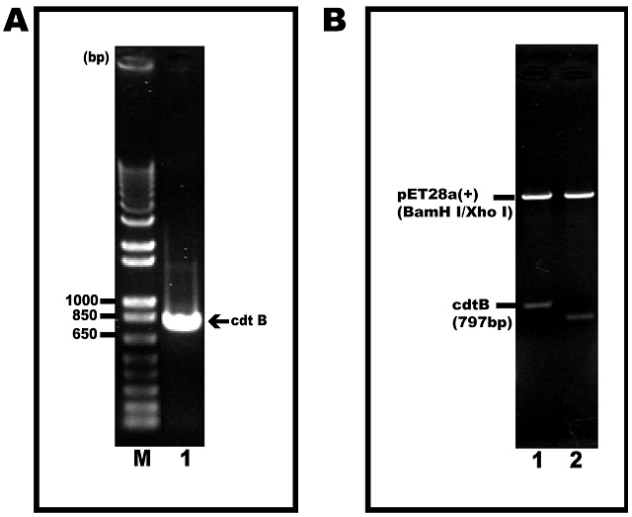

Figure 1. Cloning of CdtB gene of A. actinomycetemcomitans Y4. (A) The gene for CdtB was amplified by PCR using the genomic DNA of A. actinomycetemcomitans. Lane M shows size marker. Lane 1 indicates PCR product 797bp ot cdtB gene. (B) The presence of inserted cdtB gene in the expression vector was confirmed by digestion with restriction enzymes, BamHI and XhoI. Lane 1 and 2 indicate cdtB gene and other gene.

5. *In vitro* DNase activity

Supercoiled DNA nicking activity was determined with modification as described previously[23]. Briefly, 1 ug of supercoiled pCY2 DNA was incubated with re-constituted CdtB proteins (0~1ug of protein per re-action) in a buffer consisting of 25 mM HEPES (pH 7.0), 0~10mM $MgCl_2$ and/or 0~10mM $CaCl_2$ or 0~20mM EDTA. For conditions containing only Mg^{2+}, 0.4mM EGTA was also included in the reaction mix. At various time intervals, aliquots of the reaction mix were stopped with 10mM EDTA, 10mM EGTA, 6% glyc-erol and bromophenol blue and loaded directly on a 1% agarose gel. Gels were run 25min at 100V in TAE, stained with etidium bromide, and individual bands were quantified with a BioRad Gel Doc system. Figures were prepared by using Adobe Photoshop 7.0.

Results

1. Cloning and expression of *A. actinomycetemcomitans* cdtB gene

In order to check the activity of *A. actino-mycetemcomitans* CdtB, we constructed a clone of the *A. actinomycetemcomitans* CdtB.

A single product of approximately 797 bp, the *A. actinomycetemcomitans* cdtB gene, was amplified (Fig. 1A) and ligated into the BamHI and XhoI sites of the pET28a expression vector. Restriction endonucleases mapping and sequencing were used to confirm the in-sertion of the DNA fragments in the proper ori-entation (Fig. 1B).

Cloned pET-cdtB plasmid was transformed into the BL21 (DE3). The recombinant CdtB was expressed with an apparent molecular mass of 33 kDa after induction by 1 mM IPTG at 37°C (Fig. 2). Also, to confirm the recombinant CdtB protein co-expressing the 6xHis, Ni-NTA coupled to calf intestinal alkaline phosphatase was used for the direct detection of the recombinant protein with an accessible 6xHis tag. As shown in Fig. 3, the recombinant His6-tagged CdtB proteins were readily detected in an insoluble fraction on Western blots using the AP conjugated Ni-NTA.

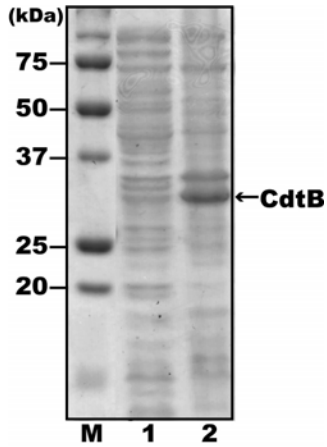

Figure 2. *SDS-PAGE analysis of induced CdtB. CdtB was induced and the resulting bacterial lysate was subjected to 12% SDS-PAGE gel followed by staining with Coomassie brilliant blue. Lane M indicates molecular size markers. Lane 1 indicates E. coli bacterial lysate before induction and Lane 2 indicates E. coli bacterial lysate after induction. Arrow indicates the position of recombinant CdtB protein (33kDa).*

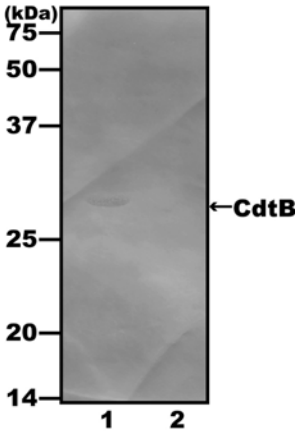

Figure 3. *Western blotting of the induced CdtB protein. Whole-cell lysate from the transferred bacteria harboring pET28a-cdtB was applied to the gel followed by nitrocellulose transfer. The transferred blot was probed with AP conjugated Ni-NTA at 1:3000 dilution. Lane 1 indicates insoluble fraction of BL21 containing pET28a-cdtB and lane2 indicates soluble fraction of BL21 containing pET28a-cdtB. Arrow indicates the position of recombinant CdtB protein (33kDa).*

2. Reconstitution of biologically active recombinant CdtB subunit

As shown in Fig. 4, recombinant 6xHis tagged CdtB protein was confirmed in an insoluble fraction of BL21 (DE3) cell lysate containing the pET28a-cdtB. To obtain the recombinant 6xHis tagged CdtB from the insoluble fraction, we optimized the modified refolding system as described previously[22,23]. In our system, the yield of inclusion bodies with recombinant CdtB was around 1mg of protein per liter of cell culture (Fig. 4). After refolding, purification, and final dialysis using D.W., fraction analysis by SDS-PAGE revealed that the pure recombinant CdtB subunit had eluted from the Ni-NTA column (Fig. 5), indicating that the protein was now soluble in a mild detergent solution.

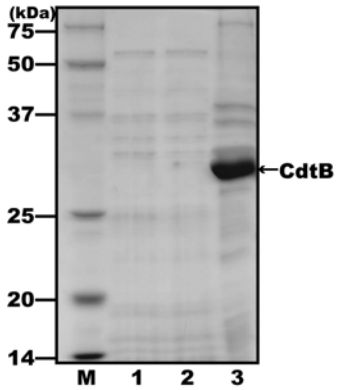

Figure 4. *SDS-PAGE analysis of insoluble fraction containing recombinant CdtB subunit. Whole-cell lysate from the transferred bacteria harboring pET28a-cdtB was applied to the 12% SDS-PAGE gel followed by staining with Coomassie brilliant blue. Lane 1 and 2 indicate soluble fraction of BL21 containing pET28a-cdtB and lane 3 indicates insoluble fraction of BL21 containing pET28a-cdtB. Arrow indicates the position of recombinant CdtB protein (33kDa).*

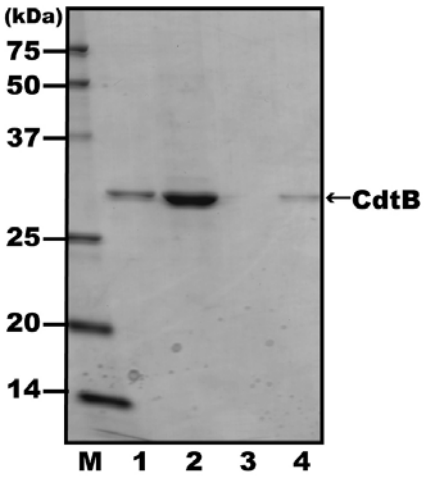

Figure 5. SDS-PAGE analysis of refolded CdtB before and after Ni-NTA column chromatography. All samples were applied to a 12% SDS-PAGE gel. Lane 1 indicates 10ul of solubilized inclusion bodies prior to dialysis. Lane2 indicates 10 ul of aggregation fraction after dialysis. Lane3 and 4 indicate 10ul of consecutive protein containing fractions from the Ni-NTA column.

3. Functional studies of the recombinant CdtB subunit

Given the striking similarity of CdtB to DNase I, we tested reconstructed CdtB for DNase activity. As shown Fig. 6, DNase activity for the CdtB subunit, which nearly eliminated the supercoiled DNA substrate, was observed in the sample containing 1ug of CdtB 1h after incubation. Also, when time dependent observations were made of samples, significant DNase activity was observed in the sample containing 0.05ug of CdtB as early as 10min after incubation (Fig. 7) and significant DNase activity was observed in the sample containing 1ug of CdtB 30min after incubation. Accordingly, our reconstructed CdtB subunit contained functional CdtB with sufficient DNase activity to completely degrade the 1ug DNA substrates as quickly as 30min after incubation.

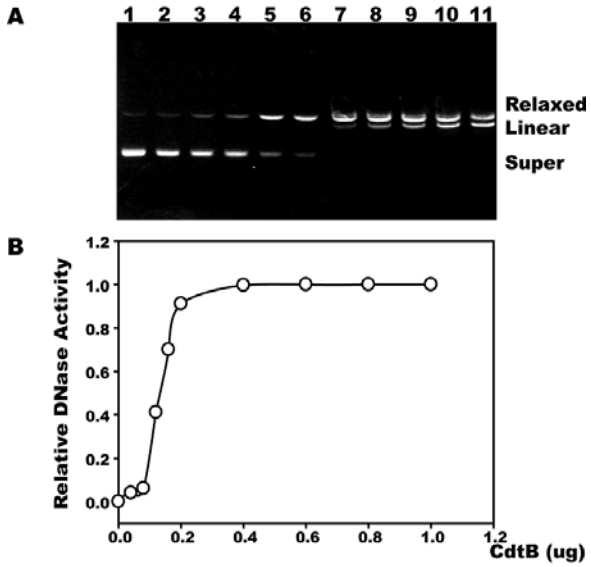

Figure 6. Analysis of DNase activity of recombinant CdtB subunit. (A) Increasing amounts of recombinant CdtB subunit was incubated with supercoiled pCY2. Lane 1 indicates no recombinant CdtB subunit; Lane 2 to 11, 0.04, 0.08, 0.10, 0.12, 0.16, 0.2, 0.4, 0.6, 0.8, and 1 (ug), respectively, of recombinant CdtB subunit (B) Relative DNase activity (a value of 1.0 represents the total disappearance of supercoiled substrate DNA). The 1% agarose gel was stained with ethidium bromide and visualized by UV transillumination. The numeric data used to generate the activity curve shown in panel B was generated as described in the text.

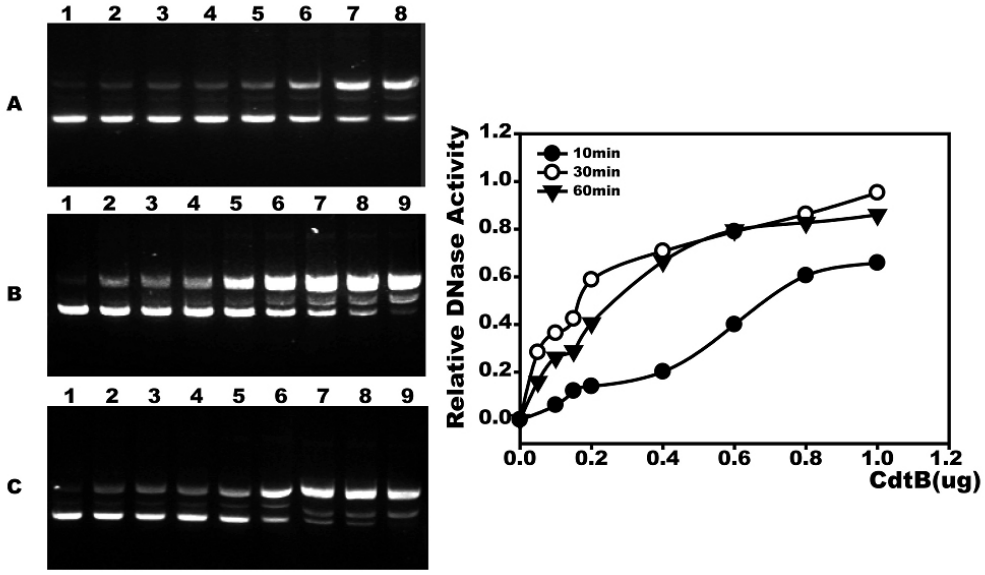

Figure 7. *The time-dependent DNase activity of recombinant CdtB subunit. Increasing amounts of recombinant CdtB subunit was incubated with supercoiled pCY2 for 10, 30 and 60min. Lane 1 indicates no recombinant CdtB subunit; Lane 2 to 9, 0.05, 0.1, 0.15, 0.2, 0.4, 0.6, 0.8 and 1 (ug), respectively.*

Also, we investigated the effects of storage periods and temperature on the DNase activity of CdtB. As might be expected, 10 days after refolding, the recombinant CdtB subunit showed an activity of 60% compared with the activity of fresh the recombinant CdtB subunit (Fig. 8), and recombinant CdtB subunits stored at -20°C in PBS lost their activity (Fig. 9). We thought that one of the biggest reasons for the inactivity of this stored recombinant CdtB subunit was the storage buffer condition.

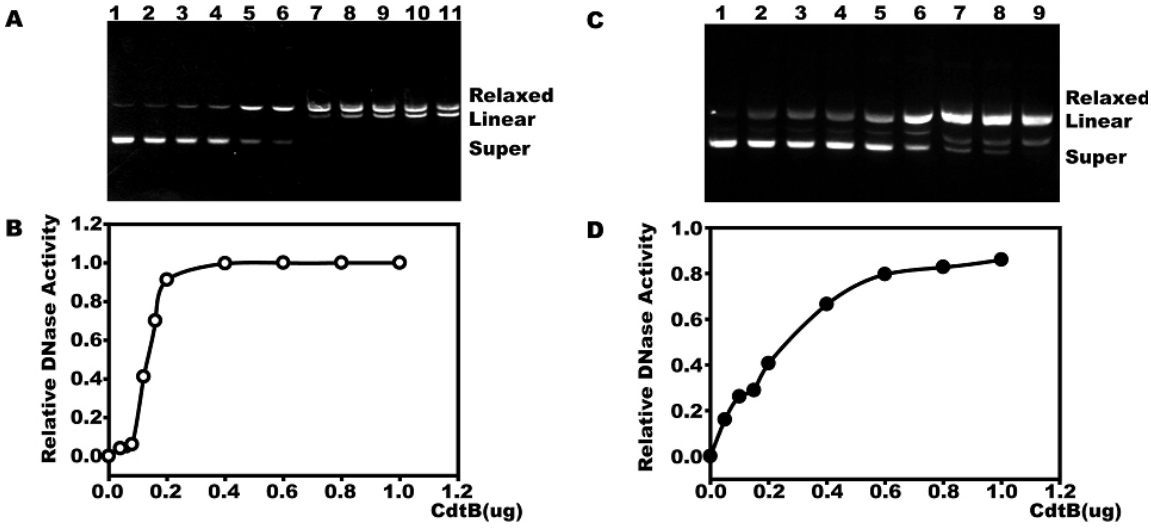

Figure 8. *DNase activity of fresh or stored recombinant CdtB subunit. Increasing amounts of recombinant CdtB subunit was incubated with supercoiled pCY2 as described in the text. Lane 1 indicates no recombinant CdtB subunit; Lane 2 to 11, 0.04, 0.08, 0.10, 0.12, 0.16, 0.2, 0.4, 0.6, 0.8, and 1 (ug), respectively, of fresh (A) or stored (C) recombinant CdtB subunit. (B) and (D).*

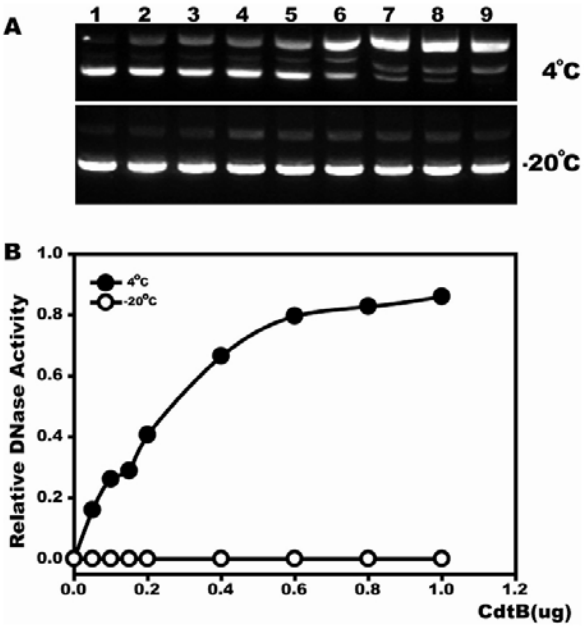

Figure 9. Effect of temperature on the DNase activity of recombinant CdtB subunit as 7 days after reconstruction. Increasing amounts of recombinant CdtB subunit was incubated with supercoiled pCY2 as described in the text. (A) Lane 1 indicates no recombinant CdtB subunit; Lane 2 to 9, 0.05, 0.1, 0.15, 0.2, 0.4, 0.6, 0.8 and 1 (ug), respectively, recombinant CdtB subunit stored at 4°C or −20°C for 7 days. (B) Relative DNase activity of recombinant CdtB subunit stored at 4°C or −20°C.

We investigated the effect of the Mg^{2+} and Ca^{2+} ions on the DNase activity of the recombinant CdtB of *A. actinomycetemcomitans* (Figs. 10 and 11). When only $MgCl_2$ or $CaCl_2$ was added to a sample containing the pCY2 plasmid and recombinant CdtB subunit, supercoiled DNA was not shifted in the agarose gel. However, when each 5mM $MgCl_2$ and 5mM $CaCl_2$ was added in sample, we do not found the supercoiled DNA and then, all of them located linear plasmid site in agarose gel. In addition, we investigated the effect of decreasing the $CaCl_2$ concentration using 0~20mM of EDTA. As shown Fig. 11, the decrease in the linear DNA band depended on the increase in the EDTA concentration. When 10 or 20mM of EDTA was added to the sample, the relative DNase activity of the recombinant CdtB subunit was shown to be 65%. So, we can conclude that the Mg^{2+} and Ca^{2+} ions were one of the critical factors in the DNase activity of the CdtB subunit of *A. actinomycetemcomitans*, as in a previous study for DNase I.

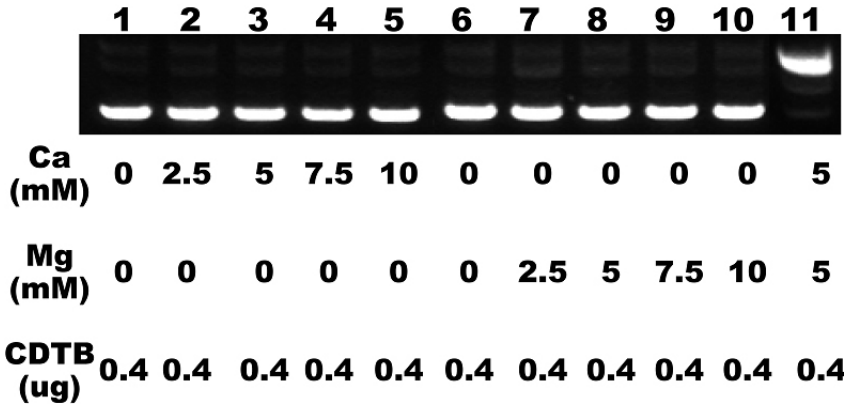

Figure 10. The metal-dependent DNase activity of recombinant CdtB subunit. The recombinant CdtB subunit (0.4ug) was added in sample which contains Ca^{2+} ion 0~10mM or Mg^{2+} ion 1-10mM and incubated for 1 h at 37°C.

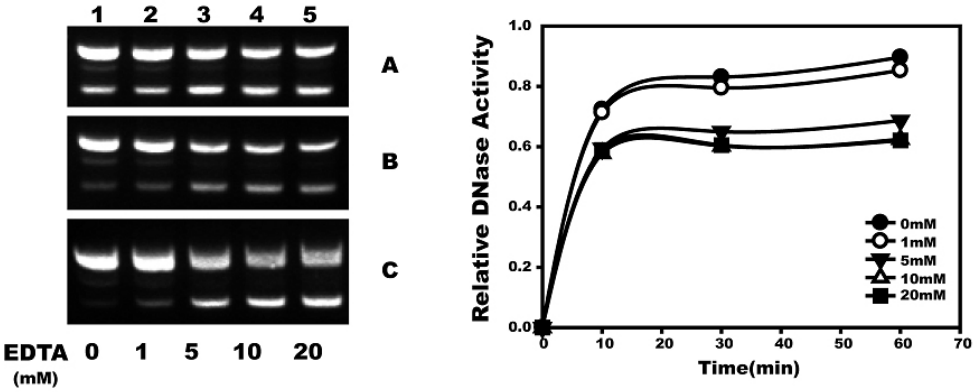

Figure 11. *The Ca^{2+} ion-dependent DNase activity of recombinant CdtB subunit. The EDTA 0~20mM was added in sample containing the $MgCl_2$ 5mM, $CaCl_2$ 5mM, HEPES 250mM, pCY2 plasmid and recombinant CdtB (0.4ug) as 10min, 30min or 60min at 37°C after incubation (A, B, C).*

Discussion

Aggregatibacter actinomycetemcomitans cytolethal heat-labile factor that is present in the culture supernatant, has been shown to inhibit the growth of human and murine fibroblasts and human keratinocytes, and to have an immunosuppressive activity toward human T and B cells[10]. Also, similar toxic factors have been reported to inhibit human gingival fibroblasts, Chinese hamster ovaries, monkey fibroblasts (Cos1), human cervical carcinoma (HeLa), human laryngeal carcinoma (HEp2), murin B-cell hybridoma, human periodontal ligament cells, JY B-lymphoblastoid, human oral epidermoid carcinoma (KB), and Primary T cells in the G2 phase of the cell cycle[8,18,24]. Recent studies have found that this cytotoxic factor of *A. actinomycetemcomitans* was associated with the CDT of *Escherichia coli*, *Haemophilus ducreyi*, *Campylobacter jejuni*, and *Helicobacter hepaticus*, and consisted of 3 subunits, CdtA, CdtB, and CdtC[25]. Above all, the CdtB subunit of each member of the CDT family has DNase I homologous residues that are critical for cytolethal distending toxin-mediated cell cycle arrest. Moreover, the DNase I homologous residues contained in the CdtB subunit are associated with catalysis, DNA binding, and metal ion binding, which are well known for their importance in DNase activity[15,18].

We constructed recombinant CdtB from *A. actinomycetemcomitans* Y4 using the PCR method and expressed it from the *E. coli* BL21 (DE3) harboring pET28-cdtB plasmid. As a result, we obtained 20mg of recombinant CdtB inclusion bodies from a one L culture cell lysate of *E. coli* BL21 containing the pET28-cdtB plasmid. Since CdtB does not have a disulfide bond, we used the CdtB refolding system with the dialysis method to obtain the functional recombinant CdtB subunit. We reconstructed 100 ug of CdtB from the 20mg of the inclusion bodies containing the recombinant CdtB. An assay for DNase activity showed that our reconstructed CdtB had a plasmid DNA digestion function and in particular that the metal ions, Mg^{2+} and Ca^{2+}, played pivotal roles, dependent on their concentrations.

In conclusion, as suggested by previous reports showing that DNase I activity is influenced by the metal ions, Mg^{2+} and Ca^{2+}, it was shown that the CdtB subunit of *A. actinomycetemcomitans* has similar acitivity[26,27]. We therefore suggest that studies of the recombinant CdtB active site and influencing factors may contribute to an understanding of CDT associated diseases, such as periodontitis, endocarditic, meningitis, and osteomyelitis.

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