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Preparation of Diphtheria Toxin A Chain from *Escherichia coli*

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Abstract : An expression vector was constructed containing the gene encoding diphtheria toxin A (DTA) which was placed after a T7 promoter. Cytoplasmic expression of the DTA gene resulted in the formation of an insoluble inclusion body. The inclusion body was collected after the complete lysis of the cell, and subsequent washing with 0.1% Triton X-100 released 16~30% of DTA protein from the inclusion body along with other contaminating proteins. The released DTA protein was purified by dialysis. The remaining pellet was dissolved in 8 M urea containing 5% β-mercaptoethanol, and the denatured DTA was renatured by the dilution-dialysis method. The total yield was 35%, and about 5 mg DTA was obtained from 1 L culture. The DTA protein has a free sulfhydryl group exposed to the protein surface, and was shown to have a tendency to dimerize through disulfide formation in the absence of β-mercaptoethanol. The utility of the sulfhydryl group was tested for the construction of recombinant toxins.
Keywords : diphtheria toxin A chain, *Escherichia coli*, gene expression, inclusion body, recombinant toxin.

Bacterial toxins with ribosome-inactivating activity are typically composed of three domains; the ligand domain which aids a toxin to bind a target cell, translocation domain for the efficient translocation of a toxin from the endosome to the cytosol, and toxin domain possessing an enzymatic activity to cause cell death. They are extremely toxic, and only one molecule of diphtheria toxin A (DTA) chain is sufficient to kill a cell (Yamaizumi *et al.*, 1978). The toxicity is threatening for human health, but could be beneficial if it had specificity toward diseased cells. A typical example of such applications is the recombinant toxin, where the ligand domain of the toxin is replaced by other tissue-specific ligands such as a monoclonal antibody or a growth factor. The resulting recombinant toxin is highly specific and effective in killing a target cell.

A recombinant toxin can be prepared either by chemical cross-linking the ligand protein to a truncated form of toxin which lacks the ligand domain, or by producing a fusion protein from a recombinant DNA. The latter approach is preferred in that the produced recombinant toxin is homogeneous, but is hampered by the folding problem. The expression of foreign proteins in *Escherichia coli* (*E. coli*) frequently results in the formation of insoluble inclusion bodies. Then the target protein should be obtained by the problematic denaturation and renaturation procedure. Although various approaches have been reported to improve the procedure, the renaturation efficiency is extremely low with multi-domain proteins or disulfide-rich proteins. In such cases, chemical cross-linking should be an alternative method.

Diphtheria toxin is one of the toxins most commonly used for the construction of recombinant toxins. It is synthesized by *Corynebacterium diphtheriae* as a single polypeptide of 58 kDa, and posttranslationally cleaved into two polypeptide chains A (21 kDa) and B (37 kDa) which are then connected by a disulfide bond. While the A chain is an enzyme with an ADP-ribosylation activity, the ligand binding and translocation activity is associated with the B chain. The three-dimensional structure reveals that the sulfhydryl groups of A and B chain are exposed to the surface (Choe *et al.*, 1992), being susceptible to disulfide formation. It was shown that the separately expressed A and B chain associated quantitatively through disulfide bond formation simply by mixing and dialyzing them overnight (Stenmark *et al.*, 1992). The whole diphtheria toxin (Bishai *et al.*, 1987) or its recombinant toxins (Williams *et al.*, 1990; Tatro *et al.*, 1992; Blanke *et al.*, 1996; Fisher *et al.*, 1996) have been expressed in *E. coli*. They were generally found in the cytoplasm of *E. coli* as soluble proteins except when the substance P peptide was used as a targeting ligand.

The major limitation in the clinical application of recombinant toxins is their strong immunogenicity. To avoid this problem, liposomal delivery of DTA was at-

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tempted with a fusogenic liposome containing the spike protein of sendai virus (Uchida *et al.*, 1979; Mizuguchi *et al.*, 1996). If the liposome could be improved in functions such as tissue-specificity, efficiency, and stability, the liposome approach might become an attractive method to deliver toxin proteins.

In this report, DTA was expressed in *E. coli* and purified for the purpose of utilizing it as a therapeutic agent either by the liposomal delivery or by the preparation of conjugated toxins containing targeting ligands via chemical cross-linking. The synthesized DTA was found to be associated with an insoluble inclusion body, and was solubilized by Triton X-100 in part. The remaining DTA was purified by denaturation and renaturation. The monomeric DTA had a tendency to form disulfide bonds, which might be useful for the construction of conjugated toxins by the chemical method.

Materials and Methods

Construction of an DTA expression vector

A plasmid containing DTA gene was a gift from Dr. I. H. Maxwell (Maxwell *et al.*, 1986). The DTA gene was subcloned into the vector pET5a (Novagen, Madison, USA) to construct a DTA expression vector pET5DTA as shown in Fig. 1. The molecular weight of the resulting DTA protein was calculated to be about 24 kDa from its amino acid sequence. The gene manipulation techniques followed the methods of Sambrook *et al.* (1989). All enzymes used for the cloning were purchased from Promega (Madison, USA).

Expression and purification of inclusion body

The pET5DTA vector was introduced into *E. coli* BL 21 (DE3) pLysS (Novagen, Madison, USA) carrying T7 RNA polymerase gene inducible by isopropyl β-D-thiogalactopyranoside (IPTG). The transformant was grown in 1 L of LB medium (Sigma, St. Louis, USA) containing 100 μg/ml ampicilin at 37°C until OD_{600} reached 0.8~1.0, and the expression of the DTA protein was induced by adding IPTG (Promega, Madison, USA) to a final concentration of 0.1 mM. The culture was further incubated at 37°C for 3~4 h.

The cells were harvested by centrifugation at 10000×*g* (10 min, 4°C), and resuspended in 200 ml of 10 mM Tris-Cl, pH 8.0. They were completely lysed by sonication, and the insoluble inclusion body was collected by centrifugation at 15000×*g* (30 min, 4°C). The pellet was resuspended in 20 ml of 10 mM Tris-Cl, pH 8.0, and Triton X-100 was added to a final concentration of 0.1%. The suspension was sonicated thoroughly to remove adsorbed contaminating proteins, and the inclusion body was harvested by centrifugation at 15000×*g* (30 min, 4°C). The supernatant and pellet were separately processed for further purification.

Denaturation and renaturation

The pellet was dissolved in 10 ml of 8 M urea containing 5% β-mercaptoethanol, and placed at room temperature for 1 h. The insoluble materials were removed by centrifugation at 15000×*g* (15 min, 4°C). The supernatant containing denatured proteins was diluted with 30 ml of PBS, and the precipitates were removed by centrifugation. The supernatant was dialyzed overnight at 4°C with PBS containing 0.2% β-mer-

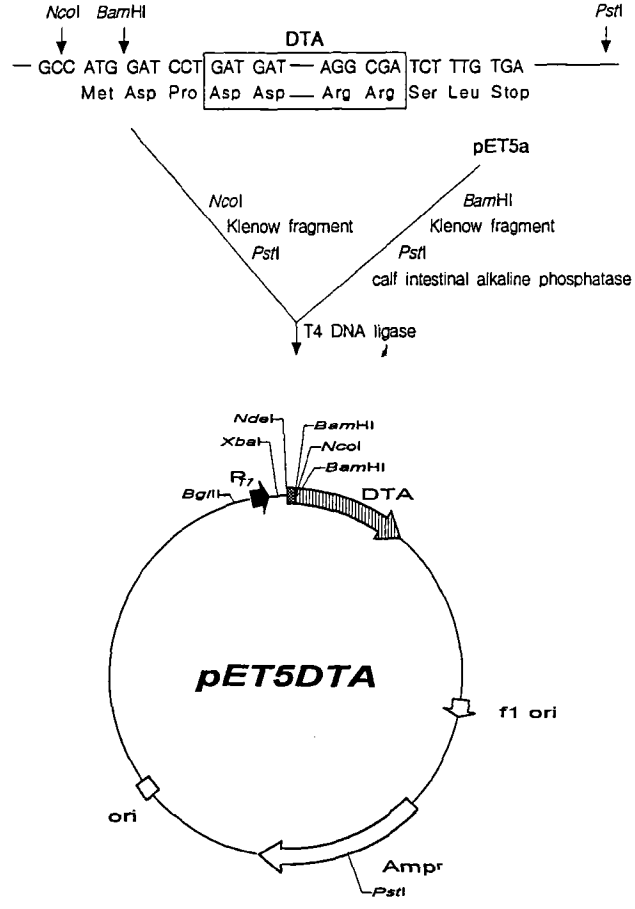

Fig. 1. Cloning scheme of the DTA expression vector. The DTA gene-containing plasmid was modified by cutting with *Nco*I followed by the treatment with four deoxyribonucleotides and the large fragment of *E. coli* DNA polymerase I (Klenow fragment) to create blunt ends. The DNA was again digested with *Pst*I, and a 2.9 kb fragment containing DTA gene was electroeluted after the agarose gel electrophoresis. The pET5a DNA was similarly modified except that *Bam*HI was used instead of *Nco*I and 3.4 kb DNA fragment was eluted. The two DNA fragments were joined by the treatment with T4 DNA ligase at 16°C, and the ligate was used to transform *E. coli* Top 10 (Invitrogen, San Diego, USA). A positive clone was selected by the restriction site analysis. The hatched diagonal region in pET5DTA represents the leader sequence of pET5a consisting of 14 amino acids.

captoethanol, and centrifuged to remove precipitates. The supernatant was concentrated with Centriprep-10 (Amicon, Beverly, USA).

Because a substantial amount of DTA was solubilized by washing with Triton X-100, the solution was dialyzed in the same way as above to remove Triton X-100. The precipitate was removed by centrifugation, and the clear supernatant was concentrated with Centriprep-10.

Chemical cross-linking

N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP from Pierce, Rockford, USA) solution was prepared in dimethylsulfoxide at a concentration of 20 mM, and 5 mg of human transferrin (Sigma, St. Louis, USA) was dissolved in 800 μl of PBS. The transferrin was derivatized with 50 μl of SPDP solution by shaking at room temperature for 1 h. The unreacted SPDP was removed by dialysis against PBS. The DTA solution was also dialyzed against PBS to remove β-mercaptoethanol which was added to prevent dimerization of DTA. For the conjugation of the two proteins, about 1 mg of SPDP-transferrin and 0.4 mg of DTA were mixed in 800 μl PBS. The tube containing the reaction mixture was flushed with nitrogen gas to suppress the DTA dimerization caused by air-oxidation, and shaken at room temperature for 18 h.

SDS-PAGE and densitometry

Protein samples were analyzed by SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) using 10% or 15% gel. In some cases, β-mercaptoethanol was omitted from the sample loading dye to make a non-reducing condition. The gel was stained with 0.25% Coomassie Brilliant Blue R250 (Sigma, St. Louis, USA) in methanol/H_2O/acetic acid (4.5:4.5:1) solution. The band intensity was estimated with ImageCalc software which was a gift from Dr. T. H. van Kuppevelt (van de Lest *et al.*, 1995) after scanning the gels.

Cell culture and cytotoxicity test

FRTL-5 cell was grown in F-12 Coon's modification medium (Sigma, St. Louis, USA) supplemented with BME amino acids, 2 mM L-glutamine, 5% bovine calf serum, 1 IU/L thyrotropin, 10 mg/L insulin, 10 nM hydrocortisone, 10 mg/L somatostatin, 5 mg/L transferrin, 10 mg/L glycyl-L-histidyl-L-lysine at 37°C in 5% CO_2. The amino acid solution was purchased from Gibco BRL (Gaithersburg, USA), and bovine calf serum and hormones were obtained from Sigma (St. Louis, USA).

To determine the cytotoxicity of proteins, about 4×10^4 FRTL-5 cells were plated on a 96-well plate. Appropriate amounts of samples were added to the culture, and the plate was incubated at 37°C for 3 days within CO_2 incubator. MTT solution (50 μl of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide in isopropanol containing 0.1 N HCl) was added to each well, and the plate was further incubated at 37°C for 5 h. The medium was removed, and the precipitate was dissolved in 200 μl of dimethylsulfoxide. The OD_{570} was measured with a microplate reader.

Results and Discussion

Expression and purification

The induction with IPTG led to the appearance of a new protein band with a molecular weight comparable to the expected value of DTA (Fig. 2A). The expres-

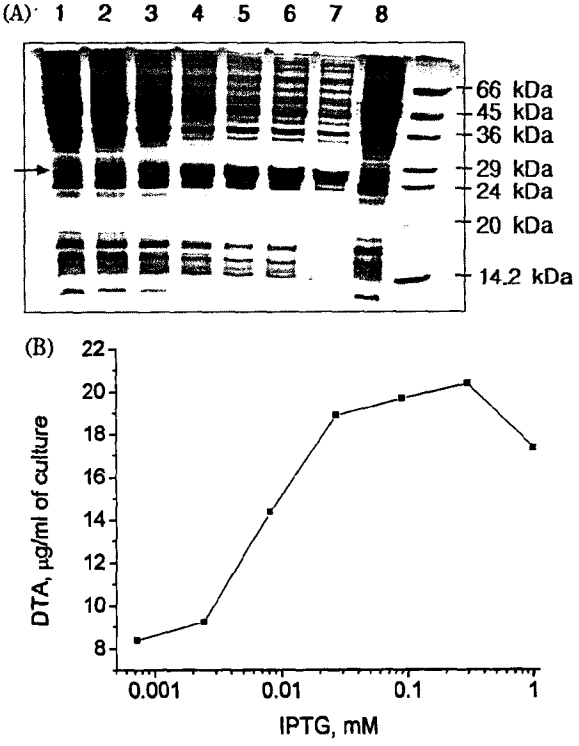

Fig. 2. The effect of IPTG concentration on the expression level of DTA. (A) *E. coli* BL21 (DE3) pLysS containing pET 5DTA was grown in LB supplemented with 100 μg/ml ampicilin until OD_{600} reached about 0.8, and divided into 8 tubes. The expression of DTA was induced by adding IPTG to each tube at concentrations of 0.00073, 0.00243, 0.0081, 0.027, 0.09, 0.3, and 1 mM (lanes 1~7). The lane 8 shows the control sample to which no IPTG was added. The whole cell samples were analyzed by SDS-PAGE in the reducing condition using 15% gel. The molecular weights of standard proteins are shown in the right side of the gel, and the DTA protein band is indicated by an arrow. (B) The band intensity was estimated with ImageCalc program after scanning the gel, and the amount of protein in each band was calculated by comparing its intensity with those of standard proteins (about 1.7 μg of protein per each band).

sion level was maximum at IPTG concentration of 0.03 ~0.3 mM where DTA protein amounted to about 16% of total cellular proteins (Fig. 2A and B). When the induction was carried out at 37℃, no detectable amount of DTA was found in the soluble fraction at all IPTG concentrations after lysis of the cell as judged by SDS-PAGE (data not shown).

There was a report describing a soluble expression of several DTA fusion proteins containing hexahistidine, hexalysine, hexaglutamate, or other hexapeptides on its N-terminus in *E. coli* (Blanke *et al.*, 1996). The fusion proteins were expressed in a condition similar to ours, and the typical yield was 1~10 mg of proteins per liter of culture depending on N-terminal peptides. Their chimeric DTA genes have a notable difference with ours in the amino acid sequences of N-terminal fusion peptides which consist of polar amino acids such as histidine, lysine, glutamate, or serine. Sometimes, small changes in the protein's primary structure can have drastic effects on stability and solubility. Replacement of the hydrophobic C-terminal amino acids of *E. coli* penicillin binding protein 5 with a shorter hydrophilic sequence made the protein water soluble (Ferreira *et al.*, 1988).

It has been suggested that the principal cause of inclusion body formation is a local high concentration of the expressed foreign protein, leading to a non-specific aggregation (Kiefhaber *et al.*, 1991). By this reasoning, expression of heterologous proteins at low inducer concentration or at low induction temperature was attempted, and was proved to be useful in some cases (Bishai *et al.*, 1987; Piatak *et al.*, 1988; Schein *et al.*, 1988; Takagi *et al.*, 1988). In our case, however, the DTA protein was not detected in the soluble fraction as identified by SDS-PAGE when the induction was carried out at 20 ℃ or 30℃ with 10^{-3}~1 mM IPTG (data not shown).

Therefore, we decided to obtain DTA protein by the denaturation-renaturation process from the inclusion body. After complete lysis of cells, the majority of the expressed DTA proteins was found in the insoluble fraction along with some contaminating proteins (Fig. 3, lane 3). Much of the protein contaminations were effectively removed by sonication in the presence of Triton X-100 (Fig. 3, lane 5) as had been reported by others (Marston *et al.*, 1984; Lin and Cheng, 1991). By this step, about 16~30% of DTA was also released into the supernatant (Fig. 3, lane 4), and over 70% of the released DTA remained soluble even after removal of Triton X-100 by dialysis (Fig. 3, lane 9). The sarkosyl detergent was shown to be useful for the generation of soluble proteins from the inclusion body in some cases where bacterial outer membrane components were suggested as a principal cause of the inclusion body formation (Frankel *et al.*, 1991; Grieco *et al.*, 1992). However, Triton X-100 has been known to have no effect on the solubilization of foreign proteins from the inclusion body, being used as a washing agent (Marston *et al.*, 1984; Lin and Cheng, 1991; Grieco *et al.*, 1992). This implies that the insoluble DTA proteins found in the pellet after lysis of the cells may be in two distinct states; a part of them is tightly aggregated to form inclusion bodies as found in other examples, and the remainder is loosely associated by hydrophobic interactions and is prone to solubilization by Triton X-100.

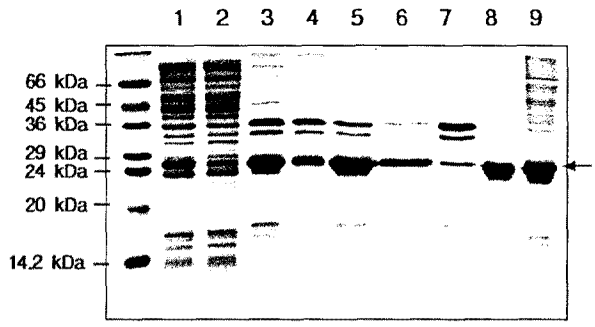

Fig. 3. Purification of DTA protein. Lane 1, whole cell after induction with 0.1 mM IPTG; lane 2: supernatant after the lysis of the cell; lane 3: pellet after lysis of the cell; lane 4: supernatant after washing with Triton X-100; lane 5: pellet after washing with Triton X-100; lane 6: supernatant after the dilution step; lane 7: pellet after the dilution step; lane 8: supernatant after dialysis of the diluted protein (lane 6); lane 9: supernatant after dialysis of the released proteins (lane 4) in the washing step. The samples were analyzed by SDS-PAGE in the reducing condition using a 15% gel. The molecular weights of standard proteins are shown in the left side of the gel, and the DTA protein band is indicated by an arrow.

Renaturation

In theory, renaturation may be accomplished by removal of the denaturing agents through dialysis. However, in practice, the problem is far more complex and suboptimal conditions can often lead to aggregation probably caused by intermolecular interactions at high protein concentrations during the folding process. In an attempt to avoid this problem, the denatured protein solution was rapidly diluted (typically at a ratio of 100:1) with a denaturant-free buffer (Brinkmann *et al.*, 1992; Tait *et al.*, 1995; Newton *et al.*, 1996). The major drawback in this approach is the inconvenience and loss of protein accompanied by the concentration procedure. Therefore, we devised a combined method of dilution and dialysis as described in Materials and Methods. After four-fold dilution (to a final urea concentration of 2 M), over 80% of the applied DTA protein was recovered at a concentration of about 0.1 mg/ml as a soluble protein, and the precipitated proteins

were mainly composed of contaminants (Fig. 3, lanes 6 and 7). Finally, urea was removed by dialysis producing pure DTA proteins (Fig. 3, lane 8). The total yield was about 35% (15% from the solubilization by Triton X-100 and 20% from the denaturation-renaturation process), and about 5 mg of pure DTA was obtained from 1 L culture. This method is very simple by eliminating the need of chromatographic steps, and is safer than the conventional purification method from the culture of *Corynebacterium diphtheriae*.

Chemical cross-linking and cytotoxicity test

Because an additional band was observed which was proposed to be dimeric DTA, when the purified DTA was analyzed by non-reducing SDS-PAGE, the reducing agent was removed by dialysis and the protein solution was stored at a refrigerator to see if the purified DTA had a tendency to dimerize by its free sulfhydryl group. The result shows the dimer percent gradualy increased over time (Fig. 4), implying that the sulfhydryl group may be utilized for the formation of recombinant toxins through chemical cross-linking. The idea was tested by coupling the DTA with SPDP-derivatized human transferrin, and the result is shown in Fig. 5. In this experiment, SPDP was used in ten-fold molar excess over transferrin, thus yielding transferrin molecules containing multiple number of 2-pyridyl disulfide groups. This would result in the formation of heterogeneously conjugated proteins of transferrin and DTA. The gel shows the migration of the bands corresponding to DTA and transferrin into the higher molecular weight

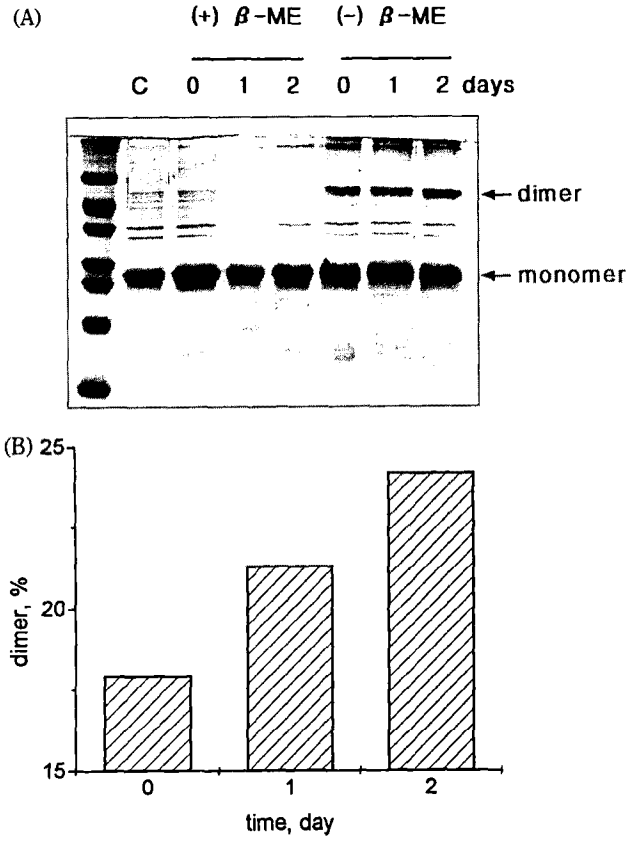

Fig. 4. Dimerization of the DTA protein. (A) The reducing agent was removed from the purified DTA protein, and aliquots were taken and frozen at time interval. They were analyzed by SDS-PAGE in the reducing [(+)β-ME] or non-reducing [(−)β-ME] condition using a 15% gel. The molecular weights of standard proteins are same as in Fig. 3. (B) The percent dimer was calculated by comparing the band intensity of dimer to the total intensity of monomer and dimer.

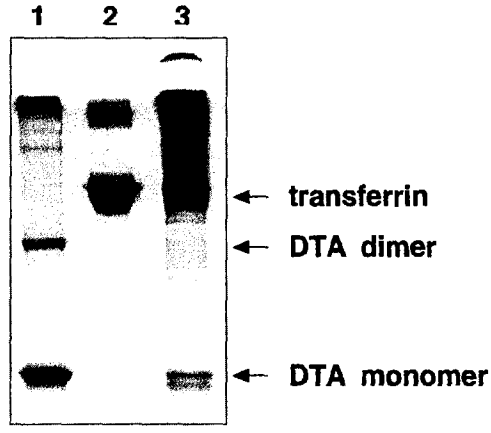

Fig. 5. Chemical coupling of DTA to the SPDP-transferrin. Lane 1: the purified DTA; lane 2: SPDP-derivatized human transferrin; lane 3: product of the coupling reaction. The protein samples were analyzed by SDS-PAGE in the non-reducing condition using a 10% gel.

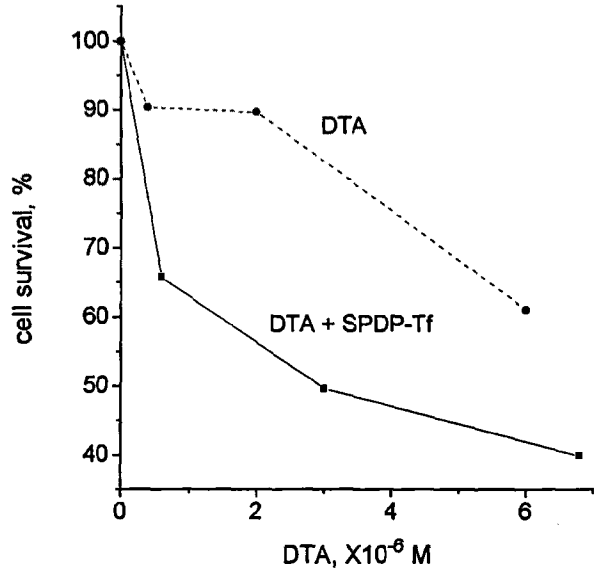

Fig. 6. Cytotoxicity test of proteins. Different amounts of the purified DTA (dotted line) or the coupling reaction product (solid line) were added to the FRTL-5 cell plated on a 96-well plate, and the cytotoxicity was estimated as described in Materials and Methods.

region.

In order to verify the renaturation and chemical coupling process, the DTA and conjugated protein were tested for their cytotoxicity with FRTL-5 cells possessing transferrin receptors. As shown in Fig. 6, about 40% of the cells were killed by the free DTA at a concentration of 6×10^{-6} M which is similar to the results of others (Blake *et al.*, 1996). After the coupling reaction, the cytotoxicity increased about 6-fold possibly because of the targeting activity of transferrin. However, the EC50 is still on the order of 10^{-6} M, and is high compared to common recombinant toxins. The primary cause of such a low toxicity may be the lack of the translocation domain. The cytotoxic activity of the purified DTA was also demonstrated by testing with the liposome-encapsulated DTA, which showed much higher activity than the naked protein (unpublished data). Thus, it is expected that the prepared DTA may be used as a therapeutic agent if properly delivered into a target cell either by the liposomal delivery or by the preparation of a recombinant toxin.

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