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# Purification and characterization of restriction endonuclease *Cst* I from *Clostridium sticklandii*

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**Abstract.** A type II restriction endonuclease *Cst* I was purified to homogeneity from *Clostridium sticklandii* by phosphocellulose chromatography and preparative gel electrophoresis. It was found that *Cst* I was an isoschizomer of *Pst* I which is widely used in molecular cloning. Both *Cst* I and *Pst* I recognize and cleave the nucleotide sequence 5′-CTGCAG-3′ of double stranded DNA. Nucleotide sequence analysis revealed that both enzymes split the phosphodiester bond between A and G.
The molecular weight of *Cst* I determined by disc gel electrophoresis was apparently 206,000. The optimal pH, temperature, sodium chloride and magnesium ion concentrations of *Cst* I were shown to be 7-9, 37-40°C, 50-200 mM and 5 mM, respectively. *Cst* I is heat-labile. When incubated at temperature higher than 40°C for 5 minutes, the enzyme lost its activity rapidly.

**Key word:** *Clostridium sticklandii;* Recognition and cleavage sequence; Restriction endonuclease.

## Introduction

Restriction endonucleases particularly the type II enzymes are indispensable to the manipulation of DNA and have become very popular enzymes in both basic and applied researches. More than six hundreds restriction endonucleases have thus far been isolated from a wide variety of organisms (Kessler and Holtke, 1986). However only a few restriction enzymes have been reported to occur in anaerobic bacteria (Makula and Meagher, 1980, Kessler and Holtke, 1986).

Although a large number of restriction endonucleases have been found, but many of them are isoschizomers, enzymes which have identical recognition sites. Our knowledge of the relationship between isoschizomers is very limited (Whitehead *et al.*, 1986). The analysis of evolutionary relationship between restriction endonucleases requires many more recognition and cleavage specificities then are currently available. Besides, new restricton endonucleases with novel recognition and cleavage specificities are still required in order to facilitate DNA manipulations. And novel restriction endonucleases may provide new materials for investigating the mechanisms of protein-DNA interactions. Therefore, we have screened a number of anaerobic bacteria for the presence of restriction endonucleases. This paper describes the purification and the determination of the recognition and cleavage sites of a restriction endonuclease from *Clostridium sticklandii.* This enzyme is designated as *Cst* I according to the nomenclature proposed by Smith and Nathans (1973).

## Materials and Methods

*Materials*

The following were purchased from commercial sources: *Eco* R1, plasmid pBR 322, λDNA, φX 174 RF DNA, (Bethesda Research Laboratories, Inc.) *Pst* I (Toyobo), Protein standards (Pharmacia), protein assay dye reagent (Bio-Rad). Phosphocellulose P11 was obtained from Whatman Ltd. and prepared according to Greene *et al.* (1978). λDNA was also prepared by the method of Maniatis *et al.* (1982) using lysogenic *E. coli* W5 (CIts 857 Sam 7)/λ. *Clostridium sticklandii* cells were grown according to Turner and Stadtman (1973).

*Endonuclease Assay*

The reaction mixture in a final volume of 50 μl contained 1 μg of λDNA, 7 mM mercaptoethanol, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and proper amounts of enzyme. Incubations were carried out at 37°C for 1 hour and terminated by the addition of 10 μl of the stop solution (100 mM EDTA, pH 8.0/40% sucrose /0.06% bromophenol blue). Twenty μl aliquots of the reaction mixtures were analyzed for DNA digestions by electrophoresis in 1% agarose gel slabs in TBE buffer (89 mM Tris/89 mM boric acid/2 mM EDTA/pH 8.3) at 5 V/cm for 4.5 hours. The DNA fragments were visualized with ethidium bromide and the gels photographed on an UV transilluminator as described by Sugden *et al.* (1975).

One unit of restriction endonuclease activity was defined as the amount of enzyme required to completely digest 1.0 μg of λDNA in one hour under the defined assay conditions. Specific activity was expressed as units of enzymatic activity per mg protein.

*Purification of Restriction Endonuclease **Cst** I*

Extraction: Defrosted cells of *C. sticklandii* were mixed with equal volume of 10 mM potassium phosphate buffer solution, pH 7.4/0.002% PMSF/1 mM EDTA/15 mM mercaptoethanol and the cells were homogenized with sonication. The homogenate was centrifuged at 100,000×g at 4°C for 1 hour. The supernatant was purified by the following methods. All steps were performed at 4°C, except otherwise indicated.

Phosphocellulose chromatography: The supernatant was dialyzed against buffer A (10 mM potassium phosphate, pH 7.5/1 mM EDTA/1 mM $NaN_3$/7 mM mercaptoethanol/10% glycerol) containing 0.2 M NaCl and applied to a phosphocellulose column (1.6×40 cm) which was previously equilibrated with buffer A containing 0.2 M NaCl. The column was washed with buffer A and the bound proteins were eluted with a linear gradient (14 folds of bed volume) of 0.2-2.0 M NaCl in buffer A. Three ml per fraction was collected. Fractions containing endonuclease activity were pooled, concentrated and further purified by preparative polyacrylamide gel electrophoresis.

Preparative polyacrylamide gel electrophoresis: Electrophoresis was performed according to the method previously described (Chen and Lian, 1986).

*Measurement of Protein*

Protein was measured by the method of Lowry *et al.* (1951) or Bradford (1976) using crystalline bovine serum albumin as the standard.

*Determination of the Recognition and the Cleavage Sites of **Cst** I*

Since the electrophoretic patterns of λDNA digested by *Cst* I and *Pst* I were essentially the same, it is suggested that these two enzymes are isoschizomers recognizing the same oligonucleotide sequence 5′-CTGCAG-3′. To determine whether *Cst* I also cleaves the same phosphodiester linkage between A and G as *Pst* I, pUC18 DNA which has one each of *Eco* RI, *Xmn* I and *Pst* I site was used.

Labeling of 3′ ends: One μg of pUC18 DNA was digested with 10 units of *Eco* RI. After

deproteinization by extraction with phenol/chloroform/isoamylalcohol (24: 24: 1, v/v) and ethanol precipitation of the DNA, the cleaved pUC18 DNA was dissolved in 16 $\mu$l sterilized water. Then 2.5 $\mu$l of 10 × nick translation buffer (0.5 M Tris-HCl, pH 7.2/0.1 M $MgSO_4$/1 mM DTT/0.05% BSA), 5 $\mu$l of [$\alpha$-$^{32}$P] dATP (3000 Ci/mmole, 10$\mu$ Ci/$\mu$l), and 5 units of Klenow fragment were added and the mixture was incubated for 30 minutes at room temperature. Subsequently the 3′ end $^{32}$P-labeled DNA was extracted with phenol/chloroform, precipitated with ethanol and evaporated to dryness under reduced pressure.

The 3′ end $^{32}$P-labeled DNA was dissolved in 39 $\mu$l of sterilized water. Ten $\mu$l of reaction buffer (30 mM Tris-HCl, pH 8.0/30 mM NaCl/30 mM $MgCl_2$/30 mM mercaptoethanol/BSA, 10 $\mu$g per ml), and 6 units of *Xmn* I were added and the mixture was incubated for 1 to 2 hours at 37°C.

After stopping the reaction by the addition of 10 $\mu$l of 100 mM EDTA, pH 7.5/0.025% BPB/40% sucrose, the fragmented DNA was separated by low melting agarose gel electrophoresis. *Xmn* I cleaved the 3′ end $^{32}$P-labeled DNA into two fragments of 838 bp and 1848 bp. The 838 bp fragment which contains *Pst* I site was eluted from the agarose gel and used for nucleotide sequence analysis.

Sequencing of the 3′ end labeled 838 bp fragment: aliquots of the 838 bp fragment was digested with *Cst* I and *Pst* I separately. Other aliquots were subjected to base-specific chemical cleavage reactions. The digestion and the reaction mixtures were electrophoresed in a sequencing gel as described by Maxam and Gilbert (1980).

## Results

*Purification of* ***Cst*** *I*

Crude extracts were made from 10 grams of *Clostridium sticklandii* cells and the units of restriction endonuclease activity was measured by incubation with λDNA. Chromatography of the crude extracts on a phosphocellulose column is shown in Fig. 1. Aliquots of even-numbered fractions were assayed for restriction endonuclease activity (Fig. 2). It was found that *Cst* I peak (Fractions 14-26) was almost completely separated from other proteins in the crude extracts, resulting in 514 folds of purification (data not shown). The high efficiency of the phosphocellulose column to purify *Cst* I was further evidenced by the analysis of the purified *Cst* I by polyacrylamide gel electrophoresis (PAGE). There were only two protein bands found in the gel and the amounts of protein in the slower moving band was larger than that of the faster moving one (data not shown). The slower moving protein was shown to be *Cst* I as described in the following.

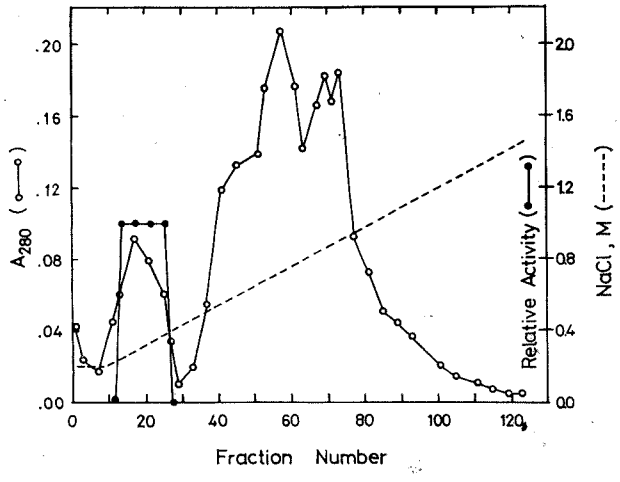



Fig. 1. Phosphocellulose chromatography of *Cst* I. The *Cst* I crude extract was applied to a phosphocellulose column (1.6 × 14 cm). After equilibrated with 10 mM potassium phosphate buffer, pH 7.6, the column was eluted with a linear gradient (14 × bed volume) of 0.2M-2M NaCl in 10 mM potassium phosphate buffer, pH 7.5. Fractions of 3 ml were collected. Samples (20 $\mu$l) of column fractions were assayed as described in "Materials and Methods". The assay mixtures were incubated at 37°C for 8 h.

The purified *Cst* I obtained from the phosphocellulose column was further purified by preparative PAGE. Fig. 3A indicates the two protein bands. Gel slices of 2-mm sections encompassing the two proteins were extracted with

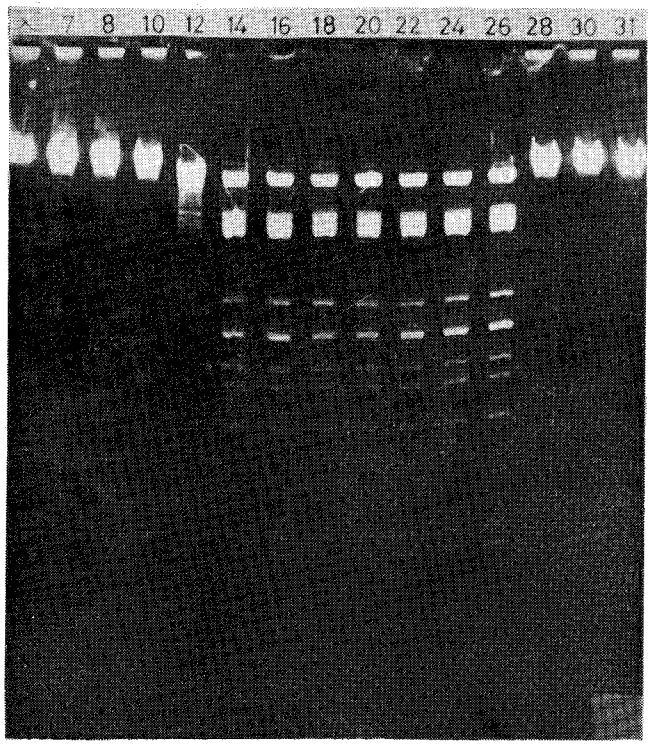



Fig. 2. Assays of the column fractions for restriction endonuclease *Cst* I activity. Aliquots (20 μl) of the solutions in even-numbered fractions were incubated with 1 μg λDNA under the standard assay condition described in "Materials and Methods." After termination of the reaction, 30 μl of the reaction mixture was loaded into a 1% agarose gel and electrophoresed at 100 V for 5 h, the gel was photographed under UV light.

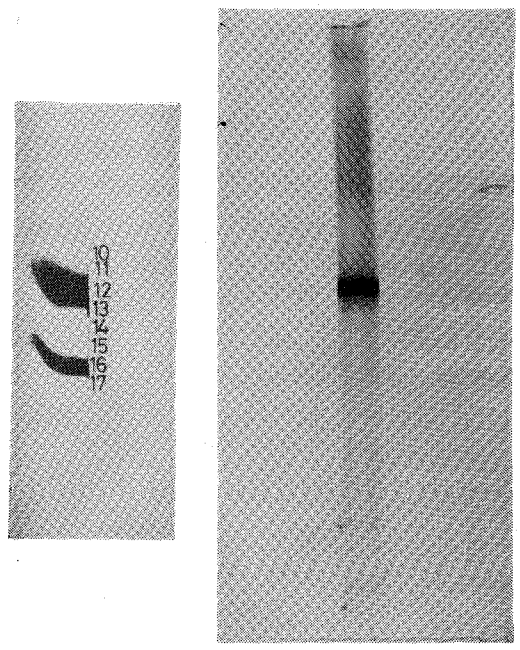
buffer A and assayed for *Cst* I activity. The results indicated that *Cst* I activity was enriched in slice No. 13 (data not shown). *Cst* I obtained from slice No. 13 was shown to be electrophoretically homogeneous (Fig. 3B).

*Determination of the Recognition Sequence and the Cleavage site*

The similar digestion patterns of *Cst* I and *Pst* I on λDNA, φX 174 RF DNA and pBR 322 suggest that these two enzymes are isoschizomers (Fig. 4). Nucleotide sequence analysis revealed that both *Pst* I and *Cst* I recognized the same oligonucleotide sequence 5′-CTGCAG-3′ and cleaved the same phosphodiester linkage between A and G (Fig. 5).

*Molecular Weight of **Cst** I*

Molecular weight of *Cst* I was determined by the method previously described (Hedrick and Smith, 1968). Log mobilities (Rm) of protein standards and *Cst* I were plotted against gel concentrations. The log (Rm) was linearly related to gel concentration (data not shown). Slope for each protein was obtained and plotted against molecular weights of protein standards (Fig. 6). Molecular weight of *Cst* I was estimated to be 206,000 from Fig. 6. This figure is larger than the previously reported molecular weights of type II




A B

Fig. 3. Preparative disc polyacrylamide gel electrophoresis of *Cst* I (A) and disc gel electrophoresis pattern of purified *Cst* I (B). A: *Cst* I preparation (680 μg) obtained from phosphocellulose chromatography was further purified by preparative PAGE as described in "Materials and Methods". One cm from the left end of the slab gel was cut and stained with Coomassie blue. Number indicated the corresponding position of gel slices in the other part of the slab gel used for extraction of enzyme. B: Forty-five μg of *Cst* I obtained from preparative gel electrophoresis was used.

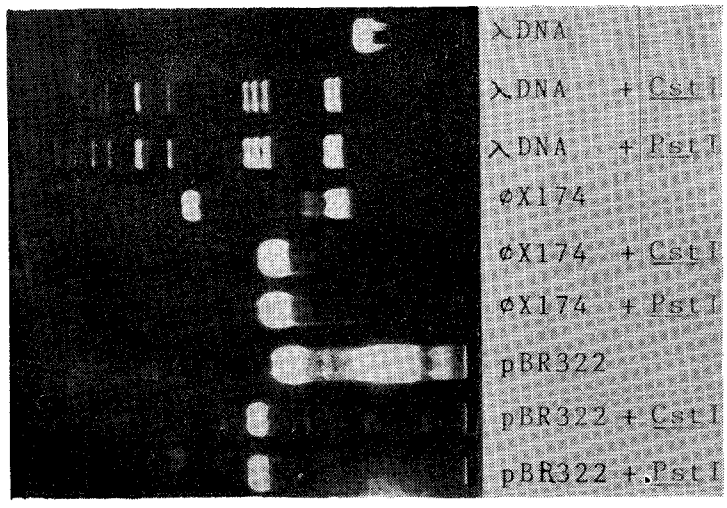



Fig. 4. Digestion patterns of *Cst* I and *Pst* I on λDNA, φX174 RF DNA, and pBR322 DNA. Each DNA sample (1 μg) was digested with 4 units of *Cst* I and *Pst* I for 1 h at 37°C. Slot 1: λDNA; Slot 2: *Cst* I on λDNA; Slot 3: *Pst* I on λDNA; Slot 4: φX174 RF DNA; Slot 5: *Cst* I on φX174 RF DNA; Slot 6: *Pst* I on φX174 RF DNA; Slot 7: pBR322 DNA; Slot 8: *Cst* I on pBR322 DNA; Slot 9: *Pst* I on pBR322 DNA.

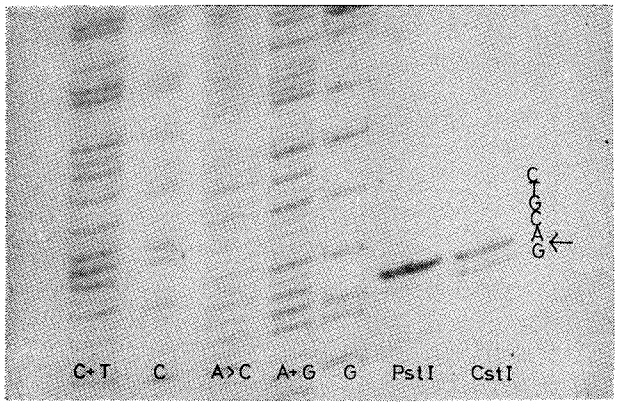



Fig. 5. Determination of cleavage site of *Cst* I on pUC18 DNA. The pUC18 DNA was cleaved by *Eco* RI, labeled with [$\alpha$-$^{32}$P] dATP and further cleaved by *Xmn* I. The DNA fragment of 838 bp was isolated and sequenced by the Maxam and Gilbert method. The autoradiogram (the lower panel) shows the electrophoretic pattern of the four chemical reaction mixtures as well as the digestion mixtures of *Cst* I and *Pst* I on the same fragment.

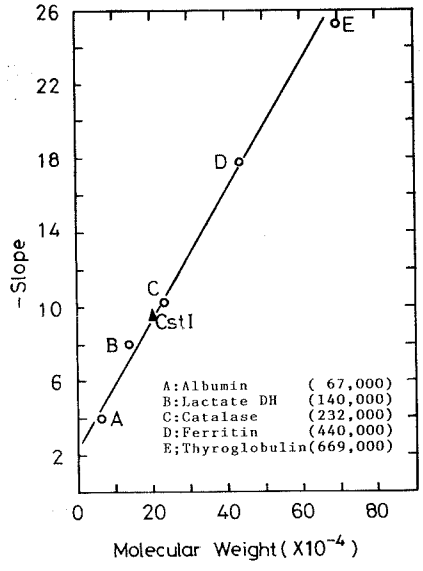
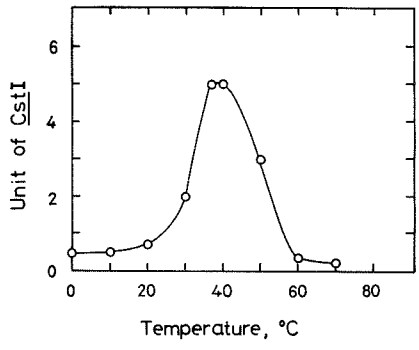



Fig. 6. Estimation of molecular weight of *Cst* I. A: Albumin, B: Lactate dehydrogenase, C: Catalase, D: Ferritin, E: Thyroglobulin. The standard curve: $y = 0.345x + 2.38$

restriction endonuclease which are in the range of 20,000 to 100,000 (Modrich, 1979).

*Effect of Temperature and pH*

*Cst* I showed sharp temperature optimum at 40°C (Fig. 7). The rapid decline of *Cst* I activity at temperature higher than 40°C suggests that this enzyme is heat-labile. This is supported by the thermostability study shown in Fig. 8. The




Fig, 7. Effect of temperature on the *Cst* I activity. One μg of λDNA was digested with 5 units of *Cst* I at various temperature for 1 h.

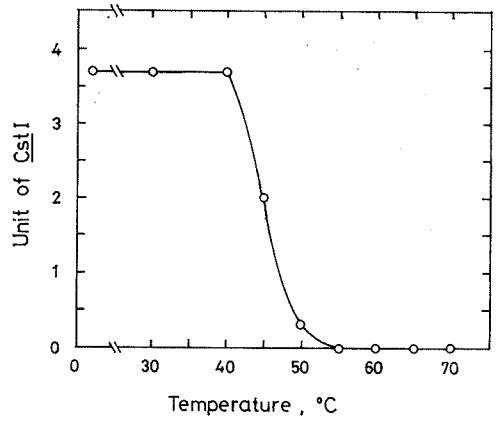



Fig. 8. Thermostability of *Cst* I at pH 7.5. *Cst* I (20 μl) was incubated at various temperature for 5 minutes and cooled down rapidly in ice bath and assayed as described in the legend to Fig. 2.

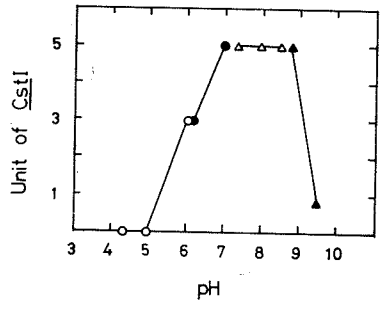



Fig. 9. Effect of pH on the *Cst* I activity. One μg of λDNA was digested with 5 units of *Cst* I at various pH for 1 h. (○—○) Citrate buffer, (●—●) Phosphate buffer, (△—△) Tris-HCl buffer, (▲—▲) NaOH-Glycine buffer.

temperature which inactivated 50% of *Cst* I activity in 5 min is about 45°C. Smith *et al.* (1976) indicated

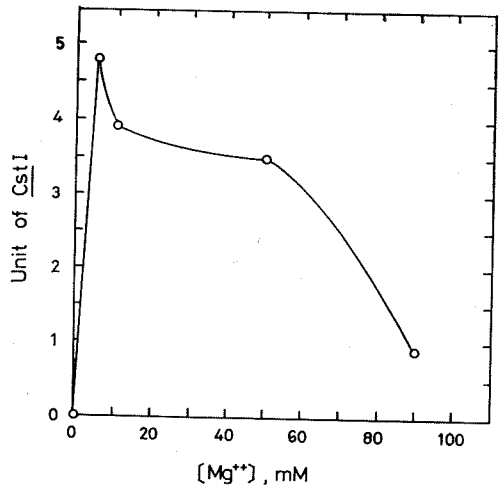



Fig. 10. Effect of magnesium ion concentration on the *Cst* I activity. One μg of λDNA was digested with 5 units of *Cst* I at various concentrations of $MgCl_2$.

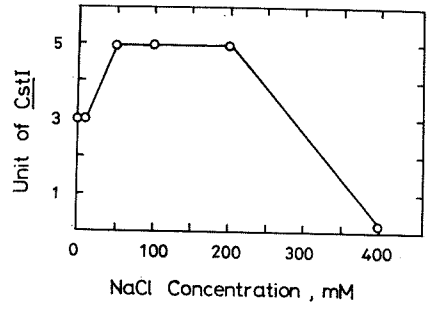



Fig. 11. Effect of NaCl concentration on the *Cst* I activity. One μg of λDNA was digested with 5 units of *Cst* I at various concentrations of NaCl for 1 h.

that partially purified *Pst* I showed almost complete loss of activity after heating for 2 min at 37°C. *Cst* I showed optimal pH between 7 and 9. The activity decreased drastically at pH higher than 9 (Fig. 9).

*Effect of Cations*

Like other type II restriction endonucleases *Cst* I also showed a requirement for $Mg^{2+}$. $Mg^{2+}$ at 5 mM gave maximal *Cst* I activity. The activity decreased slowly with the increase in $Mg^{2+}$ concentration (Fig. 10). The effect of $Na^+$ on *Cst* I activity however was not very strict (Fig. 11).

Maximal activity of *Cst* I was observed in $Na^+$ concentrations between 50 and 200 mM. The activity reduced to 60% in the absence of $Na^+$. Thus this enzyme works equally well at medium and high ionic strength (Maniatis *et al.*, 1982).

## Discussion

The restriction endonuclease of *Clostridium sticklandii Cst* I was purified to homogeneity by a simple procedure which involved phosphocellulose chromatography and preparative PAGE. Since this anaerobic bacterium can be grown quiet easily, *Cst* I may be a potential material for investigating the structural as well as the evolutional relationships among the isoschizomers.

It is interesting to note that total units of *Cst* I activity increased from 850 to 1503 after phosphocellulose chromatography (data not shown) One possible explanation is that some endogeneous factor which partially inhibited *Cst* I activity was removed after chromatography of the crude extract on the phosphocellulose column.

The recovery of *Cst* I protein from preparative PAGE was about 40% and there was substantial amounts of enzyme inactivated (data not shown). Although the preparative PAGE was run at 4°C and the gel was preelectrophoresed before application of *Cst* I sample, we suspect that heat generated during electrophoresis and the residual ammonium persulfate remained in the gel might cause severe loss of the activity of the heat-labile *Cst* I. Another possibility is that the highly purified *Cst* I might be less stable than the partially purified enzyme. Smith *et al.* (1976) indicated that more highly purified preparations of *Pst* I usually lose activity within 3 to 4 weeks, despite efforts to stabilize the enzyme. Since there was only one contaminating protein band in the *Cst* I preparation after phosphocellulose chromatography, by the use of other purification method such as chromatofocusing or preparative HPLC the above difficulty may be circumvented.

Nucleotide sequence analysis clearly shows that *Cst* I and *Pst* I have the same recognition and cleavage specificity. Careful scrutiny of the autoradiogram of the sequencing gel revealed that there was a very faint band underneath the bands which arised from the digestion of the DNA fragment of 838 bp by *Pst* I and *Cst* I (Fig. 6). This minor band, which had one nucleotide less than the normal band, is presumably due to the incomplete labeling of 3′ ends by [$\alpha$-$^{32}$P]dATP with Klenow fragment. The labeling experiment was designed in such a manner that two [$^{32}$P]-dAMP are added to each 3′ end complementary to the duplex T at the 5′ ends. If the 3′ ends were not exclusively labeled with radioactive duplex A, instead a small number of them received only one [$^{32}$P]AMP, the results like those shown in Fig. 6 could be obtained.

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# 史迪克蘭梭菌核酸內切限制酶的純化與性質

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利用磷酸纖維素管柱層析及製備式不連續膠體電泳，從嫌氣性菌史迪克蘭梭菌中純化出一種核酸內切限制酶，*Cst* I。並發現此酶和鑑識序列爲 5′-CTGCAG-3′ 的 *Pst* I 限制酶爲同形異質酶，且經核苷酸序列分析證實兩者均切割 A 與 G 間的磷酸二酯鍵。

經由不連續膠體電泳測得 *Cst* I 的分子量爲 206,000 道爾吞。*Cst* I 反應的最適溫度在 37至 40°C，最適 pH 在 7至 9，最適鎂離子濃度爲 5 mM，最適氯化鈉濃度在 50 至 200 mM，在 40°C 以上保溫 5 分鐘，酵素活性即迅速下降，顯示 *Cst* I 爲熱不安定性酵素。