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Purification of TaqI endonuclease from *Thermus aquaticus*

Mehmet Mete Altintaş, Nevra Özer, Kutlu Ö. Ülgen*

Chemical Engineering Department, Boğaziçi University, 80815 Bebek/Istanbul, Turkey

Abstract

A purification procedure for the thermostable restriction enzyme TaqI was developed using high-performance ion-exchange liquid chromatography. The effects of various operating conditions on the separation behaviour of TaqI endonuclease from the cell extracts were investigated for optimisation and scaling up. The separation of the enzyme by HPLC was found to be strongly dependent on the sample volume, slope of linear gradient and order of the ion-exchange columns. The final yield of the enzyme is also dependent to a great extent upon the number of fractionation steps employed to purify the enzyme. In the present study, 4000 U TaqI endonuclease per mg protein was recovered from 2 g *Thermus aquaticus* cells with a two-step purification protocol in one day. The purification factor was 24. Compared to other classical methods of purification reported in literature with 4000 or 32 000 U enzyme from 200 g of *Thermus aquaticus* cells, HPLC yielded 190 000 U enzyme from 200 g cells using cation and anion HPLC columns sequentially and thus resulted in a higher efficiency. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: *Thermus aquaticus*; TaqI endonuclease; Enzymes; Purification

1. Introduction

Restriction enzymes recognising unique DNA sequences are valuable tools in gene isolation and analysis as well as in the construction of novel recombinant DNA molecules. Restriction enzymes of thermophilic microorganisms are reported to be exceptionally stable to heat and to protein denaturing reagent [1]. The thermophilic bacterium *Thermus aquaticus* YT-1 possesses two restriction-modification systems, TaqI endonuclease and TaqI methylase, where the former protects the cell from infections by digesting unmodified foreign DNA and the latter protects the cell from autodigestion by modifying its DNA [2]. The thermostable enzyme TaqI endonuclease, which is of interest in the present paper, is extensively used in molecular diagnostics.

*Corresponding author. Fax: +90-212-2872460; e-mail: ulgenk@hamlin.cc.boun.edu.tr

The purification and maintenance of these enzymes can be carried out to different degrees depending upon the ultimate use. The classical procedures in general consist of cell disruption, dialysis, adsorption and several column chromatographic steps [3–6]. The purification of TaqI endonuclease was partially achieved by Sato et al. using a classical five-step protocol which included adsorption onto phosphocellulose, fractionation with ammonium sulfate, dialysis, phosphocellulose column chromatography and gel filtration chromatography. They recovered 4000 U TaqI endonuclease from 200 g *Thermus aquaticus* cells [1]. A shorter two-step purification protocol was then reported by Greene et al. using phosphocellulose column chromatography followed by hydroxyapatite chromatography with a yield of 8000 U TaqI endonuclease from 50 g *Thermus aquaticus* cells [7].

The present paper deals with the integrated development and optimization of a purification process

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by HPLC for TaqI endonuclease produced by the *T. aquaticus* YT-1 strain. In order to obtain better resolution and higher separation speed compared to conventional purification methods, the application of HPLC was incorporated into the purification process by using a Waters 650E Advanced Protein Purification system. A parametric study was conducted to investigate the factors affecting the separation behavior of TaqI endonuclease from the cell extracts. Chromatographic parameters such as the choice of column arrangement and packing material, elution type, and sample volume were investigated and optimised for the recovery of active TaqI endonuclease fractions. The results of HPLC analyses obtained with single- and two-column purification were compared with those obtained by classical methods using low-pressure chromatography.

2. Experimental

2.1. Organism and culture preparation

The bacterium *Thermus aquaticus* YT-1 obtained from the American Type Culture Collection was maintained as a frozen suspension in 50% glycerol at −20°C. The culture was grown in an orbital shaker at 70°C and 160 rpm to the late exponential phase for about a day. The growth medium included Castenholz salts supplemented with tryptone and yeast extract as the carbon and nitrogen sources. The salts included in 1 l of medium were: nitrilotriacetic acid, 0.1 g; $CaSO_4 \cdot 2H_2O$, 0.06 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NaCl, 0.008 g; KNO_3, 0.105 g; $NaNO_3$, 0.7 g; Na_2HPO_4, 0.11 g; $FeCl_3$ solution (0.03%, w/v), 1 ml, and Nitsch's trace elements (containing H_2SO_4, 0.5 ml; $MnSO_4$, 2.2 g; $ZnSO_4$, 0.5 g; H_3BO_3, 0.5 g; $CoSO_4$, 0.016 g; Na_2MoO_4, 0.025 g; $CoCl_2 \cdot 6H_2O$, 0.046 g in 1 l of water), 1 ml. After adjusting the pH to 8.2 by 5 *M* NaOH, 1 g of tryptone and yeast extract were added (Castenholz-TYE medium). The final pH was adjusted to 7.6. 3% (w/v) agar was added to solidify the Castenholz medium.

2.2. Determination of initial pH for ion exchange

Six test tubes including 0.5 ml wet volume of ion exchanger, DE 52, were equilibrated by washing five times in 0.5 *M* L-histidine (pH 5.0, 5.5 and 6.0) and 0.5 *M* Tris–HCl (pH 7.5, 8.0 and 8.5). After equilibration the anion-exchanger matrices were washed five times in 20 m*M* of the related buffers, in each tube at the pre-selected pH used in equilibration. Then 500 μl of the enzyme TaqI, which was purified by classical methods, were added to each tube and mixed for 10 min. The matrices were allowed to settle and the supernatant was assayed for enzyme activity.

2.3. Application of high-performance liquid chromatography

High-performance liquid chromatography was performed at ambient temperature on a Waters 650E Advanced Protein Purification system, with a gradient control monitoring the separation at 280 nm and with a Waters 486 tunable wavelength detector. Data were collected on a NEC Image 466 computer using Millenium 2010 software. A Bio-Rad 2110 fraction collector was integrated to the HPLC system for sample collection, where ice cold buffers and collection tubes were used. Helium gas was used for degassing the buffer solutions. All solutions and samples were prepared with Milli-Q water and filtered through Whatman Millipore membrane filters [Acrodisc poly(vinylidene difluoride) (PVDF)] with 0.45 μm pore size. The columns used in experiments were Waters Advanced Purification (AP) glass columns packed with ion exchanger: Protein Pak DEAE 15 HR AP-1 (100×10 mm) Waters weak anion-exchange column and Protein Pak SP 8 HR AP-1 (100×10 mm) Waters strong cation-exchange column. Prior to injection, HPLC columns were equilibrated with mobile phase (extract buffer) at a certain flow-rate (0.5–1 ml min^{-1}) depending on the column.

2.3.1. Purification by HPLC

After cell disruption by sonication, unlysed cells and cellular debris were removed by centrifugation for 1 h at 18 000 rpm using a Sorvall refrigerated superspeed centrifuge (fixed angle SS34 rotor). The collected supernatant (cell extract) was analysed for TaqI endonuclease activity, dialysed against extract

buffer and then passed through a 0.45-μm Millex filter to remove any remaining particulates. Dialysed and filtered cell extract (1.5–5 ml) was loaded on to the pre-equilibrated HPLC column. Adsorption and desorption (elution) periods were 20 and 60–80 min, respectively. Control and monitoring of the run was done by a gradient table in the Millenium 2010 software. The nonretained material (column flow-through) was collected at 2-min intervals. Bound material was then eluted using extract buffer containing 1 *M* NaCl. Two ml of sample fractions were collected automatically and assayed for protein and TaqI endonuclease activity. Active fractions were pooled and subjected to overnight dialysis against extract buffer so as to salt out the excess NaCl coming from the elution step. The dialysed active fractions were then applied to the second HPLC column which was equilibrated with extract buffer. Following the same procedure for adsorption and elution, active fractions were collected, assayed and stored at −80°C after 1:1 dilution with storage buffer.

2.4. Analyses

Protein concentration in samples was determined by the Bradford dye binding method [8] using bovine serum albumin as protein standard.

One unit of TaqI activity is defined as the amount of the enzyme that produces a complete digest of 1 μg of pUC18 DNA (having four recognition sites) in 1 h at 65°C in a reaction volume of 10 μl. The 20-μl digestion volume contains 2 μl sample, 2 μl pUC18 DNA (1 μg μl^{-1}), 2 μl NE buffer (100 mM Tris–HCl, pH 8.4, 100 mM $MgCl_2$ and 1 M NaCl) and 14 μl sterile water. The samples were diluted as required and the reaction mixture was incubated at 65°C for 1 h. In order to determine the least concentrated solution of the enzyme resulting in a complete digestion of the substrate, each digestion was applied to 1.2% agarose gels. The bands were examined under a UV Transilluminator. The purity of active fractions was followed by sodium dodecyl sulfate–poylacrylamide gel electrophoresis (SDS–PAGE).

Two quality tests, cut–ligate–recut and overdigestion, were performed. The initial cleavage of the substrate DNA (pUC18) was carried out with HPLC-purified TaqI endonuclease. The DNA fragments were extracted by phenol and chloroform and precipitated with ethanol. Ligation of these fragments was performed with T4 ligase at 16°C overnight. T4 ligase was inactivated by heating the reaction mixture for 5 min at 65°C. Ligated fragments were recut by using the same TaqI endonuclease preparation. For the overdigestion test, 1 μg substrate DNA (pUC18 or λDNA) was digested with purified TaqI endonuclease (10 units) for 16 h at 65°C. Digestions were investigated on 1.2% agarose gel under a UV Transilluminator.

3. Results and discussion

A Waters 650E Advanced Protein Purification system was used for rapid large scale purification of TaqI endonuclease using ion-exchange columns. The resolution of a given protein or enzyme on ion-exchange columns is a complicated function of the column and operating variables such as mobile phase velocity, packing diameter, column dimensions and gradient slope [9,10]. In the present paper, therefore, a parametric study comprising single-column and two-column purifications using anion, cation or both, as well as the effects of packing, pH, dialysis, sample volume and the type of elution (gradient versus isocratic) on separation was conducted.

In protein purification anion-exchange functionalities are most frequently used, since proteins of isoelectric point (p*I*) below 7 are more common [10]. Therefore, the purification studies were started with DEAE weak anion exchanger as the adsorbent. As pH fluctuations influence the charge on a protein and its equilibrium between stationary and mobile phases, the pH at which the TaqI endonuclease has distinctive chromatographic characteristics was first determined using two different mobile phases (L-histidine and Tris–HCl) buffered in a pH range of 5–8.5. The ion-exchange matrix was DE52 with functional group diethylaminoethyl, size-exclusion limit of M_r 500 000, and ion capacity of 0.88–1.08 mequiv. dg^{-1}. TaqI endonuclease was found to be completely adsorbed to the anion exchanger above pH 6.

3.1. HPLC application

The purification studies with HPLC were commenced with DEAE weak anion-exchange column [Protein Pak DEAE 15 HR AP-1 (100×10 mm)]. Centrifuged and filtered cell extract (1.5 ml) containing TaqI was injected on to the HPLC anion-exchange column that was equilibrated with extract buffer, pH 7.4. Adsorption took place between 0 and 18 min where the column was washed with 2–3 column volumes of extract buffer. NaCl solution (0.4 *M*) in extract buffer was fed to the system between 18 and 96 min for isocratic elution. According to the results of the enzyme assay, fractions 5–7 (flow-through between 8–14 min) and 22 and 23 (elution with 0.4 *M* NaCl at 44–48 min) showed TaqI activity. The negatively charged nucleic acids and anionic proteins are expected to bind to this anion chemistry. However, the HPLC output and protein analysis revealed that most of the protein (15 mg) did not adsorb onto the column but washed out during the adsorption stage and only 1.1 mg was obtained in the elution stage. This result indicates that the initial salt coming with the sonication buffer, i.e. NaCl, worked as the eluting agent and it was necessary to remove it. Therefore, 3 ml dialysed (against several changes of extract buffer for 4 h at 4°C), concentrated and filtered crude extract was loaded on to the HPLC anion-exchange column. The ratio of protein recovered (elution stage) to washed-out was 2 with dialysis compared to 0.07 without dialysis. Dialysis resulted in an efficient adsorption of proteins on the anion exchanger.

3.2. Comparison of gradient versus isocratic elution

In order to investigate the effect of the mode of elution on the purification of TaqI endonuclease, isocratic elution was replaced by gradient elution. A gradient table was set up by entering the operating parameters such as flow-rate, percentage of the buffers and the operation time. Following the wash with buffer (20 min adsorption stage), the column (Protein Pak DEAE 15 HR AP-1) was developed with a linear gradient of NaCl (80 ml total volume, 0.1–1 *M*) in extract buffer at a flow-rate of 1 ml/min. Fractions with TaqI activity appeared at adsorption stage between 6 and 12 min and at ca 0.4 *M* NaCl (elution with 0.38–0.44 *M* NaCl) between 15 and 20 min after the elution was started and at ca 0.6 *M* NaCl (elution with 0.58–0.62 *M* NaCl) (Fig. 1). When compared to isocratic operation, TaqI endonuclease was eluted 10 min earlier in gradient elution which prevents unnecessary dilution and long separation times. As can be seen from Fig. 1, the enzyme appeared within the two peaks eluted at 0.4 and 0.6 *M* NaCl in extract buffer possibly due to the binding to several ionogenic groups at once. In fact, this type of binding was reported for some globular

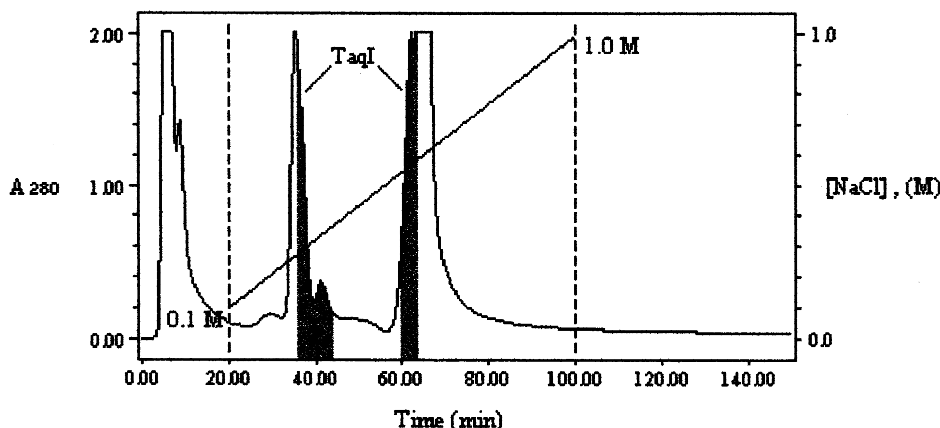


Fig. 1. HPLC output of single anion-exchange column purification using linear gradient elution [Protein Pak DEAE 15 HR AP-1 (100×10 mm)) with 3 ml sample volume.

proteins of molecular mass 50 000–100 000 and requires high salt concentrations (>0.5 M NaCl) to promote elution [10].

3.3. Effect of the slope of linear gradient

In order to further optimize the separation without changing the column length and flow-rate, the slope of the linear gradient is changed by setting the elution time between 30 and 80 min as another operating parameter. Table 1 shows the effect of the slope of the linear gradient for TaqI endonuclease elution on DEAE weak anion-exchange column [Protein Pak DEAE 15 HR AP-1 (100×100 mm)]. As the slope of the gradient decreased, the ionic strength at the peak position of the enzyme also decreased, i.e. to 0.3 *M* NaCl. The HPLC outputs showed that the resolution was better with 80 min elution. The highest purification factor was also obtained with the smallest slope of the linear gradient (80 min elution). The purification factor as a measure of the degree of purification was calculated as the ratio of the sum of the specific enzyme activities in fractions eluted to that initially loaded sample to the column.

The experiments on the effective loading capacity of the column showed a shift in peak position and a significant decrease in resolution with an increase in the amount of loaded protein from 10.72 to 21.27 mg (Fig. 2). The protein capacity of the anion-exchange column was given as 40 mg/ml of packed bed by the manufacturer and packed volume is approximately 8 ml. Taking 10% of the bed capacity as effective, at most 32 mg protein should be loaded into the column.

3.4. Cation-exchange column

The next step is to test whether cation-exchange chromatography will do better in resolving the target protein. Thus, 2 ml of cell extract was injected to the cation-exchange column (Protein Pak SP 8 HR) which has exactly the same protein binding capacity (40 mg/ml of packed bed) as the anion-exchange column. After pre-equilibration with extract buffer at 1 ml/min flow-rate, the column was washed with 3–4 column volumes of extract buffer (28 ml) to allow for the adsorption. Elution with 0.3, 0.4 and 0.5 *M* NaCl in extract buffer was performed between 28 and 58, 58 and 88 and 88 and 138 min, respectively. Column fractions were assayed for protein content and TaqI endonuclease activity as described in Section 2. Nucleic acids are expected not to bind to the cation-exchange column and are thus eliminated in the flowthrough and wash. However, the HPLC output reveals that the vast majority of 280 nm absorbing material including TaqI passed through the cation column unretained. SDS–PAGE analysis also confirmed the existence of TaqI in fractions 4–7 between 8 and 14 min (flowthrough). Therefore a dialysis step was again added prior to injection into the column. Dialysed crude extract (3 ml) was loaded on to the HPLC cation-exchange column. After 20 min of adsorption elution was performed by a linear gradient of 0.1–1 *M* NaCl in extract buffer for 80 min. TaqI endonuclease activity was observed both in flowthrough (ca 25% of loaded sample)and elution. But, 33.3% of the loaded enzyme was recovered in active fractions during elution with 0.1–0.25 *M* NaCl in extract buffer. The purification factor was 6.02. This result showed that cation-exchange column alone is not sufficient for the

Table 1
The effect of the slope of the linear gradient for TaqI endonuclease elution on weak anion-exchange column [Protein Pak DEAE 15 HR AP-1 (100×10 mm)] with 1 ml sample volume

Ionic strength (*M*)	Elution time (min)	Ionic strength at the TaqI peak(*M*)	Initial specific activity of loaded sample (U/μg protein)	Total specific activity of eluted fractions (U/μg protein)	Purification factor
0.1–1	30	0.55	0.090	0.697	7.83
0.1–1	45	0.42	0.131	0.908	6.93
0.1–1	80	0.31	0.103	0.846	8.21

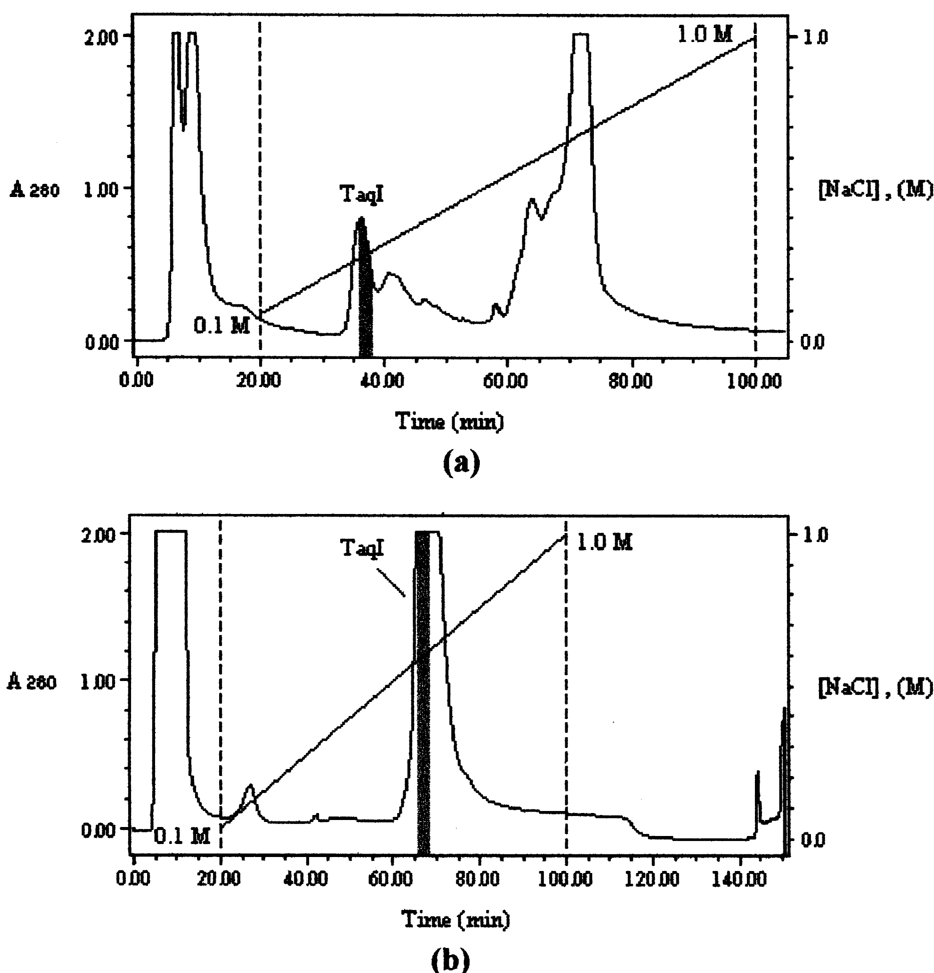


Fig. 2. Effect of sample volume on separation behavior of TaqI endonuclease [anion-exchange column using linear gradient elution, Protein Pak DEAE 15 HR AP-1 (100×10 mm)]: (a) 1-ml (10.72 mg protein) injection; (b) 2-ml (21.27 mg protein) injection.

effective separation of TaqI endonuclease from the cell extracts, as it was the case for anion-exchange column.

3.5. Development of a two-step HPLC purification protocol

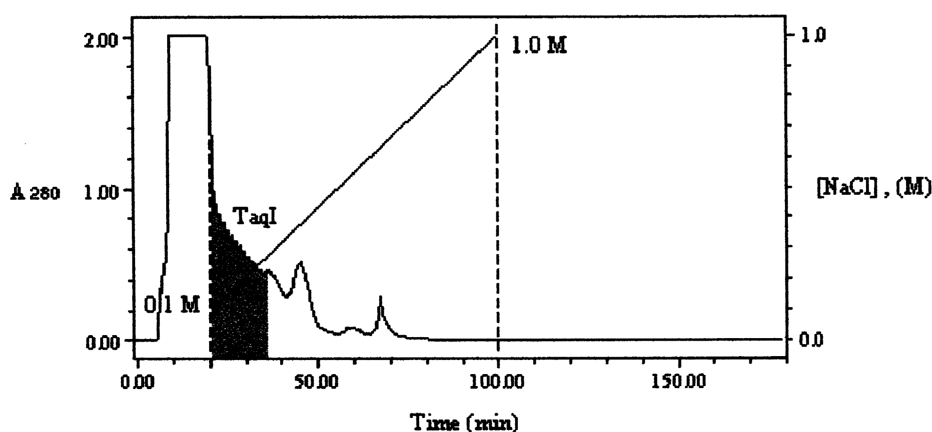
Depending on the enzyme and on the degree of purity required, it may be necessary to employ a second chromatographic step. Thus, a two-step purification protocol was developed where anion–cation, anion–anion and cation–anion columns were used in the given order. Sonicated, dialysed, filtered cell extract (3 ml) containing TaqI was loaded to the HPLC anion column that was equilibrated with extract buffer at 1 ml/min flow-rate. In each chromatographic run, adsorbed protein/enzyme was eluted using a linear NaCl gradient in extract buffer (0.1–1 *M*, 80 min). Fractions with partially purified enzyme were pooled, dialysed and filtered prior to application to the HPLC column in the second and final step. Active fractions were again collected and stored at −80°C in storage buffer. At the end of purification, ion exchangers were regenerated in a high salt concentration (1 *M* NaCl) to remove bound protein.

The results of HPLC experiments with two columns are summarized in Table 2. The total protein

Table 2
TaqI endonuclease recovery from the HPLC application

Column type arrangement	Protein recovery (%)	Specific activity of loaded enzyme (U/mg)	Total specific activity of TaqI (U/mg)	Purification factor
Anion–cation	0.32	164	2870	17
Anion–anion	13.91	169	1000	6
Cation–anion	1.18	166	4000	24

recovery is defined as the ratio of the final amount of protein (obtained from the second column) to the initial amount of protein loaded to the first column. In the two-column purification protocol, the overall protein recovery was lowest with the cation-anion HPLC system. However, the highest specific activity was obtained with the cation-anion HPLC system which resulted in 24-fold purification of TaqI endo-



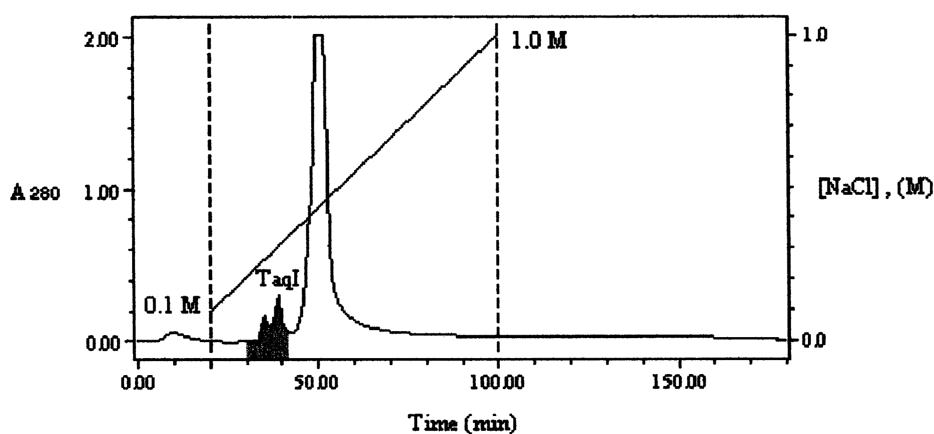


Fig. 3. HPLC output of two-column purification using linear gradient elution. (a) Cation-exchange column (Protein Pak SP 8 HR AP-1 (100×10 mm)); (b) anion-exchange column (Protein Pak DEAE 15 HR AP-1 (100×10 mm)).

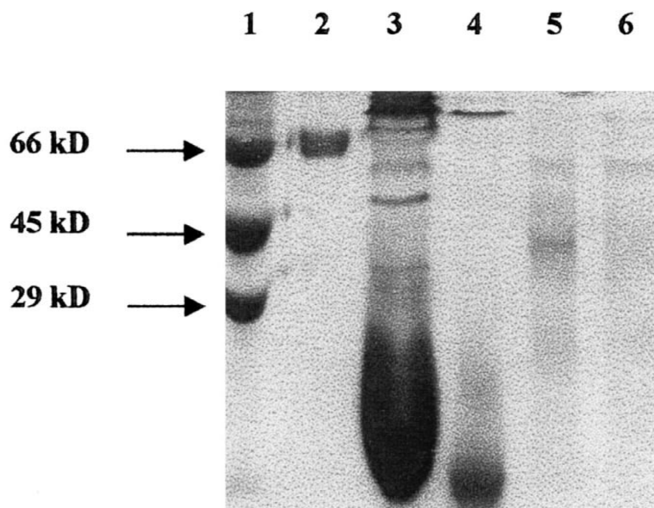


Fig. 4. SDS–PAGE analysis of the preparations. Lanes: (1) SDS molecular weight markers; (2) commercial TaqI endonuclease; (3) crude extract injected to the cation-exchange column; (4) active fractions pooled and injected to the anion-exchange column; (5,6) active fractions collected from the anion-exchange column. kD, kilodaltons.

nuclease. Elution profiles of the first and second columns of cation–anion system are presented in Fig. 3. SDS–PAGE indicated the presence of several bands together with the corresponding TaqI endonuclease band. A total of 4000 U TaqI endonuclease per mg protein were recovered from 2 g *Thermus aquaticus* cells using cation–anion HPLC columns sequentially. The classical methods reported in literature yielded 4000 U TaqI from 200 g cells with a five-step purification protocol and 8000 U from 50 g cells with a two-step protocol. HPLC, on the other hand, yielded 1900 U TaqI endonuclease from 2 g cells in 1 day. These results show that HPLC provides high efficiency and fast resolution and is therefore applicable to the enzyme purification as an alternative to conventional methods.

SDS–PAGE analysis of the HPLC-purified samples showed the presence of few contaminating proteins (Fig. 4). However, these samples were

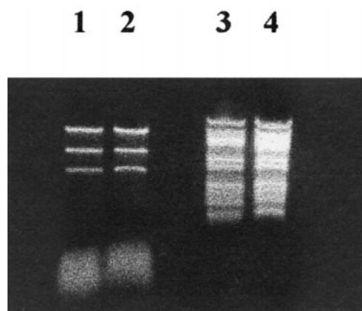


Fig. 5. Overdigestion assay of purified TaqI endonuclease. Lanes: (1) pUC18 DNA overdigested by commercial TaqI endonuclease; (2) pUC18 DNA overdigested by HPLC-purified TaqI endonuclease; (3) λDNA overdigested by commercial TaqI endonuclease; (4) λDNA overdigested by HPLC-purified TaqI endonuclease.

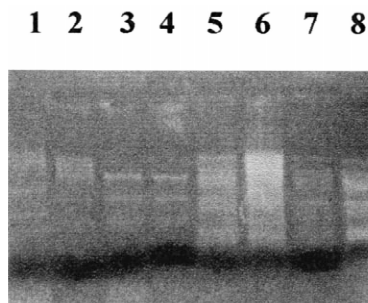


Fig. 6. Cut–ligate–recut assay of HPLC-purified TaqI endonuclease. Lanes: (1) ligation of pUC18 DNA (previously digested with commercial TaqI); (2) ligation of pUC18 DNA (previously digested with purified TaqI); (3) recut of pUC18 DNA with commercial TaqI; (4) recut of pUC18 DNA with purified TaqI; (5) ligation of λDNA (previously digested with commercial TaqI); (6) ligation of λDNA (previously digested with purified TaqI); (7) recut of λDNA with commercial TaqI; (8) Recut of λDNA with purified TaqI.

found to be free from endo- and exonucleases in overdigestion experiments (Fig. 5). The results of cut–ligate–recut assays also indicated the absence of other contaminants that would inhibit ligation or degrade termini (Fig. 6). Therefore, the preparations of the TaqI endonuclease purified from *Thermus aquaticus* by ion-exchange HPLC are suitable for use in molecular biology.

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