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Purification of industrial hydantoinase in one chromatographic step without affinity tag

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Abstract

Hydantoinase is used in industry as a biocatalyst for the production of optically pure D- or L-amino acids. Previously, homogeneous hydantoinase was obtained by multi-chromatographic purification procedures. Here, we reported a process that contained only a single chromatographic step to purify a recombinant hydantoinase to homogeneity. Hydantoinase from *Agrobacterium radiobacter* NRRL B11291 was expressed in *Escherichia coli*. The recombinant enzyme was purified following heat treatments, high concentration alcohol precipitation, and chelating Sephacel chromatography. The recombinant hydantoinase did not contain any affinity tags from the plasmid. This simplified procedure provided a convenient way to obtain hydantoinase in high yield (71%) and high purity. It should be very useful for further industrial application and for the study of the structure–function of hydantoinase. © 2003 Elsevier Science (USA). All rights reserved.

Hydantoinase (EC 3.5.2.2) is a zinc enzyme also known as dihydropyrimidinase, dihydropyrimidine hydrolase, dihydropyrimidine amidohydrolase, or imidase [1–7], which catalyzes a reversible ring-opening hydrolysis of hydantoin/pyrimidine and their 5'-monosubstituted analogues [8]. It is an industrial enzyme [9] used as a biocatalyst for the efficient production of optically pure D- or L-amino acids *via* the enantioselective hydrolysis of corresponding hydantoins [10–12]. These intermediates are widely used for the synthesis of semisynthetic antibiotics, peptide hormones, pyrethroids, and pesticides [12]. For example of these intermediates, D-*p*-hydroxyphenylglycine, is an important precursor used for the synthesis of semisynthetic cephalosporin and penicillin [11]. The preparation of D-*p*-hydroxyphenylglycine is mainly carried out in a two-step enzymatic reaction mediated by D-hydantoinase and carbamoylase [12]. Alternately, D-amino acids can be obtained by treating the *N*-carbamoyl-D-amino acids with an equimolar quantity of NaNO₂ (diazotation) in acidic process [13]. In any case, hyd-

antoinase is required to produce an optically active precursor.

Recently, one-step production of D-amino acids using whole cells of recombinant *Escherichia coli* has been developed [14–16]. The improvements of production of D-*p*-hydroxyphenylglycine have been reported to enhance the solubilization, overexpression level, and stringent regulation of hydantoinase [17–21]. Most known hydantoinases are thermostable and, therefore, are able to function at high temperature such as 60 °C or higher [13,22,23]. It appears that the growth temperature of *E. coli* could limit this bioconversion process if whole cell is to be used. To take advantage of the temperature as well as other factors, availability of hydantoinase *in vitro* seems necessary. Microbial hydantoinase has been purified in a homogeneous form from *Pseudomonas* [24], *Bacillus* [23], *Agrobacterium* [25], *Blastobacter* [26], and *Arthrobacter* [27]. However, multi-chromatographic and tedious purification procedures are required. With the increasing need for the understanding of hydantoinase structure and function [28–30], along with its industrial application [11], the research on hydantoinase has recently been proliferating. A simplified procedure for the purification of hydantoinase in high yield and high purity would be highly desirable.

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Experimental procedures

Materials

Chelating Sephacel (fast flow) was purchased from Pharmacia. Bis-Tris propane, PMSF,¹ alcohol, and phthalimide were purchased from Sigma (USA). Sodium chloride, sodium hydroxide, Tris-HCl, and zinc acetate were obtained from J.T. Baker (USA). All other chemicals were obtained commercially at the highest purity possible.

Bacterial strains and culture conditions

DNA manipulation was described before [15]. The plasmid (pHDT200) used in this study was also described previously [21]. The strain BL21 containing pHDT200 was employed for experimental work. To grow the cells, a flask culture (500 ml) was kept in an orbital shaker at 37 °C and 150 rpm. Luria-Bertani medium was used and was supplemented with ampicillin (50 µg ml⁻¹) when required for selective pressures. For inducing the cloned genes, 50 µM IPTG was added when the culture reached the mid-log growth phase. In order to obtain more soluble imidase for further purification, culture temperature was kept at 25 °C. After induction of IPTG, the culture was then allowed to grow for 20 h.

Enzyme assays

A rapid spectrophotometric assay [8] was used as the standard assay. Briefly, the decrease in absorbency at 298 nm was measured upon hydrolysis of phthalimide as the substrate at 25 °C. To start the reaction, the enzyme solution was added into 1 ml solution, containing 1 mM phthalimide and 100 mM Bis-Tris propane at pH 7.0. Under these conditions, a change in A_{298} of 2.26 represents the hydrolysis of 1 µmol of the substrate. The hydrolysis of phthalimide was monitored with a UV/VIS spectrophotometer (Hitachi U 3300).

Protein concentration

The protein concentration of enzyme solution was determined by A_{280} or BCA protein assay (Pierce, USA) using bovine serum albumin as a standard. For the homogeneous hydantoinase, 1 U A_{280} equals to 1.17 mg/ml hydantoinase based on the BCA protein assay.

Heat treatments of bacterial extract

The extract was heated at 45, 50, 55, 60, 65, or 70 °C for 10, 30, or 60 min to examine the stability of hydantoinase. The residual enzyme activity of heated extract was then determined by standard assay as described above.

Alcohol treatment of bacterial extract

Alcohol solution (95%) was directly added into the extract. The final concentration of alcohol was 10, 20, 30, 40, or 50%. The enzyme extract was incubated for 10 min, 30 min, or 60 min to examine stability of hydantoinase. The residual enzyme activity of alcohol treated extract was then determined by standard assay as described above.

Protein purification

The transformed cells (BL21 strain containing the plasmid as described above) were grown to mid-log growth phase and then induced by IPTG (50 µM) for 20 h. The cell pellet was collected by centrifugation at 20,000g for 10 min at 4 °C. All procedures for protein purification were conducted at 4 °C in a cold room or in an ice bath. The pH of buffers for enzyme purification refers to measurements taken at room temperature. An FPLC system (Pharmacia) and column (Pharmacia) were used for column chromatography.

Step 1: Extract. The cell pellet (about 3 g wet weight from 500 ml cell culture) was suspended in 10 ml buffer (10 mM Tris-HCl at pH 7.4 and 1 mM PMSF). Cells were disrupted with 550 Sonic Dismembrator (Fisher Scientific) and its microtip probe for six periods of 30 s each (2 s cycle pulses 10% power output), interspersed by cooling for 30 s in an ice bath. Then, the protein solution was collected by centrifugation at 20,000g for 20 min.

Step 2: Heat treatment I. The extract was collected and incubated in water bath at 45 °C for 1 h. The suspension was centrifuged at 10,000g for 10 min to remove the precipitate.

Step 3: Heat treatment II. The heated enzyme solution was cooled in the ice bath for 5 min and then was incubated in water bath at 65 °C for 5 min. Significant irreversible denaturation of hydantoinase may occur for longer incubation (10 min or more, Fig. 1). The suspension was centrifuged at 10,000g for 10 min to remove the precipitate.

Step 4: 40% alcohol treatment. Alcohol (95%) was directly added into the twice heat-treated enzyme solution with stirring. The ratio of 95% alcohol to enzyme solution was 2:3. The final alcohol concentration was 40% and the enzyme solution was incubated for 20 min at room temperature. Then, the enzyme solution was

¹ Abbreviations used: PMSF, phenylmethylsulfonyl fluoride; EDTA, (ethylenedinitrilo) tetracetic acid; Bis-Tris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane.

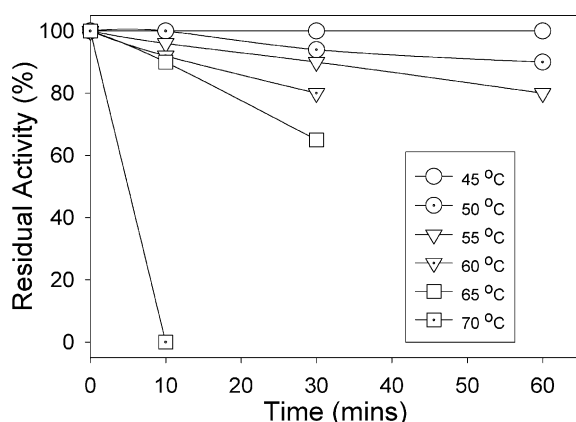


Fig. 1. Hydantoinase activity following heat treatment. The cell extract in Tris buffer (10 mM, pH 7.4) was heated at 45, 50, 55, 60, 65, or 70 °C. The enzyme activity of heated extract was then determined at 10, 30, or 60 min by standard assay described under Materials and methods.

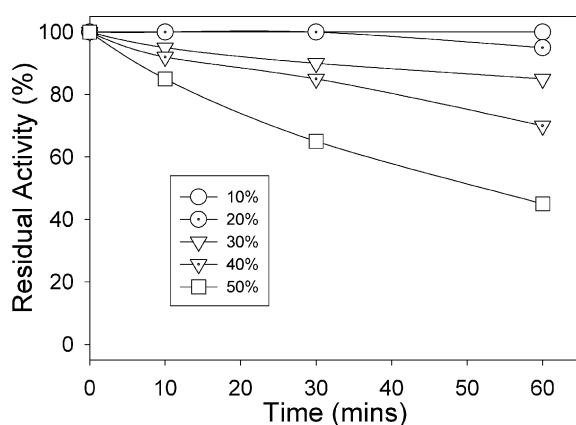


Fig. 2. Effect of alcohol treatment on hydantoinase activity. Alcohol (95%) was directly added into the extract (in Tris buffer, 10 mM, pH 7.4), and final concentration of alcohol was 10, 20, 30, 40, or 50%. The enzyme activity of alcohol treated extract was then determined at 10, 30, or 60 min by standard assay described under Materials and Methods.

diluted to contain 20% alcohol using Tris–HCl buffer (pH 7.4, 10 mM). Hydantoinase was found stable in 20% alcohol/buffer system in an hour by the standard assay (Fig. 2). The suspension was centrifuged at 10,000g for 10 min to remove the precipitate. At this time, the suspension was clear in typical experiments.

Step 5: Chelating Sephacel chromatography. The enzyme solution was applied to a column (1.6 × 10 cm) of chelating Sephacel (fast flow) that was treated with five times gel volume containing 0.2 M zinc acetate and then equilibrated with buffer A (20 mM potassium phosphate, 1 mM PMSF, and 0.5 M NaCl, pH 7). The loaded column was washed with 100 ml buffer A and the enzyme was eluted with a linear glycine gradient from 0 to 1 M with buffer A and buffer A plus 1 M glycine (total volume 500 ml). The active fractions were pooled. Approximately 10 mg purified hydantoinase (determined by BCA protein assay) was obtained per 0.5 L cell culture.

Protein purity and molecular weight determination

The purity and subunit molecular weight of hydantoinase were determined by SDS–PAGE. Gel electrophoresis was performed in a Mini-Protein II Electrophoresis Cell (Bio-Rad, USA). Protein was stained with Coomassie blue R-250 (National Diagnostics).

Results and discussion

The goal of the present project is to establish a convenient purification procedure and to obtain homogeneous hydantoinase without any affinity tag from the plasmid. Hydantoinase from *A. radiobacter* NRRL B11291 was expressed in soluble form and purified about 16-fold with 70% yield (Table 1). Based on its thermostability and stability in organic solvent (alcohol), we were able to accomplish our goal of using only one chromatographic step. The purified hydantoinase appeared to be homogeneous according to the criteria of SDS–PAGE (Fig. 4). Heat treatment has been used for the purification of hydantoinase [10,11,23]. Based on the thermostability of this enzyme as shown in Fig. 1, we developed a two-step heat treatment procedure to remove impurities and to obtain hydantoinase activity in high yield (Table 1). Alcohol precipitation was very useful for the purification of hydantoinase (Table 1). The stability of hydantoinase in alcohol is shown in Fig. 2. We found that hydantoinase was stable in 20% alcohol. The alcohol precipitation method successfully removed a fair amount of proteins (Fig. 4, lane 4). Two

Table 1
Summary of purification of bacterial hydantoinase^a

Step	Volume (ml)	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min/mg)	Yield (%)	Fold purification
Extract	14.7	1431	542	2.64	100	1.0
Heat treatment I (45 °C)	12.3	1306	339	3.85	91	1.5
Heat treatment II (65 °C)	11.2	1189	230	5.17	83	2.0
Alcohol precipitation	31.0	1152	59	19.6	80	7.4
Chelating Sephacel	2.4	1019	24	42.5	71	16.1

^a From 3 g wet weight of 0.5 L cell culture.

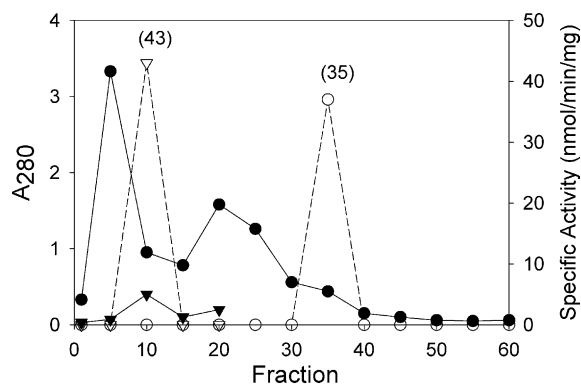


Fig. 3. Comparative study on chelating Sephacel and DEAE Sephacel chromatography. The extract after heat treatments and alcohol precipitation was directly applied into chelating Sephacel or DEAE Sephacel. The specific activity of hydantoinase is indicated in parentheses. Open symbols denote the specific activity of hydantoinase and closed symbols denote A_{280} of each fraction. Triangle symbols denote the results of purification using chelating Sephacel, and circular symbols denote that of using DEAE Sephacel. Active fractions (approximately 5 ml each) were pooled (fractions 8–12).

common chromatography resins used for the purification of hydantoinase were compared as shown in Fig. 3. Although both chromatography steps were found useful, the resulting specific activity of purified hydantoinase though chelating Sephacel was higher than that of DEAE Sephacel. We therefore report here using chelating Sephacel as chromatographic step instead of DEAE Sephacel and obtain a homogeneous enzyme (Fig. 4, lane 5). The complete procedure, including the expression and purification of hydantoinase, was summarized in Table 1 and Fig. 5.

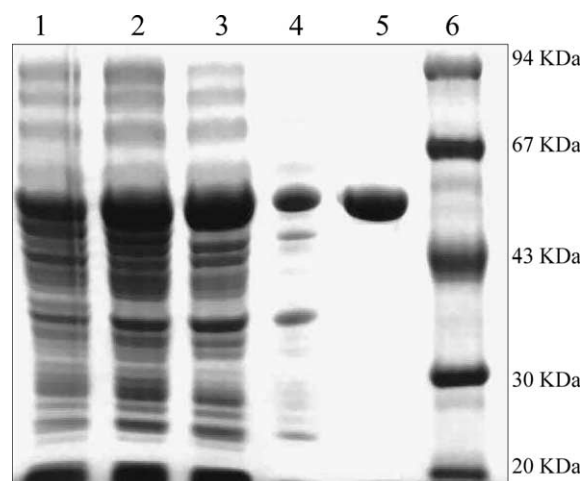


Fig. 4. The determination of molecular weight and purity of hydantoinase. SDS-PAGE (12%) of purification steps of *Agrobacterium radiobacter* hydantoinase. Lane 1, crude extracts (60 µg); lane 2, heat treatment I (60 µg); lane 3, heat treatment II (60 µg); lane 4, 40% alcohol precipitation (10 µg); lane 5, chelating Sephacel (10 µg); lane 6, protein standards (from Pharmacia) with size noted in kilodaltons: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa).

Single colony of transformed *E. coli*

- add ampicillin ($50 \mu\text{g ml}^{-1}$) in 0.5 L cell culture
- culture to mid-log growth phase (37°C)
- add IPTG ($50 \mu\text{g ml}^{-1}$)
- allow to grow for 20 hours (25°C)
- centrifuge

Induced bacterial pellet

- suspend in Tris buffer (10 mM, pH 7.4)
- add PMSF (1 mM)

Disrupt the suspended cells

- sonicate
- centrifuge

Heat Treatment

- (I) 45°C for 1 hour
- (II) 65°C for 5 mins
- centrifuge

Alcohol treatment

- 95% alcohol was directly added to suspension, and maintained at 40% for 20 mins
- dilute to 20% using Tris buffer (10 mM, pH 7.4)
- centrifuge

Chromatography

- chelating Sephacel
- pool all active fractions

Fig. 5. Expression and purification of recombinant hydantoinase from *Agrobacterium radiobacter* NRRL B11291.

Expression and purification of recombinant hydantoinase

Hydantoinase has been purified in a homogeneous form from *Pseudomonas* [24], *Bacillus* [23], bovine liver [2], calf liver [3], rat liver [4,8], pig liver [1,22] *Agrobacterium* [25], *Blastobacter* [26], and *Arthrobacter* [27]. Most purifications were conducted by three or four chromatographic steps. For the recent comparative study [31], hydantoinase from *Bacillus thermocatenulatus* GH2 was obtained in a homogeneous form by affinity purification following cleavage by factor Xa. Maltose binding protein (MBP) was fused at its N-terminus by simple end-to-end fusion. Although it is a convenient method to obtain purified hydantoinase, the specific activity of N-terminus affinity-tag-hydantoinase is lower than that of wild-type hydantoinase [31]. No enzyme activity was found when C-terminus affinity tag fused to hydantoinase (this study, data not shown). C-terminus affinity tag may affect the quaternary structure of hydantoinase because its C-terminus functions as tetramer structure core according to crystal structure information [28–30]. It may be important to express the

Table 2
Specific activity of hydantoinase^a

Substrate	λ (nm)	ϵ^b (mM ⁻¹ * cm ⁻¹)	Concentration (mM)	Specific activity ^c (μmol/min/mg)
Dihydrouracil	245	0.08	20	1.8 ± 0.1
Phthalimide	298	2.26	1	0.040 ± 0.003
Hydantoin	255	0.0049	400	3.0 ± 0.2
5-Leuciny-hydantoin	244	0.174	10	5.0 ± 0.2

^a Enzymatic activity was measured as described for the standard assay except that different substrates and buffer (100 mM Tris-HCl at pH 7.0) were used.

^b Extinction coefficient of each substrate was determined experimentally by direct measurement with a spectrophotometer.

^c Each value is the average of at least three measurements, which differ by less than 10%.

hydantoinase in its native form for the best enzyme activity. Distinct differences were observed when the recombinant cells were grown at various temperatures (25, 30, and 37 °C). The specific activity of the extracts of the cells (grown at 25 °C) was much higher than that of others. It is possible that overproduction of heterologous protein in *E. coli* results in the formation of biologically inactive aggregates, commonly referred to as inclusion bodies, deposited in bacterial cytoplasm [21].

Substrate specificity of the hydantoinase

Runser and Meyer have reported that hydantoinase purified from *Agrobacterium species* was without dihydropyrimidines (dihydrouracil and dihydrothymine) activity in different buffer systems and/or temperatures [25]. However, the lack of activity toward dihydropyrimidines was not confirmed in this study. As shown in Table 2, the recombinant hydantoinase purified from *A. radiobacter* NRRL B11291 uses several substrates including dihydrouracil. The K_m of dihydrouracil was unusually high compared to those of other related enzymes [3,4,8,26].

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