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Purification and Characterization of the D-Hydantoinase from *Bacillus Circulans*

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ABSTRACT

A D-hydantoinase (5,6-dihydropyrimidine amidohydrolase) was purified to homogeneity from *Bacillus circulans*. Purification of two hundred forty-three-fold was achieved with an overall yield of 12%. The relative molecular mass of the native enzyme is 212,000 and that of the subunit is 53,000. This enzyme is an acidic protein with an isoelectric point of 4.55. The enzyme is sensitive to thiol reagent and requires metal ions for its activity. The optimal conditions for the hydantoinase activity are pH 8.0–10.0 and a temperature of 75°C. The enzyme is the most stable in a pH range of 8.5–9.5 and up to 60°C. The enzyme is significantly stable not only at high temperatures but also on treatment with protein denaturant SDS. These remarkable properties are used for the purification procedure.

Index Entries: D-Hydantoinase; *Bacillus circulans*; thermostability; SDS-treatment.

INTRODUCTION

One of the most useful enzymatic reactions for the preparation of optically active amino acids is the hydantoinase reaction. The hydantoinase catalyze the hydrolysis of 5-substituted hydantoins to the enantiometrically pure *N*-carbamyl-amino acids and the latter are chemically or enzymatically converted into the corresponding optically active amino acids *(1)*. The hydantoinase enzyme is thought to be similar to dihydropyrimidinase (EC 3.5.2.2) and have been isolated from microbial, animal, and plant sources. However, hydantoinases for industrial applications are obtained mainly from microorganisms. The enzyme is classified as L-specific *(2)*, D-specific *(3)*, and nonspecific *(4,5)*. Among

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these, D-specific hydantoinases have been purified from several microorganisms belonging to the genera *Pseudomonas (6,7)* and *Agrobacterium (8)*. We purified D-hydantoinase to homogeneity from microorganism belonging to the genus *Bacillus*. Previuosly it was described the hydantoinase from *Bacillus brevis (2)*, but the enzyme was L-specific and only partial purification was achieved.

We have previously presented *(9)* some of the kinetic properties of the partial purified D-hydantoinase from *Bacillus circulans*. In this paper, we characterize the homogeneous enzyme from *Bacillus circulans* with emphasis on a comparison of its properties with other hydantoinases. We propose a new method for the purification of the hydantoinase based on the enzyme resistance to denaturant SDS.

MATERIALS AND METHODS

Chemicals

DL-5-substituted hydantoins were synthesized by the method of Henze and Speer *(10)*. Other chemicals used were of analytical grade and commercially available.

Culture Conditions

Bacillus circulans was cultivated aerobically at 40°C for 8–9 h in a medium (4 L, pH 7.5) containing 1.1% glicerole, 0.05% K_2HPO_4, 0.4% $KH_2PO_4 \times 3H_2O$, 0.05% $MgSO_4 \times 7H_2O$, 0.0045% $MnCl_2 \times 4H_2O$, 0.1% DL-5-(2-methylthioenthyl)hydantoin and 0.15% corn extract with a LKB fermenter (Sweden). The cells were collected in the late exponential phase by centrifugation for 15 min at 7000 g and 4°C, rinsed with distilled water and stored at –20°C.

Enzyme Activity Assay

The hydantoinase assay was performed with the colorimetric method described previously *(6)*. The reaction mixture contained, in a total volume of 1 mL, 90 μ*M* DL-5-substituted hydantoin, 90 μ*M* Tris/HCl, pH 8, 50 μ*M* $MnCl_2$ and, depending on the purity, 4–600 μg protein. After a 15–90 min incubation at 50°C, 1 mL samples were removed from the reaction medium and mixed with trichloracetic acid (0.25 mL, 12% w/vol) and dimethylaminobenzaldehyde solution (0.25 mL, 10% w/vol.) in 6*M* HCl and diluted with distilled water to 3 mL. The solution was centrifuged and the optical density measured at 420 nm. The concentration of ureidic acid was estimated from a calibration plot. Unless otherwise stated, all assays were performed in triplicate.

One unit of the enzyme activity was defined as the amount of the enzyme which produced 1 μ*M* of ureidic acid per min under the assay conditions.

Protein Assay

Protein was determined by the method of Bradford *(11)* with bovine serum albumin as a standard.

Enzyme Purification

All procedures were done at 0–5°C, and 5 m*M* Tris buffer, pH 8.5, containing 50 μ*M* $MnCl_2$ was used as the buffer throughout the purification unless otherwise noted.

Preparation of Crude Extract

The thawed cells were suspended in distilled water at a concentration of 100 mg/mL. The cells were disrupted ultrasonically at 22 kHz, pH 7.8–8.0, for a period of 5 min (ultrasound disintegrater model YZD-2, 220 V; Russia). Debris was removed by centrifugation for 30 min at 4°C and 20,000 g.

Thermal Treatment

The protein extract to which 50 m*M* of $MnCl_2$ had been added was incubated for 90 min at 60°C, pH 8.0. The precipitate was removed by centrifugation for 45 min at 4°C and 20,000g.

Sephadex G-50 Gel Filtration

The enzyme solution was passed through a Sephadex G-50 column (2.6 cm × 76 cm) equilibrated with the buffer. The enzyme was eluted at a flow rate of 4 mL/min and the active fractions were combined.

DEAE-cellulose Column Chromatography

The enzyme solution was applied to a DEAE-cellulose column (3.3 cm × 15 cm) equilibrated with the buffer. After the column had been washed with the same buffer, the enzyme was eluted at a flow rate of 20 mL/h with a linear gradient of 0.1–0.3*M* NaCl in 450 mL buffer. The active fractions, found at about 0.2*M* NaCl, were combined.

Bio-Gel TSK Phenyl-5PW HPLC (I)

The protein solution was saturated with sodium sulphate (40%) and split into two equal fractions. In two separate chromatographic runs, each sample was processed on a Bio-Gel TSK Phenyl-5PW column (0.75 cm × 75 cm), which had been equilibrated with the buffer containing 40% saturated sodium sulphate. The enzyme was eluted at a flow rate of 1 mL/min with a 60-min linear gradient of 40–0% saturated sodium sulphate. The active fractions resulting from the separate runs were combined.

Fractogel TSK HW-55 (F) Column Chromatography

The enzyme solution to which 0.5% SDS had been added was incubated for 60 min at 50°C and passed through a Fractogel TSK HW-55 (F) column (4.5 cm × 81 cm) equilibrated with the buffer containing 0.2*M* NaCl. The enzyme was eluted at a flow rate of 20 mL/h and the active fractions were combined.

Bio-Gel TSK Phenyl-5PW HPLC (II)

The enzyme solution was saturated with sodium sulphate (40%) and applied to a Bio-Gel TSK Phenyl-5PW (0.75 cm × 75 cm) column. The procedure was done as described above. The active fractions were combined and dialyzed for 24 h against 10 L of buffer. The purified enzyme was stored at –20°C.

Molecular Weight Determination

The molecular weight of the native enzyme was determined by the Sephadex G-200 column (1.6 cm × 83.5 cm) equilibrated with the 5 m*M* Tris buffer, pH 8.5, containing 0.1*N* NaCl and 0.02% (mass/vol) NaN_3. Gel-filtration chromatography under denaturating conditions was performed using HPLC system and a Ultropac TSK G 2000 SW column (0.75 cm × 60 cm) equilibrated with the 25 m*M* phosphate buffer, pH 6.5, containing 6*M* urea. The hydantoinase and standard proteins were incubated under denaturing conditions for 5–6 h at 25°C. For both types of chromatographies, the hydantoinase and standard proteins were chromatrographed individually. Protein standards were ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) (gel filtration calibration kit; Pharmacia Fine Chemicals, Sweden). The molecular weight of the subunits was also determined by SDS/PAGE in slab gels according to the method of Laemmli *(12)*. The stacking and resolving gels were 4 and 10% (mass/vol) acrylamide, respectively. Protein standards were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) (electrophoresis calibration kit; Bio-Rad; Hercules, CA). Native gel electrophoresis was performed in a 7.5% acrylamide gel. Gels were stained with silver nitrate.

Determination of the Isoelectric Point

Isoelectric focusing on 4% polyacrylamide gels containing 2% ampholyte and 10% glicerol was done with carrier ampholytes in the pH range of 4.0–6.5 (calibration kits for pI determinations using isoelectric focusing; Pharmacia).

Table 1
Purification of the D-Hydantoinase from *Bacillus circulans*[a]

Step	Total proteins (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
1. Crude extract	2326	423.7	0.18	1	100
2. Thermal treatment	684.8	348.8	0.51	2.8	82
3. Sephadex G-50	183.3	280.0	1.53	8.5	66
4. DEAE-cellulose	57.0	224.5	3.94	22	53
5. Bio-Gel TSK Phenyl-5PW (I)	13.2	139.2	10.5	58.5	32.8
6. Fractogel TSK HW-55(F)	2.2	58.1	26.4	146.6	13.7
7. Bio-Gel TSK Phenyl-5PW (II)	1.2	52.5	43.8	243.0	12.4

[a]Average values of ten separate batches.

RESULTS

Purification of the D-Hydantoinase

The results of the purification of the hydantoinase from *Bacillus circulans* are summarized in Table 1. Approximately 243-fold purification was achieved with an overall yield of 12.4%. The hydantoinase was purified to homogeneity by sequential application of physico-chemical treatments and chromatographies. Previously, it was reported *(13)* heat stability of the enzyme and we used this remarkable property for the purification. The protein solution was first heat-treated, then processed on gel-filtration, anion-exchange, and hydrophobic-interaction chromatographies. We found that the enzyme is stable in the presence of SDS, which causes com-

Fig. 1. SDS-PAGE analysis of the gel-filtration on Fractogel column of the D-hydantoinase. 1, gel-filtration without SDS-treatment; 2, gel-filtration after SDS-treatment.

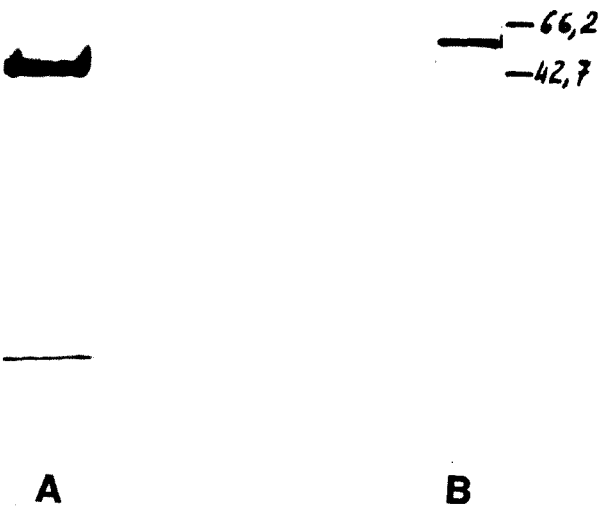

Fig. 2. Gel electrophoresis of the purified D-hydantoinase. **(A)** Native gel electrophoresis; **(B)** SDS gel electrophoresis. Protein standards: bovine serum albumin (66.2 kDa) and ovalbumin (42.7 kDa).

plete denaturation of ordinary proteins. It was determined that enzyme incubation with 0.5% SDS at 50°C for 60 min is the most effective for the fractionation on Fractogel column (Fig. 1). The last step, repeated hydrophobic-interaction chromatography, yielded electrophoretically homogeneous preparation (Fig. 2). Hydrophobic-interaction chromatography was used twice not only as an effective step of D-hydantoinase purifi-

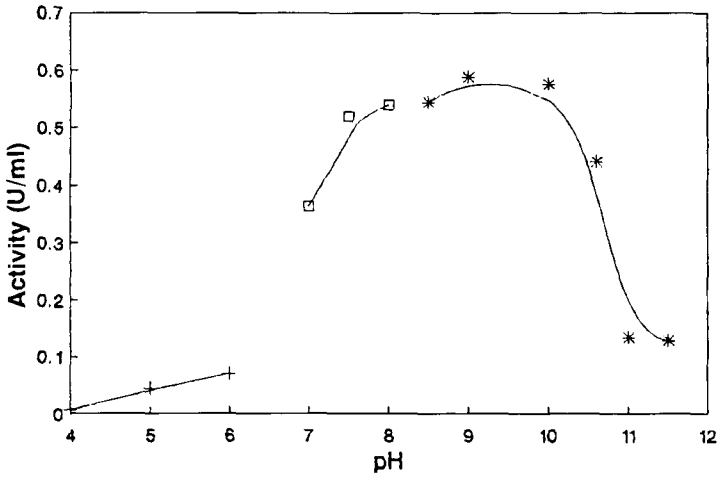

Fig. 3. Effect of pH on the activity of the D-hydantoinase. The enzyme activity was assayed under the standard conditions exept that the following 100 m*M* buffers were used: acetate buffer pH 4.0–6.0 (+), potassium phosphate buffer pH 7.0–8.0 (□), glycine-NaOH buffer pH 8.5–10.5 (✳).

cation, but also as a concentration method of the active fractions (approx 10-fold concentration effect was achieved).

The purified enzyme catalyzed the hydrolysis of D,L-5-(2-methylthioethyl)hydantoin to *N*-carbamyl-D-methionin at a rate of 43.8 µmol/min/mg under the assay conditions described in Materials and Methods.

Criteria for Purity

The purified enzyme gave one band on gel electrophoresis under both denaturing and nondenaturing conditions on gels having different concentrations of cross-linking agent. Furthermore isoelectric focusing yielded also one band for protein at a corresponding pI value of 4.55.

Molecular Weight and Subunit Structure

On a calibrated column of Sephadex G-200, the M_r of the native enzyme was estimated to be about 212,000. The subunit M_r was approximately 53,000 when determined by SDS/PAGE and 54,000 when determined by gel-filtration chromatography under denaturing conditions. From all these results it was suggested that the hydantoinase from *Bacillus circulans* is a homotetrameric protein of native M_r close to 212,000 and subunit M_r close to 53,000.

Effect of pH and Temperature on the Enzyme Activity

The hydantoinase has its maximum activity at pH 8–10 with D,L-5-isopropylhydantoin as a substrate (Fig. 3). The enzyme was active under alkaline conditions. Changes of the enzyme activity with temperature was

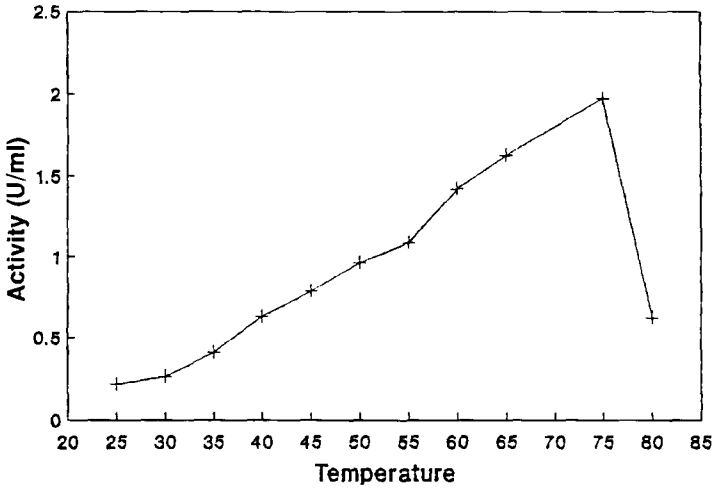

Fig. 4. Effect of the temperature on the activity of the D-hydantoinase. Assays were performed at various temperatures under the standard assay conditions.

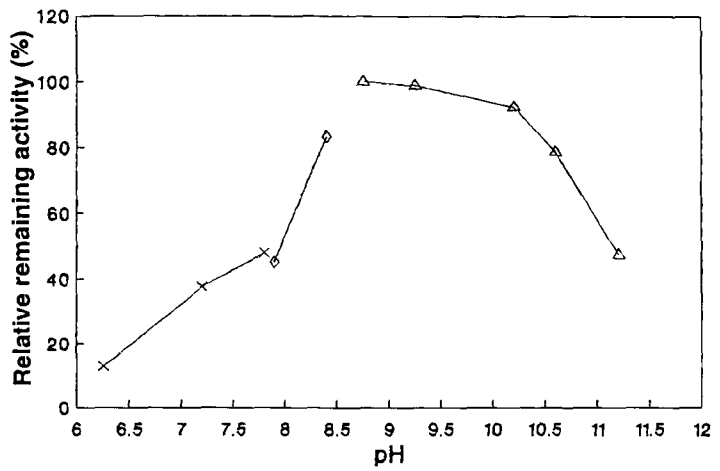

Fig. 5. Effect of pH on the stability of the D-hydantoinase. Buffers used were as follows: 100 m*M* potassium phosphate pH 6.0–8.0 (X), 100 m*M* Tris-HCl pH 8.0–8.5 (◇), 100 m*M* glycine-NaOH pH 8.5–11.0 (Δ). The relative remaining activity is expressed as a percentage of the maximum remaining activity attained under the experimental conditions.

investigated with D,L-5-isopropylhydantoin as a substrate. The optimum temperature was found to be about 75°C (Fig. 4).

Stability of the Enzyme

The enzyme was incubated at variuos pH for 90 min at 50°C, and remaining activities were assayed under the standard assay conditions. The enzyme was most stable in a pH range of 8.5–9.5 (Fig. 5). The enzyme

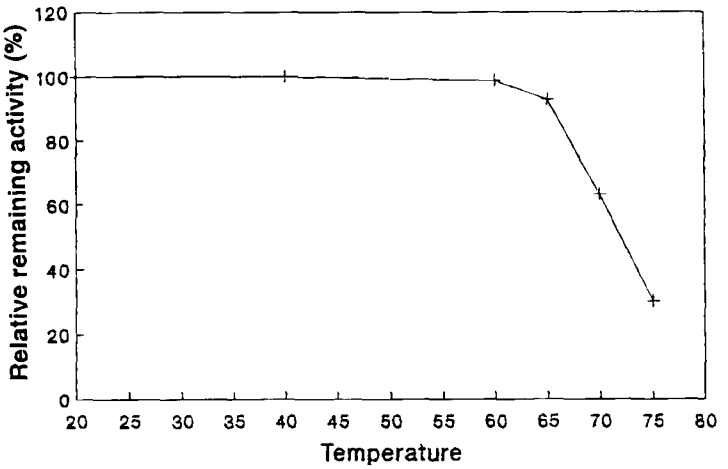

Fig. 6. Effect of temperature on the stability of the D-hydantoinase.

was incubated at various temperatures for 90 min, pH 8.5, and the remaining activities were assayed under the standard assay conditions, The enzyme was stable up to 60°C (Fig. 6).

The hydantoinase retained its full activity in 5 m*M* Tris buffer, pH 8.5, containing 50 μ*M* $MnCl_2$ at 4°C approximately one month, about 50% of initial activity were left after 6 mo storing at –20°C.

Inhibition

Some compounds were examined as to their inhibitory effects on the enzyme activity, with DL-5-(2-methylthioethyl)hydantoin as substrate. The enzyme solution to which 1 m*M* of inhibitory had been added was incubated for 60 min at 40°C and the remaining activities were assayed under the standard assay conditions, without 50 μ*M* $MnCl_2$. A sulfhydryl reagent, *p*-chloromercuribenzoate, inhibited the reaction by 100% suggesting that sulfhydryl groups of the enzyme are involved in its activity. Under the experimental conditions metal chelators such as EDTA and *o*-phenantroline caused inconsiderable inhibition by 11.5% and 12.4%, respectively.

5,5-dimethylhydantoin is not hydrolysed. It was reported *(8)* that 5,5-disubstituted hydantoins are competitive inhibitors of the hydantoinase from *Agrobacterium* sp. Though, in our paper, specific hydantoinase inhibition was not studied, but 5,5-dimethylhydantoin could probably be competitive inhibitor of the hydantoinase from *Bacillus circulans*.

The ability of the enzyme to produce only *N*-carbamyl-D-amino acids from the corresponding DL-5-hydantoins was investigated in the laboratory of Biocatalytic Processes (Institute of Biotechnology "Fermentas", Vilnius, Lithuania).

Table 2
Substrate Specificity of the D-hydantoinase from *Bacillus circulans*[a]

Substrates	V_m (μmol/min/mg)	K_m (mM)
Hydantoin	1.673	105.4
D,L-5-methylhydantoin	0.426	27.8
D,L-5-propylhydantoin	0.448	25.6
D,L-5-isopropylhydantoin	0.135	17.2
D,L-5-butylhydantoin	0.680	12.8
D,L-5-isobutylhydantoin	0.432	4.9
D,L-5-(2-methylthioethyl)hydantoin	0.678	40.7
D,L-5-benzylhydantoin	0.197	6.8
D,L-5-phenylhydantoin	1.640	58.8
Allantoin	0.085	5.8
5,5-dimethylhydantoin	-	-

[a]The K_m and V_m values were calculated from Lineweawer-Burk plots.

Substrate Specificity

The ability of the purified hydantoinase to catalyze hydrolysis of various DL-5-substituted hydantoins was investigated and kinetic parameters are shown (Table 2). The reaction medium was incubated for 180 min under standard assay conditions (*see* Materials and Methods) varying substrate concentration.

The hydrolysis of DL-5 substituted hydantoins having aliphatic side chain depends on the lengh and branching of the chain. The DL-5-substituted hydantoin with the longest chain (DL-5-butylhydantoin) is the most efficiently hydrolyzed, branching of the chain decreases the rate of the reaction, for example, V_m values for DL-5-propylhydantoin and DL-5-isopropylhydantoin are 0.448 μmol/min/mg and 0.135 μmol/min/mg, respectively. The K_m values suggested that the enzyme has higher affinity for DL-5-substituted hydantoins having long and branching aliphatic chain, for example, K_m values for DL-5-methylhydantoin and DL-5-isobutylhydantoin are 27.8 m*M* and 4.9 m*M*, respectively.

Hydantoin and DL-5-phenylhydantoin are the most easily hydrolysed compounds, but their K_m values are the highest. On the other hand, allantoin is weakly reactive, but its K_m value is low. Normal hyperbolic kinetics were observed with all compounds tested.

Effect of Metal Ions on Enzyme Activity

After the enzyme solution had been preincubated with EDTA at a concentration of 10 m*M* at 20°C for 60 min, it was dialyzed for 4–5 d against 4 × 10 L 5 m*M* Tris buffer, pH 8.5. The activity of the hydantoinase

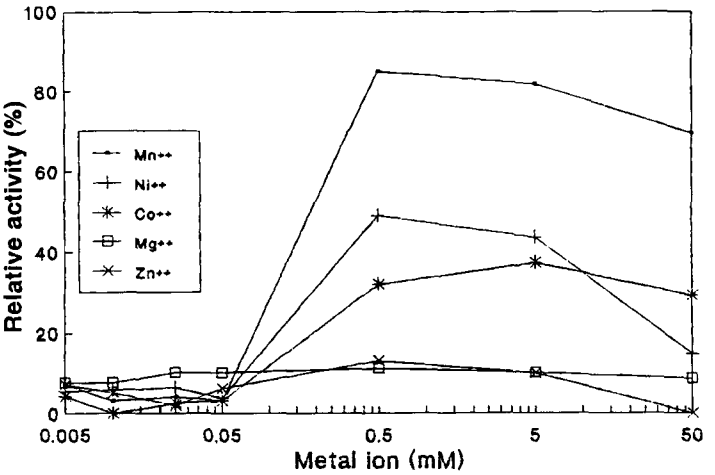

Fig. 7. Effect of metal ions on the activity of the D-hydantoinase. The relative activity is expressed as percentages of the initial activity of the native enzyme.

was considerably depressed (approx 90%). The dialyzed enzyme was incubated with different metal ions at concentrations ranging over 0.005–50 m*M* for 20 h at 20°C and the activity was assayed under the standard conditions, varying the concentrations of metal ions. It was found (Fig. 7) that Mn^{+2}, Ni^{+2}, and Co^{+2} enhanced the enzyme activity to 85, 49, and 32% of the initial activity, respectively. Zn^{+2} and Mg^{+2} did not have significant effect on the enzyme activity. Thus, our investigations indicate that the divalent metal ions are required for the catalytic activity of the pure hydantoinase.

Effect of Metal Ions on Thermal Treatment

Thermal treatment was used for the enzyme purification. It was investigated influence of metal ions on the efficiency of the thermal treatment. The protein solution was heat-treated as described in Materials and Methods with different metal ions. The results are summarised in Table 3.

It was found that the enzyme was stabilized by addition of Mn^{+2}, Ni^{+2}, and Co^{+2} ions. Other divalent metal ions tested descreased yield and purification-fold of the procedure.

DISCUSSION

D-hydantoinase was purified to homogeneity from *Bacillus circulans* and exibited a remarkable stability during the purification procedures. Our results concerning the molecular mass and subunit structure of the enzyme are in good agreement with the data determined for the hydantoinases from *Pseudomonas striata (6), Pseudomonas fluorescens (7),* and *Agrobacterium* species IP I-671 *(8).* All microbial D-hydantoinases are homotetramers of M_r 190,000–250,000.

Table 3
Effect of Metal Ions on the Thermal Treatment of the D-Hydantoinase

Metal ions	Purification (-fold)	Yield (%)
-	1.7	45.5
Mn^{+2}	2.9	78.5
Ni^{+2}	2.8	40.4
Co^{+2}	2.1	37.5
Ca^{+2}	1.4	28.4
Mg^{+2}	1.05	12.1
Sn^{+2}	0.83	17.6
Fe^{+2}	0.80	16.6
Ba^{+2}	0.6	9.9
Zn^{+2}	0.2	2.9

Concerning the catalytic properties of the enzyme, we have shown that the hydantoinase has the maximum activity at pH range of 8–10.0. These data agree well with the pH optima obtained for partial purified *(3,13,14)* and homogeneuos *(6)*. D-hydantoinases. The optimum temperature of the enzyme from *Bacillus circulans* is significantly higher than the temperature optima of hydantoinases mentioned above.

It was reported *(13)* about heat stability of the enzyme from mesophilic *Agrobacterium* sp. The enzyme purified from mesophilic *Bacillus circulans* exibits remarkable heat stability too. The partial purified hydantoinase from this microorganism *(9)* retained its full activity on heating at 40°C for 8 h and about 75% of the original activity after heating at 62.5°C for 8 h. We have found that divalent metal ions, especially Mn^{+2}, increase stability of the enzyme during heat treatment. In addition, Mn^{+2} ions stabilize the enzyme during all purification steps.

Microbial hydantoinases from *Pseudomonas* species *(6,14)* and from *Agrobacterium* sp. *(8)* require divalent metal ions for their catalytic activity. On the other hand, Moller et al. *(3)* reported that the activity of D-hydantoinase from *Arthrobacter crystallopoietes* is not influenced by metal ions. Our investigations indicate that the activity of the *Bacillus* enzyme is stimulated by Co^{+2}, Ni^{+2}, and especially by Mn^{+2} ions. We have observed a true metallo dependence and suggested that the hydantoinase is metalloenzyme with tightly bound metal ion. The inhibition effect with thiol-blocking reagent are in good agreement with the observations of homogeneous *(6,8)* and partial purified hydantoinases *(3)*, proving a role of sulfhydryl group in the catalytic process.

The D-hydantoinase resistance to denaturant SDS is the most remarkable property of the enzyme. The enzyme was not inactivated at all when incubated with 5% SDS at 50°C for 1 h (data not shown). Previously, it was

reported that some proteases, such as trypsin *(15)*, V8 protease *(16)*, lysylendopeptidase *(17)*, and carboxypeptidase B *(18)* retain their proteolytic activities in the presence of SDS. However, in contrast to D-hydantoinase, these proteases retain the full activity in the presence of up to 0.1–0.2% SDS *(16,17)*. Only one report was found *(19)*, which states stability of thermostable dipeptidase from *Bacillus stearothermophilus* at high SDS concentrations. The dipeptidase retains about 86% of its activity after incubation with 5% SDS at 55°C for 1h. But this property of the enzyme was not further discussed or applied for the purification procedures.

Extremely high resistance of D-hydantoinase to SDS is used to its purification without loss of the activity. It is obvious that during SDS treatment (*see* Results) protein complexes that have not previously been resolved are dissociated and successfully separated by subsequent gel-filtration on Fractogel column (Fig. 1). From the native gel electrophoresis and gel-filtration elution profiles (data not shown) it was concluded that D-hydantoinase was not dissociated into subunits during treatment with SDS. So the reasons of the surprising stability of D-hydantoinase at high concentrations of SDS require further study.

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