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Purification of a D-hydantoinase using a laboratory-scale Streamline phenyl column as the initial step

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Abstract

A D-hydantoinase from *Thermus sp.* was overexpressed in *Escherichia coli* and purified to homogeneity for subsequent crystallization. The purification was performed with hydrophobic interaction chromatography as the capture step followed by anion-exchange chromatography and gel permeation chromatography as intermediate purification and polishing steps, respectively. The hydrophobic interaction step was done in fluidized bed mode in a laboratory-scale Streamline column made from conventional laboratory equipment. The whole purification protocol could be finished within one day. The purified enzyme crystallizes. The crystals are suitable for X-ray protein structure analysis and diffract to at least 2.3 Å resolution. Complete data sets have been measured up to 2.6 Å resolution. The X-ray structure is currently being solved.

Keywords: Purification; Enzymes; D-Hydantoinase

1. Introduction

Hydantoinases (EC 3.5.2.2) belong to the group of cyclic amidases (EC 3.5.2). They hydrolyze one amide bond in five- or six-membered cyclic diamides. A number of bacterial hydantoinases with different stereo selectivity and substrate specificity have been described in the literature [1] (review). Most of them have D-enantio selectivity, whereas few act on L-hydantoins or have no enantio selectivity. Since the late 1950s it has been proposed that hydantoinases are the microbial counterpart of mammalian dihydropyrimidinases [2]. The sequence identity between members of both groups and the D-hydantoinase from *Thermus sp.* is in the range of 35–45%. To the other members of the cyclic amidases (allantoinases, dihydroorotases, ureases) there is a significant, however lower sequence homology [3,4] suggesting a common fold.

The biological function of hydantoinases still remains unclear. Due to the homology of dihydropyrimidinases on the level of substrate and sequence, hydantoinases are assumed to take part in the catabolic degradation of pyrimidine. Some of the hydantoinases are used technically in combination with carbamoylases for the production of D-amino acids or non-proteinogenic L-amino acids [5] (review).

Until now all structural information of hydantoinases is based on the sequence [3,4], chemical modifications [6], site directed mutagenesis [4], and reaction kinetics [7]. We are currently solving the three-dimensional X-ray structure of a D-hydantoinase from *Thermus sp.* [8]. The three-dimensional structure is expected to help in understanding the functionality and reaction mechanism and will give a

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starting point for rational protein design to alter the substrate specificity of the enzyme. Due to the high sequence homology, the tertiary structure of the D-hydantoinase can serve as a model for structure determination of other cyclic amidases by the molecular replacement technique used in protein X-ray crystallography.

During the purification the hydrophobicity, charge and size of the enzyme have been exploited. Streamline hydrophobic interaction chromatography in a laboratory scale apparature made from conventional columns was chosen as the capture step and also lead to some initial purification. Anion-exchange chromatography was used as an intermediate purification step and gel permeation chromatography as the polishing step. The optimized purification protocol could be finished within one day.

2. Experimental

2.1. Reagents and equipment

All reagents used for expression and chromatography were of analytical grade. They were purchased from Biomol (Hamburg, Germany; IPTG, Tris), Boehringer Mannheim (Mannheim, Germany; ampicillin), Degussa (Hanau, Germany; MTEH), Fluka (Steinheim, Germany; ammoniumsulphate), GibcoBRL (Paisley, UK; casein hydrolysat pepton 5), Merck (Darmstadt, Germany; HCl, $MnCl_2$, NaCl, *p*-dimethylamino-benzaldehyde, trichloroacetic acid) and Sigma (Steinheim, Germany; leupeptin, pepstatin A, PMSF, select yeast extract). The chromatographic steps were done at 283 K with an FPLC-system (Amersham Pharmacia Biotech, Uppsala, Sweden) equipped with: controller LCC-500 plus, pump P-500, UV-monitor UV-M, conductivity detector and fraction collector FRAC 100. All columns, chromatography media and electrophoresis equipment were also from Amersham Pharmacia Biotech. All buffers were prepared by solving the substances in water and adjusting the pH at room temperature with HCl or NaOH, respectively. The chromatographic mobile phases were sterile filtered (0.2 μm; cellulose acetate filter, Satorius, Göttingen, Germany).

2.2. Expression

The expression was done at 310 K in *E. coli* BL21 cells (Amersham Pharmacia, Uppsala, Sweden). The transcription of the gene for D-hydantoinase is under control of the IPTG-inducible T5-promoter and is performed by the endogenous *E. coli* RNA-polymerase. One ml of an overnight preculture in Luria broth (LB) supplemented with 150 μg/ml ampicillin was used to inoculate 1 l of the same medium additionally supplemented with 0.1 m*M* $MnCl_2$. The cells were grown to an OD_{600} of 0.8 after which they were induced with 0.1 m*M* IPTG. After 3 h induction the cells were kept for 20 min on ice and harvested by centrifugation at 5900 *g* for 25 min. The cells were resuspended in 30 ml 150 m*M* NaCl, 50 m*M* Tris/HCl pH 8.0 and kept for further use at 253 K.

2.3. Cell lysis

The frozen cells were thawed on ice after adding a protease inhibitor cocktail (35 μg/ml PMSF, 0.7 μg/ml pepstatin A and 0.5 μg/ml leupeptin). For lysis the cells were passed three times through an ice cold French Pressure cell (SLM-Aminco with FA-030; SLM-Instruments, Rochester NY, USA) operating at an internal pressure of 170 MPa.

2.4. Hydrophobic interaction chromatography

The hydrophobic interaction chromatography was performed with 45 ml Streamline phenyl sepharose resin filled in a C 16/40 column with one adaptor at the top. With the adaptor the volume could be varied from 45 ml in compressed mode to 75 ml in expanded mode. The unclarified lysate was mixed with the same volume of 1 *M* ammoniumsulphate, 50 m*M* Tris–HCl pH 8.0, 0.1 m*M* $MnCl_2$ and loaded in upward flow at 3.5 ml/min (1.7 cm/min) on the column. The column was washed in expanded mode at the same flow rate with 500 m*M* ammoniumsulphate, 50 m*M* Tris–HCl pH 8.0, 0.1 m*M* $MnCl_2$ until the absorbance at 280 nm had decreased again. Then the bed was allowed to settle, the adapter at the top was lowered manually to the top of the compressed bed and the flow direction was changed to downwards. For washing and elution a step gradient

with decreasing ammoniumsulphate concentrations (500, 250 and 0 m*M* ammoniumsulphate was performed. Active fractions were pooled and desalted in the following step.

2.5. Desalting

The active pool from the previous step (45 ml) was desalted in three fractions with a HiPrep 26/10 desalting column at 5 ml/min (1 cm/min) in 50 m*M* Tris–HCl pH 8.0, 0.1 m*M* $MnCl_2$.

2.6. Anion-exchange chromatography

The desalted eluate from the Streamline column was loaded on a MonoQ HR 10/10 column at a flow rate of 2 ml/min (2.5 cm/min). The column was washed with 20 m*M* Tris–HCl pH 7.5, 0.1 m*M* $MnCl_2$. A segmented gradient with increasing NaCl concentrations was performed in the same buffer and at the same flow rate. The segmented gradient consisted of the following steps: step from 0 to 125 m*M* NaCl, shallow linear gradient from 125 to 160 *M* NaCl, two steps at 160 and 195 m*M* NaCl and a steep linear gradient to 500 m*M* NaCl. The active peak was pooled and used for gel permeation chromatography.

2.7. Gel permeation chromatography

The eluate from the anion-exchange column was concentrated to 1 ml by ultrafiltration (centriplus 50; Amicon, Beverly, USA). Gel permeation chromatography was performed with Superdex 200 prep grade resin in a XK 70/16 column in 150 m*M* NaCl, 20 m*M* Tris–HCl pH 8.0, 0.1 m*M* $MnCl_2$ at a flow rate of 0.5 ml/min (0.25 cm/min).

2.8. Assay for hydantoinase activity

The method is described in [10] and has been modified. In brief: 200 μl 40 m*M* MTEH, 100 m*M* Tris–HCl pH 8.0 were mixed with 50 μl enzyme solution and incubated for 10 min at 310 K. The reaction was stopped by addition of 62.5 μl 12% (w/v) trichloroacetic acid followed by the addition of 62.5 μl Ehrlich reagent (10% (w/v) *p*-dimethylamino-benzaldehyde in 12% (w/v) HCl) and 375 μl water. After centrifugation (15 000 *g*, 2 min) the concentration of *N*-carbamoyl-methionine was determined at 435 nm.

2.9. Analysis

Protein concentration was determined by the bicinchinoic acid (BCA) method using the kit from Pierce (Pierce, Rockford IL, USA) and bovine serum albumin (BioRad, München, Germany) as the standard.

Analytical SDS–PAGE, native PAGE and isoelectric focusing PAGE were done with 60 ng protein on the Phast System. For SDS–PAGE and native PAGE 8–25% gradient gels, for isoelectric focusing PAGE pH 3–9 gels were used. The gels were silver-stained following standard protocols. For SDS–PAGE the marker kit combithek (Boehringer Mannheim, Mannheim, Germany) was used which contains the following proteins: M_r 170 000 α-macroglobuline, 116 000 β-galactosidase, 85 200 fructose-6-phosphate-kinase, 55 500 glutamate-dehydrogenase, 39 200 aldolase, 26 600 triosephosphate-isomerase, 20 100 trypsine-inhibitor, 14 300 lysozyme.

Dynamic light scattering was done at protein concentrations of 1.5 mg/ml with sets of 35 measurements at 829.6 nm (DynaPro 801, Protein Solutions, Charlottesville VA, USA). The data were interpreted with the Dynamics software provided by the manufacturer. The molecular weight was calculated from the hydrodynamic radius using the curve model for globular proteins.

MALDI-TOF mass spectrometry was performed on a Biflex III (Bruker, Bremen, Germany) using sinapinic acid as the matrix and bovine serum albumine as the standard for molecular weight determination.

3. Results

3.1. Chromatography

The D-hydantoinase from *Thermus sp.* was purified according to the protocol described above in the Experimental section. The results are summarized in Table 1. The total yield of hydantoinase activity was

Table 1
Summary of the chromatographic steps

Step	Activity [nkat]	Yield	Protein [mg]	Spec. activity [nkat/mg]	Purification factor
Cell lysate	75 500	100%	250	300	1.0
Streamline	65 500	86%	41	1600	5.3
Desalting	62 000	82%	38	1630	5.4
Anion exchange	41 000	54%	15	2730	9.1
Gel permeation	28 800	38%	10	2880	9.6

38% in relation to the crude extract, the final purification factor was 9.6.

On the chromatograms of the hydrophobic interaction step (Fig. 1), the anion-exchange step (Fig. 2) and the gel permeation step (Fig. 3) the peaks that show D-hydantoinase activity and the pooled fractions are marked. During the hydrophobic interaction chromatography the D-hydantoinase elutes in the step from 250 to 0 m*M* ammoniumsulphate. In the anion-exchange step the D-hydantoinase elutes in the sharp peak at 175 m*M* NaCl, in the gel permeation step in the first peak at 70 ml.

3.2. Analysis

The SDS–PAGE with samples of each purification step is shown in Fig. 4. The D-hydantoinase shows an apparent molecular weight of approximately 50 000. The native PAGE and the isoelectric focussing PAGE are shown in Figs. 5 and 6, respectively.

The MALDI-TOF spectrum is shown in Fig. 7. Quasimolecular ions with one (M_r=50 589) and two (M_r=25 274) positive charges are marked. This is in correspondance with the molecular weight calculated from the sequence (M_r=50 675). The broad peak at

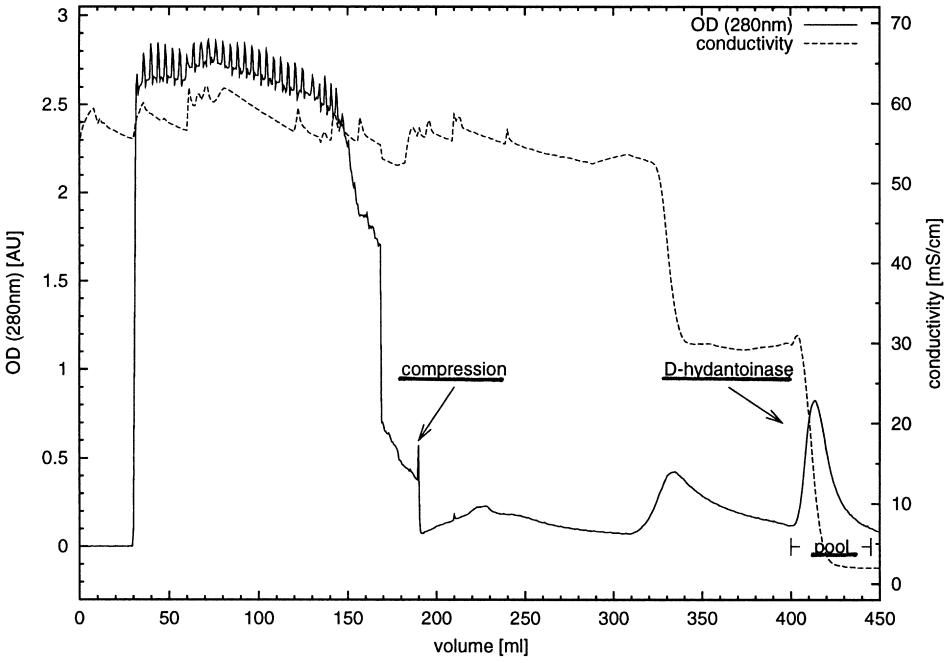

Fig. 1. Chromatogram Streamline hydrophobic interaction chromatography; the active peak, the pooled fractions and the compression of the column are labelled.

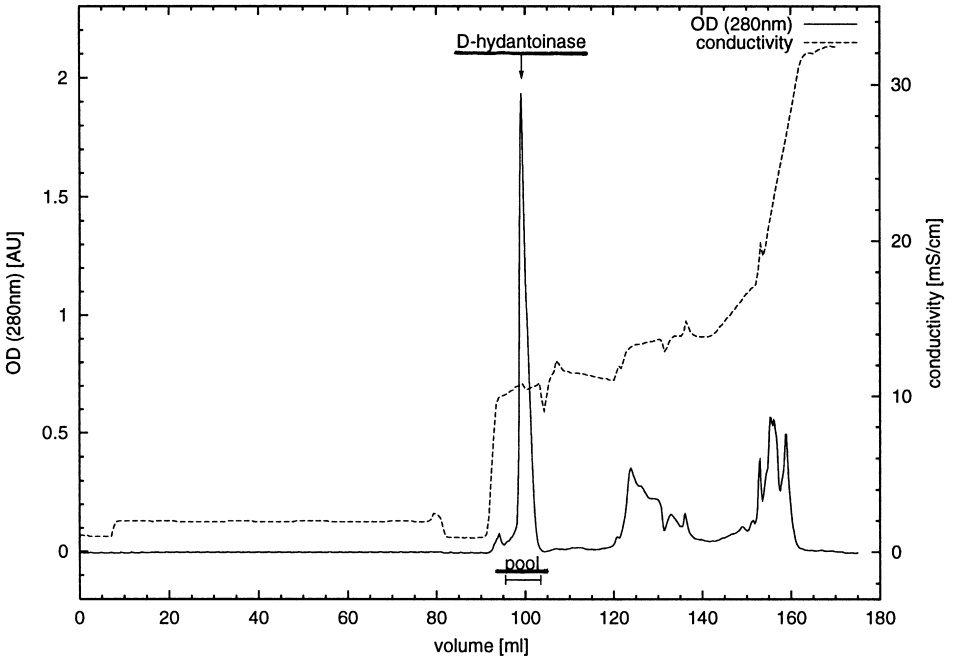

Fig. 2. Chromatogram anion-exchange chromatography; the active peak and the pooled fractions are labelled.

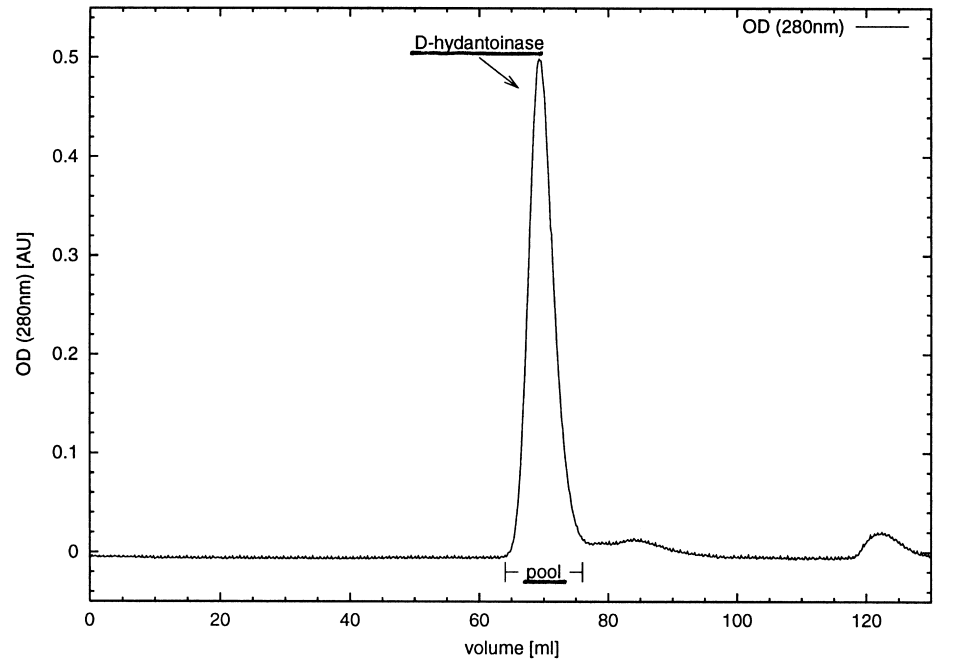

Fig. 3. Chromatogram gel permeation chromatography; the active peak and the pooled fractions are labelled.

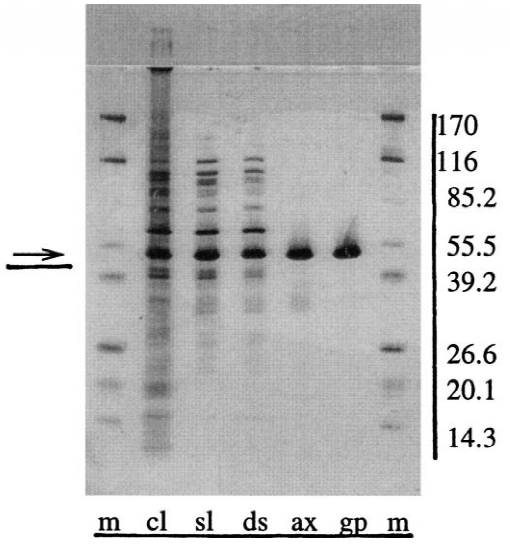

Fig. 4. SDS–PAGE of different chromatographic steps: marker (m), centrifuged cell lysate (cl), Streamline pool (sl), desalted pool (ds), anion-exchange pool (ax), gel permeation pool (gp), marker (m). The molecular weights of the maker proteins on the left are in 0.001 M_r. The position of the D-hydrantoinase is labelled with an arrow.

$m/z \approx 100\,000$ is interpreted as a single charged dimer.

With dynamic light scattering a hydrodynamic radius of $r_H = 4.5$ nm and a polydispersity value of 11% could be determined. From the hydrodynamic radius a molecular weight of $M_r = 113\,000$ for the native protein was calculated.

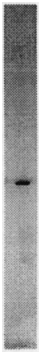

Fig. 5. Native PAGE running from the top of purified D-hydantoinase.

Fig. 6. Isoelectric focussing PAGE of purified D-hydantoinase with pH 3 at the bottom and pH 9 on the top.

4. Discussion

The D-hydantoinase from *Thermus sp.* could be expressed at high levels in *E. coli* BL21 cells. The expression of a considerable amount of D-hydantoinase in inclusion bodies could not be avoided. This was tolerated since the amount of soluble protein was sufficient. Because of the high expression the final purification factor (9.6) in relation to the crude extract was quite small.

The aim of the purification was to obtain D-hydantoinase suitable for crystallization. As a homogenous sample in general is most suitable for crystallization [9] purity and homogeneity was the ultimate goal of this purification protocol. However as crystallization requires milligram amounts of protein a good recovery of protein was also necessary.

Hydantoinases are known to contain divalent metals, predominantly zinc and manganese [1]. A functional role of zinc for the catalysis has been reported in some cases [6,7]. The D-hydantoinase from *Thermus sp.* is known to depend on manganese. Therefore 0.1 m*M* manganese was added to the medium for bacterial growth and to all chromatographic media. This leads to a higher recovery of activity than without manganese.

The Streamline phenyl column proved to be a good capture step. Due to the relatively high affinity of the D-hydantoinase to the phenyl resin comparatively low salt concentrations could be used. The lysate was clarified and some degree of purification was achieved. Step gradients were used in order to

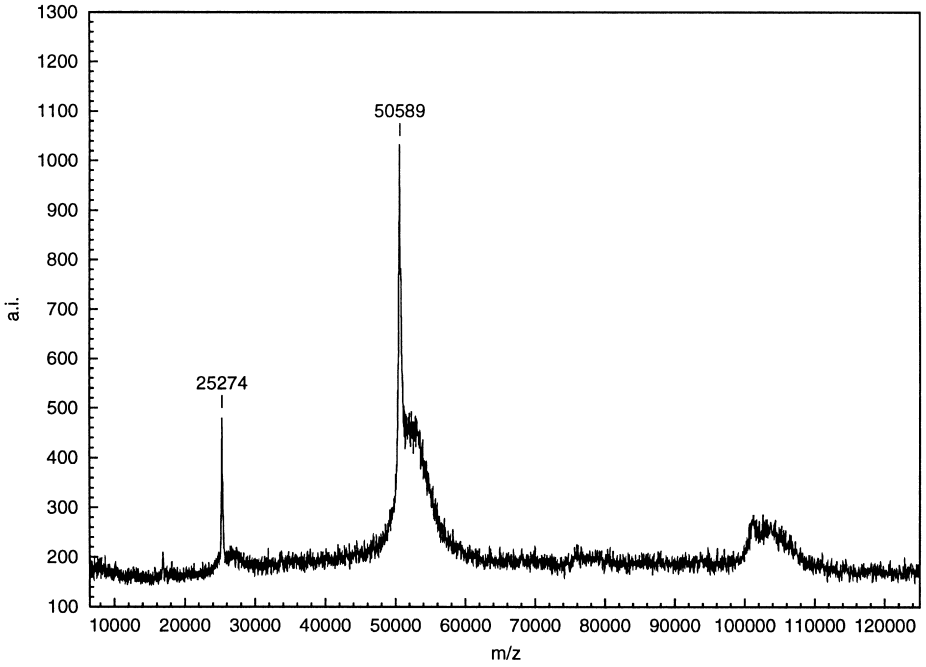

Fig. 7. MALDI-TOF mass spectrum of purified D-hydantoinase. The m/z of the single and double charged quasimolecular ion are labelled.

speed up this step. Different steps have been tested, the step from 250 m*M* to 0 m*M* ammoniumsulphate was the best regarding yield and purification. The Streamline resin which is preferred for industrial application could be used with success on laboratory scale with conventional laboratory equipment.

Since the eluate from the Streamline column still had too high a salt content for direct application on the anion-exchange column, the sample had to be desalted. The gel permeation was a rapid and reliable method.

Anion-exchange chromatography with MonoQ served as the intermediate purification step, which could remove most of the impurities. During optimization of this step buffers with different pH (6.5–8.5) were tested with a 1 ml MonoQ HR 5/5 column. The lower the pH the better was the separation of the active peak from the other peaks. As the D-hydantoinase has the highest activity at higher pH-values pH 7.5 was chosen. For a better peak separation a linear gradient was replaced by a segmented gradient. Not only purification but also an increased concentration of the protein was achieved. Hence the sample just had to be concentrated slightly for the following gel permeation chromatography.

As for crystallization purposes the highest purity possible is desired, the eluate of the anion-exchange column was purified further. Since the residual impurities had a relatively small size ($M_r \leq 40\,000$) and the D-hydantoinase forms dimers, gel permeation was chosen as the polishing step.

The purified protein showed homogeneity in different analyses: SDS–PAGE (Fig. 4), native-PAGE (Fig. 5), isoelectric focussing (Fig. 6) and MALDI-TOF mass spectrometry (Fig. 7).

A narrow unimodal distribution of particle size could be determined by dynamic light scattering. The polydispersity value is the standard deviation of the spread of particle sizes around the average hydrodynamic radius. As for protein crystallization a monomodal size distribution is necessary [11] polydispersity values of proteins preparations which are suitable for crystallization should be below 30%. For the purified D-hydantoinase a prolydispersity value of 11% was determined. The molecular weight calculated from the hydrodynamic radius ($r_H = 4.5$ nm)

using the curve model for globular proteins (M_r = 13 000) is in accordance with a dimer. Whereas most hydantoinases are described to consist of tetramers, the D-hydantoinase from *Bacillus stearothermophilus SD-1* is also reported to build dimers [12].

A further proof for the homogeneity of the purified protein consists of the fact that the D-hydantoinase from *Thermus sp.* could be crystallized [8]. X-ray data sets have been measured on a rotating anode X-ray source. The crystals diffract to at least 2.3 Å resolution and complete data sets to 2.6 Å resolution have been measured [8]. The solution of the structure using MAD and MIR methods is currently under progress.

5. Abbreviations

FPLC	Fast protein liquid chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
MAD	Multiwavelength anomalous diffraction
MIR	Multiple isomorphous replacement
MTEH	D,L-(2-Methylthiothyl)-hydantoin
OD_{600}	Optical density at 600 nm
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethanesulfonylfluoride

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