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# Enzymatic activity assay of D-hydantoinase by isothermal titration calorimetry. Determination of the thermodynamic activation parameters for the hydrolysis of several substrates

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**Abstract**

Isothermal titration calorimetry (ITC) has been applied to the determination of the activity of D-hydantoinase (EC 3.5.2.2) with several substrates by monitoring the heat released during the reaction. The method is based on the proportionality between the reaction rate and the thermal power (heat/time) generated. Microcalorimetric assays carried out at different temperatures provided the dependence of the catalytic rate constant on temperature. We show that ITC assay is a nondestructive method that allows the determination of the catalytic rate constant ($k_{cat}$), Michaelis constant ($K_M$), activation energy and activation Gibbs energy, enthalpy and entropy of this reaction.


*Keywords:* D-hydantoinase; Isothermal titration calorimetry; Activity assay; Activation parameters

*Abbreviations:* ITC, Isothermal titration calorimetry; $k_{cat}$, catalytic rate constant; $K_M$, Michaelis constant; $E_a$, activation energy; LB, Luria Bertani; $\Delta H_m$, molar enthalpy.

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## 1. Introduction

Hydantoinases, alternatively named dihydropyrimidases (EC 3.5.2.2.), belong to the group of cyclic amidases. These enzymes catalyse the reversible hydrolysis of hydantoins and dihydropyrimidines. Besides their physiological function in the reductive catabolism of pyrimidines [1], in biotechnology hydantoinases are used in combination with carbamoylases for the production of optically pure amino acids from racemic hydantoin derivatives [2]. Due to different enantioselectivities on the substrates, hydantoinases have been traditionally classified into D-, L- or non-enantioselective hydantoinases [3–5]. D-hydantoinase has attracted much attention because it is employed for the production of optically pure D-amino acids that are intermediates for semi-synthetic antibiotics, peptide hormones, pyretroids, and pesticides [6]. For this important economic reason, D-hydantoinase properties such as stability and activity as a function of temperature and pH, substrate specificity, dependency on metal ions and reaction kinetics for the corresponding *N*-carbamoyl-D-amino acids have been studied [7–9].

To investigate D-hydantoinase activity several qualitative and quantitative methods have been developed. Qualitative analyses based on deproteinization of D-hydantoin into *N*-carbamoyl-D-amino acid by D-hydantoinase enzyme were detected by thin layer chromatography or by colour production using *p*-dimethylamino-benzaldehyde [10,11]. This compound was used for quantitative determination of D-hydantoinase activity by photometric methods or in combination with an automatic amino acid analyser [5,10]. These qualitative and quantitative methods present the disadvantage of having low precision and sensitivity. Moreover, the *p*-dimethylamino-benzaldehyde method cannot be applied to aromatic *N*-carbamoyl-D-amino acids, because the corresponding D-hydantoins also react [6]. More recently studies have applied High Performance Liquid Chromatography (HPLC) methods on reversed phase column to D-hydantoinase activity analysis [12,13]. This method has solved the quantification of aromatic compounds compared with the *p*-dimethylamino-benzaldehyde one, and is currently used as the standard D-hydantoinase activity analysis.

In this work we propose a new method to measure D-hydantoinase activity using isothermal titration calorimetry (ITC). Modern isothermal power compensation calorimeters supply thermal power to the sample cell which is directly proportional to the reaction rate [14–16] and low enzyme concentrations are needed because of their high sensitivity. Another advantage is that multiple rate determinations can be carried out in a single experiment by successive additions of substrate, under pseudo-first-order conditions. Thus, ITC activity assay is a direct and highly precise method to measure the reaction rate.

## 2. Materials and methods

### 2.1. General protocols and reagents

Standard methods were used for the cloning and expression of the different genes [17,18] *E. coli* BL21 [19] was used to clone and express the D-hydantoinase gene. The D- and L-5-monosubstituted hydantoins and *N*-carbamoyl-D and L-amino acids used in this paper were synthesised according to previous works [20,21]. Restriction enzymes, T4