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Estimation of the overall kinetic parameters of enzyme inactivation using an isoconversional method

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ABSTRACT

An isoconversional method is proposed in order to calculate the kinetic parameters of enzyme inactivation. The method provides an efficient and low-cost procedure to describe both operational and thermal inactivation. Unlike the ordinary kinetic assays performed at constant enzyme concentration and at various substrate concentrations, the isoconversional method requires several extended kinetic curves for constant initial substrate concentration and different enzyme concentrations. The procedure was tested and validated using simulated data obtained for several kinetic models frequently discussed in the literature. After the validation, the isoconversional method was used for the investigation of the thermoinactivation of urease during urea hydrolysis in self buffered medium and the operational inactivation (destructive oxidation by excess peroxide) of catalase at high concentration of hydrogen peroxide. The results showed that the isoconversional method gives good results of global inactivation constant for both simple and more complex models.

1. Introduction

Enzyme stability is one of the limiting factor in most bioprocesses, biocatalyst stabilization being then a central issue of biotechnology [1]. Beside the production of intrinsically stable biocatalysts, enzyme stabilization involves studies regarding strategies for operational stabilization and mathematical modelling of the biocatalyst inactivation during operation. The application of enzymes to synthesis of desired products, in analysis, or in manufacturing of sensors requires stable, well-characterized activity. Being relatively fragile structures, enzymes display a short catalytic lifetime due to their inactivation by side reactions or at increased temperatures [2]. The enzyme inactivation is most commonly due to structural rearrangements or modification of reactive groups [1]. Less widely recognized is the instability of enzymes in vivo, where they are continuously broken down and reused. The study of inactivation kinetics of the enzyme systems in vitro represents a useful tool in understanding the dynamic behaviour of in vivo enzyme systems.

There are plenty examples in the literature of enzyme inactivation in different experimental conditions: various substrates, temperature, pH and ionic strength [3]. Thus, there was a need to develop efficient and low-cost methods to set off the enzyme inactivation during a technological process.

There are few methods to describe enzyme inactivation; some of them are qualitative, like the Selwyn test [4], which uses the extended progress curves of the product concentration vs. the product of initial enzyme concentration and time. Other methods are based either on the presumption that the kinetics obeys the Michaelis-Menten model [5], the inactivation parameters being obtained from the values of Michaelis constant, or by using specific [2,6] or phenomenological models [7] that leads to certain definite kinetic equations.

In this paper, an isoconversional method is described and applied to the thermal/operational inactivation of enzymes. Isoconversional methods used in chemical kinetics imply that, for a definite number of kinetic runs, the measurements are performed at the same concentration of reactants. If the initial substrate concentration is kept constant during all runs, the isoconversional restriction becomes simpler: the measurements are performed at the same unreacted substrate concentration or at the same product concentration. The procedure ensures a constant composition of the reaction mixture leading to a substantial simplification of the kinetic equation. The method is used to estimate the overall kinetic inactivation constant for several literature models, emphasizing on the experimental conditions required for the best estimates. After validation for several models, the method is also applied for two real systems: the thermal inactivation of urease in the hydrolysis of urea and the suicide inactivation of catalase by the substrate in the reaction of decomposition of the hydrogen peroxide.

Catalase is a component of the antioxidative defence system acting in human and animal tissues against hydrogen peroxide, which is a

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potentially deleterious reactive species that destroys (oxidizes) cellular molecules. The mechanism of the catalytic decomposition of H_2O_2 is not entirely elucidated so far. Several compounds, including its natural substrate, H_2O_2, at concentrations exceeding 0.1M. [8], reduce the activity of catalase. In the catalase — H_2O_2 system the activity of catalase is strongly dependent on the initial concentration of the substrate $[S]_0$. Thus for $[S]_0$ up to 0.1M, H_2O_2 undergoes decomposition till total conversion. For concentration $[S]_0$ exceeding 0.1M the conversions are not total, proving an inactivation effect (irreversible inhibition) of the enzyme by H_2O_2, the effect increasing with the concentration of substrate. [9].

Urease catalyzes the hydrolysis of urea, the final step of organic nitrogen mineralization. Many pathologies are associated with the activity of ureolytic bacteria, and the efficiency of soil nitrogen fertilization with urea is severely decreased by urease activity. Therefore, the development of urease inhibitors would lead to a reduction of environmental pollution, to an enhanced efficiency of nitrogen uptake by plants [10], and to improved therapeutic strategies for treatment of infections due to ureolytic bacteria [11]. The thermostability of ureases of different origin either in free or immobilized forms has often been a subject of investigation. There are several studies for jack been urease where the inactivation curve was measured at high temperatures [12,13].

2. Experimental

2.1. Materials

Catalase from bovine liver (EC 1.11.1.6, 312000U/mL) was purchased from Flucka (Switzerland) and sword bean urease (E.C. 3.5.1.5, lyophilized 5U/mg), from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and were products of Merck – the hydrogen peroxide and Chimopar (Bucharest, Romania) – urea.

2.2. Catalase assay

The solution of H_2O_2 with concentration 0.5M in distilled water was prepared from a bulk solution and the concentration was determined by titration with permanganate. The experiments were carried out for catalase concentrations ranging within $1.8\cdot10^{-7}$M and $7.2\cdot10^{-7}$M. The decomposition of the H_2O_2 in presence of catalase was studied using a gas-volumetric displacement method, in isobaric-isothermal conditions (p_0=1 atm; T=298 K), by measuring the volume of oxygen evolved in the reaction.

2.3. Urease assay

The hydrolysis of urea in the presence of sword bean urease was followed in a self-buffered system. The experiments were carried out in a batch reactor, at initial concentration of urea $[S]_0=4\cdot10^{-2}$M and several initial concentrations of urease (in protein) ranging within $1\cdot10^{-7}$M and $7.5\cdot10^{-7}$M. For each kinetic run, the conductance (C) of solution was monitored until its change became negligible; the products concentrations were calculated according to a calibration curve obtained from a stock solution by several dilutions. The stock solution was obtained through total hydrolysis of an urea solution of known concentration in a sealed vessel. The thermal inactivation of urease was studied at 343K.

3. Results and discussion

3.1. Theoretical considerations of isoconversional method

As long as the enzyme concentration is much lower than the substrate concentration, a quasi-steady state is attained and the time evolution of the system can be described by a single reaction rate. Consequently, an analytical solution can be obtained for the overall reaction rate. The corresponding kinetic equation, based on the quasi-steady state or quasi-equilibrium assumptions, coupled with the enzyme conservation equation takes the general form:

$$\frac{d[P]}{dt} = [E]_0 \cdot f([S]_0, [P], T, [M]_0, J, \mathrm{pH}) \quad (1)$$

where $[E]_0$ is the total initial concentration of present active enzyme, $[S]_0$ is the initial substrate concentration, $[M]_0$ concentration of any modulator present, $[P]$ is the concentration of product at time t, T is the temperature and J the ionic strength. Provided that in a series of assays $[S]_0$, T and $[M]_0$ are kept constant and other variables like J and pH reach the same level, the rate equation can be written in the simpler form:

$$\frac{d[P]}{dt} = [E]_0 \cdot f([P]). \quad (2)$$

This product form in the right-hand term suggests the use of an isoconversional procedure to detect the changes of enzyme concentration for a chosen conversion and various initial enzyme concentrations, when $f([P])$ remains constant.. Such "model free" methods have been extensively used for numerous kinetic analysis of reactions involving solids, where the reaction rate is also given by a product between a temperature and a conversion factor [14–20]. The proposed procedure belongs to a group of kinetic methods, like "constant velocity plot" [21] and "competition plot" [22] where the judicious restrictions imposed to kinetic measurements result in a safer analysis of experimental data.

The time values (t_1, t_2, t_3 etc.) corresponding to a settled conversion of substrate (a certain concentration of reaction product $[P]$) can be estimated from the kinetic curves $[P]=f(t)$ at different enzyme initial concentrations $[E]_0$ as shown in Fig. 1.

The first derivative with respect to time in each point of product concentration $(d[P]_1/dt)_1$, $(d[P]_1/dt)_2$ etc. can be estimated by numerical methods in the points t_1, t_2, etc., after fitting a proper function, selected for the best fit, on the extended kinetic curves.

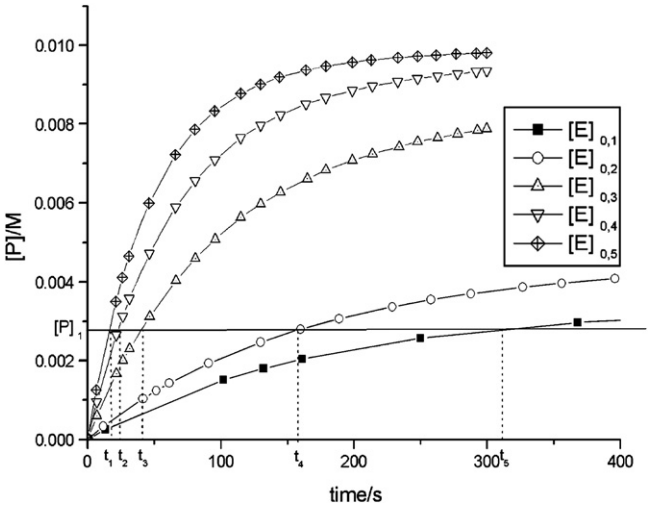

Fig. 1. Estimation of isoconversional times for various initial enzyme concentration ($[E]_{0,1}<[E]_{0,2}<[E]_{0,3}<[E]_{0,4}<[E]_{0,5}$).

Since $[P]_1$ is constant due to the isoconversional conditions, results that $f([P]_1)$=constant and Eq. (2) can be written as:

$$\left(\frac{d[P]_1}{dt}\right)_i = f([P]_1)\cdot [E]^*_{0,i} \tag{3}$$

where $[E]^*_{0,i}$ is the total enzyme concentration for run i, active at time t_i. For a first order inactivation it is given by:

$$[E]^*_{0,i} = [E]_{0,i}\cdot e^{-k_{in}t} \tag{4}$$

The first derivatives $(d[P]_1/dt)_i$ for a certain value of $[P]_1$ depend then on the corresponding isoconversional times t_i according to the following equation:

$$\left(\frac{d[P]_1}{dt}\right)_i = f([P]_1)\cdot [E]_{0,i}\cdot e^{-k_{in}\cdot t_i} \tag{5}$$

From Eq. (5), the overall inactivation constant k_{in} can be calculated either from several pairs of i and j:

$$k^{ij}_{in} = \frac{\ln\left([E]^*_{0,i}/[E]^*_{0,j}\cdot [E]_{0,j}/[E]_{0,i}\right)}{t_i - t_j} \tag{6}$$

where $[E]_{0,i}/[E]_{0,j}$ can be calculated as $(d[P]_1/dt)_i/(d[P]_1/dt)_j$, or from the linear regression

$$\ln\left(\left(\frac{d[P]_1}{dt}\right)_i \frac{1}{[E]_{0,i}}\right) = \ln f([P]_1) - k_{in}\cdot t_i \tag{7}$$

when the slope is equal to the overall inactivation constant.

For more than three experiments, the last procedure is more suitable, since the estimated slope includes also the experimental errors [3].

It is obvious that the same procedure can be applied for other values of [P]. If the inactivation does not obey a first order kinetic, the results for the inactivation constants at different values of [P] should be different. Small differences between low and high conversions can be attributed to changes in composition during reaction.

Since the first order inactivation is more frequently encountered [2], it was tested for simulated data, including both thermal and operational inactivation. A similar procedure will be hereafter applied to different orders of inactivation. The kinetic models used for testing this method are based on the simple Michaelis-Menten model, with different steps of inactivation. The models were chosen to see if the isoconversional method with first order inactivation can be used to obtain a global inactivation parameter for more complex kinetic models.

3.2. Kinetic simulation of data

Several kinetic models, based on simple Michaelis-Menten model, were used; the models are presented in Table 1.

Table 1
Kinetic models used for data simulation

No.	Name	Kinetic model
1	Simple Michaelis-Menten model + monomolecular inactivation	$E+S \underset{}{\overset{1}{\rightleftarrows}} ES \xrightarrow{2} E+P$ $E \xrightarrow{in} E_{in}$
2	Substrate inhibition + monomolecular inactivation	$E+S \overset{1}{\rightleftarrows} ES \xrightarrow{2} E+P$ $ES+S \overset{3}{\rightleftarrows} ESS$ $E \xrightarrow{in} E_{in}$
3	Product inhibition + monomolecular inactivation	$E+S \overset{1}{\rightleftarrows} ES \xrightarrow{2} E+P$ $E+P \overset{3}{\rightleftarrows} EP$ $E \xrightarrow{in} E_{in}$
4	Two steps monomolecular inactivation	$E+S \overset{1}{\rightleftarrows} ES \xrightarrow{2} E+P$ $E \xrightarrow{in1} E_{in,1} \xrightarrow{in2} E_{in,2}$
5	Lumry-Eyring model for thermoinactivation [23]	$E+S \overset{1}{\rightleftarrows} ES \xrightarrow{2} E+P$ $E \xrightarrow{in.1} E_{in,1} \xrightarrow{in2} E_{in,2}$

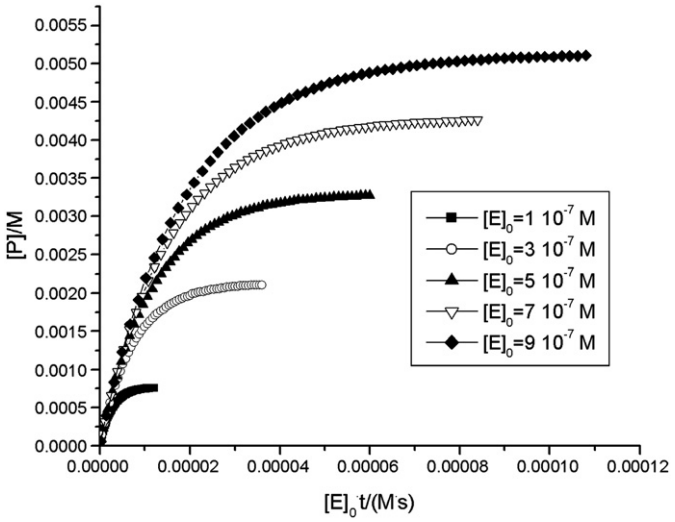

Fig. 2. Selwyn test of inactivation for the model (1) (simple model with one inactivation step).

Model number 1 is the "reference model" since it includes only one step for the enzyme inactivation. This model was used for verifying the isoconversional methods and for determining the best experimental conditions for estimating the inactivation constant. Models 2 and 3 include also a step for inhibition by substrate or products; they were used to investigate if the calculated inactivation constant is the same as in model 1, or if the inhibition step gives a false result. Models 4 and 5 are more difficult models and were used in order to see if the isoconversional method can be applied for the estimation of a global parameter for inactivation.

To simulate the extended progress curves, the differential kinetic equation was derived for each model and several sets of parameters were chosen. The equation was solved numerically obtaining the time variation of product concentration. The simulated data, having negligible errors, were provided with random errors corresponding to a Gaussian distribution and an imposed standard deviation [24]. The final results were used as real extended curves. The kinetic models outlined in Table 1 were used to simulate the experimental data, starting from the following parameters: $[S]_0$=0.01 M, $[E]_0$ varying from 10^{-7} to $9\cdot 10^{-7}$M, K_M=0.025M, K_3=0.1M, k_{in}=0.005s^{-1}. For models 4 and 5 the values of k_{in} were fixed around 0.005s^{-1}. The

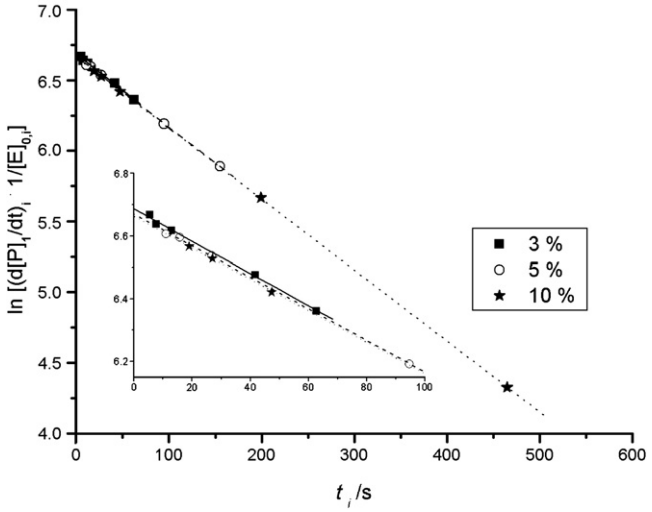

Fig. 3. Results for linear fit of Eq.(7) on model 1 for several substrate conversions (3%: y=6.68–0.00515·x;r^2=0.9985; 6%: y=6.66–0.00503·x;r^2=0.9998; 10%: y=6.66–0.00502·x;r^2=0.9999).

Table 2
Results of the inactivation constant for reference model at different initial substrate concentrations and conversions

$[S]_0$/M	Conversion %	$k_{in}\cdot10^3/s^{-1}$	Std. dev $10^5/s^{-1}$	Error %
0.005	3	5.07	5.39	1.06
	6	5.05	1.74	0.34
	10	4.92	1.60	0.32
0.01	3	4.76	19.8	4.15
	6	5.00	4.07	0.81
	10	5.00	3.16	0.63
0.02	3	5.02	4.41	0.86
	6	4.99	4.47	0.89
	10	5.02	1.01	0.20
0.1	3	5.15	16.3	3.17
	6	5.02	4.26	0.84
	10	5.02	1.32	0.26

kinetic simulations were performed with KINTECUS [25]. To confirm the existence of an inactivation process a qualitative Selwyn test is necessary as a first step. For one of the presented models, this is illustrated in Fig. 2.

3.3. Application of the isoconversional method for calculating the overall inactivation parameters

In order to estimate the inactivation constant, first model 1 was taken into account, since it is the most simple model which includes an inactivation step. From the simulated plots $[P]=f(t)$ at constant substrate concentration and 5 different enzyme concentrations, the time values for several conversions and the corresponding derivatives $(d[P]_1/dt)_i$ were estimated by fitting a Chebyshev polynomial of 20th degree and evaluating the roots at certain points and their first derivatives. The obtained values were used to estimate the inactivation constant from the $\ln\left(\left(\frac{d[P]_1}{dt}\right)_i\frac{1}{[E]_{0,i}}\right)=f(t_i)$ plot. The results for 3 different conversions are shown in Fig. 3.

As it can be seen in Fig. 3, for all conversions the plots are linear and the points are superposed; this is an indication of the fact that the isoconnversional method is very precise, since the correlation coefficients are very good and the slope values are very close to the used inactivation constant in the simulation.

The method was verified for different initial substrate concentrations and initial enzyme ranges of concentrations. The estimated values for inactivation constant are presented in Table 2, together with the standard deviations and errors, calculated as std.dev/estimate 100.

The best estimates for inactivation constant are obtained for substrate concentrations closed to Michaelis constant (0.025M in this kinetic model) and for conversions that exceed 5%. Since the absolute errors are superimposed over the simulated kinetic curves, the estimated parameters have larger errors for lower conversions.

Studies regarding the influence of experimental errors showed that the errors in inactivation constants do not exceed 14%, even when the experimental errors were 20%. The range of initial enzyme concentrations used does not influence the value or precision of the estimates. However the extension of enzyme concentration range over a decade has some experimental limitations: the time required for a selected conversion is very long for lower enzyme concentrations or becomes very short for higher concentrations.

Table 3
Rate constants of inactivation for several simulated kinetic models

Model	$k_{in}\cdot10^3/s^{-1}$	Std. dev $10^3/s^{-1}$
2	4.996	0.002
3	5.001	0.009
4	5.002	0.026
5	4.646	0.144

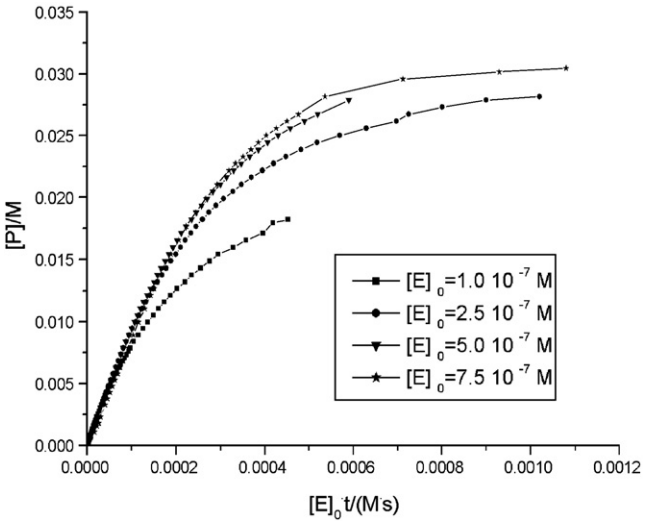

Fig. 4. Selwyn plots of products concentration versus $(t\cdot[E]_0)$ for urea 0.04 M hydrolysis in the presence of different initial concentrations of urease at 343 K.

The same procedure was applied in order to estimate an overall inactivation constant for more complex models (2 to 4 from Table 1). The time values for the selected conversion and the derivatives $(d[P]_1/dt)_i$ were estimated from the plots $[P]=f(t)$ for each kinetic model from Table 1; the rate constants of inactivation were calculated as the slope of the $\ln\left(\left(\frac{d[P]_1}{dt}\right)_i\frac{1}{[E]_{0,i}}\right)=f(t_i)$line, assuming that inactivation follows a first order kinetics. The results, with their standard deviations are given in Table 3.

The results showed that the value obtained for inactivation constant for models 2 and 3, that includes also an inhibition step is the same as the one used in simulation, so the isoconversional method does not give false results due to inhibition by substrate or products. The values obtained for models 4 and 5, which includes two steps of inactivation are also very good, indicating that this method can be applied for estimation of the rate constant for the first inactivation step. The proposed method, based on the monitoring of the active enzyme, is not able to describe the subsequent transformations of the enzyme.

The results proved that the isoconversional method is suitable for calculating the inactivation parameters, since the estimated rate constant was the same, within the limits of experimental errors, as the used one; the rate constant can be estimated with a reasonable

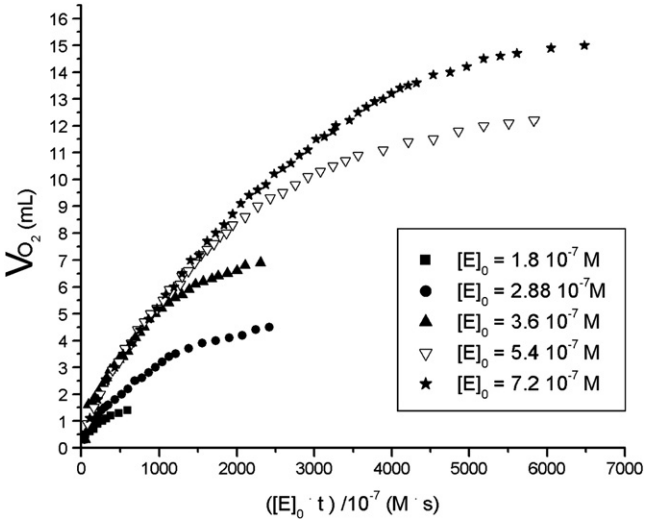

Fig. 5. Selwyn plots of O_2 versus $(t\cdot[E]_0)$ for H_2O_2, 0.5 M, decomposition in the presence of different initial concentrations of catalase at 298 K.

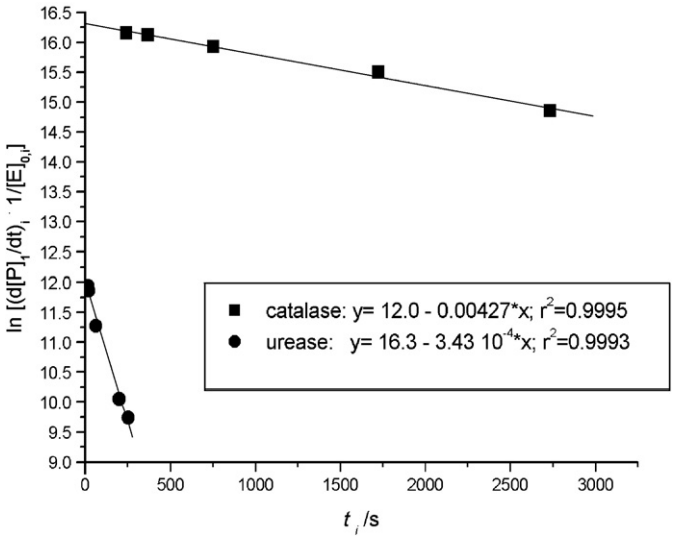

Fig. 6. Results of linear regression on Eq.(7) for inactivation of catalase and urease.

precision even in the case of more complex mechanisms involving reversible inhibition steps or several steps of inactivation.

3.4. Application of isoconversional method to real systems

The isoconversional method was applied for two different types of inactivation: thermal inactivation of urease and substrate inactivation of catalase. The experimental conditions were chosen in such a way that the enzymes were significantly inactivated in. At 343K for urease catalyzed urea hydrolysis as well as for the decompostion of a 0.5M hydrogen peroxide in the presence of catalase, the typical pattern offered by the Selwyn tests (Figs. 4 and 5) indicated the occurrence of enzyme inactivation.

The plots $[P]=f(t)$ for urease and $V_{O_2}=f(t)$) for catalase were used for the estimation of the inactivation constant as described before. The conversions used were 10%, where the results are more precise.

The values of rate constants of inactivation, estimated by linear regression on Eq.(7) are:

$k_{in} = (4.27 \pm 0.12) \cdot 10^{-3} s^{-1}$ for catalase at $[S] = 0.5$M and 298K

$k_{in} = (3.43 \pm 0.14) \cdot 10^{-4} s^{-1}$ for urease at $[S] = 0.5$M and 343K.

The results indicate that both inactivations (thermal inactivation of urease and substrate inactivation of catalase) can be rationalized as first order processes, the plots corresponding to Eq.(7) being linear, as illustrated in Fig. 6. The values of the rate constants were in the range with the literature data: $7 \cdot 10^{-3}$ s^{-1} for catalase in buffered media [9], and $5 \cdot 10^{-4}$ s^{-1} for urease in buffered media [13]. The differences can be attributed to the experimental conditions.

As compared to other methods used to analyse the enzyme inactivation, the isoconversional method has several benefits: is a "model free" method (do not need to adopt a specific kinetic model), can be used for different conversions allowing the identification of inactivation dependence on medium composition and can be extended for inactivation processes of higher orders.

4. Conclusions

The analysis of the extended progress curves for detecting the enzyme inactivation can be made qualitatively by using the Selwyn test and quantitatively by using the proposed isoconversional method. The overall constants of first order inactivation can be estimated using the extended kinetic curves at different initial enzyme concentrations. The accuracy of the results obtained on the simulated data indicated that the method can be successfully used for estimating the overall inactivation constant without the assumption of a certain model for enzyme inactivation.

The method was also applied to the thermoinactivation of urease in the hydrolysis of urea and to substrate inactivation of catalase in the decomposition of hydrogen peroxide. The results were in good agreement with literature data.

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References

[1] S. Yoshioka, K. Izutsu, Y. Aso, Y. Takeda, Inactivation kinetics of enzyme pharmaceuticals in aqueous solution, Pharm. Res. 8 (1991) 480–484.
[2] S. Ajit, Enzyme deactivation, Biotech. Adv. 6 (1988) 349–446.
[3] A. Cornish-Bowden, Detection of errors of interpretation in experiments in enzyme kinetics, Methods 24 (2001) 181–190.
[4] M.J. Selwyn, A simple test for inactivation of an enzyme during assay, Biochim. Biophys. Acta 105 (1965) 193–195.
[5] S. Schnell, S.M. Hanson, A test for measuring the effects of enzyme inactivation, Biophys. Chemist. 125 (2007) 269–274.
[6] P. Vrabel, M. Polakovic, V. Stefuca, V. Bales, Analysis of mechanism and kinetics of thermal inactivation of enzymes: evaluation of multitemperature data applied to inactivation of yeast invertase, Enzyme Microb. Technol. 20 (1997) 346–354.
[7] C. Aymard, A. Belarbi, Kinetics of thermal deactivation of enzymes: a simple three parameters phenomenological model can describe the decay of enzyme activity, irrespectively of the mechanism, Enzyme Microb. Technol. 27 (2000) 612–618.
[8] M.V. Potapovich, A.N. Eryomin, D.I. Metelitza, Ultrasonic and thermal inactivation of catalases from bovine liver, the methylotrophic yeast *Pichia pastoris* and the fungus *Penicillium piceum*, Appl. Biochem. Microbiol. 41 (2005) 529–537.
[9] O.M. Lardinois, M.M. Mestdagh, P.G. Rouxhet, Reversible inhibition and irreversible inactivation of catalase in presence of hydrogen peroxide, Biochim. Biophys. Acta 1295 (1996) 222–238.
[10] F. Sannino, L. Gianfreda, Pesticide influence on soil enzymatic activities, Chemosphere 45 (2001) 417–425.
[11] A. Radomska, S. Glab, R. Koncki, Spectrophotometric bioanalytical flow-injection system for control of hemodialysis treatment, Analyst 126 (2001) 1564–1567.
[12] V. Illeova, M. Polakovic, V. Stefuca, P. Acai, M. Juma, Experimental modelling of thermal inactivation of urease, J. Biotechnol. 105 (2003) 235–243.
[13] F. Kara, G. Demirel, H. Tumturk, Immobilization of urease by using chitosan–alginate and poly(acrylamide-co-acrylic acid)/j-carrageenan supports, Bioprocess Biosyst. Eng. 29 (2006) 207–211.
[14] H. Friedman, Kinetics of thermal degradation of char-forming plastics from thermogravimetry. Application to a phenolic plastic, J. Polym. Sci. 6 (1964) 183–195.
[15] T. Ozawa, New method of analysing thermogravimetric data, Bull. Chem. Soc. Japan 38 (1965) 1881–1886.
[16] J.H. Flynn, L.A. Wall, A quick direct method for the determination of activation energy from thermogravimetric data, J. Polym. Sci. 4 (1966) 323–328.
[17] H.E. Kissinger, Reaction kinetics in differential thermal analysis, Anal. Chem. 29 (1957) 1702–1706.
[18] S. Vyazovkin, C.A. Wight, Model-free and model-fitting approaches to kinetic analysis of isothermal and nonisothermal data, Termochim. Acta 340-341 (1999) 53–68.
[19] S. Vyazovkin, L. Vincent, N. Sbirrazzuoli, Thermal denaturation of collagen analyzed by isoconversional method, Macromol. Biosci. 7 (2007) 1181–1186.
[20] F. Rodante, S. Vecchio, M. Tomasetti, Kinetic analysis of thermal decomposition of penicillin sodium salts: model-fitting and model-free methods, J. Pharm. Biomed. Anal. 29 (2002) 1031–1043.
[21] E.A. Whitehead, Dynamics of biochemical systems, in: J. Ricard, A. Cornish-Bowden (Eds.), Subunit interactions and enzyme kinetic: the linkage approach, vol. 81, Penum, New York, 1984, pp. 45–64.
[22] C. Chevillard, M.L. Cardenas, A. Cornish-Bowden, The competition plot: a simple test of whether two reactions occur at the same active site, Biochem. J. 289 (1993) 599–604.
[23] R. Lumry, H. Eyring, Conformational chages of proteins, J. Phys. Chem. 58 (1954) 110–120.
[24] M.D. Johnston Jr., Computational Chemistry, An Emphasis on Practical Clacula-tions, Elsevier, Amsterdam, 1988.
[25] J. Ianni, KINTECUS, Windows Version 3.1, 2003 http://www.kintecus.addr.com/.