Enzyme-Catalyzed Decomposition of Dibenzoyl Peroxide in Organic Solvents

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Immobilized Catalase, Aprotic Solvents, Catalytic Activity

Catalytic activity of catalase (CAT, EC 1.11.1.6), immobilized on carbon black NORIT and soot PM-100, with respect to decomposition of dibenzoyl peroxide (BPO) in non-aqueous media (acetonitrile and tetrachloromethane), was investigated with a quantitative UV-spectrophotometrical approach. Progress of the above reaction was controlled by selected kinetic parameters: the apparent Michaelis constant (K_m^{app}), the specific rate constant (k_{sp}), the activation energy (E_a), the maximum reaction rate (V_{max}), and the Arrhenius' pre-exponential factor (Z_0). Conclusions on the tentative mechanism of the catalytic process observed were drawn from the calculated values of the Gibbs energy of activation (ΔG^*), the enthalpy of activation (ΔH^*), and entropy of activation (ΔS^*).

Introduction

The ability of native catalase to decompose hydrogen peroxide to water and oxygen in aqueous environment is well known. Besides, immobilization on various materials affords the opportunity for increasing stability and repeated use of the enzyme. Amperometric biosensors based on immobilized catalase have been proven useful analytical tools for the specific determination of either, H_2O_2 (Akgol and Dinckaya, 1999) or some catalase inhibitors -cyanides and fluorides (Stein and Hain, 1995). Catalase, co-immobilized with H_2O_2-producing, or consuming, oxidoreductases such as: glucose oxidase (Liu *et al.*, 1979; Wingard *et al.*, 1983), lactate oxidase (Scheller *et al.*, 1985), peroxidase (Tatsuma *et al.*, 1994), glutamate oxidase (Madaras *et al.*, 1997; Niwa *et al.*, 1998), L-lysine-alpha oxidase (Vrbova *et al.*, 1992) or choline oxidase (Vrbova *et al.*, 1993), had also been used for that purpose.

The recently demonstrated capabilities of catalase to act as a biocatalyst in non-aqueous media (Magner and Klibanov, 1995), as well, have led to the development of organic-phase enzyme electrodes (OPEE's) for H_2O_2 – monitoring in various anhydrous systems (Campanella *et al.*, 1996), or in water-saturated chloroform (Campanella *et al.*, 1998). The occurrence of solvent-induced changes in the enzyme substrate specificity under such a conditions, provides the principle opportunity for determining of water-insoluble organic peroxides (Wang *et al.*, 1995). A pronounced dependency of enzyme activity on the nature of solvent employed has been proven. It was suggested that, as a rule, enzymatic activity should be related directly to hydrophobicity and inversely to solvent polarity, assuming that in order to reveal its catalytic activity an enzyme, even working in an organic solvent, needs a minimal quantity of water forming its water microenvironment (Campanella *et al.*, 1996; Campanella *et al.*, 1998). Therefore, hydrophilic solvents may remove water needed by the enzyme and consequently tend to substantially enhance conformational inflexibility of the enzyme molecule, which results in a loss of catalytic activity.

Although enzyme behavior and catalytic activity in a non-aqueous medium, as dependent on the type of the solvent used, represent considerable interest in optimizing enzymatic reactions and biosensor research, the problem still has been poorly studied. Accordingly, the present paper deals with immobilized (on carbon black NORIT and soot PM-100) catalase active to decompose dibenzoyl peroxide in some organic solvents (acetonitrile and tetrachloromethane) that differ in their hydrophobicity.

Materials and Methods

Catalase (CAT) was (EC 1.11.1.6) from *Penicillium chrysogenum* 245 (Biovet – Peshtera, Bul-

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garia). The specific activity of the enzyme was 1000 U×mg^{-1} (International enzyme unit: 1U = μmol of substrate reacting or product produced per minute). The reagents for the buffer solutions: $Na_2HPO_4 \times 12H_2O$, KOH, H_3PO_4, citric acid, were of analytical grade.

Acetonitrile and tetrachloromethane for UV spectroscopy (Fluka), were used as reaction medium; dibenzoyl peroxide – BPO, $(C_6H_5CO)O_2$, with analytical grade qualification was purchased from Fluka.

The carbon materials used were: carbon black NORIT-NK and soot PM-100. The two types of carbon materials differ in their structure. The carbon black (NORIT, Amersfoort, The Netherlands) has a fine-grain structure, with an average size of particles of 5×10^3 – 45×10^3 nm and the PM-100 are built up of larger globular particles with an average size of 2.1×10^4 – 3.4×10^5 nm. The soot PM-100 was kindly provided by Prof. V. A. Bogdanovskaya, Institute of Electrochemistry, Moscow, Russia.

Catalase immobilization was performed by adsorption of the enzyme on both kinds of carbon materials under static conditions. 10 mg of adsorbent were added to a 1-ml reaction volume including catalase with a start concentration of the enzyme of 10^{-4} M in phosphate-citrate buffer (pH = 7.0). The amount of the enzyme adsorbed was determined spectrophotometrically by decrease of the catalase concentration in the solution after adsorption. The spectrophotometer used was Specord UV VIS (Carl Zeiss, Jena, Germany). The amount of the catalase in the buffer solution was determined by its 280-nm optical density on the basis of a calibration graph.

The catalytic activity of catalase immobilized on both types of carbon materials was estimated by the rate of BPO decomposition in both non-aqueous solvents. The enzyme reaction kinetics was monitored by quantitative UV-spectrophotometry at λ_{max} = 235 nm (in acetonitrile) and λ_{max} = 276 nm (in tetrachloromethane). All experiments were performed 3–5 times with a standard deviation of 3%.

For maintaining constant temperature a thermostat UH (VEB MLW Prüfgeräte Werk, Medingen, Freital, Germany) was used. A pH-meter OP-208 (Radelkis, Budapest, Hungary) was used in the preparation of the buffer solutions.

Results and Discussion

Fig. 1. depicts the substrate concentration changes with time in the heterogeneous decomposition of dibenzoyl peroxide in acetonitrile (Fig. 1a) and tetrachloromethane (Fig. 1b) catalyzed by immobilized catalase. From these kinetic curves it is seen that the rate of the catalytic process observed depends specifically on the nature of the support used for catalase immobilization (Fig. 1a, b; curves 1, 1′ and 2, 2′) and on temperature (Fig. 1a, b; curves 1, 2 and 1′, 2′). The dependencies presented at Fig. 1 show the typical pattern of enzyme-catalyzed reactions. The kinetic parameters of the enzyme process – the apparent Michaelis constant K_m^{app} and maximum reaction rate V_{max}, calculated from the dependency of the initial rate of the enzyme-catalyzed process on

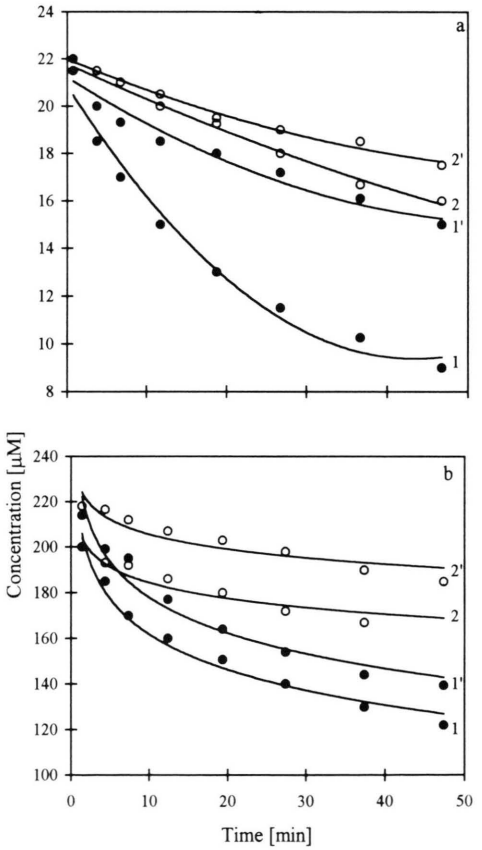

Fig. 1. Kinetic curves of enzyme-catalyzed decomposition of dibenzoyl peroxide in acetonitrile (a) and tetrachloromethane (b); catalyst: CAT/NORIT (1, 1′); CAT/PM-100 (2, 2′); temperatures, °C: 10 (1, 2); 25 (1′, 2′); substrate start concentrations, μM: 24.4 (a); 220 (b).

substrate start concentration (Lineweaver-Burk method), are presented in Table I. Different dispersity of the support used for enzyme immobilization has no major effect both on the apparent Michaelis constant and V_{max}. These parameters are independent on the immobilization support and its values are very similar in acetonitrile as well as in tetrachloromethane.

In the other hand, the influence of the nature of the reaction medium (and particularly its polarity) on kinetic parameters is significant. Comparing the apparent Michaelis constants for dibenzoyl peroxide decomposition catalyzed by CAT/NORIT, it could be seen that its value is 8 times lower in polar solvent – acetonitrile (DEC = 37.50) than in tetrachloromethane (DEC = 2.24). Analogously, for the same process catalyzed by CAT/PM-100 the value of K_m^{app} is 7 times lower in acetonitrile than in tetrachloromethane. The higher value of the Michaelis constant in non-polar reaction medium is probably due to the reduced enzyme affinity for the substrate dibenzoyl peroxide in tetrachloromethane. The lower values of the Michaelis constants in acetonitrile are probably due to the higher enzyme affinity toward the substrate. Such an inverse proportionality between K_m^{app} and catalytic rate constants (k_{sp}, determined graphically according to the first order kinetic equation, Fig. 2.) were found for the process catalyzed by CAT/NORIT: the specific rate constant detected in acetonitrile is higher than that observed in tetrachloromethane. Inversely, the corresponding value of the apparent Michaelis constant in the first case is lower than in CCl_4. This finding could be explained by the effect of substrate distribution between the bulk solution and the matrix for enzyme immobilization. The apparent Michaelis constant K_m^{app} for a reaction catalyzed by the immobilized enzyme is given by: $K_m^{app} = K_m \frac{[S]_b}{[S]_m}$, where: K_m is the Michaelis constant for the reaction catalyzed by the native enzyme; $[S]_b$ and $[S]_m$ are the substrate concentrations in the bulk solution and at the matrix surface, respectively.

Probably, in the polar hydrophilic solvent acetonitrile, the substrate is more concentrated onto the surface of the support for catalase immobilization and the ratio $[S]_b/[S]_m$ is less than 1 which leads to the decrease of K_m^{app} value, and consequently to increase in the specific rate constant according to the equation: $v = \frac{V_{max}}{K_m^{app} + [S]}$, where K_m^{app} is the apparent Michaelis constant; [S] is the substrate concentration; V_{max} is the maximum reaction rate.

In the non-polar hydrophobic medium – tetrachloromethane however, the higher value of apparent Michaelis constant and reduced rate con-

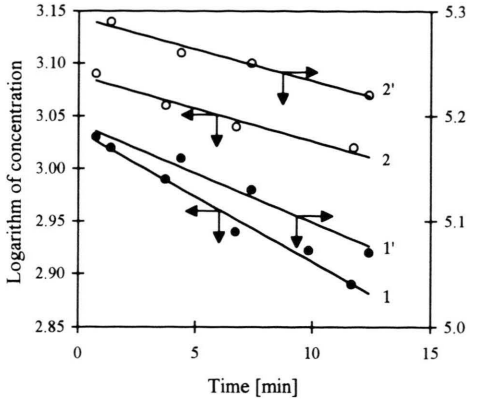

Fig. 2. Dependence of the logarithm of substrate concentration on time for enzyme-catalyzed decomposition of dibenzoyl peroxide in organic solvents; catalyst: CAT/NORIT (1, 1′); CAT/PM-100 (2, 2′); reaction medium: acetonitrile (1, 2) – primary axis; tetrachloromethane (1′, 2′) – secondary axis; temperature: 25° C. The arrows indicate corresponding *X*-*Y* axis.

Table I. Kinetic parameters* of the enzyme-catalyzed decomposition of dibenzoyl peroxide in organic solvents at 298 K.

Acetonitrile	Tetrachloromethane
Catalyst CAT/NORIT	
K_m^{app} = 23.7 μM	K_m^{app} = 189.7 μM
V_{max} = 5.3×10^{-2} μM×s^{-1}×mg^{-1}	V_{max} = 15.5×10^{-2} μM×s^{-1}×mg^{-1}
k_{sp} = 7.7×10^{-4} s^{-1}×mg^{-1}	k_{sp} = 4.8×10^{-4} s^{-1}×mg^{-1}
E_a = 25.1 kJ×mol^{-1}	E_a = 34.2 kJ×mol^{-1}
Z_0 = 19.5 s^{-1}×mg^{-1}	Z_0 = 479 s^{-1}×mg^{-1}
Catalyst CAT/PM-100	
K_m^{app} = 31.0 μM	K_m^{app} = 212.1 μM
V_{max} = 2.1×10^{-2} μM×s^{-1}×mg^{-1}	V_{max} = 13.1×10^{-2} μM×s^{-1}×mg^{-1}
k_{sp} = 2.4×10^{-4} s^{-1}×mg^{-1}	k_{sp} = 2.4×10^{-4} s^{-1}×mg^{-1}
E_a = 15.8 kJ×mol^{-1}	E_a = 18.6 kJ×mol^{-1}
Z_0 = 0.2 s^{-1}×mg^{-1}	Z_0 = 0.4 s^{-1}×mg^{-1}

* K_m^{app} – the apparent Michaelis constant; V_{max} – the maximum rate of enzyme-catalyzed reaction; k_{sp} – the specific rate constant; E_a – the activation energy; Z_0 – the pre-exponential factor in the Arrhenius relation; CAT/NORIT – catalase immobilized on carbon black NORIT; CAT/PM-100 – catalase immobilized on soot PM-100.

stant is probably due to the higher dibenzoyl peroxide concentration in the bulk solution so that the ratio $[S]_b/[S]_m$ is higher than one.

Regardless of the different values of K_m^{app} in acetonitrile and tetrachloromethane, catalytic rate constants of the process catalyzed by CAT/PM-100, are practically identical. In this case we can conclude that substrate distribution between bulk solution and immobilization matrix has no effect on the rate of the process.

The maximum rates V_{max} also depend on the nature of the solvent employed and correlate with respective K_m^{app} values (Table I).

To clarify the basic laws of catalysis with catalase adsorbed on two different carbon matrices (carbon black NORIT and soot PM-100) the effect of the temperature on the rate of dibenzoyl peroxide decomposition was investigated in both reaction media.

Effect of the matrix for catalase immobilization on catalytic activity

Because of the different nature of the support used we can expect some variance in the kinetic characteristics of catalytic systems: CAT/NORIT and CAT/PM-100. The catalytic activity of immobilized catalase on dibenzoyl peroxide decomposition within the temperature range of 283 to 303 K was considered.

For the process catalyzed by CAT/PM-100 within the specified temperature interval, in acetonitrile the specific rate constant increased 1.5 times: at T = 283 K; $k_{sp} = 1.7\times10^{-4}$ $s^{-1}mg^{-1}$; at T = 303 K; $k_{sp} = 2.5\times10^{-4}$ $s^{-1}mg^{-1}$. The activation energy E_a = 15.8 kJ×mol^{-1} (Table I) was determined graphically from an Arrhenius plot (Fig. 3) and probably represents a diffusion-limited process.

In the same reaction medium the specific rate constant of dibenzoyl peroxide decomposition catalyzed by CAT/NORIT doubled within this temperature range: at T = 283 K; $k_{sp} = 0.8\times10^{-3}$ s^{-1} mg^{-1}; at T = 303 K; $k_{sp} = 1.7\times10^{-3}$ $s^{-1}mg^{-1}$. The higher activation energy E_a = 25.1 kJ×mol^{-1} (Table I) indicates that most probably the rate-limiting step of the process is enzyme-catalyzed reaction. Comparing data for the specific rate constants at 298 K (Table I) it is seen that the rate of BPO decomposition catalyzed by CAT/NORIT

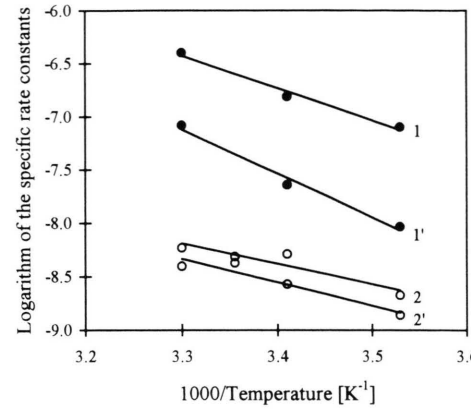

Fig. 3. Arrhenius plot of the enzyme-catalyzed decomposition of dibenzoyl peroxide in organic solvents; catalyst: CAT/NORIT(1, 1′); CAT/PM-100 (2, 2′); reaction medium: acetonitrile (1, 2); tetrachloromethane (1′, 2′).

is 3 times higher than the rate of the same process, but when catalyzed by CAT/PM-100.

Similar regularities were observed in tetrachloromethane on BPO decomposition by immobilized catalase. The rate constant of the process catalyzed by CAT/PM-100 increased 1.6 times within temperature range investigated: at T = 283 K; $k_{sp} = 1.4\times10^{-4}$ $s^{-1}mg^{-1}$; at T = 303 K; $k_{sp} = 2.3\times10^{-4}$ $s^{-1}mg^{-1}$, and the activation energy value E_a = 18.6 kJ×mol^{-1} (Table I) show that the process is probably controlled by diffusion. Under the same conditions (temperature range and reaction medium) the specific rate constant of the BPO decomposition catalyzed by CAT/NORIT increased 2.6 times: at T = 283 K; $k_{sp} = 3.3\times10^{-4}$ $s^{-1}mg^{-1}$; at T = 303 K; $k_{sp} = 8.4\times10^{-4}$ $s^{-1}mg^{-1}$. The activation energy determined E_a = 34.2 kJ×mol^{-1} (Table I) imply that most probably the process is controlled by the enzyme-catalyzed reaction. Comparing values of the specific rate constant of BPO decomposition in tetrachloromethane (Table I) it is obvious that catalytic activity of catalase immobilized on carbon black NORIT is ~2 times higher than that of the catalytic system CAT/PM-100.

Summarizing, on the basis of the values for E_a and the temperature effect on the rate of the process it can concluded that the decomposition rate of BPO exhibit the typical pattern of a process which is either diffusion-limited (catalyzed by CAT/PM-100) or located in the kinetic range of catalysis (using the catalytic system CAT/NORIT). The difference in the rate-determining step of

BPO decomposition, depending on the adsorbent for catalase immobilization, can possibly be explained with the difference in the structure of the two kinds of carbon materials. As reported (Berezin *et al.*, 1987) the rate of reactions with immobilized enzymes decreases with the growth of the size of the support particles.

Effect of the reaction medium on catalytic process

As it is seen from the data presented in Table I in both organic media the first order rate constants k_{sp} for BPO decomposition catalyzed by CAT/PM-100 are identical. The independence of the reaction rate on the nature of the solvent employed is probably a result of diffusion control over the process indicated by the poor dependence on temperature as well as the low values of activation energy.

The effect of the solvent is clearly demonstrated on BPO decomposition catalyzed by CAT/NORIT: the specific rate constant is almost two times higher in acetonitrile than in tetrachloromethane (Table I). In both reaction media the activation energy as well as the stronger dependency of the rate on temperature show that the process takes place in the kinetic range of catalysis. The lower value of E_a for the process in acetonitrile explains the higher catalytic activity of CAT/NORIT in this case.

From the data presented in Table II it is seen that the Gibbs energy of activation does not depend substantially on the solvent employed, but the values of ΔH^* and ΔS^* for both catalytic systems (CAT/NORIT and CAT/PM-100) are lower in acetonitrile than in tetrachloromethane. This finding can be explained with different dielectric constants of the solvents: lower dielectric constant of the solvent (CCl_4, DEC = 2.24), leading to increase of ΔS^* and ΔH^*, is most probably a result of smaller solvatation of the substrate in this case than in acetonitrile. As a possible result of enhanced solvatation, the values of the steric factor P also indicate stronger steric interferences in the polar solvent acetonitrile (DEC = 37.50).

Table II. Activation parameters* of the decomposition of dibenzoyl peroxide in acetonitrile and tetrachloromethane catalyzed by immobilized catalase.

Acetonitrile	Tetrachloromethane
Catalyst CAT/NORIT	
$\Delta G^* = 90.7$ kJ×mol^{-1}	$\Delta G^* = 91.9$ kJ×mol^{-1}
$\Delta H^* = 22.6$ kJ×mol^{-1}	$\Delta H^* = 31.8$ kJ×mol^{-1}
$\Delta S^* = -228.5$ J×K^{-1} ×mol^{-1}	$\Delta S^* = -201.9$ J×K^{-1} ×mol^{-1}
$P = 1.2\times10^{-12}$	$P = 2.8\times10^{-11}$
Catalyst CAT/PM-100	
$\Delta G^* = 93.6$ kJ×mol^{-1}	$\Delta G^* = 88.1$ kJ×mol^{-1}
$\Delta H^* = 13.3$ kJ×mol^{-1}	$\Delta H^* = 16.1$ kJ×mol^{-1}
$\Delta S^* = -269.4$ J×K^{-1} ×mol^{-1}	$\Delta S^* = -241.5$ J×K^{-1} ×mol^{-1}
$P = 8.4\times10^{-15}$	$P = 2.4\times10^{-13}$

* The activation parameters: entropy of activation (ΔS^*), enthalpy of activation (ΔH^*) and Gibbs energy of activation (ΔG^*), were calculated according to the basic equation of the transition state theory. The steric factor P (dimensionless), was calculated by the expression:
$P = e^{(\Delta S^*/R)}$.

Conclusions

The results discussed may be summarized as follows:

- The apparent Michaelis constant K_m and the maximum reaction rate V_{max} of BPO decomposition catalyzed by immobilized catalase depend on the nature of the reaction medium and particularly, on its polarity. Higher values of these kinetic parameters were detected in tetrachloromethane (non-polar solvent) than in acetonitrile (polar solvent).
- The effect of the support for catalase immobilization on decomposition of dibenzoyl peroxide both in tetrachloromethane and acetonitrile was investigated, using two catalytic systems: CAT/NORIT and CAT/PM-100. On the basis of the values for E_a and the temperature effect on the rate of the process it was concluded that the decomposition rate of BPO exhibited the typical pattern of a process which is either diffusion-limited (catalyzed by CAT/PM-100) or produced in the kinetic range (using CAT/NORIT) of catalysis.
- The effect of the reaction media for the same process was studied.
  - It was found that the solvent employed does not influence on the rate of BPO decomposition catalyzed by CAT/PM-100, probably because of the diffusion control over the process.
  - The effect of reaction medium is demonstrated on BPO decomposition catalyzed by CAT/NORIT. A higher catalytic rate constant was found in acetonitrile as a conse-

quence of lower activation energy of the process.

- The values of the steric factor P, calculated on the basis of ΔS^*, indicate stronger steric interferences in acetonitrile probably as a result of enhanced solvatation of the substrate in this case.

Acknowledgements

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