Kinetic study of catalase adsorption on disperse carbonaceous matrices

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Abstract: The effect of the main factors known to govern the kinetic regularities of enzyme adsorption, such as enzyme solution concentration, temperature, pH, specific surface of the adsorbent, etc., were studied. Two kinds of disperse carbonaceous materials - activated carbon NORIT and carbon black PM-100, were used as matrices for enzyme immobilization. For both immobilization matrices studied, the amount of the adsorbed enzyme was found to reach saturation at catalase (CAT) enzyme concentrations exceeding 20 mg·mL⁻¹ (∼100 µM). The pH of the solution affected the adsorption capacities of the selected immobilization matrices; larger amounts of CAT adsorbed were estimated in neutral and alkaline solutions than under acidic conditions for enzyme immobilization. UV-spectrophotometry was employed as a basic analytical approach in this study.

Keywords: Catalase, enzyme adsorption, optimization

1 Introduction

Enzymes, being exceptionally selective biocatalysts of processes and chemical changes both within and outside of living cells, represent considerable interest not only for the theory of heterogeneous catalysis, but also for solving particular practical problems of electrochemistry, enzymology, bio- and sensor technologies.

The immobilization of enzymes on a solid surface or their inclusion in polymeric matrices are effective methods of increasing their stability [1, 2]. Under these conditions, enzymes retain their molecular integrity, a conformation close to their native one, and high activity.

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The immobilization of enzymes by means of adsorption on insoluble support is distinguished by its exceptional simplicity, and is achieved when aqueous solution of the enzyme contacts the support. The relative rate of interactions (van der Waals, electrostatic, hydrogen links, and hydrophobic ones) between the support and the enzyme depends on the chemical nature of the matrix, on the functional groups on the surface of the enzyme molecule and on the conditions under which the immobilization is performed.

Catalase is one of the most efficient biocatalysts, and its practical use in an immobilized state has enormous prospects in medicine, food industry, and biochemical analysis. The immobilized catalase is used successfully in the food industry for the removal of hydrogen peroxide from food products after cold pasteurization; in the medical field as a component of biosensor systems for analysis of hydrogen peroxide [2-5].

In this regard, numerous studies report catalase immobilization in different ways on various solid substrates, such as artificial membranes [6, 7], alum \( \text{AlK(SO}_4\text{)}_2 \cdot 12\text{H}_2\text{O} \), gelatin, polyacrylamide, egg shells [8], carbon materials [9], and alumina pellets [10].

Despite the numerous theoretical models of protein adsorption on solid/liquid, liquid/liquid or liquid/air interfaces, the enzyme immobilization via adsorption in terms of the practical aspects of its optimization still remains rather poorly discussed in current literature [11].

The present paper deals with the optimization of the enzyme adsorbtional immobilization, and study of the objective regularities of this process through examining the influence of various factors (the specific surface and dispersiveness of the support, temperature, pH of the enzyme solution and its concentration) on the immobilization of the enzyme upon selected carbonaceous matrices.

2 Experimental

2.1 Reagents

Catalase (CAT; EC 1.11.1.6) purified from \textit{Penicillium chrysogenum} 245 (Biovet-Peshtera, Bulgaria) with relative molecular weight of \(~245 \text{ kDa} \) (pI \( ~7.0 \)) and a specific homogeneous activity of the native enzyme of 1000 \( \text{U/mg} \) protein was used. (1U = 1 \( \mu \text{mol of substrate consumed or 1} \mu \text{mol of product formed per minute at 25}^\circ\text{C and optimum pH}).

Buffer solutions were prepared with \( \text{Na}_2\text{HPO}_4\cdot12\text{H}_2\text{O}, \text{KOH, H}_3\text{PO}_4 \), and citric acid, with all reagents being of analytical grade.

The disperse carbon materials employed were: activated carbon NORIT-NK and carbon black PM-100. The differences in the structure of the two types of carbon materials were as follows: the activated carbon (NORIT, Amersfoort, The Netherlands) has a fine-grain structure, with an average size of particles of 5 - 45 \( \mu \text{m} \) and a specific surface area \( S_{\text{spec}}=1500 \text{ m}^2\cdot\text{g}^{-1} \), while the PM-100 are built up of larger globular particles with an average size of 21 - 340 \( \mu \text{m} \) and \( S_{\text{spec}}=100 \text{ m}^2\cdot\text{g}^{-1} \). The carbon black PM-100 was kindly provided by Prof. V.A. Bogdanovskaya, Institute of Electrochemistry, Moscow, Russia.
2.2 Procedure and apparatuses

Catalase immobilization on both kinds of disperse carbonaceous materials was performed by adsorption under static conditions (i.e. without stirring), according to the following immobilization protocol: 20 mg of adsorbent were added to a 2-mL enzyme solution (in phosphate-citrate buffer with pHs 3.02, 7.02 or 11.21) for 24 h. For investigating the dependence of the amount of the catalase adsorbed on its start concentration, the enzyme solution concentration was varied from 2 to 200 µM. The amount of the enzyme adsorbed was estimated by using the differences of 280-nm absorbance of the enzyme solution before and after adsorption. The spectrophotometer used was Specord UV VIS (Carl Zeiss, Jena, Germany).

Kinetic studies of the adsorption process were performed at 4 °C, at ambient temperature (24±1 °C) or thermostated at 32 or 40 °C. For the low-temperature experiments the samples were incubated in a refrigerator at 4 °C between measurements. During the measurements, a constant temperature in the reactor was achieved by means of a thermostat UH (VEB MLW Prüfgeräte-Werk, Sitz Freital, Germany), the pH of the buffer for enzyme solution was adjusted using a pH-meter OP-208 (Radelkis, Budapest, Hungary).

All experiments were performed 3-5 times with a standard deviation of 5–8 %.

3 Results and discussion

The dependence of the amount of adsorbed catalase (CAT) upon the examined adsorbents on the concentration of the enzyme in the solution (pH = 11.21) is depicted on Fig. 1. As the concentration of the start enzyme solution increases, the quantity of adsorbed CAT increases hyperbolically to \( C_{\text{CAT}} = 20 \text{ mg·mL}^{-1} \), afterwards it remains virtually constant and is characterized for NORIT and PM-100, accordingly, by the maximum values of 60 mg·g\(^{-1}\) and 50 mg·g\(^{-1}\). Similar dependencies were noticed at both other pHs examined.

Besides the type of the adsorbent, the maximum amount of adsorbed CAT also depends on the pH of the initial enzyme solution (Table 1). With the three examined pH values, the adsorption capacity of NORIT is higher than that of PM-100. This is probably related to the specific surface of NORIT being about 10 times higher than that of PM-100.

| Adsorbent              | Adsorption capacity, mg·g\(^{-1}\) |
|------------------------|-----------------------------------|
|                        | pH=3.02  | pH=7.02  | pH=11.21 |
| Activated carbon NORIT | 40       | 60       | 58       |
| Carbon black PM-100    | 28       | 40       | 50       |

*Table 1* Capacity of adsorption of the disperse carbonaceous matrices on catalase immobilization at acidic, neutral and alkaline pH of the solution for enzyme immobilization.

The data presented in Table 1 reveals that both supports have a higher adsorption capacity in CAT immobilization by start solutions with neutral and alkaline pH. The probable reason for the increase of the adsorbed catalase on both substrates under study,
when immobilization is performed with solutions with pH of 7.02 and 11.21, is the occurrence of electrostatic interactions between the enzyme molecule and the supports. As discussed in the literature [12, 13], electrostatic interaction, facilitating the process of adsorption, occurs between the enzyme molecules and the immobilization matrix.

For the average levels of completion, the amount of CAT adsorbed on both matrices depends linearly upon the logarithm of enzyme concentration estimated at equilibrium (Fig. 2), according to the equation [14]:

$$\Gamma = \text{const} + \frac{1}{f} \ln C$$  \hspace{1cm} (1)

where $\Gamma$ is the amount of catalase adsorbed; $f$ is usually called non-homogeneity factor; and $C$ is the concentration of the enzyme at equilibrium. The above equation expresses Tyomkin’s adsorption isotherm, indicating that for both matrices investigated, an adsorption upon energetically heterogeneous surfaces takes place.

It should be pointed out that the enzymatic activity of catalase immobilized on both supports was tested at all pHs specified of the reaction medium [9, 15]. At the optimum pH (7.02), the activity of catalase adsorbed on NORIT and PM-100 towards its typical substrate -hydrogen peroxide, was found to be 4.8 and 10.8, times lower than the homogeneous enzyme activity (dissolved CAT), respectively. Within six repetitive stability tests of both biocatalytic systems of interest (CAT/NORIT and CAT/PM-100), their specific catalytic rate constants were found to decrease about three times [9]. Furthermore, the heterogeneous catalytic systems discussed have been successfully used for decomposition of aryl-, acyl- and hydro-peroxides in acetonitrile [16-18] and tetrachloromethane [18, 19], as well.

It has been shown that at pHs below 3.5 and above 11, catalase enzyme loses partially or completely its ability to decompose hydrogen peroxide, but shows peroxidase-like
activity, i.e. it is capable of oxidating hydrogen donors (phenols, aromatic amines etc.) in the presence of hydrogen peroxide [20]. Under our experimental conditions, both in acidic (pH 3.02) and alkaline (pH 11.21) solutions [15], alteration of the enzyme substrate specificity induced by the reaction medium has been detected only for immobilized on NORIT catalase.

Fig. 3 (a, b) represents the kinetic curves of CAT adsorption of $2\cdot10^{-4}$ M enzyme solution with pH=11.21 on the examined adsorbents - NORIT (Fig. 3a) and PM-100 (Fig. 3b). These curves depict the change of the enzyme concentration in the solution of immobilization, depending on the time and temperature of performing the process. It is obvious that on both adsorbents the CAT concentration in the solution decreases with time. The linear progress of the dependency $\ln C - t$ (Fig. 4) proves that the CAT adsorption on both kinds of carbon matrices is in accordance with the kinetic equation of a first order reaction. The graphically determined rate constants of adsorption are depicted in Table 2. Apparently the values of the specific rate constants of CAT adsorption depend both on the dispersiveness of the support and on the pH of the solution for immobilization. The rate of CAT adsorption on PM-100 carbon black, in immobilization both by alkaline enzyme solutions (pH=11.21) and by neutral solutions (pH=7.02), is twice ($T_{ads}$=277 K) and 1.2 times ($T_{ads}$=297 K) as high as that on NORIT, respectively. Most probably, the coarser-grained structure of carbon black PM-100 (average size of the particles - 21-10^4 - 45-10^4 Å) is more favorable for retaining a conformation, close to the native one of the catalase macromolecules on the surface, than that of NORIT, composed of finer-grained particles (5-10^4 - 45-10^4 Å), which accounts for the higher rate of immobilization on that adsorbent.

As the temperature increases from 4 °C to 24±1 °C, the rate of adsorption also rises.
Fig. 3 Kinetic curves of catalase adsorption on activated carbon NORIT (a) and on carbon black PM-100 (b) at temperatures: 1) 4 °C; 2) 24±1 °C; 3) 32 °C. Start concentration of the enzyme solution 200 µM; pH = 11.21.

(Fig.3 a and b). At $T_{ads} = 32$ °C, the kinetic curve of CAT adsorption on NORIT overlaps with that at $T_{ads} = 4$ °C (Fig. 3a, curves 1 and 3), whereas on PM-100 at $T_{ads} = 32$ °C the adsorption of CAT is performed much slower (Fig. 3b, curve 3). Since the overall immobilization process possesses a complex and multistep mechanism, including mass transport, conformation changes, attachment to the surface, the reverse to adsorption process, desorption, stages etc., we could only hypothesize the most probable reasons for the temperature effect noticed on the adsorption rate. The rise of the adsorption rate between 4 and 24 °C could be attributed either to the enhanced thermal motion of the molecules, or to conformational changes of the protein globules promoted by the temperature increase [1].
Fig. 4 Dependencies of $\ln C$ on time for catalase adsorption on: (a) activated carbon NORIT; (b) carbon black PM-100. The adsorption process was carried out at temperatures: 1) 4°C; 2) 24±1°C; 3) 32°C; start concentration of the enzyme solution 200 µM; pH = 11.21.

The decrease of the detected adsorption rate at $T_{ads}$ above 24-30°C could be due to the fact that as temperature increases - desorption of CAT (which is favored by higher temperatures due to thermodynamic reasons) predominates. It is determined spectrophotometrically that after an hour of incubation at 40°C of PM-100 samples with catalase, the enzyme concentration increases 30%, and of the catalase adsorbed on NORIT - 25%. After incubating the samples under the same conditions for one more hour, no additional increase of catalase concentration is observed in the PM-100 samples, indicating that an adsorption-desorption equilibrium is reached, while those of the NORIT support desorb another 27% of enzyme.

The data for the specific rate constants, depicted in Table 2, also illustrate the in-
fluence of pH of the enzyme solution on the CAT adsorption rate. The adsorption of CAT on activated carbon NORIT at $T_{ads} = 297$ K takes place at a rate 7.2 times higher, when the immobilization is performed by a solvent with $pH = 11.21$, as that performed by a solvent with $pH = 7.02$. An analogous regularity is observed with regard to the rate of CAT adsorption on PM-100 carbon black, which is 6 times as high as the adsorption of CAT by alkaline solutions. As it was discussed above, the observed regularity is probably due to the presence of electrostatic interactions between the immobilization matrices and the enzyme.

| Adsorbent | Kinetic parameters | Activation parameters at pH 11.21 |
|-----------|--------------------|-----------------------------------|
|           | $k_{sp} \cdot 10^2$, min$^{-1}$·g$^{-1}$ | $E_a^*$, kJ·mol$^{-1}$ | $\Delta G^*$, kJ·mol$^{-1}$ | $\Delta H^*$, kJ·mol$^{-1}$ | $\Delta S^*$, J·K$^{-1}$·mol$^{-1}$ |
| NORIT     | pH 7.02            | pH 11.21                         |                   |                       |                      |
|           | 0.77$^b$           | 1.33$^a$                         | 51.26             | 79.56                  | 48.78                 | -103.9               |
| PM-100    | 0.93$^b$           | 2.50$^a$                         | 23.28             | 79.72                  | 20.82                 | -198.9               |

* The activation energy $E_a$ was calculated according to the Arrhenius equation: $\ln \frac{k_2}{k_1} = \frac{E_a}{R T_2} - \frac{E_a}{R T_1}$ for the temperature range 277 – 297 K.

** The activation parameters: entropy of activation ($\Delta S^*$), enthalpy of activation ($\Delta H^*$), and Gibbs energy of activation ($\Delta G^*$), were calculated at temperature 297±1 K by using the basic equation of the transition state (activated complex) theory: $k = \frac{k_B T}{h} e^{\left(\frac{\Delta S^*}{R}\right) - \left(\frac{\Delta H^*}{RT}\right)}$, where $k_B$ is the Bolzmann constant; $h$ is Planck constant; $\Delta S^*$ is the activation entropy change, $\Delta H^*$ is the activation enthalpy change; $R = 8.314$ J·K$^{-1}$·mol$^{-1}$ is the gas constant.

$E_a = \Delta H^* + RT$

$\Delta G^* = \Delta H^* - T \Delta S^*$

$
\Delta G^*$ is the Gibbs energy of activation change.

$^a,b$ the values are determined at temperatures 277±1 and 297±1 K, respectively.

Table 2 Kinetic and activation parameters** at catalase adsorption onto disperse carbon materials (activated carbon NORIT and carbon black PM-100) depending on the immobilization matrix, the pH of the enzyme solution and the temperature of immobilization.

The activation energy ($E_a$) determined within the temperature range 4-24˚C (Table 2) shows that the process of adsorption of CAT is limited differently, depending on the dispersiveness of the support. Because of the poor dependency of the adsorption rate constants on temperature, immobilization of CAT on PM-100 carbon black is most probably limited by diffusion ($E_a = 23.28$ kJ·mol$^{-1}$), while on NORIT, it is taking place in the kinetic regime ($E_a = 51.26$ kJ·mol$^{-1}$).

The data concerning the activation parameters of the process (Table 2) clearly show that the free energy of activation does not depend on the type of the adsorbent, whereas the values of $\Delta H^*$ and $\Delta S^*$ for NORIT and PM-100 are different. The lower values of $\Delta H^*$ and $\Delta S^*$ in the adsorption of CAT on PM-100 indicate that the immobilization of CAT is accompanied by less significant conformation changes of the protein molecule than its adsorption on NORIT.

The steric factor $P = e^{\Delta S^*/R}$ of the adsorption process was calculated using the
presented in Table 2 values of the entropy of activation change. For both immobilization matrices $\Delta S^*$ is negative, i.e. the values calculated for $P$ are much smaller than 1: $P = 3.73 \cdot 10^{-6}$ for catalase adsorption on NORIT; $P = 4.07 \cdot 10^{-11}$ for catalase adsorption on PM-100. These values ($P \ll 1$) indicate that the adsorption process on both immobilization matrices is impeded by steric reasons that are much larger in the case of carbon black PM-100.

4 Conclusions

The regularities observed at studying the effect of various factors on catalase adsorption onto disperse carbonaceous matrices could be summarized as follows:

- For both immobilization matrices studied, the amount of the adsorbed enzyme reaches saturation at catalase concentrations exceeding 20 mg.ml$^{-1}$ ($\sim$100 $\mu$M). The maximum adsorption capacities at the enzyme isoelectric point (pH 7.02) were found to be 60 mg.g$^{-1}$ and 40 mg.g$^{-1}$ for NORIT and PM-100, accordingly.

- The pH of the enzyme maternal solution was found to affect the following parameters of the immobilization process:
  (a) Larger adsorption capacities for both carbonaceous matrices were determined in neutral (pH 7.02) and alkaline (pH 11.21) medium;
  (b) The rate of the enzyme adsorption increases with the pH of the solution for enzyme immobilization. In alkaline medium the rates of catalase adsorption on both carbonaceous matrices, determined at room temperature, are practically identical. Yet, in neutral solutions at the same temperature, the adsorption of the enzyme on carbon black takes place 1.2 times faster than on the activated carbon.
  (c) The enzyme load on NORIT activated carbon is larger than on PM-100 carbon black under all investigated pHs of the maternal enzyme solution.

- Catalase adsorption on both carbonaceous matrices obeys the Tyomkin adsorption isotherm, i.e. it is indicative for adsorption upon energetically non-homogeneous surface. The rate limiting stage of adsorption depends on the adsorbent nature; the process is activation-limited on NORIT activated carbon, while on PM-100 carbon black is limited by diffusion.

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