Influence of macromolecular crowding on the oxidation of ABTS by hydrogen peroxide catalyzed by HRP

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ABSTRACT

The interior of the living cell is highly concentrated and structured with molecules having different shapes and sizes. However, almost all experimental biochemical data have been obtained working in dilute solutions that do not reflect *in vivo* conditions. In this paper, we study *in vitro* the effect of macromolecular crowding on the reaction rates of the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by hydrogen peroxide (H_2O_2) catalyzed by Horseradish Peroxidase (HRP), by adding Dextrans of various molecular weights to the reaction solutions as crowding agents. The results indicate that the volume occupied by the crowding agent, regardless its size, plays an important role in the rate of this reaction. Both Michaelis-Menten parameters, v_{max} and K_m, decrease when the Dextran concentration in the sample increases, which might be due to a crowding-induced effect in the catalytic constant, k_{cat}, of this enzymatic reaction. Thus, our results suggest that there is an activation control of the enzymatic reaction in this particular system. In our opinion, this work could facilitate the understanding of biomolecules behavior *in vivo* and be useful for biotechnology *in vitro* applications, since HRP is widely used in the development of biosensors.

Keywords: enzyme kinetics, HRP, macromolecular crowding

INTRODUCTION

The cell cytosol is an aqueous medium that is crowded with macromolecules and solutes, which occupy up to 40% of its total volume [1]. Therefore, biochemical reactions *in vivo* progress in solutions containing high concentrations of macromolecules. However, studies of biochemical processes *in vitro* have usually been performed in dilute solutions (typically concentrations of macromolecules less than 1 mg/mL). In the cellular environment, the rate of diffusion is reduced [2-8], and can even be anomalous at short time. In addition, the macromolecular crowding agent promotes processes such as protein folding, self-association and protein binding [9-22]. Hence, the presence of large concentrations of inert solutes can affect the enzymatic activity.

However, quite a few studies have explored the effects of crowding on enzyme catalysis, even *in vitro*. The first study on enzymatic reactions in macromolecular crowded media was carried out by Laurent in 1971 [23]. He studied several reactions in polymer media as an initial attempt to describe how the environment affects the intracellular enzyme function, and in all cases studied, the presence of the macrolecules produced a moderate decrease in the apparent Michaelis-Menten (MM) constant, K_m. Some years later, Minton and Wilf [9] studied the effect of macromolecular crowding on the various kinetic steps in the enzymatic processes of glyceraldehyde-3-phosphate dehydrogenase. They predicted that the rate of an enzymatic reaction will decrease when there is an increase in the concentration or size of the crowding agent. In other words, the excluded volume produces a decrease of both the MM constant, K_m, and the catalytic constant, k_{cat}, when the enzymatic reaction follows the Michaelis-Menten mechanism. Nevertheless, most subsequent studies reported that a high concentration of neutral polymers only had a moderate influence on enzyme reactions. Briefly, a slight decrease in K_m is frequently found, regardless of the properties of the crowding agent

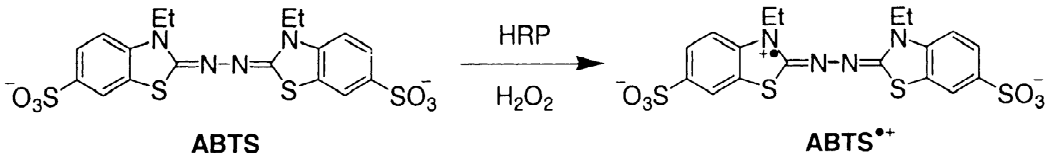
[9, 23-30]. However, the effect of the crowding agent on k_{cat} is diverse: in some cases, k_{cat} increases [24, 28-32], whereas in other cases it decreases [9, 26-27, 32]. In a previous work, we studied the crowding effect of Dextrans of various molecular weights on the reaction rates of the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin [34]. Our results pointed out that the volume occupied by the crowding agent, independent of its size, had an important role in the rate of this reaction. A v_{max} decay and a K_m increase were obtained when the Dextran concentration in the sample was increased. The rise of K_m could be attributed to a slower diffusion of the protein due to the presence of crowding, whereas the decrease in v_{max} could be explained by the effect of mixed inhibition by product, which is enhanced in crowded media.

Although considering the results revealed by the previous studies, the range of experimental conditions covered is not wide enough to fully understand the phenomenology of enzymatic kinetics in crowded media. In order to understand better the effect of macromolecular crowding brought on the enzyme kinetic, we have chosen a reaction catalyzed by Horseradish Peroxidase (HRP). HRP is a protein widely used in the field of biosensors due to its high specificity for hydrogen peroxide (H_2O_2) and we consider that our results could be interesting in this field, too. Therefore, within this paper, we studied the way in which the kinetics of the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by H_2O_2 catalyzed by HRP is affected by the presence of crowding agents of several concentrations and sizes. Thus, we have investigated the effect of the excluded volume in this enzymatic reaction due to the presence of crowders. We chose this reaction as a model process, because it is a well-known reaction [35] and there is only a minimal change in the excluded volume due to the small size of the substrates and products. Macromolecular crowding was mimicked

using Dextrans from 5 to 150 kDa. Some advantages of using HRP protein in this research have been identified: on the one hand, the absence of known interactions with Dextrans (crowding agents); and on the other hand, the protein size (hydrodynamic radius, r_h = 3.0 nm), which is intermediate between those of the selected crowding agents. Within this paper, we examine in what manner this known enzymatic reaction is affected by the presence of Dextrans of different sizes at distinct concentrations. In particular, we analyzed the effect of macromolecular crowding on the values of v_{max} and K_m parameters of this reaction.

RESULTS AND DISCUSSION

In order to carry out a study to understand the effect of macromolecular crowding on biological reaction, it is important that such a reaction to be accompanied by a minimal change in the excluded volume. Therefore, the substrates and the products must be tiny compared with the size of the protein and of the crowding agents. Hence, the effect of these molecules (i.e. substrates and products) on the excluded volume could be neglected. As a result, the effect of macromolecular crowding on the reaction can be interpreted mainly in terms of the presence of crowding agents. This represents one of the reasons for which we chose a well-known peroxidase-catalyzed reaction [36], namely the one electron oxidation of ABTS in the corresponding radical-cation, $ABTS^{\bullet+}$, catalyzed by HRP, shown in equation 3



(3)

Another reason is that this reaction can be easily monitored by UV-spectroscopy. Since 70s, ABTS has been proposed to serve as a chromogen for H_2O_2 assay using HRP, because it has a well-defined absorption maximum at 340 nm (ε_{340} = 36,000 M^{-1} cm^{-1}) [37]. The radical-cation $ABTS^{\bullet+}$ has an absorption maximum at 414 nm (ε_{414} = 36,000 M^{-1} cm^{-1}); it also absorbs at 340 nm (ε_{340} = 5400 M^{-1} cm^{-1}). The ABTS method allows the easy quantification of the initial rates of the enzymatic reaction. Most of the commercially-available peroxidase assays are now using this method [38]. As we investigate within this study the effect of macromolecular crowding on enzymatic reactivity, we need a well-behaved reaction of which kinetics can be interpreted with confidence. The oxidation of ABTS by H_2O_2 is such a process. The values of Michaelis-Menten parameters, v_{max} and K_m reported in diluted solution and the values that we obtained using similar conditions are comparable [39].

Regarding the enzymatic system chosen, we have investigated the effect brought by different concentrations and sizes of the crowding agent (Dextran). In all the cases, we observed a typical initial rise and a subsequent plateau in the absorbance/time plot of the released $ABTS^{\bullet+}$. An example of the kinetic curves obtained is shown in Figure 1. The initial velocity (v_0) was obtained by fitting the initial slope of these curves. The fitted values show a significant decrease of the initial velocity of the reaction with the increase of Dextran concentration. The dependence of v_0 on the substrate concentration for different Dextran concentrations and dimensions is illustrated in Figure 2. Each figure corresponds to a given Dextran concentration and shows the variation of v_0 with the concentration of ABTS. The Dextran concentration value given in mg/mL is proportional to the volume occupied by this crowding agent. Figure 2 reveals the dependence of v_0-substrate concentration curves on the concentration of Dextran. For the same occupied volume, the v_0-substrate concentration curves are similar irrespective

of the obstacles dimension considered. Thus, the value of v_0 does not change with the size of Dextrans, but varies with their concentration, i.e. with the excluded volume. Based on this similarity, for each Dextran concentration, the curves corresponding to the distinct Dextran sizes considered can be grouped into a single average v_0-substrate concentration curve (Figure 3).

The absent dependence of the initial velocity on obstacle dimension is in accordance with the results of our previous work [34]. In addition, these results are also in agreement with Minton et al. [2, 8-12], as these authors predicted that the excluded volume plays an important role in the enzymatic reactions that take place in macromolecular crowded media. We have found that, at least in two cases: the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin [34] and the oxidation of ABTS by H_2O_2 catalyzed by HRP, the total volume excluded by the crowding agents brings a greater impact on the velocity of the reaction than the size of these agents.

In order to better understand in what way the excluded volume affects the hydrolysis reaction of HRP in crowded media, the values of K_m and v_{max} were calculated by fitting the curves in Figure 3 using equation 2. Table 1 shows the values obtained for these parameters as a function of the excluded volume. Our results indicate that both, the value of $v_{max}$and K_m decay as increasing the obstacle concentration in the sample.

Firstly we analyzed the K_m behavior. We found (Table 1) that the values of K_m for the oxidation of ABTS by H_2O_2 catalyzed by HRP decreased with the rise of Dextran concentration, in other words, K_m diminished with the obstacle excluded volume. This result contrast with that reported in our previous work [34]. Within the

study of hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin in crowding media, an increase of K_m with the concentration of Dextran was found. As was explained in detail by Pastor et al. [34] and previously reported by Gellerich et al. [24] and Wenner and Bloomfield [25], this behavior corresponded to a diffusion controlled enzymatic process for the case of alpha-chymotripsin. However, our actual results are in agreement with the major part of the results presented in other studies on crowding effects in enzymatic reactions [9, 24-31]. Majority of these studies report a slight decrease of K_m with respect to dilute solution. The decrease of K_m with crowding could be ascribed to an increase in the ratio of activity coefficients between the native enzyme and the enzyme-substrate complex, or to an increase in chemical activity of the small molecules of substrate in highly non ideal crowded solution [27-29]. The Michaelis-Menten constant depends on the activity coefficients of the free substrate in solution, so it can be expected to be sensitive to solution composition. In addition, substrate binding also requires the dehydration of both the active site and the substrate, and consequently may be dependent on water activity, which affects the k_{cat} value. Therefore, in cases in which the value of K_m decays with the rise of obstacle concentration, the catalytic constant (k_{cat}) exhibits a greater role, as it can be affected by the environmental surroundings.

Secondly we analyzed the behavior of v_{max}. We found (Table 1) a continuous diminishing of the values of this parameter while the concentration of Dextran increases. According to the enzymatic mechanism described in equation 1, v_{max} is defined as $v_{max} = k_{cat}[E]_T$, with $k_{cat} = k_2$. Therefore, its decrease could be interpreted in terms of crowding-induced variations of the catalytic rate constant or of the effective enzyme concentration. This result is in agreement with the major part of previous studies [9, 27-29, 33] and also with the fact that in this work we found a

decrease of K_m values when the concentration of Dextran increases. A drop in v_{max} is usually explained as a result of conformational changes of the enzyme active site that are produced by crowding-induced modifications of its surroundings [9, 27-29, 31], which affects the k_{cat} value. However, we cannot estimate k_{cat} from v_{max}, since it is known that the effective enzyme concentration increases with the excluded volume in crowded media [2, 8-12]. With regard to our results, we can say that, for the investigated reaction catalyzed by HRP in *in vitro* crowded media, the catalytic constant (k_{cat}) exhibits a greater role and it might be affected by the changes in the environmental surroundings due to the presence of crowding agents.

In conclusion, we have studied the kinetics of the oxidation of ABTS by H_2O_2 catalyzed by HRP as a model enzymatic reaction occurring in different *in vitro* crowded media, produced by Dextran of various concentrations and dimensions. Our results reveal that the volume occupied by the crowding agent has a significant impact on the rate of this reaction. We obtained a v_{max} and K_m decay along with the growth of obstacle concentration. Concerning this reaction, the data presented suggest an activation control of the enzymatic reaction in the studied system, meaning that the catalytic constant (k_{cat}) brings a significant contribution as it can be affected by the environmental surroundings. This contribution could be due to an increase in the ratio of activity coefficients between the nature enzyme and the enzyme-substrate complex due to presence of crowding agents, or an increase in chemical activity of water in highly crowded solution, or a crowding-induced conformational change in the enzyme active site, or could be the sum of all these factors. In our opinion, this must be taken into account when studying enzyme-catalyzed reactions that occur within the crowded physiological environment of the cell and also in biotechnology applications like biosensors development.

MATERIALS AND METHODS

Chemicals

Peroxidase (E.C. 1.11.1.7; from horseradish, 1310 Umg^{-1}), which was used without further purification, diammonium salt of ABTS, and 33% aqueous hydrogen peroxide were purchased from Sigma-Aldrich Chemical (Milwaukee, WI, USA). Dextran (from *Leuconostoc mesenteroides*) of molecular weight of 5, 50 and 150 kDa was purchased from Fluka (Buchs, Switzerland). The polydispersities of the Dextrans were less than 2.0, as reported by the manufacturer. All other chemicals were of analytical or spectroscopic reagent grade. Concentrations of ABTS (ε_{340} = 36,000 M^{-1} cm^{-1}) and H_2O_2 (ε_{240} = 39.4 M^{-1} cm^{-1}) in stock solutions were determined by ultraviolet-visible (UV-VIS) measurements.

Oxidation of ABTS

All reactions were carried out at room temperature in phosphate buffer (0.1 M, pH = 7.4). The reaction mixture contained a fixed concentration of 10 nM of HRP and 10μM of H_2O_2, the concentration of ABTS varied between 0 and 23 μM, and the concentration of Dextran varied between 0 and 200 mg/mL. The reaction was initiated by the addition of HRP to a sample mixture of ABTS and H_2O_2 and Dextran in phosphate buffer, using a syringe to favor mixing. The reaction progress was followed by monitoring the release of ABTS radical-cation ($ABTS^{\bullet+}$) (absorbance at 414 nm) with a UV spectrophotometer (UV-1603 Shimadzu). At the beginning of the reaction, the product absorbance increased linearly with time, and the velocity of the reaction was constant at short periods of time (Figure 1). Thus, the initial velocity, v_0, was chosen as an experimental parameter, and it was calculated in all experiments as the slope of the linear fitting of

the first 10s of the absorbance/time data. To reduce the standard error of v_0, experiments were repeated from 3 to 5 times under identical conditions. This enzymatic reaction can be considered a single enzyme-substrate reaction and can be studied using the Michaelis-Menten equation following the irreversible reaction scheme that was first proposed by Henri [36]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \quad (1)$$

where k_1, k_{-1}, and k_2 are rate constants. The difficulty of following an enzymatic reaction was largely solved when Michaelis and Menten showed that, under certain conditions, e.g. an excess of substrate, enzyme activity could be studied by measuring the initial rate of product formation. Since then, enzyme kinetic parameters have usually been determined using an expression for the velocity of product formation that is known as the Michaelis–Menten equation

$$v = \frac{v_{max}[S]_0}{K_m + [S]_0} \quad (2)$$

where v_{max} is the maximum velocity defined as $v_{max} = k_{cat}[E]_T$ and, for the classical enzymatic mechanism depicted in equation 1, $K_m = (k_{-1} + k_2)/k_1$ is the Michaelis-Menten constant and $k_{cat} = k_2$.

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Table 1. Kinetic constants of the oxidation of ABTS by peroxide hydroxide catalyzed by HRP for different concentrations of Dextran (in mg/mL). The value of the fitting parameter r^2 is also given.

[Dextran] (mg/mL)	v_{max} **(μM/s)**	K_m **(μM)**	$\mathbf{r^2}$
0	0.023 ± 0.003	32 ± 7	0.9946
25	0.016 ± 0.001	29 ± 6	0.9965
50	0.012 ± 0.001	26 ± 2	0.9978
100	0.006 ± 0.001	22 ± 9	0.9967

FIGURE 1. Example of kinetic curves for different concentrations of ABTS (6, 10, 13, 16, 20 and 23 μM, respectively) in samples with 25 mg/mL of 50 kDa Dextran, 10 μM of H_2O_2 and 10 nM of HRP.

FIGURE 2. Michaelis-Menten plot that relates the reaction rate v_0 to the substrate concentration for the oxidation of ABTS by H_2O_2 catalyzed by HRP in Dextran crowded media with different Dextran concentration: (**A**) 25 mg/mL; (**B**) 50 mg/mL; and (**C**) 100 mg/mL. In each figure, the curves corresponding to three Dextran sizes are plotted: M_w = 5 kDa (open circle); M_w = 50 kDa (solid up-triangle); M_w = 150 kDa (open down-triangle) and average value (solid square).

FIGURE 3. Michaelis-Menten plot that relates the reaction rate v_0 to the substrate concentration for the oxidation of ABTS by H_2O_2 catalyzed by HRP. The curves corresponding to four Dextran concentrations are plotted: 0 mg/mL (solid circle), 25 mg/mL (open circle), 50 mg/mL (solid up-triangle) and 100 mg/mL (open down-triangle).

Figure 1

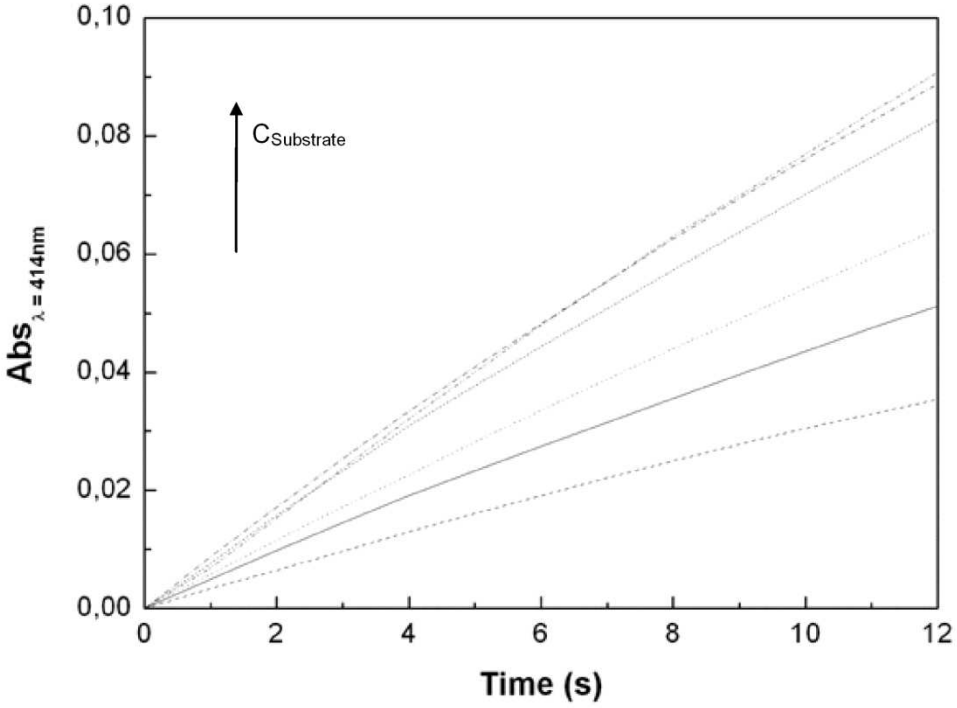
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Figure 2

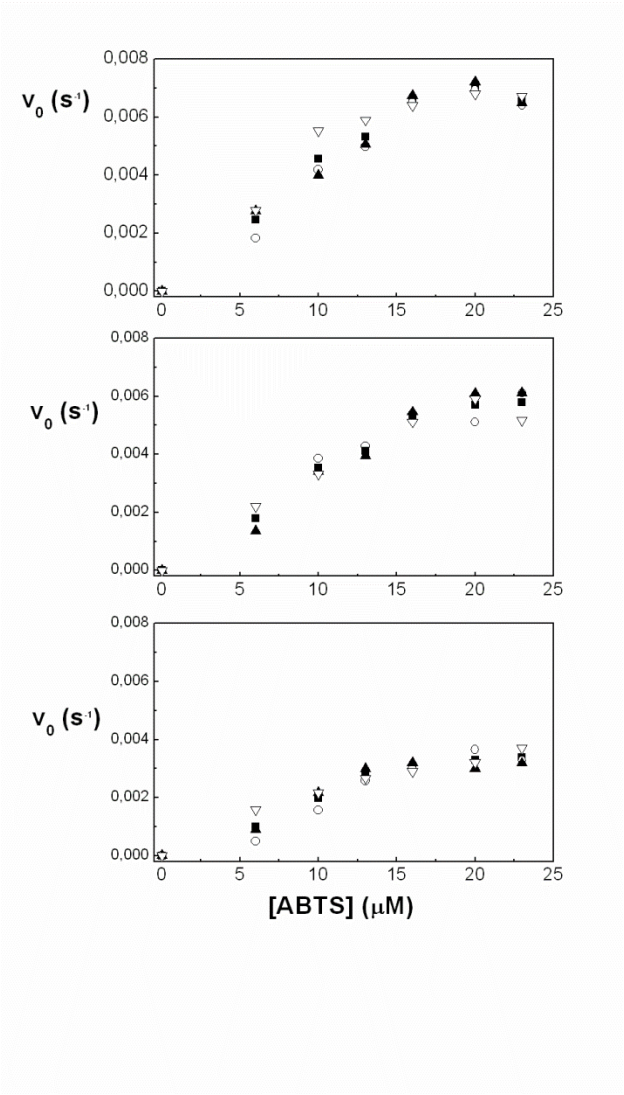

Figure 3