

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

Laura Pitulice<sup>†, #</sup>, Isabel Pastor<sup>\*, ‡, §</sup>, Eudald Vilaseca<sup>‡</sup>, Sergio Madurga<sup>‡</sup>, Adriana Isvoran<sup>†</sup>, Marta Cascante<sup>§</sup> and Francesc Mas<sup>‡</sup>.

<sup>†</sup> *Departament of Biology-Chemistry, West University of Timisoara, Timisoara (Romania).*

<sup>#</sup> *University “Al.I.Cuza” from Iași, 11 Carol I, Iasi (Romania) (temporary affiliation)*

<sup>‡</sup> *Department of Physical Chemistry and the Research Institute of Theoretical and Computational Chemistry (IQTCUB) of the University of Barcelona (UB), Barcelona (Spain)*

<sup>§</sup> *Department of Biochemistry and Molecular Biology and the Institute of Biomedicine (IBUB) of the University of Barcelona (UB) and IDIBAPS, Barcelona (Spain)*

\* Corresponding author:

Isabel Pastor

Address: C / Martí i Franquès, 1, 08028 BARCELONA (SPAIN)

E-mail: [i.pastordelcampo@gmail.com](mailto:i.pastordelcampo@gmail.com)

Fax: (+34) 934021231

## 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<sub>2</sub>O<sub>2</sub>) catalyzed by Horseradish Peroxidase (HRP), by adding Dextran 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 macromolecules 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

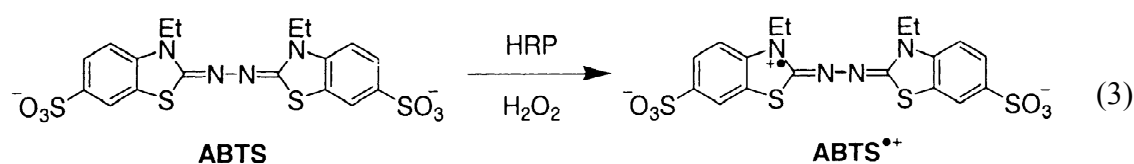
[9, 23-30]. However, the effect of the crowding agent on  $k_{cat}$  is diverse: in some cases,  $k_{cat}$  increases [24, 28-32], whereas in other cases it decreases [9, 26-27, 32]. In a previous work, we studied the crowding effect of Dextrans of various molecular weights on the reaction rates of the 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,  $ABTS^{*\cdot+}$ , catalyzed by HRP, shown in equation 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<sub>2</sub>O<sub>2</sub> assay using HRP, because it has a well-defined absorption maximum at 340 nm ( $\epsilon_{340} = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) [37]. The radical-cation ABTS<sup>•+</sup> has an absorption maximum at 414 nm ( $\epsilon_{414} = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$ ); it also absorbs at 340 nm ( $\epsilon_{340} = 5400 \text{ M}^{-1} \text{ cm}^{-1}$ ). 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<sub>2</sub>O<sub>2</sub> 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<sup>•+</sup>. 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 Dextran, 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-chymotrypsin. 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 U $\text{mg}^{-1}$ ), 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 ( $\epsilon_{340} = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) and  $\text{H}_2\text{O}_2$  ( $\epsilon_{240} = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$ ) 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 10  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$ , the concentration of ABTS varied between 0 and 23  $\mu\text{M}$ , 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  $\text{H}_2\text{O}_2$  and Dextran in phosphate buffer, using a syringe to favor mixing. The reaction progress was followed by monitoring the release of ABTS radical-cation ( $\text{ABTS}^{*\cdot}$ ) (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]



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  $k_{cat} = k_2$ .

### Acknowledgements

For LP and AI, this work was supported by the Transnational network for integrated management of postdoctoral research in the field of Science Communication (POSDRU/89/1.5/S/63663) and Institutional set up (postdoctoral school) and scholarship program (CommScie). This study was also supported by the Spanish Ministry of Science and Technology (Projects CTM2009-14612 and SAF2008-00164),

by the Red Temática de Investigación Cooperativa en Cáncer, Instituto de Salud Carlos III (ISCIII-RTICC, RD06/0020/0046 and RD06/0020/1037) and by the Generalitat de Catalunya (grants 2009SGR465, 2009SGR1308 and XRQTC). IP thanks the Juan de la Cierva Program of the Spanish Ministry of Science.

## REFERENCES

1. Ellis RJ (2001) Macromolecular crowding: obvious but underappreciated. *Trends Biochem Sci* **26**, 597-604.
2. Muramatsu N, Minton AP (1988) Tracer diffusion of globular proteins in concentrated protein solutions. *Proc Natl Acad Sci USA* **85**, 2984-2988.
3. Wachsmuth M, Waldeck W, and Langowski J (2000). Anomalous diffusion of fluorescent probes inside living cell nuclei investigated by spatially-resolved fluorescence correlation spectroscopy. *J Mol Biol* **298**, 677-689.
4. Arrio-Dupont M, Foucault G, Vacher M, Devaux PF, Cribier S (2001) Translational diffusion of globular proteins in the cytoplasm of cultured muscle cells. *Biophys J* **78**, 901-907.
5. Verkman AS (2002) Solute and macromolecular diffusion in cellular aqueous compartments. *TRENDS Biochem Sci* **27**, 27-32.
6. Weiss M, Elsner M, Kartberg F, Nilsson T (2004) Anomalous subdiffusion is a measure for cytoplasmic crowding in living cells. *Biophys J* **87**, 3518–3524.
7. Dix J, Verkman A (2008) Crowding effects on diffusion in solutions. *Annu Rev Biophys* **37**, 247-263.
8. Zhou HX, Rivas G, Minton AP (2008) Macromolecular crowding and confinement: biochemical, biophysical and potential physiological consequences. *Annu Rev Biophys* **37**, 375-397.

9. Minton AP, Wilf J (1981) Effect of macromolecular crowding upon the structure and function of an enzyme: Glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry* **20**, 4821–4826.
10. Minton AP (1981) Excluded volume as a determinant of macromolecular structure and reactivity. *Biopolym* **20**, 2093-2120.
11. Zimmerman SB, Minton AP (1993) Macromolecular crowding: biophysical, biochemical, and physiological consequences. *Annu Rev Biophys Biomol Struct* **22**, 27-65.
12. Rivas G, Fernandez JA, Minton AP (1999) Direct observation of the self-association of dilute proteins in the presence of inert macromolecules at high concentration via tracer sedimentation equilibrium: theory, experiment, and biological significance. *Biochem* **38**, 9379-9388.
13. Van den Berg B, Ellis RJ, Dobson CM (1999) Effects of macromolecular crowding on protein folding and aggregation. *EMBO J* **18**, 6927-6933.
14. Minton AP Implications of macromolecular crowding for protein assembly. *Curr Opin Struct Bio* **10**, 34-39.
15. Rivas G, Fernandez JA, Minton AP (2001). Direct observation of the enhancement of non-cooperative protein assembly by macromolecular crowding: indefinite self-association of the bacterial cell division protein FtsZ. *Roc Natl Acad Sci USA* **98**, 3150-3155.
16. Koser N, Schreiber G (2004) Effect of Crowding on Protein–Protein Association Rates: Fundamental Differences between Low and High Mass Crowding Agents. *J Mol Biol* **336**, 763-774.

17. Munishkina L A, Cooper EM, Uversky V, Fink A (2004) The effect of macromolecular crowding on protein aggregation and amyloid fibril formation *J Mol Recognit* **17**, 456-464.
18. Chebotareva NA, Kurganov BI, Livanova NB (2004) Biochemical effects of molecular crowding. *Biochemistry (Mosc)* **69**, 1239–1251.
19. Zhou HX (2004) Protein folding and binding in confined spaces and in crowded solutions. *J Mol Recognit* **17**, 368-375.
20. Minton AP (2005) Influence of macromolecular crowding upon the stability and state of association of proteins: predictions and observations. *J Pharm Sci* **94**, 1668-1675.
21. Schreiber G, Haran G, Zhou HX (2009) Fundamental Aspects of Protein-Protein Association Kinetics *Chem Rev* **109**, 839-860.
22. Dong H, Qin S, Zhou HX (2010) Effects of Macromolecular Crowding on Protein Conformational Changes. *PLoS Comput Biol* **6**, e1000833.
23. Laurent TC (1971) Enzyme Reactions in Polymer Media. *Eur J Biochem* **21**, 498-506.
24. Gellerich FN, Laterveer FD, Korzeniewski B, Zierz S, Nicolay K (1998) Dextran strongly increases the Michaelis constants of oxidative phosphorylation and of mitochondrial creatine kinase in heart mitochondria. *Eur J Biochem* **254**, 172-180.
25. Wenner JR, Bloomfield VA (1999) Crowding Effects on EcoRV Kinetics and Binding. *Biophys J* **77**, 3234-3241.
26. Derham BK, Harding JJ (2006) The effect of the presence of globular proteins and elongated polymers on enzyme activity. *Biochim Biophys Acta* **1764**, 1000-1006.

27. Olsen SN (2006) Applications of isothermal titration calorimetry to measure enzyme kinetics and activity in complex solutions. *Thermochim Acta* **448**, 12-18.
28. Sasaki Y, Miyoshi D, Sugimoto N (2007) Regulation of DNA nucleases by molecular crowding. *Nucleic Acids Research* **35**, 4086-4093.
29. Olsen SN, Ramløv H, Westh P (2007) Effects of osmolytes on hexokinase kinetics combined with macromolecular crowding Test of the osmolyte compatibility hypothesis towards crowded systems. *Comp Biochem Physiol A* **148**, 339-345.
30. Moran-Zorzano MT, Viale A, Muñoz F, Alonso-Casajas N, Eydalm G, Zugasti B, Baroja-Fernández E, Pozueta-Romero J (2007) Escherichia coli AspP activity is enhanced by macromolecular crowding and by both glucose-1,6-bisphosphate and nucleotide-sugars *FEBS Lett* **581**, 1035-1040.
31. Jiang M, Gou ZH (2007) Effects of Macromolecular Crowding on the Intrinsic Catalytic Efficiency and Structure of Enterobactin-Specific Isochorismate Synthase. *J Am Chem Soc* **129**, 730-731.
32. Sasaki Y, Miyoshi D, Sugimoto N (2006) Effect of molecular crowding on DNA polymerase activity. *Biotechnol J* **1**, 440-446.
33. Asaad N, Engberts J BFN (2003) Cytosol-Mimetic Chemistry: Kinetics of the Trypsin-Catalyzed Hydrolysis of p-Nitrophenyl Acetate upon Addition of Polyethylene Glycol and N-tert-Butyl Acetoacetamide. *J Am Chem Soc* **125**, 6874-6875.
34. Pastor I, Vilaseca E, Madurga S, Garces JL, Cascante M, Mas F (2011) Effect of Crowding by Dextran on the Hydrolysis of N-Succinyl-L-phenyl-Ala-p-nitroanilide Catalyzed by  $\alpha$ -Chymotrypsin. *J Phys Chem B* **115**, 1115-1121.
35. Pütter J, Becker R. (1983), *Peroxidases*, Vol. 3, 3<sup>rd</sup> Edition, Verlag Chemie, Deerfield Beach, FL.

36. Cornish-Bowden A (2004). *Fundamentals of Enzyme Kinetics*. 3<sup>rd</sup> edition. Published by Portland Press Ltd. London (UK).
37. Childs RE, Bardsley WG (1975) The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. *Biochem J* **145**, 93-103.
38. Zheng L, Brennan JD (1998) Measurement of intrinsic fluorescence to probe the conformational flexibility and thermodynamic stability of a single tryptophan protein entrapped in sol-gel derived glass matrix. *Analyst* **123**, 1735-1744.
39. Ryabov AD, Goral VN, Gorton L, Csöregi E (1999) Electrochemically and Catalytically Active Reconstituted Horseradish Peroxidase with Ferrocene-Modified Hemin and Artificial Binding Site. *Chem Eur J* **5**, 961-967.



**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)	$v_{max}$ ( $\mu\text{M/s}$ )	$K_m$ ( $\mu\text{M}$ )	$r^2$
0	$0.023 \pm 0.003$	$32 \pm 7$	0.9946
25	$0.016 \pm 0.001$	$29 \pm 6$	0.9965
50	$0.012 \pm 0.001$	$26 \pm 2$	0.9978
100	$0.006 \pm 0.001$	$22 \pm 9$	0.9967

FIGURE 1. Example of kinetic curves for different concentrations of ABTS (6, 10, 13, 16, 20 and 23  $\mu\text{M}$ , respectively) in samples with 25 mg/mL of 50 kDa Dextran, 10  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  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

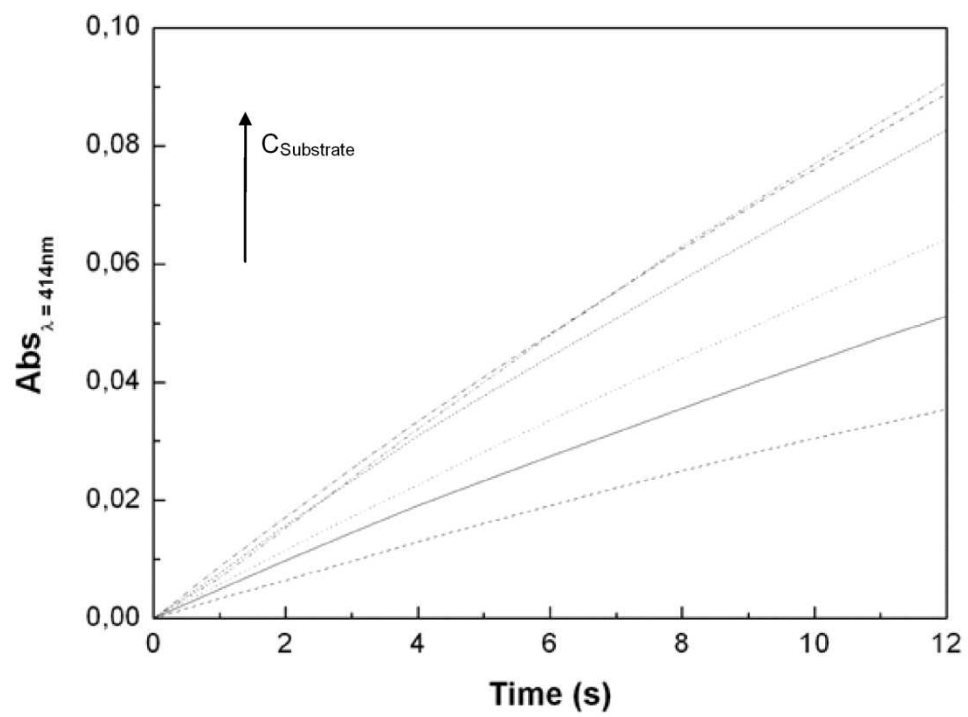


Figure 2

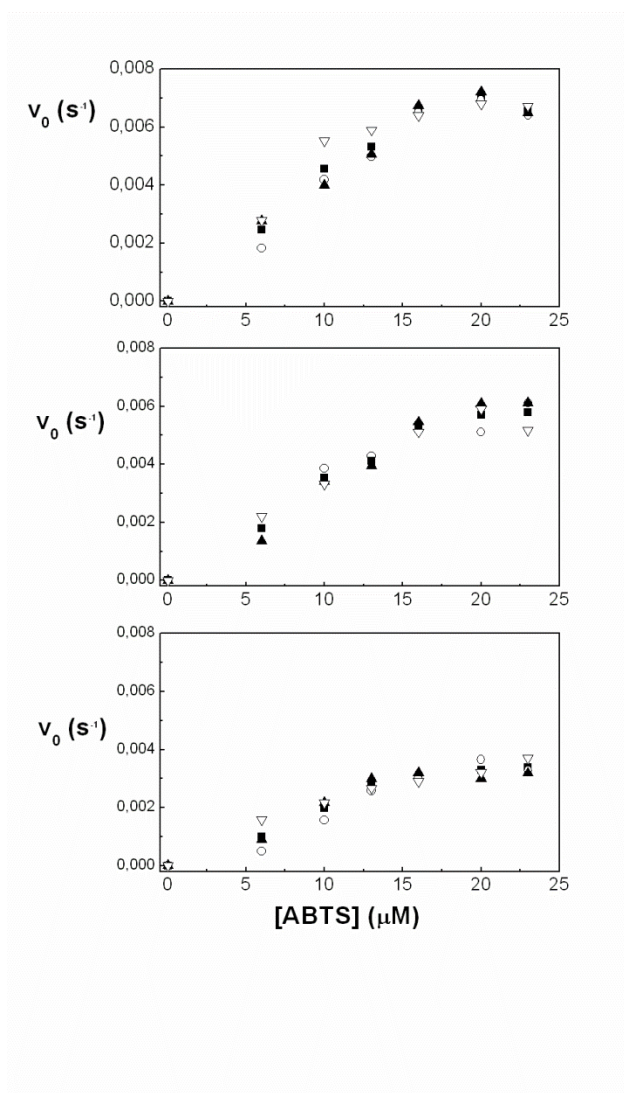


Figure 3

