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Effect of crowding by Dextrans in enzymatic reactions

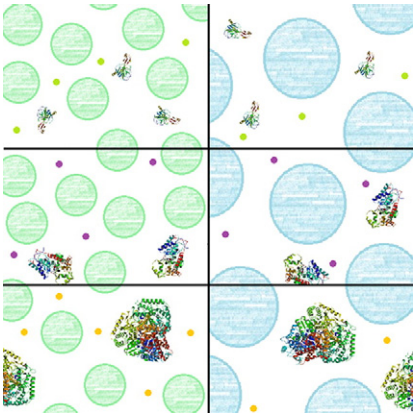
Isabel Pastor [a,b,*], Laura Pitulice [c,1], Cristina Balcells [a,b], Eudald Vilaseca [a,b], Sergio Madurga [a,b], Adriana Isvoran [c], Marta Cascante [d,e,f], Francesc Mas [a,b]

[a] Department of Physical Chemistry, University of Barcelona (UB), Barcelona, Spain
[b] Research Institute of Theoretical and Computational Chemistry (IQTCUB) of the University of Barcelona (UB), Barcelona, Spain
[c] Department of Biology-Chemistry, West University of Timisoara, Timisoara, Romania
[d] Department of Biochemistry and Molecular Biology, University of Barcelona (UB), Barcelona, Spain
[e] Institute of Biomedicine (IBUB) of the University of Barcelona (UB), Barcelona, Spain
[f] IDIBAPS, Barcelona, Spain

HIGHLIGHTS

- We studied and compared the velocity rates of three reactions in different crowded media.
- The crowded media is produced by Dextran of different concentrations and sizes.
- The volume occupied by the crowding agent plays an important role on tiny enzymes reactions.
- The rate of the reactions of large enzymes depends on both the occupied volume and dimension of the crowding agent.

GRAPHICAL ABSTRACT



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ABSTRACT

The interior of the living cell is highly concentrated and structured with molecules that have different shapes and sizes. Almost all experimental biochemical data have been obtained working in dilute solutions, situations which do not reflect the in vivo conditions. The consequences of such crowding upon enzymatic reactions remain unclear. In this paper, we have studied and compared the initial velocity of the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin, the oxidation of ABTS by H_2O_2 catalyzed by HRP and the oxidation of NADH in presence of pyruvate catalyzed by LDH. These reactions were chosen as model enzymatic processes occurring in different in vitro crowded media. The systems crowding has been built by introducing Dextran of several concentrations and sizes. Our results indicate that the volume occupied by the crowding agent, but not its size, plays an important role on the initial velocity of reactions involving tiny enzymes. However, the enzyme size is another important factor influencing the velocity of the reactions of large enzymes occurring in Dextran crowded media. In this situation, the reaction initial velocity depends on both occupied volume and dimension of the crowding agent that is present in the reaction media.

* Corresponding author at: C/Martí i Franquès, 1, 08028 Barcelona, Spain. Fax: +34 934021231.
E-mail address: i.pastordelcampo@gmail.com (I. Pastor).
[1] University "Al.I.Cuza" from Iaşi, 11 Carol I, Iasi, Romania (temporary affiliation).

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1. Introduction

Molecular crowding is by now an extensively studied issue in the micro world of cells. The intracellular fluid is a complex matrix in which a dense mixture of macromolecules and solutes is present up to a large percentage (40%) of total cellular volume [1]. Molecular crowding is one of the cell features that accounts for the distinct way biochemical reactions progress in vivo than in laboratory assays. Well-mixed dilute solutions, with less than 1 mg/mL macromolecules content, represent the typical environment for in vitro experiments [2]. Various crowding effects have been put into evidence over the last decades related to changes of diffusion rate [3–9], protein folding, self-association and protein binding enhancement [10–23], enzymatic activity alteration [24–27] and reaction kinetics modification [28–30].

In recent years, the effects of crowding on enzyme catalysis have been explored by different works, excellently depicted by Zhou et al. [9] and Noris and Malys [24]. Most of them indicate that the effect of excluded volume due to the presence of crowding agent is the major player in modulating enzymatic behavior. From the very first study on enzymatic reactions in crowded media developed by Laurent in 1971 [31], the presence of macromolecules had been revealed to produce a moderate decrease in the apparent Michaelis–Menten (MM) constant, K_m. Some years later, Minton and Wilf [10], studying the enzymatic processes of glyceraldehyde-3-phosphate dehydrogenase, predicted that the initial velocity of an enzyme-catalyzed reaction will decrease if the concentration or the size of the crowding agent is increased. In other words, the excluded volume produces a decrease of both MM constant, K_m, and catalytic constant, k_{cat}, when the enzymatic reaction follows the Michaelis–Menten mechanism. However, most subsequent studies reported that a high concentration of neutral polymers only had a moderate influence on enzyme reactions. Briefly, a slight decrease of K_m is frequently found, regardless of the properties of the crowding agent [32–37]. However, the effect of the crowding agent on k_{cat} is diverse: in some cases, k_{cat} increases [24,32,36–39], whereas in other cases it decreases [10,34,35,39]. It should be noted that these quantitative studies used different polymers as crowding agents but only of a fixed size (usually small). The effect of the crowding agent size and shape on enzymatic reactions has been also analyzed [40]: large obstacles with irregular shape reduce the reaction velocity, but large and compact obstacles have minor effects on it. In their work, Homchaudhuri and coworkers [40] studied the hydrolysis of p-nitrophenyl phosphate catalyzed by alkaline phosphase as model reaction and macromolecular crowding was mimicked using inert polymers such as Dextrans and Ficolls of molecular weights ranging from 15 to 500 kDa. They results revealed a steeper decrease of the reaction velocity as a function of fractional volume occupancy with larger Dextrans compared with smaller Dextrans. In the presence of 20% Dextran (w/w), a typical concentration of macromolecules inside the cytoplasm, the reaction rates were 2-folds slowed by smaller Dextrans and between 5- or 7-folds by larger Dextrans. Ficolls of similar size to Dextran had a comparatively smaller influence on the reaction rates (2-folds). In other words, the extent of the crowding effect may strongly depend on both concentration and relative size of the crowding species.

Therefore, our working group has investigated the influence degree of both factors on the initial velocity of different enzymatic processes. First, we studied the crowding effect of Dextrans of various molecular weights on the reaction initial velocity of the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin [41]. Our results pointed out that the volume occupied by Dextran, independent of its size, had an important role on the initial velocity of this reaction. A v_{max} decay and a K_m increase were obtained when bigger Dextran concentration was used in the sample. The rise of K_m could be attributed to a slower diffusion of the protein [42] 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.

Second, we have studied the kinetics of the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by hydrogen peroxidase (H_2O_2) catalyzed by horseradish peroxidase (HRP) as a model enzymatic reaction occurring in different in vitro crowded media [43]. The crowding was generated by Dextran of various concentrations and dimensions. Our results revealed that the reaction initial velocity was also significantly influenced by the crowding agent excluded volume, regardless of its size. Both v_{max} and K_m decayed along with the growth of obstacle concentration. Concerning this particular case, the presented data suggest an activation control of the enzymatic reaction in the studied system. In other words, the catalytic constant (k_{cat}) brings a significant contribution as a result of the environmental surroundings influence. This contribution may be the consequence of one of the following aspects or their additive effect: the rise of the ratio value between the activity coefficients of natural enzyme and enzyme–substrate complex due to the presence of crowding agents; the enhancement of water chemical activity favored by highly crowded solution; and the crowding-induced conformational change of the enzyme active site.

Besides the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin and the oxidation of ABTS by H_2O_2 catalyzed by HRP, we have also studied the oxidation of NADH in the presence of pyruvate catalyzed by lactate dehydrogenase (LDH) [Balcells et al., unpublished work]. When choosing these particular reactions as model processes, several reasons have been put forth: first, they are well-known reactions; second, these reactions can be easily monitored by UV-spectroscopy; and third, all reactions are accompanied by a minimal change in the excluded volume. In fact, the substrate and product molecules of each investigated system are tiny compared to the size of involved proteins and crowding agent. Hence, the effect of both molecules (i.e. substrate and product) on the excluded volume can be neglected. As a result, the effect of macromolecular crowding on these particular reactions can be mainly interpreted in terms of the crowding agent presence.

Moreover, the usage of these specific proteins in our investigation also presents some advantages: on the one hand, the absence of known interactions with Dextran and on the other hand, the size of the proteins (alpha-chymotrypsin of Mw = 25 kDa, HRP of Mw = 42 kDa and LDH of Mw = 140 kDa). The protein dimensions are within the size range of the crowding agent (Fig. 1).

The ideal crowding agents must provide nature-like microenvironments. The best approach to mimic macromolecular crowding would be using cell extracts [1]. However, the heterogeneity of chemical, geometrical, and physical properties of cell extracts makes difficult the experimental data collection and their interpretation. Therefore, a variety of purified macromolecules were used as crowding agents in most experimental studies of crowding effects [9,25]. Among these purified macromolecules, Dextran is one of the most commonly used since it is inert, water-soluble and resembles more closely the types of macromolecules encountered in the natural state of the cell [9]. It is also readily available in various sizes and large quantities [44]. Dextran is a very flexible polymer, represented by a random coil with spherical shape in aqueous solution, especially when present in high concentration [45]. Due to these reasons, Dextrans with molecular weight ranging from 5 to 410 kDa are used to generate the crowding complexity.

Based on the previous findings, the present paper aims at assessing and comparing, in the interest of completeness, the way in which the kinetics of three enzyme-catalyzed reactions is modulated while they are carried out in vitro, under similar crowding conditions. The systems are crowded with Dextran of various sizes and concentrations. The comparative analysis proposed here tries to emphasize the crowding induced effects on enzymatic reaction initial velocities in systems targeting the complexity of natural cellular media.

2. Materials and methods

Alpha-chymotrypsin (E.C. 3.4.21.1) from bovine pancreas type II (60 U mg^{-1}), Peroxidase (E.C. 1.11.1.7) from horseradish

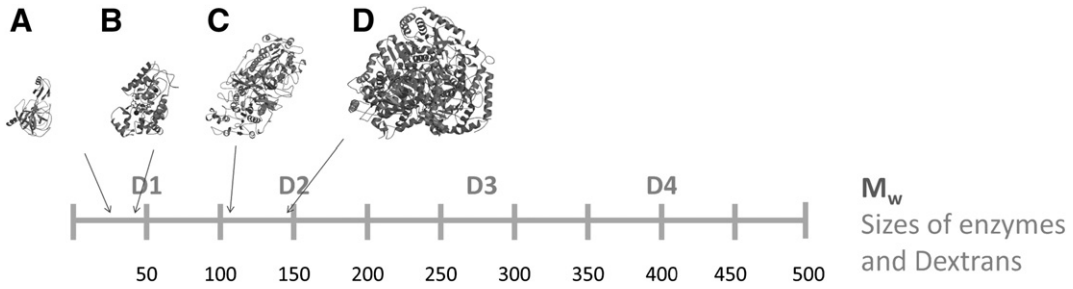

Fig. 1. Schematic representation of the protein sizes relative to the size of the used Dextrans: alpha-chymotrypsin (A), HRP (B), alkaline phosphatase (C) and LDH (D).

(1310 U mg^{-1}), L-Lactate Dehydrogenase (E.C. 1.1.1.27) from rabbit muscle (140 U mg^{-1}), N-succinyl-L-phenyl-Ala-p-nitroanilide, diammonium salt of ABTS, 33% aqueous hydrogen peroxide, sodium pyruvate and β-NADH were purchased from Sigma-Aldrich Chemical (Milwaukee, WI, USA). The proteins were used without further purification. Dextran (from *Leuconostoc mesenteroides*) of molecular weight of 5, 50, 150, 270 and 410 kDa was purchased from Fluka (Buchs, Switzerland). The polydispersities of the Dextrans were less than 2.0, as reported by the manufacturer. 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. All other chemicals were of analytical or spectroscopic reagent grade.

All activity measurements were performed using a UV-1603 Shimadzu spectrophotometer. In order to reduce the standard error, all the experiments were repeated from 3 to 5 times under identical conditions.

Further information and details of performed experiments are presented elsewhere for alpha-chymotrypsin [41,42], horseradish peroxidase (HRP) [43] and lactate dehydrogenase (LDH) [Balcells et al., unpublished work] reactions (see also Table 1).

3. Results and discussion

For a proper evaluation of the effect of macromolecular crowding on enzyme kinetics, one must consider at least two important features of the study. First, the reactions under investigation should be well-behaved processes, of which kinetics can be interpreted with confidence. In our case, all three selected reactions are well-known enzymatic processes following a Michaelis–Menten mechanism. Additionally, they can easily be monitored by UV-spectroscopy. Second, the crowding induced effect should be accounted only by the changes incurred by the crowding agent with regard to its size and concentration. This requirement is also met. For all three considered systems, the excluded volume does not significantly change during the reaction evolution due to the small particle size of each corresponding substrate and product.

The experimental investigations allowed us to record the absorbance/time plot of each corresponding released product (results not shown). For the reactions involving alpha-chymotrypsin and HRP, we observed a typical initial rise of the absorbance with time and a subsequent plateau. Regarding the LDH reaction, the absorbance drops with time until the plateau is reached. The initial slope of these curves was fitted by linear regression in order to obtain the initial velocity (v_0) for each experimental condition. After that, the average value of the initial velocity for repeated experiments was plot against substrate concentration in order to obtain a typical MM-plot. For each MM-plot a non-linear regression was applied to determine the MM parameters (K_m and v_{max}). As an example, Fig. 2 illustrates the v_0 dependence on substrate concentration normalized by K_m, for all three enzyme reactions considering Dextran of two sizes (50 and 150 kDa) and different concentrations, as explained in figure caption. The Dextran concentration, given in mg/mL, is proportional to the volume it occupies in the sample (excluded volume). Moreover, Fig. 2 also shows another important difference among the three enzymes. For the two first reactions, the plateau of the curve is achieved for values of $[S]/K_m > 1$, and theses values increase as the size of the enzyme increases. However, as it can be seen in Fig. 2, the LDH enzyme shows a different behavior depending on the relative size of the Dextran, as crowding agent, with respect to the protein. For lower relative Dextran sizes, the plateau of the initial velocity follows a behavior similar to the other two enzymes, but for equal or higher relative Dextran sizes, the plateau can achieve lower values of $[S]/K_m$, and in some cases (high Dextran concentrations) even smaller than 1.

Regarding the first two reactions (of alpha-chymotrypsin and HRP), it can be observed that the v_0–substrate concentration curves are similar for the same occupied volume, regardless of the crowding agent dimension. Thus, the v_0 value does not change with Dextran sizes, but varies with its concentration, so with the excluded volume. Based on this similarity, the curves for each Dextran concentration corresponding to distinct Dextran sizes can be grouped into a single average v_0–substrate concentration curve (Fig. 3) for the reactions involving alpha-chymotrypsin and HRP. The single average values of MM parameters can be found in Table 2.

However, the v_0 behavior is different for the reaction catalyzed by LDH. It changes with both Dextran size and concentration. Fig. 4 shows the variation of v_0 with the substrate concentration for different concentrations of Dextran. There it can be seen a clear dependence of v_0–substrate concentration curves on both the concentration and the Dextran size. Both, Fig. 4 and the values shown in Table 2, reveal that reaction initial velocity decreases as a function of Dextran size when high Dextran concentrations (>50 mg/mL) are used. As a result, the oxidation of NADH in presence of pyruvate catalyzed by LDH presents an initial velocity, v_0, dependent on the crowding agent size and concentration. In this case, the enzyme reaction behavior depends not only on the

Table 1
Summary of the experimental conditions, enzymes, reactants and buffer solution used.

Enzymes	Substrates		Buffer solution	Reaction tracing
Alpha-chymotrypsin from bovine pancreas type II	N-succinyl-L-phenyl-Ala-p-nitroanilide ($0–4.8 \cdot 10^{-4}$ M)		Tris–HCl 0.1 M pH = 8.0 10 mM $CaCl_2$	Monitored by UV–vis spectroscopy at λ = 410 nm (25 °C)
Peroxidase from horseradish	ABTS diammonium salt ($0–23 \cdot 10^{-4}$ M)	H_2O_2 (33% aq.) ($10 \cdot 10^{-4}$ M)	Phosphate buffer 0.1 M pH = 7.4	Monitored by UV–vis spectroscopy at λ = 414 nm (25 °C)
L-Lactate dehydrogenase from rabbit muscle	Sodium pyruvate ($0–5.4 \cdot 10^{-4}$ M)	β-NADH ($1.17 \cdot 10^{-4}$ M)	Imidazole–acetic acid 30 mM, pH = 7.5 60 mM CH_3COOK 30 mM $MgCl_2$	Monitored by UV–vis spectroscopy at λ = 320 nm (25 °C)

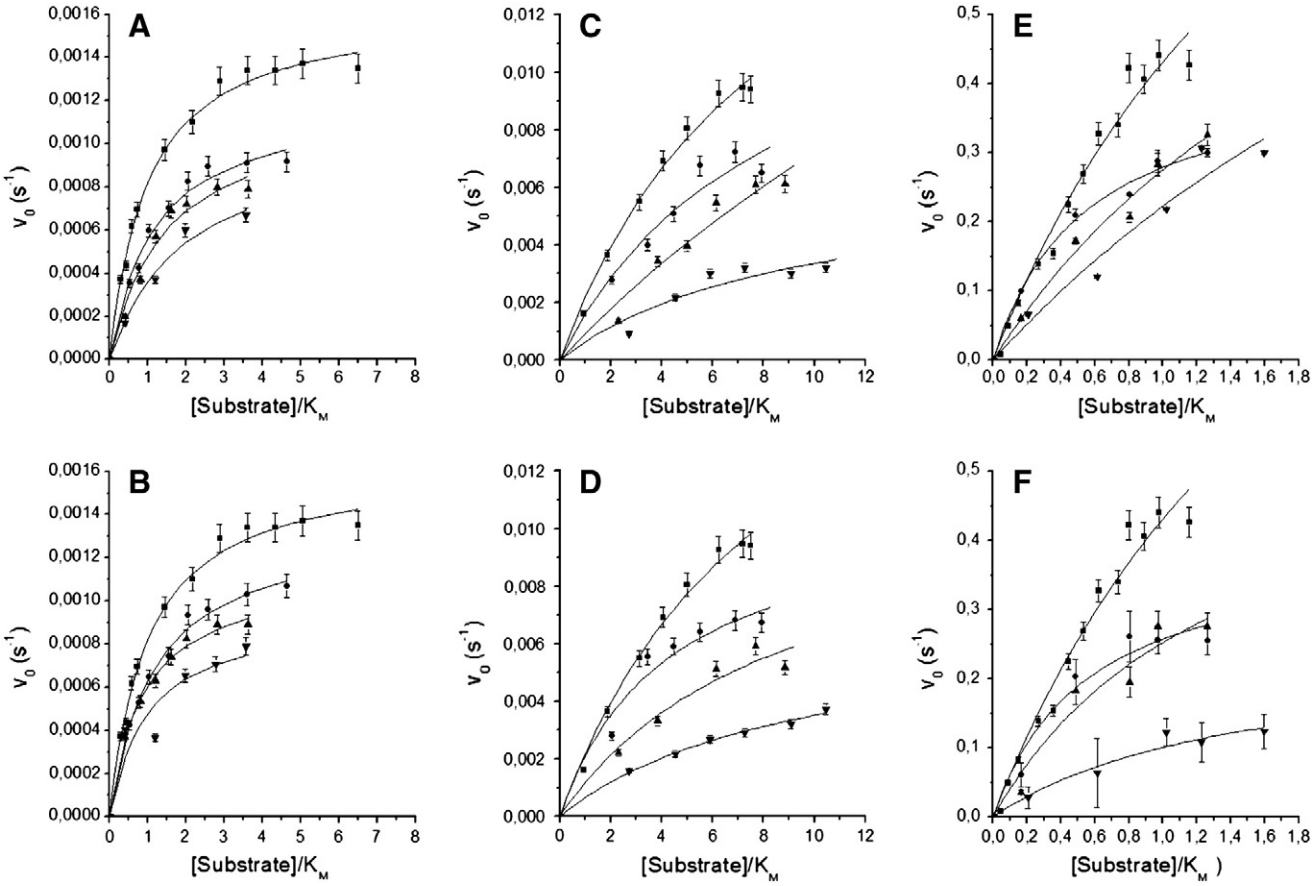

Fig. 2. Plots of reaction velocity v_0 versus substrate concentration normalized by K_m considering two different Dextran sizes (upper figures for 50 kDa and lower figures for 150 kDa Dextrans) for the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin: A and B figures; for the oxidation of ABTS by H_2O_2 in presence of HRP: C and D figures; and for the oxidation of NADH in presence of pyruvate catalyzed by LDH: E and F figures. The plotted curves correspond to four Dextran concentrations: 0 mg/mL (square), 25 mg/mL (circle), 50 mg/mL (up-triangle) and 100 mg/mL (down-triangle).

excluded volume but also on the dimension of the obstructive particles that are present in the reaction media.

The absence of the initial velocity dependence on crowding agent size for the first two investigated enzymatic reactions (hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin and the oxidation of ABTS by H_2O_2 in presence of HRP) is in accordance with the results of Minton et al. [3,9–13]. They predicted that excluded volume is a major factor influencing enzymatic reactions occurring in

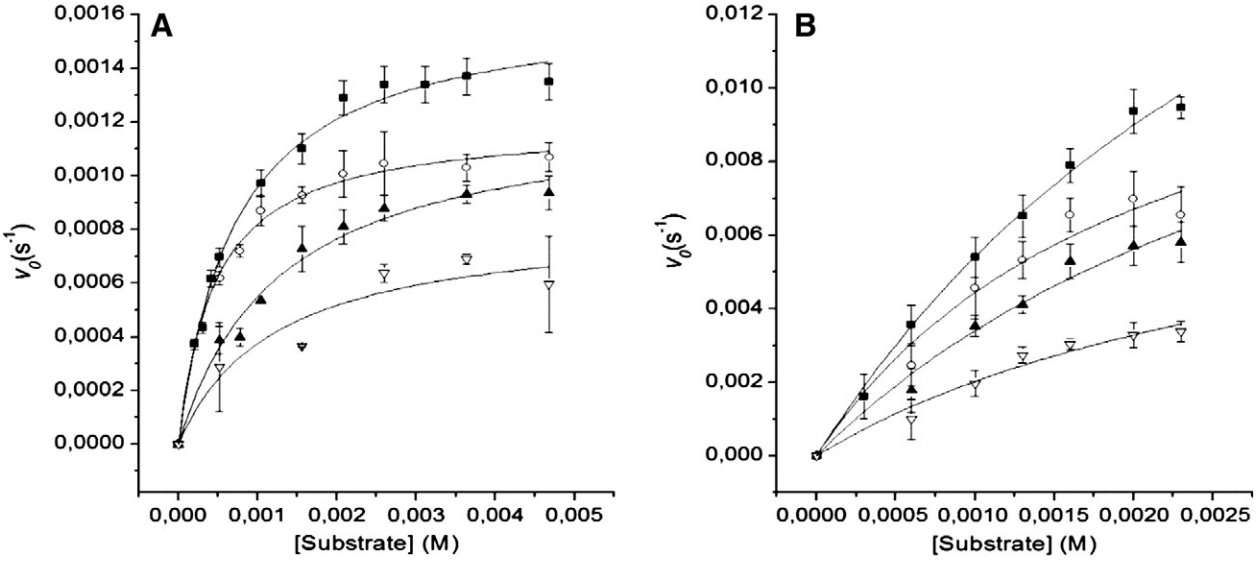

Fig. 3. Averaged velocities v_0 for different Dextran size assays versus substrate concentration for the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin (A) and for the oxidation of ABTS by H_2O_2 in presence of HRP (B). The plotted curves correspond to four Dextran concentrations: 0 mg/mL (solid square), 25 mg/mL (open circle), 50 mg/mL (solid up-triangle) and 100 mg/mL (open down-triangle).

Table 2
Kinetic parameters obtained by Michaelis–Menten fittings. Average values considering all Dextran sizes for HRP and alpha-chymotrypsin, and values considering the 150 kDa Dextran and 410 kDa Dextran for LDH.

Enzymatic system	[Dextran] (mg/mL)	v_{max} (mM/s)	K_m (mM)	r^2
Alpha-chymotrypsin	0	1.64 ± 0.04	0.72 ± 0.06	0.9927
	25	1.28 ± 0.04	1.01 ± 0.05	0.9984
	50	1.24 ± 0.04	1.29 ± 0.12	0.9958
	100	0.91 ± 0.05	1.31 ± 0.23	0.9933
HRP	0	$(2.3 \pm 0.3) \cdot 10^{-5}$	0.032 ± 0.007	0.9946
	25	$(1.6 \pm 0.1) \cdot 10^{-5}$	0.029 ± 0.006	0.9965
	50	$(1.2 \pm 0.1) \cdot 10^{-5}$	0.026 ± 0.002	0.9978
	100	$(0.6 \pm 0.1) \cdot 10^{-5}$	0.022 ± 0.009	0.9967
LDH	0	0.08 ± 0.01	0.47 ± 0.01	0.9825
	25 (150 kDa)	0.53 ± 0.03	0.43 ± 0.01	0.9879
	50 (150 kDa)	0.53 ± 0.03	0.43 ± 0.01	0.9820
	100 (150 kDa)	0.21 ± 0.01	0.34 ± 0.01	0.9918
	25 (410 kDa)	0.59 ± 0.02	0.43 ± 0.01	0.9925
	50 (410 kDa)	0.39 ± 0.03	0.43 ± 0.01	0.9855
	100 (410 kDa)	0.11 ± 0.07	0.37 ± 0.01	0.9970

macromolecular crowded media. In other words, the initial velocity of an enzyme-catalyzed reaction will experience a monotonic decrease with the increase of the fractional volume occupancy of the crowding agent.

The initial velocity lack of dependence on crowding agent size exhibited by the reactions involving alpha-chymotrypsin and HRP is in contrast with the results obtained for the oxidation of NADH in presence of pyruvate catalyzed by LDH. However, the behavior of LDH catalysis is in agreement with the results of Homchaudhuri et al. [40]. As explained in the Introduction section, these authors studied the effect of crowding of different-sized Dextrans and Ficolls on the rate of the hydrolysis of p-nitrophenyl phosphate catalyzed by alkaline phosphase. Their results revealed a steeper decrease of the reaction rate as a function of fractional volume occupancy with larger Dextrans (between 200 and 500 kDa) than with smaller Dextrans (between 15 and 70 kDa). In fact, the authors found that for a presence of 20% Dextran (w/w) the reaction rates slowed ~2-folds for small Dextrans (15–70 kDa), ~5-folds for 200 kDa Dextran and ~7-folds for 500 kDa Dextran.

It has to be acknowledged that the size of alkaline phosphatase (105 kDa) is comparable with that of LDH (140 kDa) (see Fig. 1). As a result, the initial velocity of the reactions they catalyze has similar behavior in crowded media unlike the velocity of other investigated processes catalyzed by much smaller proteins: alpha-chymotrypsin (25 kDa) and HRP (42 kDa). As revealed by Homchaudhuri et al. [34], as a consequence of the protein large size, large obstacles reduce the encounters between enzyme and substrates. In contrast, with small obstacles the effect of crowding is partially offset by the enhancement of enzyme activity owing to a caging effect, determining a minor decrease of the reaction initial velocity.

Our results underline the fact that the degree of crowding effect relies on both size and concentration of the obstructive particles. However, the size seems to exert a significant influence when larger molecules are present in the system. This observed behavior is in good agreement with other published data.

Recently, an experimental study was performed by Vopel and Makhatadze [47] investigating the effects of Ficoll as crowding agent on the kinetic parameters of three enzymes: yeast phosphoglycerate kinase (PGK), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and human acylphosphatase

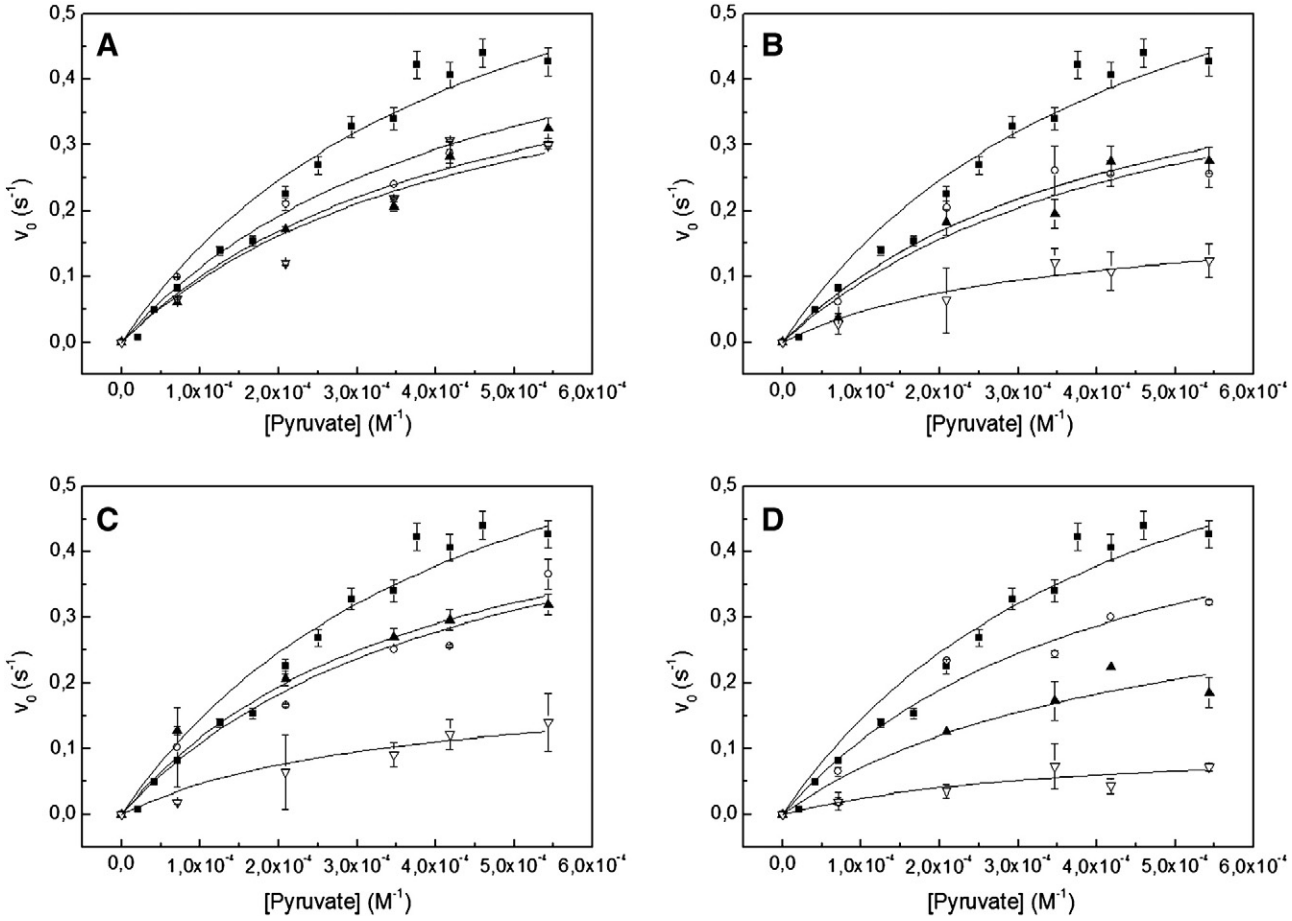

Fig. 4. Michaelis–Menten plot that relates the reaction rate v_0 to the substrate concentration for the oxidation of NADH in presence of pyruvate catalyzed by LDH in Dextran crowded media with different Dextran sizes: (A) M_w = 50 kDa; (B) M_w = 150 kDa; (C) M_w = 275 kDa and (D) M_w = 410 kDa. In each figure, the curves corresponding to four Dextran concentrations are plotted: 0 mg/mL (solid square), 25 mg/mL (open circle), 50 mg/mL (solid up-triangle) and 100 mg/mL (open down-triangle).

1 (ACYP). Even though similar to our study approach, each reaction was carried out in the presence of Ficoll of only one size (PM70) and only in one concentration (200 g/L). As such, it was found that K_m and k_{cat} values of these three enzymes do not significantly change in the presence of the crowding agent.

By comparison, our findings are based not on a single scenario, but on a systematic investigation of the influence of crowder various sizes and concentrations displays over the enzymes behavior. Additionally, two aspects have to be regarded here: 1) that Ficoll is definitely different in shape, more compact and rigid than Dextran of similar size, and therefore Dextran brings a greater effect on the reaction initial velocity [40] and 2) the size of their enzymes (PGK Mw ~ 47 kDa, GAPDH Mw ~ 36 kDa, ACYP Mw ~ 11 kDa) are small against Ficoll size. Besides, at such a high concentration used by Vopel and Makhatadze (200 g/L) it is reported [40] that Ficoll 70 presents a compressed structure and consequently a smaller excluded volume effect than expected.

In conclusion, we have studied and compared the initial velocity of three reactions in different in vitro crowded media produced by Dextran of different concentrations and sizes. Our results illustrate different behaviors. First, the initial velocity of the reactions involving alpha-chymotrypsin and HRP has been found to be greatly influenced by the volume occupied by the crowding agent. Second, the initial velocity of the reaction catalyzed by LDH depends on both size and concentration of Dextran that is present in the media. As a result, the enzyme size represents another significant factor for the initial velocity of reactions occurring in Dextran crowded media. When enzymes are small (in our case alpha-chymotrypsin and HRP) the reaction initial velocity mainly depends on the excluded volume. However, for large enzymes (in our case LDH), the reaction initial velocity is also influenced by the dimension of obstacles which are present in the reaction environment.

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