End-of-MSc Project

Crowding Effects on Oligomeric Enzymes:

Kinetic Analysis of the ALKP-Catalyzed Hydrolysis

Molecular Biotechnology MSc

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Finally, thanks to my parents, my sister and my family for their unconditional support, also my friends for always being there.

Thank you very much.

ABSTRACT

Studying enzymatic reactions in a medium that models the excluded volume within cells using synthetic polymers, provides us an insight on how metabolism is altered by the high concentrations of neighboring macromolecules surrounding any reaction. This issue is commonly referred as macromolecular crowding.

Kinetic behavior of Alkaline Phosphatase (ALKP) has been studied in dextran crowded media. Results suggest that the effect of macromolecular crowding on this enzyme is both excluded volume and size-dependent, in accordance to what has been reported for other oligomeric enzymes such as Lactate Dehydrogenase (LDH) or Malate Dehydrogenase (MDH).

In particular, it has been found that v_{max} in crowded media is always lower than in dilute solution, regardless of the concentration (from 50 to 100 mg/mL) and size of the crowding agent (from 60 to 500 kDa). Moreover, the influence of macromolecular crowding is dependent on dextran size showing its maximum effect when the size is similar in both dextran and crowder.

OBJECTIVES

The objective in this work is to study the kinetic behaviour of Alkaline Phosphatase (AKLP) in crowded media. It has been carried out in different conditions of excluded volume by adding increasing concentrations and sizes of crowding agents. Analysis of kinetic parameters allows us to know in a quantitative way how macromolecular crowding affects mainly the reaction rates.

To get data resemblance and to obtain a realistic description of the cellular environment, involves taking into account mainly two aspects: the heterogeneity of different sizes and shapes from a large variety of enzymes and diffusion processes of proteins in crowded media, considering not only the effect of the occupied volume but also the effect of how that volume is distributed.

The overall objective, set on horizons further than the present work, is to study the crowding effect in different enzyme systems applied to the field of enzyme kinetics. Individual analysis of each system in an *in-vivo*-like environment allows us to study enzymatic reactions from both a physicochemical and a biochemical point of view in order to gain a better understanding of metabolism. It also provides a lot of information which can be useful and easily applicable to a wide variety of science fields such as biochemistry, biomedicine or biotechnology and R+D projects.

OUTLINE

1. INTRODUCTION

1.1. Principles

1.1.1. Excluded Volume and Macromolecular Crowding Phenomena

The intracellular environment contains a myriad of biological macromolecules (such as proteins, nucleic acids, ribonucleoproteins, polysaccharides, *etc.*) within a range of 80-400 mg/mL which means 5-40 % (w/w) of total occupied volume [1, 2]. This implies that the intracellular environment is extremely crowded rather than concentrated because no individual macromolecular specie is present at high concentration but taken together occupy a certain fraction of volume in the medium (as is shown in *Figure 1*). This phenomenon also occurs in extracellular matrix of tissues such cartilage or even blood plasma (indeed contains about 80 mg/mL of proteins) [3]. This issue is commonly referred as macromolecular crowding.

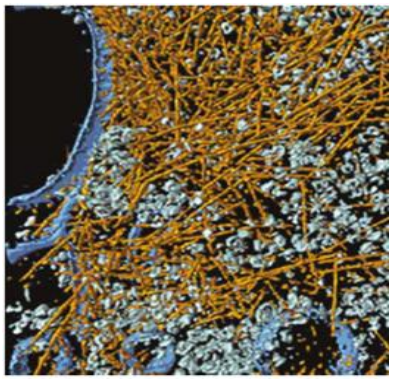


Figure 1. *3D reconstruction of a cytoplasm from Dictyostelium discoideum [2].*

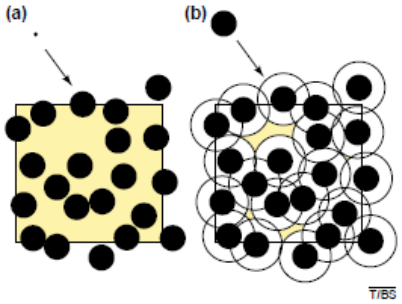


Figure 2. *The squares define volumes containing spherical macromolecules occupying ~30% of the available space (in yellow). (a) Small molecule has access to virtually all of the remaining 70% of the space. (b) Molecule similar in size to the macromolecules of background is indeed more excluded from this 70% [2].*

Any biochemical reaction that depends on available volume can be affected by macromolecular crowding effects. The thermodynamic consequences of the unavailable volume are called excluded volume effect [4]. The unavailability of volume caused by the presence of other molecules in the system (*Figure 2*) results in a decrease of entropy due to decreased randomness of the particle distribution. As a consequence, enzyme kinetics as well as several other physiological processes in which macromolecules involved can be affected by macromolecular crowding.

Despite this, crowding has not been taken into account generally in most studies *in-vitro*. In most cases, they do not properly represent macromolecular properties and reactions taking place

in physiological fluid media since *in-vitro* assays have been traditionally carried out in dilute solution conditions in concentrations less than 1 mg/mL [5].

1.1.2. *In-vivo-like* Strategies to Mimic Macromolecular Crowding

Studying any biological process at the molecular scale within living cells, that is *in vivo*, involves a careful task because of the high heterogeneity of the media and all the variables that have to be taken into account. Moreover, results could be difficult to interpret since a great number of processes share common features, reagents or products which cannot be distinguished when studied in the same system. For instance, following the reduction of pyruvate by lactate dehydrogenase *in vivo*, monitoring the change in concentration of either NADH coenzyme or pyruvate would be challenging, since both of them are also present in many other reactions throughout metabolism.

Hence, macromolecules in their native environment have to be studied separately in a model system. *In-vivo*-like media is an approach that allows us to study the behaviour of macromolecules in a physiological environment owing to successfully imitate effects due to non-specific interactions and occupied volume by background species. These media mimics the excluded volume within cells using crowding agents avoiding complex and intrinsically variable that take place *in-vivo*.

But *in-vivo-like* media also entails problems since it does not reflect exactly the physicochemical properties of the cellular environments. It is taken into account the molecular nature of the individual reactants species but background species are represented by simplified particles that resemble in general shape and size and the interactions between them [6, 7]. Despite this it was shown that adding crowding agents to model excluded volume can lead to different effects: stabilize proteins, accelerate protein aggregation, increase enzyme catalysis rates and cause refolding of disordered proteins [8].

Crowding agents must have certain properties to ensure a successful model being the main criterion to meet to be inert, so not to interact with the system components under test, except via steric repulsions. There are many types of them to simulate crowding effects, from some proteins (Haemoglobin, Lysozyme or Bovine Serum Albumin (BSA)) to synthetic polymers (Ficoll, Polyethylene glycol (PEG) or Dextran among others). Either to obtain reliable results or to select the type of crowder to work with there are some features to consider as water soluble, non-self-assembly or not alter physiologically significant factors (pH, ionic strength or redox

potential) [3]. Notice that the use of a certain crowder or another affects to the results because their properties are not equal in all cases, so generally the quantitative approach is applied by providing results analysed in equal stringency and conditions for a comparison with several experimental trials.

Another option used by researchers in this field to modelling the intracellular environment *in-vitro* is the encapsulation of proteins in silica glass using sol-gel techniques. The base of this approach is to entrap proteins into the matrix which its pore size is imposed by them. Advantage in this method is that optically transparent glass products may be analysed by a lot of spectroscopic techniques (*e.g.* fluorescence or circular dichroism) [1]. Actually, this case mimics confinement rather than macromolecular crowding. Confinement within cells is due to fibres and membrane structures within cytosol so both phenomena are imposed by occupied volume.

1.1.3. History of Macromolecular Crowding

The first studies in heterogeneous media were carried out by Ogston and Preston in 1966 [9] measuring solutions of hyaluronic acid in absence and presence of BSA by light scattering. They saw an excluded volume effect on protein mainly due to steric causes. In order to quantify the extent to which a dense heterogeneous medium could affect to enzymatic function, Laurent in 1971 [10] was the first to study several enzymatic systems in crowding environments composed by polymer media. Parameters in terms of Michaelis-Menten kinetic in all cases were altered, a decrease of Michaelis constant was found. Minton and Wilf several years after, in 1979 [11] also under macromolecular crowding conditions studied the specific activity of tetrameric protein, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPD). They showed that the addition of high concentrations of unrelated globular proteins does promote the formation of tetramer due to space-filling properties of the added species. They predicted that the rate of an enzymatic reaction would decrease when the concentration or size of crowding agents increased.

1.1.4. Relevance of Macromolecular Crowding

Excluded volume is one of the more relevant entropic effects within cells so it can influence on macromolecules involved in a wide variety of processes. It may have an impact not only on enzyme kinetics, issue that concerns us, but also it can influence diffusion, promote protein

oligomerization, aggregation and fibrillation and it can shift the conformational equilibria to protein folding and so enhancing structural stability.

Some experiments have been carried out to study the diffusion of alpha-chymotrypsin in crowded media by Fluorescence Recovery After Photobleaching (FRAP) [12]. It displayed a hindered diffusion, in an excluded volume-dependent and obstacle size-dependent manner. Hence, it is expected that the effects of excluded volume differ between diffusion-controlled reactions and activation-controlled reactions since diffusion was affected [13]. In this case, computer simulations supported experimental trials and results were in agreement since an anomalous diffusion was obtained by both means, a limiting diffusion coefficient dependent on the amount of excluded volume imposed [14].

Regarding the simulations to integer diffusion and enzymatic reactions in crowded media, ReaDDy software package [15] was developed. It is based on the application of a Brownian motion algorithm for stochastic movement of particles and Monte Carlo simulations on 3D lattice for the reaction steps [16]. The analysis of the system evolution over time allows studying reaction and diffusion processes in different kinetic and excluded volume conditions in terms of Michaelis-Menten.

Often proteins have multiple conformations, in this case macromolecular crowding also has an important role. It shifts conformational equilibria towards folded conformations by favouring compaction. Besides, it also enhances protein stability against denaturation by heat, pH or denaturing agents [6, 8].

In monomeric enzymes the effect of excluded volume is expected to affect on enzymatic activity if catalysis is accompanied by changes in size or shape but in oligomeric proteins the mechanism of the macromolecular crowding action is different. The formation of oligomers is favoured in crowded media because in this case the system changes to minimize the overall crowding by enhancing the association of macromolecules, reducing by this way the excluded volume and steric repulsions [1]. Moreover, fast associations are typically diffusion-controlled and slow associations reaction-controlled therefore macromolecular crowding is expected to decelerate fast protein associations and accelerate the slow ones [8].

Thereby all processes that depend on non-covalent associations and/or conformational changes are affected. Some examples are DNA replication and transcription, protein and nucleic acid

synthesis, intermediary metabolism and cell signalling, gene expression or the functioning of dynamic motile systems [1, 8, 12, 17, 18].

1.2. Enzyme Kinetics in Crowded Media

1.2.1. Michaelis-Menten Formalism

Enzymatic reaction kinetics was defined by Michaelis-Menten in 1913. They mathematically described the behaviour of a two-steps reaction which nowadays is known as fundamental equation of enzyme kinetics based on scheme proposed by Henri in 1902:

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

It involves considering a first step where enzyme (E) and substrate (S) combine as reactants to form a bimolecular enzyme-substrate complex (ES). This is the affinity step, when the substrate binds to the active site of the enzyme in a fast equilibrium. Consecutively, the catalytic step takes place in which complex dissociation yields the product (P) and the enzyme is recovered. This step is irreversible and it is the limiting one, slower than the previous since implies chemical transformation and product release.

The whole reaction is defined from limiting step whose kinetic constant is k_2 in terms of product formation.

$$v = \frac{d[P]}{dt} = k_2[ES] \qquad Eq.\,1.1$$

Measuring velocity in terms of enzyme-substrate complex concentration is not possible because it is very difficult to know this concentration over reaction progress. It is necessary to define the velocity equation according to species the concentration of which is known, such as total enzyme concentration $[E]_T$, or can be monitored over time, such as the variation of substrate concentration over time, $[S]$.

Moreover, another assumption was taken into account in order to reach Michaelis-Menten equation: at the beginning of the reaction the concentration of ES complex is very low, but afterwards, provided that the substrate concentration is much higher than the enzyme's, it remains constant over time. This is not an equilibrium situation since it is an unidirectional flow, instead it is a steady-state. This is known as Steady-State Assumption (SSA).

$$\frac{d[ES]}{dt} = k_1[E][S] - k_2[ES] - k_{-1}[ES] \approx 0 \qquad Eq.\,1.2$$

By assuming that $[ES]$ reaches equilibrium and so rate of formation of ES complex is equal to that of decomposition, Michaelis-Menten constant is obtained:

$$K_m \equiv \frac{k_2 + k_{-1}}{k_1} = \frac{[E][S]}{[ES]} \qquad Eq.\,1.3$$

It is a pseudo-dissociation constant since it is equivalent to dissociation constant for complex decomposition to release substrate and free enzyme. Moreover K_m, represents the enzyme affinity to the substrate.

Finally, taken into account previous assumptions and initial velocities, Michalis-Menten equation is obtained.

$$v_0 = \frac{v_{max}\,[S]_0}{K_m + [S]_0} \qquad Eq.\,1.4$$

where v_0 is the initial velocity, $v_{max} \equiv k_2[E]_T$. This formalism holds while Reactant Stationary Assumption (RSA) $[E]_0 \ll K_m + [S]_0$ is met.

Notice that k_2 does not represent a one-step reaction since the mechanism may be more complex then it must be replaced by catalytic constant, k_{cat}, which defines the capacity of enzyme-substrate complex to form product. It is also sometimes called turnover number which represents the number of catalytic cycles the enzyme can undergo in unit time [19].

From slope values at initial reaction rates, interval where reaction meets a first-order kinetic, a curve fitted by Michaelis-Menten equation is displayed. The hyperbola shown in *Figure 3* represents the dependence of initial rate v_0 on the substrate concentration [S].

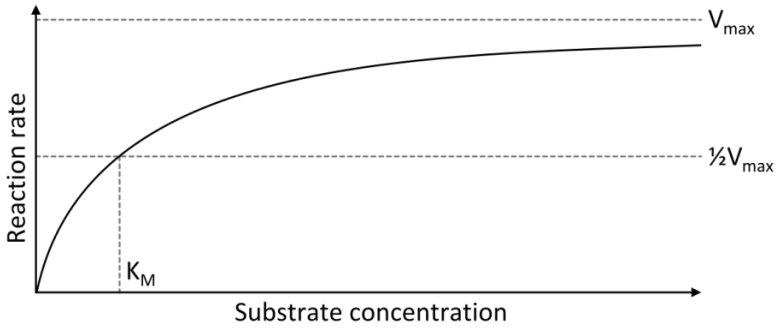


Figure 3. *Hyperbola defined by the Michaelis-Menten equation.*

At the beginning of the reaction the enzyme is present mainly in its free form. While the reaction progress happens the concentration of enzyme-substrate complex is increasing. At initial velocities, reaction rate increases linearly with concentration. Over time it reaches a saturating condition reaching an asymptotic value ($v_{\max}$) and it means that increasing the substrate concentration does not affect the reaction rate. Michaelis constant value is given by the substrate concentration at which the velocity takes the value of half the asymptotic value of $v_{\max}$.

1.2.2. Crowding Effect on Enzyme Kinetics

Enzyme kinetics is defined by Michaelis constant (K_m) and catalytic constant (k_2), and since they are defined in ideal conditions, neglecting activity coefficients, their values are usually affected by excluded volume.

Currently many researches are focused on the study of diverse enzymatic systems under crowding conditions in order to study the effect of excluded volume that reflects in kinetic parameters. Different variations have been found but in most cases maximum velocity, v_{max} decreases [5, 20-24] although there are exceptions where it increases [23, 25-27]. Whereas K_m can increase [22, 25, 27], decrease [5, 20, 21, 23, 24, 26] or remain constant [21].

Since macromolecular crowding modifies protein conformations and the conformational equilibria is intrinsic to each protein, the variation of K_m cannot be easily predicted. This parameter represents the affinity of the enzyme to bind the substrate so it depends on whether the active site is affected or not in crowding conditions, mainly depending on to what extent the catalytic site is exposed to the bulk of the solution. Regarding, k_2 and v_{max} values, they are expected to decrease in crowded media due to the hindered diffusion for diffusion-limited reactions [13] and to increase for activation-limited reactions, provided that the active site remains unaltered, since total effective enzyme concentration is enhanced. This can be attributed to the fact that a significant fraction of the reaction volume is occupied by crowding agents, and therefore the total reaction volume diminishes.

1.3. Oligomeric Enzyme to Study: Alkaline Phosphatase

The studied enzyme is **bovine intestinal alkaline phosphatase (ALKP).** It is a dimeric enzyme formed by two identical subunits as shown in *Figure 4* [28]. It is a membrane-derived glycoprotein which catalyzes the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) yielding a

yellow end-product, *p*-nitrophenol, [29] as shown *Figure 5* below. This transformation can be easily monitored by spectroscopic measurements.

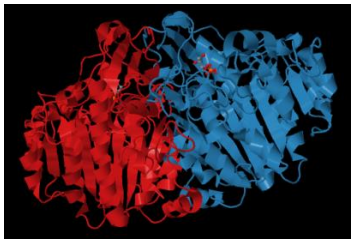


Figure 4. *Crystal structure which shows identical subunits of E. Coli AKLP from PDB (4YR1 entry, JSmol Image).*

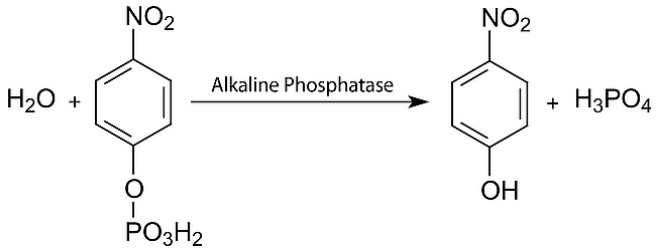


Figure 5*. Hydrolysis of pNPP catalyzed by ALKP.*

It has been chosen since its size is in the middle of the other systems studied previously by the working team: alpha-chymotrypsin ($M_W = 25\ kDa$), horseradish peroxidase (HRP, $M_W = 42\ kDa$) and L-lactate dehydrogenase (LDH, $M_W = 140\ kDa$) Alkaline phosphatase (ALKP) has a molecular weight of 104 kDa. The choice of ALKP to continue this size-dependent investigation allows us to assess not only the enzyme/obstacle size ratio but also how macromolecular crowding affects differently the reactions catalysed by enzymes of different sizes and different number of subunits.

ALKP is present in a variety of tissues, but since some of its substrates are glycerophosphate and fructose-1-6-diphosphate among others, is present in the highest amount in the intestinal mucosa [30]. Actually, ALKP has a broad specificity for phosphate esters of alcohols, amines, pyrophosphate and phenols.

Typically, the catalytic mechanism proceeds through a covalent serine-phosphate intermediate to produce inorganic phosphate and an alcohol. Inorganic phosphate is also a strong competitive inhibitor of the enzyme and fills the entire volume of the shallow active-site pocket [31] (active site is shown in *Figure 6*).

ALKP is routinely used to dephosphorylate proteins and nucleic acids. Its activity in human serum is an enzymatic signal for a variety of disease states involving particularly the liver and bone.

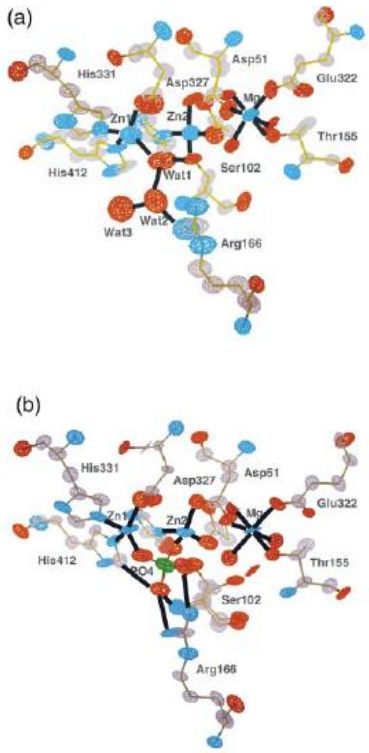


Figure 6. *Stereoview of the anisotropic refinement of the active-site residues of the alkaline phosphatase from E.Coli (a) in the absence of phosphate and (b) in the presence of phosphate [31].*

To study the function and some aspects (crystallographic structure or mechanism) it is useful to focus on ALKP from *Escherichia Coli* as a model system which is easier to obtain and characterize. It can be compared reliably to the intestinal enzyme because the *E.Coli* enzyme has similar catalytic properties, similar pH-rate profile and size and indeed it forms the same phosphoseryl intermediate [30]. They share a 25–35 % of identical sequences in structure, in which the active site and binding site for divalent ions (Zn^{2+} and Mg^{2+}), necessary for catalytic activity, is conserved [32].

To characterize the AKLP mechanism *E. Coli* ALKP was also used. In its structure, the active site contains three metal-binding sites; two of them are occupied by zinc ions and the last one by a magnesium ion. The mechanism involves two sequential in-line nucleophilic attacks at phosphorous. As described in *Figure 7*, Zn_2 in addition to substrate/ phosphate binding, coordinates the hydroxyl group of Ser102, activating it for a nucleophilic attack on the phosphate monoester in the first step. Zn_1 facilitates departure of the alcohol leaving group of the substrate in the this same step, and then activates a water molecule for the second nucleophilic attack on the covalent serine-phosphate intermediate [30, 31].

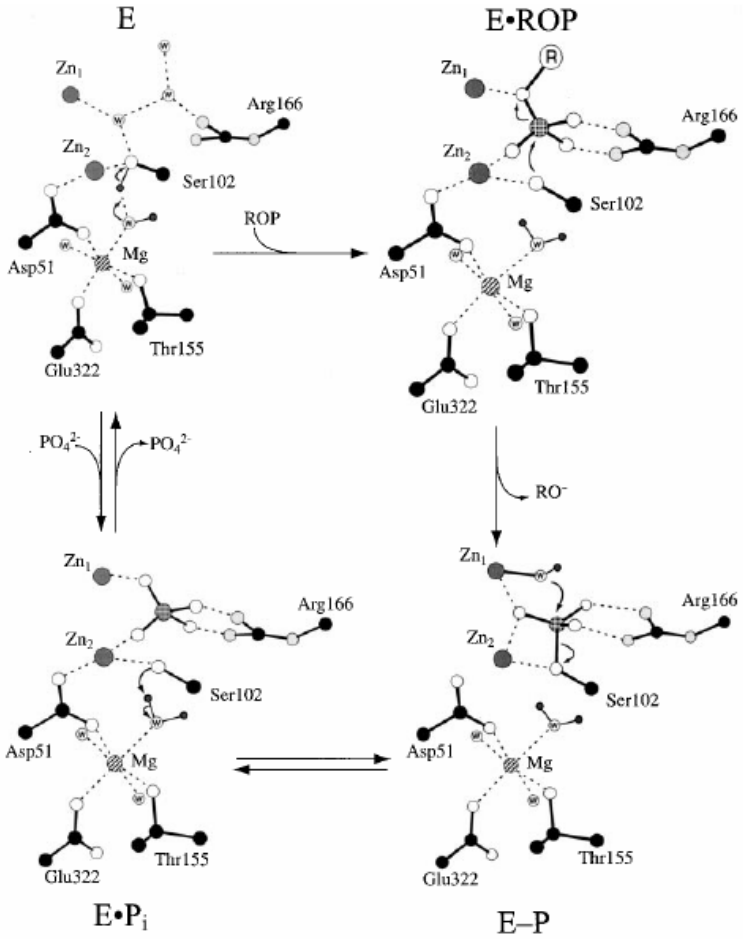


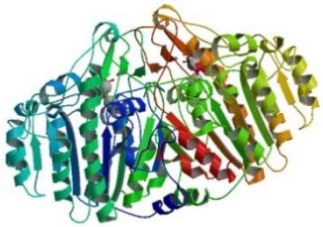
Figure 7. *The catalytic mechanism of alkaline phosphatase from E.Coli [31].*

2. MATERIALS AND METHODS

2.1. Alkaline Phosphatase Reaction System

Enzyme systems to study macromolecular crowding effects have been chosen as models based on the following issues: the reactions involved must be accompanied by a minimal change in the excluded volume due to the conversion of reagents into products, they cannot present any kind of specific interactions with crowding agents and finally, the range of hydrodynamic radii of the crowding agents has to comprise the enzyme size in a central part. That is, the size of the enzyme must be in between the range of sizes of the crowding obstacles in order to carry out a successful obstacle size-dependent investigation. Enzyme which will be described below has been chosen mainly because of its molecular weight.

Alkaline phosphatase (ALKP) from bovine intestinal mucosa (E.C. 3.1.3.1, M_w=104kDa), was purchased already purified from Sigma-Aldrich Chemical (Milwaukee, WI, USA). ALKP catalyses the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) which yields to a yellow end-product, *p*-nitrophenol. Its release can be followed spectroscopically using UV-1800 Shimadzu spectrophotometer coupled to a stopped-flow analytical system. Experimental conditions were undertaken in glycine-NaOH buffer 0.1M adjusted to pH=9.5 at 25ºC [32].



E.C. 3.1.3.1
Mw = 104 kDa
PDB Entry: 4YR1

2.2. Dextrans as Crowding Agents

Dextrans are neutral, inert and random-coil synthetic polymers of glucose polysaccharide (*Figure 8, 9*) which are commonly used as crowding agents. Variety of molecular weights for crowder was D50 (50 kDa), D150 (150 kDa), D275 (275 kDa) and D500 (500 kDa), which were obtained from Fluka (Buchs, Switzerland).

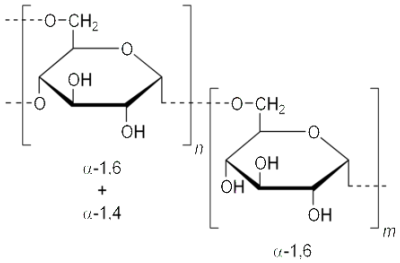


Figure 8. *Chemical structure of dextran. It presents α-1,4 and α-1,6 linkages between molecules.*

Dextrans are one the most widely used crowders, which makes them particularly suitable in order to compare experimental results within different studies. Particularly in this work, previous experiments also were carried out in the same conditions of crowder.

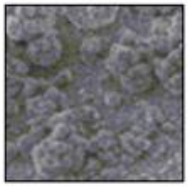


Figure 9. *Cryo-SEM image for D50 (200 mg/mL).*

2.3. Stopped-Flow Method

By using an automatic flow-based analytical system (*Figure 10*), fast enzymatic reactions and so enzyme activity can be measured at low-volume, real-time initial lineal part of the kinetic avoiding the potential errors inherent in manual operation [33]. Operated manually implies lack of homogeneity, loss of initial information of reaction and low-reproducibility in results.

Stopped-flow is consists on a mixing chamber that allows to thermostat the reaction to get homogeneous conditions all over the experiments. Two drive syringes introduce the reacting species homogenously into the cell with control of the final reaction volume. Spectrophotometer detects changes in absorbance from the beginning of the reaction, with a deadtime of 5 ms.

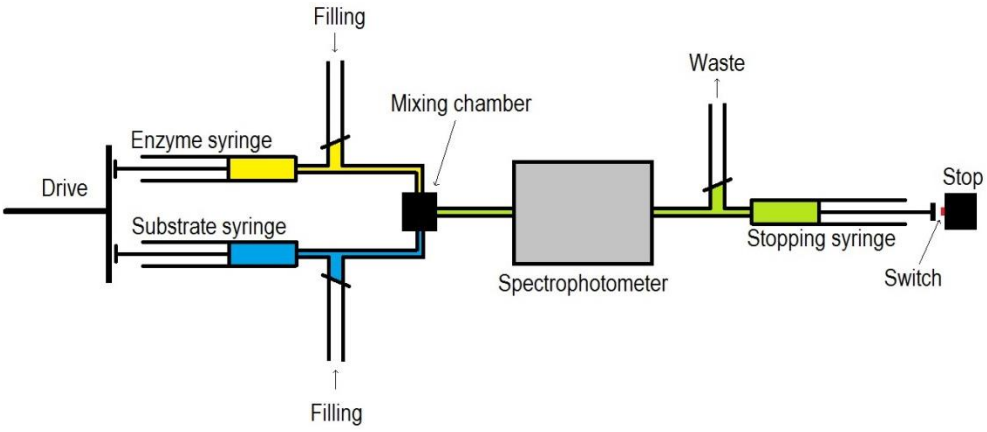


Figure 10. *Stopped-flow system scheme.*

2.4. Experimental Setup and Data Fitting

To study macromolecular crowding effects over an enzymatic system several experiments varying excluded volume conditions were carried out. The initial rates at different substrate concentrations were measured by following product release spectroscopically. The reaction was performed in dilute solution conditions and in crowded conditions by adding different concentrations and sizes of crowding agents. Hence, the effect of occupied volume and also the effect of how volume distribution could be analysed.

Plotting the initial rate against substrate concentration allowed to obtain kinetic parameters v_{max} and K_m by fitting the obtained curve to Michaelis-Menten equation.

Spectrophotometric measurements were carried out remaining constant ALKP concentration ($4 \cdot 10^{-6}$ M) while *p*NPP concentration was increased from 0.135 to 1.62 mM. Measurements were made both in dilute solution conditions and in several fractions of occupied volume by dextrans (from 50 to 100 mg/mL), (more details about reaction conditions are specified in ***Table 1***).

		Mw (kDa)	Experimental Conditions (at 25ºC)
Enzyme	Alkaline phosphatase	140	Fixed concentration $4 \cdot 10^{-6}$ M
Substrate	*p* NPP	371.14	Increasing from 0 and $1{,}62 \cdot 10^{-3}$ M
Buffer solution	**Dextrans** D60	60	Increasing from 0 to 100mg/mL
Glycine - NaOH	D150	150	
0,1M	D250	250	
pH = 9.5	D500	500	

Table 1. *Experimental conditions assayed in crowded media for ALKP reaction [34].*

Enzyme activity is detected from the rate of change in the concentration of product formed. A linear increase in absorbance with time is obtained which means that the reaction rate increases linearly with substrate concentration and it is constant at short times. As the reaction progresses over time the reaction rate decreases up to the plateau due to the saturation of the enzyme. In the range in which the SSA is valid the reaction can be treated under the Michaelis-Menten approximation according to literature [10]. It allows us to fit the initial slope of each curve linearly to obtain values for initial velocities (up to 20-25 seconds). Afterwards, initial velocity values were plotted against substrate concentration and these values were fit to Michaelis-Menten equation to obtain kinetic parameters v_{max} and K_m by Origin software.

Each measurement was repeated at least three times to minimize error and ensure reproducibility. Experiments were carried out with a stopped-flow system coupled to the spectrophotometer and also by manual mixture, depending on substrate concentration. Both kinds of measurements were found to yield identical results at low substrate concentrations. The reaction presents a large linear section therefore by comparing with slopes obtained from Stopped-flow system that ensures that values obtained manually really belongs to the linear part.

2.5. Dynamic Light Scattering (DLS)

Supplementary experiments were carried out in order to enhance information about macromolecules shapes and size in crowded media as well as to try to discern ALKP monomer-dimer equilibrium.

By measuring the diffusivity of macromolecules undergoing Brownian motion using Dynamic light scattering (DLS) using a Malvern Zetasizer equipment, also called photon correlation spectroscopy (PCS), molecules and particles in suspension can be characterized. It is a non-invasive technique that measures on a time-dependent manner the scattered light intensity due to the Brownian motion of molecules. Random motion causes laser light to be scattered at different intensities so the analysis of these intensity fluctuations yields to the velocity of the Brownian motion that is related to the diffusion coefficient leading to the particle size (in terms of hydrodynamic diameter, D_h) using the Stokes-Einstein relationship ($Eq.$ 2.6.1) [35].

$$D_h = \frac{k_B T}{3\pi\eta D_t} \qquad Eq.\ 2.1$$

where k_B is Boltzmann constant, T is thermodynamic temperature, D_t translational diffusion coefficient that is given by DLS measurements and η is the dynamic viscosity. All of this parameters can be controlled by Zetasizer Software.

In order to obtain reliable results it is necessary to take into account viscosity corrections since crowded media density differs upon simple buffer and water solution viscosity. Viscosity corrections are realised by first determining the relative viscosity (η_r) to the solvent and so using Huggins equation ($Eq.$ 2.6.2) that gives the relation to the relative viscosity increase at different macromolecule concentration (c).

$$\frac{\eta_i}{c} = [\eta] + k_H[\eta]^2 c \qquad Eq.\ 2.2$$

where $\eta_i = \eta_r - 1$ is inherent viscosity and k_H is the Huggins constant.

In order to correlate hydrodynamic radius of a particle to the intensity of the scattered light it produces, the molecular weight (M_w) of the macromolecule is required for DLS measurements. The relationship between $M_w$and the intrinsic viscosity is given by the Mark-Houwink equation ($Eq.$ 2.6.3), where k_{MH} and a are Mark-Houwink constants.

$$[\eta] = k_{MH}\ (M_w)^a \qquad Eq.\ 2.3$$

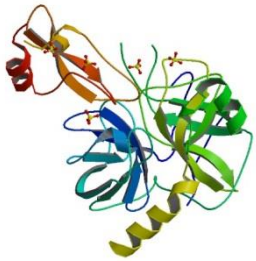
The parameters obtained by fitting this equation to the measured viscosity data were used to obtain proper values of macromolecular size in nm, as will be presented in results section.

Hence, some different conditions were probed with the aim to see the polydispersity variation of protein in the presence of crowded media and crowding agents. Measurements of ALKP in simple Glycine/NaOH buffer and also in presence of Dextrans (from D60 to D500) at different concentrations (25, 50 and 100 mg/mL) was carried out. Moreover, crowding agents were measured alone in the buffer solution at the same concentrations [36].

3. EXPERIMENTAL BACKGROUND

Previous to this work the effect of macromolecular crowding in different systems was studied within the research group where this work was performed. Bovine pancreas alpha-chymotrypsin, horseradish peroxidase and rabbit muscle L-lactate dehydrogenase. It is worth mentioning in detail these systems and their results, as they have been carried out in comparable experimental conditions and will be compared in the discussion section of this work. They also studied reactions with a kinetic behaviour where Michaelis-Menten approximation was applicable [37].

Alpha-chymotrypsin from bovine pancreas type II (E.C. 3.4.21.1) catalyses the hydrolysis of *N*-Succinyl-L-phenyl-Ala-*p*-nitroanilide in Tris-HCl buffer, (more details about reaction conditions are specify in *Table 2*). The rate of appearance of the product, *p*-nitroanilide, was monitored by UV-Vis spectroscopy at $\lambda = 405$ nm, performed at increasing substrate concentrations and for different concentrations and sizes of dextran [22].



E.C. 3.4.21.1
Mw = 25 kDa
PDB Entry: 1P2M

		Mw (kDa)	Experimental Conditions (at 25°C)
Enzyme	Alpha-chymotrypsin	25	Fixed concentration $8.5 \cdot 10^{-6}$ M
Substrate	*N*-Succinyl-L-phenil-Ala-*p*-nitroanilide	385.37	Increasing from 0 to $4.68 \cdot 10^{-4}$ M
Buffer solution	**Dextrans** D50	48.6	Increasing from 0 to 450mg/mL
Tris-HCl 0.1M	D150	150	
pH = 8.0	D275	275	
10mM $CaCl_2$	D410	409.8	

***Table 2**. Experimental conditions assayed in crowded media for alpha-chymotrypsin reaction.*

The kinetic parameters obtained suggest a dependency regarding obstacle concentration as there is a significant decrease in terms of initial rates. So, it is sensitive to excluded volume changes but not to variations in obstacle size (*Figure 11*). By increasing obstacle concentration to reaction media (*i.e.* increasing the excluded volume) leads to a v_{max} decrease and K_m increase (*Figure 12, 13*).

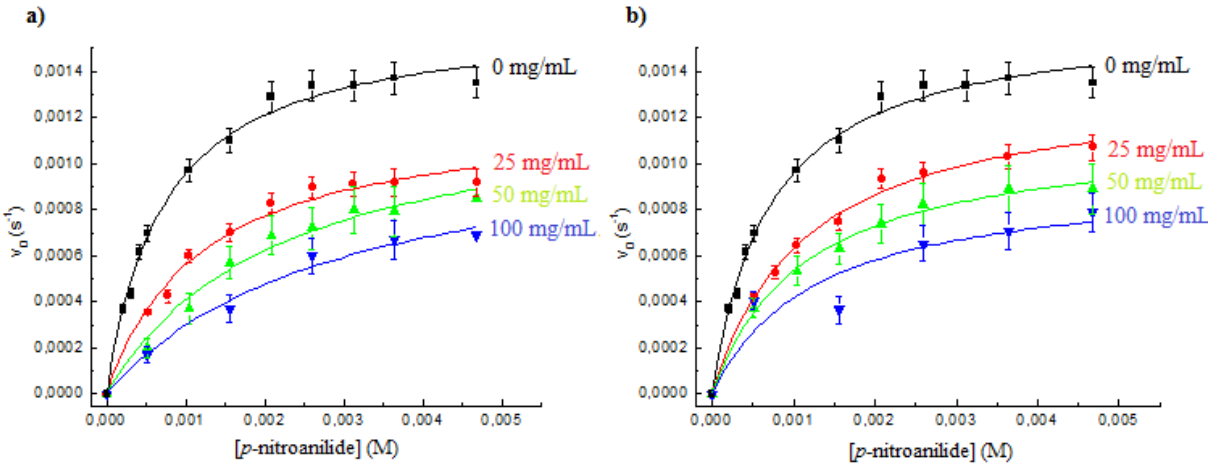


Figure 11. *Kinetic curves showing crowding effect as a function of dextrans concentrations (a) D50 and (b) D150 [22].*

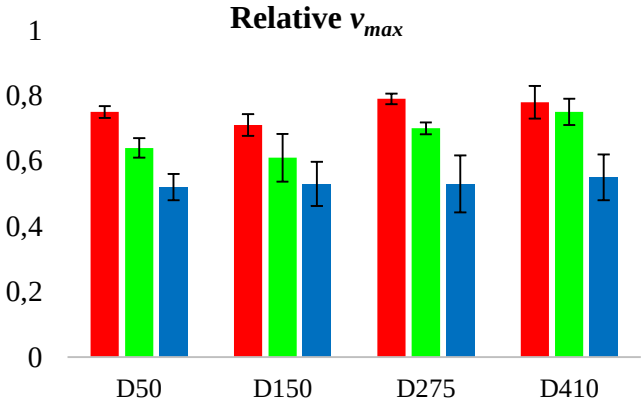


Figure 12. *Relative v_{max} to dilute solution for several sizes of dextrans (from D50 to D410) at different concentrations (25 mg/mL, 50 mg/mL and 100mg/mL).*

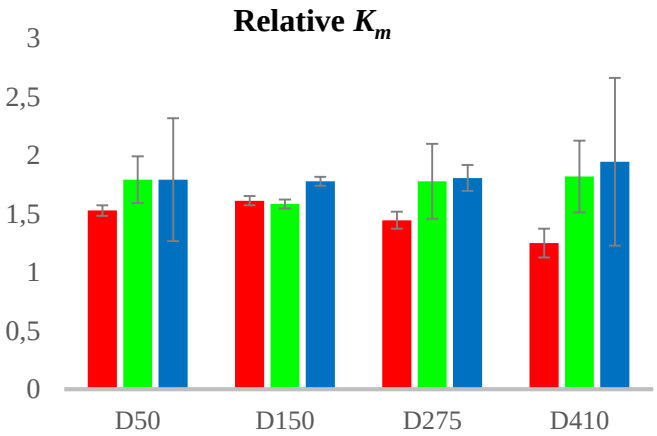
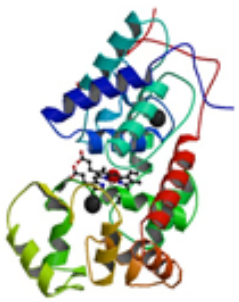


Figure 13*. Relative K_m to dilute solution for several sizes of dextrans (from D50 to D410) at different concentrations (25 mg/mL, 50 mg/mL and 100mg/mL).*

Horseradish peroxidase (**HRP**, E.C. 1.11.1.7) is a monomeric enzyme which catalyses the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by H_2O_2 in phosphate buffer (more details about the reaction conditions are specified in *Table 3*). The reaction progress was followed measuring the release of ABTS radical-cation ($ABTS^{\cdot+}$) by UV-Vis spectroscopy at 414 nm, performed at increasing substrate concentrations and for different concentrations and sizes of dextran [5].



E.C. 1.11.1.7
Mw = 42 kDa
PDB Entry: 1HCH

		Mw (kDa)	Experimental Conditions (at 25°C)
Enzyme	HRP	42	Fixed concentration $8.5 \cdot 10^{-6}$ M
Substrate	ABTS diammonium salt	548.68	Increasing from 0 and $23 \cdot 10^{-4}$ M
	H_2O_2 (33% aq.)	34.01	Fixed concentration $10 \cdot 10^{-4}$ M
Buffer solution	**Dextrans** D50	48.6	Increasing from 0 to 100mg/mL
Phosphate	D150	150	
0,1M	D275	275	
pH = 7.4	D410	409.8	

***Table 3**. Experimental conditions assayed in crowded media for HRP reaction.*

The results obtained for HRP show that crowding has a major effect in reaction rates, dependent on the amount of excluded volume. Thus, as in the alpha-chymotrypsin case, this enzyme's behaviour is also obstacle size-independent (*Figure 14*). Moreover, in this case both v_{max} and K_m decrease as the dextran concentration is increased (*Figure 15*, *16*).

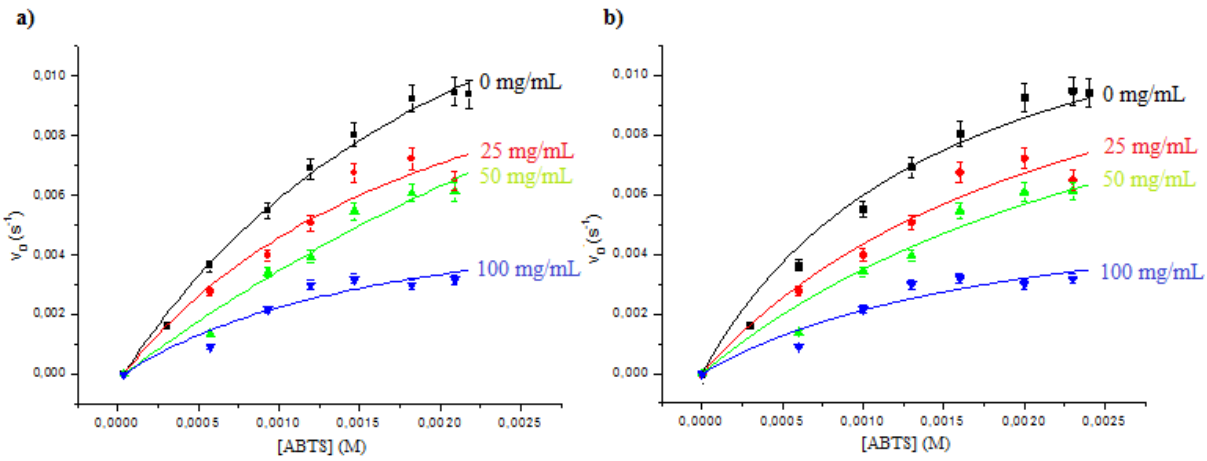


***Figure 14.** Kinetic curves showing crowding effect as a function of dextrans concentrations (a) D50 and (b) D150 [5].*

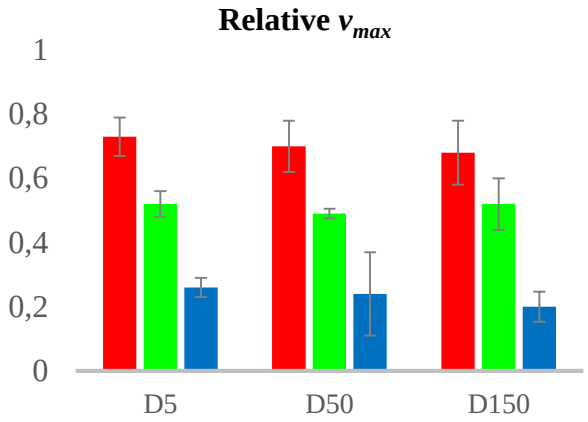


Figure 15. *Relative* v_{max} *to dilute solution for several sizes of dextrans (from D50 to D410) at different concentrations (25 mg/mL, 50 mg/mL and 100mg/mL).*

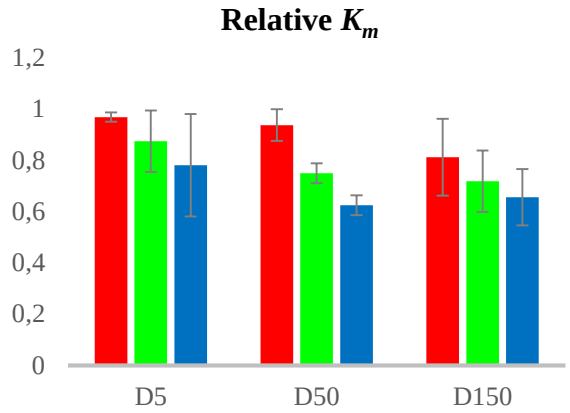
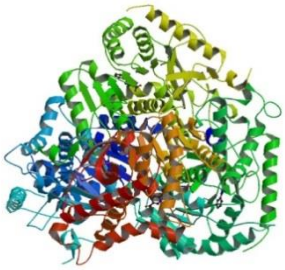


Figure 16. *Relative* K_m *to dilute solution for sevral sizes of dextrans (from D50 to D410) at different concentrations (25 mg/mL, 50 mg/mL and 100mg/mL).*

L-lactate dehydrogenase (**LDH,** E.C.1.1.1.27) from rabbit muscle is a tetrameric enzyme which atalyses the oxidation of NADH in imidazole-acetic buffer (more details about reaction onditions are specified in *Table 4*). Activity measurements were followed spectroscopically at 340 nm monitoring the loss of absorbance of NADH while it is oxidized into NAD^+ [21].

		Mw (kDa)	Experimental Conditions (at 25ºC)
Enzyme	L-Lactate dehydrogenase	104	Fixed concentration $8.2 \cdot 10^{-13}$ M
Substrate	Sodium piruvate	110.04	Increasing from 0 to $5.4 \cdot 10^{-4}$ M
	β-NADH	709,4	Fixed concentration $1.17 \cdot 10^{-4}$ M
Buffer solution	**Dextrans** D50	48.6	Increasing from 0 to 100g/L
Imidazole 30Mm	D150	150	
pH=7,5	D275	275	
60mM CH_3COOH	D410	409.8	
30mM $MgCl_2$			

Table 4*. Experimental conditions assayed in crowded media for LDH reaction.*



E.C. 1.1.1.27
Mw = 140 kDa
PDB Entry: 4I8X

Macromolecular crowding effect on LDH reaction was found to be both excluded volume and size dependent (*Figure 17*). As in the previous cases, v_{max} decreases when increasing dextran concentration (*Figure 18*) and K_m decreases (*Figure 19).* However, it remains constant in low concentrations of small dextrans, and the decrease is more pronounced for large dextrans at high concentrations. Interestingly, these results suggest a shift in the control of the reaction from activation-limited to diffusion-limited as the amount of excluded volume in the media is increased, as explained in detail elsewhere [21].

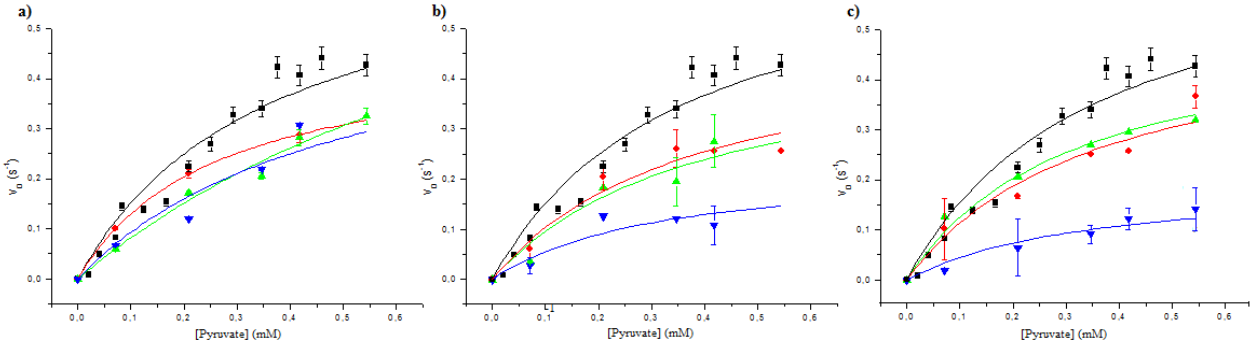


Figure 17. *Kinetic curves showing crowding effect as a function of dextrans concentrations (a) D50 and (b) D150 (c) D275 [21].*

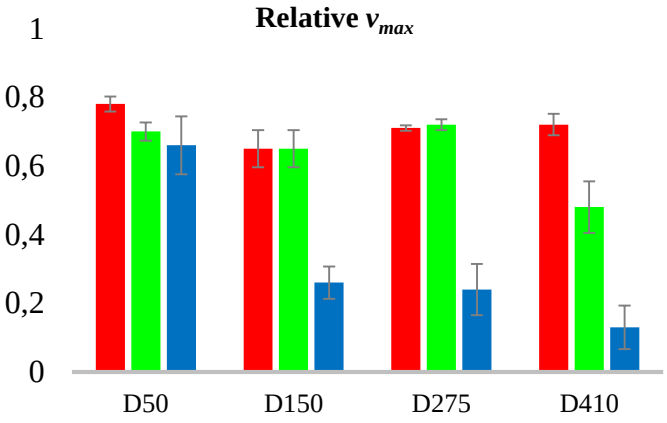


Figure 18. *Relative* $\boldsymbol{v_{max}}$ *to dilute solution for several sizes of dextrans (from D50 to D410) at different concentrations (25 mg/mL, 50 mg/mL and 100mg/mL).*

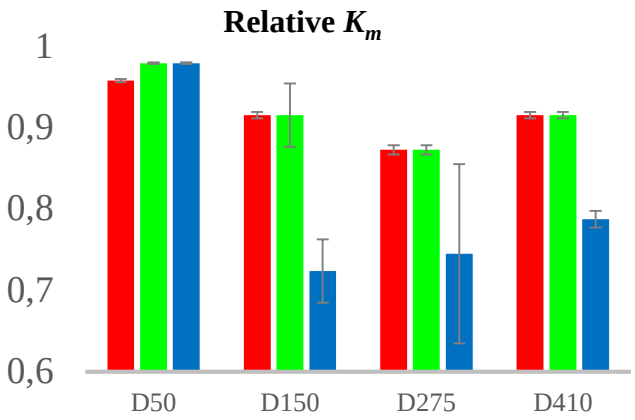


***Figure 19**. Relative K_m to dilute solution for several sizes of dextrans (from D50 to D410) at different concentrations (25 mg/mL, 50 mg/mL and 100mg/mL).*

4. RESULTS

4.1. Alkaline Phosphatase Results

Macromolecular crowding effects on ALKP catalysis [34] display dependence on both with size of dextran and mount of excluded volume (*Figure 20*). This trend was shown before in LDH case. Kinetic parameters show a v_{max} decrease (*Figure 21*) and K_m increase (*Figure 22).*

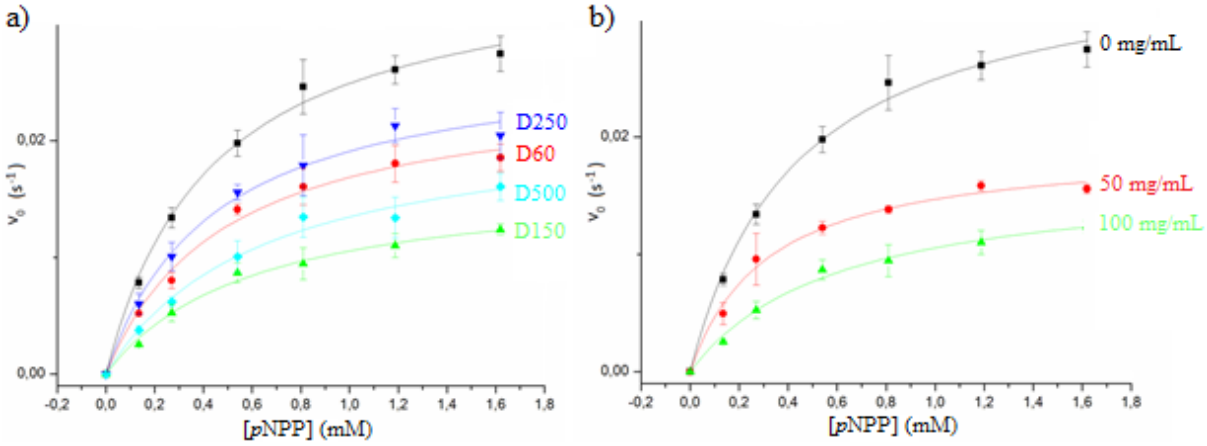


Figure 20. *a) Kinetic curve showing the crowding effect as a function of the size of dextrans at same concentration (100g/L) b) Kinetic curve showing the crowding effect as a function of the concentration of dextrans (D150) at several concentrations of crowding media [34].*

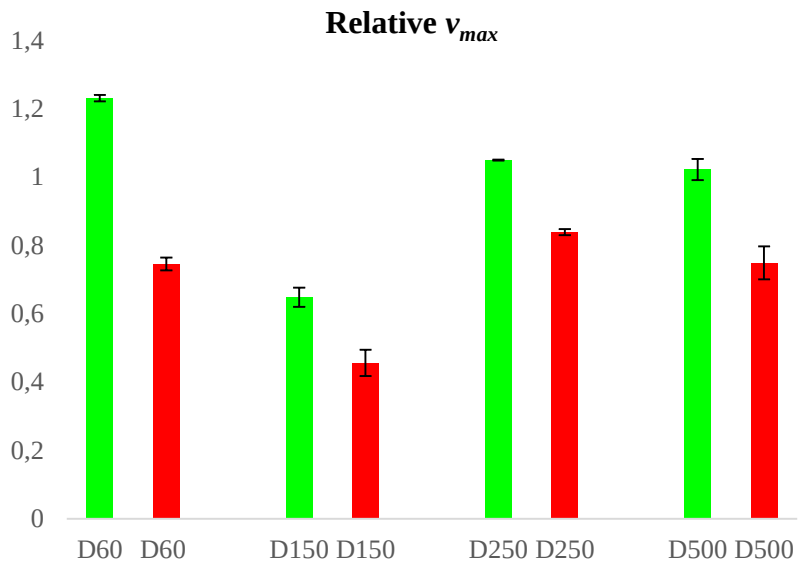


Figure 21*. Relative* v_{max} *to dilute solution for sevral sizes of dextrans at different concentrations (50mg/mL and 100mg/mL) [34].*

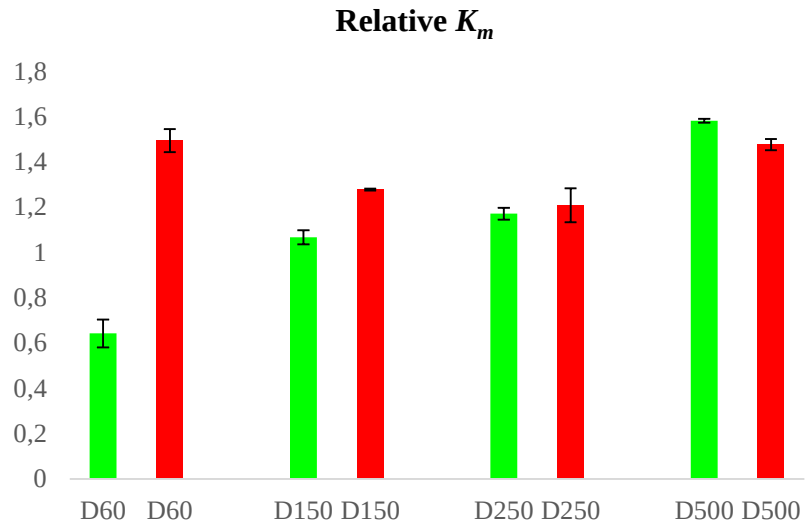


***Figure 22**. Relative K_m to dilute solution for several sizes of dextrans at different concentrations (50mg/mL and 100mg/mL) [34].*

Below, the detailed kinetic parameters are shown:

Dextran	**Dextran Concentration (mg/mL)**	v_{max} **(M·s^{-1})**	K_m **(mM)**	v_{max}/K_m
Solution	0	$(20.2 \pm 0.8) \cdot 10^{-7}$	0.37 ± 0.04	$5{,}5 \cdot 10^{-3}$
D60	50	$(24.9 \pm 0.9) \cdot 10^{-7}$	0.24 ± 0.03	$10.4 \cdot 10^{-3}$
	100	$(15.8 \pm 0.7) \cdot 10^{-7}$	0.56 ± 0.07	$2.7 \cdot 10^{-3}$
D150	50	$(13.1 \pm 0.7) \cdot 10^{-7}$	0.40 ± 0.06	$3.3 \cdot 10^{-3}$
	100	$(9.2 \pm 0.5) \cdot 10^{-7}$	0.48 ± 0.07	$1.9 \cdot 10^{-3}$
D250	50	$(21.2 \pm 0.8) \cdot 10^{-7}$	0.44 ± 0.05	$4.8 \cdot 10^{-3}$
	100	$(16.9 \pm 0.4) \cdot 10^{-7}$	0.45 ± 0.03	$3.8 \cdot 10^{-3}$
D500	50	$(20 \pm 1) \cdot 10^{-7}$	0.5 ± 0.1	$4.1 \cdot 10^{-3}$
	100	$(15 \pm 2) \cdot 10^{-7}$	0.5 ± 0.1	$3.0 \cdot 10^{-3}$

***Table 5**. Detailed values of v_{max} and K_m.*

4.2. Supplementary Experiments Results

Analyzing the results, it was concluded that Zetasizer could discern the molecular weight of both dextrans as enzyme when they were measured in an individually way. Results for mixed solution measures with dextrans and enxyme were inconclusive, since both yield intensity peaks of light scattering in the same region, which could not be disconvoluted. When ALKP was measured in Glycine/NaOH buffer Zetasizer resolution was not enough to discern between monomer-dimer.

However, interesting results were found for dextrans measures in simple Glycine/NaOH buffer since it was possible to discern different sizes of dextran and different concentrations within

each type. As dextran concentration is increased, polidispersity decreases since peaks become narrower and higher. Besides, they also tend to shift to the left, which means that the conformation of the polymer gets less branched and thus more compact. Moreover, this self-compacting effect seems to be more pronounced as the M_w of the dextran is increased. In the next Figures it is shown the effect mentioned above.

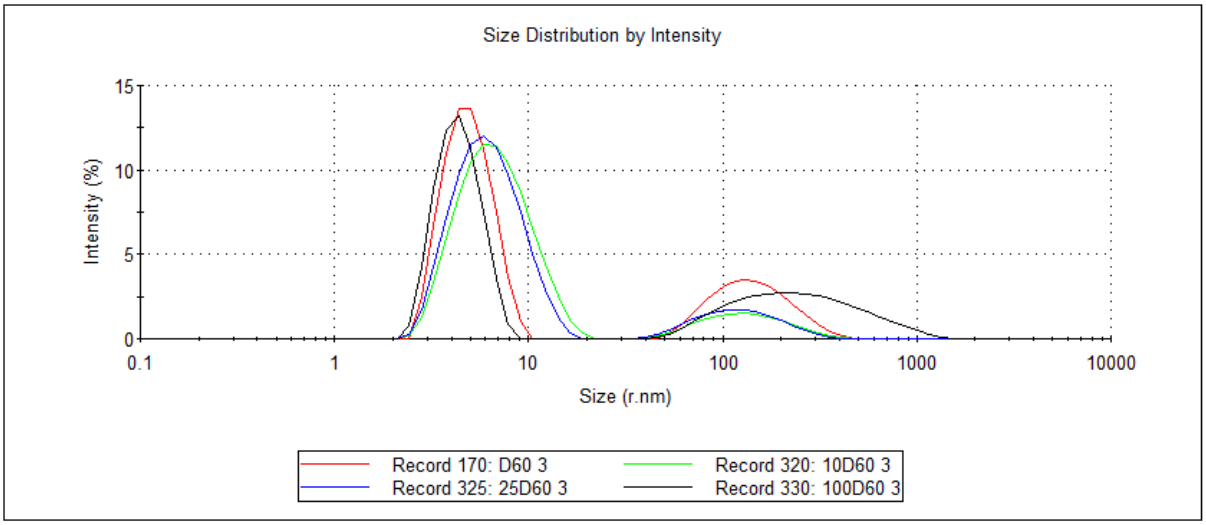


***Figure 23**. Peaks measured by DLS for D60 at different concentrations (10, 25, 50 and 100 mg/mL).*

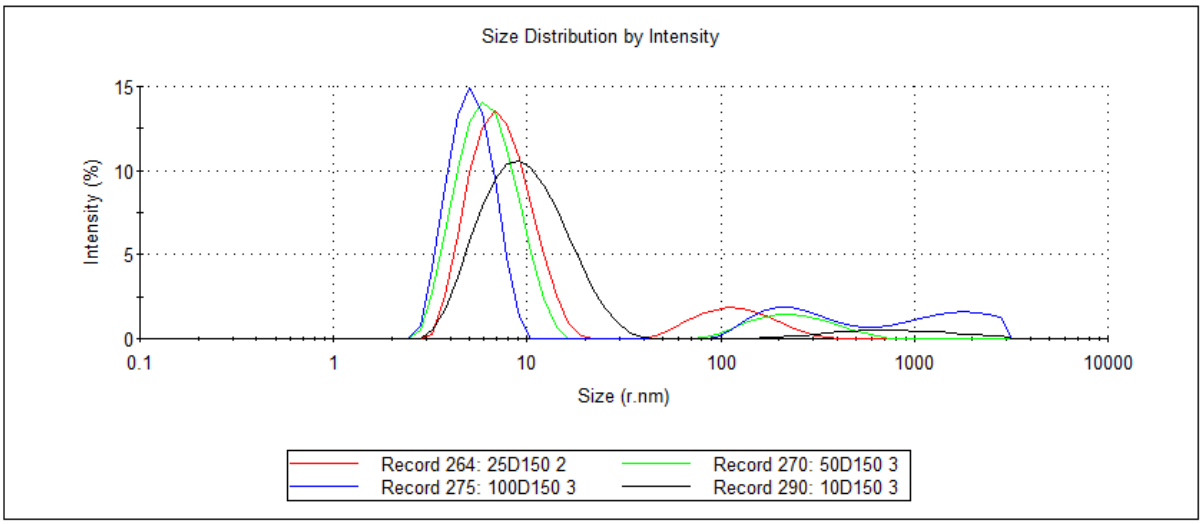


***Figure 24**. Peaks measured by DLS for D150 at different concentrations (10, 25, 50 and 100 mg/mL).*

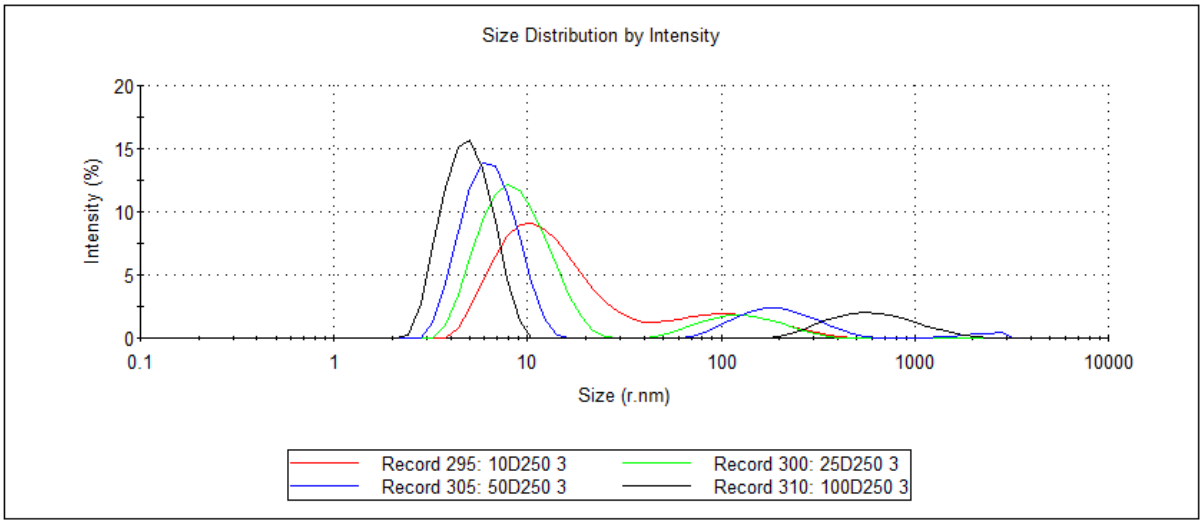


***Figure 25**. Peaks measured by DLS for D250 at different concentrations (10, 25, 50 and 100 mg/mL).*

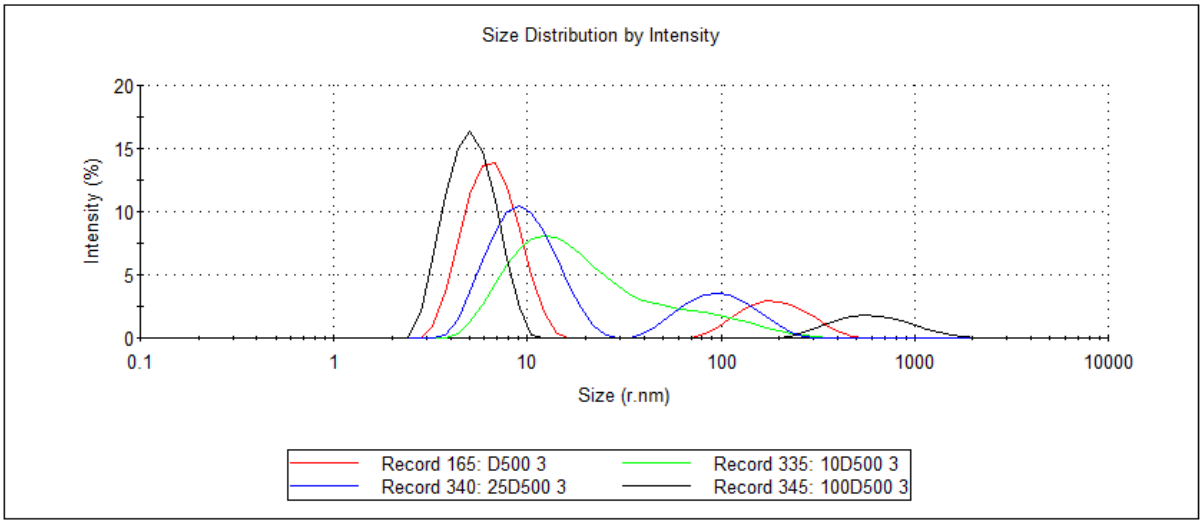


***Figure 26**. Peaks measured by DLS for D500 at different concentrations (10, 25, 50 and 100 mg/mL).*

5. DISCUSSION

5.1. Crowding Effect on Dextrans Solution

Results obtained from DLS measures show a significant decrease in polydispersity of dextrans as occupied volume is increased. Although it is necessary in the future to study this issue to gain more in-depth knowledge to interpret results carefully, it may be explained due to an autocrowding phenomenon. Dextrans are branched polymers which, at certain conditions, adopt random coil structures. When excluded volume increases, the global branched structure of the molecule becomes more compact. When dextran size increases, branched structure of the polymer is bigger and the compactation in large dextrans is more relevant than in small ones, as shown in *Figures 23-26*.

This phenomenon of a non-linearity of excluded volume to the concentration in weight of crowding agent could help to explain the non-monotonic tendency of v_{max} versus obstacle size, as will be discussed hereafter.

5.2. Crowding Effect on Monomeric and Oligomeric Enzymes

Behaviour of monomeric enzymes in crowded media seems to display obstacle size-independence [13, 37] which differs from oligomeric proteins that display an obstacle size-dependent behaviour (*Figure 27*) [13]. Thus, in oligomeric enzymes, although excluded volume is one of the factors that affects the kinetic behaviour of the enzyme, the ratio of obstacle-protein size for the same excluded volume is another factor which may affect the kinetic parameters of the enzymatic reaction. This phenomenon is still being studied, to the extent that it is still unclear whether this size-dependence is due to the protein size or to its oligomeric nature.

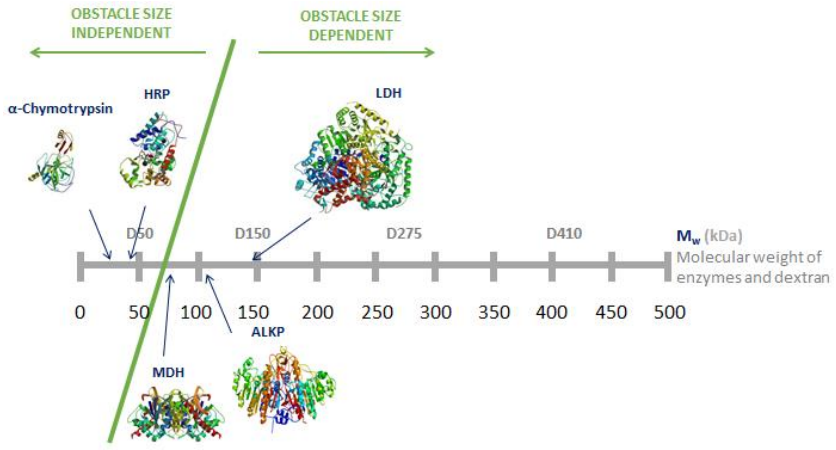


***Figure 27**. Range of enzymes and dextran to study ordered according its molecular weight.*

5.3. Enzyme/Obstacle Size Ratio

Homchaudhuri et al. [38] found a similar behaviour for ALKP kinetics in crowded media of both obstacle size and concentration dependence than in this work. Whereas, in a concentration of 20% (w/w) they found a moderate reduction on reaction rates for small dextrans (15-70 kDa) and for large dextrans (200-500 kDa) the crowding effect was much higher, it slows reaction rate considerably. However, Homchaudhuri et al. only studied ALKP system focused on v_0 values, which does not provide a complete kinetic characterization of the system. In this work, the variation of v_0 with substrate concentration and for different concentrations and sizes of dextrans is studied. An interesting phenomenon that it is obtained is that the maximum effect of macromolecular crowding was shown when the crowding agent used has a similar size than ALKP, displaying that the minimum of enzyme activity in crowding media is obtained for an enzyme-obstacle size ratio similar to enzyme studied. This behaviour has recently reported for MDH [20], which is a dimer similar in size to ALKP (*Figure 28*).

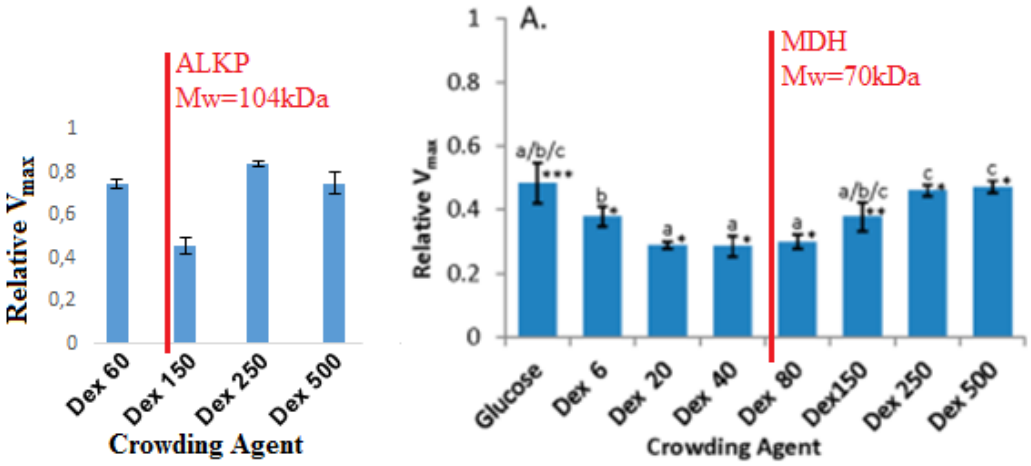


Figure 28. *Macromolecular crowding effect on ALKP and MDH at several sizes of dextrans.*

5.4. Crowding Effect on ALKP

The kinetic behaviour of ALKP in crowded media displays a minimum enzymatic activity for obstacles with a similar size to dextrans used as obstacles (*Figure 28*).

It displays a K_m increase respect to dilute solution, which it is not clear whether it is a size-dependent or excluded volume-dependent effect. Thus, the reaction must be diffusion limited which yields a decrease in k_1 and therefore, an increase in K_m, as found for the case of alpha-chymotrypsin [22].

Regarding v_{max}, it presents a size-dependent behaviour with a minimum of activity located when the enzyme and the obstacle have similar sizes. The cause of this phenomenon is still

unclear but a possible hypothesis for it would be the rise of depletion forces, entropic forces due to the size difference between enzyme and crowders. On the other hand, v_{max} decreases as a function of excluded volume for each dextran size. This can be ascribed to a conformational change of the enzyme which alters the conformation of the active site or either that k_2 is modified by the highly non-ideal environmental surroundings. Moreover, the v_{max}/K_m ratio (*Table 5*) decreases as excluded volume increases, for each obstacle size. This behaviour is found in other cases when product inhibition is present [22].

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CONCLUSIONS

As a general conclusion, macromolecular crowding effect is extensive to a wide variety of reaction-diffusion processes within cells. Excluded volume and nonspecific interactions can modulate kinetics of a large number of enzymatic reactions taking place in physiological fluid media. Measuring the way in which the kinetics of a certain enzyme is altered by a range of crowded conditions provides useful information to understanding the effect of the occupied volume and the different enzyme-obstacle size ratios.

Analysing the whole compilation of results from enzymes studied before to ALKP results, a difference between monomeric and oligomeric enzymes is encountered: an obstacle size-independence is found for monomeric enzymes and oligomeric enzymes display an obstacle size-dependent behaviour.

It is found that kinetic parameters of ALKP are altered in presence of macromolecular crowding. K_m values increase respect dilute solution and v_{max} values decrease as a function of excluded volume for each dextran size. The increase in K_m with respect to dilute solution, independently on crowder size and concentration, is ascribed to a diffusion control of the reaction. The decrease of v_{max} could be explained in terms of a conformational change in the active site or an environment-driven k_2 modification.

As excluded volume increase, a decrease on v_{max}/K_m ratio is displayed, which is typically found in product inhibition cases. Results suggest that macromolecular crowding effects on ALKP depend both on the size of dextrans and the amount of excluded volume, displaying a minimum of activity located when the enzyme and the obstacle have similar sizes, possibly due to dextran compaction and the occurrence of depletion forces.