Effect of crowding by Dextrans on the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin

Isabel Pastor [†, *], Eudald Vilaseca[†], Sergio Madurga[†], Josep Lluís Garcés [‡], Marta Cascante[§] and Francesc Mas[†]

[†]*Department of Physical Chemistry and the Research Institute of Theoretical and Computational Chemistry (IQTCUB) of the University of Barcelona (UB), C/ Martí i Franquès, 1, E-08028 Barcelona (Spain)*

[‡]*Department of Chemistry, University of Lleida (UdL), Lleida (Spain)*

[§]*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

E-mail: i.pastor@ub.edu

Fax: (+34) 934021231

ABSTRACT

Traditionally, studies on the diffusion-controlled reaction of biological macromolecules have been carried out in dilute solutions (*in vitro*). However, in an intracellular environment (*in vivo*), there is a high concentration of macromolecules, which results in non-specific interactions (macromolecular crowding). This affects the kinetics and thermodynamics of the reactions that occur in these systems. In this paper, we study the crowding effect of large macromolecules on the reaction rates of the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin, by adding Dextrans of various molecular weights to the reaction solutions. The results indicate that the volume occupied by the crowding agent, but not its size, plays 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 increase in K_m can be attributed to the slowing of protein diffusion, 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. As far as we know, this is the first reported experiment on the crowding effect in an enzymatic reaction with a mixed inhibition by product.

Keywords: enzyme kinetics, alpha-chymotrypsin, macromolecular crowding, mixed inhibition by product

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]. However, studies of biochemical processes have usually been performed in dilute solutions, in which the environment is markedly different from the cytosol. In the cellular environment, the rate of diffusion is reduced[2-8], and can even be anomalous at short times. 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*. Laurent carried out one of the first studies on enzymatic reactions in macromolecular crowded media in 1971[23]. He studied enzyme reactions in polymer media as an initial attempt to describe how the environment affects the intracellular enzyme function. This was nevertheless a complete study, because Laurent examined the effect of the polymer presence in several situations: the degradation of hyaluronic acid by hyaluronate lyase in the presence of polyethylene glycol; the lactate dehydrogenase reaction in Dextran (40 kDa) solution, and the cleavage of benzoyl DL-arginine p-nitroanilide by trypsin and its inhibition by serum albumin in Dextran solution. In all these cases, the presence of the polymer produced a moderate decrease in the apparent Michaelis-Menten (MM) constant, K_m, or in the apparent inhibition rate constant, k_1, in the case of inhibition. In 1981, Minton and Wilf[9] studied the effect of macromolecular crowding on the various kinetic steps in the enzymatic processes (enzyme [E] and substrate [S] meet and form the ES complex; the formation of an enzyme-product complex from the ES complex; and the release of product from the

enzyme-product complex) 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 the size of the crowding agent. In other words, the excluded volume decreases both the MM constant, K_m, and the 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 in K_m is frequently found, regardless of the properties of the crowding agent[24-30]. 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]. It should be noted that these studies were carried out in the presence of a single kind of polymer (e.g. Dextrans or Ficoll) of a fixed size (usually small).

The range of experimental conditions covered by these studies is not wide enough to fully understand the phenomenology of enzymatic kinetics in crowded media. In particular, two important aspects have to be investigated in depth. Firstly, to obtain a more realistic description of the cell cytosol environment, a large variety of macromolecules of different sizes and shapes should be considered. We should also take into account that each type of macromolecule may have a different influence on the enzymatic reaction. Secondly, when the diffusion process of proteins in macromolecular crowded media is studied, it is very important to consider not only the volume occupied by the crowding agent, but also how this volume is distributed. Diffusion has been found to depend on both the concentration and the size of the obstacles[33-35]. Both aspects must be studied to improve our understanding of enzymatic reactions in cell cytosol.

In this paper, we studied how the kinetics of an *in vitro* enzymatic reaction was affected by the concentration and the size of the crowding agents. We chose the alpha-chymotrypsin hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide as a model reaction, because there is only a minimal change in the excluded volume due to the small size of substrates and products. Thus, we studied the effect of the excluded volume in the reaction due to the presence of crowding agents. Macromolecular crowding was mimicked using Dextrans from 5 to 410 kDa. There were three advantages of using alpha-chymotrypsin protein in this study: the absence of known interactions with Dextrans (crowding agents); the protein size (hydrodynamic radius, r_h = 2.33 nm), which is intermediate between those of the selected crowding agents; and the protein's isoelectric point of 5.4, which implies that it was negatively charged in the buffer that was used (pH 8). This reduces the risk of aggregation, which is higher in crowded media. In this paper, we examine how this enzymatic reaction is affected by the presence of Dextrans of different sizes at different concentrations. In particular, we analyzed the effect of macromolecular crowding on the values of the v_{max} and K_m parameters of this reaction.

MATERIALS AND METHODS

Chemicals

Alpha-chymotrypsin (*E.C.* 3.4.21.1) from bovine pancreas type II (60 Umg^{-1}), which was used without further purification, and N-succinyl-L-phenyl-Ala-p-nitroanilide were purchased from Sigma-Aldrich Chemical (Milwaukee, WI, USA). Dextran (from *Leuconostoc mesenteroides*) of molecular weight 5, 50, 150, 275 and 410 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.

Hydrolysis of N-succinyl-ʟ-phenyl-Ala-p-nitroanilide

All reactions were carried out at 25ºC in Tris-HCl buffer (0.1 M, pH = 8, containing 10 mM of $CaCl_2$). The reaction mixture contained a fixed concentration of 8.5 10^{-6} M of alpha-chymotrypsin, the concentration of substrate (N-succinyl-ʟ-phenyl-Ala-p-nitroanilide) varied between 5.4 10^{-5} and 4.8 10^{-4} M, and the concentration of Dextran varied between 0 and 450 mg/mL. The reaction was initiated by the addition of alpha-chymotrypsin to a sample mixture of substrate and Dextran in Tris-buffer, using a syringe to favor mixing. The reaction progress was followed by monitoring the release of p-nitroanilide (absorbance at 410 nm) with a UV spectrophotometer (UV-1603 Shimadzu). As at the beginning of the reaction, the product absorbance increased linearly with time, and the velocity of the reaction was constant at short times (Fig. 1). Thus, the initial velocity, v_0, was chosen as an experimental parameter, and it was calculated in all the experiments as the slope of the linear fitting of the first 30 s of the absorbance/time data. To reduce the standard error of v_0, experiments were repeated from 3 to 5 times under identical conditions. Blank solutions containing the corresponding concentration of substrate and Dextran without the protein were used with each sample. This enzymatic reaction can be considered a single enzyme-substrate reaction and can be studied using the Michaelis-Menten equation following the irreversible reaction scheme that was first proposed by Henri[36]

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

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

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

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

RESULTS AND DISCUSSION

Alpha-chymotrypsin catalyzes the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide to p-nitroanilide and N-succinyl-L-phenyl-alanin. We chose this hydrolysis as a model reaction to investigate the influence of macromolecular crowding on biological reaction rates for two reasons. Firstly, the reaction is accompanied by a minimal change in the excluded volume. In fact, the substrate and the products are tiny compared with the size of the protein and of the crowding agents. Hence, the effect of both molecules (i.e. substrate and product) on the excluded volume can be neglected. As a result, effect of macromolecular crowding in the reaction can be interpreted mainly in terms of the presence of crowding agents. Secondly, this reaction can be easily monitored by UV-spectroscopy. In addition, it is important to stress that, in all the media studied, the presence of crowding agents may affect the diffusion of the substrate and enzyme

molecules. It is known that alpha-chymotrypsin (Mw = 25 kDa) diffusion depends on the size and concentration of obstacles. This diffusion is time dependent (anomalous subdiffusion) at short times and normal at long times, with a diffusion coefficient lower than that found in dilute solution[34]. In contrast, the diffusion of N-succinyl-L-phenyl-Ala-p-nitroanilide (Mw = 385.37 Da) in crowded media is observed to be normal for all times but slower than in dilute solution[37].

We studied the effect of different concentrations and different sizes of crowding agent (Dextran) in this enzymatic system. In all cases, we observed a typical initial rise and a subsequent plateau in the absorbance/time plot of the p-nitroanilide released during the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide. The kinetic curves for different concentrations of substrate ($5.4 \ 10^{-5}$ – $4.8 \ 10^{-4}$ M) in samples with 50 mg/mL of 50 kDa Dextran are shown in Fig. 1. The initial velocity (v_0) in all the experiments was obtained by fitting the initial slope of these curves. The fitted values show a significant decrease in the initial velocity of the reaction with increasing Dextran concentration.

The dependence of v_0 on the substrate concentration for different Dextran concentrations and sizes is shown in Fig. 2. Each figure corresponds to a given Dextran size and shows the variation of v_0 with the substrate concentration for different concentrations of this size of Dextran. The Dextran concentration value given in mg/mL is proportional to the volume occupied by this crowding agent. Fig. 2 shows a clear dependence of v_0-substrate concentration curves on the concentration of Dextran, but not on Dextran size. For the same occupied volume, expressed in units of inverse w/v, the v_0-substrate concentration curves are similar for all the obstacle sizes considered. Fig. 3 shows the variation of v_0 with the size and the concentration of Dextrans for a

8

fixed concentration of substrate. The value of v_0 remains the same for different sizes of Dextrans, but varies with their concentration, i.e. with the occupied volume. Because of this similitude, all the curves corresponding to the different Dextran sizes can be grouped into a single average v_0-substrate concentration curve for each Dextran concentration (Fig. 4).

This obstacle size independence of the initial velocity curves is, at first, in contrast to the results of Homchaudhuri et al.[38]. These authors studied the effect of crowding by different-sized Dextrans and Ficolls on the rate of the hydrolysis of p-nitrophenyl phosphate by alkaline phosphase. Their results revealed a steeper decrease in the rate as a function of the 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 in the crowded presence of 20% Dextran (w/w) the reaction rates slowed ~ 2-fold among smaller Dextrans (15-70kDa), ~ 5-fold for 200 kDa Dextran and ~ 7-fold for 500 kDa Dextran. One of the major differences with our study is the larger size of the protein studied. The size of alkaline phosphatase is 105 kDa, while that of alpha-chymotrypsin is 25 kDa. These authors showed that, as a consequence of the large size of alkaline phosphatase, large obstacles reduced the encounters between enzyme and substrates. In contrast, with small obstacles the effect of crowding is partially offset by enhancement of the enzyme activity owing to a caging effect, which results in a minor decrease in rate. However, both Homchaudhuri et al.[38] and our results are in agreement in that, as stated by Minton et al.[2, 8-12], 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 the case of the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin,

the total volume excluded by the crowding agents has a greater impact on the velocity of the reaction than the size of these agents. In a similar way, in a previous study we observed that the diffusion of alpha-chymotrypsin in crowded media was highly dependent on the occupied volume but only slightly dependent on the size of the crowding agents[34]. We also observed the same behavior in Monte Carlo simulations of diffusion in crowded media[35, 39]. In all these cases, the effect of the total excluded volume on the diffusion of a tracer particle prevails over the effect of the specific size of the crowders.

To better understand how the excluded volume affects the hydrolysis reaction of alpha-chymotrypsin in crowded media, the values of K_m and v_{max} were estimated by fitting Eq. 2 to the curves in Fig. 4. Table 1 shows the values obtained for these parameters as a function of the excluded volume. Our results indicate that the value of v_{max} decays with increasing obstacle concentration, whereas K_m values increase with the concentration of Dextran in the sample.

K_m analysis

Firstly we analyzed the K_m behavior. We found (Table 1) that the value of K_m for the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin increased with the concentration of Dextran, in other words, K_m increased with the obstacle excluded volume.

As stated above, there are few studies[23-28, 30] on crowding effects in enzymatic reactions, even *in vitro*. The majority of them report a slight decrease in K_m with respect to dilute solution. The decrease in 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 molecule substrate in highly non ideal crowded solution[26-28]. 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 may therefore be dependent on water activity, which affects the k_{cat} value.

In contrast, some studies do not report a decrease in K_m. For example, Assad and Engberts[32] show that the presence of 8 kDa of PEG, even at high concentration, does not modify the K_m value of the hydrolysis of p-nitrophenyl acetate by trypsin. They considered that substrate binding is unaffected by the crowder. Therefore, PEG does not compete for the active site and does not distort its structure.

However, Wenner and Bloomfield[24] and Gellerich et al.[40] reported that, as in our case, a high concentration of crowding agents increased the value of K_m of the EcoRV in Ficoll solutions and of the mitochondrial creatine kinase in Dextran solutions, respectively. They suggested that the K_m increase with Dextran concentration could be explained by an increased diffusion resistance in the sample. In our opinion, both diffusion resistance and changes in the active site must be taken into account. For the classical enzymatic mechanism depicted in Eq. 1, the MM constant is defined by $K_m = (k_{-1} + k_{cat})/k_1$. If there is diffusion control of the enzymatic reaction, the bimolecular constant (k_1) may decrease with the crowding[41-43] and lead to an increase in K_m. Moreover, studies that have observed a decrease in K_m rule out diffusion control of the enzymatic reaction and the modification of k_1 with crowding (e.g. reference 30).

Therefore, in cases in which the value of K_m decays with increasing obstacle concentration, the activity coefficient of the free substrate in solution or the ratio between the activity coefficients of the native enzyme and the enzyme-substrate complex play an important role, as both can be affected by the environmental surroundings.

In our experiments, we consider that diffusion control of the enzymatic reaction is possible for several reasons. Firstly, we know that alpha-chymotrypsin diffusion depends on the size and concentration of obstacles, and that the diffusion is time-dependent at short times and normal with a smaller diffusion coefficient at long times[34]. However, it should be noted that our experiments did not detect the short time anomalous diffusion regime of alpha-chymotrypsin, as kinetic measurements were taken at a longer time scale and in larger sample sizes than in the diffusion experiments. Secondly, it is also known that, regardless of a molecule's size, its diffusion coefficient is smaller in crowded media than in dilute solution at long times[35, 43-47], thus both diffusion coefficients, for the enzyme and for the substrate, decrease with crowding, and consequently, an enzymatic reaction can be expected to be dependent on diffusion. Thirdly, Kawai et al.[48] carried out a kinetic study of the hydrolysis of p-nitroanilides catalyzed by alpha-chymotrypsin. They examined the effect of medium viscosity on the hydrolysis of different p-nitroanilides and found that in the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide the value of K_m increased with the viscosity of the medium. This result is in agreement with diffusion control of the enzymatic reaction. Moreover, in a recent review of Schreiber et al.[21], and in references quoted therein, similar conclusions on the effect of diffusion-controlled of protein-protein association kinetics, are expressed, in particular, those relative to the effect of the medium viscosity on the

long-range translational diffusion of proteins. Finally, another test of the diffusion control of the enzymatic reaction can be made using the recent proposed spectrum of protein-protein association rate constant[21, 49]. When the enzymatic reaction follows the classical enzymatic mechanism depicted in Eq. 1 and is diffusion-controlled, $k_{-1} << k_2$, then, $k_1 \gtrsim k_2/K_m$, the consideration of the experimental values of Table 1 and the fact that $k_2 = k_{cat} \approx v_{max}/[E_T]$ yields $\frac{v_{max}/[E_T]}{K_m}$ as an estimation of the lower bound limit for k_1. Thus, as in our experiments, $[E_T] = 8 \times 10^{-6}$ M, we obtain a lower bound limit of $k_1 \approx (1\text{-}3) \times 10^5$ M^{-1} s^{-1}, which lies in the region of diffusion-limited reaction of this spectrum (see Fig.1 of reference 49).

v_{max} analysis

Secondly we analyzed the behavior of v_{max}. We found (Table 1) a decrease in the values of this parameter when the concentration of Dextran increases. According to the enzymatic mechanism described in Eq. 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 and the effective enzyme concentration. Although k_2 is a unimolecular rate constant, it is reasonable to assume that as long as the active site of an enzyme is shielded from the bulk solution, the catalytic rate constant would be unaffected by the addition of the cosolutes or crowding agents, until they are able to cause denaturation or affect the environment of the active site[22-24, 26-28, 31, 50-51]. However, it is known that the effective enzyme concentration increases with the excluded volume in crowded media[2, 8-12]. In fact, several authors explained increases of v_{max} at crowding concentration in terms of an increase in the effective enzyme

concentration due to crowding[24, 29, 31]. A decrease in v_{max} is normally explained as a result of conformational changes to the enzyme active site that are produced by crowding-induced modifications of its surroundings[26-28, 31, 51].

In our case, the environment of the active site of alpha-chymotrypsin remains constant in viscous media[48]. In addition to other explanations, we consider that effective inhibition by product is another important factor to be considered in enzymatic reactions that may show a decrease in v_{max} with crowding.
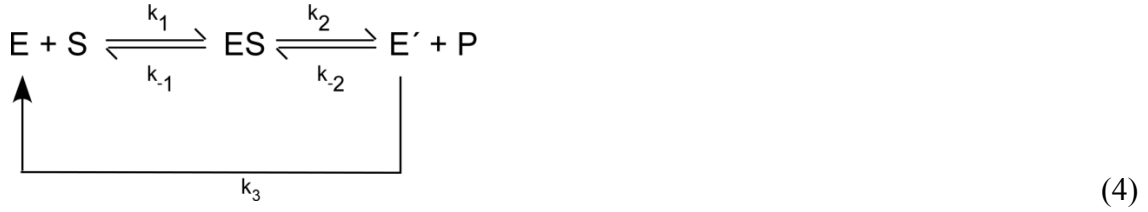
To analyze the hypothesis of inhibition by product, we repeated the enzymatic reaction experiment using samples with and without crowding, in which a high concentration of product was previously added. The experiments showed no increase in absorbance when the initial concentration of substrate was increased (data not shown). This result confirmed that there is inhibition by product in the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin in Dextran-crowded media. We think that when the Dextran concentration increases, the effective inhibition of the reaction due to the presence of product also appears to increase.

In view of this result, the expressions used so far are not appropriate. However, it is possible to write v in terms of $[S]_0$ in a similar way as in the classical expression (Eq. 2) but using apparent constants

$$v = \frac{v_{max}^{app}[S]_0}{K_{mS}^{app}+[S]_0} \tag{3}$$

The definition of the apparent constants v_{max}^{app} and K_{mS}^{app} depends on the mechanism of the reaction. The simplest inhibition mechanism that can explain the

features observed in our experimental study, called mixed inhibition mechanism[36], is depicted by

$$E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightleftharpoons[k_{-2}]{k_2} E' + P$$

$$\downarrow k_3$$

(4)

where the product P acts as a mixed inhibitor rather than as a competitive inhibitor because it binds to a different form of enzyme from substrate S.

For this mechanism, the expression of Eq. 3 holds with the following meaning of the apparent constants:

$$v_{max}^{app} = \frac{v_{max}}{\left(1 + \frac{[P]}{K_{iu}}\right)}$$

(5)

$$K_{mS}^{app} = \left(\frac{1 + \frac{[P]}{K_{ic}}}{1 + \frac{[P]}{K_{iu}}}\right) K_{mS}$$

(6)

where $K_{mS} = K_m$, the classical MM constant, and K_{ic} and K_{iu} are the inhibition constants defined by:

$$K_{ic} = \frac{(k_{-1} + k_2) k_3}{k_1 k_{-2}}$$

(7)

$$K_{iu} = \frac{k_2 + k_3}{k_{-2}}$$

(8)

In addition, it is possible to define the relation of $v_{max}^{app}/K_{mS}^{app}$ as:

$$\frac{v_{max}^{app}}{K_{mS}^{app}} = \left(\frac{1}{1 + \frac{[P]}{K_{ic}}}\right) \frac{v_{max}}{K_{mS}}$$

(9)

15

The expression of v_{max}^{app} given by Eq. 5 explains that an increase in the effective concentration of product in a crowded medium may produce a decrease in the value of this parameter. In addition, the decrease in v_{max}^{app} with crowding is accentuated by an increase in the inhibition constant K_{iu}, due to the decrease in the bimolecular diffusion-controlled rate, k_{-3} (Eq. 8). Thus, in the case of the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide by alpha-chymotrypsin, the decrease in the v_{max}^{app} values in the presence of increasing concentrations of Dextran (Table 1) can be explained as a consequence of an increase in effective inhibition by the product. In fact, the effective product concentration increases with the volume occupied by the crowding agent. It should be noted that we do not dismiss the possibility of a change in the k_{cat} because of a conformational change induced by the crowding. We believe that both factors, the change in k_{cat} and the increase in inhibition by product, should be taken into account in these kinds of studies on the crowding effect.

In addition, to prove that the mixed inhibition mechanism is the correct one for our study, we checked the results of the rest of the parameters: K_{mS}^{app} and the ratio $v_{max}^{app}/K_{mS}^{app}$.

The expression of the apparent MM constant, K_{mS}^{app}, given by Eq. 6 explains that the value of this parameter increases with crowding, as K_{mS} only depends on one bimolecular rate constant, k_1. As this is a diffusion-controlled rate constant, it decreases with crowding. Moreover, the effective product concentration increases with crowding, due to the increase in the excluded volume. The inhibition constants K_{ic} and K_{iu} can also increase with crowding, due to the decrease in the two bimolecular diffusion-controlled rate constants k_1 and k_{-3} (Eqs. 7 and 8). These relations imply that, independently of the

real value of the $[P]/K_{ic}$ and $[P]/K_{iu}$ quotients and their variation with crowding, the term in parentheses in Eq. 6 is always greater than 1, and this yields a total increase in the apparent MM constant with crowding, as shown in Table 1.

In addition, the decreasing variation of $v_{max}^{app}/K_{mS}^{app}$ when crowding increases, as shown in Table 1, can be explained by Eq. 9 in a similar manner as above. Thus, the mixed inhibition mechanism can explain all the crowding-induced parameter changes observed in our study (Table 1). Consequently, we consider that the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin in crowded media is at least dependent on diffusion in the crowded media and on effective inhibition by product, which is enhanced by the presence of these crowding agents.

CONCLUSIONS

We have studied the kinetics of the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin as a model enzymatic reaction in different in vitro crowded media produced by Dextran of different concentrations and sizes. First, our results reveal that the volume occupied by the crowding agent plays an important role in the rate of this reaction. We obtained v_{max} decay with increasing obstacle concentration, whereas K_m values increased with the concentration of Dextran in the sample. Second, the increase in K_m could be due to the diffusion control of the enzymatic reaction. Third, the decrease in v_{max} can be explained by taking into account the effect of inhibition by product, which is enhanced in crowded media. And finally, after a comparison of our results with other similar studies, it appears that each enzymatic reaction behaves in a particular way in *in vitro* media. This must be taken

into account in studies of enzyme-catalyzed reactions that occur within the crowded physiological environment of the cell.

Acknowledgements

REFERENCES

1.   Ellis R. J. *Trends Biochem. Sci.* **2001**, *26*, 577.

2.   Muramatsu, N.; Minton, A. P. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 2984.

3.   Wachsmuth, M.; Waldeck, W.; Langowski, J. *J. Mol. Biol.* **2000**, *298*, 677.

4.   Arrio-Dupont, M.; Foucault, G.; Vacher, M.; Devaux, P. F.; Cribier, S. *Biophys. J.* **2001**, *78*, 901.

5.   Verkman, A. S. *TRENDS Biochem. Sci.* **2002**, *27*, 27.

6.   Weiss, M.; Elsner, M.; Kartberg, F.; Nilsson, T. *Biophys J.* **2004**, *87*, 3518.

7.   Dix, J. A.; Verkman, A. S. *Annu. Rev. Biophys.* **2008**, *37*, 247.

8.   Zhou, H. X.; Rivas, G.; Minton, A. P. *Annu. Rev. Biophys.* **2008**, *37*, 375.

9.   Minton, A. P.; Wilf, J. *Biochem.* **1981**, *20*, 4821.

10.   Minton, A. P. *Biopolym.* **1981**, *20*, 2093.

11.   Zimmerman, S. B.; Minton, A. P. *Annu. Rev. Biophys. Biomol. Struct.* **1993**, *22*, 27.

12.   Rivas, G.; Fernandez, J. A.; Minton, A. P. *Biochem.* **1999**, *38*, 9379.

13.   Van den Berg, B.; Ellis, R. J.; Dobson, C. M. *EMBO J.* **1999**, *18*, 6927.

14.   Minton, A. P. *Curr. Opin. Struct. Biol.* **2000**, *10*, 34.

15.   Rivas, G.; Fernandez, J. A.; Minton, A. P. *FtsZ. Roc. Natl. Acad. Sci. USA* **2001**, *98*, 3150.

16.   Koser, N.; Schereiber, G. *J. Mol. Biol.* **2004**, *336*, 763.

17.   Munishkina, L. A.; Cooper, E. M.; Uversky, V.; Fink, A. *J. Mol. Recognit.* **2004**, *17*, 456.

18.   Chebotareva, N. A.; Kurganov, B. I.; Livanova, N. B. *Biochem. (Moscow)* **2004**, *69*, 1239.

19.   Zhou, H-X. *J. Mol. Recognit.* **2004**, *17*, 368.

20. Minton, A. P. *J. Pharm. Sci.* **2005**, *94*, 1668.

21. Schreiber, G.; Haran, G.; Zhou, H-X. *Chem. Rev.* **2009**, *109*, 839.

22. Dong, H.; Qin S.; Zhou, H-X. *PLoS Comput. Biol.* **2010**, *6*, e1000833.

23. Laurent, T. C. *Eur. J. Biochem.* **1971**, *21*, 498.

24. Wenner, J. R.; Bloomfield, V. A. *Biophys. J.* **1999**, *77*, 3234.

25. Derham, B. K.; Harding, J. J. *Biochim. Biophys. Acta* **2006**, *1764*, 1000.

26. Olsen, S. N. *Thermochim. Acta* **2006**, *448*, 12.

27. Sasaki, Y.; Miyoshi, D.; Sugimoto, N. *Biotechnol. J.* **2006**, *1*, 440.

28. Olsen, S. N.; Raml¢v, H.; Westh, P. *Comp. Biochem. Physiol. A*. **2007**, *148*, 339.

29. Moran-Zorzano, M. T.; Viale, A.; Muñoz, F.; Alonso-Casajas, N.; Eydaltm, G. et al. *FEBS Lett.* **2007**, *581*, 1035.

30. Jiang, M.; Gou, Z. H. *J. Am. Chem. Soc.* **2007**, *129*, 730.

31. Sasaki, Y.; Miyoshi, D.; Sungimoto, N. *Nucleic. Acids. Res.* **2007**, *35*, 4086.

32. Asaad, N.; Engberts, J. B. F. N. *J. Am. Chem. Soc.* **2003**, *125*, 6874.

33. Banks, D. S.; Frandin, C. *Biophys. J.* **2005**, *89*, 2960.

34. Pastor, I.; Vilaseca, E.; Madurga, S.; Garces, J. L.; Cascante, M.; Mas, F. *J. Phys. Chem. B* **2010**, *114*, 4028.

35. Vilaseca, E.; Isvoran, A.; Madurga, S.; Garces, J. L.; Pastor, I.; Mas, F. *PCC.* **2010**, *submitted*.

36. Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*. 3rd edition. Published by Portland Press Ltd. London (U.K.) 2004.

37. Kao, H. P.; Abney, J. R.; Verkman, A. S. *J. Cell Biol.* **1993**, *120*, 175.

38. Homchaudhuri, L.; Sarma, N.; Swaminathan, R. *Biopolymers* **2006**, *83*, 477.

39. Isvoran, A.; Vilaseca, E.; Garcés, J. L.; Ortega, F.; Cascante, M.; Mas, F. *European Biophys. J.* **2005**, *34*, 781.

40.   Gellerich, F. N.; Laterveer, F. D.; Korzeniewski, B.; Zierz, S.; Nicolay, K. *Eur. J. Biochem.* **1998**, *254*, 172.

41.   Berry, H. *Biophys. J.* **2002**, *83*, 1891.

42.   Schnell, S.; Turner, T. E. *Progr. Biophys. Mol. Biol.* **2004**, *85*, 235.

43.   Pitulice, L. *Aspecte fractale ale structurii şi dinamicii biomoleculelor*. PhD thesis. Vest Timişoara University, Timişoara (Romania), 2009.

44.   Saxton, M. J. *Biophys. J.* **1987**, *52*, 989.

45.   Saxton, M. J. *Biophys. J.* **1990**, *58*, 1303.

46.   Saxton, M. J. *Biophys. J.* **1993**, *64*, 1053.

47.   Saxton, M. J. *Biophys. J.* **1994**, *66*, 394.

48.   Kawai, Y.; Matsou, T.; Ohno, A. *J. Chem. Soc., Perkin Trans.* **2000**, *2*, 887.

49.   Alsallaq, R.; Zhou, H-X. *Proteins* **2008**, *71*, 320.

50.   Lonhienne, T. G. A.; Winzor, D. *J. Mol. Recognit*. **2004**, *17*, 351.

51.   Minh, D. D.; Chang, C.; Trylska, J.; Tozzini, V.; McCammon, J. A. *J. Am. Chem. Soc.* **2006**, *128*, 6006.

Table 1. Kinetic constants of the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide

catalyzed by alpha-chymotrypsin for different concentrations of Dextran (in mg/mL).

The value of the fitting parameter r^2 is also given.

[Dextran] (mg/mL)	v_{max}^{app} (mM/s)	K_{mS}^{app} (mM)	$v_{max}^{app}/K_{mS}^{app}$ (s^{-1})	r^2
0	1.64 ± 0.04	0.72 ± 0.06	2.3 ± 0.2	0.9927
25	1.28 ± 0.04	1.01 ± 0.05	1.3 ± 0.1	0.9984
50	1.24 ± 0.04	1.29 ± 0.12	1.0 ± 0.1	0.9958
100	0.91 ± 0.05	1.31 ± 0.23	0.7 ± 0.1	0.9933

FIGURE 1. Example of kinetic curves for different concentrations of substrate (5.4×10^{-5}, 1.04×10^{-4}, 1.56×10^{-4}, 2.08×10^{-4}, 2.60×10^{-4}, 3.64×10^{-4} and 4.68×10^{-4} M, respectively) in samples with 50 mg/mL of 50 kDa Dextran.

FIGURE 2. Michaelis-Menten plot that relates the reaction rate v_0 to the substrate concentration for the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin in Dextran crowded media with different Dextran sizes: (**A**) $M_w = 5$ kDa; (**B**) $M_w = 50$ kDa; (**C**) $M_w = 150$ kDa; (**D**) $M_w = 275$ kDa and (**E**) $M_w = 410$ 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).
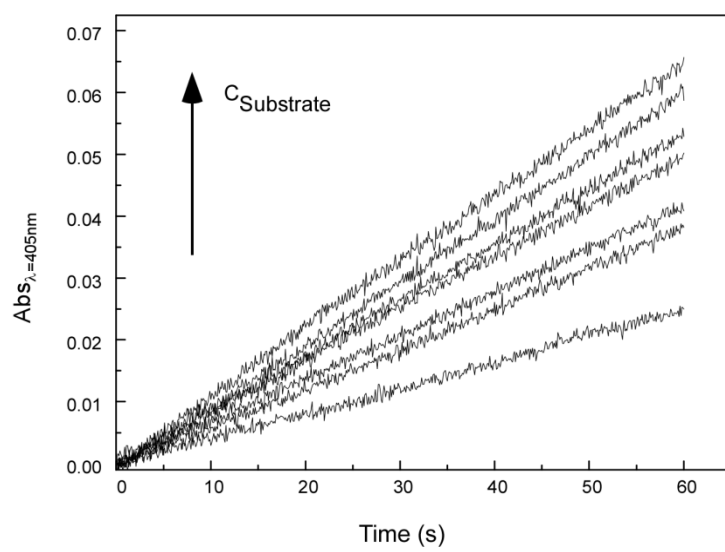
FIGURE 3. Example of variation of v_0 with the size and the concentration of Dextrans for a fixed concentration of substrate (2 mM). In the figure, three Dextran concentrations are plotted: 25 mg/mL (solid square), 50 mg/mL (open circle) and 100 mg/mL (solid up-triangle).

FIGURE 4. Michaelis-Menten plot that relates the average reaction rate v_0 to the substrate concentration for the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin. 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).
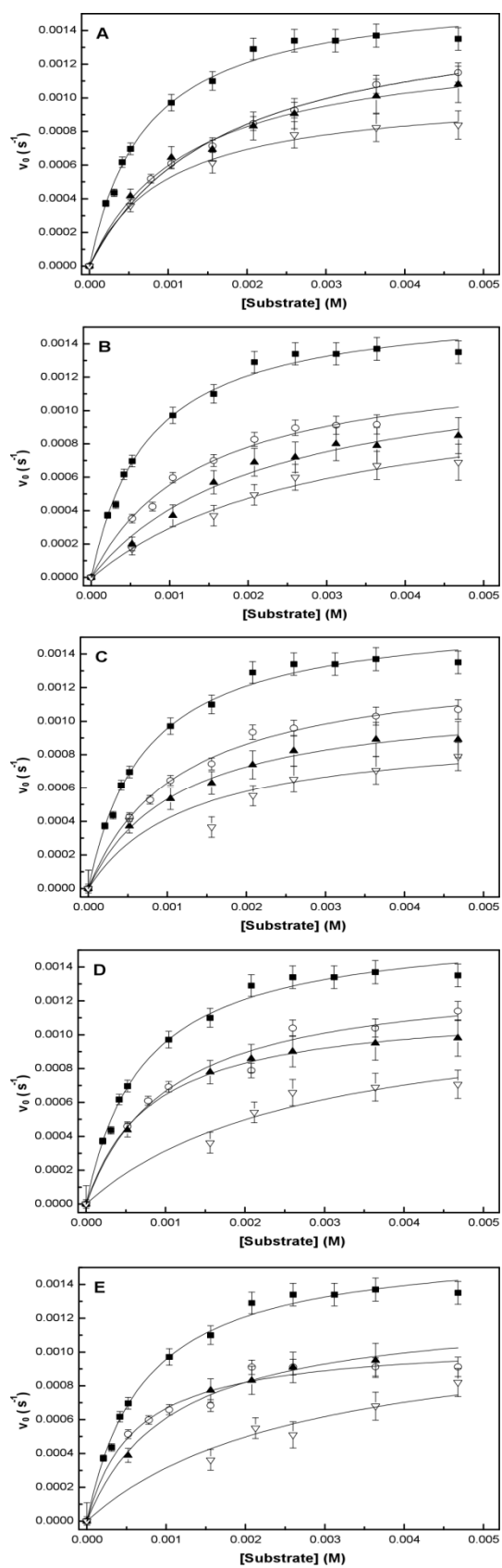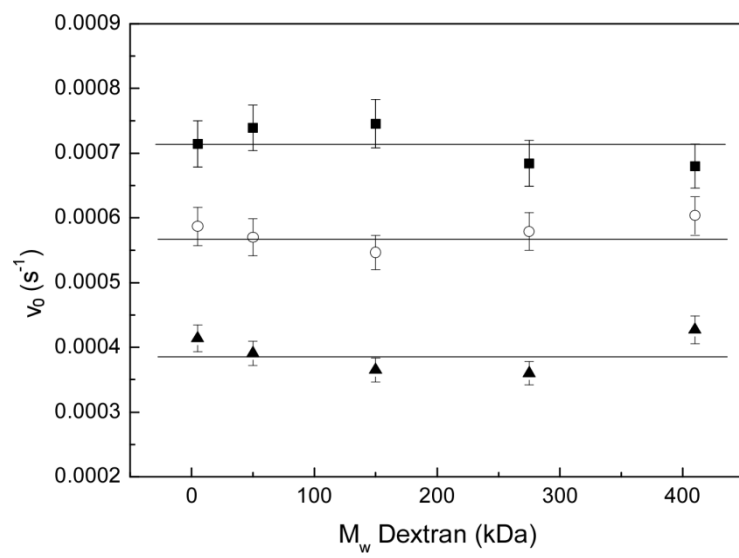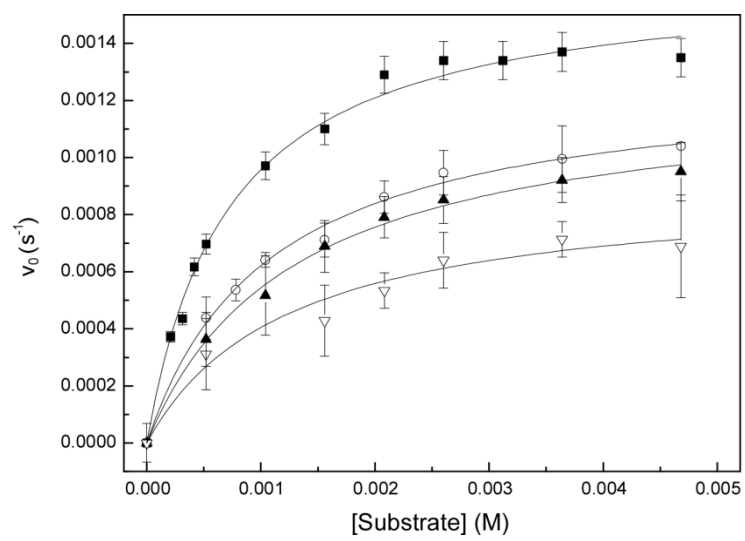
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

TOC