# The catalytic action of enzymes exposed to charged substrates outperforms the activity exerted on their neutral counterparts

Alessandra Stefan[a,b], Alejandro Hochkoeppler[a,b,*]

[a]Department of Pharmacy and Biotechnology, Viale Risorgimento 4, 40136 Bologna, Italy

[b]CSGI, University of Firenze, Via della Lastruccia 3, 50019 Sesto Fiorentino, FI (Italy)

*To whom correspondence should be addressed:

Alejandro Hochkoeppler
Department of Pharmacy and Biotechnology
University of Bologna
Viale Risorgimento 4
40136 Bologna
Italy
Tel.: +39 051 2093671
e-mail: a.hochkoeppler@unibo.it

## Abstract

Enzymes perform their catalytic action according to mechanisms featuring exquisite specificity, up to the selection of substrate conformers. However, regardless of this high specificity enzymes are able to deal with a repertoire of substrates, whose conversion into reaction products can occur with markedly different rates. Among the factors affecting the velocity of enzyme-catalyzed reactions, the presence in the substrate of an electrostatic charge could be of importance. Here we report on the kinetic parameters of four enzymes (bovine carbonic anhydrase and α-chymotrypsin, *Escherichia coli* β-galactosidase, and sweet almond β-glucosidase) determined using a $NO_2$-containing charged substrate or its neutral counterpart. Remarkably, all the considered enzymes were found more effective when exposed to the charged substrates, featuring $K_m$ and $k_{cat}$ values respectively lower and higher than those determined using the neutral substrates. Furthermore, by means of ultrafiltration experiments we detected the binding of *o*-nitrophenyl-β-D-galactopyranoside to a multiplicity of sites in *E. coli* β-galactosidase. Overall, our observations suggest that the unspecific binding of substrate to enzyme surface aids the cycling of subsequent catalytic turnovers.

**Key words:** bovine carbonic anhydrase; bovine α-chymotrypsin; *Escherichia coli* β-galactosidase; sweet almond β-glucosidase; charged and uncharged substrates; unspecific binding.

## 1. Introduction

The catalytic action of enzymes translates into reaction rates outcompeting by several orders of magnitude those occurring under uncatalyzed conditions [1]. Nevertheless, the catalytic effectiveness of enzymes spans a quite wide interval [2], at one extreme of which reside enzymes denoted as perfect. Remarkably, the performance of these perfect enzymes is limited by diffusion, as indicated by the values of their second order rate constant ($k_{cat}/K_m$) which are comparable to the rate constant for the diffusional encounter of spherical particles, i.e. $10^{10}$ $M^{-1}s^{-1}$ [3]. This extraordinary catalytic capability means that every encounter of substrate and enzyme does essentially translate into a very fast generation and release of the reaction product. A notable example of a perfect enzyme is superoxide dismutase (SOD), whose surface electrostatic potential is well conserved among prokaryotic and eukaryotic representatives [4]. Interestingly, the surface of SOD does contain a cationic funnel steering the anionic substrate into the enzyme active site, therefore limiting to a minimal extent the frequency of non-productive enzyme-substrate encounters [4]. Quite recently, the kinetic parameters ($k_{cat}$ and $K_m$) available in the BRENDA database [5] for a large number of enzymes have been surveyed [6]. Not surprisingly, this analysis revealed that only a few enzymes feature catalytic perfection, with the great majority of them performing with far lower efficiencies [6]. In particular, the median $k_{cat}$ and $K_m$ were found equal to 10 $s^{-1}$ and 100 µM, respectively. Accordingly, the $k_{cat}/K_m$ of the average enzyme equals $10^5$ $M^{-1}s^{-1}$, i.e. a value 4-5 orders of magnitude lower compared to the corresponding kinetic parameter determined for perfect enzymes. This means, in turn, that the mean enzyme is exposed to a high number of futile encounters [4], with a tiny fraction of the substrate molecules that interacted with the enzyme being converted into reaction product. However, it should also be considered that

the performance of enzymes can be largely affected by the particular substrate acted on. In particular, the occurrence of electrostatic interactions between enzyme and substrate could be of importance to enhance the encounter rate constant, which for superoxide dismutases was estimated from (0.86±0.11)•$10^9$ to 3.76±0.22) •$10^9$ $M^{-1}s^{-1}$ [4]. As previously mentioned, electrostatic interactions can also be of help to steer substrate to the enzyme active site. In addition, it has been proposed that the $K_m$ values of an enzyme for different substrates could be significantly affected by electrostatic interactions engaging the substrate and the surface of the enzyme [3]. Therefore, we considered of interest to compare the catalytic action of enzymes exerted at the expense of substrates bearing or not a charged functional group. Accordingly, here we report on the catalytic performance of four hydrolases (bovine carbonic anhydrase and α-chymotrypsin, *Escherichia coli* β-galactosidase, and sweet almond β-glucosidase) exposed to charged substrates or to their neutral counterparts, under identical conditions of temperature, pH, and ionic strength. Furthermore, we also report on the binding of *o*-nitrophenyl-β-D-galactopyranoside to the surface of *Escherichia coli* β-galactosidase, a moderately efficient enzyme featuring a value for $k_{cat}/K_m$ equal to 6.8•$10^6$ $M^{-1}s^{-1}$.

## 2. Materials and Methods

### 2.1 Materials

All reagents and enzymes were purchased from Merck-Millipore (St.Louis, MO, USA).

### 2.2 Enzyme activity assays

All enzymes considered (bovine carbonic anhydrase and α-chymotrypsin, *Escherichia coli* β-galactosidase, and sweet almond β-glucosidase) were used without further purification. Carbonic anhydrase was assayed in 50 mM Tris-HCl (pH 8.5) using *p*-nitrophenylacetate or phenylacetate as substrate, in the presence of 1 and 20 μM enzyme, respectively. Stock solutions of substrates were prepared in absolute ethanol at concentrations equal to 150 mM and 1M for *p*-nitrophenylacetate and phenylacetate, respectively. Bovine α-chymotrypsin was dissolved in 10 mM Bis-Tris (pH 6.5) immediately before its use, and reaction mixtures were buffered with 50 mM Tris-HCl (pH 8). The rate of hydrolysis of *p*-nitrophenylacetate and phenylacetate was determined in the presence of 5.2 and 13 μM α-chymotrypsin, respectively.

*Escherichia coli* β-galactosidase was assayed in 10 mM Tris-HCl, 30 mM KCl, 1mM $MgSO_4$, pH 7.5 (buffer A), using *o*-nitrophenyl-β-D-galactopyranoside (ONPGal) or phenyl-β-D-galactopyranoside as substrate, in the presence of 4.3 and 43 nM enzyme, respectively.

In addition, the hydrolysis of lactose catalyzed by *E. coli* β-galactosidase was assayed. To this aim, the catalytic action of 4.3 nM β-galactosidase was coupled to that of galactose dehydrogenase (GDH). In particular, reaction mixtures contained (in buffer A) 2.5 mM β-$NAD^+$, 2.3 Units of galactose dehydrogenase, and the reaction kinetics was determined by observing the Absorbance increase at 340 nm linked to the consumption of galactose by

GDH. One Unit of GDH is defined as the amount of enzyme catalyzing the conversion of 1 μM of galactose per second in buffer A.

The activity of sweet almond (*Prunus dulcis*) β-glucosidase was determined at pH 5.5 (50 mM MES) at the expense of *p*-nitrophenyl-β-D-glucopyranoside (PNPGluc) or phenyl-β-D-glucopyranoside (PGluc) as substrate. In agreement with previous observations [7], the β-glucosidase preparation was found to contain both monomeric and dimeric enzyme. The reaction velocities of PNPGluc and PGluc hydrolysis were determined using enzyme at 60 and 300 μg/mL, respectively.

For the different enzymes considered here, the rates of Absorbance changes observed during the assays were converted into concentration changes per unit time using the following extinction (or difference extinction) coefficients (in units of $M^{-1}cm^{-1}$): i) 5540 for *p*-nitrophenol at 348 nm [8]; ii) 1090 for phenol minus phenylacetate at 268 nm [8]; iii) 2050 for *o*-nitrophenol minus *o*-nitrophenyl-β-D-galactopyranoside at 373 nm [9]; iv) 550 for phenol minus phenyl-β-D-galactopyranoside at 280 nm [9]; v) 3500 for *p*-nitrophenol minus *p*-nitrophenyl-β-D-glucopyranoside at 347 nm [10]; vi) 1090 for phenol minus phenyl-β-D-glucopyranoside at 268 nm; vii) 6220 for β-NADH at 340 nm [11].

All the enzyme assays were performed at 20 °C using a Cary Bio 300 spectrophotometer and quartz cuvettes featuring path-length of 0.5 or 1 cm. Protein concentration was determined according to Bradford [12].

**2.3 Substrate binding assays**

To test the binding, if any, of *o*-nitrophenyl-β-D-galactopyranoside to multiple sites of *E. coli* β-galactosidase we used disposable ultrafiltration cells (Amicon Ultracel, $M_r$ cutoff 100 kDa, Merck-Millipore). First, to extensively inhibit the enzyme, β-galactosidase was incubated with 10 mM EDTA for 1.5 h, at room temperature (under these conditions, β-

galactosidase retained 0.3 % activity). Afterwards, 1 μM β-galactosidase was mixed with 1 mM ONPGal in 10 mM Tris-HCl, 10 mM EDTA, pH 7.5. Then, the enzyme-substrate mixture was transferred to a Ultracel ultrafiltration cell, and was subjected to centrifugation (15,000xg) for 1.5 minutes at 20 °C. Finally, the absorption spectrum of the collected ultrafiltrate was determined and compared with that of a control sample devoid of enzyme and subjected to the same ultrafiltration procedure. Accordingly, the absorption spectrum of the collected ultrafiltrate was corrected by subtracting the absorption spectrum of an equimolar solution of pure *o*-nitrophenolate (ONP) in 10 mM Tris, pH 7.5. It should be noted that in samples containing both ONPGal and ONP, the concentration of the latter can be exactly determined, since the absorption band centered at 410 nm is exclusively due to ONP. Therefore, by means of this correction, we were able to determine the concentration of the residual free ONPGal, and to compare the value accordingly determined with that of the initial substrate concentration.

## 3. Results and Discussion

To prime our analysis, we decided to test the catalytic action of bovine carbonic anhydrase on *p*-nitrophenylacetate and phenylacetate, at pH 8.5. However, at this slightly alkaline pH both these substrates are known to undergo hydrolysis even in the absence of catalysts. Therefore, the rate of uncatalyzed reactions was determined and subsequently subtracted from the reaction velocity observed at an equimolar substrate concentration in the presence of enzyme. First, the spontaneous hydrolysis of *p*-nitrophenylacetate was found to obey a linear dependence on ester concentration, up to 6 mM (Fig. 1A). Accordingly, by fitting a linear equation to the experimental observations we estimated a $k_{obs}$ equal to (65±2)•$10^{-6}$ $s^{-1}$ (Fig. 1A). A similar output was detected when the uncatalyzed hydrolysis of phenylacetate was analyzed. Indeed, we observed a linear dependence of reaction velocity on ester concentration up to 20 mM, yielding a $k_{obs}$ equal to (10.3±0.2)•$10^{-6}$ $s^{-1}$ (Fig. 1B). When the hydrolysis of *p*-nitrophenylacetate catalyzed by carbonic anhydrase was analyzed, a hyperbolic dependence of the initial reaction velocity on substrate concentration was determined (Fig. 1C). In particular, using these observations the $k_{cat}$ and $K_m$ of the enzyme were estimated as equal to 5.0±0.2 $s^{-1}$ and 9.3±0.5 mM, respectively, corresponding to a $k_{cat}/K_m$ value of 538 $M^{-1}s^{-1}$ (Tab. 1). Notably, these values are in good agreement with those previously reported by Pocker and Stone for the kinetic parameters of bovine carbonic anhydrase, i.e. 3.5 $s^{-1}$ and 13.2 mM for $k_{cat}$ and $K_m$, respectively [13]. The catalytic action of carbonic anhydrase on phenylacetate was definitely different when compared to the activity of the enzyme at the expense of *p*-nitrophenylacetate. In the presence of the unsubstituted ester, the initial reaction velocities did indeed obey a linear dependence on substrate concentration, which was raised up to 20 mM (Fig. 1D). Fitting a linear equation to these experimental observations

we obtained a $k_{obs}$ equal to (51±2)•$10^{-6}$ $s^{-1}$, which translates into a $k_{cat}/K_m$ value of 2.6 $M^{-1}s^{-1}$ by taking into account that the final concentration of enzyme in these assays was 20 μM (Tab. 1). Overall, the assays performed with *p*-nitrophenylacetate and phenylacetate indicate that: i) the uncatalyzed reaction is significantly, albeit moderately, affected by the presence in the reacting ester of the *p*-nitro substituent, which is responsible for increasing $k_{obs}$ 6.5 times; ii) on the contrary, the reaction catalyzed by carbonic anhydrase is strongly affected by the substrate, with $k_{cat}/K_m$ being enhanced 200 times by the presence of the *p*-nitro group in the phenylacetate moiety. It should be noted that this large increase of $k_{cat}/K_m$ implies a considerable decrease of the fraction φ of futile enzyme-substrate encounters, with φ defined as $1 - k_{cat}/K_m \bullet k_d^{-1}$, where $k_d$ represents the diffusion rate constant [2].

The substrates *p*-nitrophenylacetate and phenylacetate were also used to assay the activity of bovine α-chymotrypsin. First, the uncatalyzed hydrolysis of both esters was tested, and a linear dependence of the reaction rate on their concentration was observed. Fitting a linear equation to the experimental observations, we determined $k_{obs}$ values equal to (82±1)•$10^{-6}$ and (18.9±0.6)•$10^{-6}$ $s^{-1}$ for *p*-nitrophenylacetate and phenylacetate , respectively (Fig.s 2A and 2B). Interestingly, when the catalytic action of α-chymotrypsin at the expense of *p*-nitrophenylacetate or phenylacetate was assayed, both reactions were found to obey a Michaelis-Menten kinetics (Fig.s 2C and 2D). However, a large difference was determined between the $K_m$ values for the two substrates, with values being equal to 1.5±0.2 and 13.4±2.4 mM for *p*-nitrophenylacetate and phenylacetate, respectively (Tab. 1). On the contrary, identical $k_{cat}$ values for the two esters were estimated, i.e. 0.044±0.002 and 0.044±0.005 $s^{-1}$ (Tab. 1). Notably, the kinetic parameters of α-chymotrypsin reported here for *p*-nitrophenylacetate do reasonably agree with the

values previously reported for $k_{cat}$ and $K_m$, being respectively equal to 0.025 $s^{-1}$ and 1.9±0.4 mM [14,15]. It is also important to note that under steady-state conditions, the $k_{cat}$ of α-chymotrypsin for *p*-nitrophenylacetate refers to the enzyme deacylation step [14,15], i.e. to a stage subsequent to the release of the first reaction product. Therefore, we propose that this kinetic peculiarity accounts for the identity of the two $k_{cat}$ values determined here for α-chymotrypsin. In addition, we propose that the divergence between the $K_m$ values for *p*-nitrophenylacetate and phenylacetate is related to a decrease of enzyme-substrate futile encounters when the enzyme is exposed to the substituted ester.

To consider an additional type of bond subjected to the action of hydrolases, we assayed the activity of *Escherichia coli* β-galactosidasde, using as substrates *o*-nitrophenyl-β-D-galactopyranoside, phenyl-β-D-galactopyranoside, and lactose. When the enzyme was exposed to *o*-nitrophenyl-β-D-galactopyranoside, we detected, as expected, Michaelis-Menten kinetic (Fig. 3A). In particular, in the presence of this substrate $k_{cat}$ and $K_m$ were determined equal to 502±7 $s^{-1}$ and 285±11 μM, respectively, corresponding to a $k_{cat}/K_m$ of $1.76 \cdot 10^6$ $M^{-1}s^{-1}$ (Tab. 1). This means a lower catalytic efficiency when compared to that previously determined by Tenu et al. and equal to $7.3 \cdot 10^6$ $M^{-1}s^{-1}$, with this value representing the output of $k_{cat}$ and $K_m$ whose magnitude was estimated as 800 $s^{-1}$ and 0.11 μM, respectively [9]. However, it should be noted that our assays, contrary to those performed by Tenu et al., were carried out in the absence of NaCl, which is known to activate *E. coli* β-galactosidase by increasing $k_{cat}$ and lowering $K_m$ [16]. When phenyl-β-D-galactopyranoside was used as substrate, $k_{cat}$ and $K_m$ were determined as equal to 167±7 $s^{-1}$ and 809±63 μM, respectively (Fig. 3B, Tab. 1), yielding a $k_{cat}/K_m$ of $0.21 \cdot 10^6$ $M^{-1}s^{-1}$. Remarkably, in the presence of 160±20 mM sodium the $k_{cat}$, $K_m$, and $k_{cat}/K_m$ of the enzyme

for phenyl-β-D-galactoside were previously determined as equal to 44 $s^{-1}$, 100 μM, and $0.44 \bullet 10^6$ $M^{-1}s^{-1}$ [9]. In addition to the assays performed with the phenylgalactosides we considered of interest to test the activity of β-galactosidase at the expense of lactose. In the presence of this disaccharide, we determined the enzyme $k_{cat}$ and $K_m$ as respectively equal to 62±2 $s^{-1}$ and 5.1±0.6 mM (Fig. 3C, Tab. 1). Intriguingly enough, when similar assays were performed in the presence of 140 mM NaCl the $k_{cat}$ and $K_m$ for lactose of *E. coli* β-galactosidase were estimated equal to 60±10 $s^{-1}$ and 1.4±0.3 mM, respectively [17]. Overall, the values of $K_m$ that we determined for β-galactosidase in the absence of NaCl are significantly higher than the values previously estimated in the presence of sodium [9,17]. This systematic divergence is likely due to the interaction that a sodium ion engages with the galactosyl 6-OH in the enzyme active site [18]. Nevertheless, the outcomes of our assays indicate $K_m$ as the kinetic parameter of β-galactosidase which is mainly affected by the different substrates considered.

To further compare substrates provided or not with a charged substituent we selected sweet almond (*Prunus dulcis*) β-glucosidase. In particular, we tested the catalytic action of this enzyme at the expense of *p*-nitrophenyl-β-D-glucopyranoside and phenyl-β-D-glucopyranoside. It is important to mention that sweet almond β-glucosidase features a lower $K_m$ for the *p*-nitrophenyl-substituted substrate than for its *o*-nitrophenyl counterpart [19]. Moreover, it is known that the activity at the expense of *p*-nitrophenyl-β-D-glucopyranoside is rather pH-insensitive [20]. By performing activity assays at pH 5.5 we observed that sweet almond β-glucosidase features $K_m$ values for *p*-nitrophenyl-β-D-glucopyranoside and phenyl-β-D-glucopyranoside equal to 2.7±0.7, and 7.2±3.2 mM, respectively (Supplementary Fig.s S1A and S1B, Tab. 1). In addition, we estimated that the $V_{max}$ for the substituted glucoside is 200 timers higher than that for phenyl-β-D-

glucopyranoside (Supplementary Fig.s S1A and S1B, Tab. 1). Notably, we determined that the value of the $V_{max}/K_m$ ratio for *p*-nitrophenyl-β-D-glucopyranoside is 550 times higher than the corresponding parameter for phenyl-β-D-glucopyranoside, with this difference being 2 times larger than those previously reported as equal to 233-243 [19,20].

Overall, the observations reported here on the kinetic parameters of four different hydrolases suggest that these enzymes take advantage of charged substrates to reduce the frequency of futile encounters. To investigate this point in detail, we selected *E. coli* β-galactosidase as a model system. In particular, we reasoned that using *o*-nitrophenyl-β-D-galactopyranoside we could spectroscopically evaluate the binding of this substrate to enzyme sites other than the catalytic pocket. Indeed, *o*-nitrophenyl-β-D-galactopyranoside does exclusively absorb UV light (Fig. 4A), whereas the reaction product *o*-nitrophenolate features an absorption band in the visible region (Fig. 4B). Therefore, and as previously mentioned (see Methods), using disposable ultrafiltration cells to separate extensively inactivated β-galactosidase from reaction mixtures containing substrate and product it is possible to evaluate if the detected residual substrate concentration equals or not its initial concentration minus the concentration of the generated product. Accordingly, any recovery of substrate (in ultrafiltrated samples) lower than expected would be diagnostic of its binding to enzyme sites not engaged in the generation of reaction product, e.g. to the enzyme surface. To perform quantitatively this analysis, we determined the molar extinction coefficients at appropriate wavelengths of both *o*-nitrophenyl-β-D-galactopyranoside and *o*-nitrophenol (Fig.s 4A and 4B), namely at 263 and 320 nm for the substrate and at 410 nm for the reaction product (Supplementary Fig.s S2 and S3). We then prepared reaction mixtures containing 1 μM of inactivated (by EDTA treatment) β-galactosidase and 200 μM *o*-nitrophenyl-β-D-galactopyranoside in 10

mM Tris-HCl, pH 7.5. It should be noted that to contain as much as possible the residual enzyme activity the assay mixtures were devoid of NaCl, KCl, and $MgSO_4$. Remarkably, by means of this strategy we were able to detect the binding of *o*-nitrophenyl-β-D-galactopyranoside to multiple sites of *E. coli* β-galactosidase (Fig. 4C, Supplementary Tab. ST1). Quantitatively speaking, in the presence of 1 μM enzyme (with this concentration value referring to subunits) we estimated the occurrence of binding events to 9 sites additional to the catalytic pocket (Fig. 4C, Supplementary Tab. ST1). Furthermore, by performing three independent experiments the mean value of these binding events was determined as equal to 10.7±1.5 (Supplementary Fig.s S4 and S5, Supplementary Table ST1). Accordingly, we would like to propose that the binding of *o*-nitrophenyl-β-D-galactopyranoside to the surface of *E. coli* β-galactosidase is instrumental in minimizing the $K_m$ for this substrate, the value of which is definitely lower when compared to that for the natural substrate lactose.

## 4. Concluding remarks

We have shown here that the catalytic efficiency of four different hydrolases takes advantage of electrostatic charges present in substrates. In addition, we observed that *o*-nitrophenyl-β-D-galactopyranoside binds to a multiplicity of sites of *Escherichia coli* β-galactosidase.

We would like to propose that the effects reported here are related to the binding of charged substrates to the enzymes' surfaces, and it is our hope that this suggestion will trigger further investigations on this appealing issue.

**Funding sources**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Declaration of competing interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## References


[1] R. Wolfenden, Benchmark reaction rates, the stability of biological molecules in water, and the evolution of catalytic powers in enzymes, Annu. Rev. Biochem. 80 (2011) 645-667.

[2] A. Bar-Even et al., The moderately efficient enzyme: futile encounters and enzyme floppiness, Biochemistry 54 (2015) 4969-4977.

[3] R.A. Alberty, G.G. Hammes, Application of the theory of diffusion-controlled reactions to enzyme kinetics, J. Phys. Chem. 62 (1958) 154-159.

[4] D.R. Livesay et al., Conservation of electrostatic properties within enzyme families and superfamilies, Biochemistry 42 (2003) 3464-3473.

[5] I. Schonburg et al., BRENDA, the enzyme database: updates and major new developments, Nucleic Acids Res. 32 (2004) D431-D433.

[6] D. Davidi et al., A bird's-eye view of enzyme evolution: chemical, physicochemical, and physiological considerations, Chem. Rev. 118 (2018) 8786-8797.

[7] S. Caramia et al., Dual role of imidazole as activator/inhibitor of sweet almond (*Prunus dulcis*) β-glucosidase, Biochem. Biophys. Rep. 10 (2017) 137-144.

[8] A. Thorslund, S. Lindskog, Studies on the esterase activity and the anion inhibition of bovine zinc and cobalt carbonic anhydrases, Eur. J. Biochem. 3 (1967) 117-123.

[9] J. Tenu et al., pH dependence of the activity of β-galactosidase from *Escherichia coli*, Eur. J. Biochem., 20 (1971) 363-370.

[10] M.P. Dale et al., Reversible inhibitors of β-glucosidase, Biochemistry 24 (1985) 3530-3539.

[11] C. Bernofsky, S.C. Wanda, Formation of reduced nicotinamide adenine dinucleotide peroxide, J. Biol. Chem. 257 (1982) 6809-6817.

[12] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[13] Y. Pocker, J.T. Stone, The catalytic versatility of erythrocyte carbonic anhydrase. III. Kinetic studies of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate, Biochemistry 6 (1967) 668-678.

[14] H. Gutfreund, J.M. Sturtevant, The mechanism of the reaction of chymotrypsin with *p*-nitrophenyl acetate, Biochem. J. 63 (1956) 656-661.

[15] F.J. Kédzy, M.L. Bender, The kinetics of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate, Biochemistry 1 (1962) 1097-1106.

[16] R. Strom et al., The activation of β-galactosidase by divalent and monovalent cations. Transient- and steady-state studies, Eur. J. Biochem. 23 (1971) 118-124.

[17] M. Martinez-Bilbao et al., A highly reactive β-galactosidase (*Escherichia coli*) resulting from a substitution of an aspartic acid for Gly-794, J. Biol. Chem. 266 (1991) 4979-4986.

[18] D.H. Juers et al., A structural view of the action of *Escherichia coli* (*lacZ*) β-galactosidase, Biochemistry 40 (2001) 14781-14794.

[19] R.L. Nath, H.N. Rydon, The influence of structure on the hydrolysis of substituted phenyl β-D-glucosides by emulsin, Biochem. J. 57 (1954) 1-10.

[20] β-glucosidase: substrate, solvent, and viscosity variation as probes of the rate-limiting steps, Biochemistry, 25 (1986) 2522-2529.

| | | | | | | |
|---|---|---|---|---|---|---|
| Carbonic anhydrase (Bovine) | *p*-nitrophenyl acetate | | | phenyl acetate | | |
| | $K_m$ (mM) | $V_{max}$ (μM/s) | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{obs}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}s^{-1}$) |
| | 9.3±0.5 | 5.0±0.2 | 5±0.2 | - | $(51\pm2)\bullet10^{-6}$ | $2.6\bullet10^{-6}$ |
| α-chymotrypsin (Bovine) | *p*-nitrophenyl acetate | | | phenyl acetate | | |
| | $K_m$ (mM) | $V_{max}$ (μM/s) | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $V_{max}$ (μM/s) | $k_{cat}$ ($s^{-1}$) |
| | 1.5±0.2 | 0.23±0.01 | 0.044 | 13.4±2.4 | 0.57±0.06 | 0.044 |
| β-galactosidase (*Escherichia coli*) | *o*-nitrophenyl-β-D-galactoside | | | phenyl-β-D-galactoside | | |
| | $K_m$ (μM) | $V_{max}$ (μM/s) | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $V_{max}$ (μM/s) | $k_{cat}$ ($s^{-1}$) |
| | 285±11 | 2.16±0.03 | 502 | 809±63 | 7.2±0.3 | 167 |
| β-glucosidase (*Prunus dulcis*) | *p*-nitrophenyl-β-D-glucoside | | | phenyl-β-D-glucoside | | |
| | $K_m$ (mM) | $V_{max}$ (μM/s•mgP) | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $V_{max}$ (μM/s•mgP) | $k_{cat}$ ($s^{-1}$) |
| | 2.7±0.7 | 285±60 | - | 7.2±3.2 | 1.4±0.5 | - |

**Table 1. Kinetic parameters of the enzymes considered here.**

With the exception of the action of carbonic anhydrase at the expense of phenylacetate, all hydrolases tested were found to obey Michaelis-Menten kinetics. All enzyme assays were performed at 20 °C.

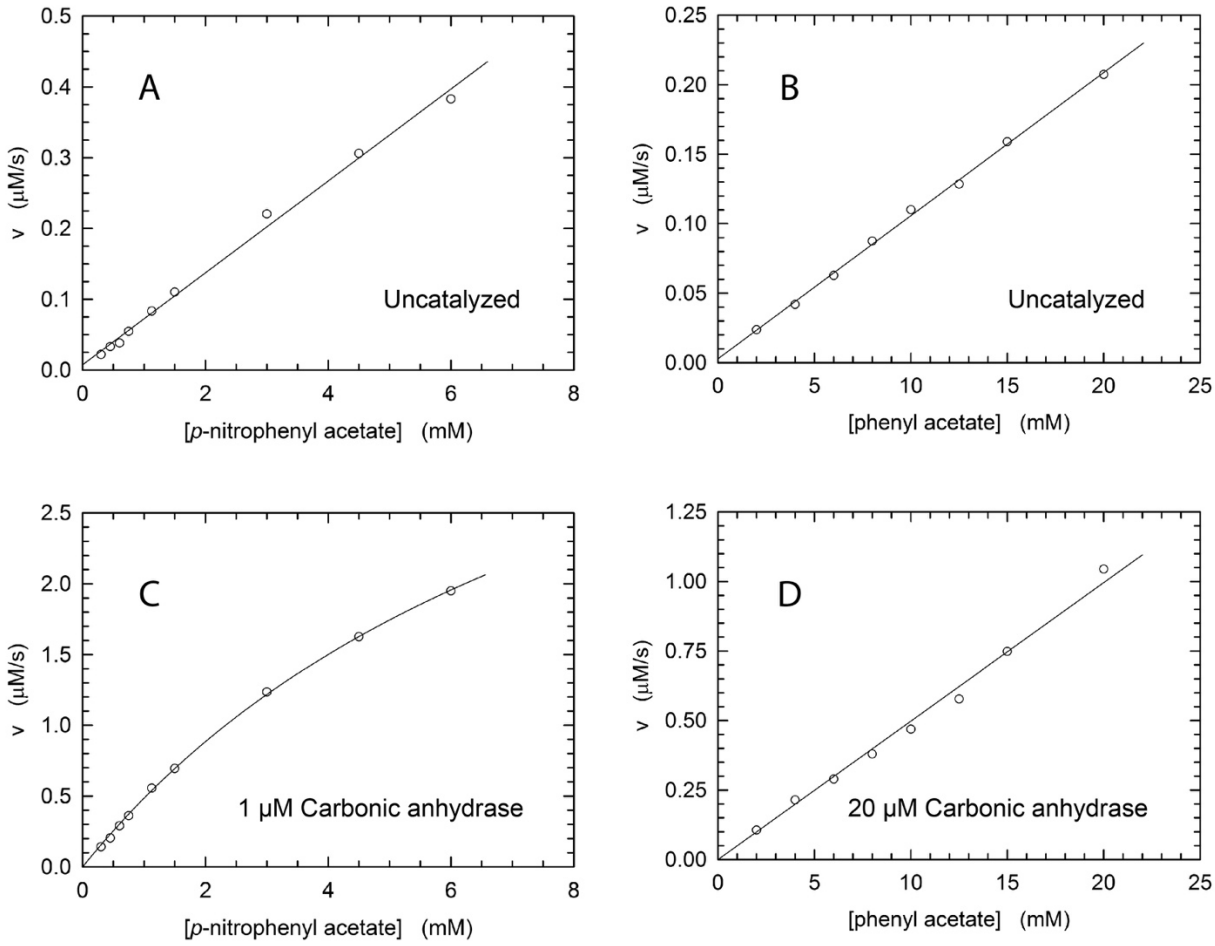


**Figure 1. Kinetics of the hydrolysis of *p*-nitrophenylacetate and phenylacetate.**
(A,B) Initial velocity of the uncatalyzed hydrolysis of *p*-nitrophenylacetate (A) and phenylacetate (B) as a function of ester concentration. The continuous lines represent the best fit of a linear equation to the experimental observations. (C,D) Initial velocities of reactions catalyzed by bovine carbonic anhydrase at the expense of *p*-nitrophenylacetate (C) and phenylacetate (D). The continuous lines represent the best fit of the Michaelis-Menten equation to the experimental observations. All reactions were assayed at pH 8.5 (50 mM Tris-HCl), in the absence (A,B) or in the presence of carbonic anhydrase at 1 (C) or 20 (D) μM.

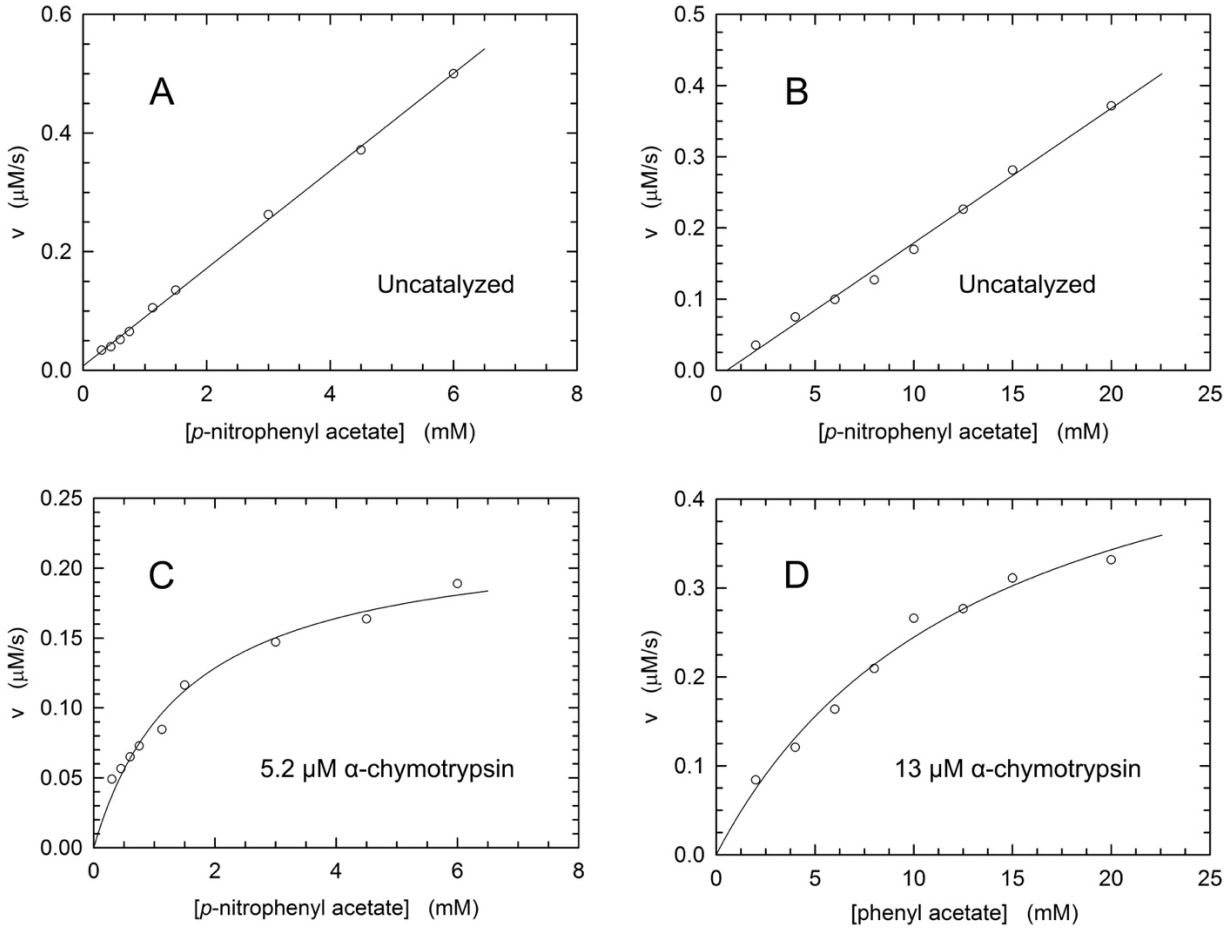


**Figure 2. Kinetics of the hydrolysis of *p*-nitrophenylacetate and phenylacetate.**
(A,B) Initial velocity of the uncatalyzed hydrolysis of *p*-nitrophenylacetate (A) and phenylacetate (B) as a function of ester concentration. The continuous lines represent the best fit of a linear equation to the experimental observations. (C,D) Initial velocities of reactions catalyzed by bovine $\alpha$-chymotrypsin at the expense of *p*-nitrophenylacetate (C) and phenylacetate (D). The continuous lines represent the best fit of the Michaelis-Menten equation to the experimental observations. All reactions were assayed at pH 8 (50 mM Tris-HCl), in the absence (A,B) or in the presence of $\alpha$-chymotrypsin at 5.2 (C) or 13.1 (D) μM.

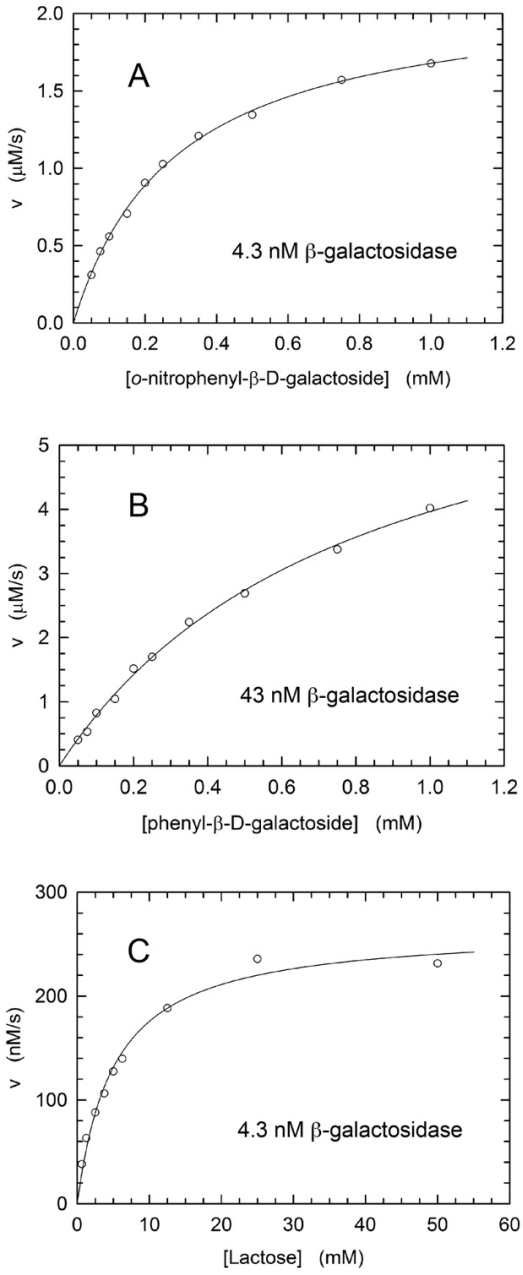


**Figure 3. Catalytic action of *Escherichia coli* β-galactosidase at the expense of different substrates.**

(A) Initial reaction velocities of the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside catalyzed by 4.3 nM β-galactosidase. (B) Kinetics of hydrolysis of phenylgalactoside catalyzed by 43 nM β-galactosidase. (C) Initial reaction velocities of lactose hydrolysis determined in the presence of 4.3 nM β-galactosidase, and 2.3 Units of galactose dehydrogenase.

All reactions were assayed at pH 7.5 (10 mM Tris-HCl, 1 mM $MgCl_2$, 30 mM KCl). The continuous lines represent the best fit of the Michaelis-Menten equation to the experimental observations.

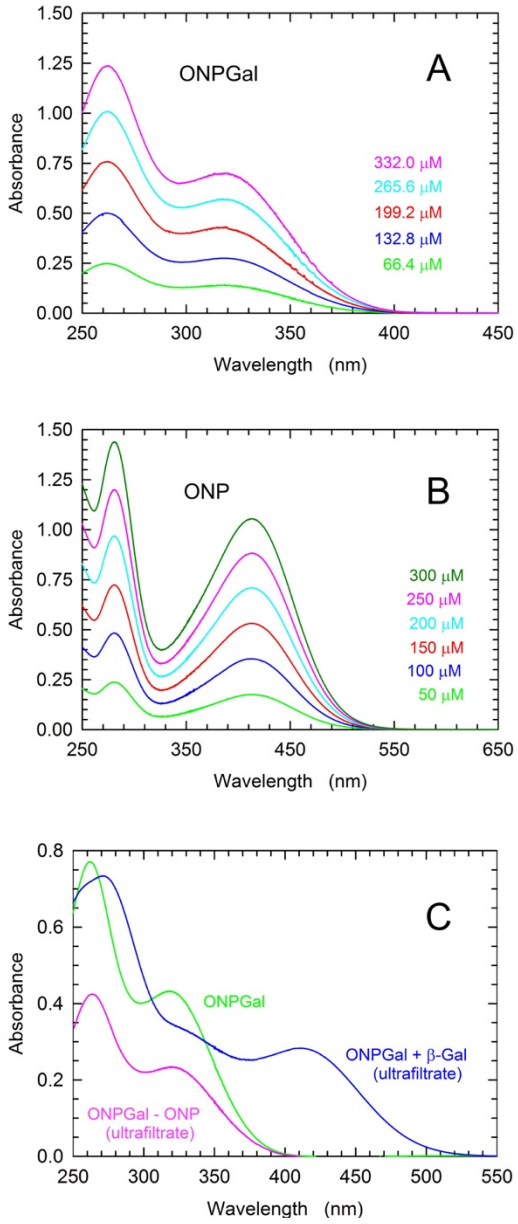


**Figure 4. Binding of *o*-nitrophenyl-β-D-galactopyranoside to multiple sites of β-galactosidase.**

(A) Absorption spectra of solutions containing the indicated concentrations of *o*-nitrophenyl-β-D-galactopyranoside. (B) Absorption spectra of 50-300 μM *o*-nitrophenol. (C) Absorption spectra observed with: i) the initial concentration of *o*-nitrophenyl-β-D-galactopyranoside (200 μM) to be exposed to 1 μM β-galactosidase (green line); ii) the sample obtained by collecting the ultrafiltrate ($M_r$ cutoff 100 kDa) of a solution containing 1 μM enzyme and 200 μM substrate (blue line). The magenta line represents the difference absorption spectrum obtained by subtracting to the absorption of the ultrafiltrated sample (blue line) the contribution of the *o*-nitrophenol present (the concentration of which was determined with the band centered at 410 nm). All spectra were recorded at pH 7.5 (10 mM Tris-HCl).