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Unravelling the role of ultrasonic energy in the enhancement of enzymatic kinetics

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ABSTRACT

The enzymatic hydrolysis of anabolic androgenic steroids excreted to urine as glucuronide conjugates has been recently reported to be improved by applying ultrasonic energy to the reaction medium. The hydrolysis time using β-glucuronidase from *Escherichia coli* K12 was reduced by a factor of six when ultrasonic energy was employed to enhance the enzymatic kinetic. In this study, the effect of ultrasonic energy on the enzymatic hydrolysis kinetic parameters, as well as on the enzymatic activity of β-glucuronidase from *E. coli* K12 was assessed. The study was conducted using the compounds 4-nitrophenyl-β-D-glucuronide and 4-nitrophenol. Experimental data suggested that the reaction follows the Michaelis–Menten kinetics type. In addition it was found that the ultrasonic energy affects the initial velocity of reaction, which is higher when ultrasound waves are employed when compared to the classical method of incubation at 55 °C. Moreover the values of V_{max} and k_{cat} are higher for the ultrasonic essay ($V_{max(US)} = 17.1 \pm 0.8\ \mu M\ min^{-1}$; $k_{cat(US)} = 340{,}523\ min^{-1}$; $V_{max(55\,^{\circ}C)} = 14.8 \pm 0.7\ \mu M\ min^{-1}$; $k_{cat(55\,^{\circ}C)} = 295{,}187\ min^{-1}$) whilst the Michaelis–Menten constant obtained for the two methodologies showed similar values ($K_{M(US)} = 94.7 \pm 7.2\ \mu M$; $K_{M(55\,^{\circ}C)} = 92.5 \pm 6.6\ \mu M$). Deactivation of the enzyme was also observed under ultrasonic energy, which was found particularly evident for experimental conditions with excess of substrate; enzymatic activity half-life time increased with the increase of the E/S ratio. This research work seems to support the idea that the use of ultrasonic energy affects the transition state of the enzymatic reaction and that mass transfer processes are also most likely enhanced.

1. Introduction

The administration of anabolic steroids has been the most frequent offence to fair competition inside sports. Their use was first introduced as agents supporting the athlete recuperation after extreme stress and fatigue, but rapidly became the main group in doping abuse [1]. In the World Anti-Doping Agency (WADA) statistic report of 2009, the anabolic agents, in which is included the group of androgenic anabolic steroids (AAS), represented 64.9% of all adverse analytical findings reported by WADAs' accredited laboratories [2].

Due to their non-polar character, most AAS are modified in the human body by phase-I and phase-II metabolic reactions, prior to their excretion in urine [3]. Phase-I metabolism consists in enzymatic reactions (e.g., oxidation, reduction, or hydroxylation) that inactivate the drug and increase its polarity by adding or exposing functional sites into the AAS structure, where phase II metabolism can subsequently occur [1,3,4]. The phase II metabolism can be characterized as the conjugation reactions that change the physicochemical properties of the compounds in order to promote its elimination from the body [1,5]. The main phase-II reactions are the conjugation with glucuronic acid or sulfate moiety [1,3]. However, for the AAS, the formation of glucuronate conjugates is the major metabolic pathway of conjugation and inactivation of these compounds [1,3,6,7]. The result of both phase-I and phase-II reactions is the synthesis of hydrophilic compounds that are more easily excreted.

Doping control of AAS is based on the detection of such compounds and its metabolites in urine samples from athletes [8,9]. Currently, the routine procedure for the detection of these compounds, comprising both screening and confirmatory analysis, is typically carried out by gas chromatography–mass spectrometry (GC–MS) methodologies [10–13]. It is important to stress out that to be analysed by GC–MS techniques, conjugated steroids must be hydrolysed and derivatised to fit GC analysis pre-requisites and improve its mass spectrometric characteristics.

Of crucial importance, is the step of the enzymatic hydrolysis of the steroid glucuronides that is carried out directly on the urine sample with the enzyme β-glucuronidase (GUS, EC 3.2.1.31) from

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Escherichia coli, which is a hydrolase highly specific to β-linked-D-glucuronides [14]. Within anti-doping laboratories, the cleavage of the glucuronide moiety to produce the free compound is normally performed by conventional warming at 55 °C for 1 h, which was found to be the optimum temperature to achieve maximum yield [15–19].

Over the past years, the increasing complexity of doping control, mainly due to the continuously introduction in anti-doping regulations of new banned substances and methods and also due to the increasing workloads inside anti-doping laboratories, has led scientists to develop new strategies for fast and high throughput sample treatments and analysis.

Modern approaches to sample preparation show a growing interest in ultrasonic energy as an alternative to conventional methods. The ability of ultrasonic irradiation methods to accelerate and, occasionally, to increase the chemical reactions yield have attracted the scientific community for its use over the last decades. Despite the widespread use of ultrasonic energy in various research disciplines, only recently the synergetic use of ultrasonication and enzymes to enhance reactions has been described [20–23].

Briefly, the vibrational motion transmitted to the liquid medium induced by ultrasound waves causes alternately expansion and compression of the medium [24]. At sufficiently high power, the strength of the acoustic field may exceed the attractive forces of the particles in the expansion or rarefaction cycle and create cavities in the liquid medium called cavitation bubbles, which can grow, oscillate, split and implode [25–28]. It is this phenomenon, of cavitational bubble implosion, that produces the powerful shearing, causing the molecules in the liquid to become intensely agitated, as extreme temperatures and pressures are generated, acting like micro-reactors inside the liquid media [25,26]. Moreover, the formation of highly reactive chemical radicals may also take place inside the liquid media [25,26].

Although the improvement of chemical reactions is easily explained by the cavitation phenomena, the understanding of the synergetic effect between ultrasounds and enzymes is still far from being completely understood. Nevertheless, some authors have pointed out that those phenomena created by ultrasonic energy, increases the contact area between phases, which allows a reduction of mass transfer limitations in the enzyme–substrate system [29].

In recent years, our research group has focused on the development of ultrasonic-assisted enzymatic reactions as a tool to enhance laborious and tedious reactions. In this sense and in collaboration with the WADA accredited anti-doping laboratory of Rome, in Italy, it has been recently demonstrated that high intensity ultrasonic energy enhances the enzymatic hydrolysis of AAS with β-glucuronidase [30]. Although this is a remarkable finding, there is still a lack of fundamental research about the effects of ultrasonic energy on the enzyme β-glucuronidase catalytic activity.

In the present work the effects of ultrasonic energy on the kinetics of β-glucuronidase enzyme were evaluated using the compound p-nitrophenyl–β-glucuronide, which is commonly used for β-glucuronidase enzymatic essays. A simple theoretical kinetic model, base on Michaelis–Menten equation, is introduced in order to investigate the variations in the equation kinetic parameters. The results of the ultrasonic based enzymatic studies were compared with those obtained by the routine warming incubation at 55 °C.

2. Experimental

2.1. Standards and reagents

The standards of 4-nitrophenyl-β-D-glucuronide and 4-nitrophenol were purchased from Sigma–Aldrich (Steinheim, Germany). Sodium hydrogen phosphate and sodium phosphate dibasic were purchased from Sigma–Aldrich. A commercial solution of β-glucuronidase from *E. coli* K12 with a specific activity of 185 U/mg at 37 °C and pH 7 with nitrophenyl-β-D-glucuronidase as substrate (1 mL contained 1.47 mg of protein) was purchased from Roche Diagnostic (Mannheim, Germany). Methanol (MeOH) was purchased from Sigma–Aldrich and trifluoroacetic acid (TFA, 99%) was from Riedel-de Haën.

Individual stock standard solutions of each compound with 0.05 M were prepared in 10 mL volumetric flasks with methanol. These standard solutions were stored in the dark at −20 °C. Working standard solutions were prepared by dilution of the stock standard solutions in the appropriate volume of methanol.

2.2. Apparatus

A cup horn sonoreactor (SR), model from Dr. Hielscher (Teltow, Germany), was used to accelerate the enzymatic hydrolysis procedure. A minicentrifuge, model Spectrafuge-mini, from Labnet (Madrid, Spain), and a minicentrifuge-vortex, model Sky Line, from ELMI (Riga, Latvia) were used throughout the sample treatment. A Simplicity 185 from Millipore (Milan, Italy) was used to obtain Milli-Q water. The enzymatic hydrolysis of 4-nitrophenyl-β-D-glucuronide was performed in 2 mL microtube flat cap from Delta Lab (Barcelona, Spain).

2.3. Chromatographic system and conditions

To analyse the hydrolysis of the 4-nitrophenyl-β-D-glucuronide, measurements were carried out by HPLC, using a Waters 600E gradient metering pump model equipped with a Waters dual channel UV–visible detector. Samples were injected through a 20 μL loop injector valve (Rheodyne Model 7120) and the chromatographic separation was performed on reversed phase 5 μm particle size, $L \times$ I.D. 15 cm × 4.6 mm Discovery® C18 column from Supelco (Bellefonte, Pennsylvania). The aqueous mobile phase (A) consisted of HPLC-grade water with 0.01% TFA; the organic phase (B) was MeOH. The HPLC elution was carried out under isocratic conditions, with 55% of solution A and 45% of solution B. The flow rate was 0.9 mL/min with a 10 min run time and the UV detector was set at the wavelength of 305 nm. Calibration curves were constructed by plotting peak areas against concentrations of PNP. Throughout the experimental work, data was collected and integrated using Chromeleon Software. For quantification, the peak areas were determined, and the concentrations calculated with external calibration curves. The calibration curves for PNP and PNP-G were run at the beginning of each week. The calibration curves were calculated by the least square method. Linearity was assessed by determining the coefficient of correlation (r^2) of the points of the curves, which was always higher than 0.9998. Both PNP and PNP-G showed linear response in the target concentration range of 0.4–500 μM for PNP and 5–600 μM for PNP-G.

To ensure the analytical quality of the calibration curve made at the beginning of each working week, a set of standard samples with different PNP-G and PNP concentrations were prepared and measured on each day at routine intervals.

2.4. Sample treatment

Spiked sample solutions of 4-nitrophenyl-β-D-glucuronide were prepared in sodium phosphate buffer (0.1 M; pH 6.8). The commercial solution of β-glucuronidase from *E. coli* K12 was diluted in the same buffer (1:13). To 2 mL of the spiked samples, 2 μL of the prepared enzyme solution, corresponding to 0.22 μg of enzyme, was added and the enzymatic assay was carried out using two different systems as follows: (a) using conventional warming

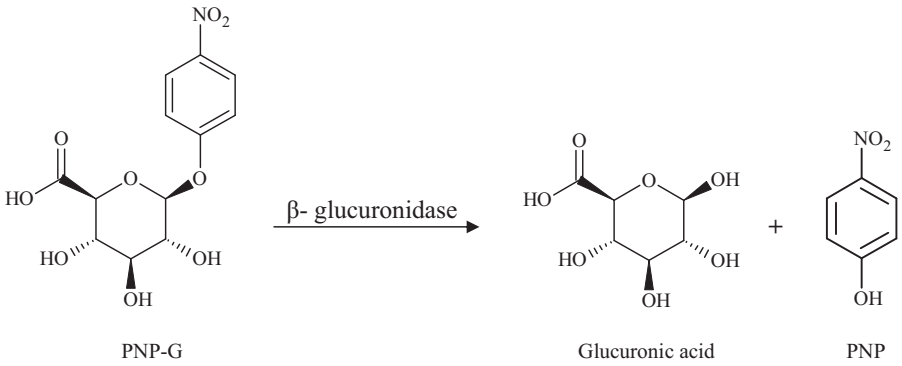

Fig. 1. Hydrolytic conversion of substrate PNP-G to the products PNP and D-glucuronic acid through the action of β-glucuronidase.

at 55 °C and (b) using ultrasonic energy provided by a cup horn sonoreactor (SR) operating at 60% of sonication amplitude. The ultrasonic amplitude was chosen based on our previous study with AAS [30]. After the hydrolysis, the reaction was quenched by the addition of 10 μL of TFA.

The calibration standards were prepared in 10 mL volumetric flasks. In order to have the same matrix than the samples, calibration solutions were made in sodium phosphate buffer (0.1 M; pH 6.8) to which was added 50 μL of TFA.

3. Results and discussion

The aim of this work was to compare the enzymatic activity of β-glucuronidase and the hydrolysis kinetic parameters under ultrasonic irradiation with the conventional reaction at 55 °C. For this purpose PNP-G was used as substrate. Fig. 1 shows the hydrolytic conversion of PNP-G to PNP. It is important to stress that for all conditions assayed, three replicates were performed and that the relative standard deviation, RSD, associated was always below 9%.

3.1. Chromatographic assay to measure the activity of β-glucuronidase

In order to assess the effect of ultrasonication on the kinetic of the enzymatic hydrolysis, both product formation and substrate disappearing rate were measured. Separation of the reaction mixture and detection of its components were carried out by HPLC, using UV detection at λ = 305 nm, which was found to be the optimal wavelength for detection of PNP-G and PNP. A representative chromatogram showing PNP-G and PNP peaks, obtained after 4 min of incubation with β-glucuronidase, is presented in Fig. 2. As it may be seen, only two well resolved peaks corresponding to PNP-G and PNP are observed, making the analysis of the reaction mixture simple to achieve.

To correctly estimate the activity of β-glucuronidase under an ultrasonic field, before the enzymatic assay, the chemical stability of both reaction components was evaluated. Experiments were carried out and the concentration of both components was measured before and after ultrasonication (10 min at 60% of amplitude). For this study five replicates were used. The results obtained showed that both compounds remain stable after application of ultrasonic energy to the sample's medium, exhibiting the same concentration before and after ultrasonication.

3.2. β-Glucuronidase activity/stability under ultrasonic irradiation

The use of ultrasonic energy to enhance enzymatic reactions is still a matter of great debate within the scientific community. Recent studies focusing on the influence of ultrasonic energy in the enzyme performance have reported both enhancement and inactivation of enzyme activity [31–34]. Based on our expertise concerning the application of ultrasonic energy to enhance a variety of reactions and, particularly, enzymatic reactions, we have found that, in fact, activation and inactivation occur and are closely linked to the ultrasonic operating conditions [23,30,35–40]. Parameters such as ultrasonic frequency and amplitude, vial shape, time of reaction, as well as enzyme amount and temperature of the reaction bulk, deeply affect the performance of the reaction. Therefore, a consistent knowledge on the ultrasonic energy performance and ultrasonic devices' characteristics is vital to carry out a successful experiment. For instance, when ultrasonication is applied to a liquid, a slow but constant increase in the bulk temperature occurs and, depending on the enzyme, denaturation may occur, not due to ultrasonic waves itself but to the warming generated.

To evaluate the effect of ultrasonic irradiation in both enzymatic activity and stability of β-glucuronidase, the appearance of PNP as a function of time under ultrasonic irradiation was compared with the conventional methodology of incubation at 55 °C. Experimental data was achieved using large excess of substrate; 450 μM of PNP-G and 0.22 μg of β-glucuronidase. Fig. 3 presents the appearance of PNP as a function of time and the correspondent relative enzyme activity, for both essays. The unit of enzymatic activity was considered as the enzyme activity that increases the rate of release of 1 μmol of PNP per minute. As it may be seen in Fig. 3b, the activity of the enzyme under ultrasonic energy is, in the first minutes of the enzymatic reaction, higher than the one obtained under thermal incubation at 55 °C. Yet, the enzyme activity quickly decreases after

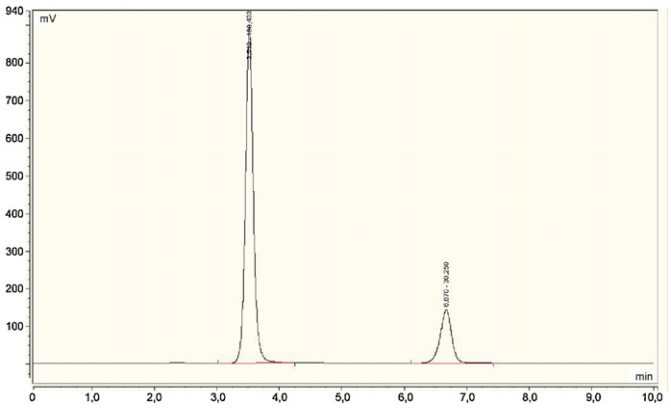

Fig. 2. Representative chromatogram obtained after 4 min of incubation of the substrate PNP-G with the enzyme β-glucuronidase. The first peak elutes at 3.51 min and corresponds to PNP-G, whereas the second peak elutes at 6.67 min and corresponds to PNP.

Table 1
Enzymatic deactivation parameters for the ultrasonic irradiation procedure at different β-glucuronidase amounts.

β-Glucuronidase amount (μg)	Time until deactivation[a] (min)	k_d (min^{-1})	$t_{1/2}$ (min)	R^2
0.04	3.34	0.32 ± 0.03	5.5 ± 0.2	0.985
0.08	4.00	0.29 ± 0.02	6.4 ± 0.2	0.993
0.16	5.03	0.33 ± 0.02	7.1 ± 0.1	0.993
0.22	5.50	0.28 ± 0.02	8.0 ± 0.2	0.998

[a] Initial reaction time with constant enzymatic activity (showing no deactivation).

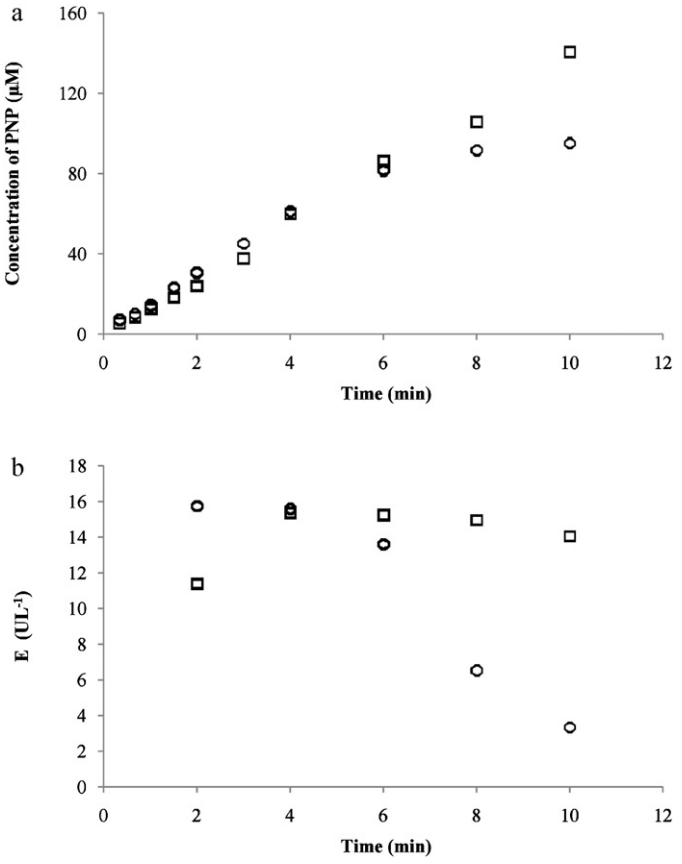

Fig. 3. (a) Appearance of PNP as a function of time for both incubation at 55 °C and ultrasonic irradiation essays. The experimental results were obtained using 450 μM of PNP-G. (b) Correspondent enzyme activity for both experiments. The unit of enzymatic activity was considered as the enzyme activity that increases the rate of release of 1 μmol of PNP per minute. Data identification: □: 55 °C; ○: ultrasonic irradiation.

6 min of reaction, unlike the reaction under thermal incubation that stays almost constant along the time studied.

Slow inactivation of the enzyme under high ultrasonic irradiation was expected; nevertheless, when compared with our previous work, in which the enhancement of the AAS glucuronates hydrolysis by ultrasonic irradiation was studied, the enzymatic inactivation of β-glucuronidase occurred faster. The major difference between the two enzymatic assay conditions, concerns the fact that the experimental procedure developed in this study was carried out with low amount of enzyme, at large excess of substrate. Concerning our previous study on the hydrolysis of AAS, the amount of enzyme was in large excess; almost 250 fold higher than the value used in the present study [30].

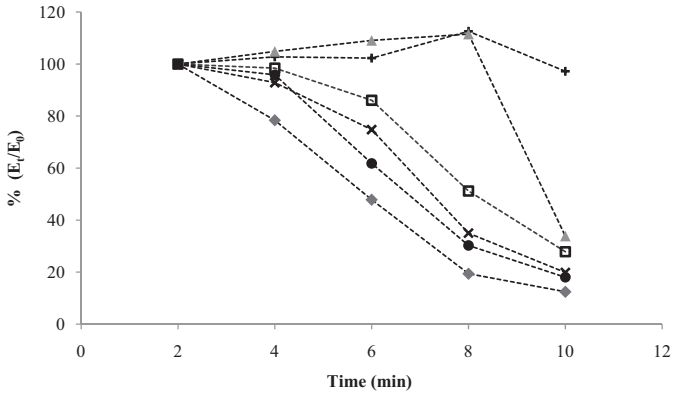

Fig. 4. Relative enzyme activity for the release of PNP from PNP-G, applying ultrasonic irradiation. The concentration of PNP-G was fixed at 450 μM and the amount of β-glucuronidase varied from 0.04, 0.08, 0.16, 0.22, 0.33 and 0.44 μg. Data identification: ◆: 0.04 μg; ●: 0.08 μg; ✖: 0.16 μg; □: 0.22 μg; △: 0.33 μg; ✚: 0.44 μg.

To get further insight into the relation between the amount of enzyme and the enzymatic deactivation by ultrasonic irradiation, a set of experiments was devised in which the amount of enzyme varied whilst the concentration of substrate was maintained constant at 450 μM. The determination of the deactivation kinetic parameters was performed using six distinct amounts of enzyme: 0.04 μg, 0.08 μg, 0.16 μg, 0.22 μg, 0.33 μg and 0.44 μg. Fig. 4 shows the relative enzyme activity for the different enzyme concentrations. As it may be seen, the results present a strong decrease in the enzymatic activity when lower amounts of enzyme were used; for higher amounts of enzyme, the enzymatic activity is kept constant for longer reaction times. With 0.33 μg of enzyme, the rate of PNP appearance increased until 8 min of reaction and then decreased abruptly. Likewise, by using 0.44 μg of enzyme, the rate of PNP still reaches its maximum at 8 min, but at 10 min, the enzymatic activity drop is not so significant and it corresponds to 86% of the maximum activity.

A first analysis of the deactivation kinetics was performed using the simple exponential model by fitting the experimental data to a first order kinetics, in which the native form is irreversibly transformed into the denaturated form. The first order equation for the enzymatic deactivation reaction is given by,

$$\frac{(E)_t}{(E)_0} = \exp(-kt)$$

where $(E)_t$ represents the enzymatic activity at time t, $(E)_0$ the initial enzymatic activity, k the deactivation rate constant and t the time.

Fig. 5 presents the semi-logarithmic plot of $(E)_t/(E)_0$ against time. It clearly shows that the stability of the enzyme is sensitive to the amount of enzyme. By increasing the enzyme amount, the enzy-

Table 2
Catalytic parameters of β-glucuronidase from *E. coli* K12 using PNP-G as substrate.

	K_M (μM)	V_m (μM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})
Incubation at 55 °C	92.5 ± 6.6	14.8 ± 0.7	295,187	3192
Ultrasonic irradiation	94.8 ± 7.2	17.1 ± 0.8	340,523	3593

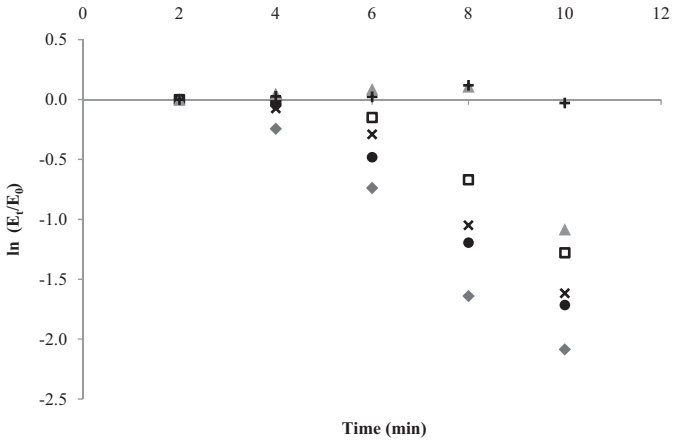

Fig. 5. Semi-logarithmic plot of $(E)_t/(E)_0$ against time. Data identification: ◆ : 0.04 μg; ●: 0.08 μg; ✖ : 0.16 μg; ◘ : 0.22 μg; △ : 0.33 μg; ✚ : 0.44 μg.

matic activity is kept constant for longer times until deactivation starts. After this holding period of constant activity the enzymatic deactivation shows a linear behaviour. The values of the deactivation constant, as well as the half-life time, were determinate for the experimental data corresponding to 0.04 μg, 0.08 μg, 0.16 μg and 0.22 μg (see Table 1). For enzyme amounts of 0.33 μg and 0.44 μg, the activity of the enzyme is kept constant for most of the experimental time and therefore, the deactivation parameters were not calculated. The deactivation rate constant values found for the different amounts of enzyme, under ultrasonic irradiation, are similar, which means that the amount of enzyme has no significant effect on the deactivation rate constant. Unlike the constant rate, the initial time at which the enzymatic activity is kept constant is sensitive to the amount of enzyme. Higher amounts of β-glucuronidase resulted in longer times at constant initial enzymatic activity, and therefore higher half-life times.

These results demonstrate that the amount of enzyme is determinant under ultrasonic irradiation. In fact, at this substrate concentration value and until 0.22 μg of β-glucuronidase, the enzyme already reached its substrate saturation point (see Section 3.3), meaning that most active sites of the enzyme are occupied. Therefore, the inactivation of the enzyme by ultrasonic waves has a deep effect on the product appearance rate. When the amount of enzyme is increased, the slow inactivation that occurs during ultrasonication is suppressed by the positive effect of ultrasonication on the enzyme activity.

Concerning our previous study on the hydrolysis of AAS, the amount of enzyme was in large excess; as a result, in the first 10 min of the AAS hydrolysis reaction, the slow inactivation of the enzyme was counterbalanced by the positive ultrasonic effects on the enzyme activity. It is important to stress that, in our previous study, the hydrolysis reaction time of AAS glucuronates was greatly improved from 60 min to 10 min by using ultrasonic irradiation together with β-glucuronidase [30].

These results clearly suggest that special attention must be taken regarding the amount of enzyme, when ultrasonic waves are employed to enhance the enzymatic reaction. To diminish the effects of enzyme denaturation on the reaction rate, a slight excess of enzyme is required.

3.3. β-Glucuronidase kinetic parameters

Through a brief literature search, in which we found several reports highlighting the use of ultrasound for numerous applications, it became evident that few attempts were made to

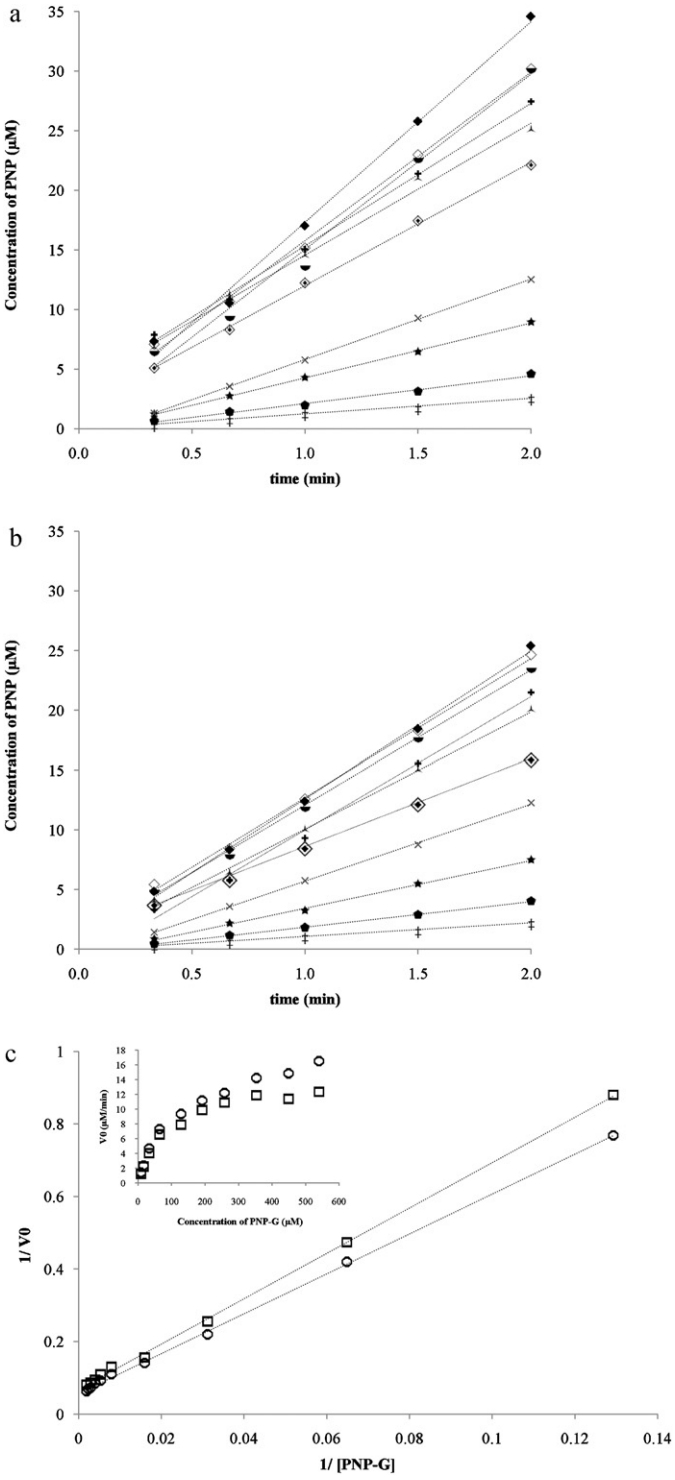

Fig. 6. Observed time courses for the hydrolytic conversion of PNP-G into PNP (a) at 55 °C and (b) under continuous ultrasonic irradiation, with increased PNP-G concentration. Data identification: ‡: 7.7 μM; ⬟ : 15.4 μM; ⋆: 32.1 μM; ×: 62.9 μM; ◈ : 127.3 μM; ⅄ : 190.1 μM; ✚ : 257.0 μM; ♢: 352.4 μM; ◡ : 448.3 μM; ♦: 539.3 μM. (c) Plots of the initial velocity values obtained as a function of the correspondent PNP-G concentration values and respective Lineweaver–Burk linearization. Enzymatic activity was assayed with 0.22 μg of enzyme at pH 6.5. For Lineweaver–Burk representation, the x and y axes indicate the reciprocals of the initial concentration of PNP-G and initial velocity, respectively. The value of V_{max} is given from the intercept and the value of K_M/V_{max} from the slope. Data identification: ◘line: incubation at 55 °C essay; $y = (6.3 \pm 0.2)x + (0.067 \pm 0.003)$, ($r^2 = 0.9992$); ○ line: ultrasonic irradiation essay; $y = (5.5 \pm 0.1)x + (0.059 \pm 0.003)$, ($r^2 = 0.9979$).

understand the effect of ultrasonic irradiation on the kinetic parameters of the mentioned enzymatic reactions.

In this work, to assess the effect of ultrasonic irradiation on the reaction kinetic parameters, the enzymatic study was performed using initial rate experiments, by varying the concentration of substrate in the presence of a fixed concentration of enzyme. The initial velocity for a given concentration of PNP-G was determined by plotting the enzyme reaction time as a function of PNP concentration. Ten PNP-G concentration values were used in this study, ranging from 7.75 to 540 μM. Although the reaction time ranged from 20 s to 10 min, the initial velocities were calculated using the first 2 min of the reaction. Fig. 6 presents the time courses for the appearance of PNP and the initial velocity values obtained as a function of the correspondent PNP-G concentration values, for both ultrasonic and incubation at 55 °C essays. As it was expected, the reaction velocity increased with the increase in PNP-G concentration until it reaches the substrate saturation for 0.22 μg of enzyme. The enzyme substrate saturation for the ultrasonic irradiation essay occurred at a higher concentration value than for the thermal incubation essay.

The kinetic parameters were determined through curve fitting, using a linear transformation of the Michaelis–Menten equation, the Lineweaver–Burk plot, in which the reciprocal of the initial rate, $1/V_0$, was considered against the reciprocal of the substrate concentration 1/[PNP-G] (see Fig. 6). The data obtained was used to display the four fundamental kinetic constants for one substrate reactions: the maximal velocity of the reaction (V_{max}), the catalytic constant (k_{cat}), the Michaelis constant (K_M), and the specificity constant (k_{cat}/K_M). With the equation line of the Lineweaver–Burk plot, the value of V_{max} can be obtained from the intercept and the value of K_M/V_{max} from the slope. The catalytic constant is obtained from the total concentration of enzyme in the reaction medium, 5.01E−05 μM, and the value of the maximal velocity of the reaction. Results are shown in Table 2. The K_M values found for both incubation at 55 °C and ultrasonic irradiation are very similar, meaning that the affinity between the enzyme and the substrate is not affected by ultrasonication. Unlike the kinetic parameter K_M, the quantity V_{max}, which reflects the limiting rate of the enzymatic reaction at substrate saturation, was positively affected by ultrasonic irradiation. Likewise, both the catalytic and specificity constants are higher under the effects of an ultrasonic field, which means that ultrasonic energy positively affects the catalytic efficiency of β-glucuronidase and, therefore, the substrate is converted into the product at an increased rate when compared with the same reaction at 55 °C.

Given these results, we hypothesised that when ultrasonic energy is delivered into the reaction medium, it has little effect on the formation of the enzyme–substrate (ES) complex, since the kinetic parameters that reflect the enzyme/substrate equilibrium, K_M, remain unaltered. Nevertheless, the ultrasonic energy must contribute to stabilise the transition state, for instance, by lowering the activation energy associated with the enzyme/substrate transition state, since the overall rate of the reaction is affected. Moreover, the shockwaves created by cavitation bubble implosion will affect the kinetic energy of the reaction medium molecules, which probably affects the mass transfer processes between the enzyme and substrate.

4. Conclusions

We have verified that ultrasonic energy enhances β-glucuronidase enzymatic kinetics by increasing both initial velocity and maximum velocity. It has been assessed that the reaction follows the Michaelis–Menten equation, remaining the respective constant unaltered. The results obtained indicate, as a first approach, that the improvement caused by ultrasonic energy is mainly due to the stabilisation of the transition state and the enhancement of mass transfer processes. It has been also proved that, at substrate excess, the positive effects of high intensity ultrasonication are quickly surpassed by the inactivation of β-glucuronidase enzyme. Hence, when ultrasonic energy is used to enhance enzymatic reactions, a concentration of enzyme superior to the one regularly used is advised.

The results obtained in this study represent an important step to understand and identify the effects of ultrasonication in the enzyme–substrate complex interaction. When compared with our previous study conducted with AAS, the effects of ultrasonication to enhance β-glucuronidase activity, using PNP-G as substrate are less accentuated. We attribute this result to the nature of substrate; the harder is the hydrolysis reaction achieved by conventional thermal incubation, more evident and advantageous is the use of high intensity ultrasonication.

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