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The effect of low-frequency ultrasound on the activity and efficiency of a commercial cellulase enzyme

Orsolya Erzsébet Szabó, Emília Csiszár*

Department of Physical Chemistry and Materials Science, Budapest University of Technology and Economics, P.O. Box 91, H-1521 Budapest, Hungary

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ABSTRACT

A commercial acidic cellulase enzyme complex was chosen in order to gain detailed information about the effect of low-frequency ultrasound (horn at 40 kHz) on the enzyme activity. The performance of the enzyme under sonication was also evaluated in a cellulose–cellulase model reaction. The filter paper activity of the enzyme and the yield of the enzyme catalysed hydrolysis were determined as a function of the parameters of the sonicated environment (treatment time, amplitude, with and without a reflector) and compared with the data measured in a non-sonicated bath. Depending on the parameters of the sonication, the enzyme is susceptible to ultrasound and its activity can significantly decrease. Despite the serious reduction of the enzyme activity, the outcome of the enzyme catalysed hydrolysis was always positive, implying that the advantageous effects of sonication impressed on the heterogeneous enzyme reaction always overcome the undesirable enzyme modifying effect of ultrasound.

1. Introduction

In the last decades ultrasound has been widely used for improving the efficiency of various chemical, physical and biotechnological processes. The effect of ultrasound can be based on a direct interaction with molecular species as well as on the cavitation phenomenon. In the field of biotechnology, low-frequency power ultrasound has recently gained much attention in the intensification of enzyme-aided processes. Ultrasound has a direct effect on the enzyme molecules and enhances the mass transfer in the heterogeneous processes by the local turbulences created by acoustic cavitation (Gogate & Kabadi, 2009; Kwiatkowska, Bennett, Akunna, Walker, & Bremner, 2011; Mason, 2007; Rokhina, Lens, & Virkutyte, 2009; Suslick, 1990).

Cavitation is the formation, growth and collapse of vapour or gas bubbles that occur with ultrasound. In a heterogeneous reaction, collapse of the bubbles near the solid surface results in high velocity micro-jets, which accelerate the transport processes and significantly improve the mass transfer (Moholkar, Nierstrasz, & Warmoeskerken, 2003). Subsequently, power ultrasound can considerably increase the reaction rate in an enzyme-aided heterogeneous system. Furthermore, during acoustic cavitation extremely high local temperatures and high pressure are created, which can have a remarkable effect on the enzyme macromolecule and its activity.

The overall effects of the power ultrasound on the homogeneous and heterogeneous enzyme-aided reactions are well-published in scientific literature. Although most of the papers confirm that the overall effect of the ultrasound is very positive (Basto, Tzanov, & Cavaco-Paulo, 2007; Moholkar et al., 2003; Yachmenev, Blanchard, & Lambert, 2004), the enzymes used in the different bioprocesses can be sensitive to ultrasound irradiation (Macleod & Dunn, 1967; Wood, Aldrich, & Ingram, 1997), and in particular cases, they can sustain damage to a certain extent. Furthermore, the enzyme-modifying effect of the ultrasound can contribute to the alteration of the enzyme activity, which can have far-reaching consequences. Changes in activity suffered by the enzyme in the ultrasonicated bath can be influenced by the parameters of the sonication and the characteristics of the enzyme. Thus, the enzyme macromolecule–ultrasound interaction has a significant effect on the efficiency of the bioprocess.

Despite the fact that ultrasound is able to alter the enzyme activity, surprisingly, only a very few publications have focused on the behaviour of enzymes exposed to power ultrasound. Interestingly, most of the papers published in this field evaluate the efficiency of the ultrasound-aided processes, but draw the conclusions on the enzyme activity modifying effect of the ultrasound, without measuring the enzyme activity itself.

Guiseppi-Elie et al. investigated a commercial glucose oxidase enzyme, which is a broadly utilized enzyme in the field of biosensors and the next generation of biofuel cell systems (Guiseppi-Elie,

* Corresponding author. Tel.: +36 1 463 1423; fax: +36 1 463 3474.
E-mail address: ecsiszar@mail.bme.hu (E. Csiszár).

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Choi, & Deckeler, 2009). The results proved that the ultrasonicated (at 23 kHz and at ice bath temperature for 10, 30 and 60 min) glucose oxidase enzyme showed a different composition with reduced α-helix and β-sheet fractions upon extended sonication compared with the pristine enzyme. Together with the changes of the secondary structure, the enzymatic activity showed a small corresponding decrease.

Özbek and co-workers investigated the stability of six different enzymes under sonication at low frequency (Özbek & Ülgen, 2000). They demonstrated that operational parameters: such as processing time, time of exposure, acoustic power, wave duty cycle, viscosity of the enzyme solution has a significant effect on the enzyme stability. Furthermore, the stability or inactivation of the enzymes can vary over a wide range depending on the enzyme properties.

A detailed study has been carried out by Souza et al. who evaluated the activity of a commercial amylase enzyme after sonication in an ultrasonic bath at 40 kHz (Souza et al., 2013). Amylases act in the hydrolysis of starch and they are applied in different areas of the food, pulp and paper, textile and bioethanol industry. The enzyme activity was measured in a wide range of temperature (from 30 to 100 °C) and at pH 4.5 with and without sonication. Results clearly proved that at lower temperatures (that is far from the optimum temperature of the enzyme) the sonication can promote enzyme reaction. But, at a higher temperature (that is closer to the enzyme's optimal conditions) sonication depresses the enzyme activity.

Since enzymes are usually used near their optimal conditions, where they exhibit maximum activity, thereby achieving their maximum reaction rate, it is therefore essential to know what the influence of the sonicated environment has on the effectiveness of the enzyme operating under ideal conditions. We therefore believe that there is a need for more research in this area in order to better understand the correlation of the 'sonication–enzyme action'. This would enable us to develop more efficient processes in the field of sono-biotechnology.

Therefore, in this study a commercial acidic cellulase enzyme complex was used in order to characterize the effect of sonication on the enzyme activity and efficiency. Cellulase refers to a group of enzymes which, acting together, hydrolyse cellulose. Cellulases are widely used in different areas of industry, agriculture, as well as in research and development. Two types of experiments were conducted. *First*, the diluted solutions of the enzyme were irradiated with cavitating power ultrasound and subsequently the enzyme activity was measured. The filter paper activity (FPA) of the enzyme was determined as a function of the parameters (treatment time, amplitude, with and without a reflector) of sonication (horn at 40 kHz). In order to ensure the optimal conditions, under which this enzyme exhibits maximum activity, the temperature and pH were maintained at 50 °C and pH 5, respectively. The activity of the enzyme in the sonicated field was compared with that of the enzyme acting in a bath mixed with a magnetic stirrer. *Second*, sonication was applied in an enzyme catalysed reaction (hydrolysis of cellulose powder by cellulase enzyme), in order to get information on the effect of sonication of the efficiency of the enzyme. By varying the above mentioned parameters of the sonication system, the reducing sugar liberation was measured continuously and the results were compared with the enzyme activity data.

2. Experimental

2.1. Cellulase enzyme and cellulose substrate

Celluclast 1.5L, a cellulase mixture produced by *Trichoderma reesei* from Sigma–Aldrich, was used in the experiments. The enzyme exhibits maximal activity near 50 °C and at pH 4.5–5.0. In this research, the filter paper activity of the enzyme was chosen exclusively for characterizing the effect of sonication on the enzyme activity. However, in a previous study (Csiszár, Urbánszki, & Szakács, 2001), beside the FPA, the 1,4-β-endoglucanase and β-glucosidase activities were also determined for a more complete characterization of the enzyme mixture (28,000 EGU/ml, 11 IU/ml, respectively).

Bleached cotton fabric obtained from Testfabrics Inc., USA, which can be seen as a pure (100%) cellulose substrate was used for the cellulose–cellulase model reaction. The fabric was ground in a ball-mill (Csiszár & Fekete, 2011), and the fraction studied in this work was composed of particles with diameters enclosed between 800 and 1000 μm, collected by sieving. All chemicals used were of analytical grade and were purchased from Sigma–Aldrich Co. LLC. and Reanal Private Ltd.

2.2. Sonication system

Fig. 1 shows the schematic illustration of the experimental setup. The ultrasonic experiments were carried out using an ultrasonic horn type reactor (Sonics & Materials, Model: Vibra-Cell VC505), with a driving frequency of 40 kHz, and a power of 500 W supplied by a piezoelectric transducer and with a 13 mm diameter replaceable tip. The horn was fitted by a 10 mm-thick rubber cork to a double walled, cylindrical glass cell with 54 mm inner diameter and 120 mm height, to achieve 1 wavelength distance ($x = 3.7$ cm) between the tip and the bottom of the vessel. In some of the experiments a stainless steel disc ($d = 52$ mm) that acted as a rigid reflector was placed at the bottom of the vessel and was used to intensify the reflectance of the ultrasound waves and to create a standing wave field (Moholkar et al., 2003). The reaction mixture was also stirred with a Teflon-coated magnetic bar rotating at 400 rpm. The system was thermostated to 50 ± 2 °C by jacket cooling, and the actual temperature and power input values were recorded at 10 s intervals. The temperature and power input sensor was connected to a computer so that the sonicated enzyme reaction was monitored by the instrument's software.

The power delivered (P_{deliv}) to the system was measured, and the actual power dissipated (P_{diss}) for each experimental configuration (i.e. at 40, 60 and 80% amplitude/49.6 μm, 74.4 μm and 99.2 μm, respectively; with continuous ultrasonication) was determined by calorimetrically (Hagenson & Doraiswamy, 1998). The intensities of the ultrasound system (I_{diss}) in the reaction mixture were calculated: the P_{diss} was divided by the area of the probe tip (1.27 cm^2).

2.3. Sonication of the enzyme solution and filter paper activity assay

For monitoring the change in enzyme activity, 250-fold dilutions of cellulase enzyme in 0.05 M acetate buffer (pH 5) were sonicated at 40, 60 and 80% amplitude, without and with a reflector, at 50 °C in a period of 0–65 min. The total volume of the reaction mixture was 125 ml. After 5, 20, 35, 50 and 65 min, 0.5 ml of the enzyme solution was taken for the filter paper activity measurement. FPA was determined as described by Ghose (Ghose, 1987). Briefly, a 1×6 cm strip (50 mg) of Whatman No. 1 filter paper was added to a total volume of 1.5 ml enzyme solution and 0.05 M acetate buffer (pH 4.8). The samples were incubated at 50 °C for 1 h. The reaction was terminated by the addition of 3 ml dinitrosalicylic acid (DNS) solution, followed by boiling for 5 min. After cooling, 20 ml distilled water was added and the absorbance was read at 540 nm. The liberated reducing sugars (glucose equivalent) were estimated according to Miller (Miller, 1959). The reducing sugar content of the fermentation media was estimated indirectly from the enzyme blanks of the FPA measurement. Filter paper unit (FPU) was calculated