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Effects of ultrasound on cellulolytic activity of cellulase complex

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Abstract

This study focused on the effects of ultrasound on the catalytic activity of cellulase preparation. Diluted Celluclast 1.5L in citrate buffer was treated by ultrasound with different variables. The catalytic activity of the treated and untreated enzymes was determined against soluble and insoluble substrates. Changes in ultrasonic intensity and sonication time significantly affected the enzyme activity. The cellulolytic activity increased when the ultrasonic intensity augmented from 0 to 6 W/mL and the treatment time prolonged from 0 to 80 sec. It was due to some slight changes in spatial structure of enzyme molecules, which supported the formation of enzyme-substrate complex and improved the adsorption of cellulase on insoluble cellulose. When the cellulase solution with the soluble protein concentration of 1.68 mg/mL was sonicated for 80 sec with the ultrasonic intensity of 6 W/mL, its catalytic activity was enhanced 18.7% against carboxymethylcellulose (CMC) and 39.4% against Whatman No. 1 filter paper. For higher ultrasonic intensity and longer treatment time, the cellulolytic activity reduced sharply.

Introduction

For many years, ultrasound has been employed as a method of enzyme inhibition but some works stated that ultrasound did not inactivate all enzymes at mild temperature condition (Feng *et al*., 2011). Although high ultrasonic intensity or long sonication time can denature enzymes, it has been shown that using ultrasonic treatment at appropriate frequencies and intensity levels can lead to an increase in enzyme activity (Mason *et al*., 1996). Consequently, ultrasound has been used in some enzymatic reactions to increase the yield or to reduce the biocatalytic time (Shah and Gupta, 2008). Cellulase preparations have extensively been used in food processing and biodegradation (Zhang *et al*., 2006). Some researchers confirmed that ultrasound could increase cellulolytic activity (Aliyu and Hepher, 2000; Li *et al*., 2005).

Crystalline cellulose is a tight fiber bundle, so the combination between enzyme and substrate occurs only on certain specific areas of the substrate surface where the enzyme can access (Henrissat *et al*., 1985). Using ultrasound as a pretreatment of substrate before cellulolytic reaction resulted in fragmentation of cellulose matrix, especially, crystalline areas. As a result, more single chains and reducing ends were released and became more accessible for enzyme to attack (Mason *et al*., 1996; Shah and Gupta, 2008; Aliyu and Hepher, 2000; Cavaco-Paulo *et al*., 1997). Pretreatment of the cellulose fibers with ultrasound prior to enzyme incubation further improved the reaction rate (Imai *et al*., 2004).

Beside the effect of substrate fragmentation, micro-mixing is another physical effect that enhances enzyme activity when ultrasound is applied to the enzymatic reactions. Ultrasonic waves at different frequencies can overcome the restricted diffusion of substrate to enzymes by inducing fluid motions to increase mass transfer, ensuring substrate availability at the enzyme and removal of products from the enzymes (Shah and Gupta, 2008). This increase in mass transfer is either obtained at lower frequencies by the generation of cavitation or at higher frequencies through the generation of turbulent microstreams and the induction of a turbulent channel flow in narrow porous structures (Sinisterra, 1992; Francis *et al*., 1995; Bengtsson and Laurell, 2004). Li *et al*. (2005) supposed that cellulase molecules, especially cellobiohydrolase and endoglucanase, diffusing into the inner layers of the fiber matrix were inactivated by

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the local concentration of released oligosaccharides. Shear stress generated by ultrasound continuously "loosened" the cellulose matrix in hydrolysis process. This effect not only provided larger surface area of substrate for cellulases but also reactivated enzyme molecules which were trapped in the inner layers of cellulose matrix (Li *et al*., 2005).

The effect of ultrasound on cellulase molecules, however, has not been clear yet. Sonication of enzyme-substrate mixture could not answer to the question if ultrasound changes enzyme structural configuration and this modification affects catalytic activity of the examined enzyme or not. The aim of this study was to investigate the effects of ultrasonic pretreatment on the catalytic activity of cellulase complex. Cellulase preparation was firstly treated by ultrasound under different conditions. Then, the cellulolytic activity of the sonicated enzyme was determined against soluble and insoluble substrates. Sonication of cellulase preparation without presence of substrate enabled us to learn about the interaction between ultrasound and enzyme cellulase more thoroughly.

Materials and Methods

Materials

Enzyme source: Commercial cellulase preparation Celluclast 1.5L from *Trichoderma reesei* (Novo Nordisk, Denmark, Cat. No. C2730) was used in this study. This preparation contains mostly endoglucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91) (Ramos et al., 1999). The cellulase activity in Celluclast 1.5L was approximately 1,500 Novo Carboxymethylcellulose Unit per gram (NCU/g) against CMC and 80 Filter Paper Unit per gram (FPU/g) against filter paper. 1 NCU is the amount of enzyme which, under standard conditions degrades CMC to reducing carbohydrates with a reduction power corresponding to 1 µmol glucose per minute. 1 FPU is the amount of enzyme which, under standard conditions degrades filter paper to reducing carbohydrates equivalent to 1 µmol glucose per minute. Cellulosic substrates: CMC (Fluka, Germany, Product No. 21902) and Whatman No. 1 filter paper (Whatman, UK) were used. Analytical chemicals: 3,5-dinitrosalicylic acid (DNS) and standard glucose were obtained from Merck (Germany), bovine serum albumin was purchased from Sigma (USA, Product No. A7030).

Experimental methods

Effect of ultrasonic intensity on cellulase activity

Celluclast 1.5L was diluted in 50 mM citrate buffer (pH 4.8) with the dilution factor of 100 times (1 mL of diluted enzyme solution contained 1.68 mg of soluble protein). Each 50 mL of the diluted enzyme solution was added into a 100 mL beaker and subsequently sonicated for 40 sec. Ultrasonic intensity was varied from 0 to 12 W/mL.

Effect of sonication time on cellulase activity

Celluclast 1.5L was diluted in 50 mM citrate buffer (pH 4.8) with the dilution factor of 100 times (1 mL of diluted enzyme solution contained 1.68 mg of soluble protein). 50 mL of the enzyme solution was added into a 100 mL beaker and then sonicated at ultrasonic intensity of 6 W/mL. Treatment time was varied from 0 to 160 sec.

Effect of initial soluble protein concentration of the cellulase preparation in the ultrasonic treatment on cellulase activity

Celluclast 1.5L was diluted in 50 mM citrate buffer (pH 4.8); the soluble protein concentration of cellulase preparation was ranged from 0.84 to 3.37 mg/mL. Each 50 mL of the diluted enzyme solution was added into a 100 mL beaker and then sonicated at ultrasonic intensity of 6 W/mL for 80 sec. Control sample was the unsonicated cellulase preparation with the dilution factor of 100 times (1 mL of the diluted enzyme solution contained 1.68 mg of soluble protein).

In all experiments, the diluted enzyme solutions were directly treated by a horn type ultrasonic probe (Mode: VC 750, Sonics & Materials Inc., Newtown, USA). This equipment operated at frequency of 20 kHz with the maximum ultrasonic power of 750 W. During the ultrasonic treatment, the beakers were placed in a water bath with chilled water to prevent excessive temperature rise. The temperature of each sample was maintained in the range from 10°C to 30°C during the treatment. After sonication, each enzyme solution was sampled for determination of cellulase activity against CMC and filter paper.

Analytical methods

Cellulase activity assays

The activity profile of the cellulase preparation was determined by using Whatman No. 1 filter paper and CMC. Reducing sugars released was monitored by the spectrophotometric method using DNS reagent. This method was proposed by Ghose (1987).

Soluble protein

Soluble protein of the cellulase solution was determined by the Bradford method, using bovine serum albumin as standard (Bradford, 1976).

Protein components of cellulase preparation

Electrophoresis was used in this study to

determine protein components of the sonicated and unsonicated cellulase preparation. Sodium dodecyl sulfate (SDS) was used to disrupt the secondary, tertiary and quaternary structure of protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol assisted the protein denaturation by reducing all disulfide bonds. This analytical procedure was proposed by Laemmli (1970).

Absorbance spectrum of protein

Before and after sonication treatment, the cellulase preparation was scanned in the wavelength from 190 nm to 380 nm, with a step size of 2 nm on Thermo Genesys 6 UV-Vis spectrometer (White bear Photonics, LLC; Minnesota; USA). All measurements were performed at 30ºC in a quartz cuvette according to the analytical procedure proposed by Greenfield (2004).

Statistical analysis

All experiments were performed in triplicate. Mean values were considered significantly different by Multiple range tests with $p < 0.05$. Analysis of variance (ANOVA) was realized using the software Statgraphics plus, version 7.0.

Results and Discussion

Influence of ultrasonic intensity on cellulase activity

As shown in Figure 1, when the ultrasonic intensity augmented from 0 to 6 W/mL, the cellulase activity against CMC and filter paper increased 14.4% and 28.5%, respectively. Enzyme binding to cellulose is considered as a prerequisite step before substrate degradation (Beldman *et al*., 1987). Walker and Wilson (1991) supposed that different cellulase proteins had different hydrolytic ability. This related to the difference in structure of cellulose binding domains of the enzyme molecules (Walker and Wilson, 1991). Ultrasound can break weak linkages like hydrogen bonds or Van der Waals interactions, and induce conformational changes in protein structure (Braginskaya *et al*., 1990; Gebicka and Gekicki, 1997). We supposed that cellulose binding domains of cellulase were changed under the effects of low intensity ultrasound, and became more flexible to link with cellulose. Those slight changes in spatial structure of enzyme molecules might also facilitate the formation of enzyme-substrate complex. That explained the increase in hydrolytic activity against both substrates.

According to Nidetzki *et al*. (1994), the adsorption capacity of cellulase on insoluble substrate was

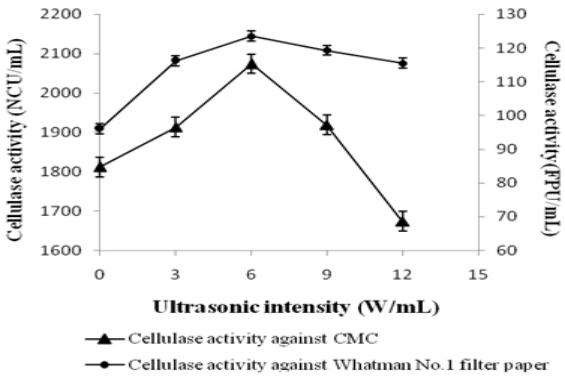

Figure 1. Influence of ultrasonic intensity on cellulase activity

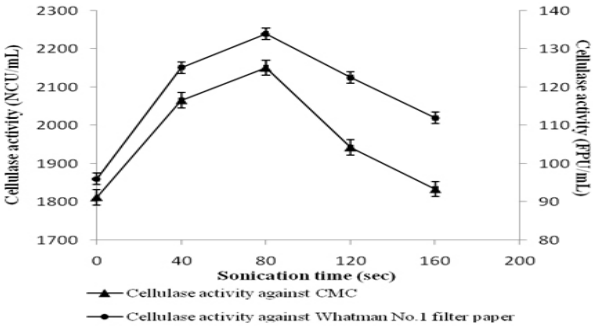

Figure 2. Influence of sonication time on cellulase activity

accompanied by some slight changes in enzyme structure (Nidetzki *et al*., 1994). Adsorption capacity of the cellulase with modified binding domains on insoluble cellulose was better than that of original cellulase; it explained the reason why increase in cellulase activity against filter paper substrate was higher than that against CMC substrate.

The activity of cellulase preparation decreased when the ultrasonic intensity was more than 6 W/mL (Figure 1). Cellulase activity against CMC of the sample sonicated with intensity of 12 W/mL reduced 19.3% in comparison with that of the sample treated with intensity of 6 W/mL. However, when filter paper was used as substrate, the decrease was just 6.5%.

High intensity ultrasound enhanced cavitation, created shock waves that caused strong shear in the adjacent liquid. Under these extreme conditions, sonication could cause great damage in the polypeptide chains, leading to the modification of the secondary and tertiary structure of the protein. In addition, the extreme localized increase in pressure and temperature also led to a generation of high energy intermediates such as hydroxyl and hydrogen-free radicals. The free radicals formed might react with some amino acid residues that participate in substrate binding domain or catalytic domain of the biocatalyst. Therefore, enzyme activity would change as a consequence (Feng *et al*., 2011).

In this experiment, ultrasonic intensity above

6 W/mL led to disadvantageous changes of spatial configuration of the cellulase molecules, hence limited the formation of enzyme-substrate complex. As a result, cellulase activity against CMC of the 12 W/mL sonicated sample reduced 7.6% compared with that of the untreated sample. However, for insoluble substrate (filter paper), the spatial configuration changes of the enzyme molecules still supported the biocatalyst to adsorb on insoluble cellulose. That resulted in a higher activity against filter paper of the sample treated with ultrasonic intensity of 12 W/mL in comparison with the catalytic activity of the untreated sample.

Influence of sonication time on cellulase activity

When sonication time prolonged from 0 to 80 sec, the cellulase activity against CMC and Whatman No.1 filter paper increased 18.7% and 39.4%, respectively (Figure 3). If the treatment time was extended to 120 and 160 sec, the enzyme activity decreased. However, the cellulase activity against filter paper reduced less than that against CMC. The enzyme activity against filter paper of the 160-sec treated sample was still 16.5% higher than that of the untreated sample while the enzyme activity against CMC of the two samples above were not significantly different ($p > 0.05$).

Soluble protein content of cellulase solutions before ultrasonic treatment, after ultrasonic treatment with intensity of 6 W/mL for 80 sec and after ultrasonic treatment with intensity of 6 W/mL for 160 sec was 1.68, 1.71, 1.67 mg/mL, respectively. There was no significant difference in soluble protein content of the samples before and after sonication. This result eliminated the possibility that the increase or decrease in cellulase activity were the consequence of the change of total soluble protein content in the preparations, including both enzyme and non-enzyme proteins.

The small bubbles simply dissolve away. But if sonication time is long enough, larger bubbles will grow over a very large number of acoustic cycles in a process referred to as rectified diffusion or form through coalescence of smaller bubbles (Lee *et al*., 2005). When collapsing, these large bubbles will generate fluctuations in velocity and pressure in the surrounding fluid (Ashokkumar *et al*., 2007). It can be supposed that when sonication was carried out during a proper time, most of enzyme molecules in the solution were slightly modified in spatial structure; and the activity of cellulase solution was enhanced. However, further increase in sonication time caused negative effects because explosion of too many microbubbles in the cellulase solution damaged substrate binding domain or catalytic domain

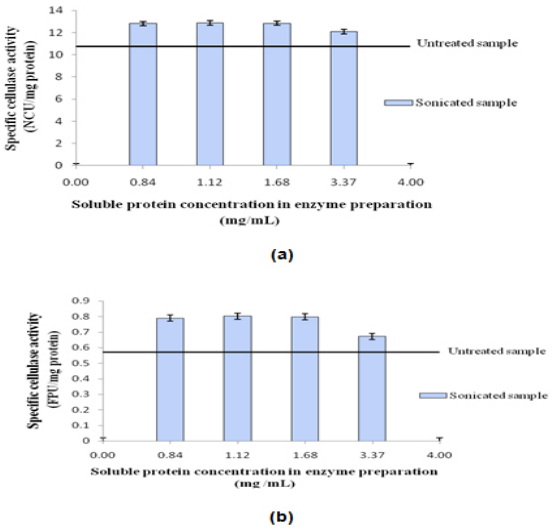

Figure 3. Influence of initial soluble protein concentration in the sonicated enzyme preparation on specific cellulase activity: (a) Specific activity against CMC, (b) Specific activity against Whatman No.1 filter paper

structure of the enzyme molecules or generated high concentration of free radicals which might react with some amino acid residues of the biocatalyst and reduce enzyme stability. Similar result was also observed by Ertugay *et al*. (2003) who stated that prolonging sonication time triggered the ultrasound inactivation effect, and caused reduction in catalytic activity of lactoperoxidase in milk (Ertugay *et al*., 2003).

Influence of initial protein concentration of the enzyme preparation in the ultrasonic treatment on cellulase activity

There was no significant difference in specific cellulase activity against CMC and filter paper ($p > 0.05$) of the samples with initial soluble protein content of 0.84, 1.12, and 1.68 mg/mL. The sample with soluble protein content of 3.37 mg/mL had the lowest specific activity among the four sonicated samples. However, its specific activity was still higher than that of the untreated sample. Therefore, the difference in initial concentration of soluble protein in the samples generated different changes in spatial configuration of protein molecules, although the ultrasonic treatment conditions were similar.

It should be noted that the cellulase activity against filter paper of a sonicated enzyme preparation always increased in higher level or decreased in lower level in comparison with the cellulase activity against CMC of the same sample. It is apparent that cellulolytic activity is closely linked to the structural characteristics of the substrates (Mansfield *et al*., 1999). CMC has a generally amorphous structure, whereas Whatman No.1 filter paper has 40% crystalline structure with parallel cellulose

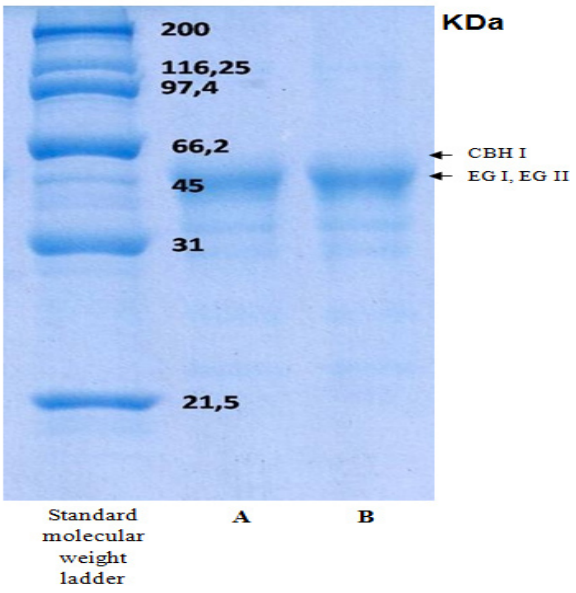

Figure 4. Electrophoresis analysis of cellulase preparation before and after sonication. (A) Cellulase preparation before ultrasonic treatment; (B) Cellulase preparation after ultrasonic treatment with intensity of 6 W/mL for 80 sec; CBH I: Cellobiohydrolase I; EG I, II: Endoglucanase I, II

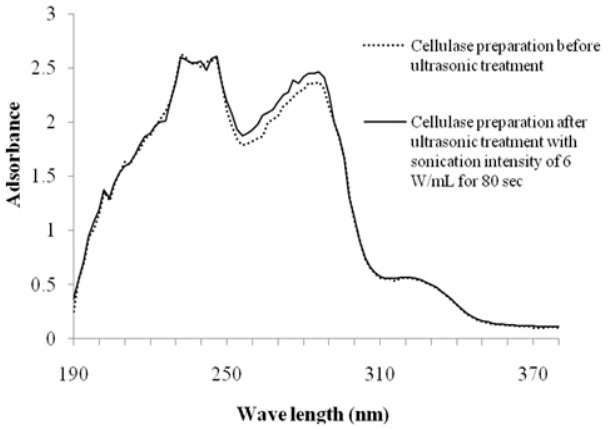

Figure 5. UV-vis spectrum of enzyme samples before and after sonication

chains tightly held together by hydrogen bonding (Zhang *et al*., 2006). Many researchers affirmed that crystalline parts of the substrate made the cellulose less accessible to the enzymes; this could explain why crystalline substrates were more resistant to enzymatic degradation than amorphous substrates (Zhang *et al*., 2006; Aliyu and Hepher, 2000; Imai *et al*., 2004; Ramos *et al*., 1999; Walker and Wilson, 1991; Fan *et al*., 1980).

According to Klyosov (1990), the rate of crystalline cellulose hydrolysis by endoglucanase was directly proportional to enzyme activity and the ratio of the concentration of enzyme adsorbed onto the substrate and the concentration of free enzyme in the solution, but the reaction rate of amorphous cellulose hydrolysis by endoglucanase only depended on the enzyme activity (Klyosov, 1990).

The results in two sections about the influence of ultrasonic intensity and sonication time on cellulase activity showed that under certain sonication conditions, adsorption of the sonicated enzyme on the insoluble cellulose was significantly improved. This led to increased penetration of enzymes into the interfibrillar space and enhanced the cellulase activity against filter paper (Gama *et al*., 1997). On the contrary, amorphous cellulose or soluble substrate had many positions on the molecule readily accessible to enzyme attack. So, the adsorption ability of cellulase on CMC became less important for improvement in catalytic activity (Henrissat *et al*., 1985).

In order to clearly understand how ultrasonic pretreatment affected catalytic activity of the cellulase complex, protein components and absorbance spectrum of cellulase solutions were examined. The results are presented in Figure 4 and 5. Electrophoresis analysis stated that protein components of cellulase preparation before sonication (sample A) and those after sonication with intensity of 6 W/mL for 80 sec (sample B) were absolutely similar (Figure 4). This result proved that ultrasound did not cause any dramatic damage of cellulase molecules that made them break down or change their primary structure. The changes might occur in spatial structure of cellulase molecules. The UV-visible spectra of cellulase preparation before and after sonication show similar peaks from 190 to 340 nm wavelength (Figure 5). No apparent differences exist between these two spectra. Consequently, this ultrasonic treatment did not cause significant modification of protein structure. The similarity of the far-UV spectra (190-250 nm) showed that ultrasound did not affect the secondary structure of cellulase molecules (Sinisterra, 1992). However, a slight difference was observed in 250-290 nm region. Signals in the near-UV spectral region are diagnostic of the microenvironments of aromatic residues (phenylalanine, tyrosine and tryptophan). Signals in the 250–270 nm region are attributable to phenylalanine, and those in the 270–290 nm region are attributable to tyrosine (Kelly *et a*l., 2005). The spectra showed that ultrasonic pre-irradiation led to perturbation of tyrosine and tryptophan environments, which caused a slight modification of protein tertiary structure (Kelly *et al*., 2005). According to Renikainen *et al*. (1992), hydrogen bonds probably accounted mainly for the interactions between the β-1,4-glycosidic chains and the cellulose binding domain (Kyriacou *et al*., 1989), and this seemed to be mediated mostly by tyrosine (Teeri *et al*., 1998) and tryptophan residues (Reinikainen *et al*., 1992).

The results in Figure 4 and 5 confirm that the

sonication conditions with ultrasonic intensity of 6 W/mL for 80 sec for cellulase pretreatment in this study did not make enzyme irreversibly denatured but slightly changed the spatial configuration of the biocatalyst and increased cellulolytic activity.

Conclusions

Under certain conditions, sonication did not denature the enzyme but improved the activity of cellulase preparation. Ultrasonic intensity, sonication time and initial protein concentration in the cellulase solution affected the catalytic activity against both soluble and insoluble cellulose. Pre-irradiation of cellulase preparation before use was a potential treatment for improvement in cellulase activity.

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