Cellulase Activity on Thin Films of Cellulose by QCM and SPR

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Abstract

We monitored the enzymatic hydrolysis on thin films of cellulose, in situ and real time by using a piezoelectric sensing device (Quartz Crystal Microbalance, QCM) and Surface Plasmon Resonance (SPR). Cellulose thin films were deposited on piezoelectric resonators using spin coating technique. Films of different crystallinity were also prepared by using self assembly of cellulose-thiol derivatives on gold substrates. By using QCM and SPR we elucidated the role of hydration water in view of the dynamics of enzyme binding and substrate degradation. We focused on the activity of isolated endoglucanases as compared to multi-component commercial enzyme mix. The activity of each kind of cellulase was determined under different conditions of temperature, pH and enzyme dose.

Introduction

Many research efforts have been dedicated during last decades to fully understand the interaction between enzymes and natural substrates such as lignocellulosic materials. Research has been carried out to manipulate and exploit enzymatic hydrolysis with the aim of producing fermentable sugars mainly driven by concerns over the escalating cost and reduced availability of fossil fuels. Such interest in cellulose conversion to energy and chemicals is not surprising since cellulose is the most abundant carbohydrate in the biosphere. Enzymes are also widely used in other industrial activities including wood pulping (1) and delignification (2); cellulose pulp bleaching (3,4,5); textile printing (6) and cotton pre-treatment (7); treatment of waste liquid effluents and detergency (8).

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1. Zhao J, Li XZ, Qu YB, et al. Alkaline peroxide mechanical pulping of wheat straw with enzyme treatment. Applied Biochemistry and Biotechnology, 2004, 112 (1), 13-23.

2. Costa SA, Goncalves AR, Esposito E. Ceriporiopsis subvermispora used in delignification of sugarcane bagasse prior to soda/anthraquinone pulping. Applied Biochemistry and Biotechnology, 2005, 121, 695-706.

3. Sigoillot C, Camarero S, Vidal T, et al. Comparison of different fungal enzymes for bleaching high-quality paper pulps. Journal of Biotechnology, 2005, 115 (4): 333-343.

4. Roncero MB, Torres AL, Colom JF, et al. The effect of xylanase on lignocellulosic components during the bleaching of wood pulps. Bioresource Technology, 2005, 96(1): 21-30.

5. Jimenez L, Ramos E, De la Torre MJ, et al. ECF and TCF bleaching methods as applied to abaca pulp. Afinidad, 2005, 62(515), 14-21.

6. Kokol V, Heine E. Effective textile printing using different enzyme systems. Coloration Technology, 2005, 121(4), 209-215.

Most of the current work dealing with enzymes is focused on their adsorption and formation of enzyme-substrate complexes and on the development of enzyme binding domains and specificity (9). Active areas of research also include the three-dimensional structure of cellulolytic enzymes and the mechanisms and kinetics of cellulose binding and hydrolysis (10).

Cellulases

In this area cellulases are the most important enzymes due to their ability to cleave cellulose polymers into smaller units or simple sugars via hydrolysis reactions. Different oxidoreductases such as laccases and peroxidases which are involved in lignin degradation and other hydrolytic enzymes such as xylanases are widely used in industrial processes, especially in pulp and paper industries.

Some microorganisms, as fungi and bacteria produce various types of cellulase components. In several industrial applications genetic material is transferred to a host organism for commercial production. Because the original organism evolved to metabolize a mixed substrate containing cellulose, hemicellulose, lignin and extractives, the lower cost commercial enzymes are often a mixture of different specific isozymes.

Commercial cellulase mixes usually contain one or more exoglucanases such as cellobiohydrolase (CBH) which will proceed from either the reducing end or non reducing end of the cellulose chain and produce a shortened chain and cellobiose. The cellulase mixture may also contain several endoglucanase (EGI, EGII, etc.), which cleave randomly the internal β-1,4-glycosidic bonds on the cellulose chain along its length to produce free chain ends that will be acted upon by exoglucanases. Most cellulases also contain both β-1,4-glycosidase, which hydrolyze cellobiose units to glucose monomers, and various hemicellulases, which may have side chain cleaving capabilities. For complete hydrolysis of cellulosic material the synergistic combination of endoglucanase, exoglucanase and β-1,4-glycosidase is required.

Most cellulolytic enzymes are composed of two functionally distinct domains. The cellulose-binding domain is responsible for the close association of the enzyme with solid cellulose through strong binding to crystalline cellulose. The catalytic domain is responsible for catalyzing cellulose hydrolysis through an acid mechanism.

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7. Pyc R, Sojka-Ledakowicz J, Bratkowska H. Biosynthesis of enzymes by Aspergillus niger IBT-90 and an evaluation of their a application in textile technologies. Fibres & Textiles in Eastern Europe, 2003, 11(4), 71-77.

8. Stebbing DW, Zhang X, Soong G, et al. Fungal enzyme treatment of newsprint mill white water: Impact on white water and paper properties. Journal of Pulp and Paper Science, 2005, 30(1), 3-8.

9. Meyer M, Wohlfahrt G, Knablein J, Schomburg D. Aspects of the mechanism of catalysis of glucose oxidase: A docking, molecular mechanics and quantum chemical study. Journal of Computer-aided Molecular Design, 1998, 12 (5), 425-440.

10. Woodward J, Affholter KA, Noles K, Troy NT, Gaslightwala SF Does cellobiohydrolaseii core protein from trichoderma-reesei disperse cellulose macrofibrils? Enzyme and Microbial Technology, 1992, 14 (8), 625-630.

Cellulosic Substrates

Cellulose fibers are a heterogeneous porous substrate with both external and internal surfaces. The external surface area is determined by the shape and size of the cellulosic fibers, while the internal surface area depends on their capillary structure. Cellulose fibers contain both amorphous and crystalline regions. Crystalline regions are considered to be more difficult to degrade than amorphous regions and therefore significantly affect the rate and extent of enzyme hydrolysis. Interactions of cellulases with cellulose play an important role in determining the efficiency of the enzymatic hydrolysis. Therefore, an understanding of enzyme interactions with the substrates is of great importance in processing wood and cellulosic fibers.

The rate of enzymatic hydrolysis and its yield are dependent on the adsorption of enzyme onto the substrate surface. The accessibility of cellulose to the cellulase seems to be controlled by the physicochemical properties of the substrate, the multiplicity of the cellulase complexes, and reaction parameters including those associated with mass transport and temperature. Various kinetic models have been developed to describe the hydrolysis rate of cellulase. The models are based on parameters such as the amount of adsorbed enzyme on the cellulosic surface, the structural characteristics of the substrate including pore size distribution, crystallinity index and specific surface area, and cellulase-cellulose adsorption rates.

EXPERIMENTAL

Materials and Methods

A multi-component commercial-available cellulase mix (was used. Mono-component cellulases and endoglucanases from *Trichoderma reesei* (Sigma-Aldrich) were employed in this investigation. Sodium bicarbonate, sodium hydroxide, hydrochloric acid and acetic acid (all from Fisher Scientific), sodium acetate trihydrate (Fluka) and tris (hydroxymethyl) aminomethane (Acros) were used in the preparation of the buffer solutions of pH 4.5, 7 and 10. Water was obtained from a Milli-Q® Gradient system (resistivity >18MΩ).

Cellulose thin films and related methods

We used cellulose thin films as models for cellulose. The process for preparing these surfaces starts with either silicon wafers, SPR gold slides or gold or silica QCM-D (Quartz Crystal Microbalance with Dissipation Monitoring) electrodes which are cleaned by standard chemical treatment and UV-ozone plasma. The QCM-D electrodes consisted of quartz crystals (Q-sense) coated with a conductive gold layer and a top 50-nm silica/gold layer.

Cellulose solution was prepared by dissolving micro-crystalline cellulose (MCC) in 50% wt water/N-Methylmorpholine-N-oxide (NMMO) at 115°C. Dimethyl Sulfoxide

(DMSO) was added to adjust the concentration of cellulose (0.05%) in the mixture. The cellulose solution was then spin coated (spin coater from Laurell Technologies model WS-400A-6NPP) on the substrates (wafer, SPR slides or QCM electrodes) at 5000 rpm. The substrate was removed from the spin coater and placed in a milli-Q water bath to precipitate the cellulose. The cellulose-coated substrate was then washed thoroughly with milli-Q water, dried in a vacuum oven at 80 ºC and stored at room temperature in a clean chamber for further use.

Enzyme/Cellulose Surface Interactions

Quartz Crystal Microbalance

A Quartz Crystal Microbalance with Dissipation monitoring, QCM-D (Q-sense D-300, Sweden) was used to study enzyme binding and activity on cellulose thin films deposited on quartz/gold electrodes which are coated with a 50 nm SiO2 layer. Temperature was controlled within ± 0.02 ºC of the respective set point via a Peltier element.

QCM-D consists of a thin plate of a piezoelectric quartz crystal, sandwiched between a pair of electrodes. It measures simultaneously changes in resonance frequency, f, and dissipation, D (the frictional and viscoelastic energy losses in the system), due to adsorption on a crystal surface. f is measured before disconnecting the driving oscillator, and D is obtained by disconnecting the driving field and recording the damped oscillating signal as its vibration amplitude decays exponentially.

Surface Plasmon Resonance

Surface Plasmon Resonance, SPR (SR7000 Reichert) was used to study enzyme binding and activity on cellulose thin films deposited on glass slides coated with a thin gold layer. SPR is an optical technique for determining refractive index changes at surfaces. This surface is typically an interface between a solid support and a liquid phase. Surface Plasmon Resonance offers a good opportunity to observe surface phenomena, including molecular binding in real-time.

RESULTS AND DISCUSSION

Enzymatic Degradation of Cellulose Films

Figure 1 depicts a typical plot of QCM frequency and dissipation signal for a cellulose film subject to enzyme treatment. The adsorption of the enzyme onto the cellulose surface is clearly shown in the form of a (transient) reduction in the QCM-D frequency. Enzyme binding occurs rather fast (less than 5 min in most cases). After binding, a reduction in mass of material on the surface is observed as judged by the increase in oscillation frequency. After some long time the frequency signal reaches a plateau which indicates no further change in the mass of the film.

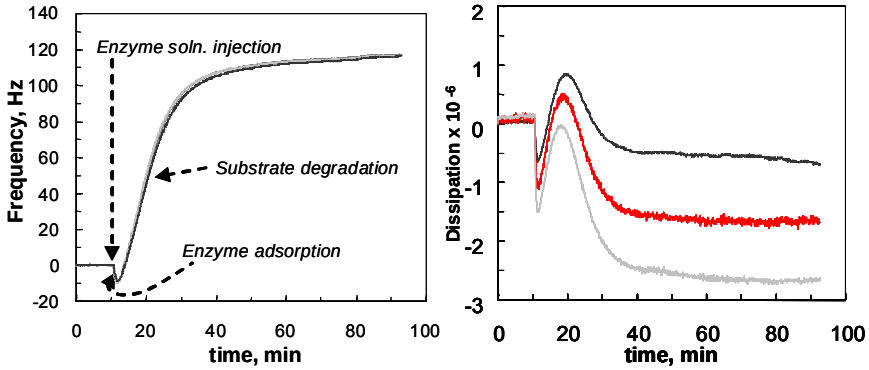

Figure 1. Change in frequency (left) and dissipation (right) by enzyme. The different lines represent the different overtones (or harmonics): f3/3 (black line), f5/5 (red) and f7/7 (light grey). The driving frequencies correspond to 15, 25 and 35 MHz for f3, f5 and f7, respectively.

Replacement of the enzyme solution by buffer solution (rinsing) did not produce any noticeable change in the frequency response. This indicates that there is no further change in the adsorbed mass and also that the possible effect of variations in bulk density and viscosity is negligible. The QCM dissipation seen in Fig. 1 (right) mirror the behavior observed for the frequency except for an initial dip after injecting enzyme solution (after 10 minutes operation) that we attribute to changes in temporal excess flow pressure on the crystal. Initially (< 10 min) the dissipation response for the buffer solution at the various overtones (D3, D5 and D7) are very similar. This reveals the existence of an initial thin, relatively rigid, film of cellulose. After about 10 min of enzyme solution injection (at ca. 20 min run time), the energy dissipation starts to decrease and more distinctive differences for the various overtones are observed. This indicates structural changes in the cellulose film upon enzyme attack.

Figure 2 illustrates a typical plot of SPR for a cellulose film subject to enzyme treatment. The adsorption of the enzyme onto the cellulose surface is clearly shown in an increase of the response measured (increase in Refractive Index Units, RIU). As detected with QCM, enzyme binding occurs rather fast. After binding, a reduction in mass of material on the surface is observed as indicated by the increase in the SPR response. After a certain time the plot reaches a plateau which indicates no further change in the mass of the sample. A second injection of enzyme was performed to ensure a complete degradation and depletion of the cellulose film.

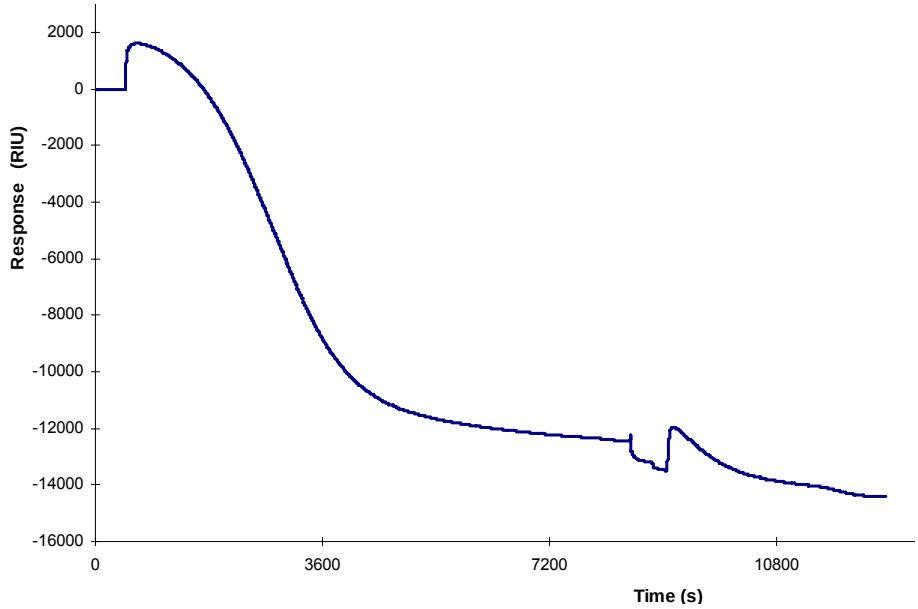

Figure 2. Change in Refractive Index Units (RIU) as the response of a SPR to endoglucanase binding and cellulose degradation

CONCLUSIONS

A piezoelectric and an optical sensing technique were used to investigate the activity of cellulase based on the change of the respective signals using a cellulose-coated sensor during enzymatic degradation. Both techniques demonstrated to be suitable tools to monitor enzyme activity on thin films of cellulose in-situ and real time. The initial slope of both instruments responses (time curves) is related to the initial reaction rates, and is useful to estimate enzyme performance under different conditions of temperature, pH and concentration. QCM-D and SPR are valuable tools to study the mechanisms involved in enzyme activity. The study of the same kind of substrate under the same conditions of use (i.e. pH, temperature, type of enzyme and concentration) with both techniques gives complementary information. The obtained results allow an interpretation of the mechanisms involved in the enzymatic degradation of cellulose.

Acknowledgements

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