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Enzymatic Polymerization to Cellulose by Crosslinked Enzyme Immobilized on Gold Solid Surface

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Mutant endoglucanase II was locally immobilized on a gold substrate by crosslinking, and enzymatic polymerization to cellulose was analyzed. The crosslinked enzyme showed an enhanced polymerization activity to produce cellulose with high crystallinity, which is due to the high local concentration of the enzyme on the substrate surface.

Cellulose is a renewable resource and useful in terms of green chemistry. Plants and microorganisms afford cellulose by using cellulose synthase complexes, which decide the intrinsic and physical properties of cellulose such as fibrous size (dimension), crystallinity, and tension strength. In vitro synthesis of cellulose has been accomplished by both organic chemical methods[1] and enzymatic polymerization.[2] However, cellulose synthesis under the control of physical properties remains to be challenged.

We have examined some mutant endoglucanase II (EGII) from *Trichoderma viride* having one or two active sites to analyze the enzymatic polymerization.[3] We found that the crystallinity of the produced cellulose was influenced by the enzyme structure and immobilization on a solid substrate (monolayer).[3d] Moreover, the fibrous morphology of cellulose was attained by using crosslinked enzymes.[3e] In the present study, we immobilized a mutant EGII on a gold surface to be locally immobilized densely on the surface by crosslinking, and the polymerization activity was analyzed.

The mutant enzyme, $EGII_{core2H}$, possesses sequentially two catalytic core domains with two His-tags (hexameric histidine) on the N- and C-terminals. $EGII_{core2H}$ was shown to be immobilized on a Ni-modified surface via the two His-tag moieties.[3c] $EGII_{core2H}$ was prepared as a secreted enzyme from transformed yeast and purified by metal-immobilized affinity chromatography and gel permeation chromatography. The purity of the prepared $EGII_{core2H}$ was confirmed by SDS-PAGE. A hetero-bifunctional molecule having a nitrilotriacetic acid (NTA) moiety and an OSu (*N*-hydroxysuccinimide) moiety for crosslinking two enzymes was designed (Figure 1). NTA is an well-known ligand for oligomeric histidine residues with an intervening Ni ion.[4] OSu is used for covalent binding to the enzyme via a Lys residue.[5] The synthesis of NTA–OSu was confirmed by ^{1}H NMR. The crosslinked $EGII_{core2H}$ on a gold surface was prepared by the following two different routes, and the immobilization process was monitored in real time by SPR measurements.[3c] One way is two-step crosslinking where $EGII_{core2H}$ was treated in advance by NTA–OSu, and the obtained NTA-modified $EGII_{core2H}$ was immobilized on the Ni-chelated NTA surface (Figure 1(i)). The NTA-modified $EGII_{core2H}$ was chelated with Ni ion (Figure 1(ii)), followed by further binding of NTA-modified $EGII_{core2H}$ (Figure 1(iii)). The

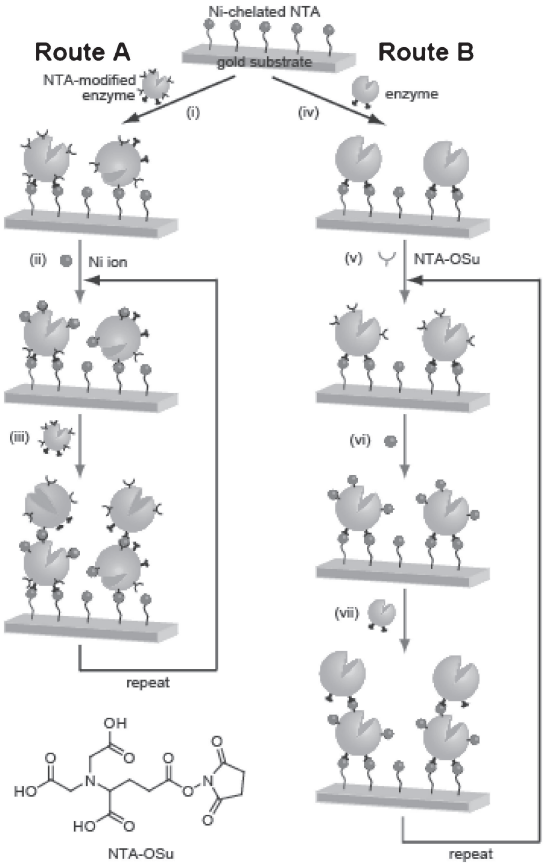

Figure 1. Schematic illustration of preparation of the crosslinked enzyme on the solid substrate and chemical structure of NTA–OSu.

cycles of (ii) and (iii) were repeated (Figure 1, route A). In the other way, $EGII_{core2H}$ was modified with NTA–OSu in situ on a solid surface (Figure 1, route B). $EGII_{core2H}$ was immobilized on the Ni-chelated NTA surface (Figure 1(iv)). Subsequently, NTA–OSu solution was added to introduce NTA moieties to the immobilized $EGII_{core2H}$ (Figure 1(v)). Ni ions were chelated to the NTA moieties (Figure 1(vi)), followed by crosslinking with newly added $EGII_{core2H}$ (Figure 1(vii)). The cycles of (v) to (vii) were repeated to obtain the crosslinked $EGII_{core2H}$ on a solid substrate. The incubation time of the enzyme was set for 10 min, and the cycle was repeated ten times in both routes.

Figure 2 shows the results of SPR measurement from the second to tenth enzyme incubation cycles.[9] The amount of immobilized enzyme at first cycle was set as 100 arbitrary units, and the increase of the immobilized amount at each cycle was relatively compared. In all the cycles, the increase of the amount

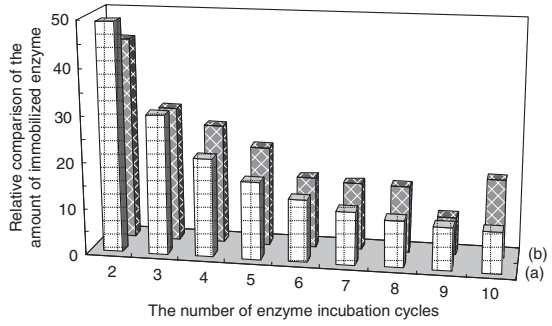

Figure 2. Results of SPR measurements of the immobilization behavior using NTA-modified $EGII_{core2H}$ (route A, a) and $EGII_{core2H}$ (route B, b) as enzyme from second to tenth incubation cycles.

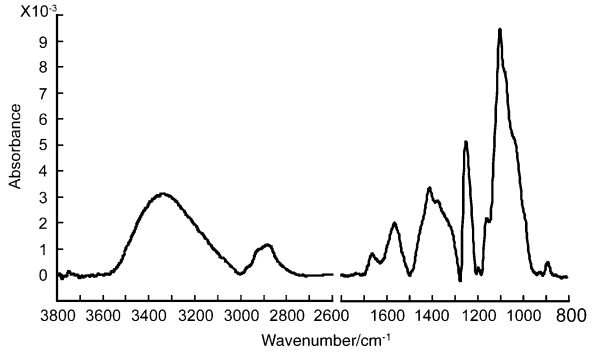

Figure 3. FTIR-RAS spectrum of the product on the surface catalyzed by crosslinked $EGII_{core2H}$. The spectrum before polymerization was subtracted.

of immobilized enzyme on the substrate was observed as a result of crosslinking of $EGII_{core2H}$ on the solid surface. However, the immobilized amount decreases with the cycle time, suggesting that the enzyme layer should have many defects. Without the crosslinking molecule (NTA–OSu), the increase of $EGII_{core2H}$ on the surface was less than those attained by the present two methods, indicating that $EGII_{core2H}$ enzymes were indeed crosslinked on the substrate via NTA–Ni–His-tag chelation in both routes of A and B. Immobilization by the route B made more $EGII_{core2H}$ immobilized on the substrate than the route A (Figure 2).

Enzymatic polymerization with using the crosslinked $EGII_{core2H}$ on the substrate was analyzed by FTIR-reflection absorption spectroscopy (RAS). For the measurements, we needed a large substrate, which required a large amount of the mutant enzyme for preparation. Because of the limitation of the obtainable mutant, we prepared the enzyme layer by route B with increase of the enzyme incubation time up to 3 h from 10 min in the case of the SPR measurements, and the immobilization cycle was repeated four times. The prepared substrate was incubated with 25 mM β-cellobiosyl fluoride in acetonitrile/acetate buffer (50 mM, pH 5.0) (3/1 v/v) at 30 °C. After 10 min polymerization, the substrate surface was analyzed by FTIR-RAS measurement.[9] All peaks of FTIR-RAS spectrum were assigned to characteristic peaks of cellulose (Figure 3),[6] indicating that the crosslinked enzyme on the substrate retained polymerization activity. The yield of cellulose using the crosslinked $EGII_{core2H}$ was higher than that using the monolayer of $EGII_{core2H}$, which was prepared without using any crosslinking molecules.[3d] It should be pointed out the density of the crosslinked $EGII_{core2H}$ on the gold surface was one third of the $EGII_{core2H}$ monolayer. The difference in the immobilized amounts between the two preparation methods can be partly explained by the different incubation times (12 h in total for the crosslinked $EGII_{core2H}$ and 24 h for the monolayer). The polymerization activity of the crosslinked $EGII_{core2H}$ apparently increases from the immobilized $EGII_{core2H}$ without crosslinking.

The degree of crystallinity of cellulose was calculated on the basis of the fingerprint region of IR spectra by the lateral order index (LOI) (absorbance ratio of CH_2 symmetric bending at 1415 cm^{-1}/C–O–C stretching at 895 cm^{-1})[7] and the total crystallinity index (TCI) (CH bending at 1379 cm^{-1}/CH stretching at 2883 cm^{-1}).[8] Both indices of the synthesized cellulose with using the crosslinked $EGII_{core2H}$ (LOI, 6.11; TCI, 2.62) are much higher than those by the monolayer of $EGII_{core2H}$ after 15 min polymerization (LOI, 2.33; TCI, 1.14). Taken together, the crosslinked $EGII_{core2H}$ efficiently affords cellulose with high crystallinity.

We consider the high efficiency in synthesizing crystalline cellulose as a result of local high concentration of $EGII_{core2H}$ by crosslinking. In the case of the crosslinked $EGII_{core2H}$, the chain-growing sites should be nearby to facilitate association of the produced cellulose chains, leading to high crystallinity of the synthesized cellulose. Association of the growing chains may also promote dissociation of cellulose from the enzymes, resulting in enhancement of polymerization activity. However, we should find optimized conditions to prepare more densely packed layers of $EGII_{core2H}$ to understand the unique properties of the crosslinked $EGII_{core2H}$.

In conclusion, the crosslinked enzyme on the solid substrate was prepared by the hetero-bifunctional molecule via NTA–Ni–His-tag linkage. As a result, the polymerization activity was increased, and highly crystalline cellulose was synthesized by this crosslinked enzyme on the substrate because of high local concentration of $EGII_{core2H}$.

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