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ARTICLE TYPE

Preparation of fibrous cellulose by enzymatic polymerization using cross-linked mutant endoglucanase II†

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A cross-linked mutant endoglucanase II was prepared for enzymatic polymerization to cellulose. The cross-linked enzyme was composed of three mutant enzymes showing polymerization activity. A characteristic point of the polymerization with this cross-linked enzyme was formation of cellulose fibriles in contrast to plate-like crystals obtained by using a free enzyme.

Cellulose is the most abundant polysaccharide on the earth. In nature, cellulose is biosynthesized by cellulose synthase in land plants, algae, and microorganisms using uridin-5'-diphospho (UDP)-glucose as substrate.[1] Cellulose synthase is classified as glycosyltransferase, and forms a complex named terminal complex (TC).[2] One TC subunit is considered to be composed of 3–10 cellulose synthases.[3] And these single TC subunits are arranged in characteristic shapes depending on their origins such as a linear type or a cyclic type (rosette) with single or multiple rows.[4] Moreover, discriminative cellulase complex with enormously high molecular weight was found in some cellulolytic anaerobic bacteria. This macromolecular complex is named as cellulosome[5] and shows tremendously higher hydrolysis activity comparing with cellulase (1–2 order) because of multi-site interaction of the cellulosome and a substrate due to proximity of multiple enzymes.[6]

The morphology of biosynthesized cellulose by these cellulose synthase is microfibril. The molecular packing in crystals and the crystalline form are considered to be determined by the spatial arrangements of the enzymes in TCs. The size of microfibril is decided by the configuration of cellulose synthase, and larger TC is known to produce larger width microfibrils.[7] In terms of crystalline form, linear type TCs mainly synthesize triclinic crystalline cellulose, whilst the rosette type TCs produce largely monoclinic crystalline cellulose.[8] On the other hand, in the case of *in vitro* cellulose syntheses, the observed morphologies are mostly spherical and lamellar.[9] Recently, productions of fibrous cellulose or crystalline cellulose were reported.[10] However, they were synthesized from wood fiber or switchgrass by enzymatic hydorolysis.

We have been studying on the properties of synthetic cellulose obtained by enzymatic polymerization[11] using the mutant endoglucanase II (EGII) from filamentous fungi *Trichoderma viride* composed of only catalytic core domain.[12] It is reported that the increase of the number of the catalytic core domain in mutant EGII from one to two, both hydrolysis and polymerization activities per a catalytic core domain is increased.[12b] Moreover, the crystallinity of synthetic cellulose became higher by the increase of the number of the catalytic core domain.[12b] Therefore, the coexistance of the catalytic core domains in close proximity seems to be a key factor for the activity of enzymatic polymerzation and the morphology of the product by the *in vitro* synthesis. It is thus expected that the morphology of synthetic cellulose can be controlled by the arrangement of the catalytic core domains in the polymerization system, which is designed in resemblance to the *in vivo* sythesis of cellulose by cellulose synthase complex.

In the present study, mutant EGIIs were cross-linked to assemble, and the enzymatic polymerization to cellulose using the cross-linked EGII as a catalyst was investigated. The cross-linking molecule used is bisNTA which has two nitrilotriacetic acid (NTA) moieties on both terminals of

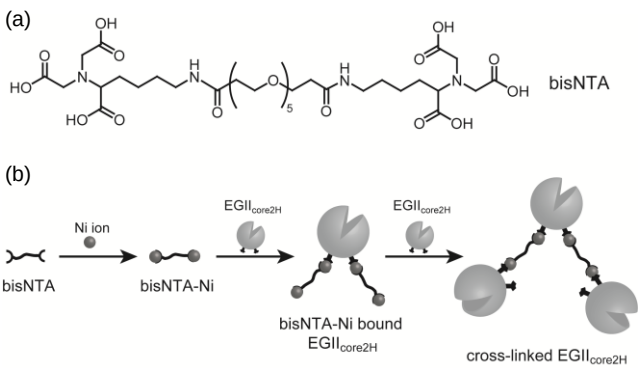

Fig. 1 (a) Chemical structure of bisNTA. (b) Schematic illustration of cross-linking of EGII$_{core2H}$.

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polyethylene oxide (PEO) (Fig. 1a). The NTA moiety is one of the most utilized ligand which is known to interact with oligo histidine residues (His-tag) through transition metal ion such as Ni.[13] BisNTA was synthesized by conventional organic chemical reactions and confirmed by ^{1}H NMR. Mutant EGII used for cross-linking is $EGII_{core2H}$ which is composed of two sequentially aligned catalytic core domains with two His-tags (hexameric histidine residues) on both chain terminals. Because of these two His-tags, one $EGII_{core2H}$ is able to bind to two other $EGII_{core2H}$s. $EGII_{core2H}$ was obtained as a secreted protein from the transformed yeast cell bearing plasmid encoding $EGII_{core2H}$ and purified by a metal immobilized affinity chromatography (Ni-NTA agarose beads) and a gel-permeation chromatography (Sephacryl S-200) as described before.[12b] Both molecular weight and purity of the purified $EGII_{core2H}$ were confirmed by SDS-PAGE.

Cross-linking of $EGII_{core2H}$ was performed as follows (Fig. 1b). First, bis-NTA was chelated with Ni ion (bisNTA-Ni). Then, an excess amount of bisNTA-Ni was incubated with $EGII_{core2H}$ to obtain the bisNTA-Ni-bound $EGII_{core2H}$ in Fig. 1b. Another fresh $EGII_{core2H}$ was added to bind to the free Ni moiety of the bisNTA-Ni-bound $EGII_{core2H}$ to yield the cross-linked $EGII_{core2H}$ in Fig. 1b. The obtained products were analyzed by gel-permeation chromatography (Sepahcryl S-200). In the elution pattern, a broad peak with elution volume from 30 to 40 ml was observed in advance of the peak of the free $EGII_{core2H}$ (Fig. 2, a and b). These fractions showed absorption at 395 nm due to the presence of Ni ions, supporting formation of the cross-linked $EGII_{core2H}$ by the bisNTA-Ni molecules. In comparison with the elution pattern of catalase from bovine liver (Fig. 2c, molecular weight is 232 kDa),[14] the cross-linked $EGII_{core2H}$ is considered to be composed of at least three molecules of $EGII_{core2H}$ (the molecular weight of $EGII_{core2H}$ is 72.3 kDa). We repeated the cross-linking reactions to confirm the major product of the cross-linked $EGII_{core2H}$ composed of three molecules of $EGII_{core2H}$ (see supporting information). The fractions which eluted from 30 to 40 mL of elution volume were collected to eliminate non-cross-linked $EGII_{core2H}$ and concentrated by ultrafiltration. We used the partially purified cross-linked $EGII_{core2H}$ for the following polymerization experiments.

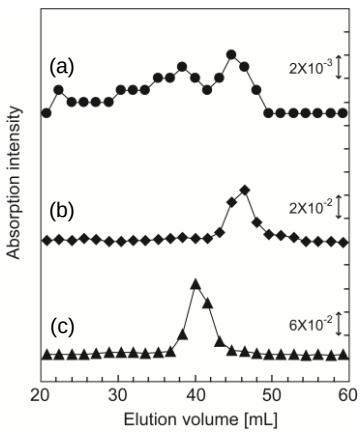

Fig. 2 Elution Pattern of gel-permeation chromatography of (a) cross-linked $EGII_{core2H}$, (b) non-cross-linked $EGII_{core2H}$ and (c) catalase from bovine liver.

Subsequently, enzymatic polymerization of β-cellobiosyl fluoride to synthesize cellulose was carried out in the acetonitrile/acetate buffer (50 mM, pH 5.0) = 3/1 v/v mixture solution using the cross-linked $EGII_{core2H}$ as a catalyst. After 0.5 h, white precipitation was suddenly observed in the reaction solution, whose appearance was different from the case of the artificial cellulose synthesis using the mutant EGII as a catalyst, where the polymerization solution became turbid gradually with time.[12a,b] The different precipitation behaviors between the two enzymes suggest different crystalization of the products.

After 48 h, the precipitates were collected, and the soluble fraction in acetonitrile was analyzed by MALDI-TOF MS. In Fig. 3, peaks equivalent to 8, 10 and 12 glucose units of cellulose with Na adducts with an equal interval of m/z 324 which corresponds to molecular weight of a cellobiose unit are observed (Fig. 3), indicating formation of cellulose. Even though the observed peaks were up to 12 glucose units, synthetic celluloses of higher molecular weight should be produced as an insoluble fraction in acetonitrile. Notably, the products are composed of glucosyl units of even numbers, which is reasonable for polymerization with using a disacchride monomer. However, enzymatic polymerization generally yielded cellulose of glycosyl units of odd numbers as well as even numbers, since the products were subjected to in situ hydrolysis by the enzyme as previously reported.[12a,15] In the present case, the synthesized cellulose was not ameanable to hydrolysis probably due to the fast

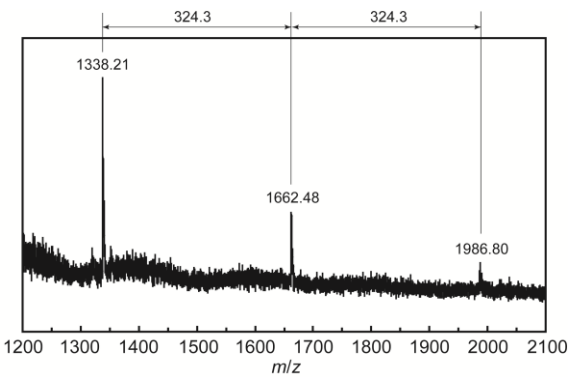

Fig. 3 MALDI-TOF MS spectrum of the product using cross-linked $EGII_{core2H}$.

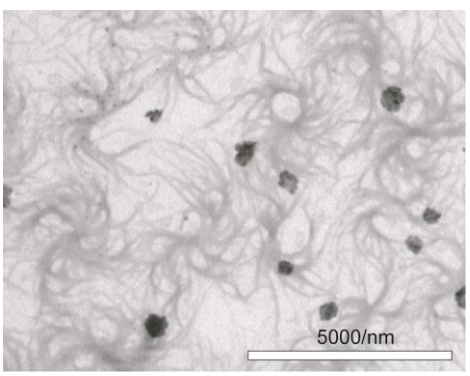

Fig. 4 TEM image of polymerization products from cross-linked $EGII_{core2H}$.

crystallization induced by the concentrated active sites in the cross-linked $EGII_{core2H}$, which does not bind to the crystalline cellulose because of lack of cellulose-binding domain.[11a] Taken together, the polymerization should proceed extremely fast and the insoluble part of synthetic cellulose with high molecular weight is produced at once as described above.

The morphology of synthesized cellulose was studied by TEM observation. In the present case, fibrous cellulose is formed (Fig. 4). The length of fibril is over μm length and its width is in the range of 50–120 nm. In our previous work, the synthetic cellulose by enzymatic polymerization yielded only plate-shape crystals.[12b] These different results seem to be the consequence of the different polymerizatoin processes. In the previous work utilizing mutant EGII as a catalyst, cellulose was synthesized progressively, so that the crystal formation of synthetic cellulose seemed to proceed under thermodynamical control, resulting in formation of plate-shape crystals. On the other hand, the cross-linked $EGII_{core2H}$ should generate multiple cellulose chains due to the dense active sites locally, cellulose chains may be assembled under kinetical control, leading to fibrile formation. Therefore, the configuration of enzyme *in vitro* synthesis to cellulose seems to influence the morphology of synthetic cellulose as is the case of *in vivo* synthesis. However, in depth analysis for formation of fibrous cellulose is now under way.

In summary, we prepared a cross-linked mutant endoglucanase II inspired from the natural cellulose synthase complex, and the effect of cross-linking of the enzymes on enzymatic polymerization was examined. The cross-linked enzyme showed higher polymerization activity than non-cross-linked enzyme due to the synergy effect of the local concentration of the enzymes. The morphology of synthetic cellulose catalyzed by the cross-linked mutant EGII was fibril. This result clearly indicates that the enzyme local arrangement of *in vitro* synthesis has potential to regulate enzyme activity as well as the morphology of synthetic cellulose.

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