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Class I hydrophobin fusion with cellulose binding domain for its soluble expression and facile purification

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ABSTRACT

Hydrophobins, highly surface-active proteins, have the ability to reverse surface hydrophobicity through self-assembly at the hydrophilic-hydrophobic interfaces. Their unique structure and interfacial activity lead hydrophobins to have potential applications on surface functional modifications. However, class I hydrophobins are prone to self-assemble into highly insoluble amyloid-like rodlets structure. Recombinant hydrophobins could be produced by *Escherichia coli* but generally as an insoluble inclusion body. To overcome this insoluble expression limitation, cellulose-binding domain (CBD) from *Clostridium thermocellum* was fused to the N-terminal of class I hydrophobin HGFI to enhance its soluble expression in *E. coli.* Approximately, 94% of expressed CBD fused HGFI (CBD-HGFI) was found as soluble protein. The fused CBD could also bind specifically onto bacterial cellulose (BC) nanofibrils produced by *Komagataeibacter xylinus* to facilitate rapid isolation and purification of HGFI from crude extract. Lysostaphin (Lst), known as GlyGly endopeptidase could successfully cleave the flexible linker $(GGGGS)_2$ between CBD and HGFI to recover HGFI from BC-bound CBD-HGFI. CBD-HGFI purified by immobilized metal-chelated affinity chromatography (IMAC) and Lst cleaved BC-CBD-HGFI still retained interfacial activity of hydrophobin and its effect on accelerating PETase hydrolysis against poly(ethylene terephthalate) (PET) fiber.

1. Introduction

Hydrophobins are a class of small (~100 amino acids) cysteine-rich proteins produced by filamentous fungi. These surface-active proteins have the unique characteristic of spontaneously self-assembling at hydrophilic-hydrophobic interfaces and then changing the hydrophobicity of the substrate [1,2]. Recently, hydrophobins have gained great attention among researchers for applications in food, medical, cosmetic, and biomaterial where interfaces need to be altered, bridged, or stabilized [3,4]. Two groups of hydrophobins have been identified based on their differences in hydropathy patterns and solubility. Class I hydrophobins assembly are extremely stable, forming amyloid-like rodlets structures which are soluble only in strong acids. Meanwhile, class II hydrophobins films are soluble in ethanol or sodium dodecyl sulfate (SDS) [5–7].

In previous works, two recombinant class I hydrophobins have been successfully expressed but as inclusion bodies in *Escherichia coli* and denaturing agent was used for solubilizing the insoluble proteins prior to purification [8]. The soluble expression of hydrophobins in bacterial systems remains a challenge because of many conserved cysteine residues present in their structure [9,10]. Tedious renaturation methods have been developed for the recovery of the insolubly expressed proteins. For example, denaturing agents (guanidine hydrochloride and urea) have to be employed for solubilizing the insolubly expressed proteins first, then the solubilized and denatured proteins are refolded back to its natural form by carefully removing denaturing agent. Usually, the renaturation processes are very inefficient and with low yield [11–13]. Therefore, cost-effective and easy purification methods for the production of active recombinant class I hydrophobins deserves to be investigated.

Cellulose binding domain (CBD), a non-catalytic domain of cellulase from cellulolytic microorganisms, known as an affinity fusion tag effective for the purification and immobilization of CBD-fused proteins using a cellulose matrix with several advantages: (a) specific binding affinity toward cellulose, (b) low non-specific protein binding, (c) higher protein yield, and (d) commercial availability of cellulose in various inexpensive forms [14–17]. CBD is rarely recognized as solubility enhancer tag compared to commonly used tags such as glutathione-S-transferase (GST), N-utilization substance (NusA), and thioredoxin (TrxA) [18]. However, some authors reported that CBD from *Clostridium*

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cellulovorans endoglucanase-xylanase D (EngD) and *Clostridium thermocellum* could significantly increase the soluble expression of fusion protein in *E. coli* due to its high solubility [19,20]. The fusion of CBD from *C. thermocellum* with fructosyl peptide oxidase (FPO) exhibited an increase in the FPO solubility up to 84% when expressed at 25 °C [20].

Herein, in this work CBD from *C. thermocellum* was designed to be fused with class I hydrophobin HGFI for the expectation to obtain effective soluble expression of HGFI in *E. coli.* CBD by nature has strong affinity interaction toward cellulose. In addition, nanofibrils of bacterial cellulose (BC) has very high specific cellulosic surface area (~60 m^2/g). Therefore, by taking advantage of the strong affinity between CBD and BC, direct isolation and purification of CBD fused HGFI (CBD-HGFI) from cells crude extract using BC produced by *Komagataeibacter xylinus* as affinity matrix could be achieved. Six histidine tag was also designed at C-terminal of the CBD-HGFI fusion for its purification by immobilized metal-chelated affinity chromatography (IMAC). The interfacial activity of CBD-HGFI complexed with BC nanofibrils (BC-CBD-HGFI) was also compared with that of free CBD-HGFI purified by IMAC. Lysostaphin is known as a GlyGly endopeptidase can cleave the crosslinked glycine peptide bridges of peptidoglycan in the cell wall of *Staphylococci*. In our previous work [21], recombinant lysostaphin (Lst) was successfully expressed and demonstrated to be capable of effectively cleaving the GGGGS flexible linker of fusion proteins. Therefore, in this work recombinant Lst was employed to cleave the flexible linker $(GGGGS)_2$ of CBD-HGFI for the recovery of HGFI. The biochemical properties of CBD-HGFI, BC-CBD-HGFI, and Lst cleaved BC-CBD-HGFI were characterized using thioflavin T assay, water contact angle, and TEM analysis.

Recently, enzymatic hydrolysis of poly(ethylene terephthalate) (PET) has drawn a lot of research attentions [22,23] due to the expectation of mild and ecofriendly PET upgrading recycle. The effects of hydrophobins on accelerating PET hydrolysis have been reported on several PET hydrolysis enzymes such as cutinase [24] and PETase [8,25]. In this work, the activity of purified CBD-HGFI and Lst cleaved BC-CBD-HGFI were also evaluated by their effect on enhancing PETase hydrolysis against PET fiber.

2. Materials and methods

2.1. Plasmids, strains, and media

Hydrophobins HGFI gene from *Grifola frondosa* (Genbank EF486307.1) was synthesized and constructed into plasmids pET-24a-hgfI by Yao-Hong Biotechnology Inc. (Taipei, Taiwan). Plasmid pET-24a(+) carrying CBD gene from *Clostridium thermocellum* and plasmid pET-21b(+) harboring *Ideonella sakaiensis* 201-F6 PETase (GenBank GAP38373.1) were obtained from Prof. Shen-Long Tsai (Department of Chemical Engineering, NTUST, Taiwan).

Komagataeibacter xylinus strain ATCC 11142 which produced bacterial cellulose was purchased from BCRC (Hsinchu, Taiwan). The bacteria were grown at 30 °C on Hestrin and Schramm (HS) medium composed of 5 g/L yeast extract, 20 g/L glucose, 2.7 g/L Na_2HPO_4, 5 g/L peptone, and 1.15 g/L citric acid. DNA fragment of lysostaphin (Lst) (GenBank accession no: KF724949.1) containing *Nde*I and *Sal*I restriction sites was codon-optimized and synthesized by Yao-Hong Biotechnology Inc. (Taipei, Taiwan). Lst expression vector was constructed by inserting Lst gene fragment into pET-24a after digested with *Nde*I and *Sal*I to generate pET-24a-Lst-6xHis as reported previously [21]. *E. coli* DH5α and BL21 competent cells were purchased from Yeastern Biotech (New Taipei City, Taiwan) as cloning and expression host. All genotypes of the *E. coli* strains and the primers used in this study are listed in Tables S1 and S2.

2.2. Construction of recombinant CBD-HGFI

The gene fragment encoding hgfI was amplified with forward primer (*FSacI_hgfI*) and reverse primer (*RSalI_hgfI*) that generated a product with length of 318 bp. Amplification was performed by initial denaturation at 98 °C for 30 s and a subsequent series of 35 cycles at 98 °C for 10 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 30 s (extension). To identify the PCR product, PCR amplification was mixed with loading buffer for DNA electrophoresis on a 1% agarose gel. The *hgfI* gene fragment was purified by Zymoclean™ Gel DNA recovery kit. Then, *hgfI* fragment and pET-24a-cbd-gfp plasmid carrying cbd-linker were digested with *SacI* and *SalI* at 37 °C overnight and ligated using T4 ligase enzyme at 16 °C overnight. The ligation product (pET-24a-cbd-hgfI) was transformed into competent cells and positive clones were confirmed by *taq* PCR and DNA sequencing. The fusion protein was designed with C-terminal $(His)_6$-tag for protein purification by IMAC.

2.3. Expression and purification of fusion protein using IMAC

Plasmid of pET-24a-cbd-hgfI from *E. coli* DH5α was extracted using Plasmid miniprep kit (Yeastern Biotech, Taiwan) and transformed into *E. coli* BL21 for protein expression. The fusion protein production cells were grown in Luria Bertani (LB) medium supplemented with 50 μg/mL kanamycin and incubated at 37 °C overnight with shaking. The cell culture was scaled up to 100 mL of medium and incubated at 37 °C until the optical density of culture (OD_{600}) achieved ~0.5 before the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) for induction. The CBD-HGFI expression was performed at 20 °C for 18 h. Then, the cell pellet was harvested using centrifugation at 8000 rpm for 15 min, washed twice with ddH_2O, and resuspended in 10 mM phosphate-buffered saline (PBS) pH 7.4 consist of 0.1 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor. *E. coli* cells pellet was lysed with an ultrasonicator on ice for 15 min and the lysate was centrifuged at 8000 rpm, 4 °C for 15 min to obtain the supernatant as soluble cell fraction.

The soluble fraction of CBD-HGFI was purified by IMAC under non-denaturing conditions. The crude protein extract was loaded into the IMAC column filled with 0.1 M $NiSO_4$, washed with binding buffer containing 5 mM imidazole, and eluted with elution buffer containing 500 mM imidazole. The eluted protein was dialyzed against PBS using Vivaspin column (30,000 MWCO) to remove imidazole and other salts. The molecular weight of protein fractions was evaluated by SDS-PAGE analysis. Protein concentration was analyzed by Bradford assay using bovine serum albumin (BSA) as standard.

2.4. Production of bacterial cellulose

A single colony of *K. xylinus* was grown in 5 mL of HS medium at 30 °C for 24 h. Then, the seed culture was transferred into 45 mL of fresh medium and incubated at 30 °C for 3–7 days under static condition. The BC pellicle formed at air-liquid interface was harvested and treated with 1 N NaOH at 90 °C for 20 min to lyse the cells. Then, the treated pellicle was washed thoroughly with distilled water until neutral pH. The pellicle was homogenized in 10 mM PBS by ultrasonicator to obtain well-suspended BC nanofibrils dispersion.

2.5. Isolation and purification of CBD fusion on BC

One mL of CBD-HGFI crude extract of various protein concentrations (1.5, 1, 0.5, and 0.1 mg/mL) was mixed in 0.5 mL of BC dispersion (20 and 50 mg BC pellicles/mL). The mixtures were incubated in an ice bath with shaking at 100 rpm for 120 min. Then, each sample was centrifuged at 14000 rpm for 10 min to separate unbound protein in the supernatant and bound protein in the pellet. The pellets were washed using 10 mM PBS buffer and centrifuged for 10 min. Finally, the bound proteins were resuspended in PBS buffer and all protein fractions were analyzed by SDS-PAGE. Moreover, recombinant Lst was produced by growing *E. coli* BL21 transformed with our previous construct [21] at 30 °C and IPTG induced for 3 h. The IMAC purified Lst was used for the cleavage of linker $(GGGGS)_2$ in CBD-HGFI captured on BC at 4 °C for 7 days. The cleaved protein fractions were analyzed by using SDS-PAGE.

2.6. Characterization of purified CBD-HGFI

Water contact angles (WCA) of PET fibers treated with IMAC-purified CBD-HGFI, BC-CBD-HGFI, and Lst cleaved BC-CBD-HGFI, respectively were measured to evaluate the interfacial activity of hydrophobins. The recombinant HGFI obtained in our previous study [8] was used as a control. Same amount of PET fibers were flatly fixed on glass surface by using double-side tape. The flatten PET fibers surface was then drop-coated with 50 μL of CBD-HGFI or HGFI (50 μg/mL), rinsed twice with distilled water, and incubated at 30 °C overnight prior to water contact angle (WCA) analysis using Goniometer 100SB (Sindatek Instruments Co., Ltd., Taiwan).

Thioflavin T (ThT) assay was performed to investigate amyloid-like rodlets of class I hydrophobin. Protein samples (50 μg/mL) prepared in PBS buffer pH 7.4 were incubated with ThT to a final concentration of 40 μM. The amyloid fibril formation was induced by shaking at 300 rpm for 1 h. Control samples containing PBS buffer and 40 μM ThT were also prepared. The assay was carried out in a Synergy H1 hybrid reader (BioTek Instruments, Inc., USA) with emission measurement at 470–650 nm and excitation at 442 nm.

Transmission electron microscope (TEM) (JEOL JSM-2000FX II, Japan) was used to analyze rodlet structure of class I hydrophobin fusion protein. Sample preparation involving TEM grid (formvar/carbon 300 mesh, Ted Pella Inc., USA) floating method on protein droplet. Fresh protein droplet (100 μg/mL) dissolved in 25% ethanol was incubated on a parafilm surface for 20 min to form hydrophobin assembly at air-water interface. Then, a TEM grid was floated over the protein droplet for 1 min and the excess solution was wiped away with filter paper. The grid was washed by floating it on the distilled water droplet before negatively stained with 2% phosphotungstic acid for 10 min. Finally, the excess stain was removed and the grid was dried at 30 °C overnight before visualized using TEM instrument at 120 kV.

2.7. Enhancement of PETase hydrolysis against PET fiber

The ethanol cleaned PET fibers of 3 mg was added into 200 μL of 50 mM phosphate buffer of pH 8 containing 20 μg/mL of HGFI containing samples. After 3 h of incubation at 30 °C, the HGFI containing solutions were removed by centrifugation. PETase (20 μg/mL) dissolved in phosphate buffer was then added to HGFI pretreated PET fibers for hydrolysis at 30 °C for 5 days. The main hydrolysis products, terephthalic acid (TPA) and mono(2-hydroxyethyl) terephthalate (MHET) in the supernatant were analyzed by HPLC and calculated based on monomer standard curves established in our previous study [8].

3. Results and discussion

3.1. Cloning and expression of CBD-HGFI fusion

HGFI fused with CBD was successfully expressed in *E. coli* BL21 as a soluble protein after IPTG induction at 20 °C for 18 h. As analyzed by SDS-PAGE in Fig. 1a, an apparent band at ~35 kDa was clearly observed in the total and soluble fraction. In contrast, protein bands in the insoluble fraction were barely notable. ImageJ software was used to evaluate the soluble fraction of the expressed CBD-HGFI which is defined by the ratio of soluble band intensity of CBD-HGFI to that of total CBD-HGFI fractions. Approximately, 94% of the expressed CBD-HGFI existed as a soluble protein after IPTG induction at 20 °C. In contrast, the soluble fraction decreased to 56% when expression temperature increased to 30 °C (Fig. 1b). It is well-known that lower expression temperature benefits the soluble expression of a heterologous protein because low temperature slows down cell growth as well as the protein expression rate that allow the nascent protein to correctly fold into its native form [26,27]. As shown in our previous work [8] (Fig. S1), the soluble expression of HGFI alone in the same host *E. coli* BL21 was not possible even at the lowered expression temperature of 20 °C.

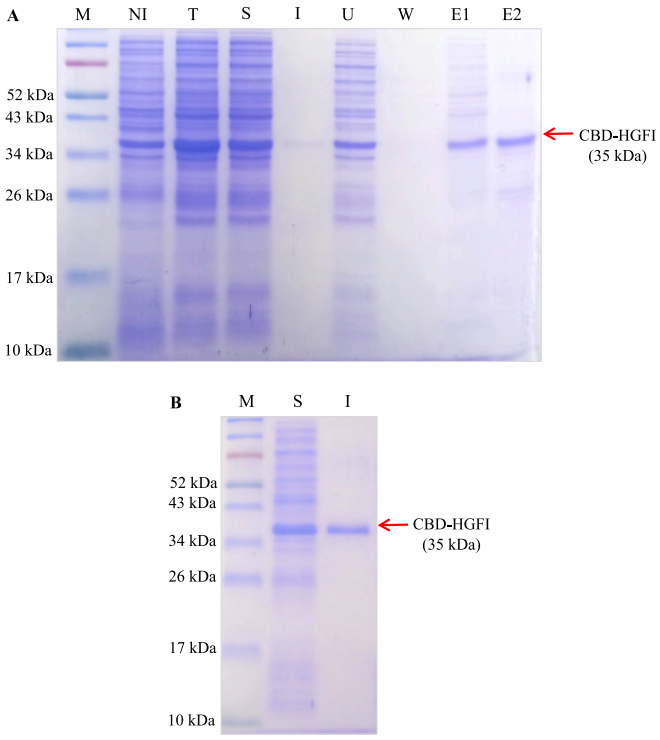

Fig. 1. Expression and purification of CBD-HGFI in *E. coli* BL21 harboring pET-24a-cbd-hgfI after induction at (a) 20 °C for 18 h and (b) 30 °C for 4 h. M: marker, NI: Non-induced cells, T: total protein, S: soluble fraction, I: insoluble fraction, U: unbound protein, W: wash, E: elution fraction.

Evidently, CBD is an effective soluble expression enhancing fusion partner.

3.2. Purification and immobilization of CBD fusion on BC

The soluble expressed CBD-HGFI could be effectively purified by IMAC because of the presence of 6xHis tag at C-terminal of its structure. The protein yield showed that 12.7 mg of purified CBD-HGFI was obtained per L of recombinant *E. coli* culture. BC has a very high specific area and been used as a cellulose matrix for capturing CBD fusion proteins [28,29]. By taking advantage of the fused CBD, the feasibility of isolation and purification of CBD-HGFI from the cell crude extract by BC was explored. BC nanofibrils dispersion obtained by extensive ultrasonication of different concentration was incubated with CBD-HGFI crude extract. As shown by SDS-PAGE analysis (Fig. 2), BC concentration of 50 mg/mL shows the better performance on capturing CBD-HGFI. Incubation time of 30 min and 60 min, however, did not give a significant difference on the amount of CBD-HGFI captured. Therefore, incubation time was prolonged to 120 min with expectation to saturate the binding of CBD-HGFI on BC nanofibrils. As shown in Fig. 3, with incubation time increased to 120 min the amount of unbound CBD-HGFI reduced significantly. As expected, the amount of bound CBD-HGFI increased with the amount of crude extract loaded. Crude fusion protein concentration of 0.5 mg/mL showed an apparent band at ~35 kDa and the other impurity bands were barely observed. As compared with IMAC purification, BC as an affinity matrix can effectively purify CBD-HGFI but the captured CBD fusion cannot be freed from BC nanofibrils due to the strong affinity interaction between BC and CBD. Therefore, Lst as an GlyGly endopeptidase was employed to cleave flexible linker $(GGGGS)_2$ in CBD-HGFI to obtain the free HGFI protein. Lst expression construct of our previous work [21] was employed to produce soluble Lst in *E. coli* BL21 with a molecular weight of ~26 kDa. Lst was purified by IMAC to cleave the free CBD-HGFI purified by IMAC as shown in Fig. S2. Two apparent cleaved bands at positions of ~14 kDa and ~ 16

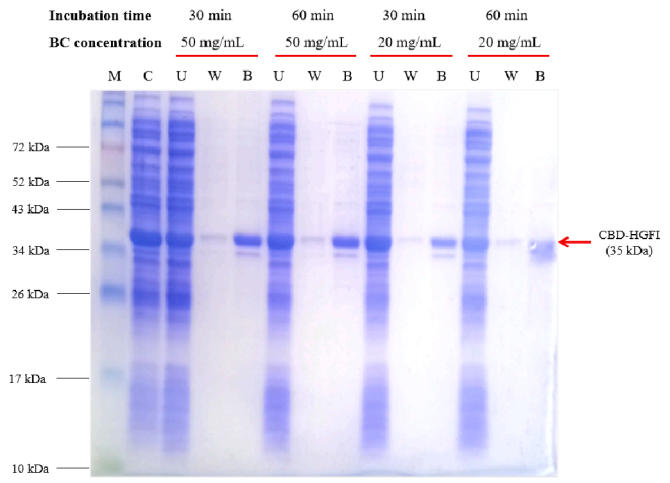

Fig. 2. Effect of incubation time and BC nanofibrils dispersion concentration on purification of CBD-HGFI from crude extract. M: marker, C: crude extract, U: unbound protein, W: wash, B: BC-bound proteins.

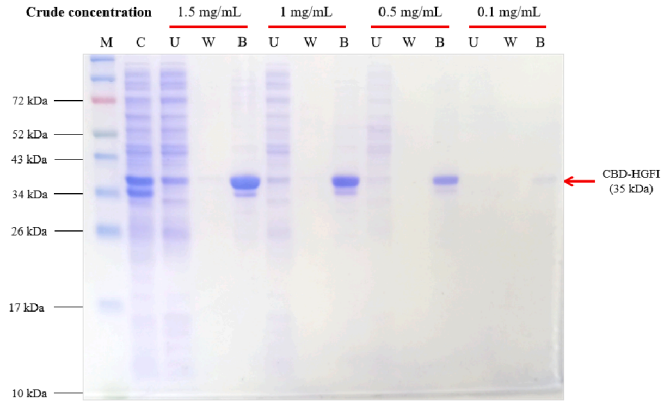

Fig. 3. Effect of crude protein concentration on purification of CBD-HGFI by BC nanofibrils (50 mg/mL) with 120 min of incubation time. M: marker, C: crude protein, U: unbound protein, W: wash, B: BC-bound proteins.

kDa could be observed. These two bands were considered to be intact HGFI and CBD fragment, respectively. Native CBD should have an estimated molecular weight of 21 kDa, however, Lst may attack the GlyGly segments located in CBD sequence (Fig. S3) that results in the generation of ~16 kDa fragment. In contrast, as shown in Fig. S4 intact CBD-HGFI and Lst bands could be well observed for CBD-HGFI captured on BC nanofibrils after 1 day of Lst attack. No cleaved fragments at position of ~14 kDa and ~ 16 kDa could be found. CBD-HGFI captured on BC nanofibrils (BC-CBD-HGFI) still can be observed by denaturation SDS-PAGE because SDS and denaturation treatment should be able to release CBD-HGFI from its affinity binding to BC. The low cleavage efficiency is probably due to the presence of BC nanofibrils which hinders Lst access to the bound CBD-HGFI. Prolonging the cleavage time is expected to increase the cleavage yield. As shown in Fig. 4, CBD-HGFI captured on BC (BC-CBD-HGFI) nearly disappeared and the desired cleavage fragments at ~14 kDa and ~ 16 kDa appeared after 7 days Lst cleavage. Evidently, Lst could successfully cleave and free HGFI from CBD-HGFI complexed with BC nanofibrils. It was found out that most of BC-CBD-HGFI complex of 0.5 mg/mL could be completely cleaved by 0.3 mg/mL Lst to release HGFI. Shorter cleavage duration and better cleavage temperature needs further optimization studies.

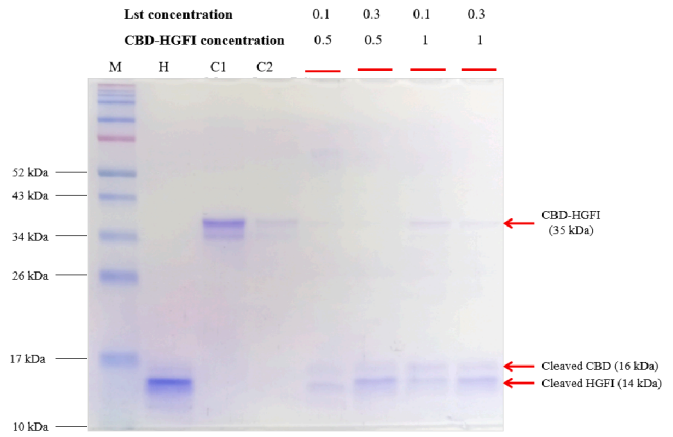

Fig. 4. Effect of Lst on cleavage of BC-bound CBD-HGFI fusion for 7 day. M: marker, H: HGFI (control), C1: BC-CBD-HGFI (1 mg/mL), C2: BC-CBD-HGFI (0.5 mg/mL), Red dash line: BC-CBD-HGFI of 0.5 to 1.0 mg/mL cleaved by Lst of 0.1 to 0.3 mg/mL.

3.3. Interfacial activity of purified CBD-HGFI

WCA analysis on the surface of PET fibers pretreated with various purified HGFI was used to evaluate their interfacial activity. As shown in Fig. 5, WCA of pristine PET fiber was 95.2 ± 2° which can be considered as a hydrophobic surface. After treated with HGFI control and free CBD-HGFI purified by IMAC on the surface of PET fibers, WCA decreased significantly to 18.7 ± 3^0 and 22.4 ± 5°, respectively. Moreover, BC nanofibrils itself also demonstrated its ability to reduce WCA to 24.6 ± 6^0 but the BC-CBD-HGFI could further lower WCA to 21.7 ± 4^o (data not shown). The significant decrease of WCA on the hydrophobic surface indicates that free CBD-HGFI still retains the similar interfacial activity as its counterpart HGFI to transform hydrophobic PET surface into hydrophilic because of its amphiphilic property [30]. In addition, the further lowered WCA (20.2 ± 3^0) observed for Lst cleaved BC-CBD-HGFI again indicates that HGFI could be freed from BC-CBD-HGFI by Lst cleavage to effectively reduce the surface hydrophobicity of PET fibers.

ThT assay is commonly used to detect the formation of amyloid fibrils because the small molecule of ThT gives strong fluorescence upon binding to amyloid structure. Class I hydrophobins are known to form amyloid-like rodlet structure due to its cross-β-secondary structure which can specifically bind ThT fluorescence dye [31,32]. As shown in Fig. 6a, ThT assay of HGFI showed a fluorescence peak ranged from 470 to 650 nm with strongest intensity (9783 RFU) obtained at 490 nm when excited at 442 nm. ThT fluorescence intensity of free CBD-HGFI and BC-CBD-HGFI were 7436 and 7071 RFU, respectively as shown in Fig. 6b. The lowered ThT fluorescence intensity as compared with free HGFI indicates that the presence of CBD and BC nanofibrils will interfere HGFI from forming amyloid fibril structure. In contrast, HGFI released from BC-CBD-HGFI by Lst cleavage showed a much higher ThT intensity of 9307 RFU. This again shows that solubly expressed CBD-HGFI could not only be isolated on BC nanofibrils but also cleaved by Lst to release HGFI. As observed by TEM (Fig. S5), the Lst cleaved BC-CBD-HGFI exhibited the presence of rodlet-like fibril structures as characteristic

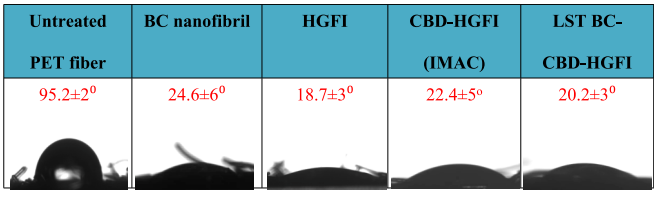

Fig. 5. Water contact angles on the surfaces of hydrophobins treated PET fibers.

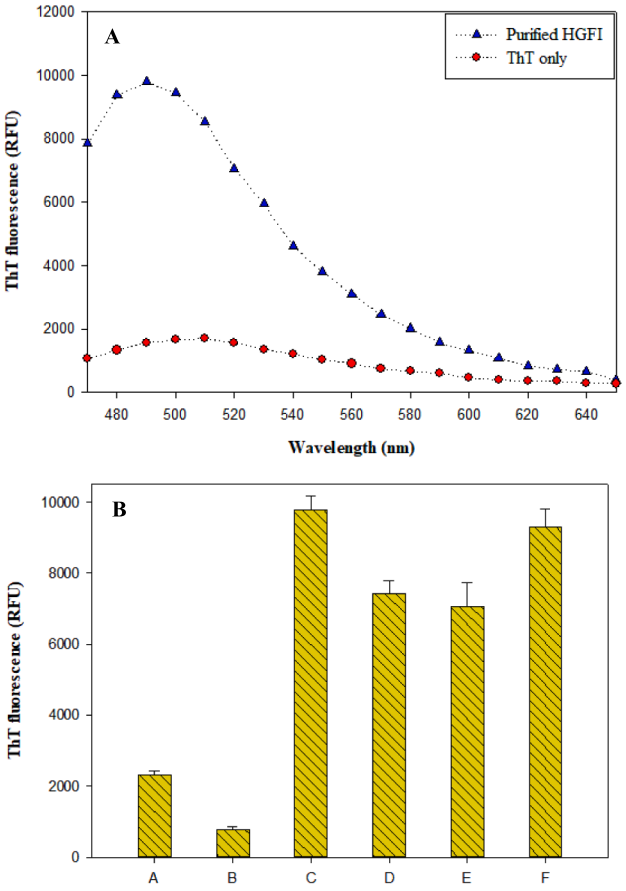

Fig. 6. (a) ThT fluorescence measurement of self-assembled recombinant HGFI (50 μg/mL) at 470–650 nm. (b) ThT fluorescence emission in presence of various recombinant hydrophobins (50 μg/mL) at 490 nm. (A) ThT only, (B) PBS buffer, (C) HGFI, (D) CBD-HGFI (IMAC), (E) BC-CBD-HGFI, (F) Lst cleaved BC-CBD-HGFI.

of class I hydrophobin.

1.1. *Effect of CBD-HGFI on enhancing PETase hydrolysis against PET*

Previously we have reported class I hydrophobin pretreatment on PET can improve PETase hydrolysis rate [8,25]. The enhancing effect on PET hydrolysis was also studied for fusion hydrophobins, IMAC purified free CBD-HGFI and BC-CBD-HGFI. As shown in Fig. 7, CBD-HGFI fusion demonstrates slightly better performance as compared with HGFI that 50% more hydrolysis products TPA and MHET were obtained when pretreated on PET fibers surface for PETase hydrolysis. In contrast, BC-CBD-HGFI did not show appreciable hydrolysis enhancement. Probably, the presence of BC nanofibrils will prevent CBD-HGFI from self-assembling on the surface which was speculated can promote PETase attaching onto PET surface that leads to an enhanced hydrolysis rate. When Lst cleaved BC-CBD-HGFI was applied, the hydrolysis enhancement was noticed that 50% higher TPA concentration was obtained as compared to control. Evidently, Lst can free HGFI from BC-CBD-HGFI to have HGFI effectively modify the surface for enhancing PETase hydrolysis. Interestingly, TPA and MHET concentrations released from CBD-HGFI fusion pretreatment was about 1.2 fold higher than that of HGFI. It is speculated that the presence of CBD in the structure of CBD-HGFI may also contribute to its self-assemble binding onto PET surface that leads to an enhanced PETase attachment, as consequence, the hydrolysis rate is enhanced. A similar effect of CBD on enhancing PET hydrolysis was also recently reported by Dai [33] using cellulose binding domain (CBM) of cellobiohydrolase I from *Trichoderma reesei* fused IsPETase^{EHA}.

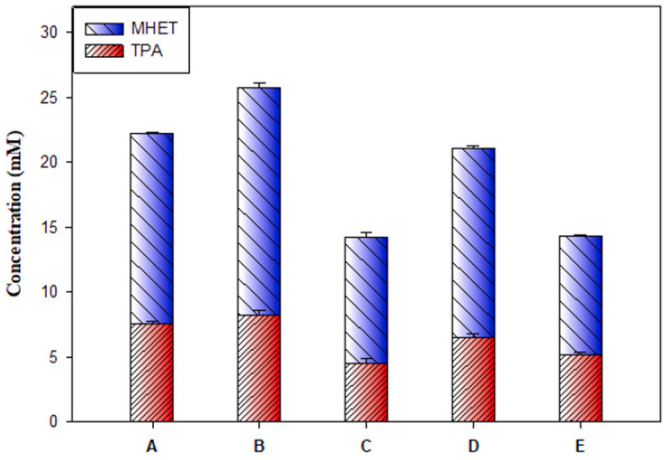

Fig. 7. Concentrations of TPA and MHET released upon PETase hydrolysis of hydrophobins-pretreated PET fibers by (A) HGFI, (B) IMAC purified CBD-HGFI, (C) BC-bound CBD-HGFI, (D) Lst cleaved BC-CBD-HGFI, (E) control (no pretreatment). The enzymatic hydrolysis was performed at 30 °C, pH 8 for 5 days with PETase loading of 20 μg/mL.

The significantly improved enzyme activity of IsPETase^{EHA}_CBM fusion was attributed to the enhanced binding between CBM and PET surface.

4. Conclusions

Insoluble expression of recombinant class I hydrophobin HGFI in *E. coli* was resolved by fusion of CBD from *Clostridium thermocellum* to the N-terminal of HGFI. CBD was demonstrated to be an effective solubility enhancer tag of HGFI. Rapid isolation and purification of CBD-HGFI fusion on BC nanofibrils was achieved. In addition, recombinant Lst could successfully cleave the flexible linker between CBD and HGFI to release HGFI from CBD-HGFI captured on BC nanofibrils. Both free CBD-HGFI fusion purified by IMAC and BC-CBD-HGFI showed the interfacial activity of hydrophobins to lower the water contact angle on PET fibers significantly. PET hydrolysis using PETase could also show at least 50% enhancements resulted from free CBD-HGFI as well as Lst cleaved BC-CBD-HGFI pretreated PET surfaces. In addition, the free CBD-HGFI could achieve approximately 20% higher hydrolysis enhancement that that of free HGFI. The enhanced binding interaction between CBD and PET might contribute to the enhanced hydrolysis that could be considered as an additional advantage of fusion hydrophobin with CBD for enhancing its soluble expression in *E. coli*.

CRediT authorship contribution statement

Nathania Puspitasari: Methodology, Investigation, Software, Validation, Visualization, Data Curation, Writing - Original Draft.

Cheng-Kang Lee: Conceptualization, Methodology, Writing - Reviewing & Editing, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2021.10.089.

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