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Mining and identification of a glucosidase family enzyme with high activity toward the plant extract indican

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ABSTRACT

The present study described that the systematic mining and identification of potentially active β-glucosidase family enzymes toward indican, which extracted from the plant *Polygonum tinctorium* as one of precursors of production for indigo-blue. Some of the mined enzymes were previously identified as glycosyl hydrolases or putative enzymes with unknown properties. In addition, there were no reports on the hydrolytic activity toward indican. In order to confirm the activity, we analyzed the activity on indican or related substrates in selective medium and amplified four genes from mined strains using PCR, then cloned into *E. coli*. Using a related fluorescent substrate MUG, we verified successful cloning through checking the expression of genes and comparing characteristics with wild-type strains. Then, using recombinant enzymes and chemically synthesized pure indican or the plant extract, it was confirmed that indican was readily converted into indigo-blue. For the overexpression of an enzyme derived from *Shinorhizobium meliloti*, which was found to be the most active through comparative analyses, we subcloned the gene in pMAL-c2X vector and expressed it as a MBP fusion protein. The resulting enzyme was overexpressed (>35% of whole cell protein) and found mainly in soluble fraction. The purified enzyme was determined to be a monomer with calculated molecular mass of 52 kDa and showed a specific activity (0.8 unit/mg protein) on the plant extract including indican. These results demonstrated that the mined enzymes not only could be an alternative resource for indigo-blue production, but also might be useful in the production of indigo from the plant indican by a single process.

1. Introduction

Natural dyes have been used widely in various areas since long ago before the manufacturing method of synthetic dye was established, and most of them have been produced from natural resources, mainly, plant extracts [1]. Different from synthetic dye, natural dyes are produced using environmentally friendly processes, thus relatively non-toxic. When used in fabric dying and cosmetics, they produce natural and harmonious color and texture that cannot be expressed with synthetic dyes. Indigo-blue is one of natural dyes for blue color, which is traditionally produced by the reaction between indican and enzyme rich in plant *Polygonum tinctorium* (Fig. 1). In this method, the plant is dipped in water for a specific period to induce the destruction of tissue, and then indigo-blue is produced through the conversion of indican by innate glucosidases [2]. The resulting indigo is precipitated with the addition of lime, recovered, and used as dye. When dye is manufactured by this method, it takes a long time, the grains are not uniform, and the quality is different according to temperature and oxidation [3]. The kind of lime used or dipping compound can also be hardly standardized. Therefore, there are problems in color fastness and reproducibility, and price is high due to difficulties in manufacturing and storage. Since these problems were overcome through chemical synthesis of indigo [4], most processes have been using synthetic indigo. Recently, however, it is reported that when synthetic indigo is added to dying process, food or cosmetics, it may cause allergies or diseases including cancers [5,6]. Environmental pollution is also being raised in connection to the chemically synthetic method. In this situation, the synthetic route to indigo by conventional process is reactivated through the reattention of consumer for natural dyes [7], and attempts are made to produce indigo by biological methods instead of conventional ones for mass and standardized production. Currently various indigo derivatives are used as the materials of cosmetics, food additives and antimicrobial drugs, and are studied for uses as medicine because they are

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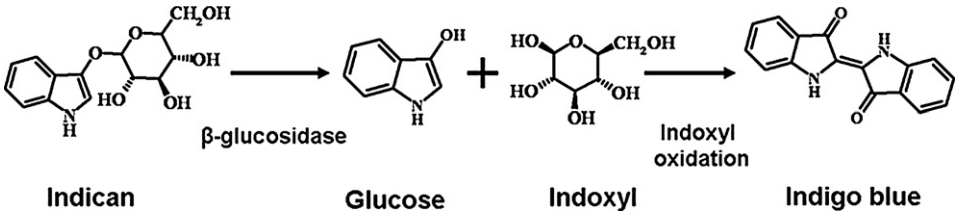

Fig. 1. Schematic representation of the enzymatic conversion of the free indican into indigo by glucosidase in plant *P. tinctorium*.

known to promote immune reactions and to have an anticancer effect [8–11].

There are several methods for attempting to produce indigo by biological processes. The first method is converting aromatic compounds, such as naphthalene and toluene, into indoxyl using bacteria, finally producing indigo-blue [12,13]. As a typical host, *E. coli* has few of enzymes involved in the conversion into indoxyl, and therefore, the method has to go through a very complicated gene cloning process. *Pseudomonas*, which has most of related enzyme, was studied as an alternative [14]. However, these chemicals with aromatic rings are not mixed well with water and their strong toxicity hinders the growth of host cells [15,16]. Additionally, these compounds must be removed completely from the produced dye. There are alternatives such as the fermentation or enzymatic conversion that produces indigo-blue from an amino acid tryptophan via indole, but they are not practically used due to low yield and cytotoxicity by both indole and indigo-blue [17]. Besides, there were reported that indican could be converted into indigo by glucosidases such as Novozyme 188 and novagram G, but these enzymes were produced no longer because of high price and low demand [18]. Alternatively, there have been many reports on the contents of indican in *P. tinctorium* and the indigo production by its own enzyme [19]. However, there have been no reports on mass-producible enzymes that can be used in the bioconversion of indican.

Here, we presented a systematic approach to mine a glucosidase family enzyme with high activity toward indican, extracted from the plant *P. tinctorium*. Then, the mined enzymes of a family were compared in terms of activity, solubility and expression level, thereby screening a potential enzyme from *Shinorhizobium meliloti*. If indigo-blue could be produced from the plant extract including indican by a method that uses only a microbial glucosidase enzyme in a single step, it might be an alternative process for indigo production.

2. Experimental

2.1. Mining of an enzyme family with hydrolytic activity on indican

In order to select β-glucosidases with activity on indican, we collected all the gene and amino acid sequences of glucosidases from the protein database of GeneBank, using three keywords (β-glucosidase, bacteria and endoglucosidase), and analyzed the evolutionary relationship of enzymes. Among the members, those with high homology (>35%) in amino acid sequence, duplicated paralogues within the same strain and orthologues with extremely close evolutionary relationship were excluded, resulting 141 genes derived from a total of 61 genus. For this purpose, we applied two different programs, Clustal W and Muscle, aligning data by amino acid sequences under default parameter options. After this, we further removed closely related neighbors by eye-inspection to construct phylogenetic data. Then, the phylogenetic tree was reorganized with 86 genes and divided into 11 subfamilies (Fig. 2). We selected 11 strains containing the gene representing each subfamily, which was an arbitrary selected sequence from whole genome sequenced strains due to ease access to gene resource. We then made comparative analysis of activity on target substrate indican

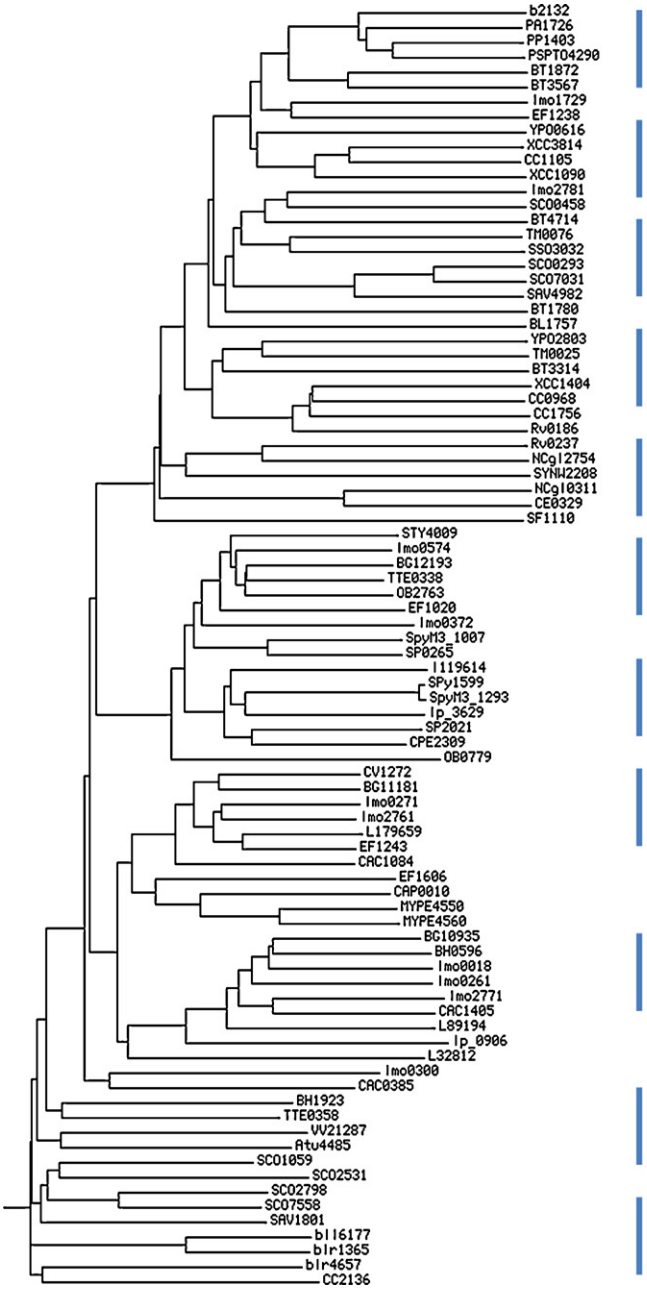

Fig. 2. Schematic representation of the phylogenetic tree of glucosidases produced by Clustal W multiple sequence alignment program using default options. Bars represent each subfamily divided.

using glucose assay kit (Sigma) and HPLC equipped with ELSD (Shimadzu). The consensus sequence of all the protein sequences of a subfamily containing the representative strain showing activity on indican was determined using the default Clustal X options for multiple sequence alignment. Then we further obtained a family sequences by the BLAST network service at NCBI using blast-p program [20]. After scanning, eight strains were finally selected as potential candidates for genes encoding indican-hydrolyzing enzymes.

2.2. Strains, plasmids and culture conditions

The finally selected strains with a putative or identified glucosidase were *Thermus caldophilus* GK24, *Thermus thermophilus* HB8 (KCCM40879), *Thermus* sp. (DSM625), *Agrobacterium tumefaciens* (ATCC33970), *Bradyrhizobium japonicum* LMG6138 (M015361), *Shewanella baltica* (M004549), *Flavobacterium johnsoniae* (KACC11414) and *Sinorhizobium meliloti* (KCTC2363). Eight strains were arbitrary chosen from query results of BLAST-P search, based on the close relationship with consensus sequences, easy cultivation and habitant associated with plants. A culture of *T. caldophilus* GK24 strain was obtained from EnzBank Co. (Daejeon, Korea) and the other strains were obtained from culture collection centers: KCCM, Korea Culture Center of Microorganisms; M, MicroBank (www.microbank.re.kr); KACC, Korean Agricultural Culture Collection; KCTC, Korean Collection for Type Cultures. *E. coli XL1-Blue* (Stratagene) was used as hosts for cloning and protein expression. Plasmid used for subcloning was pTrc99A (Pharmacia). Two plasmids, pQE30 (Qiagen) and pMAL-c2X (New England Biolabs), were used to induce for soluble expression and simple purification.

In order to confirm the activity of β-glucosidase, each strains was cultured at 25–50 °C using LB medium or the medium specified by the strain supply institutions. Indican (1 mM) was added as an inducer when needed. *E. coli* cells were cultured at 37 °C in LB or M9 media and measured the activity of β-glucosidase. The use of indican as the sole carbon source by wild-type and recombinant cells were confirmed using minimal medium containing indican (3 mg/ml) under the condition for optimal growth.

2.3. Cloning of glucosidase genes from selected strains

To confirm the activity of corresponding genes, four β-glucosidase genes out of eight strains mined were primarily cloned. Genomic DNA used as templates was purified using a prep kit (Promega). The primer used in PCR was designed based on sequences deposited in GenBank (Table 1). The amplified DNA fragments were separated by agarose gel (0.8%) and recovered using Wizard SV gel and PCR clean-up system (Promega). Glucosidase genes amplified from the genomic DNA of *T. caldophilus* and *T. thermophilus* HB8 were digested with EcoRI and HindIII restriction enzymes and cloned into the same site of pTrc99A plasmid. Glucosidase genes of *F. johnsoniae* and *S. meliloti* were subcloned into NcoI and HindIII sites of the pTrc99A plasmid. The digested plasmid and insert were ligated (4 °C, 12 h) and transformed into *E. coli XL1-Blue*. Among them, the best clone (harboring gene from *S. meliloti*) showing the highest activity toward indican was deposited at KCTC (accession number, KCTC11236BP).

Table 1
List of strains, genes and primers used in this study.

Strain	Acession no.	Primer sequence
T. caldophilus	AAO15361	5′-TAGAATTCAACGCCGAAAAGTTT-3′ 5′-TAAAGCTTTCACTCTGGCTG GGG-3′
T. thermophilus	YP145326	5′-TAGAATTCAACGCCGAAAAATTC-3′ 5′-TAAAGCTTTTAGGTCTGGGCCCG-3′
F. johnsoniae	ZP01246844	5′-GCCCATGGGTAAAATTGAAAACTCATTT-3′ 5′-GCAAGCTTTTAAGATAAAAAATCTTTAAA-3′
S. meliloti	NP386997	5′-ATCCATGGTGATCGAAGCCAAGA-3′ 5′-ATAAGCTTTCATCCCGGCTTGT-3′

2.4. Expression and zymogram analysis of recombinant β-glucosidases

In order to analyze the expression and activity of recombinant enzymes, the *E. coli* cells were spread on LB plates containing ampicillin (50 μg/ml), then overlaid 1% agarose gel (50 mM Na-acetate, pH 5.0) containing 5 mM fluorescent substrate MUG (4-methylumbelliferyl-β-D-glucopyranoside, Sigma–Aldrich). After incubation for 5 min, the functional expression was confirmed by fluorescence excited at 350–375 nm [21]. The colonies found to be active was seeded in LB medium and cultured at 37 °C for 10 h. After 1% of the pre-culture was reseeded and further grown in the same medium at 37 °C and 180 rpm to an $O.D_{600}$ of 0.5, 0.5 mM IPTG was added to induce the expression for 100 min. The induced cells were harvested using a centrifuge and resuspended in a phosphate buffer (50 mM, pH 7.0). After disruption, the resulting crude extract was loaded on a 10% SDS-PAGE and analyzed in terms of expression level and protein solubility. For zymogram assay, an aliquot (10 μl) of crude extract was mixed with 0.5 vol. of native sample buffer and resolved on a 8% PAGE under non-reducing conditions. After electrophoresis, the separating gel was washed twice with 50 mM of Acetate buffer (pH 5.0), and overlaid 1% agarose gel containing 5 mM MUG. The fluorescent protein bands were rapidly developed by incubation at 37 °C for 5 min.

2.5. Analysis of enzyme activity using pure indican and indican-containing plant extract

For the analysis of enzyme activity on target chemical, pure indican was purchased from Sigma–Aldrich. Plant extract containing indican was prepared by the procedure reported in previous work [22]. To use the freeze-dried extract, an aliquot of powder (30 mg) was dissolved in 200 μl of methanol (>99%) and then added into reaction solution or solid plate. Zymogram assay, activity staining and utilization of indican as a carbon source on solid plates were conducted by the identical procedure described in Section 2.

2.6. Identification of indican and indigo-blue and enzyme assay

The structure and properties of blue precipitate (indigo-blue) resulted from the enzymatic conversion of indican was identified by the method reported in elsewhere [23]. The concentration and content of indican and/or indigo-blue in the plant extract or reaction solution were analyzed with spectrophotometer (UV-1700, Shimadzu) and high performance liquid chromatography (LC solution, Shimadzu) using pure indican as an authentic sample according to the procedure of previous work [24]. The enzyme assay was also performed by the procedure of previous work [18]. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmole of indigo-blue per min under the specific conditions.

2.7. High-level expression and purification of a potential enzyme

In order to overexpress the potential enzyme from *S. meliloti*, the corresponding gene was further subcloned into expression vectors pQE30 and pMAL-c2X to which His-tag and MBP fusion were applicable. PCR primers used were as follows: forward primer, 5′-ATGGATCCATGATCGAAGCCAAGA-3′ and reverse primer

5′-ATAAGCTTTCATCCCG GCTTGT-3′. BamHI and HindIII were used for introducing the insert into both plasmids. As for PCR reaction, the cloned gene in pTrc99A was used as a template and *E. coli XL1-Blue* was used as an expression host.

For the protein purification, the *E. coli* cells was seeded in LB liquid medium containing ampicillin (50μ/ml) and incubated at 37 °C overnight. After 1% of the medium was reseeded in 10 ml of the same medium, it was further grown at 37 °C and 180 rpm to an $O.D_{600}$ of 0.5, then induced with 0.5 mM IPTG for 100 min. Recombinant cells were recovered using a centrifuge and washed three times with phosphate-buffered saline (PBS). The resulting cells were suspended in 4 ml buffer solution (50 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.2), and lysed using a sonicator (5 s pulse on, 10 s pulse off, amplify by 37%). After the product was centrifuged at 12,000 rpm for 30 min, the supernatant and the precipitate were separately recovered. These samples were loaded on a 8% PAGE gel and analyzed in terms of the expression level and protein solubility.

The recombinant enzyme was purified by affinity chromatography according to the instruction manual of pMAL™ purification system (New England BioLabs). After purification, β-glucosidase was isolated from MBP by cleavage with factor Xa protease. The resulting solution was reapplied onto the same resin, and the unbound protein was harvested and concentrated using centriprep YM-10 (Millipore). The protein homogeneity was confirmed by SDS-PAGE. The amount of protein was determined by the dye-binding method of Bradford (Bio-Rad) with BSA as a standard solution. The molecular mass and oligomeric structure of the enzyme was determined on an FPLC system with a gel-filtration column (Superose-12). The flow rate of the mobile phase, containing 50 mM Tris and 100 mM NaCl, was 0.4 ml/min. The enzyme properties were further analyzed in terms of optimum pH and temperature by using the purified enzyme, according to the same procedures from elsewhere [20,25].

3. Results and discussion

3.1. Selection of indican-utilization strains with β-glucosidase genes through data mining

According to the simple procedure described in our previous work, we could easily classify strains and genetic resources deduced to have responsible genes through the systematic mining process mentioned in Section 2. All of the identified genes were predicted to be a glycosyl hydrolase family enzyme, but there were few researches on the characterization of the enzymes. Moreover, there were no reports on the indican-hydrolyzing activity of these enzymes.

Along with *Agrobacterium* used as the representative strain for systematic mining, additionally selected seven kinds of strains were examined concerning whether they express glucosidase active on indican. We preliminarily analyzed the cell growth and activity of enzyme in nutrient and minimal medium using fluorescent substrate MUG and target substrate indican. In case of fluorescent MUG, most of indican-hydrolyzing enzymes found in the mining process showed a similar relative activity. Because the activity could be measured precisely by fluorescence, MUG was used together with indican in characterization procedure. When each strain grown in nutrient medium was overlaid with soft agar containing MUG, four strains showed a distinct activity, but it was relatively difficult to observe in situ activity of *Thermus*, *Bradyrhizobium* and *Shewanella*. When indican was overlaid, only two strains, *Bradyrhizobium* and *Shewanella*, did not show distinct activity. When the strains were cultured using indican as a sole carbon source in minimal medium, *Agrobacterium* and *Sinorhizobium* grew notably within 48 h, also forming blue colonies distinctly. The growth of the other strains was too slow or inhibited. When the growth was induced by adding glucose (2 mM) as a co-substrate, most of the strains were grown well and also revealed a notable activity, but the growth of *Bradyrhizobium* and *Flavobacterium* were relatively slow. The formation of blue color around colony of *Bradyrhizobium* and *Shewanella* was very weak. Accordingly, it was presumed that indican-hydrolyzing enzyme was expressed in grown cells in rich medium, and that the expression of enzyme in both *Bradyrhizobium* and *Shewanella* was not further induced even when glucose was added as a carbon source and indican as an inducer. As another result, it was plausible that the usability of indican as a sole carbon source could provide a simple route to screen the related strains expressing β-glucosidases active on indican.

It is known that indican is converted to indigo by the activity of β-glucosidase in the indican-containing plant itself [26]. The physiological meaning of the conversion of indican to indigo-blue is presumed to be related to the protective function against herbivores [27] and is also might be closely linked with toxicity to microbes, plant pathogens. On the contrary, it is believed that microbes around these plants may degrade indigo-blue or be resistant to it, or depending on case, there may be microbes that can use indican as a sole carbon source. Because most of microbes selected here are soil microbes (*Rhizobium*, *Shinorhizobium*, *Agrobacterium*, *Flavobacterium*) and are frequently found in farmlands, hot springs and aquatic environment systems (*Swanella* and *Thermus*), they are considered to hydrolyze easily the plant indican common around their habitats. All the mined enzymes were composed of amino acid (431–459) with similar size and homology of over 32%. Additionally, the putative active sites in glycosyl hydrolase were conserved well (Fig. 3).

3.2. PCR cloning and expression of β-glucosidases

β-Glucosidases selected in the mining process showed considerable activity on fluorescent substrate and indican at a whole cell level, but in each of the selected strains, there are two or more hydrolases capable of hydrolyzing the glycosidic bond on glucose. For example, in case of *Agrobacterium*, 3 fluorescent protein bands were observed in zymogram using MUG (data not shown). Accordingly, in order to determine whether the selected genes are the responsible for the activity, we decided to clone the genes mined. In consideration of the sequence similarity and the characteristics of enzymes suitable for practical application, the genes from 4 strains (*Shinorhizobium*, *Flavobacterium* and 2 strains of *Thermus*) were arbitrary selected to be cloned. The genomic DNA was extracted from each of grown cells in nutrient media to the mid-exponential phase and used as a template for PCR.

As shown in Fig. 4, it was confirmed that, if intact ORF was expressed without using tag or fusion protein, all recombinant enzymes were expressed in an insoluble state. The enzyme of *T. caldophilus* was expressed by less than 1% of the whole cell protein. The enzymes of *T. thermophilus* HB8 and *F. johnsoniae* were expressed by about 20% of the whole cell protein, but over 85% were observed in an insoluble fraction. Accordingly, enzyme activities were measured higher in insoluble fractions than in soluble fractions, without any supplement or pretreatment for solublization. As observed in previous reports, high-level expressed insoluble fraction does not mean that there is no activity. The protein with high aggregation tendency is occasionally enriched and found in insoluble fraction, but has a reasonable activity [28]. The enzyme of *S. meliloti* was expressed to be about 35% of the total cell protein but only 20% was soluble. These expression patterns were similar at lower temperature or with different hosts, and also were maintained in varying

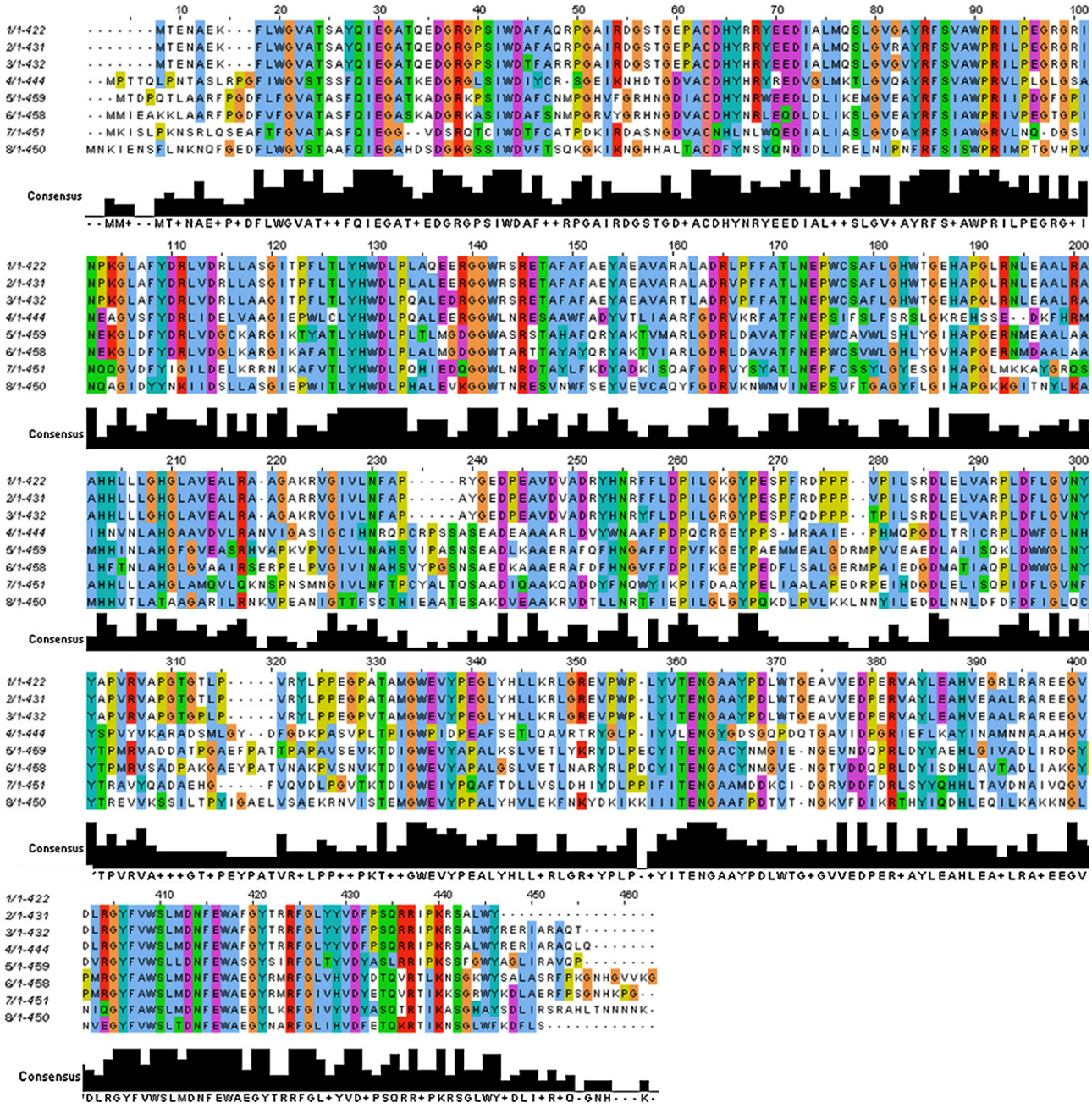

Fig. 3. Amino acid sequence alignment of the mined β-glucosidases by a systematic approach. All of the sequences were obtained from GenBank and analyzed by Clustal X program. The conserved amino acid residues were boxed. Line 1, *Thermus* sp.(BAA86923); line 2, *T. thermophilus* HB8 (YP145326); line 3, *T. caldophilus* (AAO15361); line 4, *B. japonicum* (NP771297); line 5, *A. tumefaciens* (NP534963); line 6, *S. meliloti* (NP386997); line 7, *S. baltica* (ZP01436526); line 8, *F. johnsoniae* (ZP01246844).

the concentration of inducer and induction time. These suggest that expression in *E. coli* is not stable. It was evident that the minimum-level expression by leaking without the addition of inducer was advantageous to the expression in soluble fractions for characterization of enzymes. In such a case, the relative expression level of enzyme was determined by its suitability (codon usage and folding landscape) for *E. coli* system [29,30].

3.3. Activity of recombinant enzymes on MUG and indican

In order to select a potential enzyme for practical application, we compared activity of the recombinant enzymes in *E. coli* cells, without the addition of inducer. First, the recombinant cells were analyzed for activity by using grown cells in LB agar plate overlaid with soft agar containing MUG. In all the enzymes, except clones containing the gene of *F. johnsoniae*, distinct fluorescence was observed clearly (Fig. 5A). Among them, clones with genes from *S. meliloti* showed the highest fluorescence, suggesting a relatively high activity or expression level. When MUG was exposed for a long time (>1 h) in order to detect the activity of enzyme derived from *F. johnsoniae*, weak fluorescence was observed around the colony. However, relatively faint but obvious fluorescence was also observed around clones used as a control with empty vector. Accordingly, in order to exclude the effect of host enzyme on hydrolyzing MUG and to determine whether there is the activity of enzyme from *F. johnsoniae*, zymogram assay was performed by using the crude extract of cultured cells. As shown in Fig. 5B, fluorescent bands were observed in different positions corresponding

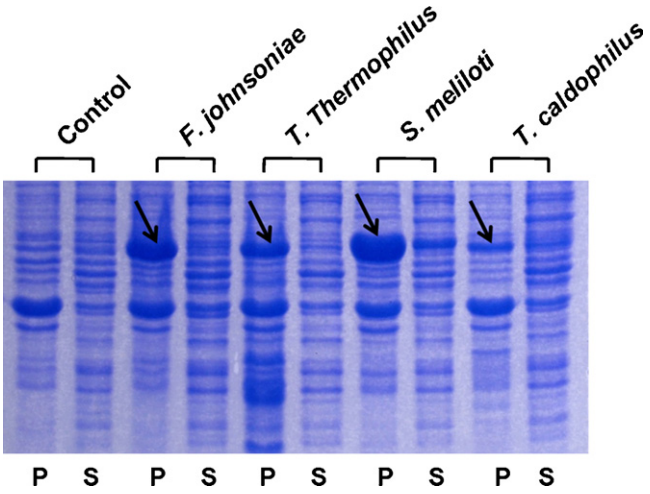

Fig. 4. SDS-PAGE analysis of the cloned β-glucosidases from four strains arbitrarily selected. *E. coli* cells harboring each recombinant enzyme were induced with 0.5 mM IPTG, and an aliquot of the crude extract was analyzed via SDS-PAGE. The proteins overexpressed at high levels are indicated by arrows: P, insoluble fraction; S, soluble fraction.

to the recombinant enzyme in clones, and difference in activity was similar to that in solid medium. Considering this, the cleavage of substrate by the same enzyme of the host was considered to be ignorable. As in solid medium, it was observed in zymogram

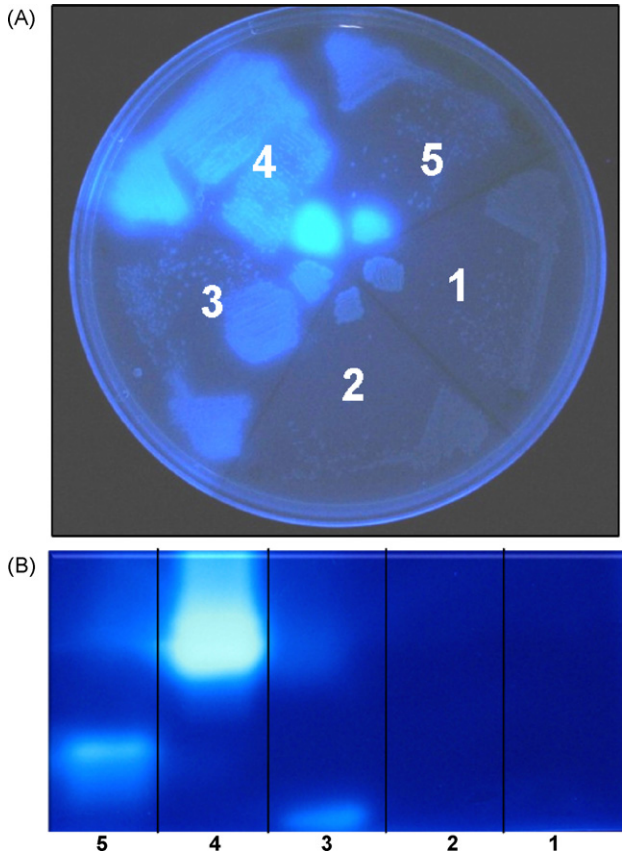

Fig. 5. Activity staining and zymogram assay using MUG as a substrate. (A) The photograph of MUG-overlaid cells under UV excitation at 375 nm. (B) The zymogram assay of crude extract on agarose gel under native conditions. Lane 1, control cells with empty vector; clones with genes from lane 2, *F. johnsoniae*; lane 3, *T. thermophilus* HB8; lane 4, *S. meliloti*; lane 5, *T. caldophilus*.

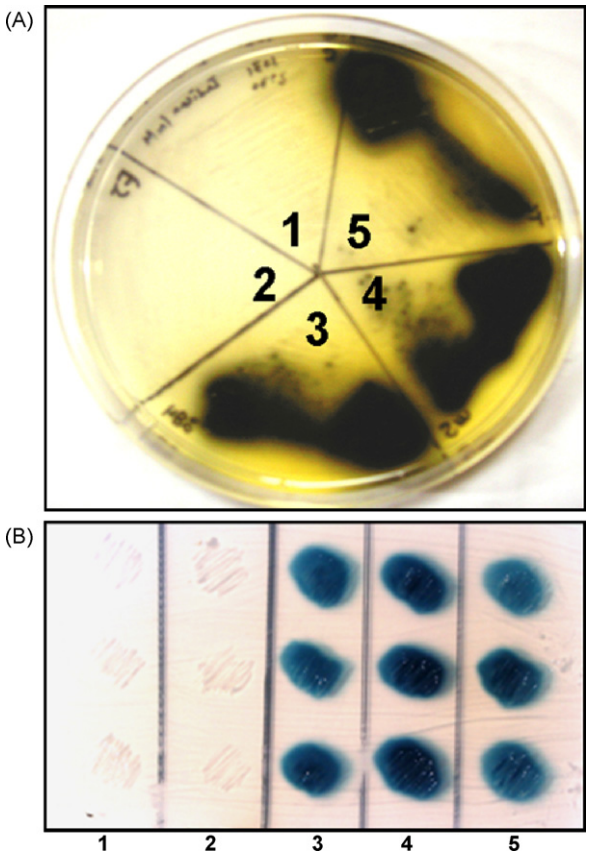

Fig. 6. Activity staining of β-glucosidases on solid plate using pure indican and plant extract containing indican as a substrate. (A) The photograph of grown cells in pure indican-supplemented M9 media as a sole carbon source. (B) The activity staining of plant extract-overlaid cells grown in LB media. Lane 1, control cells with empty vector; recombinant cells with genes from lane 2, *F. johnsoniae*; lane 3, *T. thermophilus* HB8; lane 4, *S. meliloti*; lane 5, *T. caldophilus*.

that the expression and activity of the enzyme from *S. meliloti* were outstanding. In case of enzyme derived from *F. johnsoniae*, a very weak fluorescent band was revealed, but difference from the crude extract of control cells was not clear, when the protein was loaded at high concentration (40 μg).

In order to reconfirm the results above using target substrate indican and to determine the use of indican as a sole carbon source in recombinant cells, we added indican to M9 minimal medium and examined the growth of recombinant cells. The results confirmed that, after cultivation for 45–50 h, indigo-blue was produced with the growth of recombinant cells containing the genes of *S. meliloti*, *T. caldophilus* and *T. thermophilus* HB8, except one harboring the enzyme of *F. johnsoniae* (Fig. 6A). As in M9 medium, β-glucosidase of *S. meliloti*, *T. caldophilus* and *T. thermophilus* HB8 also produced indigo-blue in LB medium. *E. coli* having the enzyme of *F. johnsoniae* produced a small amount of indigo-blue around the colony after 4 days. Expectedly, recombinant cells harboring the gene of *S. meliloti* showed the most intensive color development. A host transformed using empty vector without insert did not grow in M9 medium and its activity was not detected in LB medium.

3.4. Activity of recombinant enzymes on plant extracts containing indican

It was confirmed that the mined enzymes have clear activity on indican, but if pure indican is considered as the substrate for industrial process, it should be purified from plant cells or chemically

synthesized. These caused many difficulties in direct application of microbial glucosidase to practical application. Accordingly, in order to develop economic and environment-friendly process, we need to use substrate in the form of plant itself or crude or fractionated extract. To examine this possibility, we tested whether enzyme can utilize indican-plant itself as raw material. It was, unexpectedly, hard to detect enzymatic reaction in intact or ground leaves. This agrees well with previous result that the reason is explained by the difficulty in the access of enzyme to indican distributed separately in vacuoles [27]. To solve this problem, a solution containing indican extracted from plant leave powder was used as a substrate for activity assay. After the extract was overlaid, the deep purple color readily developed around the colony, which showed the production of indigo in plant extract mixed with other chemical co-extracted (Fig. 6B). The solution assay using whole cells as enzymes were also identical to that of solid plate. As shown in other results, clones with *S. meliloti*-derived gene showed the highest activity, and no activity was observed in the control clone.

3.5. Expression, purification and partial characterization of a potential β-glucosidase

When compared the properties of enzymes expressed by leaking in *E. coli* without inducer, *S. meliloti* β-glucosidase was found to be useful due to its high expression and activity, although the specific activity cannot be compared accurately in the state of crude extract. However, as observed in previous section, more than 80% of induced protein was found in an insoluble fraction and thus improper for overexpression and purification. To solve this problem, we attempted to express the protein using fusion tag and protein. Even though expression was induced using His-tag, solubility was very low as in the case of sole protein (Fig. 7). On the contrary, in case of MBP fusion protein, more than 85% of induced protein was soluble and also functional. Additionally, its expression level reached to be about 35% of the total cell protein.

We further conducted an experiment to analyze the general properties of enzyme by purification of the overexpressed enzyme. As shown in Fig. 7, over 2 mg of pure protein was separated through a singe step and used for partial characterization. Purified β-glucosidase was determined to be a monomer with an apparent molecular mass of 52 kDa, from the elution volume of gel filtration column chromatography. The optimal pH and temperature were found to be 7.5 and 35 °C, respectively. Under the optimal conditions, its specific activity on the plant extract containing indican was about 0.8 unit/mg protein-min.

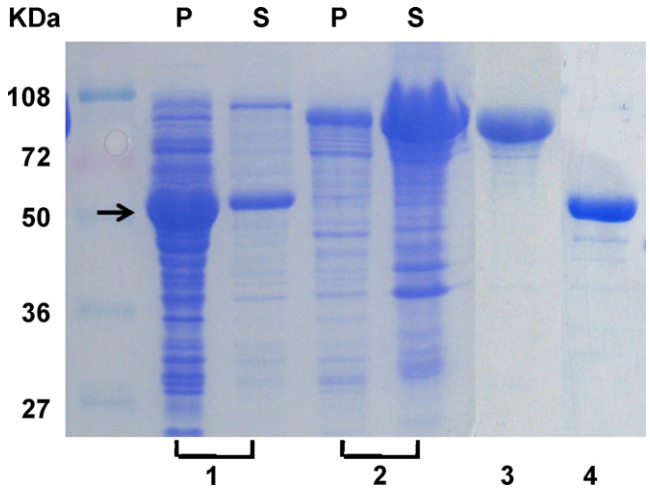

Fig. 7. SDS-PAGE analysis of His-tag, MBP fusion and purified enzyme. *E. coli* cells harboring each recombinant enzyme were induced with 0.5 mM IPTG, and an aliquot of the crude extract or purified enzyme was analyzed via SDS-PAGE. The proteins overexpressed at high levels are indicated by arrows: lane 1, expressed enzyme with His tag; lane 2, expressed enzyme with MBP in insoluble fraction (P) and soluble fraction (S); lane 3, purified enzyme with MBP; lane 4, purified enzyme after cleavage with factor Xa.

4. Conclusion

Our experiment confirmed that systematically mined enzymes have high activity on indican. This result is meaningful in that it suggests the possibility of producing indigo dyes from plant extract containing indican. Accordingly, it is expected to produce indigo using natural substrate and utilize them in various areas. Especially, because the natural substrate is used, it is considered advantageous to the development of medicine related to immune and antimicrobial activities. Furthermore, we expect that the advantage of the single enzyme process may solve problems in the traditional method such as high cost, low reproducibility and non-uniformity. This process may also replace the complex multistep process using aromatic compounds or tryptophan. For this purpose, we need to improve the properties of enzyme, such as specific activity and protein stability, when induced as sole protein without fusion partner. Therefore, the directed evolution of the enzyme is under progress, using the plant extract containing indican as a substrate.

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