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PURIFICATION OF RECOMBINANT ENDOGLUCANASE GH5-CBM72-CBM72 EXPRESSED IN *Escherichia coli*

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ABSTRACT

In a previous study, a gene GL0694641 coding for endoglucanase containing 3 domains GH5-CBM72-CBM72 was exploited from metagenomic DNA data of bacteria in Vietnamese goats' rumen. The gene (*eg5*) encoding the mature enzyme (without signal peptide coding sequence) was optimized codons, artificially synthesized, and inserted into the pET22b(+) vector at *Nco*I and *Xho*I to generate expression vector pET22-eg5 for expression of the gene in *Eschrichia coli*. In this study, the gene *eg5* was well expressed in *E. coli* BL21 and Rosetta 1 strains to produce recombinant endoglucanase of 77 kDa. The recombinant enzymes were expressed mainly in the soluble fractions of both strains. However, the enzyme expressed in *E. coli* BL21 was precipitated by imidazole at even a low concentration of 20 mM, whereas endoglucanase produced from *E. coli* Rosetta 1 strain was well soluble in buffers containing imidazole at concentrations of 20, 50, 200, and 250 mM. To our knowledge, this is the first study showing the negative effect of imidazole on recombinant protein. Endoglucanase expressed from strain *E. coli* Rosetta 1 was successfully purified by His-tag affinity chromatography using phosphate buffer saline (PBS). Protein contaminations were washed out by PBS buffer containing 20 mM and 75 mM imidazole then the target protein was harvested by the buffer containing 200 mM imidazole. After purification and desalting by the PD10 column, the recombinant endoglucanase had purity up to 97%. The pure enzyme exhibited endoglucanase activity hydrolyzing carboxymethyl cellulose in agar plate and by zymogram. The purified enzyme can be used as a material for its characterization.

Keywords: Endoglucanase GH5CBM72CBM72, *E. coli* Rosetta 1, expression, purification, zymogram.

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INTRODUCTION

Cellulose is the most abundant natural biomaterial in plant biomass and is supposed as an important resource for the development of the bioeconomy to produce high added value products such as biofuels, xylitol, fine chemicals,... However before fermentation to synthesize the final products, cellulose must be converted into monosugar glucoses by three main cellulases: endoglucanase (in other words β-1,4-endoglucanase); β-1,4-exoglucanase and β-glucosidase (Pandey et al., 2014). The endoglucanase is an important member of the cellulase family, cleaves crude cellulose fibers into shorter polysaccharides enabling beta-glucosidase and exoglucanase to convert them into glucose. Endoglucanases have been isolated from bacteria, fungi, animals and were characterized by many researches (Lynd et al., 2002).

Almost cellulose-degrading enzymes including endoglucanase have modularity that means besides the catalytic core, these enzymes also possess non catalytic domains such as the carbohydrate-binding domain (CBM). The additional domains play a very important role in enzyme function (Do et al., 2018). For example, CBM has an affinity to cellulose fibers, thus CBM assists to anchor or direct the host enzymes to targeted substrates and in some cases, CBM can disrupt crystalline cellulose microfibrils to enable cellulase access and hydrolyze cellulose (Guillén et al., 2010; Ding et al., 2008). Up to now, 88 CBMs were found and numbered from 1 to 88. The CBM72 is an important protein domain, it can bind a broad spectrum of polysaccharides including soluble and insoluble cellulose, β-1,3/1,4-mixed linked glucans, xylan, and β-mannan to assist the host enzyme to bind and hydrolyze variety ranges of substrates (Duan et al., 2016).

Due to many outstanding applications, endoglucanase gained much attention over the world to mine novel enzymes for effective and quick hydrolysis of cellulose. In recent years, with the rapid development of new generation sequencing technology, scientists tend to exploit novel genes coding for endoglucanase from unculturable bacteria. For mining novel genes coding for lignocellulase, we sequenced the metagenomic DNA of bacteria in the Vietnamese goats' rumen and exploited thousands of sequences encoding endoglucanases. From this data set, a complete gene (code GL0694641) of 2094 nucleotide coding for endoglucanase was chosen for the expression of the gene in *E. coli* because of its novel modularity. The deduced enzyme contains three domains. The catalytic domain GH5 situates at the N terminus, followed by two domains of CBM72. Thus, the enzyme was designated as endoglucanase GH5-CBM72-CBM72 (abbreviated by EG5). The gene fragment (*eg5*) coding for the mature enzyme was codon optimized and inserted into pET22b(+) at the restriction enzyme site *Nco*I-*Xho*I to generate pET22-eg5. The pET22-eg5 was transformed into *E. coli* BL21 and Rosetta 1 strains. This study described the expression, purification, and characterization of recombinant endoglucanase EG5 from the *E. coli* strain.

MATERIALS AND METHODS

Materials

Vector pET22-eg5 was designed by the Genetic engineering laboratory, Institute of Biotechnology. According to this design, the gene *eg5* was fused with a gene encoding the hexahistidine at the 3’ end to help the purification of the recombinant protein easily by affinity chromatography column.

HisTrap affinity chromatography column (GE, Healthcare, Sweden) was used for purification of the recombinant endoglucanase. The PD-10 ultrafiltration column (GE Healthcare, Mississauga, ON, Canada) was used for desalting the purified recombinant enzyme. Carboxymethyl cellulose substrate (CMC, C9481, Sigma) was used for the determination of endoglucanase activity on agar plates and zymogram. Cellulase (C9748, Sigma) was used as a positive control. Other chemicals used in experiments were purchased from Merck (Germany) and Thermo Scientific (USA).

Methods

Expression of recombinant endoglucanase EG5

The transformants *E. coli* BL21, Rosetta 1 harboring pET22-eg5 were cultured in 10 mL Luria-Bertani broth containing 100 µg/mL ampicillin (LBA medium) at 37 ºC, 180 rpm overnight. The overnight culture (0.2 mL) was transferred into 20 mL of fresh LBA and cultivated at 37 ^{o}C, 180 rpm until the optical density (OD_{600}) of the samples reached ~ 0.4–0.6. Then, the cells were induced for the endoglucanase expression by adding 0.05 mM IPTG and further cultured at 30 ^{o}C, 180 rpm for 5 hours. The cell biomass was collected by centrifugation at 8,000 rpm for 5 min and suspended in PBS buffer (1 L of PBS contained 8 g NaCl; 0.2 g KCl; 0.24 g KH_2PO_4; 1.42 g Na_2HPO_4, adjusted to pH 7) into OD_{600} of 20. The cells were kept at -20 ºC until used.

Most proteins expressed in the soluble form are generally biologically active. Therefore, the total, soluble and insoluble protein fractions of the cells were examined. Firstly 500 µL of the cells from -20 ^{o}C was quickly thawed in the water bath at 50 ^{o}C then disrupted by sonication on ice with a period of 3 seconds of sonication and 3 seconds of rest for a total of about 30 to 40 pulses at 20 kHz. Then, 50 µL of sonicated cells (total proteins) was transferred to an Eppendoft tube. The supernatant (soluble fraction) was harvested by centrifugation at 13000 rpm for 10 min. The pellet (insoluble fraction) was suspended by 450 µL sterile distilled water. All the proteins in total, soluble and insoluble fractions were analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on the polyacrylamide gel 12.6%.

To assess whether the expressed enzymes are active or not, we tested the enzymes' activity from the total, soluble phase, insoluble phases on an agar plate containing CMC as described by Joshi et al. (2021). The protein solutions (10 µL) were dripped down a 5 mm diameter filter paper placed on an agar plate containing 0.5% CMC. Positive control was the same volumes of 0.1 U cellulase (Sigma) and negative control was PBS. The plate was incubated at 37 ºC for about 15 hours. The plate was stained with Congo red 0.1% for 30 minutes and then washed with 1 M NaCl solution until a clear zone against the red background was observed (Kasana et al., 2008).

Imidazole impact on the solubility of the recombinant enzyme

Proteins that are susceptible to being precipitated by imidazole will greatly interfer their purification. Therefore, before purification, the recombinant proteins expressed from the strains were tested for precipitation in the presence of imidazole. Accordingly, the soluble proteins from the 2 expressing *E. coli* BL21 and Rosetta 1 strains were supplemented with imidazole to final concentrations of 20, 50, 200, and 250 mM, mixed well and left on ice for 5 min. The soluble fractions were then separated by centrifugation at 13,000 rpm for 5 min and transferred to a new tube. The remaining precipitate was added with an equal volume of PBS buffer, pH 7 and resuspended well. The soluble and insoluble fractions at the different imidazole concentrations were analyzed by SDS-PAGE.

Purification of recombinant endoglucanase by affinity chromatography

The recombinant endoglucanase from soluble proteins fraction was purified by affinity chromatography as described by Nguyen et al. (2019) with little change. Specifically, the cells (5 mL) at OD_{600} of 20 from -20 ^{o}C were thawed then sonicated for 9 min. The cell lysates were centrifuged at 12,000 rpm for 10 min. The EG5-containing soluble phase in PBS was added with imidazole to a final concentration of 20 mM. The sample was mixed well then applied into a 3 mL HisTrap column previously equilibrated with PBS buffer pH 7 containing 20 mM imidazole. The fluid that flowed through the column was collected to check for endoglucanase binding. The contamination

proteins were further washed with 15 mL of PBS buffer containing 20 imidazoles and then 75 mM imidazole. The EG5 bound to the column was eluted with 15 mL PBS buffer containing 200 mM imidazole (2 mL/fraction). Proteins in the fractions were examined by SDS-PAGE on 12.6% polyacrylamide gels. The purified enzyme was desalted by a PD-10 ultrafiltration column.

The purity of the recombinant endoglucanase was assessed using Quantity One software as described by Rhodes & Laue (2009). Briefly, the purified enzyme was analyzed by SDS-PAGE. Then the protein-containing polyacrylamide gel was scanned with a scanner and uploaded to Quantity One software. The software recognized protein bands including target and standard proteins, and then plotted peaks based on protein density. The quantity of target protein and total proteins in a certain lane was calculated. The purity is a ratio of the target protein to the total proteins in a lane.

Endoglucanase activity was tested by zymogram as described by Bischoff et al. (2006) with a little modification. Specifically, the 9% native polyacrylamide gel was prepared similarly to the SDS-PAGE gel the native gel contained 0.1% CMC and without SDS. The samples (50 µL) were mixed well with 10 µL of sample buffer 6x (0.1 M Tris-HCl pH 6.8; 60% glycerol; 0.06% bromophenol blue). Each 15 µL of the sample was loaded into a well and duplicated in another gel. The electrophoresis was carried at 100 V for 1 hour. One gel was stained with coomassie brilliant blue to determine the position of the protein band, the other was used to determine the endoglucanase activity by following steps: wash the gel with de-ionized water to remove SDS, incubate the gel for 30 minutes at 37 ºC. Gels were stained with 0.1% Congo red and washed with 1 M NaCl solution.

RESULTS

Expression of endoglucanase EG5

The gene *eg5* coding for mature endoglucanase derived from goats' rumen bacteria was 2,094 bp in length, encoding for the enzyme of 77.4 kDa (theoretical calculation) which contains hexahistidine at the C-terminus. Electrophoresis of total, soluble and insoluble protein fractions showed that, in the presence of inducer IPTG in the medium during culture, the recombinant *E. coli* BL21 and Rosetta 1 strains synthesized endoglucanase of 77.4 kDa at a high level as expected (Fig. 1A). While a very little band of proteins that had the same size as with the recombinant enzyme were also observed in the proteins from the strains that were not induced with IPTG (Fig. 1A). Besides the strains, we also expressed the *eg5* gene in other *E. coli* strains including JM109, C43, SoluBL21 but the enzyme was not produced in these strains (results not shown).

The investigation of the recombinant endoglucanase in soluble and insoluble fractions of the cells showed that most enzymes were expressed in the soluble phase in both recombinant *E. coli* strains BL21, Rosetta 1 and expression levels were the same in both strains. To assess whether the expressed enzyme is active or not, we tested the enzyme activity from total, soluble, insoluble phases on an agar plate containing a 0.5% CMC substrate. Under the action of cellulase, the hydrolyzed CMC will no longer be colored with the dye, forming a bright halo visible to the naked eye around the site of cellulase solution.

The results showed that PBS buffer (negative control) did not hydrolyze CMC thus there was not a hydrolytic zone occurred. In all the samples (the sonication lysates of both cells BL21, Rossetta 1 strains that were cultured and induced or not induced with IPTG) appeared bright clear zones of hydrolysis the same as the positive control. However, in the samples derived from non-induced cells, the bright zones were significantly lower than the zones observed from the samples of the induced cells. The CMC-degrading enzyme activity in the soluble proteins obtained from the two recombinant strains was similar and the

clear zones were the largest. Besides, a little protein band of endoglucanase observed in the insoluble fraction in SDS-PAGE gel also made clear zones, but the zones were much smaller than those from soluble samples (Fig. 1). Therefore, either strain can be selected for gene expression to get raw material for endoglucanase purification.

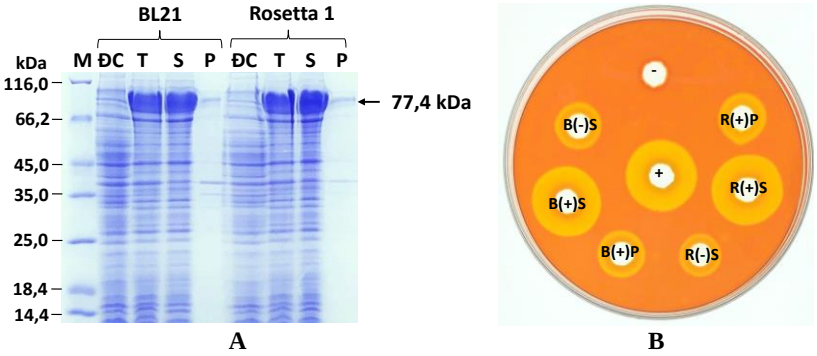

Figure 1. Analysis of proteins from *E. coli* BL21 and Rosetta 1 strains carrying *eg5* gene on 12.6% polyacrylamide gel **(A)** and cellulase activity test of the samples in a plate containing 0.5% CMC **(B).** M: protein marker (Thermo Scientific, #SM0431), ĐC: The corresponding *E. coli* strain carrying the *eg5* gene was cultured in the medium without the IPTG inducer; T: total protein; S: proteins in the soluble phase; P: proteins in the insoluble phase; B(-)S, B(+)S, B(+)P: total protein from uninduced BL21 strain, soluble and insoluble proteins from induced BL21 strain respectively. R(-)S, R(+)S, R(+)P: total protein from uninduced Rosetta 1 strain, soluble and insoluble proteins from induced Rosetta 1 strain respectively

Assessment of the susceptibility of recombinant endoglucanase with imidazole

As analyzed above, proteins that are easily precipitated by imidazole will be hardly purified successfully by affinity chromatography with hexahistidine under native conditions. Before purification, we evaluated the sensitivity of recombinant endoglucanase generated from two strains with different imidazole concentrations.

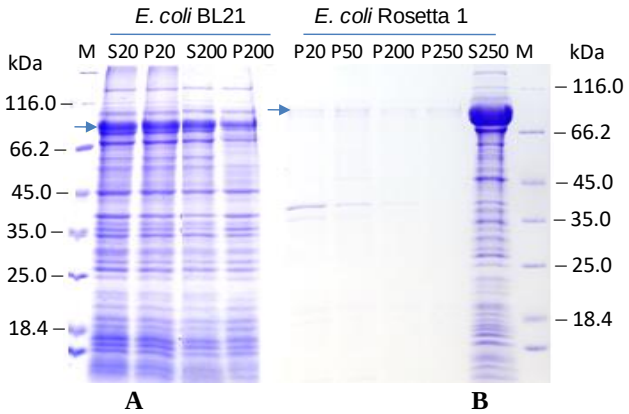

Figure 2. Effect of different imidazole concentrations on the precipitation of recombinant endoglucanase from *E. coli* BL21 (A) and Rossetta 1 (B) strains analyzed by SDS-PAGE. M: protein marker (Thermo Scientific, SM0431); S: soluble proteins; P: precipitated proteins; 20, 50, 200, 250: imidazole concentration in the samples. Arrows indicate the site of recombinant endoglucanase

The results (Fig. 2) showed that the endoglucanase from *E. coli* strain BL21 was precipitated by imidazole even at a low concentration of 20 mM (Fig. 2A) and the enzyme in *E. coli* Rosetta 1 strain was not precipitated by imidazole at a high concentration of 250 mM (Fig. 2B). Therefore, we decided to use the enzyme obtained from the *E. coli* Rosetta1 strain for purification.

Purification of recombinant endoglucanase by His-tag affinity chromatography

In theory, when the sample is loaded in the affinity chromatography column, the His-tag in the EG5 will strongly bind to the Ni^{2+} ions, so the EG5 is retained in the column, while other proteins flow out. However, the purification efficiency also depends on protein characteristics, buffer composition, salt concentration and sample pretreatment steps. In this case, the condition for EG5 purification was investigated. The results showed that, at the 20 mM imidazole, little protein with the same endoglucanase size flowed out because of the sample overload. When increasing the imidazole concentration to 100 mM, a large part of the enzyme was washed out of the column, exhibited by the thick bands in three fractions W2-4 (Fig. 3A). The target protein was eluted PBS buffer containing 200 mM imidazole and it was high purity observed in the polyacrylamide gel (Fig. 3A).

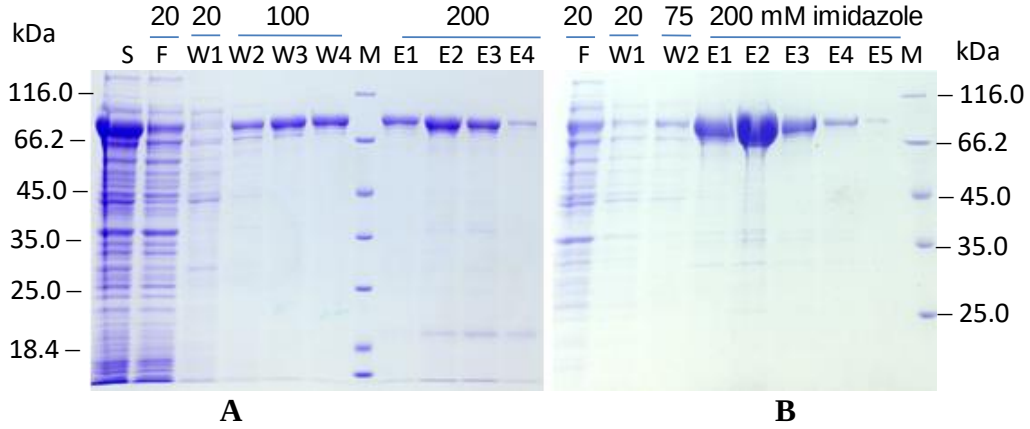

Figure 3. SDS-PAGE analysis of protein fractions during EG5 purification process 1 (A) and 2 (B). S: Soluble proteins from sonicated cell lysate; F: flowed proteins; W: washing fractions at different concentrations of imidazole; E_1-E_e: eluted fractions; M: protein marker (Thermo Scientific, SM0431)

Because by the washing with PBS buffer containing 100 mM imidazole, the enzyme was released quite a lot, therefore, we reduced the imidazole concentration for the washing step to 75 mM (Fig. 3B). The results showed that most of the contaminated proteins were washed out of the column at 20 mM and 75 mM imidazole, but a few EG5 were washed out, which is acceptable. The target proteins were obtained from eluting buffer containing 200 mM imidazole and were concentrated mainly in fractions 1 and 2, especially in fraction 2 (Fig. 3B). The target enzymes in fractions 1 and 2 were pooled together and subjected to a PD10 column for desalting against PBS buffer. The purity of the enzyme after purification and desalting was assessed by Quantity One software based on the scanning picture of the protein in polyacrylamide gel (Fig. 4).

The observed spectral image showed a single peak, which means the enzyme was pure and the purity reached 97.5% (Fig. 4). Thus, the endoglucanase enzyme has been successfully purified and has high purity.

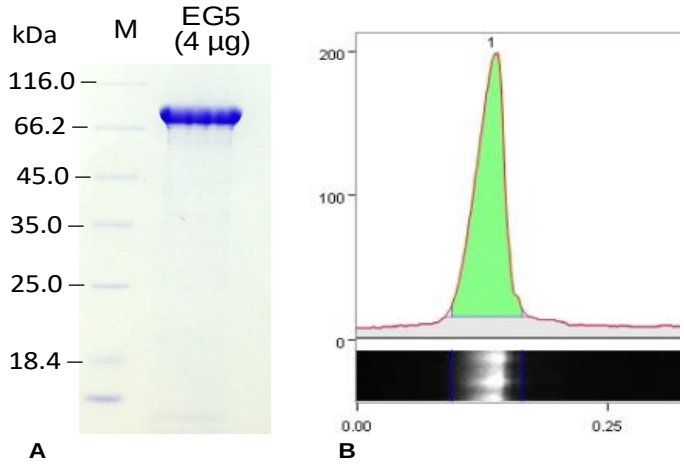

Figure 4. SDS-PAGE analysis of recombinant endoglucanase after purification and salt removal (A) and measurement of endoglucanase purity by QuantityOne software (B)

Assessment of the recombinant endoglucanase activity by zymogram

The pure endoglucanase after purification and salt removal was checked activity by zymogram. In the native polyacrylamide gel 9%, recombinant endoglucanase occurred a smear extending from over 55 kDa to approximately 100 kDa. The commercial cellulase (Sigma) contained at least 2 proteins that were visible in the coomassie brilliant blue stained gel (Fig. 5). In the gel containing CMC and stained with Congo red, hydrolysis zones were observed as bright smears extending from wells to the sites of enzymes but strong signals were seen at the enzyme positions. That means during immigration in the gel, the enzyme passed through and simultaneously hydrolyzed the substrate. This result confirmed that the recombinant endoglucanase EG5 was successfully expressed, purified from the recombinant *E. coli* Rosetta strain. This purified enzyme will be used for its characterization.

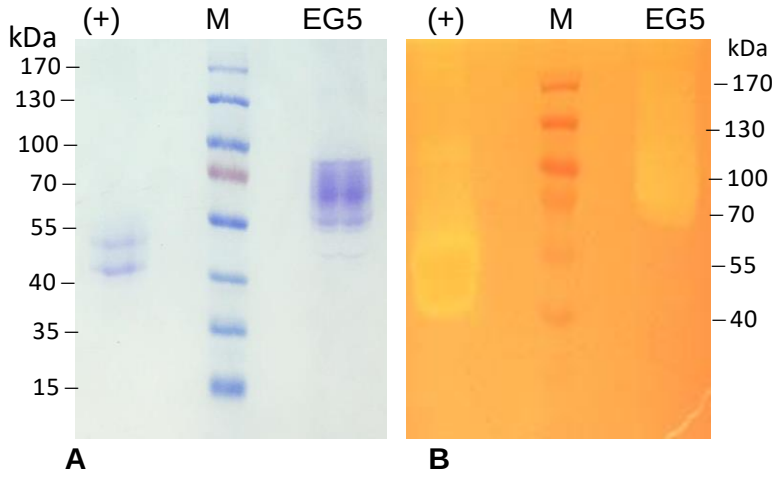

Figure 5. Analysis of desalted purified endoglucanase on native polyacrylamide gel 9% containing CMC. A. Bromophenol blue staining gel; B. Congo red staining gel.
(+): Commercial cellulase; M: Page ruler prestained protein Ladder (Fermentas);
EG5: recombinant endoglucanase

DISCUSSION

Microbial endoglucanases are useful enzymes due to their vast industrial applications in pulp and paper industries and bio-refinery. To improve enzyme production for potential industrial applications, there are many researches expressing the enzymes in *E. coli*. For seeking and screening of potential endoglucanase for application, in our laboratory, total of 5 genes were derived from metagenomic DNA data of bacteria in goats' rumen, coding for endoglucanases were expressed in *E. coli*, in which 4 enzymes belonging to glycosyl hydrolase GH5. However, 2 enzymes did not contain an additional domain besides the catalytic domain, one enzyme harbors additional CBM72 domains (that was designated as endoglucanase EG3) (Nguyen et al., 2021) and the other possessed two CBM72 domains (called endoglucanase EG5). In this study, the endoglucanase EG5 was expressed in *E. coli* and purified for enzyme characterization in the future. As the obtained results, endoglucanase EG5 was highly expressed in induced *E. coli* BL21 and Rosetta 1 strains and had endoglucanase activity to strongly hydrolyze CMC on the agar plate.

In theory, the transcription of *eg5* in the pET22-eg5 vector will be regulated by IPTG-inducing promoter T7, thus the recombinant enzymes are only produced in the cells cultured in a medium containing inducer IPTG. However, in this study, total proteins from uninduced strains also had endoglucanase activity. This could be explained by the untightly regulated T7 promoter resulting in leaking expression of recombinant enzymes as was seen in other studies (Du et al., 2021; Kang et al., 2007) because the original strains did not have the activity (data not shown).

Usually, soluble enzymes have activity because of having good structure and insoluble enzymes have not got activity because of improper structure. However, in this study, a little protein band of insoluble endoglucanase observed in the SDS-PAGE gel also made clear zones although the zones were much smaller than that from soluble samples (Fig. 1). This may be caused by the renaturation of the enzyme during the solution diffusing into CMC agar and incubation.

Imidazole is known as the factor to solubilize and stabilize recombinant proteins. For example, most RAFT3 single-chain Fv antibody fragment was expressed in *E. coli* in soluble fusion form with hexahistidine at the C terminus. This antibody fragment was specific for melanoma-associated proteoglycan and thus may assist in the clinical radio imaging study. Imidazole helped to stabilize the antibody fragment in solution (Hamilton et al., 2003). However, in this study, the presence of imidazole resulted in the precipitated recombinant endoglucanase produced in the *E. coli* BL21 strain but had no effect on the recombinant enzyme produced in *E. coli* Rosetta 1 strain (Fig. 2). The reason is that different *E. coli* strains have different capacities for recombinant enzyme synthesis and structure conformation, leading to the difference in the enzyme properties. To our knowledge, this is the first study that showed the negative effect of imidazole to precipitate recombinant enzymes expressed in *E. coli*.

The protein purification processes are based on protein size, physicochemical properties, or protein affinity interactions. By design, the recombinant endoglucanase was fussed with hexahistidine at the C terminus that is convenient for purification of the enzyme by His-tag affinity chromatography. Thus, the endoglucanase was successfully purified with a purity of over 97% (Fig. 4) by PBS buffer. There are many buffers were investigated for enzyme purification. Shi et al. (2013) expressed endoglucanase derived from *Dictyoglomus thermophilum* in *E. coli* BL21 then purified recombinant by His-tag affinity chromatography using the citrate-phosphate buffer. By this buffer, another endoglucanase also was successfully purified Shi et al. (2013). In 2015, purified recombinant endoglucanase derived from *Alicyclobacillus vulcanalis* expressed in *E. coli* DH5α by His-tag affinity chromatography using sodium phosphate

buffer, 500 mM NaCl, and obtained the enzyme by varying the pH from 4.55 to 7 (Boyce & Walsh, 2015). Vadala et al. (2021) purified recombinant cellulase expressed *E. coli* BL21 by His-tag affinity chromatography using a buffer containing 300 mM NaCl, 10 mM Tris-Cl pH 8, in which washing buffer contains 20 mM imidazole, collection buffer containing 200 and 250 mM (Vadala et al., 2021). In this study, we also investigated buffers for enzyme purification. Accordingly, the enzyme was soluble in some buffers such as Tris, PBS and phosphate buffer. But the activity of the enzyme was best in PBS buffer. Thus, the PBS buffer was chosen for the purification of this enzyme. The His-tag affinity chromatography is widely used for the purification of many cellulases derived from many microorganisms in *E. coli* such as cellulase from *Streptomyces* G12 (Amore et al., 2012), *Serratia proteamaculans* CDBB-1961 (Cano-Ramírez et al., 2016).

The purified and desalted endoglucanase was a sharp band of 77 kDa in the denatured polyacrylamide gel 12.6% (Fig. 4) but was seen as a smear extending from over 55 kDa to approximately 100 kDa in the native polyacrylamide gel 9%. This may be caused by different structures of the enzyme or by the interaction and binding of the enzyme with CMC in the native gel as observed in another study (Nguyen et al., 2021). The smear endoglucanase in the native gel exhibited activity by zymogram. This is the method widely used from the end of the 20th century up to now for detecting endoglucanase activity in polyacrylamide gel by adding a specific substrate into the composition of native gel (Sharma & Guptasarma, 2017; Holt & Hartman, 1994). By this way, enzymes usually appear in some sizes depending on the enzyme polymerization or interaction with the substrate and the hydrolysis zones are bright smear (Holt & Hartman, 1994).

CONCLUSION

Endoglucanase GH5-CBM72-CBM72 derived from goat rumen was expressed in *E. coli* in soluble form. The enzyme synthesized in *E. coli* BL21 is sensitive to be precipitated by imidazole. Endoglucanase expressed in *E. coli* Rosetta 1 was not sensitive with imidazole and was successfully purified by His-tag affinity chromatography. After desalting, the purified enzyme had a purity 97.5%. The enzyme exhibited endoglucanase activity hydrolyzing CMC to a bright halo around the enzyme band when stained the gel with Congo red by zymogram. This is the first study showing the negative effect of imidazole to precipitate recombinant endoglucanase synthesized in *E. coli* BL21. This is valuable for research applications.

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