Polyhistidine Affinity Chromatography for Purification and Biochemical Analysis of Fungal Cell Wall-Degrading Enzymes

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1. Introduction

More than 10 years ago, proteins had to be purified to homogeneity by a combination of multiple chromatography steps, and their amino-terminus sequences were determined before their genes could be cloned. Nowadays, purification of native proteins is still an important step, but the utilization of recombinant proteins, which are produced by microbes, fungi, plants and animals by means of introducing an expression vector harboring a coding DNA sequence, provides a time-saving and useful way for analyses of protein characteristics and crystal structure, and for industrial applications. For detailed biochemical characterization, purified proteins (single molecular preparations) are preferentially used in order to avoid the effects of contaminant proteins.

A significant technique for quickly obtaining purified proteins is affinity chromatography using epitope-tags and antibodies. Epitope-tags, such as polyhistidine, hemagglutinin (HA) and FLAG among others, are easily fused to recombinant proteins by preparing a DNA structure containing the nucleotide sequence of the epitope-tag at the 5' and/or 3' end of the cDNA of the protein of interest. Following expression, the epitope-tagged recombinant protein can be specifically separated by affinity chromatography using epitope-tag binding and/or antibody-linked resins. Antibodies against epitope-tags are also used for immunoblot analysis and immune-precipitation. Although polyclonal or monoclonal antibodies against recombinant proteins are also precious tools for immune-precipitation and cellular localization, they may not be suitable for use with many kinds of recombinant proteins due to the requirement for antibodies corresponding to the individual protein.

The recent development of molecular biological techniques has contributed to elucidation of the genome DNA sequence of many species. The resultant sequence databases are being used in various fields, including gene expression analyses and protein engineering. To identify the function of proteins encoded by genome DNA sequences, the production and purification of recombinant proteins has become an essential strategy, in which the addition of an epitope-tag sequence enhances both the preparation of purified recombinant proteins and the following characterization.

An example for this epitope-tagged technique can be applied on the preparation of purified recombinant cell wall-degrading enzymes and the functional analyses. Microbial cell wall-degrading enzymes play a significant role in the degradation of both their own cell walls and plant cell walls during infection (Reese, et al., 1950; Henrissat, et al., 1985; Wood, 1992). In addition, plant cell wall-degrading enzymes are also involved in wall loosening during cell expansion, cell wall biosynthesis, and countermeasures against infectious pathogens (Walton, 1994; Nicol, et al., 1998; Takeda, et al., 2002). These enzymes, especially those from microbes, are one of the most important industrial products with applications in, for example, beer and wine, animal feed, paper, textile, laundry detergent, and food ingredient industries (Bhat, 2000). Hence, reducing cellulase manufacturing costs by increasing the productivity of cellulases with high specific activities through biotechnological modification is a desired research goal. To identify cell wall-degrading enzymes that are needed for various industrial applications, it is necessary to carry out a series of steps encompassing the production, purification, characterization and molecular modification of cell wall-degrading enzymes. Here, the production, purification and characterization of recombinant epitope-tagged proteins are described, with a particular focus on fungal cell wall-degrading enzymes.

2. Production of epitope-tagged recombinant proteins

2.1 Addition of an epitope-tag to a recombinant protein

To identify recombinant proteins produced by host cells, visualization by Coomassie brilliant blue 250-R (CBB) staining and immunoblotting after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are routinely carried out. Furthermore, purifying recombinant proteins is an important procedure for determining biochemical properties, such as substrate specificity, optimum reaction temperature and pH, and stability. Epitope-tags, which consist of the amino acids shown in Table 1, are useful and convenient tool both for purifying proteins using epitope-tag affinity chromatography and for immunoblot analysis using antibody.

Epitope-tags	Amino acid sequence
Heptahistidine	HHHHHHH
HA	YPYDVPDYA
c-Myc	EQKLISEEDL
FLAG	DYKDDDDK
VSV-G	YTDIEMNRLGK
HSV	QPELAPEDPED
V5	GKPIPNPLLGLDST

Table 1. Amino acid sequences of epitope-taggs

2.2 Expression of polyhistidine-tagged protein

For the detailed analysis of cell wall degradation, *Trichoderma reesei* endo-1,4-β-glucanase (TrCel12A) was produced in *Brevibacillus choshinensis* (Takara-Bio) and purified using a polyhistidine-binding resin (Clontech). The *TrCel12A* gene was cloned by PCR using GXL DNA polymerase (Takara-Bio) and specific DNA primers, which were designed on the basis of the *T. reesei* genome DNA sequence. A DNA sequence encoding seven contiguous histidines was added to the 3' end of the *TrCel12A* gene by PCR as shown in Fig. 1.

Fig. 1. Scheme of production of recombinant His$_7$-tagged TrCel12A in *B. choshinensis*. *Nm^r*; neomycin resistance gene, *Amp^r*; ampicillin resistance gene, ColE1 *ori*; *E. coli* replication origin.

The synthesized DNA was cloned into an expression vector (pNCMO2) for protein expression in *B. choshinensis.* The construct was transformed into *B. choshinensis* cells by electroporation. The transformants were screened on MT [1% (w/v) glucose, 1% (w/v) Tryptone, 2% (w/v) Beef extract, 2% Yeast Extract, $FeSO_4$ (65 µmol/L), $MnSO_4$ (41 µmol/L), $ZnSO_4$ (6 µmol/L) and $MgCl_2$ (43 µmol/L), pH7.0] agar plates supplemented with neomycin (10 µg/mL), and the transformants obtained were cultured for producing recombinant heptahistidine-tagged (His_7-tagged) TrCel12A as previously described (Takeda, et al., 2010). *B. choshinensis*, which is a Gram-positive bacteria, secretes synthesized proteins into the culture medium when a secretion signal peptide is fused to the recombinant protein. In contrast, *Escherichia coli*, which is widely used as a host cell, accumulates synthesized recombinant proteins in the cytosol. Because the amount of proteins secreted into the culture medium is very small as compared with that of cytosolic proteins, secretion of the recombinant protein will make any subsequent purification procedure easier.

2.3 Purification of His_7-tagged TrCel12A

The culture filtrate of *B. choshinensis* expressing His_7-tagged TrCel12A was subjected to ammonium sulfate (60%, w/v) precipitation, and the precipitate obtained by centrifugation was dissolved in Equilibration buffer (10 mM sodium phosphate, pH 7.0, 50 mM NaCl) for polyhistidine affinity chromatography. The solution was applied to a polyhistidine-binding resin charged with cobalt ion: on this column, His_7-tagged TrCel12A is captured by cobalt ion and released by buffer exchange with imidazol. After washing the column with Equilibration buffer, the proteins that bound to the resin were sequentially eluted with 0.1 X Elution buffer (10 mM sodium phosphate, pH 7.0, 50 mM NaCl, 20 mM imidazol), Elution buffer (50 mM sodium phosphate, pH 7.0, 50 mM NaCl, 200 mM imidazol) and Elution buffer containing 40 mM EDTA. The fractions were subjected to SDS-PAGE followed by CBB staining to verify the purity of the eluates (Fig. 2A).

Expressed His_7-tagged TrCel12A was detected at around 24 kDa (Fig. 2A). The eluate in 0.1 X Elution buffer (Fig. 2A, lane 1) contained His_7-tagged TrCel12A and several other proteins. The eluate in Elution buffer (Fig. 2A, lane 2) consisted of mostly His_7-tagged TrCel12A and a small quantity of other proteins. In contrast, the eluate in Elution buffer containing 40 mM EDTA (Fig. 2A, lane 3) contained His_7-tagged TrCel12A without other visible proteins. Thus, His_7-tagged TrCel12A and non-specific binding proteins are gradually eluted from the resin with increasing imidazol concentration. These results imply that most His_7-tagged TrCel12A remained bound to the resin after application of the Elution buffer recommended by the manufacturer, and that His_7-tagged TrCel12A was best eluted with Elution buffer containing EDTA. EDTA chelates cobalt ion from the resin, resulting in the release of TrCel12A that bound tightly to the resin. Subsequently, EDTA and cobalt ion were removed from the protein preparation by ultrafiltration.

2.4 Immunoblot analysis using antibody against polyhistidine-tag

Recombinant proteins with an epitope-tag are recognized by an antibody against the corresponding epitope-tag. For example, His_7-tagged TrCel12A expressed in *B. brevibacillus* was subjected to SDS-PAGE, followed by immunoblotting using an antibody against

polyhistidine (Qiagen) (Fig. 2B). Immunoblot analysis showed that the molecular weight of the proteins that reacted with antibody was identical to that of the major proteins observed in the eluate from polyhistidine-binding resin by CBB staining (Fig. 2A). Purified protein in the eluate with Elution buffer containing EDTA was recognized by the antibody.
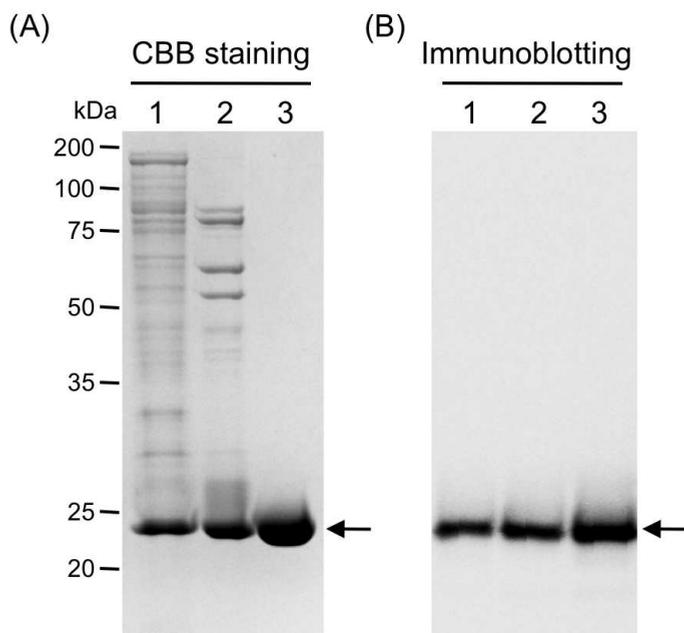

Fig. 2. Visualization of His$_7$-tagged TrCel12A with CBB (A) and immunoblotting using antibody against polyhistidine-tag (B) after separation by SDS-PAGE. Lane 1, eluate from polyhistidine-binding resin eluted with 0.1 X Elution buffer; lane 2, eluate with Elution buffer; lane 3, eluate with Elution buffer containing 40 mM EDTA. Arrows indicate His$_7$-tagged TrCel12A.

3. Biochemical analysis of His$_7$-tagged protein

3.1 Effect of EDTA on purified His$_7$-tagged TrCel12A

Native TrCel12A catalyzes the hydrolysis of 1,4-β-glucans such as carboxymethyl cellulose (CMC), crystalline cellulose, phosphoric acid-swollen cellulose (PSC), xyloglucan and 1,3-1,4-β-glucan (Sprey and Uelker, 1992). For determination of its biochemical properties, recombinant His$_7$-tagged TrCel12A must retain hydrolytic activity after elution with Elution buffer containing EDTA. To test the effect of EDTA treatment on hydrolytic activity, His$_7$-tagged TrCel12A was incubated with 1,3-1,4-β-glucan and sodium acetate (100 mM, pH 5.5) in the presence of different concentration of EDTA. Hydrolysis of 1,3-1,4-β-glucan by TrCel12A results in the increase in the number of the molecule with a reducing terminus. The reaction mixture was mixed with *p*-hydroxybenzoic acid hydrazide and incubated in a boiling water. Hydrolytic activity was determined by measuring the absorbance at 410 nm in a spectorphotometer as described

previously (Miller, 1972) (Fig 3). The hydrolytic activity of His$_7$-tagged TrCel12A was not negatively influenced by EDTA. Similarly, the hydrolytic activities of recombinant proteins that we produced previously a xyloglucan-specific endoglucanase (XEG) in *B. choshinensis*, β-glucosidases in *Aspergillus oryzae* and cellobiohydrolase in *Magnaporthe oryzae* were not negatively affected by EDTA. However, not all His$_7$-tagged recombinant proteins are applicable to EDTA elution because some enzymes, such as peroxidases, require metal ions for their catalytic reaction, and these metal ions are removed by the chelater EDTA (Cohen, et al., 2002; Lundell, et al., 2010).
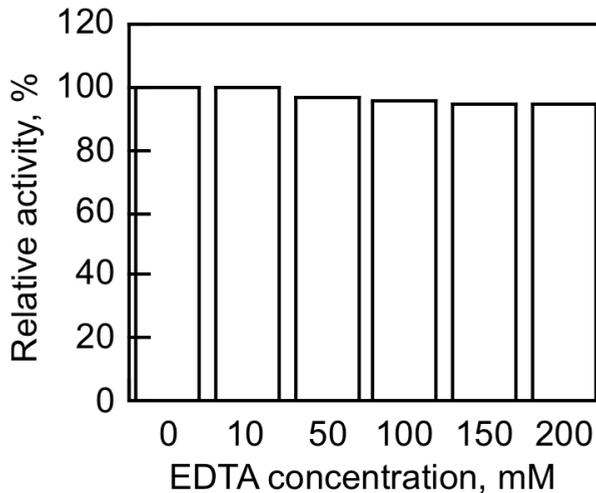
Fig. 3. Effect of EDTA on the hydrolytic activity of purified His$_7$-tagged TrCel12A. The hydrolytic activity of His$_7$-tagged TrCel12A toward 1,3-1,4-β-glucan was assayed in the presence of EDTA.

3.2 Substrate specificity and product analysis

To characterize recombinant His$_7$-tagged TrCel12A expressed by *B. choshinensis*, its hydrolytic activity was assayed as described above. His$_7$-tagged TrCel12A preferentially hydrolyzed water-soluble β-1,4-glucans such as xyloglucan and 1,3-1,4-β-glucan, and slightly cleaved crystalline cellulose, PSC and CMC, of which the greatest hydrolysis was observed toward 1,3-1,4-β-glucan (Fig. 4). HPLC (ICS-3000, Dionex) analysis of the hydrolyzed products showed that His$_7$-tagged TrCel12A produced mainly cellobiose, cellotriose and cellotetraose from PSC after a long incubation (Fig. 5). Thus, His$_7$-tagged TrCel12A purified by polyhistidine affinity chromatography was accurately characterized without effects from other contaminant proteins. Native TrCel12A in *T. reesei* culture filtrate has previously been purified to homogeneity by a combination of chromatography steps; in contrast, recombinant His$_7$-tagged TrCel12A with hydrolytic activity was purified by one-step polyhistidine affinity chromatography.
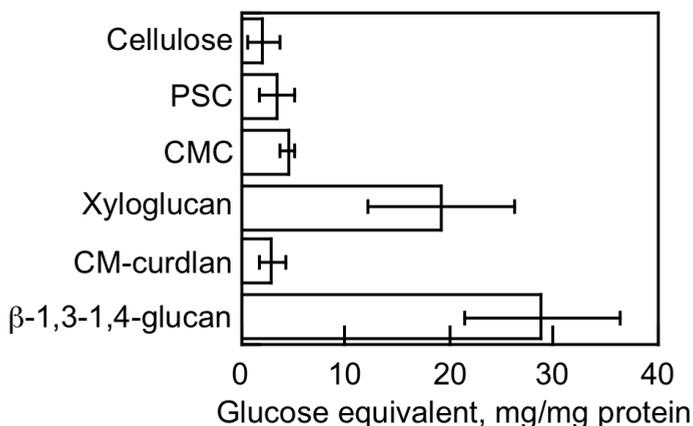
Fig. 4. Substrate specificity of His$_7$-tagged TrCel12A. Hydrolytic activity of His$_7$-tagged TrCel12A was determined using diverse polymers as indicated on the y axis. Data are the means ± SE of three determinations.
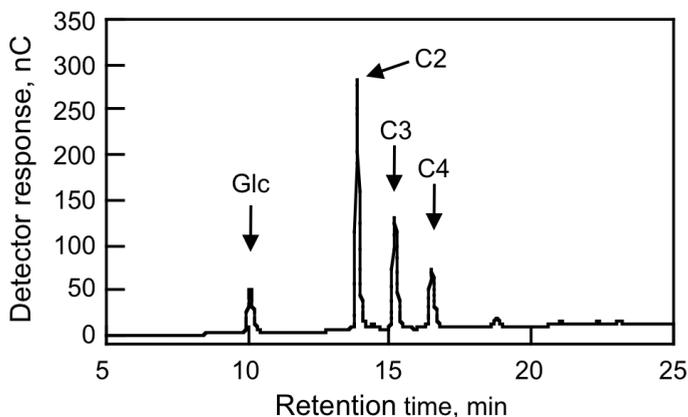
Fig. 5. Product analyses of hydrolysates from PSC. After incubation of PSC with His$_7$-tagged TrCel12A in sodium phosphate buffer (100 mM, pH 5.5), a portion of the reaction mixture was subjected to HPLC. Arrows indicate the position of glucose (Glc), cellobiose (C2), cellotriose (C3) and cellotetraose (C4) eluted during the separation.

4. Other cell wall-degrading enzymes with His$_7$-tag

Magnaporthe oryzae is the pathogen that causes rice blast, the most devastating fungal disease of rice, and it secretes a variety of cell wall-degrading enzymes during its invasion of rice. The complete genome DNA sequences of *Magnaporthe* genera have been elucidated (Dean et al., 2004; Yoshida et al., 2007). Therefore, genetic analysis and protein engineering can be facilitated by utilizing these DNA databases. Indeed, recombinant proteins with hydrolytic

activities toward cell wall polymers and oligosaccharides have been produced after cloning the corresponding DNA by PCR from complementary DNA pools. For example, cellobiohydrolase (MoCel6A), which preferentially hydrolyzes PSC, cellulose and 1,3-1,4-β-glucan, has been produced from *M. oryzae* and *Aspergillus oryzae* (Takahashi, et al., 2010). β-Glucosidases (MoCel3A and MoCel3B), which produce glucose from β-1,3- and β-1,4-glucans, have been produced from *M. oryza*e (Takahashi, et al., 2011). A specific 1,3-1,4-β-glucanase has been produced from both *B. choshinensis* and *M. oryzae* (Takeda, et al., 2010). The purified proteins were obtained by using polyhistidine affinity chromatography coupled with an EDTA-containing elution buffer. In contrast, for the preparation of β-glucosidase (UeBgl3A) from *Ustilago esculenta*, whose genome DNA sequence has not been elucidated, the partially purified β-glucosidase was first obtained in low quantity after two steps of ion affinity chromatography and then subjected to trypsin-digestion followed by MS/LC/LC analysis to identify the partial amino acid sequence. On the basis of the deduced peptide sequence, the cDNA was then cloned by using degenerate DNA primers. Lastly, His$_7$-tagged UeBgl3A was expressed by *A. oryzae* and purified by polyhistidine affinity chromatography (Nakajima, et al., 2011).

5. Conclusion

Purifying native and recombinant proteins is an important procedure for the detailed analysis of properties such as mode of action and substrate specificity, and for determining optimal reaction conditions. From this viewpoint, the addition of epitope-tags to a protein facilitates easy purification by epitope-tag affinity chromatography; however, this method is applicable only to recombinant proteins. Especially, the polyhistidine-tag has the ability to bind metals attached to a resin and is liberated by exchange with imidazol, as demonstrated in this chapter. Furthermore, tightly bound proteins can be eluted by using EDTA, which chelates metals from the resin. In addition, antibodies against epitope-tags are a useful tool to detect epitope-tagged proteins. Because antibodies bind tightly to the corresponding antigen, however, it is necessary to use strong acid or SDS to release the antigens from the antibodies in which active enzymes would become inactivated. Thus, we have to proceed with caution when using affinity resins and antibodies for the purification and detection of epitope-tagged proteins.

So far, the large-scale production of recombinant proteins that catalyze the hydrolysis of cell walls has been carried out in *B. choshinensis*, *M. oryzae* and *A. oryzae* as a host cell in our lab because the heptahistidine-tag works well for purification, and non-specific binding of intact proteins are removed by increasing concentration of imidazole. On the other hand, His$_7$-tagged and HA-tagged TrCel12A have been produced in *Nicotiana benthamiana* by transient expression as described previously (Takken et al., 2000), and verified by immunoblotting using antibody against polyhistidine and HA, respectively (data not shown). However, purification of His$_7$-tagged TrCel12A expressed by *N. benthamiana* did not work well because it was difficult to separate His$_7$-tagged TrCel12A from many proteins that bound non-specifically to the polyhistidine-binding resin. Selecting the best epitope-tag to use in terms of not only purification but also immunoblotting should be done before the protein production procedure is established.

6. References

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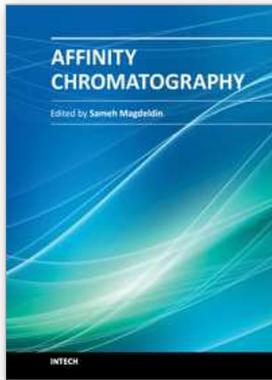
Affinity Chromatography

Edited by Dr. Sameh Magdeldin

Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific interactions on the ligand. Within this book, the authors tried to deliver for you simplified fundamentals of affinity chromatography together with exemplarily applications of this versatile technique. We have always been endeavor to keep the contents of the book crisp and easily comprehensive, hoping that this book will receive an overwhelming interest, deliver benefits and valuable information to the readers.

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