論文の内容の要旨

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論文題目

A study on endogenous beta-glucosidases from termites
and their applicability in biomass conversion
（シロアリ由来βーグルコシダーゼの生産とその利用に関する研究）

Introduction

Worries regarding to the current crisis of climate change and depletion of fossil fuels make the utilization of bioethanol as an attractive option for combating both global warming and less dependence on fossil fuels. A further increase in bioethanol production may come from lignocellulosic biomass, such as agricultural residues and wood. Lignocellulose from waste products is abundant, inexpensive, and renewable. Cellulose, which is a homopolysaccharide composed of β-D-glucopyranose units linked by β-1,4 glycosidic bonds, comprises around 40–50 wt.% of plant biomass. One of the best known cellulose decomposers is termite, which is able to hydrolyze 74-99% of the cellulose ingested. Taking advantage of this high efficiency with which termites digest cellulose, and due to the essentiality of β-glucosidase catalyzing the hydrolysis of cellobiose or cello-oligomers into the fermentable sugar, glucose, in the process of cellulose degradation, the objective of this work is to heterologously express, purify, characterize, and compare three endogenous glycoside hydrolase family 1 (GH1) β-glucosidases from termites. Further comparison was done with the commercial β-glucosidase Novozym 188. In addition, the effect of addition of termite β-glucosidases or Novozym 188 to Celluclast 1.5 L on the degradation of Avicel was studied. This work might introduce alternative and efficient enzymes to the bioethanol production field.

Chapter 1. Heterologous expression and production of endogenous β-glucosidases from termites

Despite the high efficiency of termites in the degradation of cellulosic materials, the production level is too low to be used for commercial exploitation. In this work I have used the expression systems of *Aspergillus oryzae* and *Pichia pastoris* to produce three endogenous β-glucosidases from termites. G1NkBG, an enzyme found in the salivary gland of the lower termite *Neotermes koshunensis*, was expressed in *A. oryzae*, a filamentous fungus that can produce and secrete large amounts of proteins and has a Generally Recognized as Safe (GRAS) status. Since production of two other enzymes, G1sgNtBG1 and G1mgNtBG1 from the salivary gland and midgut, respectively, of the higher termite *Nasutitermes takasagoensis*, was not successful in *A. oryzae* due to premature polyadenylation of transcripts, *P. pastoris* was used to express them. *P. pastoris* has been widely used to express heterologous proteins due to its easy manipulation and low cost of production. Both G1sgNtBG1 and G1mgNtBG1 were successfully expressed in a new vector called pBGP3, which is introduced in Chapter 4.

Chapter 2. Purification and characterization of β-glucosidases

G1NkBG was purified by ammonium sulfate precipitation followed by anion exchange, hydrophobic, and gel filtration chromatographies. G1sgNtBG1 and G1mgNtBG1 were purified by affinity (Ni^{2+}-NTA) chromatography. In the hydrolysis of lignocellulose, it is well-known that most β-glucosidases are strongly inhibited by the end-product, glucose, which may restrict the whole degradation process of cellulose. Differently from the majority of β-glucosidases, G1NkBG was not only resistant to glucose inhibition, but its activity against *p*-nitropheny β-D-glucopyranoside was stimulated by glucose: more than 90% of its maximum activity was retained in the presence of 1 M glucose, and with 0.2 M glucose the activity was stimulated by 1.3-fold. Although G1sgNtBG1 and G1mgNtBG1 did not show any stimulation by glucose, they were also very resistant to glucose inhibition. G1mgNtBG1 showed relatively high optimum temperature at 65°C. Regarding the thermostability, G1mgNtBG1 displayed more than 88% and 64% of its maximum activity, respectively, after 5 h of incubation at 55°C and 60°C. All β-glucosidases studied were active on cello-oligosaccharides. Having β-glucosidases resistant to glucose inhibition, with high thermostability, and active on cello-oligosaccharides, is very interesting for biotechnological applications, and they can be useful in bioethanol production.

Chapter 3. Comparative analysis on hydrolytic activities of purified β-glucosidases from termites with those of commercially available cellulases

For complete hydrolysis of cellulose, synergistic action of three different enzymes known as endoglucanase, cellobiohydrolase, and β-glucosidase is needed. In industry two kinds of commercial cellulases are usually used in the hydrolysis of cellulose; Celluclast 1.5 L from *Trichoderma reesei* and Novozym 188 from *Aspergillus niger*. While the content of the former is mainly endoglucanases and cellobiohydrolases, the latter is mainly composed of β-glucosidases. In this Chapter the thermostability and glucose-resistance of β-glucosidases from termites and Novozym 188 were compared. In addition, the cooperation of G1NkBG, G1sgNtBG1, G1mgNtBG1, and Novozym 188 with Celluclast 1.5 L in the hydrolysis of Avicel was examined. The results showed that G1mgNtBG1 was more thermostable than Novozym 188. All β-glucosidases from termites tested were far more glucose-tolerant than Novozym 188. When added to Celluclast 1.5 L, G1mgNtBG1 produced more reducing sugars than Novozym 188, G1NkBG, and G1sgNtBG1. This suggests that G1mgNtBG1 more efficiently removed accumulated cellodextrins in the reaction mixture which could have inhibited the activity of cellulase components in Celluclast 1.5 L. Hence, the results further suggest that G1mgNtBG1 serves as an enzyme that shows better synergism with other cellulolytic components.

Chapter 4. Vector construction for expression of N-tagged heterologous proteins in *P. pastoris*

Purification of proteins usually requires laborious works. Very often more than one chromatography step is needed, and setting/employing the best conditions for each steps is time-consuming and can lead to low yields and increased costs of production. With these issues in mind, recombinant proteins are usually expressed as fusions with extra sequences added at either N- or C-terminal end, which allows one-step purification by affinity chromatography. Hexahistidine tag is often used due to the high affinity of polyhistidines to the immobilized metal ion matrices such as Ni^{2+}, Zn^{2+}, and Co^{2+}. It is difficult, however, to predict which of the N- or C-terminal addition of tags is compatible with the correct folding, detection, and purification of the protein expressed. Hence having several options of vectors is helpful in determining the best way to express and purify expressed proteins. For this purpose, a sequence for the addition of N-terminal c-Myc and 6×His tags was inserted into pBGP1, a vector for the expression of C-terminal tagged proteins in *P. pastoris*, giving origin to pBGP2. Next, the redundant C-terminal tags in pBGP2 were removed to generate pBGP3. To test the effectiveness of pBGP3, three β-glucosidases (G1NkBG, G1sgNtBG1, and G1mgNtBG1) and

two endoglucanases (RsEG and NtEG) from termites were expressed using this vector. Western blot analysis with anti-c-Myc antibody confirmed the production of the enzymes. Purification was performed in one-step by Ni^{2+}-affinity chromatography as confirmed by SDS-PAGE analyses. These results demonstrate the efficacy of pBGP3 for the expression and one-step purification of heterologous proteins.

Conclusion

In parallel with an increasing demand for bio-based fuels, there is also a further interest for new technologies that improve lignocellulose hydrolysis. One way to reach such achievement is the research on cellulolytic enzymes which facilitate the degradation of cellulose. Particularly, as discussed in this work, β-glucosidase is a key enzyme in this process. I have worked on three endogenous β-glucosidases from termites, G1NkBG, G1sgNtBG1, and G1mgNtBG1. All of them were more glucose-tolerant than the majority of β-glucosidases including Novozyme 188, and active on cello-oligosaccharides. Moreover, G1mgNtBG1 was thermostable at 60°C. G1NkBG, G1sgNtBG1, and G1mgNtBG1 produced more reducing sugars than Celluclast 1.5 L alone, suggesting that termite β-glucosidases were effective in alleviating the inhibitory effect of cellodextrins on cellulases. Hence, they are potential enzymes that can be used as a supplement in the hydrolysis of lignocellulosic biomass.

Publications

1) Uchima, C. A., Tokuda, G., Watanabe, H., Kitamoto, K., and Arioka, M. (2011). Heterologous expression and characterization of a glucose-stimulated β-glucosidase from the termite *Neotermes koshunensis* in *Aspergillus oryzae*, Applied Microbiology and Biotechnology 89(6): 1761-1771.

2) Uchima, C. A. and Arioka, M. (in press) Expression and one-step purification of recombinant proteins using an alternative episomal vector for the expression of N-tagged heterologous proteins in *Pichia pastoris*, Bioscience, Biotechnology, and Biochemistry

3) Uchima, C. A., Tokuda, G., Watanabe, H., Kitamoto, K., and Arioka, M. (submitted) Heterologous expression in *Pichia pastoris* and characterization of an endogenous thermostable and high glucose-tolerant β-glucosidase from the termite *Nasutitermes takasagoensis*, Applied and Environmental Microbiology