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Cloning, characterization and expression of glucoamylase gene from ectomycorrhizal basidomycete, *Tricholoma matsutake*

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ABSTRACT: In order to confirm the presence of putative glucoamylase gene in *Tricholoma matsutake* genome, the genomic DNA was prepared from *T. matsutake* NBRC30773 strain and was used as template to clone the glucoamylases gene (*TmGlu1*). We obtained the nucleotide sequence of *TmGlu1* and its franking region. The coding region (from ATG to stop codon) is 2,186 bp. The locations of exons and introns were determined from the nucleotide sequences of 3'- and 5'-RACE PCR and RT-PCR products. On the other hand, to investigate the relationship between composition of medium and glucoamylase expression, we checked the expression level of glucoamylase gene by realtime reverse transcription PCR and measurement of glucoamylase enzyme activity. It was found that enzyme activity of glucoamylase was very low in different medium. Expression of glucoamylases gene appeared to not be affected by different carbon source.

KEYWORDS : Cloning, Glucoamylase gene, *Tricholoma matsutake*

Introduction

The ectomycorrhizal fungus *Tricholoma matsutake* is one of the most valuble edible mushroom in Japan. The annual production of *T. matsutake* in Japan was reportedly 12,000 tons in 1941, 211tons in 1995(Yamada, 2005), and decreased substantially to 24 tons in 2009. So far, *T. matsutake* is difficult to cultivate artificially without the host plant. Glucoamylase(1,4-a-D-glucan glucohydrolases; EC 3.2.1.3) are enzymes, which catalyze the relase of ß-D-glucose units from the nonreducing ends of amylose, amylopecting, and other polysaccharides (Sakaguchi, 1992).

However, in 1994, Ohta reported that as the ectomycorrhizal, *Lyophyllum shimeji* can form mature fruit bodies within a bottle containing a barley grain in a cultivation medium without a host plant. He mentioned that a sufficient quantity of starch used as a carbon source was able to supply the factor that allows successful fruit-body formation without raising the osmotic pressure in the medium. Glucoamylase are exohydrolases,that, are believed to be important in the utilization of starch by the basidiomycetous fungus. In 2000, in the study of *Lentinula edodes*, Zhao(2000) mentioned that glucoamylase may play an important role in the morphogenesis of the basidiomycetous fungus. In 2004, Kusuda *et al.*(2004) reported that glucoamylase activity in the medium increased markedly during fruit-body formation. Although glucomamylase-encoding genes have been cloned from several fungi including *Aspergillus awamori*, *Aspergillus niger*, *Neurospora crassa*, *L. edodes*(zhao, 2000) and so on, study on the cloning of *T. matsutake* have not been carried out.

In the present study, in order to to confirm the presence of putative glucoamylase gene in *T. matsutake* genome, we cloned and characterized glucoamylase gene from *T. matsutake*. On the other hand, to investigate the relationship between composition of medium and glucoamylase expression, we checked the expression level of glucoamylase gene and measurement of glucoamylase enzyme activity.

Materials and method

Fungal strains used in this study

Tricholoma matsutake NBRC30773 strains were used in this study.

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Medium composition and culture conditions

For experiments of the cloning of Glucoamylase gene in *T. matsutake*, the mycelium was grown on modified Hamada's agar medium(0.5% KH_2PO_4, 0.2% Yeast extract, 2% glucose, agar, 1.5%, pH 5.1) prepared with tap water at 25℃ for 60 days, after which the mycelium, along with five square agar blocks(5x5x5mm), was transferred to 20 ml of Modified Hamada's (0.5% KH_2PO_4, 0.2% Yeast extract, 2% glucose, pH 5.1) liquid medium prepared with tap water in a 100ml Erlenmeyer flask and grown at 25℃ for 60 days.

For the measurement of glucoamylase activity in different starch medium, the cultivation of *T. matsutake* was used in different starch medium containing 1% starch(glucose, starch corn, starch soluble, starch wheat, starch potato, amylose separately), 0.2% Yeast extract, 0.05% Hyponex, pH 5.1. The liquid medium prepared with tap water in a 100 ml Erlenmeyer flask and grown at 25℃ for 30 days.

For the investigate the expression of glucoamylase gene in *T. matsutake* in different starch medium, the mycelium was grown on 20ml of Modified Hamada's liquid medium prepared with tap water in a 100ml Erlenmeyer flask and grown at 25℃ for 30 days, was transferred to 10ml different starch medium prepared with tap water in a 50ml Erlenmeyer flask and grown at 25℃ for 2 days.

DNA and RNA preparation

To prepare genomic DNA from the *T. matsutake* NBRC30773 strain, Genomic DNA from lyophilized mycelium of *T. matsutake* strain NBRC30773 was prepared using a GENEALL Plant SV mini kit(Toyobo Co., Osaka, Japan) according to the manufacturer's instructions.

To prepare total RNA from *T. matsutake* NBRC30773 strain, the mycelia were harvested by filtration from Modified Hamada's liquid medium(for experiments of the cloning of glucoamylase gene) and different starch medium(for the investigate the expression of glucoamylase gene), frozen in liquid nitrogen, and ground to a fine powder in a mortar and pestle. Total RNA was extracted from the frozen powdered mycelia using an Rneasy plant Mini kit(Qiagen, Tokyo) according to the manufacturer's instructions.

Amplification of glucoamylase genes

All amplified DNA fragments were subcloned into the pT7Blue (R) T-vector(Novagen, Madison, WI, USA), and all plasmids were sequenced. PCR was performed with a Takara PCR Thermal Cycler Personal(Takara Bio Co., Shiga, Japan). Initially, fragments of genomic DNA encoding the putative glucoamylase protein(*TmGlu1*) were amplified by PCR with degenerate oligonucleotide primer pairs F15-GP2-AF/F15-GP2-BR. The F15-GP2-AF and F15-GP2-BR primers were designed based on the amino acid sequences GLGEPKF and FDLWEEI, respectively, which are conserved in the glucoamylase protein of *Lentinula edodes*(Zhao *et al*. 2000). PCR was carried out in a 50㎕ reaction containing 1 x Ex Taq buffer(Takara Bio Co.), 50ng of extracted genomic DNA, 50pmol of each primers, each dNTP at a concentration of 0.2 mM, and 1.25U of Ex Taq polymerase(Takara Bio Co.). PCR was carried out with an initial denaturation for 3 min at 95℃, followed by 30 cycles of 30s at 94℃, 2 min at 50℃, and 30 s at 72℃. Finally, the reaction mixture was maintained at 72℃ for 10 min. This produced fragments of approximately 400bp, which was subcloned, generating p*TmGlu1*. Partial sequences for *TmGlu1* were obtained from these fragments, and complete nucleotide sequencing of the coding region of *TmGlu1* was carried out using cassette amplification by PCR with two primer sets(30773-GP2-A1/30773-GP2-A2, 30773-GP2-S1/30773-GP2-S2). These primer sets were designed based on the partial nucleotide sequences of *TmGlu1* obtained from the first PCR. Template DNAs for cassette PCR were prepared with a TaKaRa LA PCR In Vitro Cloning Kit(TaKaRa bio co.) according to the manufacturer's instructions. Genomic DNA from *T. matsutake* was digested with *Hin*dIII and *Sal*I, and the fragments were ligated with nucleotide linkers and used as templates for PCR. For *TmGlu1*, we cloned and sequenced an approximately 2.0kbp PCR product from 30773-GP2-A2 to a *Hin*dIII site containing the 5'-untranslated region and a 2.0-kbp product from 30773-GP2-S2 to a *Sal*I site containing the 3'-untranslated region. To amplify the whole genomic clone of *TmGlu1*, oligonucleotide primers T-M 30773 GP2 F/T-M 30773 GP2 R were designed based on the nucleotide sequence of DNA fragments amplified by the cassette PCR method. PCR was carried out in a 100㎕ reaction containing 1xExTaq buffer, 100ng of extracted genomic

DNA, 100pmol of each primers, each dNTP at a concentration of 0.2mM, and 2.5U of Ex Taq polymerase. PCR was carried out using an initial denaturation at 94℃ for 1 min, followed by 30 cycles of 30s at 94℃ and 5 min at 68℃. The amplified DNA fragments for *TmGlu1* was purified with a QIAquick PCR Purification Kit(Qiagen, Tokyo, Japan) according to the manufacturer's instructions and used as a DNA template for direct sequencing with oligonucleotide primers.

DNA sequencing and computer analysis of nucleotide and protein sequences

DNA sequencing was carried out in an ABI PRISM 3100 Genetic Analyzer(Applied Biosystems, Tokyo, Japan) using the chain-termination procedure with a Big-Dye Terminator Cycle Sequencing version 3.1 kit(Applied Biosystems) according to the manufacturer's instructions. Nucleotide and protein sequence data were analyzed using GENETYX 9.0(Genetyx, Tokyo, Japan). Protein motifs in amino acid sequence of the Glucoamylase were discovered with MOTIF Search program on the web site(http://motif.genome.jp/). Subcellular localization of the Glucoamylase was predicted by PSORTII, which is available on the internet(Nakai and Horton, 1997; http://psort.ims.u-tokyo.ac.jp/form2.html) and SOSUI program, which is also available on the internet(Hirokawa *et al.*, 1998; http://sosui.proteome.bio.tuat.ac.jp/ sosuiframe.html).

Reverse transcription-PCR (RT-PCR) procedure

Total RNA from the *T. matsutake* NBRC30773 was used as template for all RT-PCR in this study. Amplification of full-length cDNA by RT-PCR and 3'-rapid amplification of cDNA ends (RACE)-PCR were performed using a Takara RNA LA PCR Kit(AMV) version 1.1(Takara Bio Co.). The 5'-RACE PCR was performed using a 5'-Full RACE Core set(Takara Bio Co.). The all- reverse transcription reaction and PCR were carried out according to the manufacturer's instructions. The amplified fragments generated with these methods were subcloned and sequenced.

Glucoamylase assays

For purification of the glucoamylase, after 30 days incubation, the culture medium was filtered through a filter paper for mycelia removal, and then the culture filtrate was vacuum freeze-dried. The freeze-dried filtrate was dissolved by MES buffer(50mM, pH 5.5). glucoamylase activity was checked with a Glucoamylase and alpha-Glucosidase Assay Kit(Kikkoman Co., Tokyo, Japan). The data analysis was performed according to manipulation's instruction.

Total protein assays

For purification of the total protein, we estimated the total protein in freeze-fried filtrate of the different culture medium by lowry method.

Real-time PCR assay

We used the actin gene as the housekeeping gene. A partial actin gene in P.microspora was cloned by the degenerate PCR primers Univ Act F1 and Univ Act R1 Primers for *TmGlu1*, and actin were designed according to their cDNA sequences by using GENETYX 9.0(Genetyx). The primers were designed according to the principles of primer design, and 3 to 6 bp of the 3' site were designed to cross the intron in the primer spanning the intron. All primers were tested to ensure amplification of single bands with no primers-dimers. Plasmid extraction was performed according to the method modified by Birnboim(1983). Four 10 fold dilutions of plasmid were performed to construct standard curves. Real-time PCR was conducted using the RNA-direct SYBR green Real-time PCR master mix(Toyobo, Osaka, Japan) and Linegene(BioFlux, Hangzhou, China). Each reaction was run twice. The cycling parameters were 90℃ for 30s to activate thermostable DNA polymerase, 61℃ for 20 min to reverse transcription, 95℃ for 30s predenaturation, and then 35 cycles of 95℃ for 15s, 60℃ for 15s, and 74℃ for 30s. Melting curves were determined according to the manufacturer' s instructions. After realtime RT-PCR, samples were also run on a 1.5% agarose gel to confirm amplification specificity. The data analysis was performed according to manipulation's instruction.

Results

Structure of the glucoamylase gene (*TmGlu1*) in *T. matsutake*. In order to confirm the presence of putative

Table 1. Character of TmGlu1 in *T. matsutake*

	ORF	amino acids	Mol. mass(kDa)	Signalpeptides
TmGlu1(*Tricholoma matsutake*)	2183 bp	576	61.4	15

glucoamylase gene in *T. matsutake* genome, the *TmGlu1* was amplified and sequenced. The character of *TmGlu1* in *T. matsutake* are shown in Table 1. The coding region (from ATG to stop codon) is 2,183bp. The locations of the exons and introns were determined from the nucleotide sequences of the PCR products amplified by 3'- and 5'-RACE PCR and by RT-PCR. All of the introns started with GT and ended with AG. The coding region was split into nine exons by eight introns. This gene encodes a protein of 576 amino acids and contains four family 15 glycoside hydrolase signature and two motifs of the starch-binding domain in glycoside hydrolase. PSORTII program predicted that this protein is extracellular protein (probability=55.6%) including cell wall and first 15 amino acids is putative signal peptide which was found in N terminal region of this protein. Moreover, SOSUI program predicted that this protein is water soluble protein.

The deduced protein sequence of glucoamylase has 68% identity and 82% similarity with the Glycoside hydrolase family 15 protein of *Laccaria bicolor*(Martin *et al*. 2008) and 66% amino acid identity and 79% similarity with the glucoamylase protein of *Lentinula edodes*(Zhao *et al*., 2000). Phylogenetic tree constructed by the UPGMA(unweighted pair group method with arithmetic mean) method based on the protein sequences of the fungal glucoamylases is shown in Table 1. From this, the protein sequence glucoamylase from *T. matsutake* and L. bicolor which belong to ectomycorrhizal basidomycete and Tricholomataceae family are divided different clade. Therefore evolution of glucoamylase has no relationships with differentiation between saprophytic fungi and ectomycorrhizal fungi. Because the myceliums of both ectomycorrhizal fungi are able to grow on artificial agar medium containing starch, i.e. they can grow saprophytic ally.

Discussion

Although there have been many reports on glucoamylase from fungi, no glucoamylase gene from *T. matsutake* has been reported. Here we report for the first time the nucleotide sequence of glucoamylase gene(*TmGlu1*) and its franking region in *T. matsutake*. The expression of it was also described. It would be useful in the further study of gene regulation and expression.

The major function of the fungal glucoamylase is to degrade polymeric starch and thereby to provide a soluble simple carbon source for nutrition. In the study on the regulation of glucoamylase, there were some researchers found that composition of medium can affect glucoamylase activity. Fowler *et al*.(1990) proved that glucoamylase was strongly induced when A. niger was grown on starch as the carbon source. In the study on *Aspergillus terreus*, Ventura *et al*.(1995) found that carbon catabolite repression of the synthesis of glucoamylase. In the study on regulation of the glucoamylase from *L. edodes*, Zhao *et al*.(2000) considered such carbon catabolite regulation is also working in *L. edodes*. In their resaerch, they found that a low, constitutive level of glucoamylase was observed in the mycelium grown in the medium without glucose or starch. They show that expression of glucoamylase gene was induced by starch and increased during the process of fruiting body formation, which indicates that glucoamylase may plays an important role in the morphogenesis of the basidiomycetous fungus.

In this article, the relationship between composition of medium and glucoamylase expression was been investigated. As a result, enzyme activity of glucoamylase was very low in different medium. Glucoamylase has no relationships with differentiation between saprophytic fungi and ectomycorrhizal fungi. Enzyme activity of glucoamylase was very low in different medium. Expression of glucoamylases gene appeared to not be affected by different carbon source.

From the present research, carbon source can not effect on glucoamylase in *T. matsutake*. In the future research, we will investigate other factors affecting on the expression of glucoamylase. On the other hand, in order to clarify the change regularity of glucoamylase

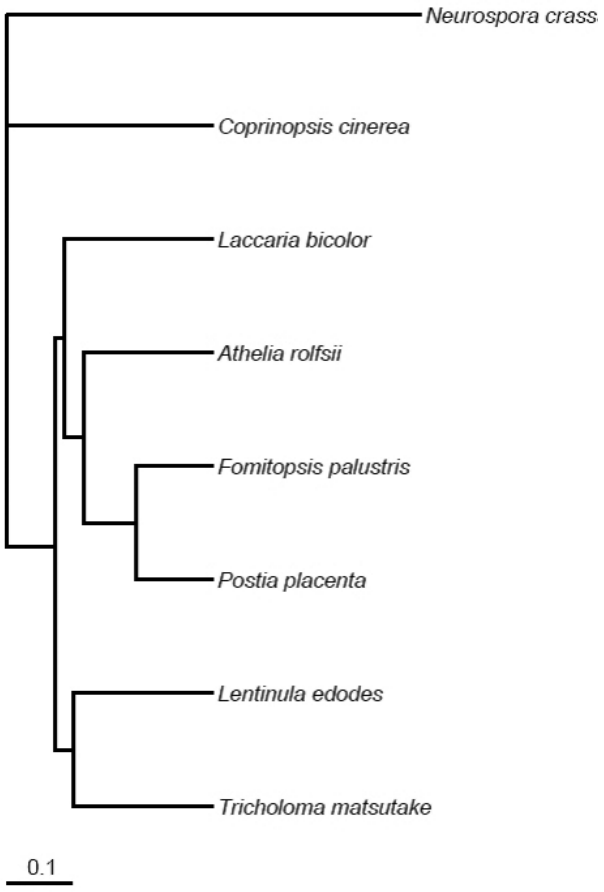

Fig. 1. Phylogenetic tree constructed by the UPGMA method based on the protein sequences of the fungal glucoamylases. Protein sequence of Glucoamylase protein from *Lentinula edodes* (Zhao et al. 2000), *Laccaria bicolor* (Martin et al. 2008), *Coprinopsis cinerea*, *Athelia rolfsii* (Nagasaka et al. 1995), *Fomitopsis palustris* (Yoon et al. 2006), *Postia placenta* (Martinez et al. 2009) and *Neurospora crassa* (Stone et al. 1993) appeared in the DNA database under accession numbers Q9P4C5, B0CVJ1, A8NSG1, Q12596, Q33CE4, B8PI57 and P14804, respectively.

in process of fruiting-body formation, we will investigate the glucoamylase activity from vegetative stage to ripening stage in *L. shimeji* and *Pholota nameko*. These will provide basic research for artificial cultivation of *T. matsutake*.

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References

Birnboim, H. C. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. **100** : 243-255

Fowler, T, Berka, R. M., Ward M. 1990. Regulation of the glaA gene of *Aspergillus niger*. Curr Genet. **18** : 537-545

Hirokawa, T., Boon-Chieng, S. and Mitaku, S., 1998. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics **14** : 378-379

Horton, P. and Nakai, K. 1997. Better prediction of protein cellular localization sites with the knearest neighbors classifier. Proc Int Conf Intell Syst Mol Bio. **15** : 147-152

Martin, F., Aerts, A., Ahren, D., Brun, A., Danchin, E. G. J., Duchaussoy, F., Gibon, J., Kohler, A., Lindquist, E., Pereda, V., Salamov, A., Shapiro, H. J., Wuyts, J., Blaudez, D., Buee, M., Brokstein, P., Canbaeck, B., Cohen, D., Courty, P. E., Coutinho, P. M., Delaruelle, C., Detter, J. C., Deveau, A., DiFazio, S., Duplessis, S., Fraissinet-Tachet, L., Lucic, E., Frey-Klett, P., Fourrey, C., Feussner, I., Gay, G., Grimwood, J., Hoegger, P. J., Jain, P., Kilaru, S., Labbe, J., Lin, Y. C., Legue, V., Le Tacon, F., Marmeisse, R., Melayah, D., Montanini, B., Muratet, M., Nehls, U., Niculita-Hirzel, H., Oudot-Le Secq, M. P., Peter, M., Quesneville, H., Rajashekar, B.., Reich, M., Rouhier, N., Schmutz, J., Yin, T., Chalot, M., Henrissat, B., Kuees, U., Lucas, S., Van de Peer, Y., Podila, G. K., Polle, A., Pukkila, P. J., Richardson, P. M., Rouze, P., Sanders, I. R., Stajich, J. E., Tunlid, A., Tuskan, G. and Grigoriev, I. V. 2008. The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature **452** : 88-92

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D., Schmoll, M., Kubicek, C. P., Ferreira, P., Ruiz-Duenas, F. J., Martinez, A. T., Kersten, P., Hammel, K. E., Vanden Wymelenberg, A., Gaskell, J., Lindquist, E., Sabat, G., Bondurant, S. S., Larrondo, L. F., Canessa, P., Vicuna, R., Yadav, J., Doddapaneni, H., Subramanian, V., Pisabarro, A. G., Lavin, J. L., Oguiza, J. A., Master, E., Henrissat, B., Coutinho, P. M., Harris, P., Magnuson, J. K., Baker, S. E., Bruno, K., Kenealy, W., Hoegger, P. J., Kues, U., Ramaiya, P.,

Lucas, S., Salamov, A., Shapiro, H., Tu, H., Chee, C. L., Misra, M., Xie, G., Teter, S., Yaver, D., James, T., Mokrejs, M., Pospisek, M., Grigoriev, I. V., Brettin, T., Rokhsar, D., Berka, R. and Cullen, D. 2009. Genome, transcriptome, and secretome analysis of wood decay fungus Postia placenta supports unique mechanisms of l ignocellulose conversion. Proc Natl Acad Sci USA **106** : 1954-1959

Nagasaka, Y., Muraki, N., Kimura, A., Suto, M., Yokota, A. and Tomita, F. 1995. Cloning of *Corticium rolfsii* glucoamylase cDNA and its expression in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. **44** : 451-458

Stone, P. J., Makoff, A. J., Parish, J. H. and Radford, A. 1993. Cloning and sequence analysis of the glucoamylase gene of *Neurospora crassa*. Curr Genet. **24** : 205-211

Sakaguchi, K., Takagi, M., Horiuchi, H. and Gomi, K. 1992. Fungal enzymes used in oriental food and beverage industries. Applied molecular genetics of filamentous fungi 100-130.

Ventura, L, González-Candelas, J, Pérez-González and Ramón D. Molecular cloning and transcriptional analysis of the *Aspergillus terreus* gla1 gene encoding a glucoamylase. Appl Environ Microbiol. **61** : 399-402

Yoon, J. J, Igarashi, K., Kajisa, T. and Samejima M. 2006. Purification, identification and molecular cloning of glycoside hydrolase family 15 glucoamylase from the brown-rot basidiomycete *Fomitopsis palustris*. FEMS Microbiol Lett **259** : 288-294

Zhao, J, Chen, Y. H., Kwan, H. S. 2000. Molecular cloning, characterization, and differential expression of a glucoamylase gene from the basidiomycetous fungus *Lentinula edodes*. Appl Environ Microbiol. **66** : 2531-2535