Developing *Ophiostoma floccosum* as a novel expression system

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Abstract

Ophiostoma spp. belong to the *Ophiostomataceae* family, a large group of ascomycetes, which are the most frequent blue stain fungi isolated from stained wood. Most *Ophiostoma* species do not compromise the strength properties of wood, but do reduce the aesthetic quality of timber and therefore decrease the economic value of lumber. Some albino variants of *O. floccosum* and *O. piliferum* have been used as biological control agents to prevent blue staining. This successful whole organism approach plus the added capability of extracellular protein secretion makes *Ophiostoma* spp. attractive for industrial application. In addition, *Ophiostoma* produces only a small range of abundantly secreted proteins in liquid culture, which can facilitate downstream purification of any recombinant gene product introduced into the system. Genes encoding efficiently secreted proteins provide a potential source for strong promoters for high-level gene expression. These characteristics provide an excellent starting point for the development of a novel expression system.

In this study, UV-mutagenesis was applied to improve protein secretion in *Ophiostoma floccosum*. Amylase activity was used as an indicator for enhanced protein secretion after repeated rounds of mutagenic treatment. Several mutants of *O. floccosum* derived by UV mutagenesis were isolated and the total amount of secreted protein was increased by 4 to 6 times. The amylase activity in the culture supernatant of the best mutant (MQ.5.1) was increased by more than 240-fold compared to the initial parental strain. At the same time, the amount of total secreted protein was about six times greater to that of the parental strain. Proteinase profiles in the culture supernatants of several key mutants were characterized for the future matching of an expression host with a particular gene product. N-terminal sequencing of the five dominant proteins separated by SDS-PAGE from the culture supernatant was conducted. Two of the proteins identified were subtilisin-like proteinases and one was a pepsin-like proteinase. In addition, one protein was identified as an α-amylase and one remained unidentified. A 6.5 kb DNA fragment was isolated by Genomic Walking PCR using primers based on the α-amylase amino acid sequence. The amplified fragment contained the entire gene encoding α-amylase (*amy1*) and its regulatory

sequences. Analysis showed that multiple transcripts were generated from the single α-amylase gene locus.

A series of expression vectors containing the α-amylase regulatory sequences and partial *amy1* gene were constructed. Several selection markers were screened and the *hph* gene conferring hygromycin resistance under the regulation of the *Aspergillus nidulans gpd* promoter was chosen and inserted into the *amy1* expression vectors. The gene encoding a red fluorescent protein DsRed-E5 was used as a reporter gene to test the expression system using mutant MQ.5.1 as host. However, no transformants were obtained by either biolistic transformation or protoplast transformation. Subsequently, an alternative strategy was developed using a thermostable xylanase B as a reporter. Thermostable xylanase activity was detected in the culture supernatants of several transformants. Production of xylanase by transformant SS41 which exhibited high secreted xylanase activity was investigated. Xylanase activity in the culture supernatant of SS41 was visualized by a zymogram gel assay. Two active proteins with molecular masses of around 27 and 30 kDa, which were larger than the predicted *Mr* of 25 kDa were detected. This is the first report describing successful expression of a recombinant thermostable bacterial enzyme in *Ophiostoma*.

Declaration

The research presented in this thesis is original work conducted between March 2003 and November 2006 by the author. This material has not been submitted as part of the requirement for any other degree or course to any other institution. To the best of my knowledge it contains no material previously published or written by any other person except where due reference is made in the text.

Caiyan Wu

List of publications

This thesis is based on the following articles, referred to in the text by the Roman numerals given below. In addition, some unpublished results are presented.

I Caiyan Wu, Junior Te'o, Robeta Farrell, Peter Bergquist and Helena Nevalainen (2006) Improvement of the secretion of extracellular proteins and isolation and characterization of the amylase I (*amy1)* gene from *Ophiostoma floccosum*. Gene 384: 96-103.

II Caiyan Wu, Qiang Xu, Fei Liu and Helena Nevalainen (2007) Activity-based identification of secreted serine proteases of the filamentous fungus *Ophiostoma*. Accepted by Biotechnology Letters, DOI 10.1007/s10529-007-9333-6.

III Caiyan Wu, Junior Te'o, Peter Bergquist and Helena Nevalainen (2007) Expression of a thermostable bacterial xylanase in the filamentous fungus *Ophiostoma floccosum*. Submitted to Letters in Applied Microbiology in July 2007.

Abbreviations

Abbreviations frequently used in the text are:

bp	Base pair
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DsRed	Red fluorescent protein
EDTA	Ethylenediamine-tetra-acetic acid
ERAD	Endoplasmic reticulum associated degradation
GWPCR	Genomic walking PCR
Kb	Kilobase pair
kDa	Kilo Dalton
MCS	Multiple cloning site
min	Minute
Mr	Molecular weight
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
RLM-RACE	RNA ligase mediated rapid amplification of cDNA ends
RNA	Ribonucleic acid
s	Second
SBD	Starch binding domain
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSF	Solid state fermentation
TSS	Transcription starting site
UPR	Unfolded protein response
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

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