Monitoring Global Gene Expression of Proteases and Improvement of Human Lysozyme Production in the \textit{nptB} Gene Disruptant of \textit{Aspergillus oryzae}

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Received September 12, 2007; Accepted November 5, 2007; Online Publication, February 7, 2008

Aspergillus oryzae has numerous protease genes that might cause proteolytic degradation of heterologously-produced proteins. The productivity of the heterologous protein can be improved by protease gene disruption, but it is difficult to select disruption targets efficiently. In this study, we monitored the expression of 132 protease genes by DNA microarray. A group of protease genes up-regulated during cultivation was identified by clustering analysis. In this protease group, the \textit{nptB} gene encoding neutral protease II was included as well as the \textit{alpA}, \textit{tppA}, and \textit{pepA} genes, disruption of which has improved human lysozyme (HLY) production. The \textit{nptB} gene was disrupted to investigate its involvement in HLY production, and \textit{nptB} disruptants showed an improvement in the production. These observations suggest that monitoring the expression of protease genes is an efficient strategy in screening potential disruption targets for heterologous protein production in \textit{A. oryzae}.

Key words: \textit{Aspergillus oryzae}; protease gene expression; DNA microarray; heterologous protein production; neutral protease II

Aspergillus oryzae serves as an excellent host for enzyme and protein production, with its safety assured by a long history of use in food fermentation in Japan. The whole genome of \textit{A. oryzae} was sequenced, and was predicted to have approximately 12,000 genes.\textsuperscript{11} In our previous studies, we bred a quadruple auxotrophic host that allows multiple insertion and/or disruption of genes of interest.\textsuperscript{21} We also developed a plasmid construction system enabling various forms of gene expression in \textit{A. oryzae}.\textsuperscript{37} These genetic manipulation systems facilitate efficient expression and/or disruption of the genes for the improvement of protein production in \textit{A. oryzae}.

In the heterologous protein production of filamentous fungi, proteolytic degradation of the product is one of the bottlenecks limiting the yield.\textsuperscript{4,5} In \textit{Aspergillus niger}, PepA (aspergillopepsin A) is associated with the degradation of heterologous proteins,\textsuperscript{6,7} and a triple disruptant of aspartic protease genes (\textit{pepA}, \textit{pepB}, and \textit{pepE}) has been obtained.\textsuperscript{8} In \textit{Aspergillus awamori}, deletion of \textit{pepB} greatly increased thaumatin production in combination with a \textit{pepA} defective mutant.\textsuperscript{9,10} In \textit{A. oryzae}, the anti-sense RNA technique repressed serin-type carboxypeptidases and increased human lysozyme (HLY) production.\textsuperscript{11} In addition, we have employed systematic gene disruption of five protease genes (\textit{pepA}, \textit{pepE}, \textit{alpA}, \textit{tppA}, and \textit{palB}), and double disruption of \textit{pepE} and \textit{tppA} genes successfully increased HLY production in \textit{A. oryzae}.\textsuperscript{12} Hence, it is evident that disruption or repression of protease genes enhances the productivity of the heterologous protein.

In a comparative analysis of \textit{Aspergillus} genomes, however, it was found that \textit{A. oryzae} has a total of 134 protease genes, more than \textit{Aspergillus nidulans} or \textit{Aspergillus fumigatus}.\textsuperscript{1,13} Selection of disruption targets is difficult because a variety of protease genes exist, presumably having complex expression profiles. Recently, DNA microarray permitted global gene expression analysis in \textit{A. oryzae},\textsuperscript{14,15} and its potential use has been suggested for monitoring gene expression during fermentation. It is likely that protease genes up-regulated when productivity decreases are associated with proteolytic degradation. Since \textit{A. oryzae} has numerous protease genes in its genome, DNA microarray is advantageous in comprehensive expression analysis of the protease genes and allows screening for the disruption target.

In this study, we monitored the expression of \textit{A. oryzae} protease genes in three different culture phases. The \textit{nptB} gene encoding neutral protease II was found to be one of the up-regulated genes during cultivation, in which HLY production decreases. We report here that disruption of the \textit{nptB} gene successfully improved HLY productivity in \textit{A. oryzae}.

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Materials and Methods

Strains, media, and culturing conditions. A. oryzae RIB40 was grown on a potato dextrose agar (Nissui Pharmaceutical, Tokyo) plate for 5 d at 30°C. The conidia were harvested and the suspension of 10⁶ conidia was inoculated into each flask containing 100 ml of 5 x DPY (10% dextrin, 5% polypepton, 2.5% yeast extract, 0.5% K₂HPO₄, and 0.05% MgSO₄·7H₂O, pH 8.0). The culture was incubated at 30°C with shaking for the indicated times. In harvesting the mycelia from the culture, vacuum filtration was used, followed by washing with distilled water.

NAR-2L-7 (niaD⁻ sC⁻ ΔargB adeA⁻) pA2LS[P-amyb::amyB-(HLY)::T-amyB::A. nidulans sC]), an HLY-producing strain having a tandem copy of HLY with inserted Kex2 cleavage sites, was used as the parent strain in the nptB gene disruption experiment. In HLY production, NA-2L (niaD⁻ sC⁻ ΔargB adeA⁻) pA2LS[P-amyb::amyB-(HLY)::T-amyB::A. nidulans sC] pAdA[adeA]), an adeA complemented strain constructed from NAR-2L-7, was used as a control, and was inoculated as described above.

Total RNA extraction and real-time RT-PCR. The harvested mycelia were frozen in liquid nitrogen and thoroughly ground to powder by mortar and pestle. Total RNA samples from A. oryzae RIB40 were prepared from culture grown in 5 x DPY (pH 8.0) at 2 d, 4 d, and 6 d respectively. In the DNA microarray experiment, they were extracted using RNeasy Plant Mini Kit spin columns (Qiagen Sciences, Maryland, MD) and dissolved in RNase-free water. In real-time RT-PCR, total RNA was further treated with DNase (Qiagen Sciences, Maryland, MD) and cDNA was amplified using ReverTra-Ace reverse transcriptase (Toyobo, Osaka) with oligo(dT)₁₂₋₁₈ primer (Invitrogen, Carlsbad, CA). Real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN) as instructed by the manufacturer. The primers used in this study are listed in Table 1.

DNA microarray and clustering analysis for protease genes of A. oryzae RIB40 strain. DNA microarray experiments were conducted following the Affymetrix GeneChip manual with custom DNA microarray (NimbleGen Systems, Madison, WI) covering 12,048 genes of the A. oryzae RIB40 strain. Briefly, one cycle cDNA synthesis was performed and biotin-labeled cRNA was amplified, followed by fragmentation. The fragmented cRNA was hybridized with probe sets, each containing 11 probe pairs consisting of a perfect match probe (PM) and a mismatch probe (MM) with 25 mer oligonucleotides. After washing and staining, the probes were scanned with GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA).

Normalization of microarray data was performed by GROS v1.4 (Affymetrix) as well as calculation of signal intensity and the detection p-value. The trimmed mean signal of the array was scaled to the target signal of 500 with the All Probe Sets scaling option. Detection call was used in detection of a particular transcript, with a detection p-value of p < 0.05 as present (P), 0.05 ≤ p < 0.065 as marginal (M), and p ≥ 0.065 as absent (A). K-means clustering and hierarchical clustering based on protease gene expression were carried out using GeneSpring GX 7.3 (Agilent Technologies, Santa Clara, CA), in which Pearson correlation was used as a similarity measure. The signal intensities of 132 protease genes were extracted from the normalized data sets, and were further normalized to the median for each gene (per-gene normalization). Protease genes with the detection call of A at all culture phases were excluded from the clustering analysis.

nptB gene disruption and Southern blotting analysis. Approximately 2.0 kb up- and downstream of the nptB gene were amplified from the A. oryzae RIB40 genome by PCR. A plasmid containing the nptB gene disruption fragment was constructed with the MultiSite Gateway system (Invitrogen) and subjected to PCR amplification with a forward primer for the 5’ upstream region and a reverse primer for the 3’ downstream region (Table 1). Subsequently, the PCR-amplified 6.0-kb fragment was transformed into an HLY producing strain (NAR-2L-7) (Fig. 3A), and transformant candidates were screened by PCR for adeA marker integration. The adeA-integrated transformants were subjected to

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<table>
<thead>
<tr>
<th>Target region</th>
<th>Primer sequence (forward and reverse)</th>
<th>Amplicon size (bp)</th>
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<tr>
<td>Upstream of nptB</td>
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<tr>
<td>Downstream of nptB</td>
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<td>2040</td>
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<tr>
<td>Southern blotting</td>
<td>5'-tctggtagagtttgcgcgt-3', 5'-ctggtagagtttgcgcgt-3'</td>
<td>999</td>
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<tr>
<td>Real-time RT-PCR</td>
<td>5'-taacctgaaccacggacca-3', 5'-gcgcgattgcgtg-3'</td>
<td>397</td>
</tr>
<tr>
<td>adeA</td>
<td>5'-gcgcttcaagaccgcggttttgg-3</td>
<td>398</td>
</tr>
</tbody>
</table>
Southern blot analysis. The genomes of the transformants were phenol-extracted and digested with BamH I and Xho I respectively. The probe for Southern analysis was PCR-amplified with the primers listed in Table 1, and purified using GeneClean II Kit (Funakoshi, Tokyo).

**Lysozyme activity assay.** The culture supernatants of the nptB gene disruptants (NA-2L-ΔnptB-1 and -2) and NA-2L were collected for lysozyme activity assay, in which the activity of HLY was measured by the OD change at 450 nm.

### Results

**Global gene expression of A. oryzae proteases during the cultivation in 5 × DPY (pH 8.0) using DNA microarrays**

In our previous study of heterologous protein production using A. oryzae, HLY production was higher under alkaline conditions (pH 8.0) than under acidic conditions (pH 5.8), and a dramatic increase in production was achieved in 5 × DPY (pH 8.0), which is 5-times concentrated rich medium. Maximal production under this condition was obtained at 4 d, and productivity decreased in the late phase, indicating degradation of the product by the proteases from A. oryzae.

Based on this, we focused on three culture phases: the early (2 d), the middle (4 d), and the late (6 d). When A. oryzae RIB40 (the wild type) was grown in 5 × DPY (pH 8.0), the culture pH dropped as the biomass increased in the early phase, but it returned to neutral pH in the late phase (Fig. 1A). To screen the protease genes potentially involved in the degradation of the heterologous protein under this condition, expression of the protease genes in A. oryzae RIB40 was monitored using DNA microarrays that cover 12,048 genes containing 132 protease genes. Expression of approximately 18% (24/132) of the protease genes was not detected (detection call = A, see "Materials and Methods") at any culture phase examined, and hence, these transcriptionally inactive protease genes were excluded from further analysis.

To facilitate the screening process, K-means clustering was applied to a total of 108 expressed genes, and they were divided into six groups based on the expression patterns (Fig. 1B). First, we focused on the protease groups up-regulated in the late phase, because HLY production decreases in that phase. There were two protease groups up-regulated in the late-phase culture. The protease genes in set 4 contained genes with increased transcription only in the late phase (Fig. 1B). In this protease population, for example, the dppV gene, encoding alanyl dipeptidyl peptidase, which is specifically expressed in solid-state culture, was included. On the other hand, the protease group of set 3 contained genes up-regulated both in the middle and the late phase cultures (Fig. 1B). We selected this group for further analysis, since it included protease genes up-regulated not only in the late phase, but also in the middle phase, in which the decrease in HLY production starts. Next we screened secretory protease genes by analyzing the N-terminal signal sequences of the protease genes. Set 3 was predicted to have nine protease genes with signal peptide and seven non-secretory protease genes.

**Confirmation of nptB gene expression by real-time RT-PCR**

Since the DNA microarray data were from single experiments and the normalized signal intensities for the nptB gene were near the detection limit, real-time RT-PCR was performed to confirm its expression in each culture phase (Fig. 2). Relative expression to actin gene expression was performed with the probe indicated in Fig. 1C. The hierarchical clustering is suggestive of the similar transcriptional behavior of these genes (Fig. 1D). Hence, we speculated that NptB was also involved in the degradation of the product under this culture condition, and the nptB gene was selected as the disruption target.

**Improvement of HLY production in A. oryzae by nptB gene disruption**

Disruption of the nptB gene was conducted by replacing it with the adeA marker gene by double homologous recombination (Fig. 3A). HLY-producing strain NAR-2L-7 was used as host and was transformed with the disruption fragment. After the transformation, a total of 10 transformants were subjected to PCR screening for adeA integration, and two homokaryotic transformants were obtained (data not shown). To confirm nptB gene disruption, their genomes were digested with BamH I and Xho I, and Southern blot analysis was performed with the probe indicated in Fig. 3A. The BamH I-digested genome from NAR-2L-7 contained a 5.6-kb fragment whereas the transformants contained only 2.7-kb fragments (Fig. 3B). Similarly, the Xho I-digested genome from NAR-2L-7 contained a 5.5-kb fragment whereas the transformants contained only 4.5-kb fragments. Hence, the nptB gene was replaced with the adeA marker gene, and disruption of the nptB gene was confirmed.

In order to assess the efficacy of nptB gene disruption on HLY production, HLY production of nptB disrup-
tants in 5 × DPY (pH 8.0) was measured (Fig. 4). There was no apparent growth defect in the nptB disruptants. Maximal production, a 22% increase, was obtained in the middle phase culture (4 d) of NA-2L-nptB-2. NA-2L-nptB-1 also increased productivity, as compared with the control strain. Therefore, nptB gene disruption successfully improved HLY production in 5 × DPY (pH 8.0).

**Discussion**

Efficient selection of disruption targets potentially involved in the degradation of the product is difficult, since A. oryzae has various and numerous protease genes in its genome. In this study, we took advantage of DNA microarray and monitored the expression of 132 protease genes of A. oryzae in 5/C2 DPY (pH 8.0) in three different culture phases. Judging by the DNA microarray results, there was a distinct group with elevation of the transcripts both in the middle and the late phase cultures (Fig. 1B), in which a decrease of HLY production was observed. The nptB gene had an expression pattern similar to those of the protease genes disruptants of which previously improved HLY produc-

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**Fig. 1.** DNA Microarray Analysis of A. oryzae Protease Genes in 5 × DPY (pH 8.0).

A, Biomass and pH changes. The wet weight (●) was measured as well as the pH (○) of the filtrate after vacuum filtration of A. oryzae RIB40 culture at the indicated times. B, K-means clustering for 108 expressed protease genes. Signal intensities in the early (2 d), the middle (4 d) and the late (6 d) phases were normalized to the median for each gene (per-gene normalization), and the protease genes were divided into six groups. The per-gene normalized signal intensities on a log scale were plotted on the Y axis against cultivation days on the X axis. The number of genes in each set is indicated in parentheses. C, Signal intensities of the tppA, alpA, nptB, and pepA genes in early (black), middle (white), and late (stripe) phase cultures. D, Hierarchical clustering of 108 expressed protease genes. A dendrogram based on their expression patterns was generated, in which the signal intensities were per-gene normalized. The cluster of protease genes in set 3 is enlarged. Blue rectangles represent down-regulation and red rectangles represent up-regulation as compared to the median value (yellow). The protease genes with low trust (low signal intensities) are colored dark or black. The image was adopted from GeneSpring GX 7.3.
Also called deuterolysin, 18) was up-regulated during disruption (Fig. 4). Hence, it was selected as a candidate gene, and its relative transcriptional levels of Fig. 2. Relative Transcriptional Levels of nptB by Real-Time RT-PCR. Beta-actin was used as a reference gene and nptB expression was adjusted for each culture phase. The data shown are the means ± SE of two independent experiments.

tion. Hence, it was selected as a candidate gene, and its disruption resulted in an improvement in HLY production (Fig. 4).

The nptB gene encoding neutral protease II (NPII), 17) also called deuterolysin, 18) was up-regulated during cultivation (Fig. 1C, Fig. 2). In addition, the alpA gene encoding an alkaline protease 19) was up-regulated, with a similar expression pattern (Fig. 1C, Supplementary figure: see Biosci. Biotechnol. Biochem. Website). It has been reported that transcription of these secretory protease genes was induced when the culture pH changed to neutral pH from acidic pH, suggesting that their transcription is regulated by pH. 20) Our DNA microarray data indicate that the pacC transcript, encoding a transcription factor required for alkaline adaptation, 21) was up-regulated more than 2-fold in the late phase (data not shown), in which pH recovery to neutral was observed (Fig. 1A). The consensus binding sequence of PacC (5′-GCCARG-3′) was also found in the promoter regions of these genes. It is, therefore, possible that pH recovery in culture is one of the concomitant factors elevating transcription of the nptB and alpA genes during cultivation.

In A. oryzae, there are two other known neutral proteases with a metalloprotease motif, neutral protease I (NPI) and III (NPIII) (AO090011000036 and AO090138000160 respectively). From DNA microarray data, the transcript for NPI was not detected, and NPIII was expressed at extremely low levels without elevation of the transcript under the conditions examined (data not shown). On the other hand, transcription of the nptB gene encoding NPII was elevated during cultivation (Fig. 1C, Fig. 2). Disruption of the nptB gene improved the productivity of HLY, with a maximal 22% increase (Fig. 4). To our knowledge, improvement in heterologous protein production by disrupting a neutral protease gene has not previously been reported for filamentous fungi. Compared with the alpA gene, disruptant of which improved HLY production, with an approximately 8% increase, 12) the nptB transcript level was much lower (Fig. 2). This suggests that NptB (NPII) preferably degrades heterologous proteins such as HLY. It has been reported that NPII has a zinc-binding motif with preference for basic proteins as substrates. 22) Although its activity in culture could not be measured, probably due to the high polypepton content of the medium, NptB might recognize basic residues of HLY, causing the proteolytic degradation.

It is expected that disruption of the other protease genes up-regulated in the middle and/or the late phases also increases HLY production. We have found that a double disruptant of the protease genes (tppA, pepE) significantly increased HLY production. 12) Therefore, multiple disruption of protease genes up-regulated during cultivation might prevent proteolytic degradation in the late phase and further improve heterologous protein production in A. oryzae.
Acknowledgment

We thank Dr. Praveen R. Juvvadi for helpful discussion and technical assistance in the DNA micro-array experiment. We are also grateful to Dr. Kazuhiko Imagawa and Dr. Shinobu Matsuura for the technical help, and to Dr. Ichiro Matsumoto for assistance in the use of GeneSpring GX 7.3. This study was supported by a Grant-in-Aid for Scientific Research (S) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the Program for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) of Japan.

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