Efficient Expression of Mono- and Diacylglycerol Lipase Gene from *Penicillium camembertii* U-150 in *Aspergillus oryzae* under the Control of Its Own Promoter

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The gene, *mdlA*, coding for mono- and diacylglycerol lipase from *Penicillium camembertii* U-150 was expressed efficiently in *Aspergillus oryzae* under the control of its own promoter. The gene product was secreted into the culture medium with a highest productivity of 1 g/liter and correctly processed at both N- and C-termini. KEX2-like processing was suggested to occur at the C-terminus in both *A. oryzae* and *P. camembertii*. Specific activity and substrate specificity of the purified recombinant protein were also almost the same to that of native protein but the extent of N-glycosylation in the recombinant protein was about half of that of the native protein. The presence of introns did not seem to affect the gene expression.

The *mdlA* expression was induced by lipids and regulated transcriptionally in *A. oryzae* as well as *P. camembertii*. Promoter deletion analysis showed that the region between the positions at −382 and −554 bp from the translation initiation point was important to the higher expression of *mdlA*. The promoter sequence of *mdlA* was compared to that of the *Geotrichum candidum* lipase gene, which is also reported to be inducible by lipids, with three commonly observed oligonucleotide sequences.

**Key words:** lipase; gene expression; *Penicillium camembertii*; *Aspergillus oryzae*; lipid induction

Lipase is an important enzyme in industry. Many microbial lipases have been manufactured for a long time as biocatalysts for fat and oil processing, food additives, cleaners, diagnostic reagents, and digestive enzymes. Until now only limited information on the regulation of the lipase gene expression in filamentous fungi has been available. Although some lipases from filamentous fungi have been reported to be inducible by lipids, there are few reports based on gene expression. Elucidation of the gene regulation on lipase expression in filamentous fungi is important since they are lipase producers of industrial importance.

Moreover, lipase has also received increased attention recently in studies to investigate the molecular mechanism of its catalytic action. Several X-ray crystallographic structures have been identified in the triacylglycerol lipases from various origins and a mechanism of lipolysis has been proposed. Mono- and diacylglycerol lipase (MDGL) from *Penicillium camembertii* U-150 is a unique enzyme hydrolyzing mono- and diacylglycerol but substantially not triacylglycerol and its primary structure deduced from the gene, *mdlA*, and its cDNA shows sequence similarity to those of triacylglycerol lipases from other filamentous fungi. We have been interested in the protein structure of MDGL in view of its unique substrate specificity. More recently, the 3-D structure of the enzyme has been solved, revealing the close similarity of the structure to those of triacylglycerol lipases from other filamentous fungi, *Rhizomucor miehei*, *Humicola lanuginosa*, and *Rhizopus delemar*. In our previous study, site-directed mutagenesis was done using *Saccharomyces cerevisiae* as an expression host, resulting in the identification of the amino acid residues that form the catalytic center of MDGL. Since *S. cerevisiae* was, however, an insufficient host for MDGL expression because of extensive glycosylation and low productivity (about 2 mg/liter), an efficient expression system is needed for the production and characterization of mutant enzymes.

In this study, we arranged the efficient expression and secretion of MDGL by *A. oryzae* under the control of its own promoter. Characterizations of the purified recombinant MDGL, regulation of *mdlA* expression by lipids and deletion analysis of *mdlA* promoter in *A. oryzae* are reported.

**Materials and Methods**

*Strains, plasmids, and cultivation.* *A. oryzae* *niaD* mutant AO1.1 was used as a transformation host throughout this study. Transformation of *A. oryzae* was done as described by Unkles et al. (11) Plasmid pLGG300 and pLGR80, containing the *P. camembertii* *mdlA* gene and its intron-deleted gene, respectively, were described previously. (12) Plasmid pSTA14 containing the *A. oryzae* *niaD* gene and an *A. oryzae* mutant AO1.1 were the kind gifts of Dr. J. R. Kinghorn, University of St. Andrews, Scotland. The basal medium for cultivation consisted in 2% carbon source, 0.5% yeast extract, 0.3% NaNO₃, 0.1% K₂HPO₄, 0.05% KCl, and 0.05% MgSO₄·7H₂O, pH 6.0. Cultivations were done at 30°C for 72h on a reciprocal shaker (120 strokes/min, 40 mm pitch).

*Plasmid construction.* Plasmid pNLGG2 and pNLGR2, containing the *P. camembertii* *mdlA* gene and its intronless gene, respectively, and the *A. oryzae* *niaD* selection marker gene, were constructed in the following way: a 2.0-kb HindIII fragment from pLGG300 or 1.9-kb HindIII fragment from pLGR80 was blunt-ended and ligated into the SmaI site of pUC19. Into the HindIII sites of the resultant constructs, the 5.5-kb HindIII fragments containing the *niaD* gene from pSTA14 were ligated. Promoter-deleted plasmids were constructed by the treatment of pLGG300 with exonuclease III and mung bean nuclease (Stratagene, La Jolla, CA, Fig. 7, F4, F6, and F7) or by removal of the 0.18-kb SmaI fragment from pLGG300 (F3). To construct the plasmid containing 1.6-kb 5′-flanking region of *mdlA*, a 1.2-kb SmaI fragment from the 16-kb BamHI fragment of the original cloned recombinant phage (8) was replaced with a 0.18-kb SmaI fragment of pLGG300 in the appropriate direction (F1). The resultant genes with various lengths of the promoter region were cut out by HindIII and inserted into the HindIII site of the *A. oryzae*...
transformation plasmid pN3, which was constructed by inserting the blunt-ended 5.5-kb HindIII fragment from pSTA14 into the Smal site of pUC19.

**Protein purification.** A. oryzae transformants were cultivated as described above. Recombinant MDGL was purified from the culture filtrate by the three steps of chromatography as described elsewhere.\(^2\) Protein was measured by a BCA protein assay kit (Pierce, Rockford, IN, U.S.A.) using bovine serum albumin as a standard.

**Amino acid sequencing.** The N-terminal amino acid sequence was analyzed by automated Edman degradation on an Applied Biosystem (Foster City, CA, U.S.A.) gas-phase sequencer. The C-terminal amino acid sequence was analyzed by carboxypeptidase Y digestion as follows. Five nmol of desalted purified protein was dissolved in 5 μl of 10 M urea, 20 mM methylamine, 50 mM sodium citrate, pH 6.0, and denatured by heating at 80°C for 10 min. After dilution with 50 mM sodium citrate, pH 6.0, by 10-fold, 1.6 μg of carboxypeptidase Y (sequence grade, Boehringer Mannheim Biochemica, Germany) was added to the mixture. Samples were removed intermittently and the reaction was stopped by boiling. The liberated amino acids were analyzed by an amino acid analyzer (Shimadzu, Kyoto).

**Enzyme assay.** The lipolytic activity was assayed as described previously using vinylacetate as a substrate.\(^3\) Glyceride hydrolase activity was also measured as described previously\(^4\) with slight modifications. The reaction was done for 15 min and stopped by the addition of acetone in all cases. One enzyme unit (U) was defined as the activity which liberated 1 μmol equivalent fatty acid/min under these conditions.

**Southern and Northern blot analysis.** The mycelia were frozen immediately after cultivation and ground with sea sand under liquid nitrogen. Total DNA and RNA were isolated from the powder mycelia by the method of Garber and Yoder\(^1\) and Chirgwin et al.\(^2\) respectively. Blottings and hybridizations of DNA and RNA were done by the standard methods.\(^5\) DNA fragments separated by electrophoresis through a 0.8% agarose gel were transferred to Hybond N+ (Amersham, UK) and hybridized with the 32P-labeled DNA probe. For Northern blotting, RNA was separated by electrophoresis using a 1.0% agarose gel containing 18% formaldehyde. DNA probes were 32P-labeled using a multiprime DNA labeling kit (Amersham, UK).

**Endoglycosidase H treatment.** Purified protein was dissolved in 1% SDS at a concentration of 1.0 mg/ml and boiled for 10 min. Denatured protein solution was diluted 10-fold with 50 mM sodium phosphate, pH 6.0, and incubated with an equal volume of endoglycosidase H (Genzyme, MA) solution (4 U/ml) for 5 h at 37°C.

**Other methods.** Carbohydrate content was assayed by the phenolsulfonic acid method using α-mannose as a standard.\(^6\) SDS-polyacrylamide gel electrophoresis (PAGE) was done by the method of Laemmli\(^7\) using a gradient gel of 10–15% polyacrylamide (Daichi Pure Chemicals, Tokyo). Western blotting analysis was done as described previously.\(^8\)

**Results**

**Expression of mldA in A. oryzae.**

A. oryzae AO1 was transformed to nitrate prototrophy using the niaD gene on plasmid pNLGG2 and pNLGR2 (Fig. 1), which contain the MDGL-encoding mldA gene and its intronless gene, respectively. Fifty transformants each obtained from the transformation with pNLGG2 and pNLGR2 were randomly picked up and analyzed for MDGL productivity. MDGL activities were observed in the culture filtrate of 44 and 42 transformants, respectively. The other 14 strains were considered to be false transformants since no hybridization signal was detected by Southern blot analysis of DNAs from these 14 strains using mldA as a probe. The highest productivity were 11,100 U/ml, which corresponds to about 1 g/liter of MDGL protein as calculated with the specific activity of the recombinant MDGL purified from the transformant (see below).

In general, a transformation event in filamentous fungi is a chromosome integration type at different chromosome sites (homologous and nonhomologous sites) with various copy numbers. Since the copy number and integration site of the introduced gene affect the expression level of the gene, we compared distribution patterns and means of expression levels between intact mldA- and intronless mldA-introduced transformant groups to estimate the effects of introns on expression. Distributions of MDGL productivity of each transformant are shown in Fig. 2. There is no significant difference in the distribution patterns between pNLGG2- and pNLGR2-transformant groups and the mean productivities for both groups were also almost the same. From these results, the presence of the introns does not seem to affect the MDGL expression in A. oryzae.

![Fig. 1. Structures of Plasmids pNLGG2 and pNLGR2.](image)

![Fig. 2. Distributions of MDGL Productivities of the pNLGG2- and pNLGR2-Transformants.](image)

Transformants were cultivated for 3 days and MDGL activities in the culture filtrates were assayed. Average productivities for each transformant group are shown in the inside panel with standard deviations.
N- and C-Terminal processings of MDGL produced by A. oryzae transformant

MDGL secreted by the transformant, 1-26 from the pNLGR2-transformant group, was purified and characterized. Specific activity, assayed using vinyllaurate as a substrate, of the purified recombinant MDGL was 11,600 U/mg, which is identical to that of native MDGL (11,400 U/mg). The substrate specificity of recombinant MDGL was also almost the same to that of the native one, conserving its unique substrate specificity, i.e., substantially no activity toward triacylglycerol (Table). The N-terminal amino acid sequence of recombinant MDGL, analyzed by an automated protein sequencer, was identical to that of native MDGL. The released amino acids from both recombinant and native MDGL treated by carboxypeptidase Y were Phe, Pro, and Leu in this order of releasing rate, indicating the C-terminal sequence of both proteins was -Leu-Pro-Phe. These results indicate that the N- and C-termini of MDGL secreted by A. oryzae was correctly processed as in the original host, P. camembertii.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (U/mg, mean ± SD)</th>
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<tbody>
<tr>
<td>Recombinant</td>
<td>Native</td>
</tr>
<tr>
<td>1-ras-Monopalmitin</td>
<td>3469.9 ± 155.8</td>
</tr>
<tr>
<td>2-Monopalmitin</td>
<td>558 ± 1.5</td>
</tr>
<tr>
<td>1-ras-Monoolein</td>
<td>138.0 ± 15.7</td>
</tr>
<tr>
<td>1,2-ras-Diolein</td>
<td>472.6 ± 48.0</td>
</tr>
<tr>
<td>1,2-ox-Diolein</td>
<td>547.2 ± 65.1</td>
</tr>
<tr>
<td>1,3-Diolein</td>
<td>489.2 ± 56.9</td>
</tr>
<tr>
<td>Tripalmitin</td>
<td>1.0 ± 0.10</td>
</tr>
<tr>
<td>Triolein</td>
<td>1.0 ± 0.14</td>
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*Glyceride hydrolytic activities were assayed as described in Materials and Methods. Standard deviations (SD) were estimated from three independent measurements.

Lipid induction of mldA expression in A. oryzae

Two transformants, 1-26 and W-24 from the pHLGG2-transformant group, as well as P. camembertii U-150, which is the origin of mldA, were grown in the medium containing glucose or lipid as a sole carbon source and the MDGL activities in the culture filtrates were assayed. As shown in Fig. 4, the MDGL synthesis was clearly induced by lipids such as soybean oil or methylolate in both transformants and P. camembertii. The total RNAs were isolated from the transformant mycelium grown in both glucose and soybean oil medium and analyzed by Northern blotting using the mldA coding region as a probe. As shown in Fig. 5, the much larger amount of the 1.4-kb transcript for MDGL was observed in the RNA from soybean oil grown mycelium (lane 2) compared with that in the RNA from glucose grown mycelium (lane 1). The same results were obtained when the total RNAs from the glucose grown and soybean oil grown mycelium of P. camembertii were analyzed by Northern blotting (data not shown). These results indicate that the mldA expression is controlled transcriptionally in A. oryzae as well as in P. camembertii.

Deletion analysis of mldA promoter in A. oryzae

To investigate the upstream regulatory sequence required for expression of mldA, deletion analysis of the promoter was done. A series of plasmids with deletions were constructed and introduced into A. oryzae. Ten independent transformants for each plasmid were randomly selected and cultivated in the medium containing soybean oil or glucose as a sole carbon source. The levels of MDGL secreted in the culture filtrates were analyzed by the activity measurement and Western blotting. The average activities of ten transformants with each plasmid are shown in Fig. 6. Under both induction (soybean oil) and non-induction (glucose)
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Fig. 4. Effects of Carbon Sources on MDGL Productivities of *P. camembertii* and *A. oryzae* Transformants. Strains were cultivated for 3 days and MDGL activities in the culture filtrates were assayed. Relative productivities were calculated when the highest activity for each strain was taken as 100%.

Fig. 5. Northern Blot Analysis of RNAs Prepared from *A. oryzae* Transformant Grown in Glucose- and Soybean Oil-medium.

Total RNA was prepared from mycelium of transformant W-24 cultivated for 2 days in the medium containing glucose (lane 1) or soybean oil (lane 2) as a sole carbon source. Ten microgram of total RNA were separated by electrophoresis, blotted on a nylon membrane, and probed with *P*-labeled *mdlA* gene. The size marker RNA ladder was purchased from Gibco BRL (Gaithersburg, MD).

conditions, the MDGL expressions were observed in the transformants with the plasmids containing over 255 bp upstream from the translation initiation point (plasmids #1–4). The presence of MDGL protein in the filtrate of induced culture of transformant #4 and in those of non-induced cultures of #3 and #4 were demonstrated by Western blotting although the activities in these culture filtrates could not be detected due to the low activities. Higher level expressions were observed in the transformants with plasmids containing over 382 bp of the 5' upstream sequence (#1 and #2) in both induced and non-induced conditions compared to those of transformant with #3 and #4. These results indicates that (an) element(s) required for the basal expression of *mdlA* would be present in the 127-bp sequence between the position at -255 and -382 from the translation initiation point and also (an) element(s) required for the higher level expression would be in the 172-bp sequence between the position at -382 and -554. The levels of MDGL expression under the non-induction conditions were fairly low compared with those under the induction conditions. MDGL expressions under the induction conditions were about 8000-fold higher than those under the non-induction conditions, which were estimated in the cases of #1 and #2.

**Discussion**

It is well known that genes from filamentous fungi are functional in different filamentous fungi, even in different genera. Besides some genes such as *A. nidulans trpC, amds*, and *niaD* used for the transformations of other filamentous fungi as selection marker genes, many extracellular enzymes from filamentous fungi have been produced in the different filamentous fungi, each under the control of its own promoter. There are few reports, however, investigating the functions of the expression signal of the gene and the processing of the gene product in detail. In this study, the efficient expression of *P. camembertii* *mdlA* gene in *A. oryzae* was reported, revealing some facts as follows.

The presence of introns in the introduced gene did not seem to affect the expression level of the gene. The factors affecting the gene expression level in filamentous fungal transformants have been investigated. Besides the gene copy number, it was demonstrated that the integration sites of the introduced gene on the genome affected the expression of the gene. There is, however, no report concerning the effects of introns on the gene expression. We here first reported the results on such effects. It also seemed that the introns in *mdlA* were spliced correctly in *A. oryzae*. Expression of the *mdlA* gene was inducible by lipids and regulated at the transcriptional level in *A. oryzae* as well as
in *P. camemberti*. Closely similar machinery for the lipase gene expression seems to exist in both fungi. It was reported that the promoter sequences of *Geotrichum candidum* genes for lipase I (*lip1*) and II (*lip2*), which are inducible by lipids,\(^{244}\) and the transcriptionally higher expression of *lip2* than *lip1*,\(^{231}\) When the promoter sequences of *lip1*, *lip2*, and *mdlA* were compared, three oligonucleotide sequences were observed with complete (Fig. 7, region B, 8 bp) or incomplete (region A, 7 of 8 bp; region C, 8 of 9 bp) matching between *G. candidum* *lip2* and *mdlA* in the same order. These sequences were in the 172-bp region from −382 and −554 of *mdlA*, which was supposed to be important for higher level expression of *mdlA* in this study, and not observed in the less expressing *lip1*. Also, the region B includes the CCAAT sequence, which is often observed in the promoter of the higher expressing genes in filamentous fungi. It might be, therefore, that the three sequences correlate to the higher expression and/or lipid induction of the lipase gene. Since the full promoter sequences of other fungal lipase genes were not reported except for these cases, no further comparison could be done. A strain of *A. oryzae* was reported to produce a MDGL-like enzyme\(^{260}\) but the presence of lipid induction of the lipase expression has not been examined in the *A. oryzae* strain. *A. oryzae* strain used as a host in this study did not produce such a MDGL-like enzyme under the conditions we used.

With respect to the post-translational processing, both N- and C-terminal processing occurred correctly in *A. oryzae*. According to the general feature of signal peptide cleavage sites,\(^{271}\) it is considered that MDGL is synthesized as a prepro form having a 18-amino acid signal peptide and 8-amino acid pro peptide which ends with a basic amino acid, Arg. The N-terminal region of the MDGL pro form was probably processed at the C-terminal side of a basic amino acid by a trypsin-like protease in *A. oryzae* as well as in *P. camemberti*. The C-terminal amino acid sequence of both native and recombinant MDGL was -Leu-Pro-Phe by carboxypeptidase Y digestion. This C-terminal sequence of native MDGL is consistent with the observation of Isobe and Nokihara,\(^{230}\) in which trypsin-digested peptides from MDGL were sequenced. Since the C-terminal amino acid sequence deduced from the nucleotide sequences of both gene and cDNA is -Leu-Pro-Phe-Lys-Arg-Val, it is considered that the tripeptide -Lys-Arg-Val, containing a basic amino acid dipeptide, is removed by, probably, a yeast KEX2 protease-like enzyme which cleavages at a basic amino acid dipeptide pair. The presence of the KEX2-like protease have been already suggested in *A. nidulans*\(^{291}\) and *A. niger*.\(^{30,311}\) From our observations, *P. camemberti* as well as *A. oryzae* were presumed to have the KEX2-like protease. Although the N- and C-terminal processings occurred correctly in *A. oryzae*, the mode of N-glycosylation was different, i.e., the molecular mass of the carbohydrate chain attached to recombinant MDGL was about half of that of the native one. We have observed a similar phenomenon when other heterologous proteins such as bilirubin oxidase from *Myrothecium verrucaria* were produced in the same strain of *A. oryzae* used in this study.

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<tr>
<th>Plasmid</th>
<th>Soybean oil</th>
<th>Glucose</th>
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<tbody>
<tr>
<td></td>
<td>Act. (U/ml)</td>
<td>W</td>
</tr>
<tr>
<td>#1</td>
<td>859.2±584.3</td>
<td>+</td>
</tr>
<tr>
<td>#2</td>
<td>1583.0±1213.0</td>
<td>+</td>
</tr>
<tr>
<td>#3</td>
<td>3.1 ± 1.1</td>
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<tr>
<td>#4</td>
<td>0.05</td>
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<td>#5</td>
<td>0.05</td>
<td>-</td>
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<td>#6</td>
<td>0.05</td>
<td>-</td>
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<tr>
<td>#7</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>pN3</td>
<td>0.05</td>
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Fig. 6. Analysis of MDGL Expressions of Transformants Carrying *mdlA* with Various Length of 5'-Flanking Region. Average MDGL activities in the culture filtrates of ten individual transformants with each plasmid are shown with standard deviations (SD). In the column of W, + (or −) means that MDGL protein in the culture filtrate was (or was not) detected by Western blot analysis. The nucleotide numbers from translation initiation site are indicated. CCAAT, TATA, and tsp represent putative CCAAT box, TATA box, and transcription start point, respectively. Plasmid #2 means pNLGG2.

Fig. 7. Similar Sequences in the 5'-Flanking Regions of *P. camemberti* *mdlA*\(^{290}\) and *G. candidum* *lip2*.\(^{231}\)

The nucleotide numbers from translation initiation site are indicated. The distance between two similar sequences is shown as a nucleotide number in parentheses.
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(Yamaguchi *et al.*, unpublished results). These results are comparable to the other cases where overglycosylations were observed in proteins produced heterologously by filamentous fungal hosts.

Using the convenient characteristics of the *mdlA* gene, *i.e.*, 1) the efficient expression in *A. oryzae*, 2) the C-terminal processing at a basic dipeptide, 3) the functionality of the promoter *S. cerevisiae* reported previously, we have developed a novel heterologous expression system, succeeding in the production of some heterologous proteins from not only filamentous fungi but other higher eukaryotes. In another aspect, we have constructed and analyzed a mutant MDGL using the *A. oryzae* expression system described here.

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References