Rhizomucor miehei Triglyceride Lipase Is Processed and Secreted from Transformed *Aspergillus oryzae*

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The cDNA encoding the precursor of the *Rhizomucor miehei* triglyceride lipase was inserted in an *Aspergillus oryzae* expression vector. In this vector the expression of the lipase cDNA is under control of the *Aspergillus oryzae* α-amylase gene promoter and the *Aspergillus niger* glucoamylase gene terminator. The recombinant plasmid was introduced into *Aspergillus oryzae*, and transformed colonies were selected and screened for lipase expression. Lipase-positive transformants were grown in a small fermentor, and recombinant triglyceride lipase was purified from the culture broth. The purified enzymatically active recombinant lipase (rRML) secreted from *A. oryzae* was shown to have the same characteristics with respect to mobility on reducing SDS-gels and amino acid composition as the native enzyme. N-terminal amino acid sequencing indicated that approximately 70% of the secreted rRML had the same N-terminal sequence as the native *Rhizomucor miehei* enzyme, whereas 30% of the secreted rRML was one amino acid residue shorter in the N-terminal. The recombinant lipase precursor, which has a 70 amino acid propeptide, is thus processed in and secreted from *Aspergillus oryzae*. We have hereby demonstrated the utility of this organism as a host for the production of recombinant triglyceride lipases.
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The phycomycete fungus *Rhizomucor miehei* (1) secretes a triglyceride lipase (RML, triacylglycerol acylhydrolase EC 3.1.1.3). We have previously reported on the partial purification (2) of this enzyme, and we recently isolated the lipase cDNA from a *Rhizomucor miehei* cDNA library constructed in *E. coli* (3). From the DNA sequence of the lipase cDNA clones we deduced the amino acid sequence of the 363 amino acid lipase precursor. We presented evidence that RML is synthesized as a 39.4 kD precursor with a 24 amino acid signal peptide and a 70 amino acid propeptide. The propeptide is cleaved (in one or more as yet unidentified steps) from the 269 amino acid residues of the mature enzyme through a cleavage between a methionine and a serine residue in the precursor. The enzymatic mechanism in the maturation process remains unknown.

Triglyceride lipases have a number of potential industrial applications exemplified by their use in transesterification and in household detergents. Therefore, it will be important to provide a production system that will make these enzymes available at a reasonable cost. The use of recombinant DNA technology in the production of triglyceride lipases will probably be important in achieving this goal.

We now report on the heterologous expression of the *Rhizomucor miehei* lipase in another filamentous fungus —*Aspergillus oryzae*. We have recently established an efficient expression system for heterologous proteins in this organism (4). The aspartic proteinase (5) from *Rhizomucor miehei* was secreted from a transformed strain of this organism into the growth medium in high quantities. Our present experiments present evidence that the precursor of the RML is correctly processed in *A. oryzae*, and that the mature lipase is secreted into the growth medium.

MATERIALS AND METHODS

Expression of RML in Aspergillus oryzae. The 1.2 kb RML cDNA (3) was inserted by the use of synthetic adaptors and linkers into an *A. oryzae* expression vector pBoel-777 (4) using standard recombinant DNA technology (6). In the resulting construct pRML-787 (Fig. 1), the preproRML cDNA is flanked by a *Bam*HI site (GGATCC) just 5′ to the initiating methionine codon, the actual sequence reading: GGATCCACC*ATG*. Through this *Bam*HI site the 5′ end of the preproRML encoding cDNA is joined to a *Bam*HI site previously introduced at a position 9 nucleotides 5′ to the initiating methionine codon of the α-amylase gene. The 5′ untranslated region of an RML mRNA synthesized from this construct would therefore be identical in length and sequence to the 5′ untranslated region of the α-amylase mRNA from *A. oryzae*, except for the presence of the *Bam*HI linker 5′ to the methionine codon. Site specific oligonucleotide directed mutagenesis (6) in M13 vectors (7) was used to introduce

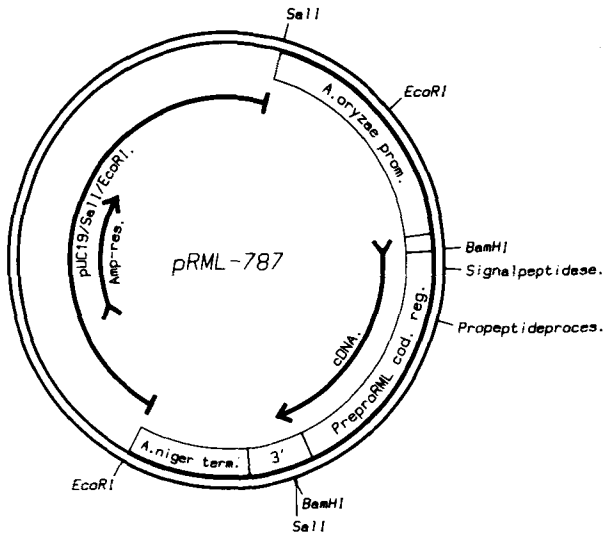

FIG. 1. The *A. oryzae* expression plasmid pRML-787 (5.7 kb) with the preproRML coding region inserted at a unique *Bam*HI cloning site between the *A. oryzae* α-amylase promoter and the *A. niger* glucoamylase terminator. "Amp-res." is the β-lactamase gene of pUC19. The position of the signalpeptide- and propeptide-processing sites in the RML precursor protein are indicated. "3′" designates a region that contains 3′ untranslated sequences from both the RML and the glucoamylase cDNA.

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Abbreviations used: bp, base pair; kb, kilobase; SDS, sodium dodecyl sulphate; LU, lipase units; RML, *Rhizomucor miehei* lipase; rRML, recombinant-RML synthesized in *A. oryzae*.

a *Bam*HI site at the 3′ end of the preproRML cDNA at a position 90 bp downstream from the terminator codon. This *Bam*HI site was ligated to a *Bam*HI linker that previously had been introduced in the *A. niger* glucoamylase gene at the *Sal*I site 5′ to the terminator codon of this gene (8). The 3′ untranslated region of the RML mRNA synthesized in *A. oryzae* is therefore composed of 90 bp from the native RML mRNA and 160 bp from the 3′ end of the glucoamylase mRNA.

This expression vector was cotransformed into *A. oryzae* wild type strain A1560 with the *AmdS* plasmid p3SR2 (9), as described (4). Transformants were selected by their ability to use acetamide as sole nitrogen source. This ability is encoded on p3RS2.

Stable transformants were picked from selective plates and maintained on Czapek-dox (10) agar slants. After 5–10 days of growth at 30°C spores were harvested from the agar slants and inoculated in 500 ml culture flasks containing 100 ml of SSP-medium (3% soybean meal, 1.5% potato starch and 0.5% Bacto peptone). The cultures were grown for 4 days at 30°C, and the lipolytic activity was assayed in the culture supernatants as the ability to cleave tributyrin (2).

Purification and characterization of rRML. An *A. oryzae* transformant that secreted lipolytic activity to the growth medium was selected for production of rRML, and was subsequentially grown in a small fermentor (4). Before the preparation of a crude rRML powder (2), the culture broth was adjusted to alkaline pH (approximately pH 10) in order to loosen the lipase protein from the mycelium. The crude rRML was chromatographed on DEAE Sepharose (2) to generate a partially purified rRML preparation, which was further purified by gel filtration chromatography (flow rate 5 ml/min) on a TSK G3000 SWG column (21.5 × 600 mm) mounted with a precolumn TSK SWPG (21.5 × 75 mm) (3). Procedures for the determination of the amino acid composition, carbohydrate content and isoelectric point of the purified rRML as well as protocols for the SDS-polyacrylamide gel-electrophoresis and tandem-crossed immunoelectrophoresis have previously been described (2,3). The N-terminal amino acid sequence of the purified rRML was determined by automated Edman degradation using an Applied Biosystems Model 470A gas phase sequencer (11). Approximately 4 nmol of purified rRML was applied to the filter of the sequencer, and 24 Edman degradation cycles were carried out.

RESULTS

The cDNA encoding the precursor (3) of the triglyceride lipase from *Rhizomucor miehei* was inserted in an *Aspergillus oryzae* expression vector (4) under control of the *A. oryzae* α-amylase gene promoter and the *A. niger* glucoamylase gene terminator to generate the plasmid pRML-787 (Fig. 1). We have recently (4) demonstrated that this type of expression plasmid directed efficient production of the aspartic proteinase from *Rhizomucor miehei* when the cDNA (5) for this protein was inserted into a similar vector. This construct was cotransformed into *A. oryzae* with the *amdS* gene (cloned on the plasmid p3SR2 [9]) from *A. nidulans* as selective marker. The *amdS* gene encodes an acetamidase enabling *A. oryzae* to grow on acetamide as sole nitrogen source. In the present study, approximately 80% of the transformants selected on acetamide expressed lipolytic activity in the supernatant. Untransformed *A. oryzae* did not produce any tributyrine hydrolyzing activity.

One such transformant was grown in a small fermentor in order to obtain enough rRML for purification and characterization. Purification was performed by consecutive steps of chromatography on DEAE Sepharose and TSK G3000 SWG columns. The 280 nm absorbance profile from the gel filtration step is shown on Figure 2. The two main peaks of eluted material represent an α-amylase secreted from *A. oryzae* and the rRML, respectively. On the SDS-polyacrylamide gel shown in Figure 3,

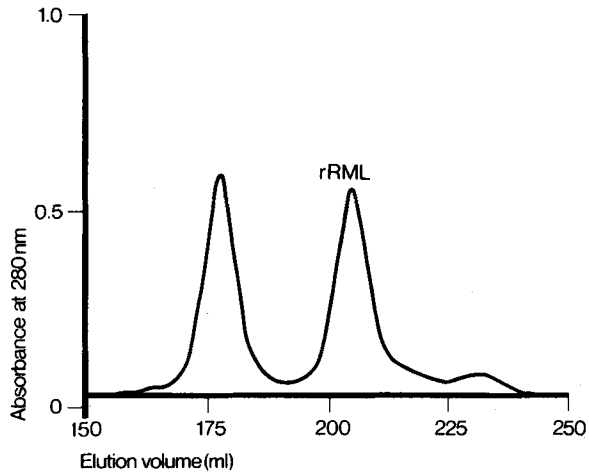

FIG. 2. Gel filtration chromatography of recombinant lipase on a TSK G3000 SWG column. The partially purified preparation of the rRML from the DEAE Sepharose chromatography was further purified on the TSK G3000 SW column (3). The peak to the left in the chromatogram represents an α-amylase endogenous to *A. oryzae*. Tributyrine hydrolyzing activity was only found in the pool represented by the peak designated rRML.

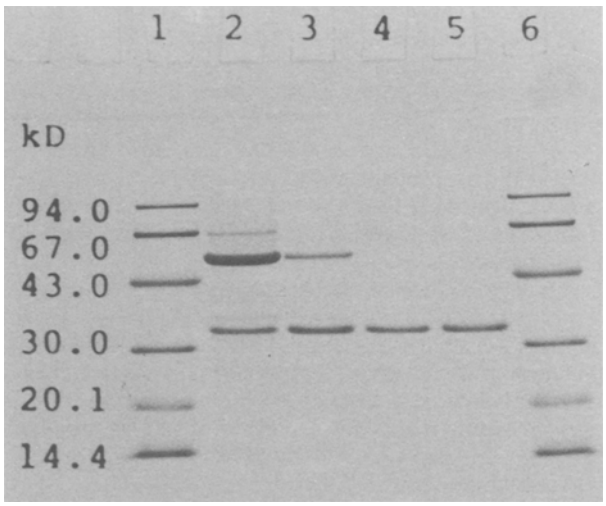

FIG. 3. SDS-Polyacrylamide gel electrophoresis of rRML. Lanes 1 and 6: molecular weight standards; Lane 2: Proteins in the crude powder from the ultra filtration step (17 μg protein applied). Lane 3: Partially purified rRML from the DEAE Sepharose chromatography (6 μg protein applied). Lane 4: Purified rRML from the TSK G3000 SWG column (3 μg protein applied). Lane 5: Purified RML-B from *Rhizomucor miehei* (3 μg protein applied). The gel was stained with Coomassie Brilliant Blue.

the results from the different purification steps were analyzed. Lanes 2–5 show the composition of the crude powder, the partially purified material from the DEAE Sepharose chromatography, the finally purified rRML from the gel filtration chromatography and the purified native RML-B (3) for comparison, respectively. The purified rRML as well as the purified native RML-B have apparent molecular weights of 32 kD. The specific activity of the purified rRML was 8810 LU/mg protein as compared to the 7500 LU/mg protein of the previously described (3) native RML-B. These values are based on protein determinations according to the Lowry method (12). The corresponding values based on protein determinations by amino acid compositional analysis were 12150 LU/mg protein of the rRML and 11200 LU/mg protein of the native RML-B. We have consistently observed a difference in the estimated amount of protein in the purified lipase as determined by the two methods.

The amino acid composition of the purified rRML is compared to the amino acid composition of RML as deduced from the cDNA sequence (3) in Table 1. Only minor deviations between the two compositions are noticed. As a further characterization of the recombinant lipase product determination of the isoelectric point was performed (Fig. 4). In this experiment the pI of rRML is compared to the pI of both native RML-A and RML-B (2). The pI of rRML is 4.3 as compared to 3.9 for RML-A and 4.3 for RML-B. We have previously shown that alkaline conditions (as employed in the purification of rRML in this study) or acidic conditions result in a partial deglycosylation of the native RML, and we have designated this partially deglycosylated variant the B form (2). The isoelectric point of rRML purified as described in this report indicates that this protein is on a form similar to the B type. The carbohydrate content of the purified rRML was approximately 1.2% as compared to 4% (w/w) for the purified native RML-B (3).

The antigenic identity between the rRML and the native RML was investigated using tandem-crossed immunoelectrophoresis. It is evident from the results shown in Figure 5 that the two lipases share a very high degree of antigenic identity.

As a final characterization we performed N-terminal amino acid sequence analysis on the purified recombinant lipase. The result of the analysis is shown in Table 2. Based on the relative yields of the amino acid derivatives in the first three cycles of the Edman degradation, we concluded the following: among the lipase molecules secreted and purified from *A. oryzae*, approximately 70% had the

TABLE 1

Amino Acid Composition of RML and rRML

Amino acid	RML[a]	rRML[b]
Asp/Asn	26	29
Thr[c]	27	25
Ser[c]	25	24
Glu/Gln	21	25
Pro	13	14
Gly	20	19
Ala	18	18
Cys-SH[d]	7	7
Val[c]	21	21
Met	1	1
Ile[c]	17	16
Leu	22	22
Tyr	15	14
Phe	10	10
Lys	7	8
His	6	6
Trp	3	4
Arg	10	10
Totals	269	273

[a] Deduced from cDNA sequence (3).

[b] rRML is the recombinant lipase secreted by *A. oryzae*.

[c] Determined as extrapolated values to 0 or infinite hydrolysis time.

[d] Determined as S-β-(4-pyridylethyl)-cysteine.

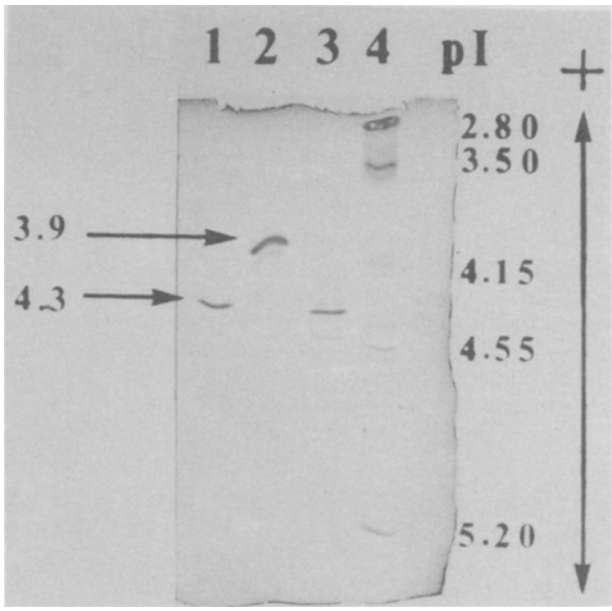

FIG. 4. Isoelectric focusing of purified rRML in comparison with the purified native RML-A and RML-B. Lane 1: rRML. Lane 2: RML-A. Lane 3: RML-B. Lane 4: Reference proteins.

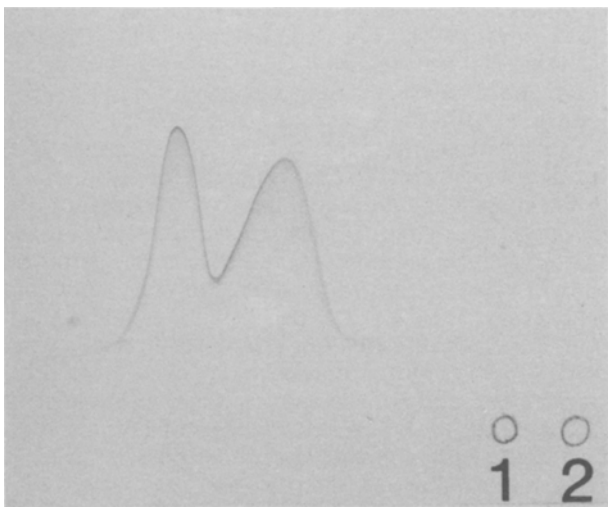

FIG. 5. Tandem-crossed immunoelectrophoresis stained with Coomassie Brilliant Blue. Well 1: the purified rRML. Well 2: a crude powder of the native RML. The rabbit antibodies applied in the electrophoresis were raised against purified native RML-B.

TABLE 2

Automated Edman Degradation of Purified rRML[a]

Cycle no.	PTH-a.a.[b]	Yield (nmol)	PTH-a.a.[b]	Yiled (nmol)
1	Ser	1.0	Ile	0.9
2	Ile	2.1	Asp	0.8
3	Asp	1.5	Gly	0.9
4	Gly	(1.6)[c]	Gly	(1.6)[c]
5	Gly	1.3	Ile	0.7
6	Ile	1.7	Arg	0.3
7	Arg	1.2	Ala	1.2
8	Ala	(2.5)[c]	Ala	(2.5)[c]
9	Ala	1.9	Thr	0.4
10	Thr	0.7	Ser	0.5
11	Ser	0.7	Gln	0.4
12	Gln	0.9	Glu	0.8
13	Glu	1.3	Ile	0.6
14	Ile	1.1	Asn	0.5
15	Asn	1.0	Glu	0.6
16	Glu	1.1	Leu	0.6
17	Leu	1.1	Thr	0.3
18	Thr	0.5	Tyr	0.7
19	Tyr	(1.2)[c]	Tyr	(1.2)[c]
20	Tyr	1.0	Thr	0.3
21	Thr	(0.4)[c]	Thr	(0.4)[c]
22	Thr	0.3	Leu	0.6
23	Leu	0.8	Ser	0.4
24	Ser	0.6	Ala	0.5

[a]The sequence determination was stopped after 24 cyles. The average repetitive yield during operation of the sequencer was 90.5%.

[b]PTH-a.a. is phenylthiohydantoin amino acid.

[c]The same residue is present in both sequences.

same N-terminal amino acid sequence as the native RML (Ser-Ile-Asp-Gly-Gly-, etc.) (3); the remaining 30% of the molecules had lost the N-terminal serine residue, and therefore had isoleucine at the N-terminal.

DISCUSSION

Recently, we demonstrated the versatility of *A. oryzae* as a potential host organism for the production of recombinant proteins (4). We demonstrated that the *Rhizomucor miehei* derived aspartic proteinase was secreted in high quantities (more than 3 grams/liter) to the growth medium. Furthermore, the specificity of the N-terminal processing of the propeptide from the zymogen of this enzyme was identical to that reported for the processing of the native enzyme precursor (13). Processing of the zymogens to the aspartic proteinases takes place through an autocatalytic mechanism, and therefore, correct processing of this type of zymogen in *A. oryzae* is not totally unexpected.

There is a rapidly growing interest in lipases from different sources. Many lipases have been purified and characterized, and an increasing number of lipase cDNAs have been cloned (14). We have continued our studies on the RML (2,3) by investigating the possibility of providing a recombinant system for production of the enzyme.

The precursor of RML has 24 amino acid residues in the signalpeptide, 70 amino acid residues in an N-terminal propeptide and 269 residues in the mature enzyme (3). The maturation of the enzyme thus involves a proteolytic cleavage between the C-terminal amino acid residue (Met) of the propeptide and the N-terminal residue (Ser) of the mature enzyme. We have no knowledge of the enzyme(s) involved in this processing in *R. miehei.* The versatility of *A. oryzae* as a host organism for the expression of recombinant triglyceride lipases will depend on the ability of this fungus to secrete and correctly process the lipase precursors. To answer that question we have purified and characterized the recombinant RML enzyme produced in *A. oryzae.*

Purified rRML showed the same mobility as purified native RML-B in a reducing SDS-polyacrylamide gel. The molecular weight of 32 kD for both enzymes compares well with 29,472 dalton, which is the calculated value for the protein backbone of the mature enzyme.

Within experimental error the amino acid composition of purified rRML was identical to the values for the RML cDNA sequence (3). The recombinant produced protein has the same pI as native RML-B, and has a high degree of antigenic identity with this molecule as determined through the tandem-crossed immunoelectrophoresis. About one third of the molecules in the secreted and purified rRML population had lost their N-terminal serine. This heterogeneous N-terminal processing of the triglyceride lipase precursor in *A. oryzae* does not influence the specific activity of the secreted recombinant molecule as compared to the native enzyme.

We anticipate that the primary translation product synthesized from the transfected construct in *A. oryzae* is the preprolipase, as it is specified by the cloned cDNA sequence (3). Thus, the simplest interpretation of our data is that *A. oryzae* and *R. miehei* share the enzymatic capacity to process both the signalpeptide of this precursor and the 70 amino acid propeptide present in the proRML molecule. The heterogeneity at the N-terminus of rRML would then be a result of degradation of the released protein. Other alternatives, however, such as formation of mature-sized rRML as a result of aminopeptidase activity in the fermentation broth of *A. oryzae,* have yet to be rigorously excluded.

In all the investigated characteristics of the recombinant-derived lipase molecule, we have noticed a high degree of resemblance to the native RML. We have thus demonstrated the ability of *A. oryzae* to synthesize, process and secrete the triglyceride lipase from *R. miehei.* The ability of *A. oryzae* to process and secrete an active heterologous triglyceride lipase significantly broadens the industrial potential of this filamentous fungus as a host organism for expression of recombinant proteins.

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