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Streptomyces rimosus GDS(L) Lipase: Production, Heterologous Overexpression and Structure-Stability Relationship

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Summary

Streptomyces rimosus lipase gene has been overexpressed in a heterologous host, *S. lividans* TK23. The maximal lipase activity was determined in the culture filtrates of the late stationary phase. Time course of lipase production was monitored by a modified plate assay. *S. rimosus* lipase gene has been located on the *Ase*I B fragment approximately 2 Mb far from the left end of the *S. rimosus* linear chromosome. Out of eight examined streptomycetes, the presence of this rare type of bacterial lipase gene was detected in two belonging to the *S. rimosus* taxonomic cluster, and in one non-related species. Comparison of protein sequences of the *Streptomyces* lipolytic enzymes was performed. The result indicated the best structural stability of the putative *S. coelicolor* lipase-2.

Key words: lipase, *Streptomyces*, plate assay, thermostability

Introduction

The genus *Streptomyces* consists of sporulating Gram-positive soil bacteria with a mycelial growth habit and a life cycle with complex morphological and physiological differentiation *(1).* According to Sztajer *et al. (2)* and Sommer *et al. (3)*, *Streptomyces* are not typical lipase producers compared to other bacteria. As mentioned by Vujaklija *et al. (4)*, there are only five cloned and sequenced lipase genes from streptomycetes described so far, of which three have about 82 % sequence identity: *S. exfoliatus* M11 *(5); S. albus* G *(6)* and *S. coelicolor* A3(2) *(7)* lipases, while two share no sequence similarity with either of the three or between each other, *i.e. S. cinnamomeus* lipase, LipA *(3)* and *S. rimosus* lipase *(4).* The regulation of formation of lipases and esterases in streptomycetes has attracted particular interest. Stationary-phase--dependent formation of lipases has been reported for lipA from *S. exfoliatus* and *S. coelicolor* A3(2) *(8).*

S. rimosus lipase gene has been recently cloned *(4).* The time course of lipase production and the purification of overexpressed lipase protein are presented here. To monitor efficiently the time course of lipase production in fermentation filtrates, a modified plate assay was developed. The position of lipase gene on *S. rimosus* chromosome and the incidence of *S. rimosus* lipase gene

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among various *Streptomyces* species was examined. A possible structure-stability relationship among different *Streptomyces* lipases was discussed.

Materials and Methods

Media and bacterial strains

The basal GR_2d medium *(9)* and culture conditions for lipase production were as described by Vujaklija *et al. (4). Streptomyces rimosus* R6-ZGL3(pDJ7) *(4)* was used as a source of lipase, while *S. rimosus* R6-ZGL3(pIJ699), not producing lipase, was a control strain. *S. lividans* TK23, a heterologous host, was transformed with plasmid pDJ7, harbouring *S. rimosus* lipase gene as described by Thompson *et al. (10).*

Assay of lipase activity

The specific lipase activity was measured spectrophotometrically with *p*-nitrophenyl palmitate (*p*NPP) as described by Abramić *et al*. (*11*) with some modifications. One unit (1 U) of lipase activity was defined as the amount of enzyme needed to liberate 1 μmol of *p*NP per minute in the described conditions.

Plate assays were performed to examine a lipolytic activity in culture filtrates on 1 % tricaprylin as a lipase substrate in 0.05 M Na_3PO_4 buffer, pH=8. The buffer and $CaCl_2$ (10 mM) were autoclaved and cooled to about 60 °C. Then tricaprylin (1 % v/v) was emulsified by vigorous stirring as described by Kouker and Jaeger (*12*). Clear haloes around 5 mm holes containing 50 μL of culture filtrates were measured after overnight incubation of plates.

Pulse field gel electrophoresis

Preparation of total DNA in agar plugs was performed according to Gravius *et al. (13).* Restriction digestion was performed with 50 U of *Ase*I enzyme. PFGE was run in 1 % agarose in 0.5 % TBE with 10 mM thiourea, using pulse programme at 200 V with ramp of the pulse time 40–50 seconds for 36 hours at 14 °C. Southern hybridisation was performed with an internal fragment of *S. rimosus* lipase gene.

Purification of overexpressed lipase

Overexpressed lipase from *Streptomyces rimosus* was purified in a three-step procedure. First step was batch-wise binding of lipase to CM-cellulose at pH=6.0 and elution from the column, performed at 4 °C. The amount of 840 mL of *S. rimosus* R6-ZGL3(pDJ7) *(4)* culture filtrate was adjusted to pH=6.0, mixed and shaken for 3×20 min with three portions of sorbens (120 mL and 2×60 mL of CM-cellulose equilibrated at pH=6.0), and sedimented on Büchner funnel. The cakes of CM-cellulose with bound lipase were resuspended in 10 mM Na_3PO_4, pH=6.0 with 10 mM EDTA (starting buffer A) and the column was filled. Packed column was eluted with 0.5 M NaCl in starting buffer. Eluted lipase was concentrated 4.5 times in Amicon concentrating cell with YM-10 membrane and desalted in four aliquotes by gel-filtration on Sephadex G-25 Medium (Pharmacia, Sweden) column (2.5×16.5 cm) in starting buffer for CM-cellulose. The next step was rechromatography on the column of CM-cellulose (2.5×32.5 cm) at 4 °C. After washing with starting buffer, elution was performed with a linear gradient of 0.00–0.35 M NaCl in buffer A, followed by 0.5 M NaCl in the same buffer. Pooled active fractions (in ~0.085 M NaCl) were concentrated 8 times in Amicon cell and desalted in aliquots by gel-filtration on a PD-10 column (Pharmacia, Sweden), equilibrated in 20 mM Na_3PO_4, pH=7.0 (starting buffer B). The final purification step was fast protein liquid chromatography (FPLC) on MonoQ column (Pharmacia, Sweden) in buffer B with 1 mM NaCl. It was performed at room temperature, but after elution (with gradient of 1–500 mM NaCl in buffer B) fractions were immediately placed on ice. Lipase was eluted from MonoQ column with ~0.059 M NaCl. Purified enzyme was stored at –10 °C.

Results and Discussion

Time course of lipase production

It has been reported that the lipase synthesis of *S. exfoliatus* and *S. coelicolor* A3(2) is growth phase dependent (*8*). Therefore the time course of lipase synthesis in *S. rimosus* R6-ZGL3(pDJ7) was analysed more carefully by quantitative measurements of lipase specific activity and biomass. As depicted in Fig. 1, very low lipase activity was found in the medium during exponential growth but lipase activity increased constantly once the cultures entered stationary phase. One possible explanation might be related to the recent finding that *Streptomyces* spp. are perhaps unique among prokaryotes in using triacylglycerols as storage compounds; it has been speculated that these serve as possible carbon sources for antibiotic synthesis in stationary phase (*14*). Horinouchi *et al.* (*15*) proposed that hydrolysing enzymes are required for the stationary phase of growth.

Optimisation of plate assay for lipase activity detection

A modified plate assay was developed to monitor lipase activity in culture filtrates. The original plate as-

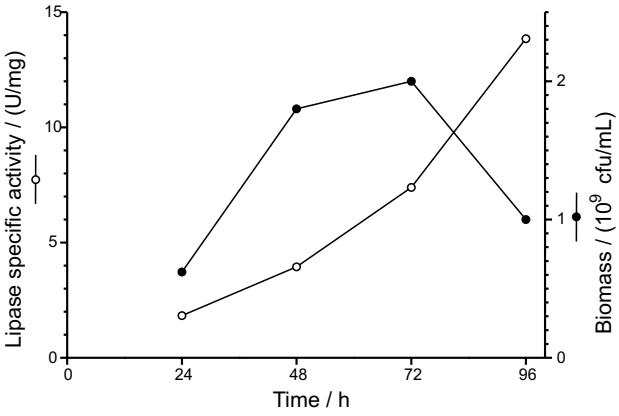

Fig. 1. Time course of lipase production by *S. rimosus* R6-ZGL3 harbouring pDJ7 possessing lipase gene. Number of colonies was obtained by plating dilutions of samples taken at indicated time intervals. Filled circles represent biomass; blank circles represent lipase specific activity.

Table 1. Incidence of *S. rimosus* lipase gene in other *Streptomyces*

Strain	Hybridization[a]	Numerical classification[b]	
		Cluster group	Group
S. coelicolor A3(2)	+	A-1	I
S. aurefaciens ATCC 23884	–	A-14	I
S. lividans TK 23	–	A-21	I
S. exfoliatus 5012	–	A-5	II
S. pheochromogenes ATCC 23945	–	A-40	II
S. rimosus ATCC 10970	+	B-42	non-grouped
S. annandii ATCC 19388	–	B-42	non-grouped
S. albofaciens ATCC 25184	+	B-42	non-grouped
S. chrestomyceticus ATCC 14947	–	B-42	non-grouped

[a] *S. rimosus* lipase gene was used to probe chromosomal DNAs
[b] Numerical clasiffication was designated as published before (*25*)

Table 2. Purification procedure for overexpressed *S. rimosus* lipase

Step	Total activity U	Specific activity U/mg	Yield %	Purification factor
Culture filtrate	67409	122.3	100.0	1.0
CM-cellulose	63162	174.0	93.7	1.4
reCM-cellulose	63162	359.2	93.7	2.9
FPLC (MonoQ)	46108	436.2	68.4	3.6

say applied olive oil as substrate and a fluorescent dye as indicator of lipolytic activity (*12).* Instead, we used tricaprylin as the optimal substrate in a buffered medium with addition of Ca^{2+} that eliminated inhibition of lipase by binding liberated caprylic acid, thus producing clear haloes. This was experimentally confirmed: when plates were incubated for several days, the Ca-caprylate crystals became visible around the haloes similar to those described by Sierra *et al. (16).* Another advantage of our method was the appearance of clear haloes already after two or several hours of incubation at 37 °C. This assay might be applied for screening of lipolytic activities in other *Streptomyces*, using various lipase substrates.

Heterologous expression of Streptomyces rimosus lipase gene

During the cultivation of the *S. rimosus* R6-ZGL3 that possesses lipase gene on a high copy number plasmid pDJ7, a significant plasmid structural instability was observed. To avoid this phenomenon common to majority of *Streptomyces* (*17*), we transferred the lipase gene into *S. lividans* TK23, declared as restriction modification negative. Besides, this strain does not possess homologous lipase gene (Table 1). The same level of lipase production was achieved in heterologous host. However, the rearrangements occurred in *S. lividans* TK23 with the same frequency as in *S. rimosus* (data not shown). Since the same level of the specific lipase activity was obtained as a result of heterologous expression in *S. lividans* TK23, the important conclusion is that all functions necessary for lipase production are on a 4 kb fragment of our construct, and that no additional chromosomal genes are required.

Enzyme purification

The original purification procedure was developed for this overexpressed extracellular lipase. In the first step, culture filtrate was bound batchwise to CM-cellulose. Starting from unconcentrated culture filtrates of *S. rimosus* R6-ZGL3(pDJ7) with 22 times higher specific activity than that of the wild-type strain (*4*), through three–step procedure described in Methods, a highly purified preparation of lipase (specific activity: 436 U/mg of protein) was obtained with a high yield of 68.4 % (Table 2).

Position of lipase gene on S. rimosus chromosome

According to the analysis of the *Streptomyces coelicolor* A3(2) chromosomal map *(18)*, all genes that are presumed to be unconditionally essential for the cell growth and division are located in the core region. On the contrary, the genes coding for probable non-essential functions, such as secondary metabolites or hydrolytic enzymes, lie at the ends of the chromosome *(18).* The production of *S. rimosus* and *S. coelicolor* A3(2) *(8)* lipases in a stationary phase indicates that these enzymes might not be essential for the basic metabolism. To get more insight into this, we decided to locate *S. rimosus* lipase gene on a linear chromosome *(19)*, and to compare its position with other lipase genes published for *S. coelicolor.* By procedure described in Materials and Methods, *S. rimosus* lipase gene was positioned on the *Ase*I B fragment approximately 2 Mbs far from the left end of the *S. rimosus* linear chromosome, similar to the position of the putative *S. coelicolor* lipase-1 gene (*4*), which is placed on the *Ase*I I fragment about 1.5 Mbs far from the same end *(18).* On the other hand, the positions of *S. coelicolor* A3(2) lipA (*7*) and the putative *S. coelicolor* lipase-2 (*4*) genes are closer to the left and the right chro-

Table 3. Distribution of cysteine residues in the mature streptomycetes lipase/esterase sequences

ENZYME		Position of cysteine in the primary structure					
Name	Total amino acids						
S. rimosus lipase	234	27	52	93	101	151	198
S. coelicolor putative lipase-1*	239	32	57	98	106	156	202
S. coelicolor putative lipase-2*	264	34	58	107	121	174	223
S. scabies esterase	306	34→	64	117→	141	197→	255
S. diastatochromogenes esterase	290	31	57	123	137	188	244
S. cinnamomeus lipase	245	15	52	140	143	203	241
S. exfoliatus lipase	262					242→	258
S. coelicolor lipase	262					242	258
S. albus lipase	262					242	258

→ Disulfide bridges proven by crystallographic data.
* Signal peptide cleavage site predicted with SignalP (*26*).

Table 4. Amino acid composition of mature lipases from *Streptomyces* strains

Amino acid	Amino acid composition									
	S. rimosus		*S. coelicolor* 1		*S. coelicolor* 2		*S. cinnamomeus*		*S. exfoliatus*	
	mol/mol	mol%	mol/mol	mol%	mol/mol	mol%	mol/mol	mol%	mol/mol	mol%
Ala	36	15.4	30	12.6	40	15.2	25	10.2	25	9.5
Leu	17	7.3	19	7.9	27	10.2	21	8.6	21	8.0
Val	15	6.4	14	5.9	17	6.4	23	9.4	13	5.0
Ile	7	3.0	9	3.8	8	3.0	6	2.4	11	4.2
Total	234		239		264		245		262	
mol% (Ala+ Leu+Val+Ile)		32.1		30.2		34.8		30.6		26.7

Data obtained from the protein sequences:
S. rimosus Q93MW7, *S. coelicolor* 1 Q9S2A5, *S. coelicolor* 2 Q93J06, *S. cinnamomeus* O33969, *S. exfoliatus* Q56008.

mosomal ends, respectively. At present, the results indicate that in both species at least one lipase gene is positioned close to essential region while the others are at the ends of nonessential regions, thus being exposed to rather frequent deletion events in *Streptomyces.*

The incidence of S. rimosus lipase gene in other Streptomyces

So far the *S. rimosus* GDS(L) lipase gene and two similar genes found by data base search in *S. coelicolor* A3(2) are the only examples of this type of lipolytic enzymes found among the streptomycetes. Furthermore, this type of lipolytic enzyme is reported only for one more bacterium, *Photorhabdus luminescens (20).* Therefore, we investigated the presence of *S. rimosus* lipase gene among other streptomycetes. Among the examined species (Table 1) we found the presence of this gene in two phenetically related and one non-related *Streptomyces.* These data are in a good agreement with observations of Cruz *et al. (6),* who found the incidence of *S. exfoliatus lip*A gene distributed in not closely related members of this genus. Similar distribution of lipase families has been found in other bacteria, like *Pseudomonadaceae (21).*

Structure-stability relationships in Streptomyces lipolytic enzymes

We have reported that *S. rimosus* lipase temperature optimum and thermal stability are similar to properties of lipases from thermophilic microorganisms *(11,22).* Different internal molecular mechanisms could contribute to that.

The correlation of enhanced stability with additional intramolecular S-S bonds has been shown for thermophilic enzymes when compared to their mesophilic counterparts *(23).* According to our knowledge, characterised streptomycete lipases contain two to six Cys residues in their molecule (Table 3). The stabilisation of 3-D structure by three disulfide bridges (Table 3) has been revealed for *S. scabies* esterase *(24).* The distribution pattern of Cys residues in the sequences of *S. rimosus* lipase and *S. scabies* esterase is similar (Table 3). Furthermore, secondary and tertiary structure prediction suggested significant similarity between them as well *(4).* This and the data on sensitivity of the *S. rimosus* lipase to reducing agent dithiothreitol *(4),* its insensitivity to thiol blocking agent *(11)* and its electrophoretic behaviour of monomeric protein (data not shown), indicate the presence of intramolecular S-S bonds in extracellular lipase from *S. rimosus.*

The content of hydrophobic amino acid residues Ala, Val, Ile and Leu was shown to be important for protein stability *(23).* The molar percentage of those four amino acids in the *Streptomyces* lipases (Table 4) is comparable to that of the lipases from thermophiles *Bacillus stearothermophilus* (24.5 %) and *B. thermocatenulatus* (37.9 %) *(22).*

Based on the results of the analysis summarised in Tables 3 and 4, relatively high stability could be expect-

ed for putative lipase *S. coelicolor*-2. Thermostability is an important requirement for industrial applications of enzymes. If the experimental results confirm our expectations, more rational approach in designing enzyme with significant biotechnological potential will be enabled.

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Biosinteza, heterologna prekomjerna ekspresija te odnos strukture i stabilnosti GDS(L) lipaze iz bakterije *Streptomyces rimosus*

Sažetak

Lipazni je gen iz bakterije *Streptomyces rimosus* prekomjerno eksprimiran u heterolognom domaćinu, *S. lividans* TK23. Maksimalna je lipazna aktivnost određena u filtratu bakterijske kulture u kasnoj stacionarnoj fazi. Za praćenje lipazne aktivnosti tijekom fermentacije modificirana je metoda na pločama. Lipazni gen iz bakterije *S. rimosus* lociran je na *Ase*I B fragmentu približno 2 Mb od lijevoga kraja linearnog kromosoma. Od osam ispitanih streptomiceta ovaj je rijetki tip bakterijskog lipaznoga gena otkriven u dvije vrste koje pripadaju istoj taksonomskoj skupini, te u jednoj nesrodnoj vrsti. Uspoređene su proteinske sekvencije lipolitičkih enzima iz streptomiceta**.** Pretpostavlja se da bi najstabilniju strukturu imala lipaza-2 iz soja *S. coelicolor A3(2).*