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Novel *Rhodococcus* esterases by genetic engineering

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Abstract

Esterases are important enzymes because of their ability to catalyze a number of hydrolytic reactions. Cloning and overexpression of esterase genes provides a useful strategy to make further esterases available for technical use. A genomic library of *Rhodococcus* sp. NCIMB 11216 was screened by activity assay using α-naphthol-acetate. Two independent esterase genes were isolated and one of them showed ability to hydrolyse esters of tertiary alcohols. By rescreening the genomic library using the bulky substrate, naphthol AS-D acetate, a few further esterase genes were isolated. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Esterases; *Rhodococcus* sp. NCIMB 11216; Allylic tertiary alcohol; Genetically engineered strains

1. Introduction

The genus *Rhodococcus* is a unique taxon consisting of microorganisms that exhibit broad metabolic diversity, particularly to hydrophobic compounds such as hydrocarbons, chlorinated phenolics, steroids, lignin, coal and petroleum. Bioprocessing systems employing various *Rhodococcus* strains are operational for industrial and environmental applications. Progress on the genetic systems of the rhodococci is rather limited, although a number of plasmids, cloning vectors, and DNA transfer systems have been reported recently [1].

The use of esterases for biotechnology has been frequently suggested, and this has been reinforced by the increasing interest in specific enantioselective hydrolysis and synthesis of esters [2–5]. In recent years, interest in asymmetric synthesis of chiral compounds has grown enormously in response to a growing awareness by the pharmaceutical and regulatory bodies that the optical antipode of a useful compound may be, at best, inactive or even harmful [6].

Rhodococcus sp. NCIMB 11216 contains several enzymes that catalyze the hydrolysis of carboxylic esters. Four different genes from *Rhodococcus* sp. NCIMB 11216 were cloned by screening for esterase activities expressed in *Escherichia coli* clones, encoding the esterases RR1, RR2, RR3, and RR4. One of these esterases, RR1, is able to hydrolyse acetate ester of the allylic tertiary alcohol linalool.

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2. Experimental

2.1. Materials and methods

2.1.1. Bacterial strains and plasmids

Rhodococcus sp. NCIMB 11216 was used as the DNA source for cloning of esterase genes. The vector for the cloning experiment was phagemid pBluescript II SK + / − (Stratagene). *E. coli* SURE or HB101 were the hosts for the recombinant plasmids.

2.1.2. Media and growth conditions

E. coli and *Rhodococcus* sp. were grown in L-broth (10 g of trypton, 5 g of yeast extract and 5 g of NaCl/l of water) at 37°C and 25°C, respectively. When needed, ampicillin was added at a concentration of 100 μg/ml.

2.1.3. Construction of the genomic library

Rhodococcus sp. NCIMB 11216 genomic DNA was partially digested with *Sau*3AI. A total of 3- to 7-kb fragments was filled in with dGTP and dATP, and ligated with pBSSK(+/−) plasmid DNA that had been previously digested with *Xho*I and filled in with dTTP and dCTP. Ligation products were used to transform *E. coli* SURE.

2.1.4. Screening for esterase positive clones

Bacterial colonies grown on LB-ampicillin (100 mg/l) agar plates were overlaid with 70 mm Whatman filter discs previously soaked in a solution of 1 ml of 0.1 M sodium phosphate (pH 7.0), 0.1 ml substrate stock solution (α-naphthyl acetate or naphthol AS-D acetate, 1% [w/v] in acetone) and 30 μl of fast blue B stock solution (2% [w/v] in H_2O). Esterase activity of bacteria was detected by development of a purple stain on the filter paper within 5 min.

2.1.5. Enzyme production

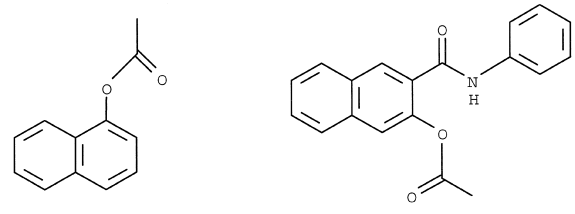
Cultivation of *E. coli* containing esterase plasmids were performed in 250 ml of LB containing ampicillin (100 mg/l). Cells were grown at 30°C for about 11 h and harvested by centrifugation ($10\,000 \times g$). Pellets were resuspended in 10 ml of 100 mM Tris–HCl (pH 7.0), stored overnight at −20°C, thawed and disrupted on ice with a sonificator (Labsonic 2000, B. Braun, FRG). After ultracentrifugation ($35\,000 \times g$) the supernatant and the resuspended pellet (100 mM Tris–HCl, pH 7.0) were stored separately, frozen at −20°C or lyophilized at 4°C.

2.1.6. Enzyme assay

Esterase patterns of protein fractions were analysed after fractionation by native polyacrylamide gel electrophoresis [7]. The samples were diluted in 2× dissociation buffer (50 ml of 20 mM Tris, pH 7.4, 50 ml of glycerol, 1 g of Triton X-100, and 0.1 g of bromophenol blue) and incubated for 10–15 min at room temperature prior to loading onto gels. Esterase activity of protein bands on gels was detected by incubating gels in a solution of 20 ml of 0.1 M sodium phosphate (pH 7.0), 2 ml substrate stock solution (α-naphthyl acetate or naphthol AS-D acetate, 1% [w/v] in acetone) and 500 μl of fast blue B stock solution (2% [w/v] in H_2O). The reaction was stopped by soaking gels in a 10% acetic acid solution. Esterase active proteins were detected as dark-red or blue bands.

2.1.7. TLC analysis

A sample of crude lyophilized enzyme preparation (50 mg) was rehydrated with shaking (30



α-naphthol-acetate naphthol AS-D acetate

Fig. 1. Substrates used for screening genomic library of *Rhodococcus* sp. NCIMB 11216.

min) in 1 ml phosphate buffer (0.1 M, pH 7.0). Then, substrate *rac*-linaloyl acetate was added (15 mg) and the mixture was agitated on a rotary shaker (200 rpm) at ambient temperature. Samples were withdrawn and analyzed by TLC using Merck Silica gel 60 F_{254} and petroleum ether/ethyl acetate (5:1) as eluent. Compounds were visualized by spraying with vanillin/H_2SO_4 conc. and heat treatment.

3. Results and discussion

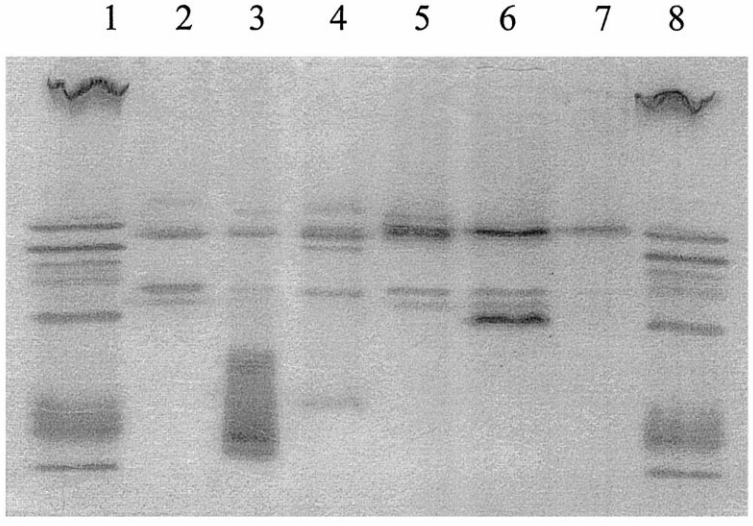
3.1. Cloning of esterase genes

For the isolation of the esterase genes, a genomic library was constructed as described in Section 2.1.3. Approximately 16 000 ampicillin-resistant transformants were screened for esterase activity using naphthol acetate substrates (Fig. 1).

By screening the library with α-naphthol acetate, two independent esterase genes were isolated and named RR1 and RR2. Another two esterases, RR3 and RR4, were isolated after screening the library with naphthol AS-D acetate.

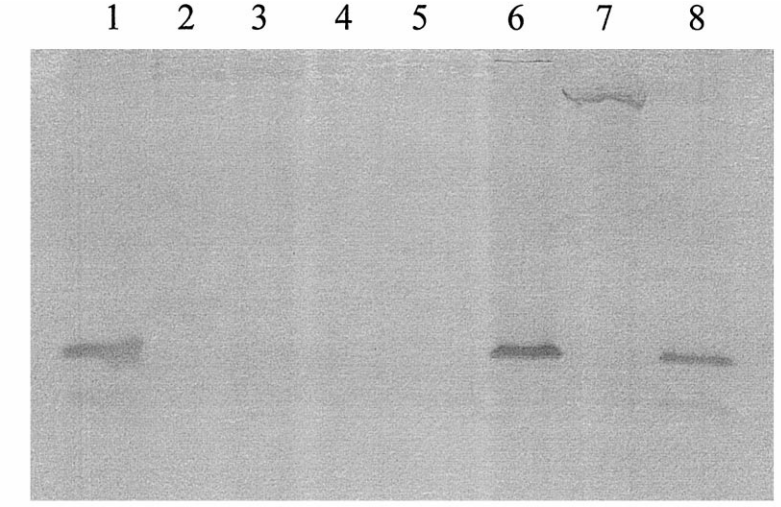
Restriction mapping of plasmids pRR1, pRR2, pRR3 and pRR4 revealed the presence of DNA inserts of 4.5 kb, 3.5 kb, 4.0 kb and 7.0 kb, respectively (results not shown). Since these clones contained inserts too big for immediate sequencing, active subclones containing 1.5–2.5 kb were constructed.

All four subclones were examined for esterase activity on native polyacrylamide gels described in Section 2.1.6. Esterase RR4 showed



Lane:
1) *Rhodococcus* sp. NCIMB 11216 – supernatant of crude lysate
2) *E.coli* HB101/pBSSK –
3) *E.coli* HB101/pRR1 –
4) *E.coli* HB101/pRR2 –
5) *E.coli* SURE/pBSSK –
6) *E.coli* SURE/pRR3 –
7) *E.coli* SURE/pRR4 – pellet of crude lysate
8) *Rhodococcus* sp. NCIMB 11216 – supernatant of crude lysate

Fig. 2. Activity gel stained with α-naphthol acetate and fast blue.



Lines:

1) *Rhodococcus* sp. NCIMB 11216 – supernatant of crude lysate
2) *E.coli* HB101/pBSSK –
3) *E.coli* HB101/pRR1 –
4) *E.coli* HB101/pRR2 –
5) *E.coli* SURE/pBSSK –
6) *E.coli* SURE/pRR3 –
7) *E.coli* SURE/pRR4 – pellet of crude lysate
8) *Rhodococcus* sp. NCIMB 11216 – supernatant of crude lysate

Fig. 3. Activity gel stained with naphthol AS-D acetate and fast blue.

Table 1
Activity of the *Rhodococcus* esterases on various chromatogenic substrates tested by staining in native polyacrylamide gels

Substrate	Esterase RR1	Esterase RR2	Esterase RR3	Esterase RR4
α-Naphthol acetate	+ + +	+ +	+ + +	+
β-Naphthol acetate	+ +	+ + +	+ + +	+ +
α-Naphthol propionate	+ + +	+	+ + +	+
β-Naphthol propionate	+ +	+ +	+ + +	+ +
α-Naphthol butyrate	+ +	+	+ + +	+
β-Naphthol butyrate	+	+	+ + +	+
β-Naphthol 2-chloropropionate	+	+	+ + +	+ +
α-Naphthol valerate	−	−	+ + +	+ +
β-Naphthol valerate	−	−	+ + +	+ +
β-Naphthol caproate	−	−	+	+ +
α-Naphthol nonaoate	−	−	−	−
α-Naphthol palmitate	−	−	−	−
Naphthol AS-D Cl-acetate	−	−	+ + +	+ +
Naphthol AS β-Cl-propionate	−	−	+ + +	+ + +
Naphthol AS-B1 butyrate	−	−	+ + +	+ + +
Naphthol AS benzoate	−	−	−	−
Naphthol AS phenylacetate	−	−	−	−

+ + + strong activity; + + active; + weak activity; − no activity.

activity in pellet and esterases RR1, RR2 and RR3 in supernatant fraction (Figs. 2 and 3).

The difference between lipases and esterases has not yet been defined with absolute clarity. One way of distinguishing is by substrate specificity [8]. While esterases preferentially split esters of shorter-chain fatty acids, lipases prefer esters of longer-chain fatty acid.

Novel *Rhodococcus* esterases were therefore tested for activity towards various chromogenic substrates. The activity was judged by comparing the colour intensity of the bands on native polyacrylamide gels. Results summarized in Table 1 show higher activity towards naphthol substrates of short-chain length (C2–C4) for esterases RR1 and RR2. Esterases RR3 and RR4 can hydrolyse some naphthol substrates of longer-chain length, as well as bulky substrates (naphthol AS substrates) which was not observed for esterases RR1 and RR2.

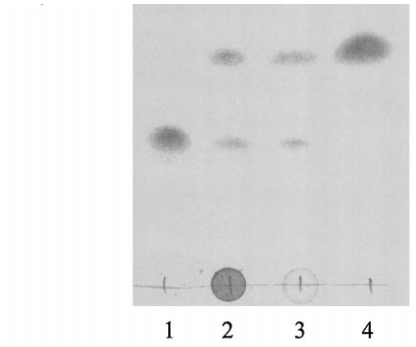


Fig. 5. Thin layer chromatography (TLC).

3.2. Hydrolysis of tertiary alcohol

The ability of esterases to hydrolyse tertiary alcohols was examined with linaloyl acetate as a substrate. *rac*-linalool was reported to be a non-substrate for several microbial lipases in esterification reactions [9,10].

The reaction (Fig. 4) was monitored by TLC as described in Section 2.1.7. Only esterase RR1 was found to be active on this substrate (Fig. 5).

Although the activity is not high, the ability of esterase RR1 to cleave sterically hindered esters of tertiary alcohol is remarkable particularly in view of the large number of lipases, proteases and whole bacterial cells tested which proved to be inactive [11].

Further studies are directed towards overexpression the structural genes and to test all esterase preparations in different hydrolysis reactions for activity, specificity and selectivity, as well as to modify the activity and selectivity using site and random directed mutagenesis.

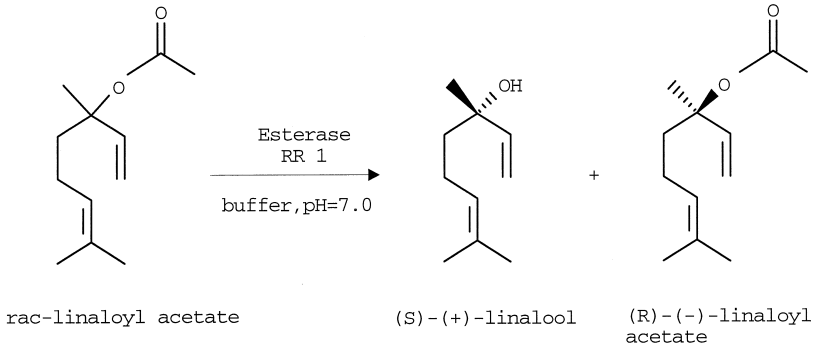


Fig. 4. Enzymatic resolution of *rac*-linaloyl acetate catalyzed by esterase RR1.

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