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Purification and Characterisation of an Ester Hydrolase from a Strain of *Arthrobacter* Species: Its Application in Asymmetrisation of 2-Benzyl-1,3-propanediol Acylates☆

S. Johri, V. Verma, R. Parshad, S. Koul, S. C. Taneja and G. N. Qazi*

Regional Research Laboratory (CSIR), Canal Road, Jammu-Tawi 180 001, India

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Abstract—An ester hydrolase (ABL) has been isolated from a strain of *Arthrobacter* species (RRLJ-1/95) maintained in the culture collection of this laboratory. The purified enzyme has a specific activity of 1700 U/mg protein and is found to be composed of a single subunit (Mr 32,000), exhibiting both lipase and esterase activities shown by hydrolysis of triglycerides and *p*-nitrophenyl acetate respectively. Potential application of the enzyme concerns the asymmetrisation of prochiral 2-benzyl-1,3-propanediol esters besides enantioselective hydrolysis of alkyl esters of unsubstituted and substituted 1-phenyl ethanols. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

Acylhydrolases (EC 3.1.1.3) commonly called lipases are ester hydrolases capable of catalysing the breakdown of ester bonds in water insoluble substrates, such as natural oils and fats, cholesterol esters and a large number of drugs, other bioactive molecules or their intermediates. They are ubiquitous in animals, plants, and microorganisms. Industrial applications of ester hydrolases range from fat conversion, accelerated cheese ripening and vegetable fermentation to detergent formulations, leather processing and in pharmaceuticals and cosmetics.[1–4] However, the enantioselectivity of many such enzymes offers interesting possibilities for their use in the processes for synthesis of optically active compounds as well as for separation of enantiomers or optical isomers in a racemic mixture.[5] Their use in the kinetic resolution of racemic carboxylic acids, halohydrins, ketorolac esters, cyanohydrins and octyl ester substrates has been reported.[6–11] Such recent interest in the potential use of microbial lipases/esterases in biotechnology has stimulated work on the purification and characterisation of several of these enzymes from microorganisms. A strain of *Arthrobacter* species (RRLJ-1/95) exhibiting lipase/esterase activity was isolated during the course of a screening programme in this laboratory. The enzyme designated as ABL shows wide substrate specificity and moderate to high enantioselectivity, characterisitics of an industrially potential lipase/esterase. The enzyme was found to be comparable to other commercially available lipases such as PPL (Porcine pancreatic), CCL (*Candida cylinderacea*) and PSL (*Pseudomonas* sp.) for its hydrolytic profile, enantioselectivity and overall reaction kinetics.[12] In this communication, besides isolation, purification and characterization of the enzyme, some examples of its application in chiral chemistry are described.

Results and Discussion

10–12 g wet cell pellet were obtained per litre of fermentation broth in a stirred tank batch reactor. The optimum cell mass and enzyme activity were obtained in 18–20 h time period (Fig. 1). >90% of the enzyme activity was found to be present on the washed cell pellet. Upon disruption of cells, the respective specific activities of lipase and esterase were of the order of 17 and 40 U/mg protein.

The majority of the bacterial lipases reported in the literature are extracellular; however, there are reports related to the presence of outer membrane bound lipolytic enzymes in bacteria.[13]

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*Corresponding author. Tel.: +91-191-578923; fax: +91-191-543829/

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The zymogram of the cell free extract of *Arthrobacter* sp. (RRLJ-1/95) under non-denaturating conditions showed several ester hydrolase active bands of varying molecular weight (Fig. 2a). However, aggregation of the specific ester hydrolase with other proteins and non-protein polymers present in the culture extract cannot be ruled out. Such a phenomenon of protein aggregation has been reported for a number of bacterial lipases.[14–16] The fast mobility of the major protein band showing the ester hydrolase activity (Fig. 2b) in native-PAGE suggest its acidic nature, which was further confirmed by binding of the enzyme with anion exchange resin.

About 52% of the activity was recoverable at 40–60% saturation of ammonium sulphate. The specific activity of over 60 U/mg protein obtained in precipitated protein at 60% saturation of ammonium sulphate was about 3-fold higher than that in crude cell extract.

Upon further purification on Phenyl-Sepharose CL-4B, specific activity of about 650 U/mg protein corresponding to over 30-fold increase was obtained. Contrary to the observations of Kordel et al. (1991),[16] the elution profile did not show tailing in the present study. Sephadex G-200 Gel filtration resulted in purification of the desired protein to homogeneity. The specific activity of the purified enzyme was of the order of 1700 U/mg protein, which corresponded to overall 100-fold purification and a final yield of about 15% (Table 1).

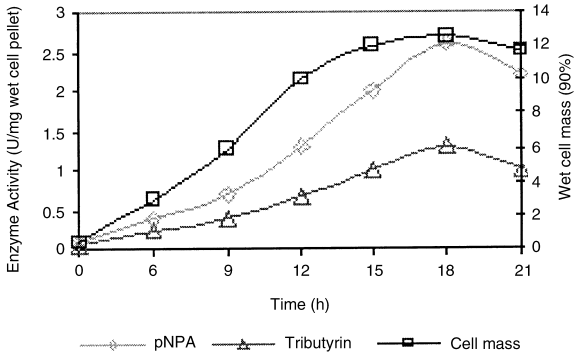


Figure 1. Growth and enzyme profile of *Arthrobacter* sp.

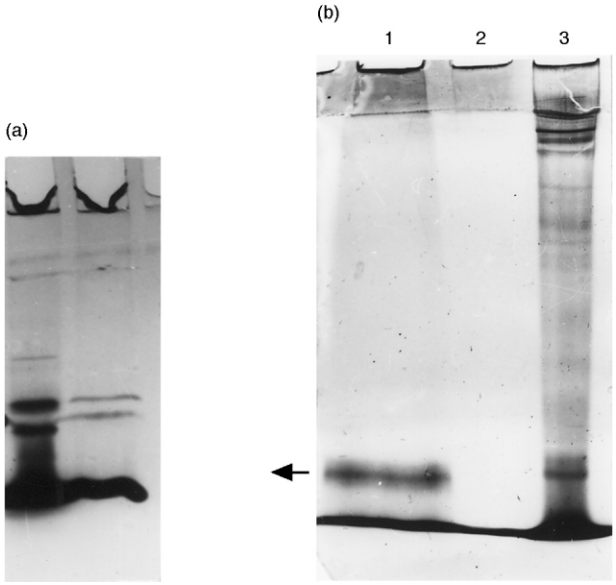


Figure 2. (a) 10% Native-PAGE electrogram of lipase from RRLJ-1/95 stained for lipase activity. (b) 11% Native-PAGE electrogram of lipase from RRLJ-1/95 stained with silver nitrate. Lane 1: purified lipase. Lane 2: blank. Lane 3: crude enzyme.

Purity of the enzyme was confirmed by the presence of a single band on non-denaturating PAGE gels (Fig. 2b), which corresponds to the position of the activity band on the zymogram. Furthermore, upon gel filtration with Sephadex G-200, the enzyme was eluted as a single peak soon after the void volume, indicating a molecular mass of >100 kD, perhaps due to the formation of aggregates, as has been also reported by Lesuisse et al.,[17] Jaeger et al.,[18] and Dharmsthiti and Ammaranond[19] for the lipases from *Bacillus subtillis*, *Pseudomonas strains* and *Trichosporon asteroides*.

SDS–PAGE of the purified protein depicted a single band corresponding to the molecular weight marker of 32 kD (Fig. 3). The low molecular weight of the enzyme (Mr 32 kD) as well as its single subunit structure is in agreement with those reported from several *Pseudomonas* sp. (29–35 kD)[20,21] but in contrast with those reported from other organisms like *Pseudomonas floresecens* MC50 (55 kD)[22] and *Staphylococcus aureus* (70 kD).[23]

The complete inhibition of the *Arthrobacter* lipase by the serine active reagent PMSF indicated that the enzyme is a member of serine hydrolases and serine residue may be easily accessible to the substrate.

The purified enzyme from *Arthrobacter* sp. (RRLJ-1/95) depicted pH and temperature optima of 8.5 and 37 °C respectively when tributyrin was used as substrate. The

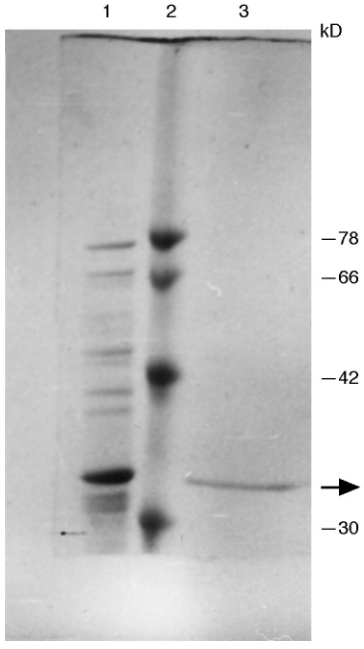


Figure 3. 10% SDS–PAGE electrogram of lipase from RRLJ-1/95. Lane 1: Fraction from phenyl sepharose. Lane 2: Molecular mass marker proteins (values shown on the right). Lane 3: Purified lipase.

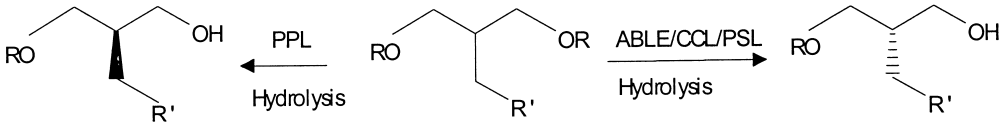
enzyme activity was not inhibited by EDTA (2 mM), indicating that the enzyme is not a metalloprotein.

Applications: asymmetrisation reaction

Lyophilised crude enzyme (ABL) prepared from *Arthrobacter* sp. (RRLJ-1/95) was earlier reported by us depicting high enantioselectivity (>98% ee) for the kinetic resolution of racemic 1-phenylethanol alkylacylates.[12] In the present study another application affecting asymmetrisation of mesodiester of 1,3-propanediol acylates is reported.

In the literature, only a few enzymes are reported to exhibit the property of generating monoester monocarboxylic or monoacetate propanol derivatives from 1,3-propanedicarboxylates or diol dialkyl acylates.[24] These chiral intermediates are useful precursors for a variety of pharmaceutical products. The dialkyl acylates of 2-benzyl-1,3-propanediol have one prochiral centre which can generate chiral monoalcohols in high yields (up to 100%) in reaction with the enzyme utilising the 'mesotrick' (see Scheme 1).

The experimental data of assays carried out with meso-2-benzyl-1,3-propanediol esters using both crude (ABL) and



Scheme 1. R = $COCH_3$, $COCO_2CH_3$, $COCH_2CH_2CH_3$; R′ = 4-methoxy, 3,4-dimethyl and 3,4-methylenedioxy phenyl.

Table 1. Purification of lipase (ABL) from *Arthrobacter* sp. (RRLJ-1/95)[a]

Fraction	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Total activity (U)	% yield
Cell free extract	210	16.8	1	3550	100
Ammonium sulphate ppt.	36.4	51.7	3	1875	52.8
Phenyl-Sepharose	1.8	655	39	1180	33.2
Sephadex G-200	0.64	1700	~100	596	14.8

[a]Activity is expressed as unit equivalents of fatty acids generated/min from tributyrin.

Table 2. Enantioselective hydrolysis of various substituted substrates with crude (ABL), purified (ABLE) *Arthrobacter* enzyme and its comparative activity with known lipases such as PPL, PSL and CCL

S. No	R	R′	Enzyme	Reaction time (h)	Isolated yield (%)	Specific[a] rotation (degrees)	ee[b]	Configuration of mono-acetate[c]
1	-$COCH_3$	3,4-methylene-dioxyphenyl	PPL	4	46	+24	87	R
2	-do—	-do—	PSL	4	37	−27.5	98	S
3	-do—	-do—	ABL	4	29	−22	74	S
4	-do—	-do—	ABLE	4	28	−20.5	72	S
5	-do—	-do—	CCL	4	38	−3.5	12	S
6	-$COCH_2CH_3$	-do—	PPL	5	57	+28.5	76	R
7	-do—	-do—	PSL	6	47	−37.5	>98	S
8	-do—	-do—	ABL	6	27	−16.5	44	S
9	-do—	-do—	ABLE	6	28	−16.4	44	S
10	-do—	-do—	CCL	6	41	n.d.	20	S
11	-$COCH_2CH_2CH_3$	-do—	PPL	6	56	+21.5	89.4	R
12	-do—	-do—	PSL	6	46	−24	>99	S
13	-do—	-do—	ABL	6	29	−16	66	S
14	-do—	-do—	ABLE	6	28	−16	66	S
15	-do—	-do—	CCL	6	43	n.d	8	S
16	-$COCH_3$	4-methoxy-phenyl	PPL	17	44	+11	54	R
17	-do—	-do—	PSL	17	35	−17.4	89	S
18	-do—	-do—	ABL	17	27	−6.7	34	S
19	-do—	-do—	ABLE	17	24	−5.8	29	S
20	-do—	-do—	CCL	17	32	n.d	8	S
21	-$COCH_3$	3,4-dimethoxy-phenyl	PPL	25	30	+17	77	R
22[d]	-do—	-do—	PSL	25	20	−28	97	S
23	-do—	-do—	ABL	25	28	−14.7	65	S
24	-do—	-do—	ABLE	25	25	−13.2	60	S
25	-do—	-do—	CCL	25	29	n.d	13	S

[a]$[\alpha]_D^{25}$ determined in chloroform.
[b]ee determined by chiral HPLC (Whelk S,S column).
[c]Configuration determined on comparison with known compounds.
[d]Itoh et al.[25]

purified (ABLE) enzyme preparations derived from *Arthrobacter* sp. (RRLJ-1/95) are given in Table 2. The table also contains the data on comparative activity analysis of the enzyme with other known commercial enzymes.[25]

Tombo et al.[26] reported asymmetrisation of meso-2-benzyl-1,3-propanediols (hydrolysis or esterification) using the crude PPL (Sigma L3126). However, they reported significant loss in activity and enantioselectivity when the purified fraction (PPL L0382) was used. In the present study, as shown in Table 2, the hydrolase activity of ABL remains intact even after purification, therefore both crude and purified protein can be utilised. Its activity in terms of degree of asymmetrisation of meso-diol ester is also comparable to other known commercial enzymes. In conclusion, ABL has all the potential to become a versatile and useful enzyme like other commercial enzymes of its class.

Materials and Methods

Preparation of enzyme

Arthrobacter sp. (RRLJ-1/95) used in this study was isolated and deposited in culture collection of the institute. 5 L volume of culture medium consisting of 1% tryptone, 1% yeast extract, 0.5% NaCl at pH 7.2 was inoculated with 0.1 L of an overnight preculture prepared in the same broth. The culture was grown at 30 °C for 18 h in an ideally mixed 10 L total volume bioreactor with aeration and agitation rates of 0.5 vvm and 500 rpm respectively. The cell pellet was separated from the broth by centrifugation at $8000 \times g$ for 10 min at 4 °C (Sorvall RC 5C). The cell pellet (~50 g) was washed with 50 mM Tris–HCl buffer (pH 7.0) and disrupted in the same buffer by ultrasonication in MSE 50 kHz ultra-sonifier at 4 °C for 5 min. The cell free extract was obtained by centrifugation of the cell homogenate at $15{,}000 \times g$ for 15 min at 4 °C (Sorvall RC 5C).

Enzyme assays

The method described by Lawrence et al.[27] was followed for spot agar diffusion assay of the enzyme during fractionation. Routinely the enzyme activity was monitored by titremetric assay using triglycerides as substrate. One unit of lipase activity was expressed as the amount of enzyme that releases 1 μmol titreable fatty acid/min.The esterase activity was assayed colorimetrically using *p*-nitrophenyl acetate as substrate as described by Ihara et al.[28] One unit of esterase activity was defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol/min. The method described by Gabriel[29] was followed for locating enzyme activity directly on polyacrylamide gels. Protein was determined photometrically by the modified method of Bradford.[30]

Purification of the enzyme

All lipase purification steps were performed at 5 °C except for the hydrophobic interaction chromatography, which was performed at 20 °C. Based on the pre-determined hydrophobic interaction of the desired protein, Phenyl-Sepharose CL-4B was employed for purification.

The protein precipitated from cell free extract at 40–60% ammonium sulphate saturation was fractionated on 6L-4B Phenyl-Sepharose (1.5 cm × 6 cm column), pre-equilibrated with 0. 6 M buffered ammonium sulphate. The bound enzyme was eluted by 1 mL/min buffered ammonium sulphate through negative linear gradient. The fractions were monitored for the peak enzyme activity by agar diffusion assay using tributyrin as substrate.

The pooled enzyme fractions thus obtained were dialysed against 10 mM Tris–HCl (pH 7.0). The concentrated protein solution was further fractionated on Sephadex G-200 (1 cm × 15 cm column) previously equilibrated with 50 mM Tris–HCl (pH 7.0) with 0.2 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 12 mL/h.

The approximate molecular weight of the protein bands was determined on SDS–PAGE performed according to the method of Laemmli.[31] The lipase active bands were identified on the gel by a zymogram using α-naphthyl acetate and Fast blue RR salt according to the method of Gabriel.[29]

Isoelectic focusing was done with Pharmacia ready to use gels with pH ranging from 3–9. The gels were run at 410Vh on Pharmacia fast gel system. Mixtures of pI markers between 3.5–9.3 were used as standard.

Preparation of substrate and enantioselectivity assay

2-Benzyl-1,3-propanediol alkyl acylates were selected as the substrates. These substrates were prepared from corresponding aryl aldehyde and diethyl malonate by Knoevenagel condensation. Unsaturated diester thus obtained was hydrogenated and subsquently reduced with lithium aluminium hydride to give prochiral 2-benzyl-1,3-propanediol. Acylation of the diol gave the required substrate. The products were purified by column chromatography and characterised by spectral and elemental analyses. The optical rotations of asymmetrised compounds were determined on Perkin–Elmer 241 polarimeter.

Enantioselectivities of the crude and purified enzyme preparations from *Arthrobacter* sp. (RRLJ-1/95) were compared with established commercial enzymes like *Pseudomonas* sp. (PSL Amano; enzyme activity-700 U/mg), *Candida cylindracea* (CCL, Sigma Chemical, USA; enzyme activity, 423 U/mg), Porcine pancreatic (PPL, Sigma Chemical, USA; enzyme activity 284 U/mg). The enzyme to substrate ratio was uniformly taken as 1:5. The reaction was carried out on 80 to 100 mg scale at 18–20 °C in 0.1 M phosphate buffer (pH 7.0) using pH stat (Metrohm).

The progress of the reaction during the assay was monitored by TLC. At the end of the reaction the contents

were extracted with chloroform (5×5 mL), washed with water (2×5 mL) and concentrated in vacuo at 35 °C. The product subjected to chromatography on SiO_2 and elution with *n*-hexane:ethyl acetate (9:1) yielded optically active monoacylates as one of the products in the first instance. Further elution with *n*-hexane:ethyl acetate (17:3) yielded the achiral diol. The chiral monoacylates were analysed by chiral HPLC using Whelk (S,S) E. Merck column and *n*-hexane:isopropanol (19:1) as mobile phase.

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