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Enantioselective hydrolysis of methoxyphenyl glycidic acid methyl ester [(±)-MPGM] by a thermostable and alkalostable lipase from *Pseudomonas aeruginosa*

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Abstract

A potent bacterial strain, *Pseudomonas aeruginosa,* has been isolated from the soil which produces extracellular lipase that can carry out the excellent stereospecific hydrolysis of *trans*-3-(4-methoxyphenyl)glycidic acid methyl ester [(±)-MPGM)] to give [(−)-MPGM], an intermediate required in the synthesis of cardiovascular drug, diltiazem. As a preliminary experiment for enzymatic resolution, we characterized the fractionated enzyme. The enzyme had a pH and temperature optima of 8.0 and 60 °C, respectively. The enzyme showed high degree of thermostability. Also, the enzyme was found to be stable in alkaline condition and in organic solvents. The activity of the enzyme increased by the addition of magnesium ions. The small-scale hydrolysis of (±)-MPGM (250 mg) with partially purified enzyme (21,000 U) gave (−)-MPGM with good isolated yield (44%) and excellent enantiomeric excess (99.9%) in a very short time (12 h).

Keywords: Lipase; Thermostable; Alkalostable; (±)-MPGM; Diltiazem

1. Introduction

Lipases (triacyl glycerol acyl hydrolases EC3.1.1.3) catalyze the hydrolysis of triglycerides to glycerol and fatty acids at oil–water interface. They are widely distributed in animals, plants and microorganisms [1]. Microbial lipases have been receiving particular attention because of their potential applications in the detergent, oil and fat and drug and pharmaceutical industries. Interest in the microbial lipases has increased marketedly in the last two decades owing to the potential industrial applications [2,3]. One of the applications is the synthesis of chirally important drugs and drug intermediates [4,5]. The stringent FDA guidelines regarding the chirally pure drugs has made it essential for the pharmaceutical industry to produce these in a more environment-friendly way.

Diltiazem hydrochloride, (2*S*,3*S*)-3-acetoxy-5-[2-(dimethylamino) ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5H)-one hydrochloride, is a typical calcium channel blocker and has been clinically used as an effective anti-anginal and anti-hypertensive agents [6,7] in more than 100 countries for over than 20 years. Conventional production was carried out using a chemical synthetic process through nine steps from 4-anisaldehyde and chloroacetic acid methyl ester [8]. However, in the chemical process, a large amount of loss in the raw material occurs, as the optical resolution was carried out at the later stage of the process using a high molecular weight compound, i.e. *threo*-2-hydroxy-3-(4-methoxyphenyl)-3-(2-nitrophenylthio)-propanoic acid. A few lipase catalyzed resolution of (±)-MPGM have been reported using the lipases from the commercial sources and using the enzyme from *Serratia marcescens* [9,10]. The process has been developed on the industrial scale using the lipase from *S. marcescens* [8]. A maximum of 48.2% yield along with an

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Table 1
Reported organisms capable of resolution of (±)-MPGM

Organism	Conversion (%)	ee (%)	Reference
Serratia marcescens sr41 8000	48.2	89	[8]
Pseudomonas putida ATCC 17426	44.2	75.1	[8]
Cornybacterium promorioxydans ATCC 31015	44.6	75.6	[8]
Pseudomonas mutabilis ATCC 31014	44.7	74.7	[8]
Cornybacterium alkanolyticum ATCC 21511	45.8	77.4	[8]
Candida cylinderacea	82	50	[8]
Bacillus licheniformis	41	57	[8]
Rhizopus japonicus	58.4	42.8	[8]
Pseudomonas sp.	49.8	68.6	[8]
Serratia marcescens ECU1010	–	97	[9]
Candida cylindradea	45	99	[18]

enantiomeric excess of >89% was obtained (Table 1). In view of the potential uses of this enzyme, it is desirable to study this enzyme from different microbial sources. In order to further improve the hydrolytic process in terms of conversion and enantiomeric excess and the reaction time, we conducted an extensive screening for the microorganisms that can carry out the stereospecific resolution of (±)-MPGM. As a result of this screening a bacterial strain, *Pseudomonas aeruginosa* was obtained that is capable of carrying out the stereospecific resolution of (±)-MPGM giving good yield (44%) and excellent ee (99.9%). We herein report the screening of the microbial strain, characterization of the enzyme and its subsequent exploitation in the stereospecific resolution of (±)-MPGM.

2. Materials and methods

2.1. Chemicals and medium

(±)-Methoxyphenyl glycidic acid methyl ester was prepared from 4-anisaldehyde and methyl chloroacetate in presence of sodium methoxide according to the reported procedure [11]. *p*-Methoxyphenylacetaldehyde was also synthesized in laboratory (M^+) (*m*/*e*) 151. ^{1}H NMR δ ($CDCl_3$, 300 MHz), 3.57 (2H, M CH_2CHO), 3.75 (3H, S, OCH_3), 6.85 (2H, d, J = 8.5 Hz, Ar–H), 7.09 (2H, d, J = 8.5 Hz, Ar–H), 9.67 (1H, S, CHO). All other chemicals were purchased from various commercial sources and were of analytical grade. Soil samples were collected from different areas of Punjab region of India. Minimal salt medium (MSM) consisting of disodium hydrogen phosphate (0.2%), potassium dihydrogen orthophosphate (0.1%), ammonium chloride (0.04%) and magnesium chloride (0.04%) was used with (±)-MPGM as the sole source of carbon and energy. Agar plates were prepared by supplementing MSM with 2 mM (±)-MPGM. The production medium consisted of peptone (0.5%), beef extract (0.15%), yeast extract (0.15%), NaCl (0.5%) and glucose (1%).

2.2. Isolation of microorganism

A soil suspension was prepared by adding 10 ml tap water to 1 g of the soil sample. It was vortexed and 1 ml supernatant was used as an inoculum in 100 ml MSM containing 2 mM (±)-MPGM medium and was incubated at 30 °C in an orbital shaker (200 rpm) for 5–7 days. Enriched samples were streaked on selective plates (the plates containing [(±)-MPGM])] in MSM agar. The organisms so obtained were purified and maintained on nutrient agar plates (0.5% peptone, 0.15% yeast extract, 0.5% beef extract, 0.5% sodium chloride, agar 1.5%, pH 8). These organisms were further grown in nutrient broth (0.5% peptone, 0.1% yeast extract, 0.5% beef extract, 0.5% sodium chloride, pH 8). The enzyme activity, the hydrolytic potential and the enantioselectivity for (±)-MPGM hydrolysis were determined. Out of these, the organisms showing the maximum hydrolytic activity and enantioselectivity were chosen for further studies. The microbial strain showing the best hydrolytic potential was identified according to the general procedures of Bergey's Manual of Systematic Bacteriology [12] at Microbial Type Culture Collection (MTCC), Chandigarh, India.

2.3. Analytical methods and enzyme assay

Enzyme assay was carried out by the method of Winkler and Stuckmann [13] with a little modification using *p*-nitrophenyl palmitate as a substrate. The substrate was dissolved in iso-propanol (3 mg/ml). It was then emulsified with aqueous solution (9 ml) of gum arabic (0.11%) and triton X-100 (0.44%). This emulsion (0.9 ml) was mixed with 1.5 ml Tris–HCl buffer (50 mM, pH 8) and 0.5 ml $CaCl_2$ (75 mM). The mixture was preincubated at 60 °C for 5 min and 100 μl of appropriately diluted enzyme was added and incubation was continued for further 10 min. The optically density was taken at 410 nm spectrophotometrically with proper enzyme and substrate blanks. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol/min of *p*-nitrophenol under the standard assay condition.

Conversion and the enantiomeric excess of the hydrolytic reaction were monitored by HPLC performed on a Shimadzu 10AVP Instrument equipped with UV detector using a Chiracel ODH column (0.46 mm × 250 mm, 5 μm, Diacel). The mobile phase used was hexane:isopropyl alcohol in the ratio of 85:15 (v/v) at a flow rate of 0.5 ml/min and detected at 254 nm. ^{1}H NMR spectra was recorded on a Bruker Advance DPX 300 NMR spectrometer. Chemical shifts were reported in parts per million (ppm, δ) using TMS as an internal standard. LCMS was performed on mass spectrometer (LCQ Finnigan MAT, England) using Chiracel ODH column (0.46 mm × 250 mm, 5 μm, Diacel) with mobile phase consisting of hexane: isopropyl alcohol