Appl Microbiol Biotechnol (1998) 49: 709–717

ORIGINAL PAPER

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Purification, characterization and immobilization of an NADPH-dependent enzyme involved in the chiral specific reduction of the keto ester M, an intermediate in the synthesis of an anti-asthma drug, Montelukast, from *Microbacterium campoquemadoensis* (MB5614)

Received: 31 October 1997 / Received revision: 8 January 1998 / Accepted: 24 January 1998

Abstract (*S*)(*E*)-2-{3-[3-[2-(7-chloro-2-quinolinyl)ethenyl]-phenyl]-3-hydroxypropyl} benzoic acid methyl ester, a key intermediate in the synthesis of the anti-asthma drug, Montelukast, was prepared from the corresponding ketone (keto ester M) by microbial transformation. The biotransforming organism, *Microbacterium campoquemadoensis* (MB5614), was discovered as a result of an extensive screening program and was used for the isolation and purification of the responsible enzyme. The enzyme is a soluble cytoplasmic protein which was purified as a complex with a low-molecular-mass molecule that had a visible-light absorption maximum at 460 nm. The purified enzyme has an apparent molecular mass of 60 kDa, when denatured, and is isolated in the native state as an oligomer. The isolated enzyme requires NADPH for its activity and reduces the keto ester M to the desired (*S*)-hydroxy ester with an enantiomeric excess greater than 95% at the optimum temperature of 30 °C and pH 8. The enzyme was immobilized on oxirane-activated acrylamide beads with some loss of activity, but it was fully active in a two-phase (water/hexane 25:75) solvent system, both as a free solution and in an immobilized form.

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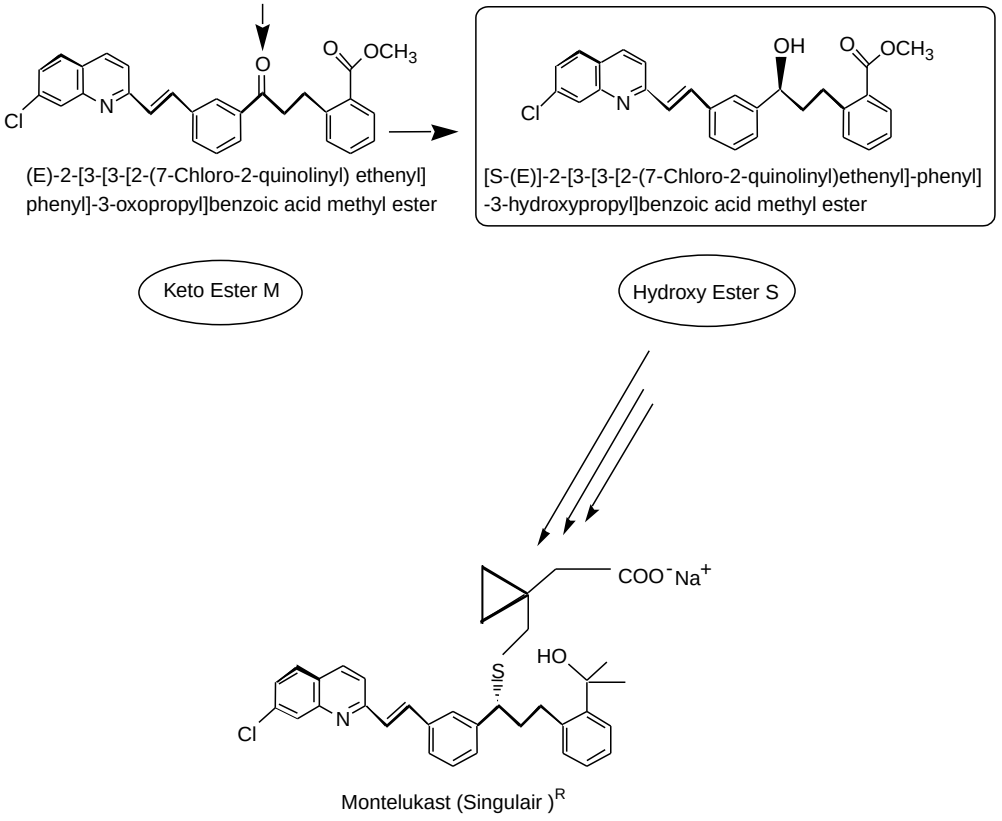
Introduction

Merck's scientists have chemically synthesized a compound, Montelukast, which functions as an antagonist toward one of the leukotrienes, LTD_4. This compound is being developed as a therapeutic agent for the treatment of asthma (currently under FDA review). The chemical process for the synthesis of multikilogram quantities of this material is well established. One of the steps of the synthesis of Montelukast involves the stereospecific reduction of a keto ester intermediate, [(*E*)-2-[3-[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-3-oxopropyl]benzoic acid methyl ester, designated here as keto ester M (Scheme 1). Much effort has been directed toward the development of a catalytic procedure for the chiral reduction of this ketone to the desired (*S*)-hydroxy ester. As a result, a method has been developed that uses Brown's β-chlorodiisopinocampheylborane for the stereoselective reduction of the ketone to the desired hydroxy ester (King et al. 1993). Despite the availability of this method, alternative economical and environmentally acceptable procedures are desired.

Issues including stereoselectivity and reusability, as well as biodegradibility of the reagents and economic factors, make microorganisms appealing catalysts in the chemical synthesis of various chemical and pharmaceutical materials (Ward and Young 1990). In view of the fact that microbial (Matsumae et al. 1995) and enzymatic (Hummel and Kula 1989) chiral reductions of ketones have shown great promise for commercial applications, we started a screening program to find microorganisms for stereoselective reduction of the ketone intermediates that are important to Merck's manufacture of pharmaceutical compounds. Consequently, keto ester M, a key intermediate in the synthesis of Montelukast, was selected as the substrate in the screening of a panel of microorganisms for evaluation of their potential stereoselective reductions.

In this paper, we report the discovery of two microorganisms, identified as *Microbacterium campoquema-*



Scheme 1

doensis (MB5614; ATCC accession number 55557) and *Mucor hiemalis* (Heimbuch et al. 1994), that are capable of stereoselectively reducing the keto ester M. In addition, we also report the isolation of the reductase responsible from the first organism, and its purification and characterisation.

Materials and methods

Preparation of the biotransformation cultures and screening procedures

A group of 80 microorganisms, consisting of yeasts, fungi, and bacteria, were randomly chosen from our culture collection and were grown in seed and biotransformation media. The seed medium comprised of 0.1% dextrose, 1% dextrin, 0.3% beef extract, 0.5% ardamine PH, 0.5% NZ amine type E, 0.005% $MgSO_4 \cdot 7H_2O$, 0.037% K_2HPO_4, and 0.05% $CaCO_3$ with the pH adjusted to 7.1 before autoclaving. The biotransformation medium contained 2% glucose, 0.5% soya meal, 0.5% yeast extract, 0.5% NaCl, 0.98% MES with the pH adjusted to 7.0 before autoclaving.

Frozen seed cultures, or isolated colonies that were stored on solid agar plates, were used for the inoculation of the seed medium. Normally, a 2-ml suspension or a loopful of seed culture was inoculated into a 50-ml conical flask containing 10 ml sterilized seed medium. These cultures were incubated at 27 °C on a shaker with a 220 rpm gyratory motion. After overnight incubation, a 2-ml sample of each of the seed cultures was transferred into 50 ml sterilized biotransformation medium contained in a 250-ml baffled conical flask. To each biotransformation culture was added a 0.5-ml acetone solution of keto ester M (10 mg/ml) and the flasks were incubated under conditions similar to those described before for the preparation of the seed cultures. Owing to the sensitivity to light of the keto ester and its reduction product, all of the biotransformation experiments were carried out in the dark.

Cultures under investigation were screened for the chirally specific reduction of keto ester M by analysis of an aliquot of the biotransformation broth as follows. At various times, a 1-ml sample was taken from each flask and mixed with 1 ml isopropanol. The resulting mixture was centrifuged after vortexing and an aliquot from each supernatant was examined by HPLC on a reverse-phase column under the conditions described below. The extracts showing reduction of the substrate were then examined for the stereospecificity and enantiomeric excess (e.e.) of the hydyroxyl ester on a chiral column (see below).

Enzyme assay

The enzymatic reaction was carried out in 1 ml solution (final volume) containing 0.1 M TRIS/HCl or 0.1 M potassium phosphate buffers, pH 8.0, 3 mM NADPH, 0.05 mM substrate (keto ester M) and the appropriate volume of enzyme solution. The reaction was initiated by the addition of the substrate and the mixture was incubated in the dark at 30 °C for 3 h. Under these conditions, the rate of the reduction was directly proportional to the amount of protein (MonoQ fraction, see below) added, up to 200 mg. With 100 mg protein in the assay mixture, the rate of the enzymatic reduction was first-order for at least 5 h. For kinetic analysis, a highly purified MonoQ fraction was utilized. Kinetic parameters (V_{max} and K_m) for the keto ester M (substrate) and

NADPH (cofactor) were obtained from the Lineweaver-Burk double-reciprocal plot under conditions of initial velocity. The parameters for the keto ester M (variable substrate, 10.96–109.6 mM) were determined at 3 mM NADPH, a saturating concentration. Similarly, the parameters for NADPH (0.12–4.8 mM) were obtained using 0.05 mM keto ester M. No inhibitory activity was observed when 0.05 mM substrate and 4.8 mM NADPH were used in the assay.

HPLC analysis of the extracts of the biotransformation cultures and enzymatic reaction products

The HPLC (Waters, Milford, Mass.) analysis of the extracts were carried out on a Whatman Partisil 10 ODS-3 analytical column (Clifton, N.J.). The column was developed with a linear gradient of acetonitrile in water in which the concentration of the acetonitrile was raised from 55% to 95% in 30 min with a flow rate of 1 ml/min at the column temperature of 45 °C. The chirality of the product was determined by HPLC analysis of the purified fraction on a Chiralcel OD column (0.46 × 25 cm; Chiral Technologies Inc., Exton, Pa.). This column was developed isocratically with hexane/isopropanol (80:20) at a flow rate of 1 ml/min. For product characterization, a methylene chloride extract of the fermentation broth was worked-up and applied to a semi-preparative silica gel TLC plate, and the plate was developed in a methylene chloride solvent system. The developed plate was examined under UV light and the fraction showing an R_F value similar to that of the standard (*S*)-hydroxy ester was localized and extracted. The extract was further purified by HPLC using a semi-preparative Whatman Partisil 10 ODS-3 (Magnum 9, Clifton, N.J.) column. This column was developed by a solvent system under the conditions described for the analytical column (see above) except for the flow rate, which was 3 ml/min.

Purification of the keto ester M reductase

Microbacterium campoquemadoensis (MB5614) was grown for 44 h, as described above, and harvested by centrifugation at 27 000 *g* for 120 min. The pellet was recovered and washed three times with 0.1 M phosphate buffer, pH 7.2, and suspended in 0.1 M TRIS/HCl, pH 8, containing, 1 mM EDTA, 2 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 10% glycerol (buffer A). The resulting suspension was passed through a French press twice under 138 MPa pressure. The cell homogenate thus prepared was centrifuged at 200 000 *g* for 2 h and the supernatant (cell-free extract) was recovered. Streptomycin sulfate (1%) was then added to the enzymatically active cell-free extract. The resulting solution was stirred overnight and centrifuged at 200 000 *g* for 2 h. The supernatant was then brought to 10% ammonium sulfate saturation, stirred for 30 min, and centrifuged as above. The small pellet was discarded and the supernatant was subjected to Q-Sepharose (Pharmacia, Piscataway, N.J.) column chromatography (2.5 cm × 75 cm). The column was washed with 450 ml buffer A and developed with a linear gradient of KCl (0–1 M) in buffer A with a flow rate of 84 ml/h. Fractions of 7 ml were collected and each fraction was examined for enzyme activity and for the light-absorption properties (200–600 nm). The enzyme activity was detected in fractions 40–47. These fractions showed characteristic visible-light absorption properties with the maximum intensity at 460–462 nm. The intensities of the light absorbed at this wavelength were proportional to the enzyme specific activity. The active fractions thus prepared were pooled, concentrated by centrifugation in a Cf-50 cone (Amicon, Mass.) and dialyzed overnight against buffer A using a dialysis membrane with a 15-kDa-molecular-mass cut-off (Spectrum Medical Ind., Tex.). The dialyzed sample was further purified by chromatography through a preparative MonoQ column (Pharmacia, Piscataway, N.J.) using a fast protein liquid chromatography system (Pharmacia, Piscataway, N.J.) as follows. The partially purified material from the Q-Sepharose column was dialyzed overnight against buffer A and applied to the previously equilibrated MonoQ column (Pharmacia HR10/10). This column was developed with buffer A containing a gradient of 0–1 M KCl, which was formed during a running time of 95 min. Fractions of 1 ml were collected and each fraction was examined for reductase activity. Enzyme activity was detected in fractions 52–58 with the specific activity of each fraction being proportional to the intensity of the light absorption at 460 nm, properties similar to those observed for the active fractions eluting from the Q-Sepharose column (see above). The active fractions from MonoQ column (52–58) were pooled, concentrated by centrifugation in a Centriflo cone (CF-50) and applied to a calibrated Superose-12 column. This column was developed with buffer A containing 0.15 M NaCl salt at a flow rate of 0.3 ml/min, and 0.3-ml fractions were collected. Fractions eluting from this column were monitored for enzyme activity and ultraviolet- and visible-light-absorption properties. The enzyme activity was detected in fractions 28–30 with the specific activity proportional to the intensity of the absorbance at 460 nm. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) of the Superose-12 fraction 29 (peak specific activity) showed a major band with a molecular mass of 60 kDa with two close minor bands.

Isolation of the material co-purifying with the keto ester M reductase

The purified keto ester M reductase was applied to a C_4 reverse-phase column (Vydac, Calif.). The column was developed with a gradient of solvent B (0.1% trifluoroacetic acid in acetonitrile) and solvent A (0.1% trifluoroacetic acid in water). The gradient was formed during a 40-min run in which the concentration of acetonitrile in water was raised from 20% to 80%. Fractions of 1 ml/min were collected and each fraction was examined for the characteristic ultraviolet/visible light absorption, which had been observed in the spectrum of the purified keto ester M reductase. Fraction 37 from this column proved to have a visible-light absorption spectrum identical to that of the purified enzyme.

Molecular mass determination

The molecular masses of the native and denatured keto ester M reductase were estimated by gel filtration on a Superose-12 column and by SDS-PAGE respectively. The gel-filtration column was equilibrated and run in buffer A containing 0.15 M NaCl at a flow rate of 18 ml/h. Under these conditions, thyroglobulin (660 kDa) ferritin (450 kDa), catalase (240 kDa), immunoglobulin G (158 kDa), egg albumin (44 kDa) and B12 (1355 Da), which were used as standard markers, were eluted with fractions 30, 39, 41, 41, 44 and 67 respectively. The SDS-PAGE was carried out in 0.1% SDS gel containing 3% and 12% acrylamide in stacking and separating layers respectively. Bovine serum albumin (66 kDa), egg albumin (44 kDa), carbonic anhydrase (30 kDa) and lysozyme (14 400 kDa) were used as standard markers during SDS-PAGE analysis.

Immobilization of the keto ester M reductase

A partially purified enzyme preparation with a specific activity of 27.22 ng mg^{-1} min^{-1} was immobilized on the ethylene-oxide-activated (oxirane) acrylic beads according to the established procedures (Cusak and Beynon 1989). Briefly, the enzyme solution, dissolved in 0.5 M potassium phosphate buffer, pH 7.4 (2 ml, 6 mg), was added to the oxirane acrylic beads (200 mg) and the mixture was incubated at room temperature for 16 h with stirring. The mixture was then moved to the cold room (4 °C) and stirred for an additional 44 h and extensively washed with ice-chilled buffer on a sintered-glass funnel. The unreacted oxirane groups on the beads were blocked by suspension in 1 M glycine solution in the same buffer and stirring overnight at 4 °C. The resulting suspension was washed as before and suspended in our assay buffer before analysis.

The keto ester M reductase activity in two-phase (aqueous/organic) solvent systems

In order to examine the effect of various organic solvents on the enzymatic activity of the keto ester M reductase, a panel of 13 organic solvents, including isopropanol, dimethylsulfoxide, acetone, dimethylformamide, acetonitrile, dioxan, tetrahydrofuran, hexane, toluene, carbon tetrachloride (CCl_4), methyl ethyl ketone, ethyl acetate and chloroform ($CHCl_3$) were chosen. Initially, an aliquot (constituting 10% of the total assay volume) of each of the organic solvents was added to the assay mixture and the enzymatic activity of the reductase was determined under standard assay conditions as described above. In cases where enzyme activity had survived the 10% solvent concentration (dimethylsulfoxide and hexane), higher solvent concentrations in the assays were also examined.

Results

Identification of the microbial cultures showing chirally specific reduction of the keto ester M

In order to screen microorganisms for their biotransforming potential toward chiral specific reduction of the keto ester M to its (*S*)-hydroxy analog, two HPLC systems, equipped with either reverse-phase or chiral columns, were developed. The reverse-phase column was used to monitor the formation of the hydroxy (*S* and/or *R*) analog in the extracts of the biotransformation cultures. The chiral column was then utilized to determine the chirality of the resultant hydroxy product. In the reverse-phase column system, the keto ester M substrate and its hydroxy analog (*R* or *S*) elute at 18.67 min and 15.86 min respectively (data not shown). In the chiral column system, however, the standard *S* and *R* hydroxy reduction products of the keto ester M elute at 16.49 min and 17.67 min respectively, while the parent keto ester emerges from the column at 15.32 min (Fig. 1).

On the basis of the HPLC analysis of the bioconversion extracts described above, two cultures, MB5614 and MF5021, now identified as *Microbacterium campoquemadoensis* and *Mucor hiemalis*, were discovered. Both of these cultures converted the substrate keto ester M to its (*S*)-hydroxy analog, the desired compound, with over 95% e.e. Milligram quantities of the reduction products were isolated from each culture extract and purified by a combination of TLC and semi-preparative HPLC. The isolated fractions were examined for their structural integrity on a chiral column and further analyzed by MS and NMR (data not shown). Owing to a faster rate of conversion of the substrate as well as higher specific activity, culture MB5614 (over 50% conversion at 47 h, see below) was chosen for further study and as the source for the enzyme isolation.

Time course of the formation of (*S*)-OH analogue by the whole broth and washed cell cultures of MB5614

Initially, a time-course study was carried out on both whole broth and washed cells from the culture MB5614

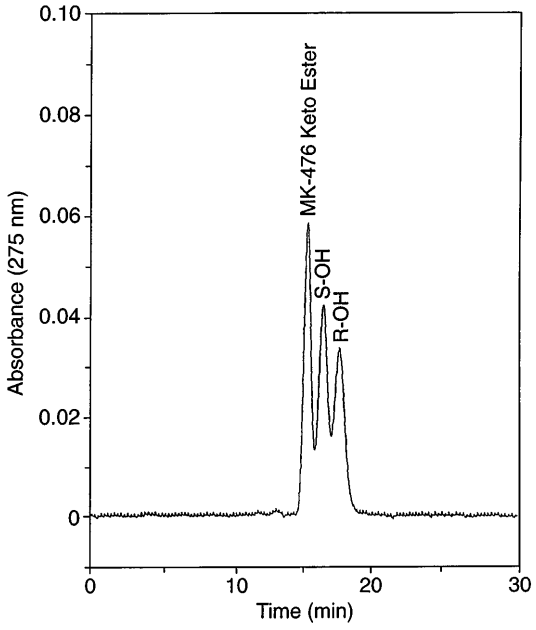


Fig. 1 HPLC elution profile of the standard keto ester M, (*S*)- and (*R*)-hydroxy esters on a chiral column (Chiralcel OD). These compounds eluted from the column with the retention times of 15.32, 16.49 and 17.67 min respectively

in order to locate (intra-/extracellular) the enzyme and determine the time for the peak appearance of the (*S*)-OH analog from the keto ester M substrate. This information was used as our guide to determine the proper time of the harvest as well as selection of the source (whole broth as compared to the washed cells) for the purpose of enzyme isolation. The results of these studies for MB5614 indicated that, with the whole broth (Fig. 2A), it took 41 h before appreciable conversion was observed (10.0 μg h^{-1} g $cells^{-1}$). In this case, the conversion was virtually complete in 93 h (12.28 μg h^{-1} g $cells^{-1}$) with some loss of the substrate and/or product (79.8% recovery). Similarly, the rate of the reduction by the washed cells that had been obtained from the 44-h-old culture of MB5614 was studied (Fig. 2B). The results not only showed a much faster rate of reduction by the washed cells (about 50% conversion of the substrate; 15.62 μg h^{-1} g $cells^{-1}$ after 40 h of incubation) but also established the intracellular presence of the enzyme. The faster rate of reduction by the washed cells of the MB5614 culture prompted us to select this preparation as opposed to the whole broth for our enzyme isolation studies.

Isolation and purification of keto ester M reductase

Enzymatically functional cell-free extract was prepared from 44-h-grown washed cells of the MB5614 culture. The extract was initially treated with 1% streptomycin and 10% ammonium sulfate to remove nucleic acid and extraneous proteins and then sequentially subjected to Q-Sepharose, MonoQ and Superose-12 chromatograp-

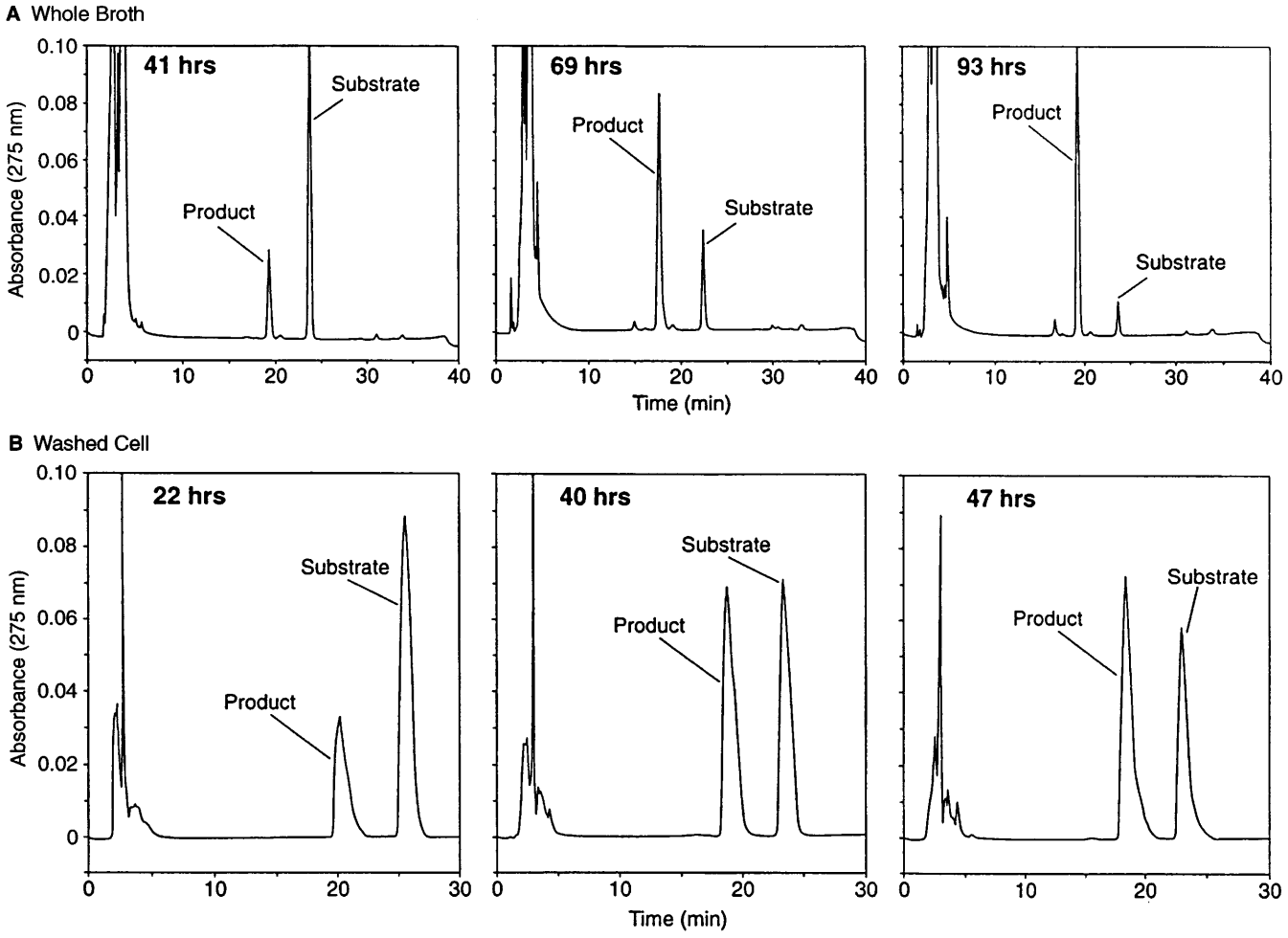


Fig. 2A, B HPLC elution profiles of the keto ester M and its reduction product from the C_{18} reverse-phase column during the time course of bioconversion in the whole broth (**A**) and washed cells (**B**)

hies for purification. Table 1 shows a summary of the results that were obtained during various purification steps. As shown, the final preparation from the Superose-12 column shows about 51-fold purification when compared to the crude extract with a 0.42% recovery of the activity. Also shown in this table is the total enzymatic activity in the crude extract (19.8 μg/min), representing 39.53% recovery of the activity when compared to the total activity (50.24 μg/min) that had been observed with 193 g washed cells. Needless to say, the low numbers for purification (51-fold) and for the recovery of the activity both from washed cells (39.5%) and after the purification (0.42%) are interpreted as either being due to the enzyme being unstable in the isolated state and/or being caused by the dissociation and loss of the co-purifying factor (see below) during the fractionations, which included extensive dialysis and gel-filtration chromatographies. Nonetheless, SDS-PAGE of the final preparation was indicative (see below) of a highly purified material, which was capable of the chirally specific reduction of the keto ester M, as illustrated in Fig. 3. In this figure, the peak with a retention time of 15.32 min shows the elution of the unused keto ester M substrate, while the second peak, with a retention time of 16.56 min, represents the elution of the enzymatically produced (*S*)-hydroxy product; the chemical structure of the enzymatically produced (*S*)-OH methyl ester was established by both mass and NMR spectral analysis.

Properties of the purified keto ester M reductase

The purified keto ester M reductase from culture MB5614 is a soluble cytoplasmic enzyme. In the Superose-12 column chromatography, the peak of enzyme activity was eluted with fraction 29, suggesting a molecular mass higher than 669 kDa when in the native state. When this fraction was analyzed by SDS-PAGE, a major band (Fig. 4) with mobility indicating a molecular mass of 60 kDa appeared. These results suggest that the keto ester M reductase has a molecular mass of 60 kDa and exists as an oligomer in the native state. This enzyme, in addition to the protein absorption at 280 nm, showed a distinct visible-light absorption profile between 400 nm and 500 nm with the maximum at 460 nm (Fig. 5A). In

Table 1 Data for the purification of the keto ester M reductase from 193 g washed cells from culture MB5614. The total enzyme activity in the washed cells (193 g) before cell-free extract preparation was determined to be 50.24 μg/min

Fraction	Protein (mg)	Specific activity (ng min^{-1} mg^{-1})	Total activity (ng/min)	Purification (-fold)	Recovery (%)
Crude extract	4250.00	4.67	19860.40	0.00	[a]
$(NH_4)_2SO_4$ (10%)	2200.00	2.02	4440.40	–	22.37
Q-Sepharose	57.54	41.61	2394.23	8.91	12.05
MonoQ-FPLC	3.10	214.00	663.40	45.82	3.34
Superose-12	0.35	239.76	83.91	51.34	0.42

[a]39.53% of the total enzyme activity (50.24 μg/min) was recovered in the cell-free crude extract (19.86 μg/min)

light of the fact that many reductases (dehydrogenases) require prosthetic groups such as FAD and FMN with a characteristic visible-light absorption profile, the isolation and characterization of the co-purifying material was deemed necessary. Toward this end, a highly purified preparation of the reductase (fraction 29 from the Superose-12 column) was subjected to reverse-phase column chromatography and a fraction was isolated on the basis of chromophore absorbance. This fraction gave a visible-light absorption profile (Fig. 5B) that was superimposable on the visible-light absorption portion of the spectrum from the purified keto ester M reductase. No enzyme activity was detected with any of the fractions that had been recovered from the reverse-phase column either before or after reconstitution of the fractions with a putative cofactor. Although, the reason for the loss of enzyme activity can not be definitely explained in our study, we speculate that it might be either due to the dissociation of the co-purifying material (see above) or because of denaturation of the protein moiety as a result of the conditions that had been used in the running of the reverse-phase column.

The purified enzyme catalyzed the specific chiral reduction of the ketone substrate (Fig. 3) using NADPH as cofactor, and showed its maximal activity at a temperature of 30 °C and pH of 8. The activity was stable for many months when the preparation (lyophilized or in solution) was stored at −80 °C. NADH (5 mM), zinc chloride (10 mg/l), mercaptoethanol (5 mM), and EDTA (1 mM) did not show any effect on the enzyme activity while mercuric chloride (37 μM), FMN (110 μM), and FAD (63 M) completely inhibited the reduction of the substrate.

Kinetic parameters for both the keto ester M reductase and NADPH were also determined for the purified enzyme under conditions of initial velocity. The results with the keto ester as the variable substrate gave values of 2.02 ± 0.45 nmol mg^{-1} min^{-1} and 147.86 ± 49.90 μM

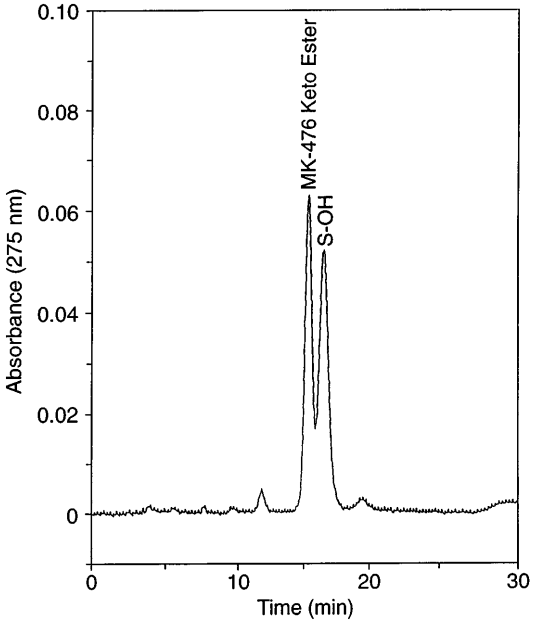


Fig. 3 HPLC elution profile of the extract of the enzymatic reaction mixture from a chirally specific column (Chiralcel). Keto ester M and its chirally specific (*S*)-hydroxy reduction product eluted with retention times of 15.32 min and 16.56 min respectively

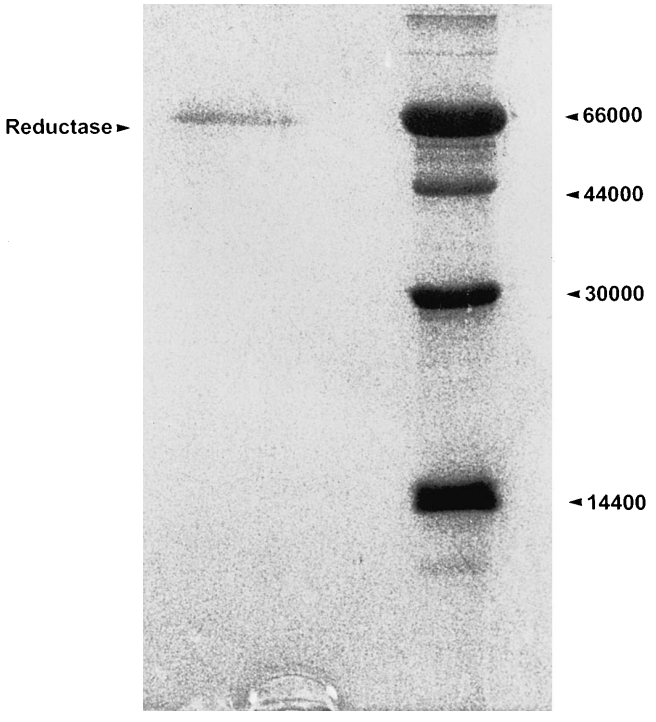


Fig. 4 Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the purified keto ester M reductase (*left*) and standard molecular mass markers (*right*). Molecular masses of the standards are shown

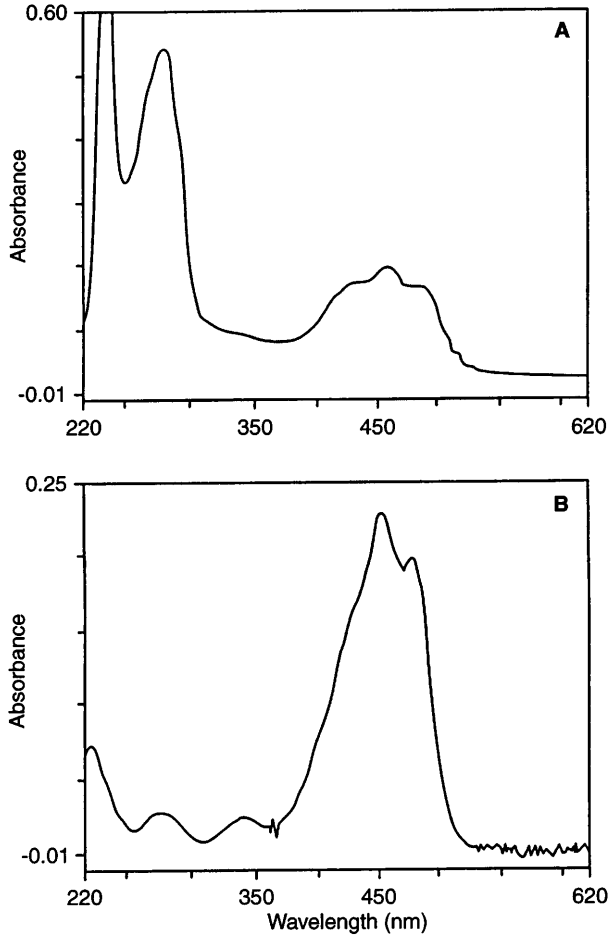


Fig. 5A, B Ultraviolet/visible-light absorption spectra of the purified native enzyme (**A**) and material isolated from the purified enzyme (**B**)

for V_{max} and K_m, respectively. Similarly, values of 0.75 ± 0.043 nmol mg^{-1} min^{-1} and 0.72 ± 0.13 mM were obtained for V_{max} and K_m of NADPH in the presence of a constant amount of the keto ester M (0.05 mM).

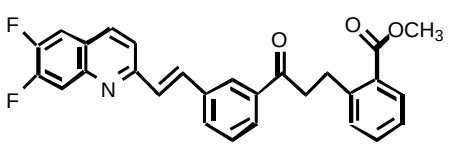
Whole-cell substrate specificity

Washed cells prepared from the *M. campoquemadoensis* and *M. hiemalis* cultures were examined for their substrate specificities using three compounds (A, B, C) with the chemical structures shown in Scheme 2. Despite the rather close sub-structural similarity of compounds A and B to that of keto ester M, no reduction was observed with a preparation from culture *M. campoquemadoensis* culture. These two ketones were, however, efficiently reduced by *M. hiemalis*. Similarly, there was only a low level of conversion of compound A (the fluoro derivative of keto ester M) by either of the cultures. These results not only point toward the structural differences between the reductases from the *M. campoquemadoensis* and *M. hiemalis*, but also suggest rather tight substrate specificities associated with both enzymes, especially with the reductase from the *M. campoquemadoensis* culture. Similar results were observed with the cell-free extracts prepared from these cultures, indicating that the transport of these compounds across the membranes is not a factor in biocatalysis.

Reductase activity in a two-phase (aqueous:organic) solvent system

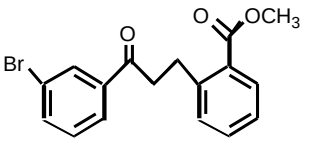
Many organic compounds have poor solubility in aqueous solvents where enzymatic catalyses are normally carried out. Consequently, the use of an aqueous solvent is a major disadvantage in the application of enzymes as catalysts in large-scale organic synthetic reactions. In order to circumvent this problem, numerous attempts (Dordick 1991; Woodley and Lilly 1992) have been made to develop conditions in which organic solvents can be utilized in enzyme-catalyzed reactions.

One such attempt is the development of the two-phase aqueous/organic solvent systems (Klibanov and Cambou 1987), which have been shown to improve the performance of some enzyme-catalyzed reactions in



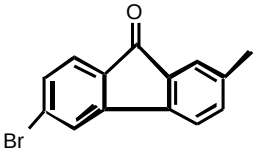
Fluoro Derivative of MK-476 Keto Ester

A



2-[3-[3-Bromophenyl]-3-Oxopropyl] Benzoic Acid Methyl Ester

B



2-Methyl-6-Bromo-9-Fluorenone

C

Scheme 2

cases where the substrates had poor solubility in the aqueous solvent. In view of the fact that keto ester M is also poorly soluble in water, and this is a limiting factor in using an enzymatic catalyst for the large-scale chirally specific reduction of this compound, the effect of various organic solvents on the purified keto ester M reductase were examined. The results of these experiments are presented in Table 2. As shown, while the partially purified reductase loses its activity in most of the organic solvents (10% concentration), it showed remarkable stability in dimethylsulfoxide and hexane. Moreover, the enzyme retained over 50% and 75% activity respectively, when as much as 20% dimethylsulfoxide and 75% hexane were used in the assay mixtures.

Immobilization of the keto ester M reductase

Immobilization of enzymes and cells for use in large-scale organic synthetic reactions as biocatalysts is an area of technology that is actively being pursued (Tanaka et al. 1993). Advantages, such as possible stabilization, use with organic solvents, ease of recovery of the reaction product and enzyme reusability, have been the reasons behind the development of this technology (Anderson et al. 1987). With these points in mind, oxirane-activated acrylic beads were used to immobilize our partially purified enzyme preparation. Of the total enzyme activity that was used for the coupling, 35.5% was recovered in the immobilized form. The immobilized enzyme thus prepared was examined for activity in a two-phase (aqueous/organic) solvent system in which the organic phase constituted 50% of the reaction mixtures. Except with hexane, where full chirally specific reduction of the substrate was observed, no activity was detected with $CHCl_3$ dimethylformamide and toluene. The results therefore indicated that the immobilized enzyme had activity similar to that of the free enzyme in two-phase solvent systems (Table 2).

Table 2 Effect of various organic solvents (10% in buffer) on the enzymatic activity of partially purified keto ester M reductase

Solvent	Specific activity (ng mg^{-1} min^{-1})
Aqueous	15.99
Isopropanol	0.00
Dimethylsulfoxide	16.92
Acetone	1.24
Dimethylformamide	4.04
CH_3CN	0.00
Dioxan	0.00
Tetrahydrofuran	0.00
Hexane	12.81
Toluene	0.00
CCl_4	0.00
Methyl ethyl ketone	0.00
Ethyl acetate	0.00
$CHCl_3$	0.00

Discussion

Because of their proven value in the chirally specific reduction of ketones, microbial reductases have attracted the attention of investigators in both academia and industry (Peters 1992). These enzymes have been found in mammalian, bacterial and fungal cells, most of the investigative effort being concentrated on yeast. This is because the reductases in the yeast show impressive specific activity in addition to having a higher specific content (Nassenstein et al. 1992). However, in this case the enzyme specificity seems to be rather broad (Peters et al. 1992). In the work presented here, a new organism, *M. campoquemadoensis*, was identified which catalyzed the chirally specific reduction of keto ester M with an enantiomeric excess better than 95%. Owing to its potential use in the large-scale production of the desired hydroxy ester, fermentation conditions for this microorganism as well as the time of addition of the ketone substrate to the culture have been optimized (Roberge et al. 1996). These investigators were able to achieve 500 mg/l production of the (*S*)-hydroxy ester of keto ester M in 280 h.

As we have reported here, in the hopes of eventually cloning, overexpressing and immobilizing the responsible reductase for a large-scale production, we isolated and purified the enzyme. The enzyme, both in solution and in immobilized forms, retained its activity as well as resisting inactivation in hexane. One drawback of this approach is the need to provide the expensive NADPH cofactor. Several methods for the regeneration of cofactors such as NADH/NADPH have been suggested (Hummel and Kula 1989). It is also possible to clone and overexpress this enzyme in an organism that has been shown to be a good source of the specific cofactor, in this case NADPH, and can be fermented on a large scale. We have previously used this approach for the overexpression of 31-*O*-demethylFK-506 methyltransferase, which requires *S*-adenosyl-L-methionine for the transfer of the methyl group to its substrate. Remarkably, the *Streptomyces lividans* that was used as the host for the expression of the enzyme provided the required *S*-adenosylmethionine for the production of a large amount (100-mg scale) of FK-506 (Motamedi et al. 1995).

Finally, the detailed characterization of the co-purified material that was observed during the purification of our reductase is of critical importance. This is because the availability of this material can help us to elucidate its role in the reduction. Furthermore, its inclusion in the reaction mixture might improve the specific enzyme activity as well as the catalytic rate of the reaction by the biocatalyst. Preliminary mass-spectral analysis of this molecule gives a molecular mass of 530 Da; detailed elucidation of the structure must await further studies.

Acknowledgements We would like to thank Dr. R. Schwartz for his editorial comments and Dr. T. Chen for his collaboration during our screening. Dr. P. Reider's support of this project is gratefully acknowledged.

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