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Comparative study of the properties of wild type and recombinant cyclohexanone monooxygenase, an enzyme of synthetic interest

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Abstract

Cyclohexanone monooxygenase (CHMO), a flavoenzyme of synthetic interest (it catalyses the NADPH-dependent enantioselective oxidation of ketones and of several heteroatoms such as nitrogen, sulfur, phosphorous and selenium present in organic compounds) previously overexpressed in *E. coli* (TOP10 pQR239), was purified to homogeneity, as demonstrated by SDS-PAGE and MALDI/TOF analysis, and characterised. The recombinant and the wild type (*Acinetobacter*) enzymes had identical molecular mass, K_m values, pH-activity profile and circular dichroism spectra, but slightly differed for pH- and thermo-stability. The latter findings might be due to a different pattern of proteases contaminating the monooxygenases isolated from the two microorganims.

Keywords: Cyclohexanone monooxygenase; Recombinant microorganism; Enzyme purification; Bicyclo[3.2.0]-hept-2-en-6-one

1. Introduction

Cyclohexanone monooxygenase (CHMO) is a xenobiotic metabolising enzyme that catalyses the oxidation of nitrogen, sulfur, phosphorous and selenium atomic centres present in a number of drugs, pesticides and other environmental chemicals [1–3]. In particular, CHMO is a soluble ∼60 kDa FAD-containing enzyme first isolated from *Acinetobacter calcoaceticus* NCIMB 9871 and *Nocardia* species grown on cyclohexanol as sole carbon source [4]. In vivo, the enzyme catalyses the NADPH-dependent oxidation of cyclohexanone to ε-caprolactone in a ring expansion reaction that occurs via Baeyer–Villiger mechanism [5,6]. This reaction is one of the several degradative steps of cyclohexanol that ultimately yield diacid precursors of acetyl-CoA [7]. In addition to its capacity to oxidize ketones and aldehydes, *Acinetobacter* CHMO is also well recognised for the selective heteroatom oxidation [8,9]. CHMO carries out both types of reactions often with high regio- and stereo-selectivity, which has prompted the use of this enzyme as a chemoenzymatic reagent for the synthesis of a variety of key chiral products [7,10–13].

The increase in interest for this enzyme has brought to its cloning in heterologous microorganisms. The production of CHMO from wild type strains has, indeed, some disadvantages such as: (1) pathogenicity of *A. calcoaceticus* which therefore is unattractive for large scale fermentation, (2) mild toxicity of the carbon source (e.g. cyclohexanol) required for induction during the fermentation process, (3) low enzyme productivity: typically 60–70 U/l culture (with cyclohexanone as the substrate) [4,14] and (4) the presence in the wild type microorganism (i.e. *A. calcoaceticus* NCIMB 9871) of a lactone hydrolase which, therefore, requires either purification of the CHMO or selective inhibition of the hydrolase, prior to the biotransformation. For these reasons the CHMO

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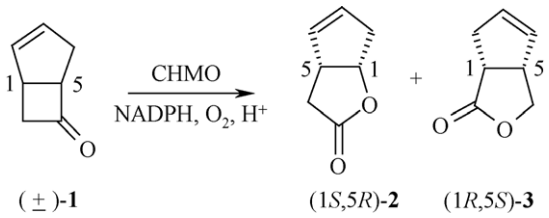

Scheme 1. CHMO-catalysed enantiodivergent oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one **1** to lactones (1*S*, 5*R*)-**2** and (1*R*, 5*S*)-**3**.

gene had been cloned in different hosts such as, for example, *E. coli* and yeast [15,16]. Among these, *E. coli* is particularly suitable for this purpose since it does not contain any lactone hydrolase activity which would lead to degradation of the products, the strain is not pathogenic and the enzyme production is induced by a cheap inducer such as L-(+)-arabinose. The recombinant *E. coli* TOP10 [pQR239] has proven to be particularly efficient, being the content of the expressed CHMO higher than that in the wild type strain by a factor of 25 [17].

The purpose of the present study was to investigate if the catalytic properties of CHMO isolated from *Acinetobacter* grown on cyclohexanol were fully maintained also in the recombinant enzyme from *E. coli* TOP10 [pQR239]. Furthermore, since CHMO lacks in stability, we aimed at knowing if the different enzymatic pools of the two microorganisms had a different influence on the properties of the biocatalyst at different purification degrees. The study was carried out using bicyclo[3.2.0]hept-2-en-6-one **1** as the substrate. This compound, is a sort of reference substrate, used also for other monooxygenases, since its oxidation yields the two regioisomeric lactones **2** and **3** with high optical purity (Scheme 1). These are chiral synthons particularly useful in the synthesis of prostaglandins and nucleosides [18].

2. Experimental

2.1. Materials

Growth medium constituents were obtained from Oxoid (Oxoid S.p.A., Garbagnate Milanese, Milano, Italy). The reagents used in the CHMO enzyme assay were bought from Sigma–Aldrich (Poole, Dorset, UK). Protease Inhibitor Cocktail (PIC) for bacterial cells was purchased from Sigma–Aldrich. All other chemicals were of analytical grade.

2.2. Growth of Acinetobacter calcoaceticus and recombinant E. coli TOP10 [pQR239]

A. calcoaceticus was grown as described by Trudgill [14] and the transformed microorganism was cultivated essentially as described previously by Doig et al. [17].

2.3. CHMO activity assay

Unless otherwise indicated, the enzyme activity was spectrophotometrically determined by monitoring NADPH consumption at 340 nm using as the assay buffer 0.05 M Tris–HCl, pH 8.6, containing 50 μm **1** and 0.12 mM NADPH [19].

2.4. Purification of cyclohexanone monooxygenases

The same purification procedure was adopted for CHMO both from *A. calcoaceticus* and from the *E. coli* TOP10 [pQR239] strain. The procedure was adapted from that already described for the wild enzyme [8]. Unless otherwise indicated, all steps of purification were carried out at 4 °C using 0.02 M potassium phosphate (KP) buffer with 0.01 M DTT, pH 7.2 (buffer A). The cells obtained from 1 l of culture medium were harvested, disrupted by sonication and cell debris removed by centrifugation. The supernatant was subjected to fractionation with $(NH_4)_2SO_4$ and the fraction which precipitated between 40 and 85% saturation was recovered by centrifugation at 6000 × *g* per 30 min. The pellet was redissolved in buffer A, dialysed overnight against the same buffer and loaded on an anion exchange column (Fractogel EMD DEAE, 10 cm × 2 cm) which was previously equilibrated with buffer A. The enzyme was eluted with a linear gradient from 0 to 0.15 M NaCl in 300 min in the same buffer, at a flow rate of 2 ml/min. Active fractions were collected and loaded on an affinity column (Matrex gel red A, agarose 5%; 10 cm × 2 cm) previously equilibrated with buffer A and eluted with the same buffer but containing NADP 0.05 M (flow rate of 1 ml/min). Active fractions were collected, dialysed overnight against buffer A and lyophilised.

2.5. Electrophoresis analysis

SDS-PAGE was performed on 10% polyacrylamide gels according to Laemmli [20] and proteins were stained with 0.25% (w/v) Coomassie Blue R-250 solution in 50% (v/v) ethanol and 10% acetic acid. Molecular mass under denaturing conditions was determined using standard proteins from Bio-Rad (myosin, 200.000 Da; β-galactosidase, 116.250 Da; phosphorilase b, 97.400 Da; serum albumin, 66.200 Da; ovalbumin, 45.000 Da; carbonic anhydrase, 31.000 Da; trypsin inhibitor, 21.500 Da).

2.6. Mass determination by MALDI-TOF/MS analysis

Matrix-assisted laser desorbtion ionization/time-of-flight mass spectrometric analyses were performed by using a Bruker Daltonics Reflex IV instrument equipped with a nitrogen laser (337 nm), and operated in linear mode with a matrix of sinaptic acid in 0.1% TFA/CH_3CN = 2/1. External standards were used for calibration (Bruker protein calibration standard) ranging from 24 to 66 kDa. Each spectrum was accumulated for 30 laser shots.

2.7. Determination of the pH dependence of CHMO activity

To test the effect of pH on the activity of the wild and the recombinant enzymes at different purification degrees, aliquots (10–50 μl) of enzyme solutions in water (1 U/ml) were added to 0.02 M KP buffer (pH 6.0, 7.0, 8.0) or 0.02 M Tris–HCl buffer (pH 8.6, 9.0, 10.0), containing 50 μM of **1** and 0.12 mM NADPH. Absorbance changes were immediately monitored at 340 nm and at 25 °C.

2.8. Determination of the pH and temperature stability profiles of CHMO

The pH and thermal stabilities of recombinant and wild type CHMOs were determined using preparations with different degrees of purification: (a) crude extract, (b) after DEAE chromatography and (c) after affinity chromatography. For pH stability, 50 μl of enzyme solutions (1 U/ml) incubated for scheduled times at 25 °C, in 0.05 M KP buffer pH 7.0, or in 0.05 M Tris–HCl buffer pH 8.6 or pH 10.0, were withdrawn and immediately checked for the residual enzyme activity. Furthermore, crude extracts from *E. coli* and *Acinetobacter* were incubated in the presence of PIC following the procedure suggested by the supplier (Sigma–Aldrich), at pH 8.6 and 25 °C. For thermostability, 50 μl of enzyme solutions (1 U/ml in 0.02 M KP, pH 7.0), incubated for 5 min at temperatures ranging from 20 to 50 °C, were withdrawn and immediately checked for the residual enzyme activity.

2.9. Absorption and circular dichroism spectra

UV–visible spectra were recorded with a Jasco V-530 spectrophotometer using a 1 mg/ml protein solution.

Circular dichroism (CD) spectra of CHMO from *E. coli* and *A. calcoaceticus* were recorded in the range 200–250 nm, at 20 °C, with a Jasco J600 Circular Dichroism Spectropolarimeter equipped with a system of computer-assisted data processing, at a scanning rate of 20 nm/min and with a time response of 4 s. The final spectra were smoothed after subtracting the buffer spectrum (blank). Protein concentration was 0.1 mg/ml in 50 mM KP buffer (pH 8.6). The optical path of the cuvette was 1 mm. Molar mean residue ellipticity (θ) values were expressed for all wavelengths as degrees centimeter squared per decimole and were calculated from the equation:

$$\theta = \frac{\theta_{\text{obs}} 110.7}{10dc}$$

where θ_{obs} is the ellipticity measured in degrees, 110.7 the mean residue molar weight of CHMO, c the protein concentration in grams per milliliter and d is the optical path in centimeters.

2.10. Determination of CHMO kinetic parameters for bicyclo[3.2.0]hept-2-en-6-one

CHMO kinetic parameters (K_m and specific activity) were determined in Tris–HCl buffer, pH 8.6, 25 °C, using bicyclo[3.2.0]hept-2-en-6-one (2.5 μM–46 mM) as the substrate. The study was carried out both on CHMO from *Acinetobacter* and from *E. coli* with different purification degrees.

3. Results and discussion

3.1. Growth of Acinetobacter calcoaceticus and recombinant E. coli TOP10 [pQR239]

CHMO is not a constitutive enzyme of *Acinetobacter* and, therefore, its expression in wild type cells depends on the presence of cyclohexanone in the culture media. Because of this, a low level of enzyme production is obtained (∼60–70 U/l culture [4,14]). On the contrary, in the recombinant host, CHMO production is under the control of a strong promoter and, in turn, the expression of the protein is regulated by the inducer L-(+)-arabinose, which allows a better control of enzyme expression. By means of this expression system, developed by Doig et al. [17], we obtained an enzyme production by *E. coli* cells which was approximately 20-fold higher than that obtained by *Acinetobacter*.

3.2. Purification of cyclohexanone monooxygenase

Recombinant CHMO, recovered from cells after sonication and ammonium sulphate precipitation, was purified to homogeneity by ion-exchange (DEAE) and affinity (Red A) chromatography. The chromatographic behaviour of recombinant CHMO was indistinguishable from that of the enzyme produced by *Acinetobacter*. The enzyme specific activity was 9.1 U/mg after affinity chromatography (Table 1). Unfortunately, the purification yield of the recombinant enzyme was quite low (12%), but slightly higher than that obtained with the enzyme from *Acinetobacter* (∼7%), which had a very similar specific activity (9.2 U/mg protein). The low stability of the purified enzyme and the large eluate volume after affinity column (the CHMO activity was broadened in numerous fractions, even though $NADP^+$ was used for elution), might be responsible for the low enzyme activity recoveries. The total recovered activity for the recombinant enzyme was at least 30 times higher than that for the wild type enzyme, for the same volume of cell culture.

3.3. Electrophoretic and MALDI/TOF analyses

After the affinity chromatography step, the recombinant enzyme appeared to be homogeneous as indicated by SDS-PAGE (data not shown). The recombinant enzyme had the same electrophoretic mobility of that of the wild type enzyme, which indicates that the two enzymes have the same

Table 1
Purification of CHMO from recombinant *E. coli* TOP10 [pQR239]

Fraction	Total protein[a] (mg)	Total activity[b] (units)	Specific activity (units/mg)	Yield (%)	Purification
Cells extract	1858	804	0.4	100	1
After 40% $(NH_4)_2SO_4$ (supernatant)	1272	684	0.5	85	1.2
After 85% $(NH_4)_2SO_4$ and dialysis	806	496	0.6	62	1.4
DEAE chromatography	60	248	4.1	31	9.6
Affinity chromatography	11	100	9.1	12	21.0

[a] Protein content was determined according to Bradford [21], using bovine serum albumin as a standard.
[b] Measurements were carried out as described in Section 2.

molecular mass. The identity of recombinant CHMO and wild type enzyme was further confirmed by the more sensitive MALDI/TOF method; r-CHMO mass: 61586.04 Da and *Acinetobacter* CHMO mass: 61583.54 Da.

3.4. Activity profiles of CHMOs as a function of pH

The effect of pH on the activity of CHMOs, using bicyclo[3.2.0]hept-2-en-6-one as substrate, was examinated in the pH 6–10 range. Fig. 1 shows that purified recombinant and wild type CHMOs have the same pH activity profiles, with optimal pH at 8.6. Analogous results were obtained with the less purified enzyme preparations, i.e. after precipitation with ammonium sulfate or after DEAE chromatography (data not shown). Interestingly, the decrease of pH causes a much more marked effect on CHMO activity than the increase. In fact, while the decrease of 1.6 pH units from pH 8.6 to 7.0 induces a loss of about 70% of activity, an analogous variation toward a higher pH value (from pH 8.6 to 10.0) causes only a 20% decrease of activity.

3.5. Stability profiles of CHMOs as a function of temperature and pH

The temperature stability profiles of CHMOs from *E. coli* and *Acinetobacter* with different purification degrees are reported in Fig. 2A and B. Both enzymes show an increase of thermostability passing from the crude preparation to the homogeneous protein. In fact, the melting temperature (T_m), namely the temperature at the transition midpoint where the concentration ratio of active and inactive states is 1, changes from 34 to 39 °C and from 35 to 36.5 °C, for recombinant and wild type CHMO, respectively. In general, for biocatalytic applications, lower protein content in the reaction media allows better oxygenation, avoids foam formation and, consequently, a less troubling downstream process. Thus, the possibility to use a biocatalyst with higher specific activity and higher stability is of particular interest.

Surprisingly, the data also evidenced a slightly higher thermostability for the recombinant CHMO preparations (Fig. 2). This behaviour was confirmed for enzyme stability as a function of pH (Table 2). In fact, the remaining activity after incubation of the purified enzymes for 5 or 48 h at pH 6.0, 8.6 and 10.0, resulted in all cases higher for the recombinant enzyme. Moreover, it can be seen (Table 2) that the enzyme from *E. coli* shows a higher stability than that from *Acinetobacter* also in the form of crude extract or after DEAE chromatography. Recombinant and wild CHMOs were also incubated in the presence of the Protease Inhibitor Cocktail (PIC). It can be seen (Table 3) that wild CHMO after 24 h incubation in buffer alone had a residual activity of 20% whereas after

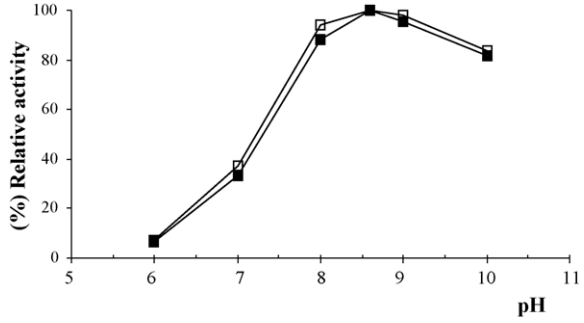

Fig. 1. Activity profiles of purified CHMOs from *Acinetobacter* (□) and *E. coli* (■) as a function of pH. For details see Section 2.

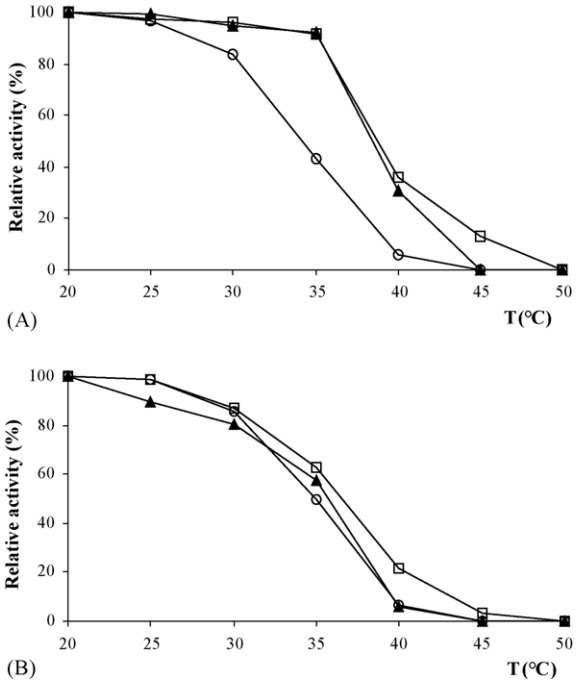

Fig. 2. Thermostability of recombinant and wild type CHMOs. The enzymes from *E. coli* (A) and *Acinetobacter* (B), with different degrees of purity, were employed: crude extract (◯); after DEAE chromatography (▲); after affinity chromatography (□). For details see Section 2.

Table 2
Stability of recombinant and wild type CHMOs as a function of pH[a]

Step	pH 6		pH 8.6		pH 10	
	E. coli	*Acinetobacter*	*E. coli*	*Acinetobacter*	*E. coli*	*Acinetobacter*
Crude extract[b]	71	66	88	65	63	61
DEAE chromatography[b]	95	89	99	89	98	91
Affinity chromatography[c]	88	76	85	76	55	18

[a] The remaining enzyme activity was expressed as a percentage of the initial enzyme activity. For details see Section 2.
[b] The remaining activity was measured after 5 h incubation.
[c] The remaining activity was measured after 48 h incubation.

Table 3
Stability of crude extract of recombinant and wild type CHMOs in the presence of PIC, at pH 8.6

Time (h)	*E. coli*		*Acinetobacter*	
	Control[a]	Plus PIC[a]	Control[a]	Plus PIC[a]
1	99	98	97	98
5	89	90	67	75
24	55	55	20	60

[a] The remaining enzyme activity was expressed as a percentage of the initial enzyme activity. For details see Section 2.

incubation with PIC the residual activity was about 60%. On the contrary the stability of the recombinant enzyme was not substantially affected by the presence of PIC. The higher stability of CHMO preparations from *E. coli* makes them more appealing for biocatalytic applications.

3.6. Absorption and circular dichroism spectra

The absorption spectra of both recombinant and wild type purified CHMOs showed maxima at 274, 382 and 438 nm, and the ratios of absorbance at 280 and 450 nm were 11.1 and 10.5, respectively. These values are in excellent agreement with that previously reported by Donoghue et al. [4] for the wild enzyme (10.8), which indicates that 1 mole of apoenzyme binds one mole of FAD. Analogously the far UV circular dichroism spectra were coincident (Fig. 3), which points to an identical conformation for the two enzymes.

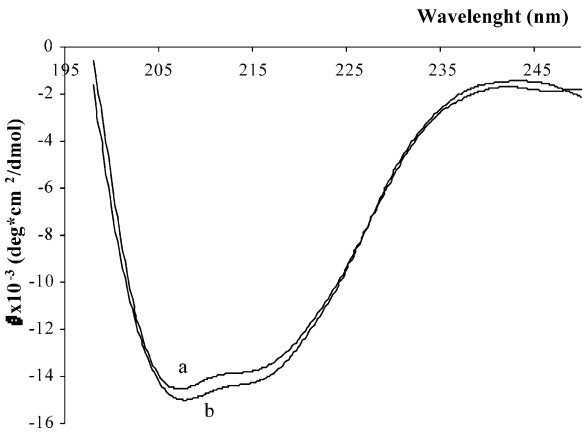

Fig. 3. Circular dichroism spectra of purified CHMO from (a) *E. coli* and from (b) *Acinetobacter*.

3.7. Kinetic parameters of CHMOs

The K_m values for the purified enzymes from *Acinetobacter* and from *E. coli* were identical, within the experimental error (1.4 ± 0.3 μM) and also the specific activities were very similar, namely 9.2 and 9.1 units/mg protein, respectively.

4. Conclusion

This study has shown that CHMO obtained by means of the expression system *E. coli* (*E. coli* TOP10 pQR239) is practically identical to that obtained from *Acinetobacter*. This was inferred by the fact that molecular weight, electrophoretic mobility, catalytic constant and pH activity profile were extremely similar for the two enzymes. Such results indicate a correct expression of the recombinant enzyme in *E. coli* and, therefore, the expression system appears suitable for the generation, through gene manipulation, of variants of CHMO with improved properties. Slight variations in the pH and thermostability profiles were observed, with the recombinant enzyme appearing more stable than the wild enzyme (Fig. 2 and Table 2). Such differences can hardly be ascribed to different structural stabilities for the enzymes obtained from the two microorganisms. The differences might rather be due to different enzymatic pools present in the two microorganisms. In particular, the presence of small traces of proteolytic enzymes, which cannot be evidenced by SDS-PAGE because their amount is below the detection limit, might be responsible for the different inactivation profiles observed for the two enzymes. This is confirmed by the finding that the pH stability of the wild enzyme becomes very similar to that of the recombinant enzyme when incubated in the presence of protease inhibitors (Table 3). However, it has been shown that purification improves biocatalyst stability (Fig. 2 and Table 2), which is particularly useful in the perpesctive of repeated use of CHMO in membrane reactors [22,23] or in the immobilised form.

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