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Enzymes of a New Modified *ortho*-Pathway Utilizing 2-Chlorophenol in *Rhodococcus opacus* 1CP

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Abstract—Chlorocatechol 1,2-dioxygenase (CC 1,2-DO), chloromuconate cycloisomerase (CMCI), chloromuconolactone isomerase (CMLI), and dienolactone hydrolase (DELH), the key enzymes of a new modified *ortho*-pathway in *Rhodococcus opacus* 1CP cells utilizing 2-chlorophenol via a 3-chlorocatechol branch of a modified *ortho*-pathway, were isolated and characterized. CC 1,2-DO showed the maximum activity with 3-chlorocatechol; its activity with catechol and 4-chlorocatechol was 93 and 50%, respectively. The enzyme of the studied pathway had physicochemical properties intermediate between the pyrocatechase of ordinary and chlorocatechase of modified pathways described earlier for this strain. In contrast to the enzymes investigated earlier, CMCI of the new pathway exhibited high substrate specificity. The enzyme had K_m for 2-chloromuconate of 142.86 μM, V_{max} = 71.43 U/mg, pH optimum around 6.0, and temperature optimum at 65°C. CMCI converted 2-chloromuconate into 5-chloromuconolactone. CMLI converted 5-chloromuconolactone into *cis*-dienolactone used as a substrate by DELH; this enzyme did not convert *trans*-dienolactone. DELH had K_m for *cis*-dienolactone of 200 μM, V_{max} = 167 U/mg, pH optimum of 8.6, and temperature optimum of 40°C. These results confirm the existence of a new modified *ortho*-pathway for utilization of 2-chlorophenol by *R. opacus* 1CP.

Key words: modified *ortho*-pathway, chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, chloromuconolactone isomerase, dienolactone hydrolase, 2-chlorophenol, *Rhodococcus opacus* 1CP

The ability of *Rhodococci* to convert xenobiotics stimulates investigations aimed at production of new strains acting on a broad spectrum of substrates. By prolonged adaptation to 2-chlorophenol, a *Rhodococcus opacus* strain 1CP, which normally utilized 4-chloro- and 2,4-dichlorophenols, was produced which grows on this substrate. This strain assimilates 2-chlorophenol via a 3-chlorocatechol branch of a modified *ortho*-pathway. Cloning and sequencing of the genes encoding the key enzymes of the new pathway revealed four enzymes involved in the conversion of 3-chlorocatechol: CC 1,2-DO, CMCI, CMLI, and DELH [1].

The modified *ortho*-pathway contains only three of these enzymes and lacks muconolactone isomerase (MLI), typical of the ordinary ortho-pathway [2].

Abbreviations: DELH) dienolactone hydrolase; MLI) muconolactone isomerase; CMCI) chloromuconate cycloisomerase; CMLI) chloromuconolactone isomerase; CC 1,2-DO) chlorocatechol 1,2-dioxygenase; 3-MC) 3-methylcatechol; 4-MC) 4-methylcatechol; 3-CC) 3-chlorocatechol; 4-CC) 4-chlorocatechol; 3,5-DCC) 3,5-dichlorocatechol.

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The objectives of this work were to isolate and characterize CC 1,2-DO, CMCI, CMLI, and DELH, the key enzymes of the 3-chlorocatechol branch of the modified *ortho*-pathway, from *Rhodococcus opacus* 1CP cells grown on 2-chlorophenol.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. *Rhodococcus opacus* 1CP was isolated from the enrichment culture maintained for a long time on medium containing 2,4-dichlorophenol as the sole source of carbon and energy [3]. The strain utilized phenol, 4-chlorophenol, and 2,4-dichlorophenol as substrates [3]. After prolonged adaptation, a strain utilizing 2-chlorophenol was obtained [4].

The cultivation medium for *R. opacus* 1CP contained (g/liter): Na_2HPO_4, 0.7; KH_2PO_4, 0.5; $MgSO_4$, 0.2; $MnSO_4$, 0.001; NH_4NO_3, 0.75; $FeSO_4$, 0.02; pH 7.2. The bacteria were cultured in Ehrlenmeyer's flasks containing 150 ml medium, at 29°C. 2-Chlorophenol was added in

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0.3 mM portions as the substrate was utilized. Culture growth was monitored by the change in absorption at 545 nm.

Biomass for the isolation and purification of the enzymes was obtained by periodic cultivation of the strain in a 10-liter bioreactor containing 7 liters of medium of the above-mentioned composition. 2-Chlorophenol added in 0.3 mM portions was used as the substrate. Culture growth was monitored by a decrease in oxygen consumption and by changes in pH and absorption at 545 nm. The pH was maintained constant by periodic additions of small volumes of 0.1 M NaOH. Cells were centrifuged at 5,000*g* for 15 min, washed with 0.05 M Tris-HCl buffer (pH 7.4) containing 2 mM $MnSO_4$, and stored at −20°C.

Preparation of cell-free extracts and enzyme activity assays. To prepare cell-free extracts, biomass was defrosted at room temperature and disrupted in a Hughes press (working pressure in the disintegration chamber of 3500 kg/cm^2 and extrusion slot of 350 μm). After disintegration, the biomass was incubated with DNase for 15 min at room temperature and centrifuged at 18,000*g* for 40 min. The pellet was resuspended in 0.05 M Tris-HCl buffer (pH 7.4) containing 2 mM $MnSO_4$ and centrifuged under the same conditions. The supernatants were pooled, centrifuged at 18,000*g* for 60 min, and used for enzyme purification.

Enzyme activities were measured on Shimadzu UV-160 and UV-2501PC spectrophotometers (Japan) in 10-mm quartz cuvettes at 25°C.

The CC 1,2-DO activity was assayed as described [5]. The reaction mixture contained 0.05 M Tris-HCl buffer (pH 7.4), 0.25 mM catechol or substituted catechol, 1.3 mM EDTA, and the enzyme. The reaction was initiated by the addition of the enzyme. The enzyme activity was calculated from the rate of formation of the reaction products absorbing at 260 nm using the following molar absorption coefficients [6, 7]: 16,800 $M^{-1}\cdot cm^{-1}$ for catechol; 17,100 $M^{-1}\cdot cm^{-1}$ for 3-chlorocatechol; 12,400 $M^{-1}\cdot cm^{-1}$ for 4-chlorocatechol; 12,000 $M^{-1}\cdot cm^{-1}$ for 3,5-dichlorocatechol; 18,000 $M^{-1}\cdot cm^{-1}$ for 3-methylcatechol; 13,900 $M^{-1}\cdot cm^{-1}$ for 4-methylcatechol.

The CMCI activity was determined as described in [8]. The reaction mixture contained 0.05 M Tris-HCl buffer (pH 7.2), 2 mM $MnCl_2$, 0.1 mM substrate, and the enzyme. The reaction was initiated by the addition of the enzyme. The enzyme activity was estimated from the rate of substrate utilization at 260 nm [8]. The substrates (3-chloro-, 3-methyl-, and 2,4-dichloromuconates) were prepared *in situ* from 4-chloro-, 4-methyl, and 3,5-dichlorocatechols using a purified chlorocatechase from *R. opacus* 1CP. The reaction was monitored spectrophotometrically. After the completion of the reaction, proteins were removed by ultrafiltration on a Centriprep YM-10 (Amicon, USA).

The CMLI activity was assayed by the increase in absorption at 280 nm characteristic of *cis*-dienolactone. With DELH, the CMLI activity was measured only qualitatively by changes in the 5-chloromuconolactone spectrum.

The DELH activity was assayed according to the procedure of Maltseva et al. [9]. The reaction mixture contained 0.05 M Tris-HCl (pH 8.4), 0.1 mM substrate, and the enzyme. The reaction was initiated by the addition of the enzyme. The enzyme activity was estimated from the rate of substrate utilization at 280 nm using the molar absorption coefficient for *cis*-dienolactone of 17,000 $M^{-1}\cdot cm^{-1}$ [7].

The amount of the enzyme catalyzing the conversion of 1 μmole of the substrate or the formation of 1 μmole of the product during 1 min was taken for 1 activity unit.

Enzyme purification. *Chlorocatechol 1,2-dioxygenase purification*. Biomass of *R. opacus* 1CP (20 g wet weight) grown in the bioreactor with 2-chlorophenol as the sole source of carbon and energy to the absorbance at 545 nm of 1.6-1.7 was used for isolation and purification of CC 1,2-DO. The cell-free extract was applied to a Q-Sepharose Fast Flow column (40 ml, Pharmacia, Sweden) equilibrated with 0.05 M Tris-HCl buffer, pH 7.4 (buffer A). The column was washed with one or two volumes of the same buffer. Proteins were eluted with a linear NaCl gradient (0-0.5 M NaCl) in 400 ml of buffer A at flow rate 0.95 ml/min. The fraction volume was 4.7 ml. The fractions with the highest chlorocatechol activity were combined. Ammonium sulfate was added to final concentration 1.5 M. After centrifugation at 18,000*g* for 40 min, the preparation was applied to a butyl-Sepharose Flow column (20 ml) (Pharmacia) equilibrated with 1.5 M $(NH_4)_2SO_4$ in buffer A. Proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (1.5-0 M) in 200 ml of the buffer at flow rate 1 ml/min. Fractions of 3.5 ml with the maximum CC 1,2-DO activity were pooled. The preparation was dialyzed against buffer A. Catechol was used as the substrate during the purification of CC 1,2-DO.

Purification of chloromuconate cycloisomerase. *R. opacus* 1CP biomass (52 g wet weight) grown in the bioreactor on 2-chlorophenol to the absorption of 2.0 was used for CMCI purification.

A cell-free extract was applied onto a Q-Sepharose Fast Flow column (40 ml) equilibrated with 0.05 M Tris-HCl buffer (pH 7.4) containing 2 mM $MnSO_4$ (buffer B). The column was washed with one or two volumes of the same buffer. Proteins were eluted with a linear NaCl gradient (0-0.5 M) in 400 ml of buffer B at flow rate 1.3 ml/min. Fractions of 5.4 ml were collected. The fractions with the highest CMCI activity were pooled. Ammonium sulfate was added to final concentration 0.8 M. After centrifugation at 18,000*g* for 40 min, the enzyme preparation was applied to a phenyl-Sepharose CL-4B column (17 ml) (Pharmacia) equilibrated with 0.8 M $(NH_4)_2SO_4$ in buffer B. Proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (0.8-0 M) in 200 ml of the buffer at flow rate 0.8 ml/min. Fractions (3.2 ml) showing the highest CC 1,2-DO activ-

ity were pooled and concentrated to 1.5 ml by ultrafiltration on an Amicon 5-ml cell (Amicon) equipped with a UM-10 membrane and centrifuged at 14,000*g* for 5 min. The resulting preparation was desalted by gel filtration on a Sephadex G-25 Superfine column (Pharmacia) equilibrated with buffer B. Ammonium sulfate was added to the enzyme preparation to final concentration 1.2 M. After 2 h, the preparation was centrifuged at 18,000*g* for 40 min and applied onto a Resource Iso column (1 ml) (Pharmacia) equilibrated with 1.2 M $(NH_4)_2SO_4$ in buffer B. Proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (1.2-0.7 M) in 20 ml of buffer B at flow rate 0.8 ml/min. The fraction volume was 1 ml. The fractions with the highest enzyme activity were pooled and dialyzed against buffer B. 2-Chloromuconate was used as the substrate during the purification of CMCI.

Chloromuconolactone isomerase purification. The cell-free extract was applied to a Q-Sepharose HR column (1.6 × 10 cm, 20 ml) (Pharmacia) equilibrated with 0.05 M Tris-HCl buffer, pH 7.4. The CMLI activity was detected in the unbound fraction. $(NH_4)_2SO_4$ was added to final concentration 1.6 M. After centrifugation at 20,000*g* for 15 min, the supernatant showing the CMLI activity was applied in three portions onto a Resource Phe column (1 ml) (Pharmacia). After washing the column, proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (1.6-0 M) in 25 ml at flow rate 1.5 ml/min. Fractions of 0.3 ml were collected. The fractions with the highest CMLI activity were pooled. The enzyme preparation desalted and concentrated on a Centricon 30U (Amicon) was heated at 70°C in a water bath for 15 min and centrifuged.

5-Chloromuconolactone was used as the substrate during the purification of CMLI.

Dienolactone hydrolase purification. DELH was purified together with CMCI. A cell-free extract was applied to a Q-Sepharose Fast Flow column (40 ml) equilibrated with buffer B. DELH did not bind to the support, the enzyme activity being detected in the unbound fraction. Proteins eluted in this fraction were first precipitated with $(NH_4)_2SO_4$ added to 25% saturation, and after centrifugation at 18,000*g* for 20 min, $(NH_4)_2SO_4$ was added to 40% saturation. The protein was desalted and concentrated by ultrafiltration (180 ml) (Amicon) using a UM-10 membrane. After saturation to 1.8 M $(NH_4)_2SO_4$ and centrifugation at 18,000*g* for 40 min, the preparation was applied to a butyl-Sepharose column (20 ml) equilibrated with 1.8 M $(NH_4)_2SO_4$ in buffer B. After washing the column with one or two volumes of the starting buffer, proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (1.8-0 M) in 200 ml of buffer B. The flow rate was 0.8 ml/min; the fraction volume was 3.2 ml. The fractions with the highest DELH activity were pooled and concentrated to 1.5 ml in an ultrafiltration cell (10 ml) (Amicon) equipped with a UM-10 membrane or using a Centriprep YM-10 (Amicon). After centrifugation at 14,000*g* for 5 min, the preparation was applied to a Sephadex G-25 superfine column equilibrated with 0.05 M Tris-HCl buffer, pH 9.0 (buffer C) for gel filtration. Further purification of DELH consisted of chromatography on a Mono Q anion-exchange column (1 ml) (Pharmacia) equilibrated with buffer C. The enzyme was applied to the column in five stages. After the protein was applied and the column was washed with the equilibration buffer, proteins were eluted with a linear NaCl gradient (0-0.2 M) at flow rate 1 ml/min in 20 ml volume. The fraction exhibiting the highest DELH activity was supplemented with $(NH_4)_2SO_4$ to concentration 1.8 M in buffer C, centrifuged, and applied to a Resource Iso column (1 ml) equilibrated with 1.8 M $(NH_4)_2SO_4$ in buffer C. Proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (1.8-0.9 M) at flow rate 0.8 ml/min in 20 ml. The fractions exhibiting DELH activity were pooled and dialyzed against 0.05 M Tris-HCl buffer, pH 8.4.

cis-Dienolactone was used as the substrate during the purification of DELH.

Determination of physicochemical properties of the enzymes. The homogeneity of the enzyme preparations and molecular masses of subunits were determined by SDS-PAGE in 12-13% polyacrylamide gels by a modification of the Laemmli procedure [10]. Gels were stained with Coomassie G-250 [11]. A low-molecular-weight kit was used as standards: phosphorylase *b* (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

To determine molecular masses of the enzymes, gel filtration on a Superdex 200 prep grade column (16 × 60) (Pharmacia) equilibrated with buffer B containing 0.1 M NaCl was used. The molecular weight standards were as follows: DNP-L-alanine (255 daltons), cytochrome *c* (12.3 kD), myoglobin (17.8 kD), chymotrypsinogen A (25 kD), ovalbumin (45 kD), BSA (67 kD), aldolase (160 kD), catalase (240 kD), and ferritin (450 kD).

To estimate pH optima of the enzymes, different buffers were used. The activity of CC 1,2-DO was measured in borate–phosphate–acetate buffer, pH 2.7-10 [12]; 0.25 mM catechol was used as the substrate. CMCI was assayed in 0.05 M bis-Tris-HCl buffer, pH 5.8-7.2, 0.05 M Tris-HCl buffer, pH 7.2-9.1, and in borate–phosphate–acetate buffer, pH 2.7-10; 0.1 mM 2-chloro-*cis,cis*-muconate was used as the substrate. DELH activity was measured in 0.05 M sodium phosphate buffer, pH 5.8-8.0, 0.05 M Tris-HCl buffer, pH 7.2-9.1, 0.05 M glycine-NaOH buffer, pH 8.6-10.6, borate–phosphate–acetate buffer, pH 2.7-10; 0.1 mM *cis*-dienolactone was used as the substrate.

The temperature optima were determined in the temperature range from 5 to 75°C using a Shimadzu temperature controller in the following buffers: the CC 1,2-DO activity was measured in 0.05 M Tris-HCl buffer, pH 7.6; the CMCI activity was assayed in 0.05 M bis-Tris-HCl buffer (pH 6.2) containing 2 mM $MnCl_2$; the activi-

ty of DELH was measured in 0.05 M Tris-HCl buffer, pH 8.6.

Determination of kinetic parameters of the enzymes. The apparent K_m and V_{max} values were estimated from double-reciprocal plots ($1/v_0$ versus $1/S$), where S is substrate concentration. To estimate the apparent K_m and V_{max} values for CC 1,2-DO (0.05 M Tris-HCl buffer, pH 7.4, containing 1.3 mM EDTA), the following substrates were used: 22-58 μM catechol, 15-200 μM 3-CC, 10-140 μM 4-CC, 30-125 μM 3-MC, 25-125 μM 4-MC. To determine the apparent K_m and V_{max} for CMCI, 2-chloro-*cis,cis*-muconate in the concentration range 12-150 μM (0.05 M Tris-HCl buffer, pH 7.2, containing 2 mM $MnCl_2$) was used as the substrate. To calculate apparent K_m and V_{max} for DELH, *cis*-dienolactone (6-150 μM) in 0.05 M Tris-HCl buffer, pH 7.8, was used.

The inhibition constant (K_i) was determined according to Dixon's procedure [13] from the relationship between $1/v$ and inhibitor concentration at two given substrate concentrations.

The specificity constants were calculated as the ratio of molecular activity to the apparent Michaelis constant k_{cat}/K_m [14].

Analytical methods. *Absorption spectroscopy*. The component composition of the reaction mixtures was analyzed on a Shimadzu UV-2501PC spectrophotometer. In the experiments on the conversion of 2-chloromuconate by CMCI, the reaction mixture contained 30 mM Tris-HCl buffer (pH 7.2), 2 mM $MnCl_2$, 0.1 mM 2-chloro-*cis,cis*-muconate, and the enzyme (1.7 μg/ml). The spectra were taken at 190-340 nm every 120 sec. The conversion of 5-chloromuconolactone by CMLI was recorded at 190-340 nm every 90 sec.

HPLC. A Grom SIL 100 C8 reversed-phase column (4.6 × 125) (Grom, Germany) was used. *cis*-Dienolactone, *trans*-dienolactone, and 2-chloromuconate were determined at 280 nm; 5-chloromuconolactone, 2-chloromuconolactone, and maleylacetate were determined at 210 nm; the flow rate was 0.9 ml/min. The mobile phase was 5% aqueous methanol (v/v) with 0.1% phosphoric acid (w/v). At the beginning of the experiment, the reaction mixture contained 0.1 mM 2-chloromuconate and 2 mM $MnCl_2$. A chromatogram obtained before the addition of CMCI was used as a control. The reaction was monitored by taking samples of the reaction mixture after certain time intervals. After the completion of the reaction (after 75 min), 0.32 μg CMLI were added; 20 μl of partially purified DELH were added to the reaction mixture 85 min after the beginning of the experiment.

Protein concentration was determined by a modification of the Bradford procedure [15] using BSA as the standard.

RESULTS AND DISCUSSION

Purification of chlorocatechol 1,2-dioxygenase. The maximum yield of the enzyme was observed with the biomass grown to the absorption of 1.6-1.7 at 545 nm. The purification procedure in given in Table 1. The enzyme was purified 9.6-fold; the recovery by activity was 66.3%. The subunit molecular mass determined by SDS-PAGE was 28 kD (Fig. 1), and the molecular mass of the enzyme estimated by gel filtration was 66 kD. Thus, CC 1,2-DO is apparently a homodimer.

Physicochemical properties of CC 1,2-DO. The pH optimum of CC 1,2-DO in Tris-HCl buffer is 7.6; in borate–phosphate–acetate buffer it is 7.4. The temperature optimum of the enzyme is 45°C.

Catalytic properties of CC 1,2-DO. The substrate specificity of CC 1,2-DO is shown in Table 2. To calculate k_{cat}, the subunit mass of 28.5 kD was taken (calculated from the nucleotide sequence [1]). Similarly to other known chlorocatechases, the enzyme exhibited high specificity to catechol. The calculated specificity constants showed that the enzyme displays high affinity for 3-CC, 3-MC, and 4-MC. In contrast to CC 1,2-DO of *R. opacus* 1CP described earlier [2], the studied enzyme is inactive towards 3,5-DCC; conversely, this compound acted as an inhibitor with K_i of 2.5 μM.

Purification of chloromuconate cycloisomerase. The procedure for CMCI purification is presented in Table 3. CMCI was purified to electrophoretic homogeneity in three steps. However, an additional purification step, heating at 65°C for 10 min, was sometimes used. The

Table 1. CC 1,2-DO purification procedure

Purification stage	Volume, ml	Total protein, mg	Total activity, units	Specific activity, U/mg	Degree of purification	Yield, %
Cell-free extract	49	392	392	1		100
Q-Sepharose Fast Flow	27.7	221.6	355	1.6	1.6	90.6
Butyl-Sepharose	27	27	260	9.6	9.6	66.3

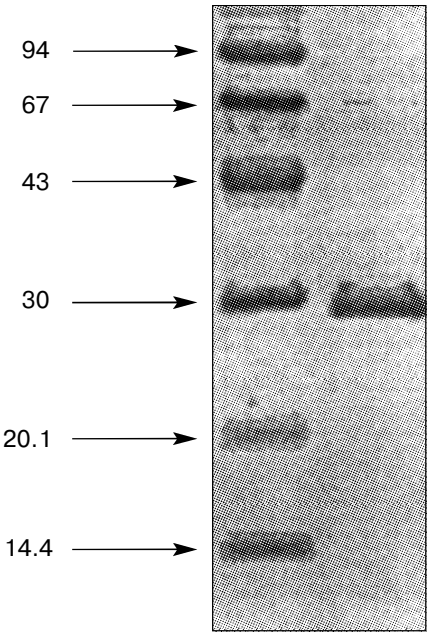

Fig. 1. SDS-PAGE of CC 1,2-DO. Molecular masses of the standards are shown at the left: phosphorylase *b* (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

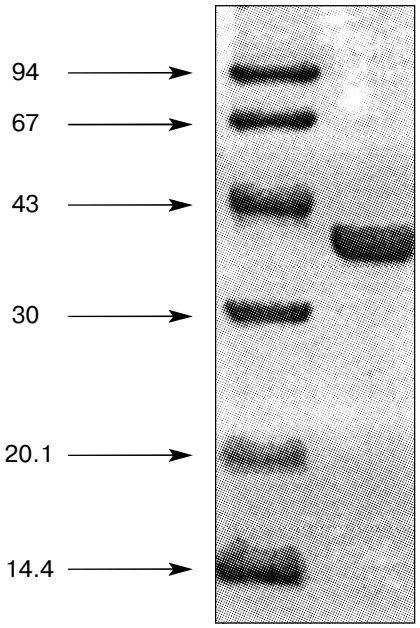

Fig. 2. Electrophoresis of CMCI. Molecular masses of standards are shown at the left: phosphorylase *b* (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

Table 2. Substrate specificity of CC 1,2-DO

Substrate	K_m, μM	V_{max}, U/mg	k_{cat}, min^{-1}	k_{cat}/K_m, $min^{-1}\cdot\mu M^{-1}$
Catechol	2.1	16.1	458.8	218.5
3-Chlorocatechol	1.4	11.5	327.7	229.2
4-Chlorocatechol	3.5	10.0	285.0	82.6
3-Methylcatechol	5.7	45.5	1295.3	227.2
4-Methylcatechol	3.4	43.5	1239.7	364.6

Table 3. CMCI purification procedure

Purification stage	Volume, ml	Total protein, mg	Total activity, units	Specific activity, U/mg	Degree of purification	Yield, %
Cell-free extract	110	990	154	0.15	1	100
Q-Sepharose Fast Flow	31	36	213	6	40	138
Phenyl-Sepharose CL-4B	20	13	146	11.2	75	95
Resource Iso	2.8	3.4	60	18	120	39

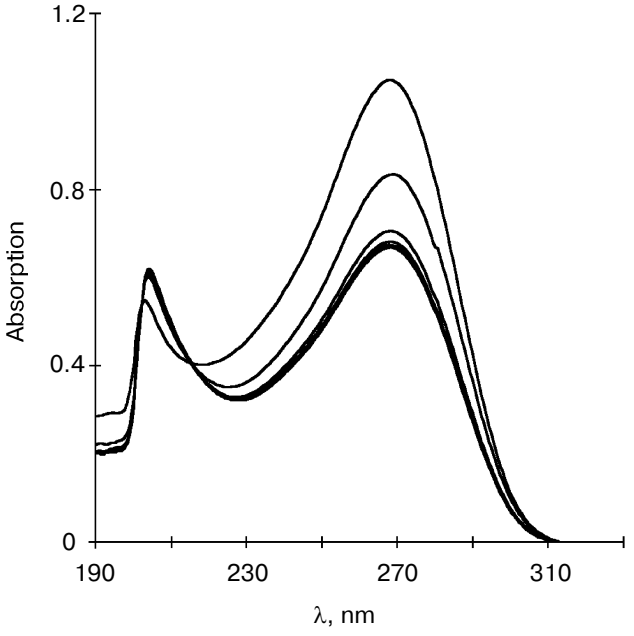

Fig. 3. UV spectra of the conversion of 2-chloromuconate by CMCI.

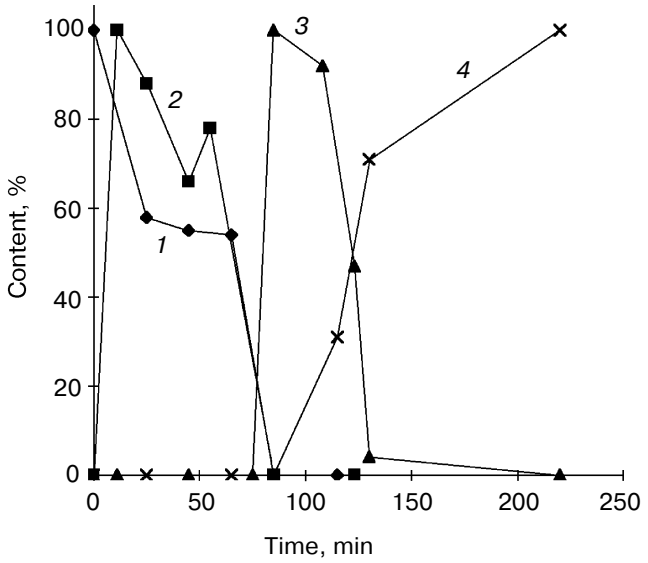

Fig. 4. Dynamics of 2-chloromuconate conversion to 5-chloromuconolactone, *cis*-dienolactone, and maleylacetate catalyzed by CMCI, CMLI, and DELH. The enzymes were added successively; CMCI was added at the beginning of the experiment followed by CMLI (after 75 min) and DELH (after 85 min): *1*) 2-chloromuconic acid; *2*) 5-chloromuconolactone; *3*) *cis*-dienolactone; *4*) maleylacetate.

enzyme was purified 120-fold; the yield of the enzyme was 39%. The final CMCI preparation contained 3.4 mg protein with specific activity of 18 units per mg protein. The subunit molecular mass estimated by SDS-PAGE is 40 kD (Fig. 2), and the molecular mass of the enzyme determined by gel filtration is 340 kD. In all probability, CMCI is a homooctamer.

Physicochemical properties of CMCI. The pH optimum of CMCI in bis-Tris-HCl and Tris-HCl buffers is 6.2, and in borate–phosphate–acetate buffer it is 5.7. The enzyme is inactive in sodium phosphate and glycine-NaOH buffers.

The temperature optimum of CMCI is ~65°C.

Catalytic properties of CMCI. The K_m for 2-chloromuconate is 142.86 μM, V_{max} = 71.43 U/mg, k_{cat} = 2850 min^{-1}, and k_{cat}/K_m = 19.95 $min^{-1}\cdot\mu M^{-1}$. The k_{cat} values were calculated from the subunit molecular mass of 39.9 kD. The CMCI from *R. opacus* 1CP involved in degradation of 2-chlorophenol showed high substrate specificity. 2-Chloro-*cis,cis*-muconate was employed as the substrate for the enzyme; *cis,cis*-muconate, 3-chloromuconate, 3-methylmuconate, and 2,4-dichloromuconate were not CMCI substrates.

The formation of 5-chloromuconolactone from 2-chloromuconate was confirmed by UV spectroscopy and HPLC (Figs. 3 and 4).

Properties of chloromuconolactone isomerase. The scheme for purification of CMLI is shown in Table 4. After three purification steps, 0.33 mg protein with specific activity 250.5 U/mg was obtained.

Table 4. CMLI purification procedure

Purification stage	Volume, ml	Total protein, mg	Total activity, units	Specific activity, U/mg
Cell-free extract	22.8	120.8	n.d.	n.d.
Q-Sepharose HP	148	4.74	n.d.	n.d.
Resourse Phe	9.3	0.37	88.4	238.97
Incubation at 70°C for 15 min	0.25	0.33	82.7	250.5

Note: n.d., not determined.

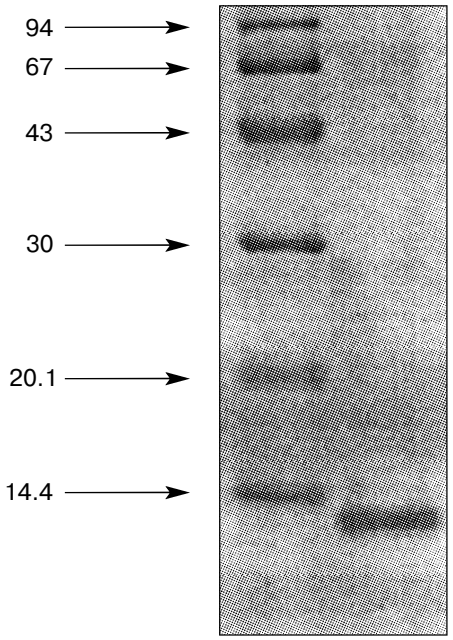

Fig. 5. SDS-PAGE of CMLI. Molecular masses of markers are shown at the left: phosphorylase *b* (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

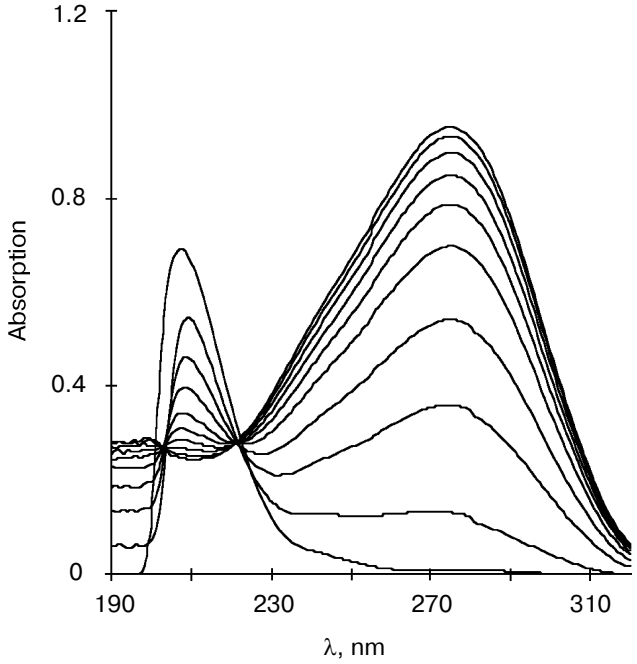

Fig. 6. UV spectrum of the conversion of 5-chloromuconolactone by CMLI.

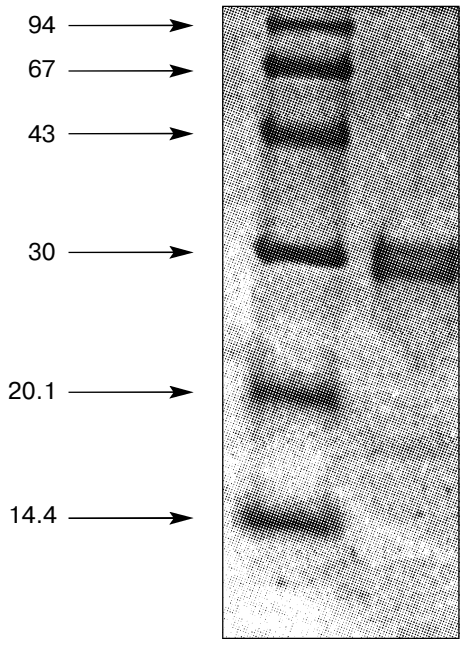

Fig. 7. SDS-PAGE of DELH. Molecular masses of markers are shown at the left: phosphorylase *b* (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

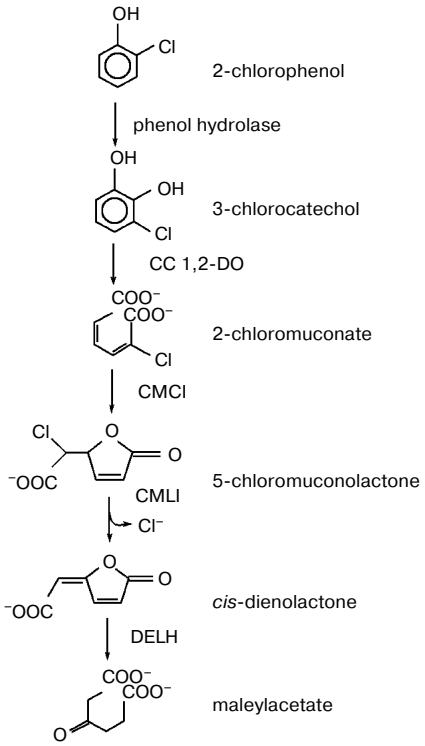

Fig. 8. A new pathway of 2-chlorophenol degradation by *R. opacus* 1CP.

Table 5. DELH purification procedure

Purification stage	Volume, ml	Total protein, mg	Total activity, units	Specific activity, U/mg	Degree of purification	Yield, %
Cell-free extract	110	990	880	0.89	1	100
Q-Sepharose Fast Flow	130	208	929.5	4.5	5	105.6
Phenyl-Sepharose	19	26	689	26.5	30	78.3
Mono Q	10	5	206.8	41.4	46	23.5
Resource Iso	3.4	1.7	102	60	67	11.6

SDS-PAGE in a 13% gel revealed a band at ~12 kD (Fig. 5). The molecular mass of the native enzyme was ~66 kD. Thus, the enzyme contains 5-6 homologous subunits.

The conversion of 5-chloromuconolactone into *cis*-dienolactone catalyzed by CMLI was analyzed spectrophotometrically by the decrease in absorption at 209 nm and by the appearance of a peak at 277 nm (Fig. 6).

Purification of dienolactone hydrolase. The procedure used for DELH purification is shown in Table 5. DELH was purified 67-fold with 11% yield.

SDS-PAGE revealed two bands corresponding to polypeptides with molecular masses slightly above and below 30 kD (Fig. 7). During gel filtration, only one peak corresponding to a 30-kD protein was detected.

Physicochemical properties of DELH. The pH optimum of DELH in different buffers is near 8.6. The temperature optimum of DELH is ~40°C.

Catalytic properties of DELH. The K_m for *cis*-dienolactone is 200 μM, V_{max} = 167 U/mg, k_{cat} = 4633 min^{-1}, and k_{cat}/K_m = 23 $min^{-1}\cdot\mu M^{-1}$. The k_{cat} values were calculated from the subunit molecular mass of 27.8 kD. DELH involved in degradation of 2-chlorophenol did not utilize *trans*-dienolactone as a substrate.

Thus, we isolated and characterized four enzymes: CC 1,2-DO, CMCI, CMLI, and DELH. The data are consistent with the suggestion that these enzymes belong to a new 3-chlorocatechol pathway (Fig. 8) predicted earlier from molecular-biological data [1] and show their difference in physicochemical and catalytic properties from previously described enzymes. Since the enzymes of the new modified *ortho*-pathway in the *R. opacus* 1CP strain occupy an intermediate position between the analogous enzymes of classical and modified pathways, the problem of their evolutionary relatedness remains unclear. In addition, among the enzymes of the new modified *ortho*-pathway, enzymes present in the modified and classical pathways (MLI) are found [16].

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