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Purification and characterization of a *Galactomyces reessii* hydratase that converts 3-methylcrotonic acid to 3-hydroxy-3-methylbutyric acid

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Cell free extracts of *Galactomyces reessii* contain a hydratase as the key enzyme for the transformation of 3-methylcrotonic acid to 3-hydroxy-3-methylbutyric acid. Highest levels of hydratase activity were obtained during growth on isovaleric acid. The enzyme, an enoyl CoA hydratase, was purified 147-fold by precipitation with ammonium sulphate and successive chromatography over columns of DE-52, Blue Sepharose CL-6B and Sephacryl S-200. During purification, hydratase activity was measured spectrophotometrically (OD change at 263 nm) for 3-methylcrotonyl CoA and crotonyl CoA as substrates. The enzyme displayed highest activity with crotonyl CoA with a K_{cat} of 1,050,000 min^{-1}. The ratio of crotonyl CoA to 3-methylcrotonyl CoA activities was constant (20:1) during all steps of purification. The K_{cat} for crotonyl CoA was also about 20 times greater than the K_{cat} for 3-methylcrotonyl CoA (51,700 min^{-1}). The enzyme had pH and temperature optima at 7.0 and 35°C, a native M_r of 260±4.5 kDa and a subunit M_r of 65 kDa, suggesting that the enzyme was a homotetramer. The p*I* of the purified hydratase was 5.5, and the N-terminal amino acid sequence was VPEGYAEDLLKGKMMRFFDS. Hydratase activity for 3-methylcrotonyl CoA was competitively inhibited by acetyl CoA, propionyl CoA and acetoacetyl CoA.
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Introduction

3-Hydroxy-3-methylbutyric acid (HMBA) is a new dietary supplement that improves animal growth and health [17,18] and increases strength and lean gains in resistance exercise training in humans [12]. HMBA is produced from leucine *via* transformation to α-ketocaproate in mammals [13]. Efficient syntheses of HMBA have become important because of the expected increasing demand for the compound. Chemical routes are known, such as the sodium hypochlorite-catalyzed oxidation of diacetone alcohol [12]. Syntheses like these can generate environmentally undesirable byproducts and residues. Thus, enzymatic or microbiological approaches to HMBA syntheses are of interest.

We have studied the microbial transformation of 3-methylbutyric acid (MBA) to HMBA, a reaction that is efficiently catalyzed by whole cells of *Galactomyces reessii*. Up to 38 g/l HMBA was obtained from MBA as substrate in a fed-batch culture process [13]. The conversion process occurs by intercepting key enzymes in *G. reessii* involved in the leucine metabolic pathway [13] (Figure 1). An analysis of enzymes in crude cell free extracts of *G. reessii* showed activities for acyl CoA synthetase, acyl CoA dehydrogenase and enoyl CoA hydratase. Of these, enoyl CoA hydratase was the dominant enzyme activity in cell free extracts, exhibiting a specific activity more than 30 times that of any other enzyme in the leucine degradation pathway.

Further work on the biocatalytic capabilities of *G. reessii* enzymes has been undertaken because this organism was the best among others examined for transforming HMB to HMBA. In whole cell cultures, the hydratase reaction appears to be substantially

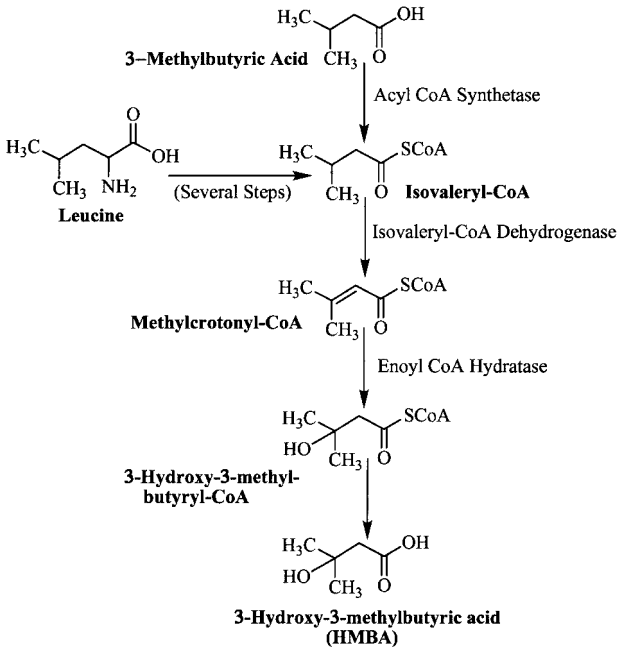

Figure 1 Metabolic pathway for the conversion of leucine or MBA to HMBA by *G. reessii*.

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different than that in other organisms [2,9,12,25]. This study was undertaken to clarify the catalytic properties of the major *G. reessii* enzyme involved in MBA-to-HMBA bioconversions. An understanding of kinetic, temperature, substrate specificities, inhibition and other aspects of the key enzyme could lead to further optimization of the whole cell process, and considerable further improvement in HMBA yields.

Materials and methods

G. reessii CBS179.60 (Central Bureau for Schimmel Cultures, Baarn, the Netherlands) was used for all the experiments. Stock cultures were maintained on Luria agar and stored in a refrigerator at 4°C prior to use. Luria broth (LB) contained, per liter: 5 g of yeast extract, 10 g of tryptone, 10 g of NaCl, and 10 g of glucose. The basal fermentation (G1) medium contained (per liter) 13 g of $(NH_4)_2HPO_4$, 7 g of KH_2PO_4, 0.8 g of $MgSO_4 \cdot 7H_2O$, 3 g of yeast extract, 1 g of NaCl, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2 \cdot 2H_2O$ and 10 ml of trace element solution [13]. For cultivation, MBA (0.3% w/v) was added to G1 medium as the sole carbon source and the pH was adjusted to 7.0 with 8 N NaOH. The two-stage process was initiated by inoculating a loopful of *G. reessii* into 25 ml of LB medium held in 125-ml conical-shaped, stainless steel-capped, DeLong culture flasks. Cultures were incubated with shaking at 250 rpm for 24 h at 30°C on an Innova 5000 gyratory tier shaker (New Brunswick Scientific, Edison, NJ, USA). A 10% inoculum from the first stage culture was used to initiate the second stage culture, which was grown in 200 ml of G1 medium containing MBA held in 1-l DeLong flasks. Second stage cultures were incubated as before for 72 h before being harvested. To study the induction of enoyl CoA hydratase, *G. reessii* was grown for 72 h in medium G1 supplemented with 3 g/l of each of the growth substrates listed in Table 1. Stock solutions of organic acids were adjusted to pH 7.0 prior to their additions to the basal medium.

Preparation of crude extracts

Cells were harvested from incubations by centrifugation at 10,000×*g* at 4°C for 20 min. Cell pellets were washed twice with cold 50 mM phosphate buffer, pH 7.0. Typical wet cell yields were approximately 15 g/l. The washed cells were stored at −70°C until needed. Cells were suspended in 50 mM phosphate buffer, pH 7.0, containing 1 mM PMSF, 1 mM DTT and 10% glycerol to a density of 300 g/l at 4°C and then sonicated at 4°C with a Branson Sonifier Cell disrupter 350 (Branson Sonic Power, Danbury, CT, USA) for a total of 6 min with 15-s cooling intervals between two 15-s pulses. The supernatant obtained by centrifugation of broken cell suspensions at 10,000×*g* for 30 min was used for enoyl CoA hydratase purification. For induction experiments, cell free extracts were prepared for each batch of *G. reessii* cells grown on different substrates.

Protein and enzyme assays

Protein concentrations were estimated by the method of Bradford [5] with bovine serum albumin as the standard. Initial rates of hydration of enoyl CoA thioesters were determined over 3 min by measuring decreases in absorbance *versus* time at 263 nm. Enzymatic addition of water abolished characteristic ultraviolet absorptions due to double bonds, which are conjugated with carbonyl groups of thioester substrates [19,20,21]. Assay mixtures contained 100 mM phosphate buffer, pH 8.0, 0.05 mg/ml ovalbumin, 50 μM 3-methylcrotonyl CoA or 100 μM crotonyl CoA or 100 μM tiglyl CoA and 2–10 μg of enzyme in a final volume of 0.5 ml. Any necessary dilution of the enzyme fractions was made with the same buffer. Under these conditions, extinction coefficients were determined for 3-methylcrotonyl CoA (ε=6700 $cm^{-1}\,M^{-1}$), crotonyl CoA (ε=6520 $cm^{-1}\,M^{-1}$) and tiglyl CoA (ε=3800 $cm^{-1}\,M^{-1}$). A unit of enzyme activity was defined as the amount of enzyme that catalyzed the hydration of 1 μmol of substrate per minute.

The activity of 3-hydroxybutyryl CoA dehydrogenase was measured spectrophotometrically at 30°C. The standard assay contained 70 μmol of glycine buffer, pH 9.0, 1 μmol of NAD^+ and 0.15 μmol of crotonyl CoA in a final volume of 1 ml. The reaction was initiated by addition of enzyme and was monitored by measuring the change of absorbance at 340 nm [16]. All assays were conducted in triplicate, and values are expressed as the mean, with standard deviations not exceeding ±5%.

Purification procedure

All steps used in purifying *G. reessii* enoyl CoA hydratase were performed at 4°C. Crude cell free extract (150 ml, 555 mg protein) was brought to 30% saturation by the addition of ammonium sulfate over a period of 30 min with constant stirring. After centrifugation at 20,000×*g* for 30 min, the supernatant was collected and brought to 65% ammonium sulfate saturation. After 2 h, this solution was centrifuged at 20,000×*g* for 30 min, and the resulting pellet was

Table 1 Effects of culture medium growth substrates on enoyl CoA hydratase-specific activities in *G. reessii* cell free extracts

Growth substrates[a]	Wet weight (g/l cells)	Specific activity[b] (U/mg) with	
		3-Methylcrotonyl CoA	Crotonyl CoA
D-glucose	23.2	0.17±0.008	3.70±0.17
L-glutamate	15.3	0.15±0.007	3.35±0.12
L-leucine	17.3	0.82±0.04	18.60±0.75
L-valine	6.2	0.50±0.022	11.10±0.42
L-isoleucine	15.8	0.41±0.018	8.80±0.38
L-isoleucine+L-valine	16.4	0.53±0.021	11.80±0.45
Butyric acid	14.4	0.62±0.028	13.60±0.66
2-Keto-3 methyl valeric acid	12.8	0.48±0.019	10.75±0.40
Isovaleric acid	15.1	1.20±0.036	26.40±1.05

[a]All substrates added to growth medium at 3.0 g/l.
[b]Incubations were conducted in triplicate using standard assay conditions.

Table 2 Purification of enoyl CoA hydratase from *G. reessii*.

Steps	Total protein (mg)[a]	Total activity units		Specific activity		Fold		Yield	
		(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Cell free extract	555	644	14,152	1.16	25.5	1	1	100	100
30–65% $(NH_4)_2SO_4$	228	580	11,514	2.54	50.5	2.2	1.9	88.5	81.3
DE-52 Pool	28.3	260	5521	9.19	195	7.9	7.6	40.3	39.0
Blue Sepharose CL6B	3.2	104	2027	32.5	633.4	28.0	24.8	16.1	14.3
Sephacryl S-200	0.35	60	1218	171.5	3480	147	136	9.3	8.6

Activities are shown for 3-methylcrotonyl CoA (1) and crotonyl CoA (2).
[a]DE-52 (fractions 80–105); Blue Sepharose CL6B (fractions 28–40); Sephacryl S-200 (fractions 56–66).

resuspended in 10 mM phosphate buffer, pH 7.0, containing 1 mM DTT and 10% glycerol (buffer A), and dialyzed overnight against 2 liters of the same buffer.

The dialyzed sample (30 ml, 228 mg protein) was loaded onto a DEAE cellulose column (DE-52) (3×30 cm) that had been previously equilibrated with buffer A. The column was washed with 150 ml of buffer A at a flow rate of 30 ml/h, before the enzyme was eluted by a linear gradient of KCl (0–0.5 M) in 500 ml of the same buffer while 2-ml fractions were collected. Fractions were assayed with both 3-methylcrotonyl CoA and crotonyl CoA as substrates. Active hydratase fractions (80–105) were pooled, concentrated by ultrafiltration through a 10-kDa (PM 10) cut-off membrane (Amicon Division, Beverly, MA, USA) and dialyzed against 1 l of buffer A.

The dialyzed enzyme solution (3 ml, 28.3 mg protein) from the DE-52 column was further fractionated over a Blue-Sepharose CL-6B column (1×15 cm) previously equilibrated with buffer A. Unbound proteins were washed off the column by 30 ml of the same buffer, and bound proteins were eluted by a 60-ml linear gradient of KCl (0–1.5 M) in buffer A at a flow rate of 30 ml/h while 2-ml fractions were collected. Fractions (28–40) containing hydratase activity were pooled, dialyzed against 1 liter of buffer A and concentrated to 1 ml.

The concentrated Blue Sepharose CL6B column preparation was subjected to gel filtration over Sephacryl S-200 (1×100 cm) equilibrated in and eluted with buffer A. Fractions of 0.5 ml were collected at a flow rate of 15 ml/h. The hydratase-active fractions (56–66) were pooled for subsequent analysis.

Properties of G. reessii enoyl CoA hydratase

The apparent molecular mass (M_r) of the denatured enzyme was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [11] carried out in minigels (Bio-Rad, Hercules, CA, USA) containing 12% acrylamide for the separating gel and 3% acrylamide for the stacking gels containing 0.1% SDS. Protein standards (Sigma, St. Louis, MO, USA) used for the estimation of subunit molecular masses were bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa) and lysozyme (14 kDa). Gels were stained with Coomassie blue.

The M_r of the active, native enzyme was determined with Sepharose 6B (Sigma) chromatography (1×100 cm) with apoferritin (443 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa) as molecular weight markers. The column was equilibrated and eluted with buffer A.

The enoyl CoA hydratase isoeletric point (p*I*) was estimated by chromatofocusing on a PBE94 (Pharmacia, Piscataway, NJ) column. The purified enzyme (100 μg) was dialyzed in 20 mM imidazole–HCl buffer, pH 7.4. The column was eluted with polybuffer 74 (Pharmacia) covering a pH range of 4–7 while 1-ml fractions were collected. The pH and enzyme activity of each fraction were measured.

For N-terminal sequence analysis, purified enoyl CoA hydratase (10 μg) was loaded onto and developed by SDS-PAGE and then transblotted onto a polyvinylidene difluoride membrane (Problott; Applied Biosystems, Foster City, CA, USA). N-terminal amino acid analysis was done by automated microsequencing by Edman degradation reaction on a 475A sequencer (Applied Biosystems) performed in the University of Iowa Protein Structure Facility.

All kinetic studies used highly purified enoyl CoA hydratase for incubation mixtures containing 10 μl of prediluted enzyme (0.06 U) in final volumes of 500 μl while concentrations of 3-methylcrotonyl CoA were varied from 0.1 μM to 1 mM. Kinetic constants (K_m and K_{cat}) were obtained by fitting experimental data with the EZ-FIT program developed by Perrella [15]. The kinetic parameters for crotonyl CoA (0.1 μM–1 mM) and tiglyl CoA (0.1 μM–1 mM) were also determined.

Optimal incubation temperature and pH conditions were estimated in assay mixtures containing 0.3 U of enzyme. Incubation temperatures were varied from 15°C to 50°C with 3-methylcrotonyl CoA as substrate. The influence of pH on reaction rates was determined in the following buffers: 100 mM phosphate (pH 6.0–8.0) and 100 mM Tris–HCl (pH 6.0–9.0).

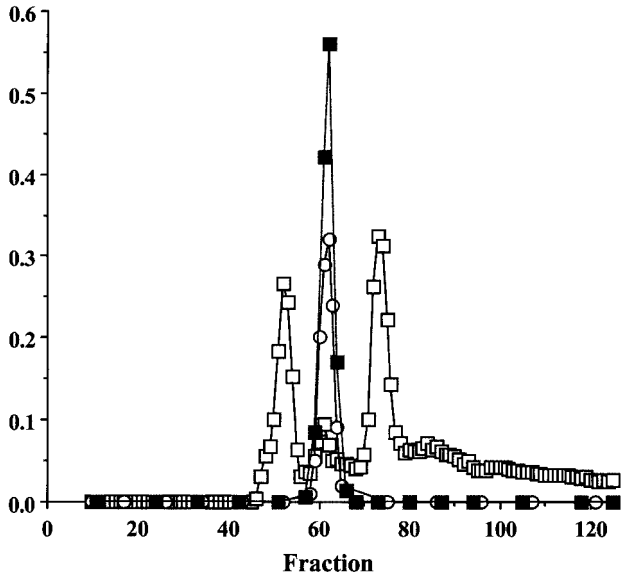

Figure 2 Elution profile for enoyl CoA hydratase from Sephacryl S-200 gel filtration. (□) OD at 280 nm. (■) Crotonyl CoA activity (U/ml). (○) 3-Methylcrotonyl CoA (U/ml).

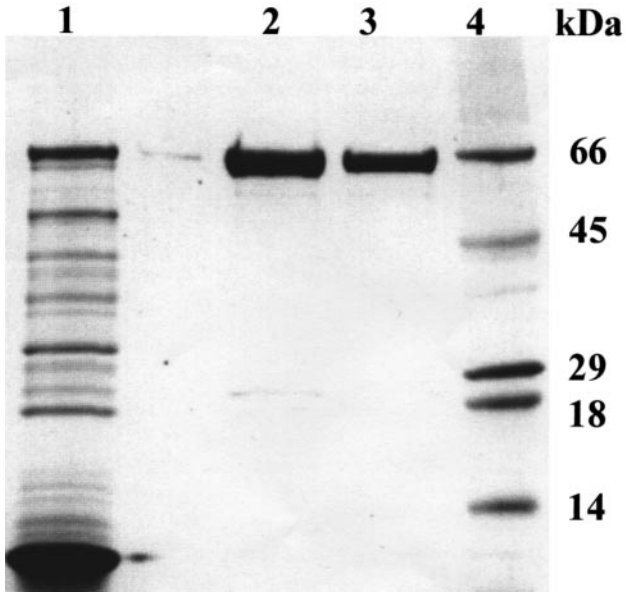

Figure 3 SDS-PAGE analysis of 10-μg samples from each purification step. Lane 1: Blue Sepharose CL6B. Lanes 2 and 3: Purified enzyme from Sephacryl S-200 pool. Lane 4: Molecular markers — bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa) and lysozyme (14 kDa). Protein samples loaded in Lanes 1, 2, 3 and 4 were 10, 10, 5 and 10 μg, respectively.

For inhibition studies, purified enzyme was preincubated with 1–10 mM *N*-ethylmaleimide, chloromercuribenzoate or metal ions in 100 μM phosphate buffer for 30 min before residual hydratase activity was measured. Incubations containing only enzyme in buffer were used as controls. Incubations were also conducted with 1 and 20 mM EDTA. K_i values for acetyl CoA, propionyl CoA and acetoacetyl CoA for enoyl CoA hydratase were determined using concentrations of 50, 75 and 100 μM for each inhibitor, while the substrate 3-methylcrotonyl CoA was varied from 5 to 40 μM. Individual K_i values were determined from computer-generated plots [15].

Results

Enzyme induction

Cell free extracts prepared from cells grown with various carbon sources were assayed for hydratase activity using both 3-methylcrotonyl CoA and crotonyl CoA as substrates (Table 1). Growth on glucose or glutamate gave extracts with the lowest specific activities for both enzymes, suggesting that either lack of induction or catabolic repression of these enzymes occurred. High levels of hydratase activities were obtained with cells grown in L-leucine or isovaleric acid, linking enzyme activities to the leucine degradation pathway. For all substrates, the ratio of the specific activities for 3-methylcrotonyl CoA to crotonyl CoA was essentially constant at 1:22, suggesting that the same enzyme catalyzed hydrations of both substrates.

Enzyme purification

A highly reproducible enoyl CoA hydratase purification procedure was established, and replicated three times. In a typical example, the crude extract prepared from cells grown in medium containing (0.3% w/v) isovaleric acid as sole carbon source was subjected to sequential purification steps. Activities for both crotonyl CoA and 3-methylcrotonyl CoA during each stage of purification are summarized in Table 2. Measurement of hydratase activity was simple because addition of water to the double bond of the crotonyl CoA substrates caused a time-dependent decrease in absorbance at 263 nm. Ammonium sulfate fractionation removed more than half of the total protein with a high recovery of activity, facilitating subsequent chromatographic steps. DE-52 anion exchange and Blue Sepharose chromatographies each gave about three- to

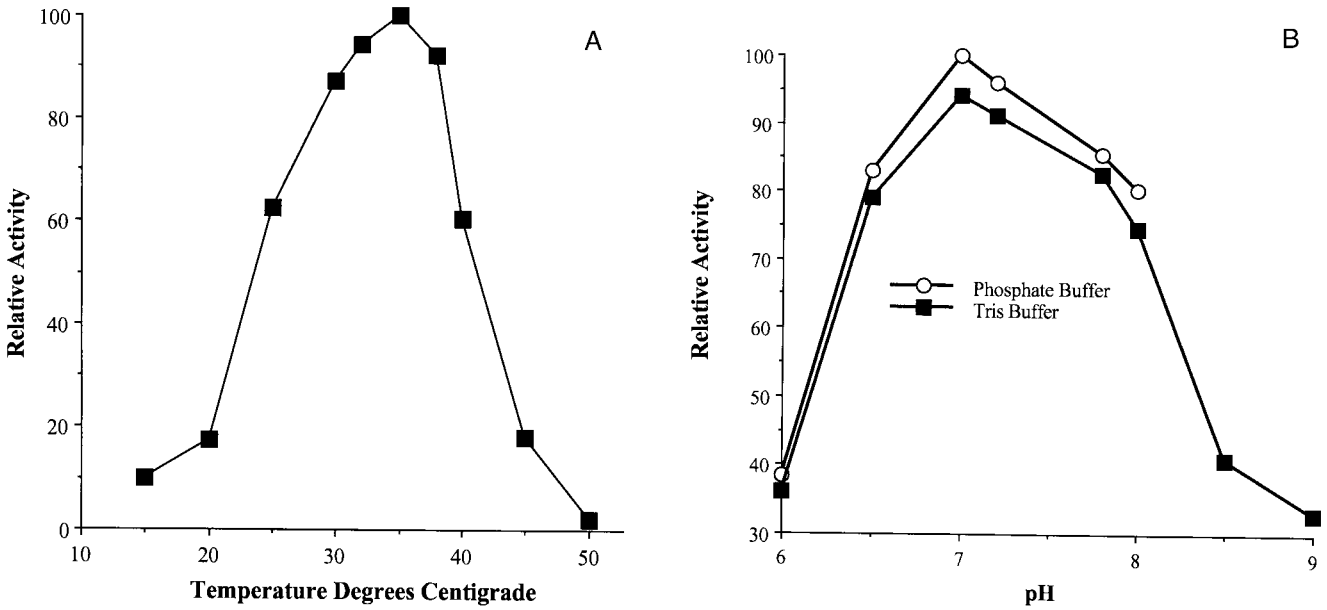

Figure 4 Responses of *G. reessii* enoyl CoA hydratase to incubation temperature (A) and pH (B).

Table 3 Catalytic properties of purified *G. reessii* enoyl CoA hydratase *versus* those from *P. putida* and *C. acetylbutylicum*

Organism	Substrate	K_m (μM)	K_{cat} (min^{-1})	CoA derivative	Inhibitor[a] K_i (μM)
G. reessii	Crotonyl CoA	74.73±3.12	1,050,000		
	3-MC CoA[b]	24.99±2.30	51,700		
	Tiglyl CoA	71.52±4.52	10,450		
	3-MC CoA			Acetyl CoA	28.90±4.65
	3-MC CoA			Propionyl CoA	22.45±6.09
	3-MC CoA			Acetoacetyl CoA	25.41±1.91
P. putida [16]	Crotonyl CoA	110	1,100,000[c]		
	3-MC CoA	110	2,300[c]		
	Tiglyl CoA	35	61,000[c]		
	Crotonyl CoA			Acetyl CoA	230
	Crotonyl CoA			Propionyl CoA	130
	Crotonyl CoA			Acetoacetyl CoA	130
C. acetobutylicum [25]	Crotonyl CoA	30	6,500,000[c]		
	Hexanoyl CoA	130	39,000[c]		

[a]All were competitive inhibitors.
[b]3-MC CoA=3-methylcrotonyl CoA.
[c]Literature values [16,25] were in mol/min/mol enzyme.

fourfold increases in enzyme-specific activity for both substrates with good overall enzyme recovery. The elution profile obtained by the final step of gel filtration with Sephacryl S-200 is shown in Figure 2. The hydratase active fractions from 56–66 were pooled to 147-fold purified enzyme in 9.3% overall yield *versus* the crude extract. The combined active fractions contained specific activities for 3-methylcrotonyl CoA and crotonyl CoA of 171.5 and 3480 U/mg protein, respectively.

Properties of G. reessii enoyl CoA hydratase

By gel filtration, the native molecular mass was 260±4.5 kDa, while SDS-PAGE (Figure 3) gave an M_r of 65 kDa. Thus, the active enzyme appears to be a homotetramer. By UV–visible spectroscopy, the purified enzyme displayed an ultraviolet absorption maximum only at 280 nm, indicating absence of prosthetic groups such as flavin or heme. The enzyme was stable at 4°C for 4 weeks and for 6 months at −70°C, retaining about 90% of its activity when stored in 10 mM phosphate buffer, pH 7.0, 1 mM DTT and 10% glycerol. Optimum enzyme activity was measured at pH 7.0 and at 35°C (Figure 4) and the p*I* was 5.5. The N-terminal amino acid sequence of the enoyl CoA hydratase subunit was NH_2-VPEGYAEDLLKGKMMRFFDS. BLAST [1] and literature comparisons of the *G. reessii* sequence showed homologies with carnitine acyltransferase (55%) from the fungus *Megnaporthe grisea* [22]; enoyl CoA hydratase (20%) from *Clostridium acetobutylicum* [4]; enoyl CoA hydratase (10%) from *Bacillus halodurans* [23]; enoyl CoA hydratase (10%) from *Aeromonas caviae* [8]; and enoyl CoA hydratase (10%) from mammalian peroxisomes [6].

Catalytic properties and substrate specificity

Purified enoyl CoA hydratase from *G. reessii* displays the highest specific activity for crotonyl CoA, and the least for tiglyl CoA (Tables 2 and 3). K_m and K_{cat} values for crotonyl CoA, 3-methylcrotonyl CoA and tiglyl CoA were determined (Table 3). The K_{cat} for crotonyl CoA was about 20 times greater than the K_{cat} for 3-methylcrotonyl CoA. K_m values for crotonyl CoA and tiglyl CoA were nearly three times higher than that for 3-methylcrotonyl CoA. However, while K_m values for tiglyl CoA and crotonyl CoA were similar, the K_{cat} for crotonyl CoA is 100 times greater than that for tiglyl CoA. K_{cat} for the hydratase for 3-methycrotonyl CoA was five times that of tiglyl CoA.

Acetyl CoA, propionyl CoA and acetoacetyl CoA were all competitive inhibitors of 3-methylcrotonyl CoA hydration (Figure 5) and the respective K_i values are listed in Table 3. The effect of crotonyl CoA on hydration of 3-methylcrotonyl CoA could not be tested because the hydration of crotonyl CoA was so much faster than 3-methylcrotonyl CoA.

Effects of metals, cofactors and inhibitors on enzyme activity

Enzyme activity was completely inhibited by 1 mM mercuric chloride, 42% by 1 mM *N*-ethylmaleimide and 56% by 1 mM *p*-chloromercuribenzoic acid, indicating the involvement of sulfhydryl groups hydratase activity. As found with other hydratases, other metal ions including Fe^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+}, all at 1 mM concentrations, had essentially no effect on purified enzyme activity. Addition of 1–20 mM EDTA

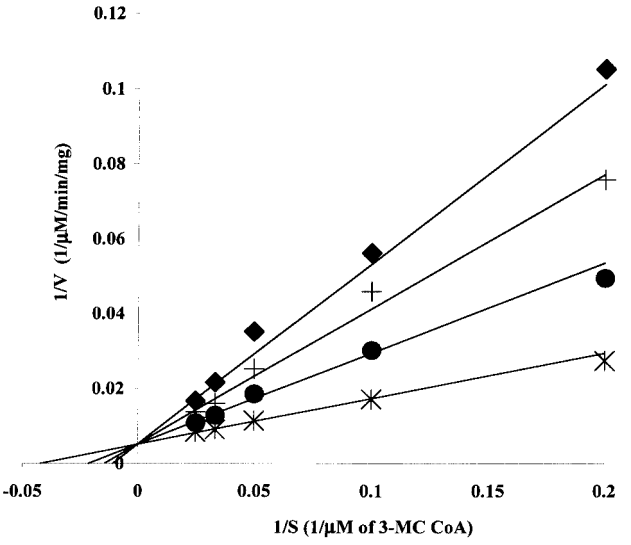

Figure 5 Double-reciprocal plots of the inhibition of *G. reessii* 3-methylcrotonyl CoA hydratase by acetoacetyl CoA. Inhibition assays were carried out under initial velocity conditions. Symbols are: (*) 0 μg acetoacetyl CoA; (●) 25 μg acetoacetyl CoA; (+) 50 μg acetoacetyl CoA; (◆) 75 μg acetoacetyl CoA.

had no effect on hydratase activity, confirming the lack of a metal requirement for catalytic activity. Likewise, addition of 1 mM concentrations of FAD, FMN, NAD, NADP, NADH or NADPH had no effect on hydratase activity.

Discussion

Enoyl CoA hydratases are known from mammalian and microbial sources. From beef liver, the enzyme participates in the oxidation of short- and long-chain fatty acids catalyzing the stereospecific hydration of *trans*-α,β-unsaturated acyl CoA derivatives to the corresponding L-(+)-3-hydroxyacyl CoA derivatives [19]. Crotonyl CoA is a substrate for this enzyme and the hydratase is known as crotonase. Purified crotonase from bovine liver [10,19,20,21] and porcine heart [7] participates in the hydration of short-chain fatty acids (C_4–C_6). A *C. acetobutylicum* hydratase is similar to mammalian crotonase but it is responsible for formation of butyrate during sugar fermentation [25], while that from *Pseudomonas putida* has a role in isoleucine metabolism [16]. In *Escherichia coli* [2] and *Candida tropicalis* [9], the enzyme is part of a multifunctional β-oxidation enzyme complex. The hydratase isolated from *G. reessii* appears to have a relatively specific function in the biosynthesis of HMBA, and it is the first such enzyme from yeast.

The hydratase was purified from crude cell free extracts of *G. reessii*. The enzyme was best induced with leucine and isovaleric acid, confirming our earlier findings that the hydratase derives from the leucine catabolic pathway [13]. The relative specific activity of *G. reessii* hydratase for 3-methylcrotonyl CoA and crotonyl CoA was constant at about 1:22 for all carbon sources examined. These results parallel that found in the induction of another enoyl CoA hydratase from *P. putida* in the presence of intermediates from the isoleucine catabolic pathway [16]. In *P. putida*, the ratio of activities among crotonyl CoA, tiglyl CoA and 3-methylcrotonyl CoA was constant over a 14- to 17-fold range, consistent with the operation of a single hydratase. The constant ratio (1:20) of hydratase activity during enzyme purification (Table 2) appears to show that a single enzyme was responsible for the hydration of crotonyl CoA and 3-methylcrotonyl CoA.

The active, undenatured enzyme is a homotetramer with an M_r of 260±4.5 kDa. The enoyl CoA hydratase from *C. acetobutylicum* is also a tetrameric protein, but with an M_r of 158 kDa [25]. The isoelectric point at 5.5 is similar to that for a *P. putida* enoyl CoA hydratase [16]. BLAST database comparison and literature analysis indicated good homology (55%) between the *G. reessii* enoyl CoA hydratase and carnitine acyl transferase. This result indicates that *G. reessii* enoyl CoA hydratase may share an evolutionary link with fatty acyl transport. The relatively low homology of the *G. reessii* enzyme with other enoyl CoA hydratases [4,8,23] supports the uniqueness of the enzyme that was also demonstrated in its catalytic properties. The catalytic properties of the *G. reessii* enzyme were compared to those for enoyl CoA hydratases from *P. putida* and *C. acetobutylicum* (Table 3). The K_{cat} values for crotonyl CoA for *G. reessii* and *P. putida* were similar, while that for *C. acetobutylicum* was six times larger. The kinetic turnover of the *G. reessii* enzyme for 3-methylcrotonyl CoA was about 20 times greater than that for the *P. putida* enzyme. In *P. putida*, the order of substrate specificity was crotonyl CoA>tiglyl CoA>3-methylcrotonyl CoA, different than that for *G. reessii*. This suggests a more specific role for 3-methylcrotonyl CoA hydratase in the biotransformation of isovaleric acid by *G. reessii*. Inhibition constants for acetyl CoA, propionyl CoA and acetoacetyl CoA were 10 to 5 times lower for the *G. reessii* enzyme *versus P. putida*.

Competitive inhibition of *G. reessii* enoyl CoA hydratase by acetyl CoA, propionyl CoA and acetoacetyl CoA *versus* 3-methylcrotonyl CoA suggests a link between the hydratase and products of fatty acid β-oxidation. Similarly, competitive inhibition of bovine crotonase [24] and a *Pseudomonas* enzyme [16] by β-oxidation intermediates indicates regulatory roles for fatty acid oxidation products and these enzymes. Thus, favoring physiological conditions that limit the levels of acyl CoA intermediates during the biotransformation of MBA to HMBA should enhance bioconversion yields. The purified enzyme showed no activity for 3-hydroxy acyl CoA dehydrogenase. Thus, the *G. reessii* enzyme appears to be different than the β-oxidation multienzyme complex from *E. coli* [3] that contained crotonase, β-hydroxyacyl CoA dehydrogenase and thiolase. From *Caulobacter crescentus* [14], crotonase activity was copurified along with 3-hydroxyacyl CoA dehydrogenase activity. Each activity could be attributed to a single separate protein that formed multimeric aggregates composed of homologous subunits. The inducible enoyl CoA hydratase from *G. reessii* is not a multifunctional protein. Rather, it is a single enzyme capable of catalyzing the hydration of crotonyl CoA and 3-methylcrotonyl CoA.

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