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High Expression, Purification, and Properties of Recombinant Homocysteine α,γ-Lyase

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Homocysteine α,γ-lyase from the anaerobic protozoan parasite *Trichomonas vaginalis* has been cloned from genomic DNA using PCR methods and expressed in *Escherichia coli* with a vector containing the T7 promoter. The recombinant homocysteine α,γ-lyase (rHCYase) is expressed as the major protein in the host *E. coli* cells. The enzyme was purified to approximately 90% purity using heat treatment at 50°C, precipitation steps with polyethyleneimine, polyethylene glycol 8000, and high sodium chloride, DEAE–Sepharose FF chromatography, and phenyl–Sepharose 6 FF chromatography. The final yield was greater than 50%, which encompassed an approximate 18-fold purification. The enzyme is a homotetramer with a monomer molecular weight of 43K and contains pyridoxal phosphate. The *Trichomonas* rHCYase is selective for homocysteine with respect to very low cysteinase activity in contrast to the α,γ-lyase from *Pseudomonas putida,* which has very high cysteinase activity with respect to homocysteine. The *T. vaginalis* and *P. putida* α,γ-lyases readily separate on a phenyl–Sepharose 6 FF column with the *T. vaginalis* enzyme eluting first. rHCYase is stable up to 50°C and active over a pH range of 6–8. These properties of high recombinant expression in *E. coli,* a simple and effective high-yield purification procedure and high relative specificity for homocysteine with respect to cysteine, make rHCYase a promising candidate to use for the diagnosis of hyperhomocystenemia, which has been demonstrated to be a major risk factor for the onset and mortality of cardiovascular disease of all types.

Methionine/homocysteine α,γ-lyases (EC 4.4.1.11) (1,2) have been studied for more than three decades. These enzymes are of interest both mechanistically and for their therapeutic and diagnostic applications. The enzymes that catalyze these reactions were first found in *Escherichia coli* and termed methioninase (3). α,γ-Lyases were found in extracts of a soil bacterium (3), *Clostridium* sp. (3), *Pseudomonas* sp. (1–4), rumen bacteria (5), and *Aeromonas* sp. (6). The enzyme was partially purified from *Clostridium sporogenes* (7), *Pseudomonas putida* (= ovalis) (6,8), and *Aeromonas* sp. (6). These enzymes have a molecular weight of approximately 170 kDa and catalyze the α,γ-elimination reaction of methionine to form α-ketobutyrate, methanethiol, and ammonia in the presence of pyridoxal 5′-phosphate. A similar mechanism has been proposed for aerobic bacteria and fungi (9,10). Methionine α,γ-lyase from *P. putida* is composed of four identical subunits of molecular weight 43 kDa with a K_m of approximately 1 mM (11). All of these lyases are pyridoxal 5′-phosphate dependent and catalyze α,γ- as well as α,β-elimination.

We have developed a simplified purification procedure that enables high-yield production of endotoxin-free methionine α,γ-lyase from *P. putida* suitable for tumor-selective, nontoxic cancer therapy of methionine-dependent tumors (12–15,19).

The gene encoding methionine α,γ-lyase in *P. putida* was cloned and expressed at relatively low levels in *E. coli* by Inoue *et al.* (16). Recombinant methioninase (rMETase) was shown to be composed of 398 amino acid residues with a calculated molecular weight of 42,626 corresponding to the subunit of the homotetrameric enzyme of native methionine α,γ-lyase (16).

Hori *et al.* also reported the cloning of a gene from *P. putida* that they termed L-methionine-γ-deamino-α-mercaptomethane-lyase (17). The peptide sequence deduced from the sequence of the gene has 398 amino acids with a molecular mass of 42,720 Da. However, this gene and corresponding protein differ significantly

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in sequence from that reported by Inoue *et al.* (16) and from native METase (16).

Toward the goal of developing methionine α,γ-lyase into an effective antitumor therapeutic, we cloned and overexpressed the *P. putida* methionine α,γ-lyase in *E. coli* using a vector with the T7 promoter. A protocol for high-yield, large-scale purification of rMETase was developed (18).

The protozoan *Trichomonas vaginalis* metabolizes L-methionine, producing methanethiol (3,20). A methionine α,γ-lyase was purified to homogeneity from *T. vaginalis* (3). The enzyme is pyridoxal 5′-phosphate dependent, has a native molecular mass of approximately 170 kDa, and consists of four apparently identical subunits of molecular mass 43–45 kDa. Two similar α,γ-lyases have been recently cloned from *T. vaginalis* (21). The *T. vaginalis* enzymes have significant homocysteine and methionine α,γ-lyase and cysteine α,β-lyase activity.

McCully in 1969 first suggested that excessive amounts of the amino acid homocysteine were implicated in the pathogenesis of arteriosclerosis (22,23) and it has now become widely accepted that hyperhomocysteinemia is a major independent risk factor for cardiovascular disease (24–29).

In a prospective study of U.S. physicians, hyperhomocysteinemia was found to increase the risk of myocardial infarction threefold (27). A strong, graded relation between plasma homocysteine levels and overall mortality was found in patients with existing cardiovascular disease (29). Intake of vitamins B_6 and B_{12} and folic acid correlate with lowered homocysteine levels and lowered risk of cardiovascular disease (30–32).

Major increases in cardiovascular disease and cardiovascular disease mortality are associated with exquisitely small increments in homocysteine levels indicating the urgent need for a widely available and accurate homocysteine diagnostic (24–32).

Araki *et al.* (33) described a detection method for free and protein-bound homocysteine in plasma that utilizes a thiol-specific fluorogenic agent, followed by detection with high-pressure liquid chromatography (HPLC) to separate thiol species.

Recently a homocysteine assay has been developed based on enzyme conversion of homocysteine to *S*-adenosylhomocysteine (AdoHcy), which is quantitatively detected by enzyme-linked immunoassay using a monoclonal antibody against AdoHcy (34).

The present report describes the expression, purification and properties of a new recombinant α,γ-lyase from *T. vaginalis* with high specificity for homocysteine relative to cysteine making it a strong candidate as a basis for a simple, widely available diagnostic for homocysteinemia. Due to the high specificity for homocysteine, we have termed this new enzyme recombinant homocysteinase (rHCYase).

MATERIALS AND METHODS

rHCYase DNA Clone

An expression clone of rHCYase was constructed using PCR techniques from the genomic DNA of the protozoan *T. vaginalis* and subcloned into a T7-7 expression vector in *E. coli* BL21 (DE3) (M. Lenz, Y. Tan, and R. Hoffman, manuscript in preparation). The use of primers, PCR reactions, and subcloning into the T7-7 expression vector were similar for the *T. vaginalis* gene to how we previously cloned the homologous, but not identical, methionine α,γ-lyase from *P. putida* and subcloned the gene into the T7-7 vector (18).

Biochemicals and Chemicals

DEAE–Sepharose FF, phenyl–Sepharose 6 FF, and the FPLC system were obtained from Pharmacia Biotech, Inc. (Piscataway, NJ). LB medium was from Difco Laboratories. Polyethylene glycol 8000 and ammonium sulfate were from Fisher. Pyridoxal phosphate; polyethyeneimine, and Lowry reagent were from Sigma. The NOVEX kit and molecular standards were from Novex Experimental Technology (San Diego, CA) (18).

Growth of Recombinant Bacteria

Each fermentation run was started with one vial of recombinant *E. coli* containing rHCYase from the master cell bank (MCB). Ten microliters of bacteria from the MCB was seeded into 5 ml of LB medium and grown at 37°C at 400 rpm overnight at 37°C. The culture was transferred to 800 ml LB medium in 6-L flasks and grown overnight at 37°C at 400 rpm at which time the OD_{600} was approximately 10. The bacteria were collected by centrifugation at 4000 rpm for 20 min. The bacteria were then transferred into 800-ml LB-medium cultures in 6-L flasks and grown at 37°C at 400 rpm for 16 h . The bacteria were harvested by centrifugation at 4000 rpm at 4°C for 20 min.

Precolumn Treatment

The bacterial pellets, derived from 72,000 ml cultures of recombinant *E. coli*, were combined and disrupted with a cavitation type homogenizer (Microfluidics Corp., Newton, MA; Model HC 8000). The homogenate was suspended in 2 vol of 20 mM potassium phosphate, pH 7.2, containing 10 μM pyridoxal phosphate, 0.01% β-mercaptoethanol, and 20% ethanol. Heat treatment of the homogenate was carried out at 50°C for 1 min. Polyethyleneimine (PEI; 1% w/v) was added to the suspension solution and mixed for 20 min. The suspension was centrifuged with an automatic refrigerated centrifuge (Sorvall, Superspeed RC 2-B) at 4°C at 12,000 rpm for 40 min to remove cell debris and nucleic acids. The supernatant was then

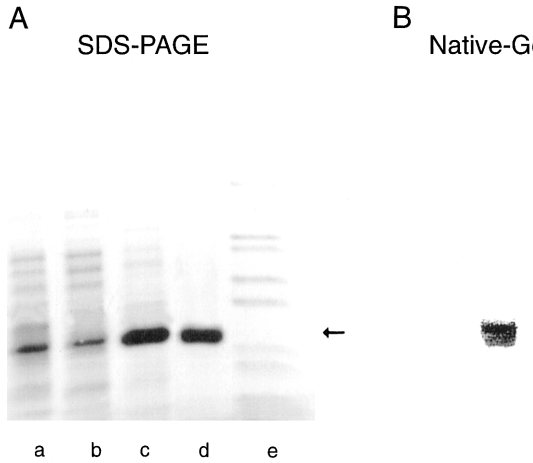

FIG. 1. (A) SDS–PAGE analysis of samples taken after each step of rHCYase purification. Approximately 10 μg protein were applied to each lane. Lane a, crude extract; lane b, after precolumn treatment; lane c, after the DEAE–Sepharose FF column chromatography step; lane d, after the phenyl–Sepharose 6 FF column chromatography step; lane e, molecular weight standards. Protein was stained with Coomassie brilliant blue R-250. Arrow indicates 43 kDa. (B) Native-gel analysis of sample taken after phenyl–Sepharose 6 FF column chromatography.

collected and mixed with polyethylene glycol 8000 (PEG8000) to a final concentration of 10–12 % (w/v) and stirred at 4°C for 60 min. The precipitate containing contaminating proteins was removed by centrifugation at 12,000 rpm for 40 min. Sodium chloride (3.0 M) was added to the supernatant to give a final concentration of 0.12 M which precipitated rHCYase. The pellet was collected by centrifugation at 12,000 rpm for 40 min and dissolved in 20 mM potassium phosphate buffer, pH 7.6, containing 10 μM pyridoxal phosphate and 0.01% β-mercaptoethanol.

DEAE–Sepharose FF Chromatography

The enzyme sample from the precolumn treatment was applied to a column of DEAE–Sepharose FF (30 × 5 cm) (Pharmacia) that was previously equilibrated with 20 mM potassium phosphate, pH 7.2. After loading the column, it was prewashed with 50 mM sodium chloride in 20 mM potassium phosphate, pH 7.2, for approximately 3 vol, until the OD_{280} dropped below 0.1. rHCYase was then eluted with a linear sodium chloride concentration gradient of 0.05–0.5 M in the same buffer for 90 min.

Phenyl–Sepharose 6 FF Chromatography

Solid ammonium sulfate (79.3 mg/ml) was added to the active fractions of the DEAE–Sepharose FF chromatography to give a final concentration of 0.6 M. Before loading the supernatant on the phenyl–Sepharose 6 FF column (20 × 2.6 cm), the column was equilibrated with Buffer A consisting of 0.6 M ammonium sulfate in 20 mM potassium phosphate, pH 7.6. Bound protein was eluted by linearly decreasing the ammonium sulfate gradient with Buffer B which contained 20 mM potassium phosphate, pH 7.6, containing 10 μM pyridoxal phosphate, 0.02% β-mercaptoethanol, and 5.0% ethylene glycol. The active fractions were concentrated by DEAE–Sepharose FF (20 × 1.6 cm) which removed ammonium sulfate and by polyethylene glycol. Purified enzyme was stored at −80°C.

rHCYase Activity Assays

Activity assay for the keto product of the α,γ- or α,β-elimination was carried out in 1 ml of 50 mM phosphate buffer, pH 8.0, containing 10 μM pyridoxal phosphate and 20 mM homocysteine for 10 min at 37°C, with varying amounts of enzyme. The reaction was stopped by adding 0.5 ml of 4.5% TCA. The suspension was centrifuged with an eppendorf centrifuge at 13 krpm for 2 min. One-half milliliter of supernatant was added to 0.5 ml of 0.05% 3-methyl-2-benzothiazolinone hydrazone (MBTH) in 1.0 ml 1.0 M sodium acetate, pH 5.2, was added, and then the mixture was incubated at 50°C for 30 min. The amount of reaction product was determined by spectrophotometry at OD_{335}. The hydrazone product has an extinction coefficient of 7.6×10^3 l/mol × cm (39). The amount of protein was determined with the Lowry reagent kit (Sigma) with bovine serum albumin as a standard. The specific activity was calculated as units per milligram of protein, with one unit of enzyme defined as the

TABLE 1

Purification of rHCYase

Steps	Protein (mg)	Activity (U)	Activity (U/mg)	Yield (%)	Purification (fold)
Homogenate	26,000	46,800	1.8	100	1.0
Precolumn	4,280	35,100	8.2	75	4.6
DEAE–Sepharose FF	1,202	30,420	25.3	65	14.1
Phenyl-Sepharose 6 FF	862	27,144	31.5	58	17.5

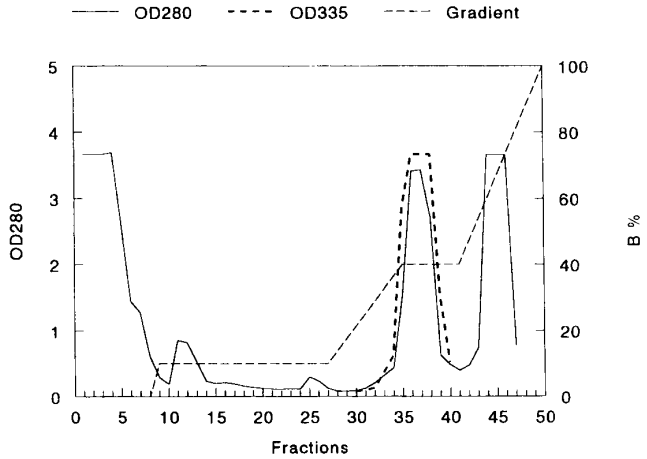

FIG. 2. DEAE–Sepharose FF chromatography. 2000 ml of partially purified precolumn extract (approximately 42.8g) was loaded on to a DEAE–Sepharose column (30 × 5.0 cm). The column was preequilibrated with 20 mM potassium phosphate buffer, pH 7.2, containing 0.01% β-mercaptoethanol and 10 μm pyridoxal phosphate, at a flow rate 10 ml/min. After loading, the column was prewashed with 50 mM sodium chloride in the same buffer and then eluted with a gradient of Buffer A (containing 0.05 sodium chloride) and Buffer B (containing 0.5 sodium chloride). The active fractions were pooled.

amount that catalyzed the formation of 1 μmol of α-ketobutyrate (8,18).

Activity assay for the γ- and β-elimination reactions of homocysteine and cysteine, respectively, were carried out at 37°C for 30 s. The resulting H_2S produced was measured using methylene blue formation at OD_{671} (35).

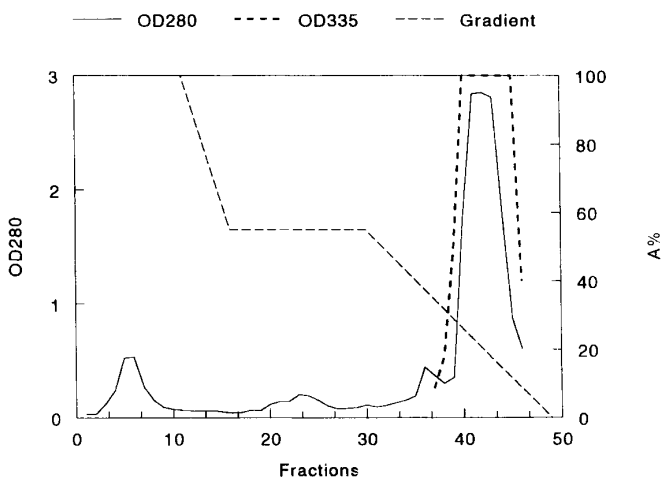

FIG. 3. Phenyl–Sepharose 6 FF chromatography. 500 ml of active fractions from the DEAE–Sepharose FF column containing 79.3 mg/ml ammonium sulfate were applied to a 20 × 2.6-cm column of phenyl–Sepharose 6 FF. The column was preequilibrated with 0.6 M ammonium sulfate in 20 mM potassium phosphate buffer, pH 7.6, containing 20 μm pyridoxal phosphate and 0.02% β-mercaptoethanol. The flow rate was 5.0 ml/min. The enzyme was eluted by decreasing the ammonium sulfate concentration from 100 to 0% using Buffer B containing 20 mM potassium phosphate, pH 7.6, 5% ethylene glycol, 10 μm pyridoxal phosphate, and 0.02% β-mercaptoethanol. The active fractions were combined, concentrated, and stored at −80°C.

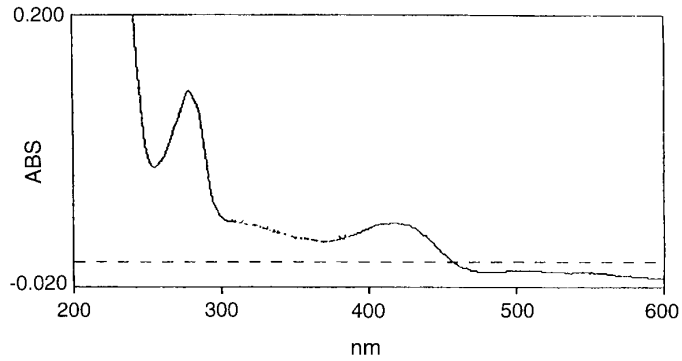

FIG. 4. Absorption spectrum of purified rHCYase. Purified enzyme was diluted in 20 mM potassium phosphate, pH 7.6, at a concentration of 75 μg/ml. The absorption spectrum was measured and recorded between 200 and 600 nm using 20 mM potassium phosphate, pH 7.6, buffer as the blank.

SDS–PAGE and PAGE

Electrophoresis was performed according to the methods described in the Novex kit (Novex Experimental Technology, San Diego, CA). A 12% Tris gel was used for SDS–PAGE. After staining with Coomassie brilliant blue, the intensity of protein bands was estimated with molecular standards (Novex Mark 12 wide range protein standard), including myosin, 200 kDa; β-galactosidase, 116.3 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66.3 kDa; carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; aprotinin, 6.0 kDa. Native gels were stained for rHCYase activity by immersion in a reaction mixture containing 3.3 mM-D,L-homocysteine, 0.33 mM lead acetate, 28.4 mM β-mercaptoethanol, and 100 mM Tris–HCl buffer, pH 7.5 (3,21). Bands containing homocysteinase activity will convert homocysteine to α-ketobutyrate, ammonia, and H_2S. Hydrogen sulfide reacts with lead acetate to form a dark brown precipitate (Pb_2S). The gels were then stained for protein with Coomassie blue.

Ultraviolet Absorption Spectrometry

To determine the UV spectrum of rHCYase, a diluted sample of protein in 20 mM potassium phosphate, pH 7.6, at a concentration of 75 μg/ml was made. The absorption spectrum between 200 and 600 nm was measured using 20 mM potassium phosphate, pH 7.6, buffer as the blank.

Effect of Temperature and pH on Purified rHCYase Activity

The enzyme was equilibrated at different temperatures for 30 min in 20 mM potassium phosphate, pH

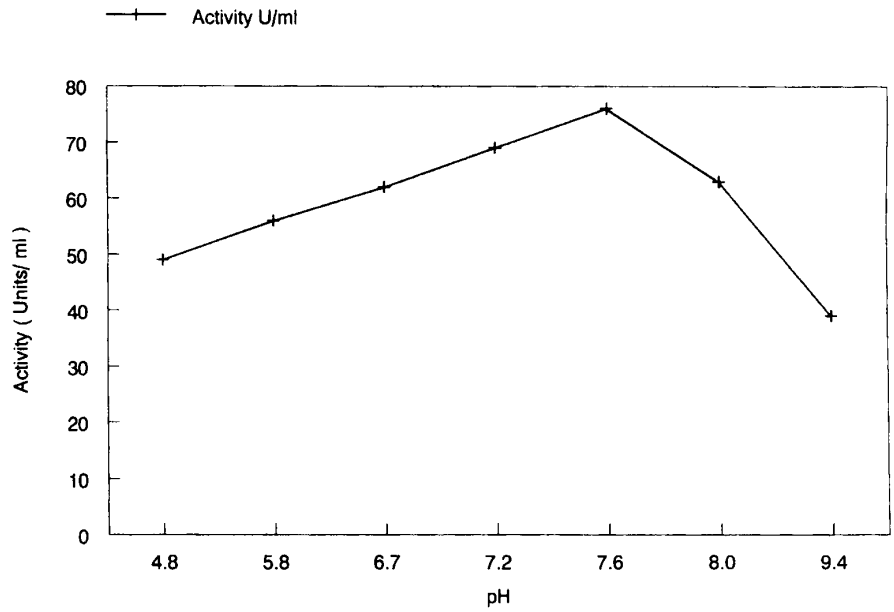

FIG. 5. Effect of pH on the activity of purified rHCYase. The purified enzyme was added to 50 mM potassium phosphate buffer at pHs 4.8 to 9.4 at 4°C for 6 h. The activity was then measured with the MBTH method with homocysteine at 20 mM as described under Materials and Methods.

7.6, 10 μM pyridoxal phosphate, and 0.02% β-mercaptoethanol. To determine the pH stability, the enzyme was incubated in the same buffer but at different pHs for 6 h at 0–4°C. Activity of rHCYase was measured as described above for the α-elimination reaction.

K_{cat} of rHCYase

The activity assays, for the α-elimination reaction, were carried out as described above in 1.0 ml of 100 mM phosphate buffer, pH 8.0, containing 10 μM pyridoxal phosphate and different concentrations (10 μM–1.6 mM) of D,L-homocysteine, methionine, and cysteine for 10 min at 37°C with 50 μl of enzyme (30 units/ml).

RESULTS AND DISCUSSION

Purification and Yield of Enzyme

rHCYase is highly expressed in *E. coli* as the major protein synthesized at approximately 5–10% of total protein (Fig. 1, Table 1). The genetic sequence (M. Lenz, Y. Tan, and R. Hoffman, manuscript in preparation) of rHCYase differs from α,γ-lyase thus far cloned from *P. putida* (16–18) and *T. vaginalis* (21).

The yield of rHCYase during the purification procedure which involved a precolumn heat step, three precipitation steps, DEAE–Sepharose FF chromatography, and phenyl–Sepharose 6 FF chromatography was 58%, as seen in Table 1. The specific activity of rHCYase for homocysteine increased from 1.8 U/mg protein in the crude extract to 31.5 U/mg protein after the phenyl–Sepharose column, a 17.5-fold purification. During the purification of the rHCYase, the enzyme solution was maintained in 20 mM phosphate buffer, pH 7.6, which contained 10 μM pyridoxal phosphate and 0.02% β-mercaptoethanol.

In the precolumn treatment, 20% ethanol was added to facilitate precipitation of contaminating proteins and also to decrease the viscosity of the homogenate. Heat treatment was used for denaturation and removal of contaminating proteins. A low concentration of polyethyleneimine (PEI) in the homogenate precipitated nucleic acids. PEG8000 was used to enrich rHCYase by precipitation of the relatively nonpolar contaminating proteins. These precolumn steps increased the specific activity from 1.8 to 8.2 U/mg protein. The advantage of PEG8000 is the shorter time required for the contaminating proteins to precipitate (36–38).

Column Chromatography

DEAE–Sepharose FF chromatography removed most of the contaminating proteins (Fig. 2). The remaining contaminating proteins were removed by phenyl–Sepharose 6 FF chromatography (Fig. 3). Phenyl–Sepharose removed relatively nonpolar contaminating proteins not removed by the DEAE–Sepharose.

SDS–PAGE and PAGE

A single major band of rHCYase was obtained with both SDS PAGE and native PAGE (Figs. 1A and 1B).

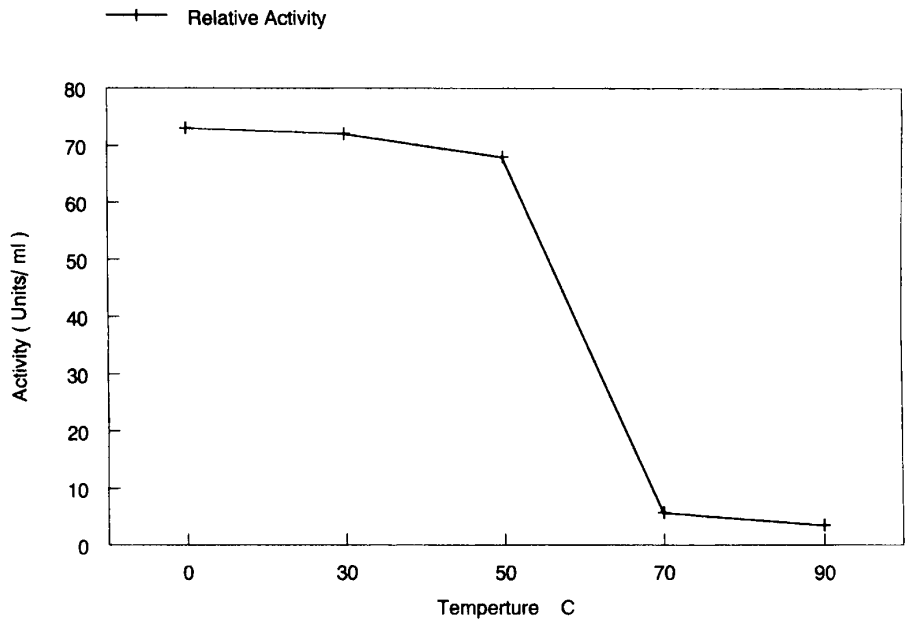

FIG. 6. Effect of temperature on rHCYase activity. The enzyme was equilibrated at different temperatures for 30 min in 20 mM potassium phosphate, pH 7.6, and containing 10 μM pyridoxal phosphate and 0.02 % β-mercaptoethanol. Activity of rHCYase was then measured using the MBTH method with homocysteine at 20 mM as described under Materials and Methods.

On SDS–PAGE, the major band could be observed at approximately 43 kDa. On native gels, one major band of MW 172 kDa was obtained after purification.

Ultraviolet–Visible Absorption Spectra

The absorption spectrum of the purified enzyme is shown in Fig. 4. The absorbance spectrum of the protein showed a maximum at 279 and 420 nm.

pH and Temperature Stability of rHCYase

The pH dependence of rHCYase is shown in Fig. 5. The optimum pH was found to be 7.8 and the enzyme was stable over a pH range of 6–8 at 4°C for 6 h. At pH 9.4, the enzyme retained 52% of its original activity.

The effect of temperature on enzyme activity is shown in Fig. 6. The enzyme was stable at 50°C for 1 min, but unstable at temperatures greater than 50°C.

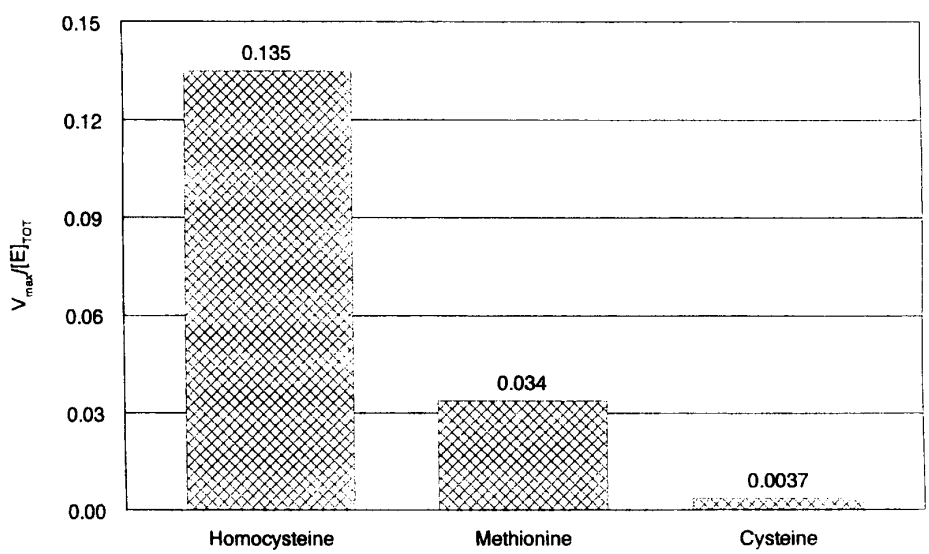

FIG. 7. K_{cat} of rHCYase for various substrates. The assay was carried out with different concentrations (10 μM–1.6 mM) of D,L-homocysteine, L-methionine, or L-cysteine, for 10 min at 37°C with 50 μl of rHCYase (30 units/ml). The amount of reaction product was determined using MBTH as described under Materials and Methods. $V_{max}/(E)_{TOT}$ was calculated.

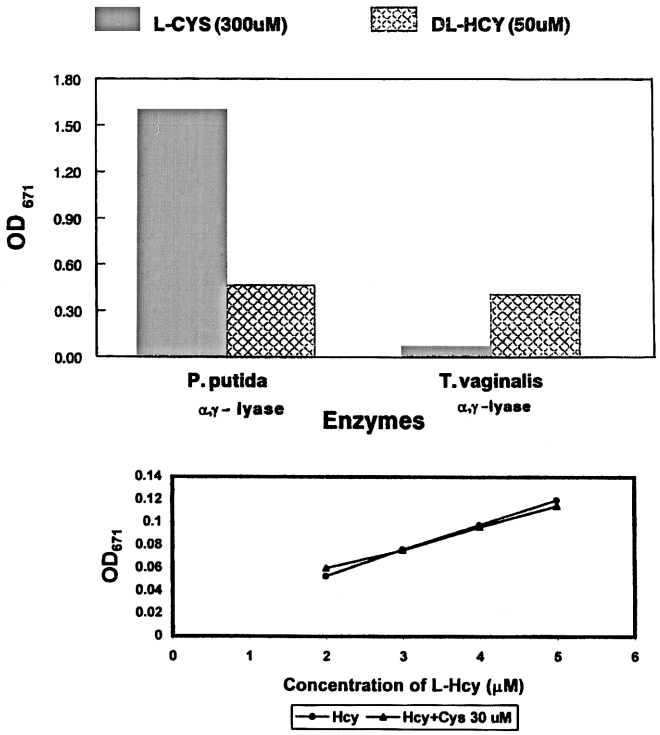

FIG. 8. (A) Comparison of lyases from *P. putida* and *T. vaginalis.* Activity assay for the γ- and β-elimination reactions of homocysteine and cysteine, respectively, were carried out at 37°C for 30 s using purified recombinant *P. putida* and *T. vaginalis* α,γ-lyase as described under Materials and Methods. The resulting H_2S produced was determined using methylene blue formation measured at OD_{671} (35). (B) Specificity of rHCYase: effect of cysteine on reaction of rHCYase with homocysteine. Cysteine (30 μM) had no effect on the reaction of rHCYase with 2–5 μM homocysteine. H_2S was measured as in A.

K_{cat} of rHCYase

The K_{cat} of the rHCYase for D,L-homocysteine as substrate compared to methionine and cysteine is shown in Fig. 7. The apparent K_{cat} value of rHCYase is 0.135 for D,L-homocysteine, 0.034 for methionine, and 0.0037 for cysteine at 37°C, pH 8.0.

Specificity for Homocysteine vs Cysteine of T. vaginalis and P. putida Lyases

Under conditions of 300 μM cysteine and 50 μM homocysteine, the *P. putida* α,γ-lyase was three times more active on cysteine than homocysteine in striking contrast to the *T. vaginalis* α,γ-lyase which had only borderline activity on cysteine but high activity on homocysteine (Fig. 8A).

Figure 8B demonstrates that 30 μM cysteine does not interfere with the *T. vaginalis* α,γ-lyase when it is incubated with 2–5 μM homocysteine in a 30-s assay (Fig. 8B).

Comparison of Elution Patterns of T. vaginalis and P. putida Lyases on Phenyl–Sepharose FF

A mixture of purified rHCYase and the *P. putida* α,γ-lyase was applied to a phenyl–Sepharose 6 FF column. The *T. vaginalis* α,γ-lyase eluted earlier than the *P. putida* α,γ-lyase indicating that the *T. vaginalis* α,γ-lyase was more hydrophilic.

Comparison of rHCYase with Nonrecombinant HCYase from T. vaginalis

Coombs *et al.* (3) purified methioninase γ-lyase from *T. vaginalis* using ion-exchange chromatography (MonoQ), hydrophobic interaction chromatography (Alkyl–Superose), and gel filtration (Superose 12). The protocol was small-scale level, the final yield was only 8.8%, and the enzyme was unstable. The disadvantage of that purification procedure was that it involved many steps with very low yield and it is thus not suitable for scale-up manufacturing of rHCYase. In contrast, the simpler protocols developed by us allow the production of rHCYase at the multigram level per batch with high yield (>50%), high purity (>90%), and high stability.

Conclusion

High recombinant expression in *E. coli*, a simple and effective high-yield purification procedure, and high specificity for homocysteine with respect to cysteine make rHCYase a promising candidate to use for the diagnosis of hyperhomocystenemia, which has been demonstrated to be a major risk factor for the onset and mortality of cardiovascular disease of all types.

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