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ORIGINAL ARTICLE

Preparation of optically active β-hydroxy-α-amino acid by immobilized *Escherichia coli* cells with serine hydroxymethyl transferase activity

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Abstract In this research, an improved method for preparation of optically pure β-hydroxy-α-amino acids, catalyzed by serine hydroxymethyl transferase with threonine aldolase activity, is reported. Using recombinant serine hydroxymethyl transferase (SHMT), an enzymatic resolution process was established. A series of new substrates, β-phenylserine, β-(nitrophenyl) serine and β-(methylsulfonylphenyl) serine were used in the resolution process catalyzed by immobilized *Escherichia coli* cells with SHMT activity. It was observed that the K_m for L-threonine was 28-fold higher than that for L-*allo*-threonine, suggesting that this enzyme can be classified as a low-specificity L-*allo*-threonine aldolase. The results also shows that SHMT activity with β-phenylserine as substrate was about 1.48-fold and 1.25-fold higher than that with β-(methylsulfonylphenyl) serine and β-(nitrophenyl) serine as substrate, respectively. Reaction conditions were optimized by using 200 mmol/l β-hydroxy-α-amino acid, and 0.1 g/ml of immobilized SHMT cells at pH 7.5 and 45°C. Under these conditions, the immobilized cells were continuously used 10 times, yielding an average conversion rate of 60.4%. Bead activity did not change significantly the first five times they were used, and the average conversion rate during the first five instances was 84.1%. The immobilized cells exhibited favourable operational stability.

Keywords D-*threo*-Amino acid · Enzymatic resolution · Immobilized cells · Serine hydroxymethyl transferase

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Introduction

With the steady growth of the importance of enantiomerically pure or enriched compounds in pharmaceuticals, agrochemicals and food additives, the so-called "chiral market" has become an expanding area of the fine chemicals industry (Reetz 2001). L-*threo*-β-(3, 4-Dihydroxyphenyl) serine, for example, is a special remedy for Parkinson's disease (Maruyama et al. 1996), L-*threo*-β-(4-methylthiophenyl) serine is an intermediate for the production of antibiotics, florfenicol and thiamphenicol (Apley 1997a,b), 4-hydroxy-L-threonine is a precursor of rizobitoxine, and 3, 4, 5-trihydroxy-L-aminopentanoic acid is a key component of polyoxins (Vassilev et al. 1995). D-Isomers are also biologically significant, because they not only exist in mature mammals (Fukushima et al. 1995) but are also constituents of a range of antibiotics, for example, Fusaricidin (Kajimura and Kaneda 1996) and Viscosin (Burke et al. 1989). Concerning the field of asymmetric synthesis of β-hydroxy-α-amino acids, Hayashi and Belokon carried out a series of fundamental and creatively research (Soloshonok et al. 1992, 1993), for example, they have investigated asymmetric aldol reactions of isocyanoacetic derivatives with fluoroaryl aldehydes, benzaldehydes and aryl ketones catalyzed by gold (I) or silver(I)/triethylamine (Soloshonok and Hayashi 1994a, b; Soloshonok et al. 1994c, 1996b). Soloshonok et al. (1996a, 1997) have also researched the aldol reactions of methyl isocyanoacetate with a wide range of prochiral ketones catalyzed by transition metal/base, giving rise to a straightforward approach to stereochemically defined β,β-dissubstituted-β-hydroxy-α-amino acids.

In addition to asymmetric systhesis, β-hydroxy-α-amino acids (except L-threonine and L-serine) are mainly produced through chemical synthesis processes, and then chemical resolution (Burke et al. 1989). But these

processes have some problems, such as chemical resolution is lengthy and inefficient, need protection and deprotection of the amino groups of the amino acids before and after the resolution process and the overuse of organic solvents results in environmental problems. Accordingly, the development of an efficient and clean enzymatic resolution process is desirable.

Extensive studies have been carried out on the biotransformation activity of serine hydroxymethyl transferase (SHMT) from different species. SHMT catalyzes the cleavage of several C^3-OH amino acids varying in substituent and stereochemistry at C^3, with most research focusing on threonine and β-phenylserine (Schirch and Gross 1968; Ulevitch and Kallen 1977). The SHMT extracted from *H. methylovorum*, *Escherichia coli* respectively, was found to have a wider substrate specificity. As for the degradation of β-hydroxy-α-amino acids, *β-threo*-phenylserine, L-serine, allothreonine, *threo*-3,4-dihydroxy-phenylserine and L-threonine were good substrates. However, D-forms of serine and threonine were not substrates of this enzyme (Miyazaki et al. 1987; Schirch et al. 1985). SHMT also showed potential as a biocatalyst for the stereoselective synthesis of β-hydroxy-α-amino acids. *Streptococcus thermophilus* YKA-184 containing SHMT catalyzes aldol addition reactions with non-natural aldehydes, such as benzyloxyacetaldehyde and (R)-N-Cbz-alaninal to corresponding β-hydroxy-α-amino acid diastereoisomers (Vidal et al. 2005). *E. coli* harbouring serine hydroxymethyl transferase catalyzes diastereospecific formation of L-*allo*-threonine (Makart et al. 2007). So, enzymatic kinetic resolution gradually plays key roles in industrial biotransformations for production of enantiomerically pure compounds from racemic precursors (Straathof et al. 2002).

It is well known that an immobilized cell system can increase cell concentration, enhance bead stability, and allow easier separation for reuse. Therefore, the immobilized cell system is a desirable technique in enzymatic resolution. In the present research, we used SHMT from recombinant *E. coli* to product some important optically pure β-hydroxy-α-amino acids, and optimized the conditions for enzymatic resolution from some novel substrates, such as β-(nitrophenyl) serine, β-(methlsulfonylphenyl) serine. Key kinetics constants and other factors such as substrate specificity, pH, temperature, and substrate mole concentration were investigated.

Materials and methods

Chemicals

Isopropyl-β-D-thiogalactoside (IPTG), L-threonine, L-*allo*-threonine were from Sigma (St. Louis, MO., USA). DL-*threo*-β-phenylserine, DL-*threo*-β-(nitrophenyl) serine, DL-*threo*-β-(methylsulfonylphenyl) serine were synthesized in our laboratory by previously described procedures (Koch et al. 1985; Shiraiwa et al. 2003). All other chemicals and reagents used in this work were of analytical grade.

Microorganisms

The gene encoding serine hydroxymethyl transferase, *gly*A, was cloned from *E. coli* K-12 MG1655. The *E. coli* strain BL21 (DE3) carrying the recombinant plasmid pET-28a-*gly*A, was constructed in our laboratory. The strain was cultured in LB broth containing antibiotic and induced with 0.4 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C.

Immobilization of cells

A modified method from Birgisson et al. (2007) was used for cell immobilization. To make a final concentration of 3% alginate (w/v), 5 g cells (wet) were mixed with 100 ml sodium alginate water solution. After stirring the components together, the mixture was added into 500 ml of 2% (w/v) $CaCl_2$ solution using a syringe to drip the mixture from a 10 cm height. The average diameter of the resulting beads was about 3 mm. Prior to use, the beads were allowed to harden for 2 h at 4°C without agitation.

SHMT activity assays

By measuring the glycine released from β-hydroxy-α-amino acid, SHMT activity was determined with amino acid analyzer. The reaction mixture (final volume, 5 ml) containing 0.20 mM β-hydroxy-α-amino acid, 5 mM Tris−HC1 (pH 7.0), 20 μmol of PLP, and enzymes (i.e. free or immobilized cells) was incubated at 37°C for 10 min. The reaction was discontinued by adding 1.5 M hydrochloride, and the amount of liberated glycine was determined from aminoacid analyzer. One enzyme unit (U) was defined as the amount of free or immobilized cells that released 1 μmol glycine per min from β-hydroxy-α-amino acid at 37°C. The specific activity is defined as units/g of free or immobilized cells.

Analytical methods

Enantiomeric compositions of residual substrate were determined as described by Zheng (2007). Enantiomeric excess (*ee*) was defined as $(A_1 - A_2)/(A_1 + A_2) \times 100$, where A_1 and A_2 are peak areas of the enantiomers, and A_1 was the larger peak area. ^{1}HNMR spectra was recorded on a Bruker Avance-400.

Table 1 Steady-state kinetics constants for the SHMT from *E.coli* K-12

Substrate	Relative activity (%)	V_{max} (μmol/min·per mg)	K_m (mM)	V_{max}/K_m
L-threonine	100[a]	15.1 ± 0.17	43.1 ± 3.31	0.3 ± 0.01
L-*allo*-threonine	291	82.8 ± 4.47	1.5 ± 0.01	55.2 ± 3.35
DL-*threo*-β-phenylserine	180	44.3 ± 3.36	19.2 ± 1.13	2.3 ± 0.07
DL-*threo*-β-(nitrophenyl) serine	143	32.4 ± 3.23	21.5 ± 2.16	1.5 ± 0.01
DL-*threo*-β-(methylsulfonylphenyl)serine	121	21.3 ± 2.15	23.6 ± 2.19	0.9 ± 0.03

[a] Activity relative to enzyme activity towards L-threonine. The reaction was carried out in a 10 ml mixture containing 50 mmol/l β-hydroxy-α-amino acid, and 1 g immobilized SHMT cells at pH 7.0 and 37°C. A gram of immobilized cells contains 600 units of enzymes

Table 2 Changes of enantiomeric excess of product (ee_P) in enantioselective SHMT-catalyzed kinetic resolution progress

Substrate	Conversion (%), with time (h) in parentheses	ee_P
DL-*threo*-β-phenylserine	97 (8)	98.2%
DL-*threo*-β-(nitrophenyl) serine	95 (10)	97.5%
DL-*threo*-β-(methylsulfonylphenyl) serine	93 (12)	97.9%

Results

SHMT substrate specificity

To investigate SHMT substrate specificity, five different serine derivatives compounds L-threonine, L-*allo*-threonine. DL-*threo*-β-phenylserine, DL-*threo*-β-(nitrophenyl) serine, DL-*threo*-β-(methlsulfonylphenyl) serine were used as substrates in the enzymatic resolution catalyzed by SHMT. As shown in Table 1, all of these compounds could serve as substrate of SHMT, whose activity was determined, as described in "Materials and methods". The values of the kinetic constants are averages ± SD of three different determinations. Table 2 shows the enantiomeric excess of product, calculated from the peak areas, as described in analytical methods.

pH and temperature effect

Enzyme activity can be affected by environmental factors such as pH and temperature. In this study, using DL-*threo*-β-(methylsulfonylphenyl) serine as substrate, a pH range from 4 to 11 was used to determine the optimal initial pH. As shown in Fig. 1, with the initial pH increasing from 4 to 7.5, SHMT activity increased significantly and reached its maximum at pH 7.5. An activity decrease was observed at pH 8. The optimal temperature for the reaction was subsequently optimized.

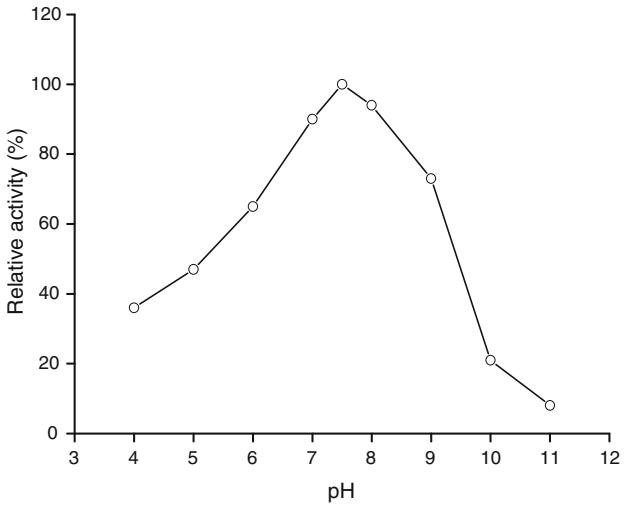


Fig. 1 Effect of pH on SHMT activity. The reaction was carried out in a 10 ml mixture containing 0.5 mM DL-*threo*-β-(methylsulfonylphenyl) serine, and 1 g immobilized SHMT cells. The mixture was kept in a plastic tube at 37°C as it was being shaken. Enzymic activity was measured after 20 min. The highest relative activity of 100% denoted 2.1 mmol/l of D-*threo*-β- (methylsulfonylphenyl) serine

When assayed at pH 7.5, the optimal temperature for the reaction was about 45°C (Fig. 2).

Substrate concentration effect

The substrate concentration effect on the conversion rate from DL-*threo*-β-(methylsulfonylphenyl) serine to D-*threo*-β-(methylsulfonylphenyl) serine was studied. The conversion rate time course under different substrate concentrations is shown in Fig. 3. The results indicate that with a substrate concentration increase, the conversion rate from DL-*threo* to D-*threo* increased. Reaction inhibition was observed at a high substrate concentration. Taking production efficiency into consideration, the optimal substrate concentration would be 200 mmol/l. The optimal reaction time was prolonged with substrate concentration increase. When the DL-*threo*-β-(methylsulfonylphenyl) serine concentration was 200 mmol/l, the optimal reaction time was 14 h.

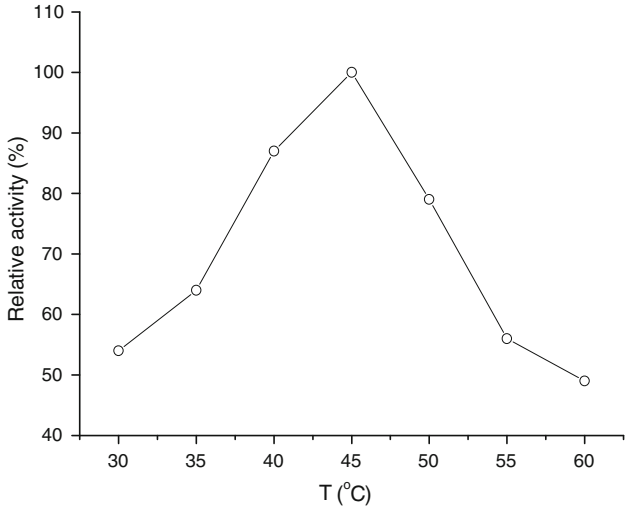


Fig. 2 Effect of temperature on SHMT activity. The mixture components were the same as those mentioned in Fig. 1. The mixture was kept in a plastic tube at pH 7.5 as it was being shaken. Enzymic activity was measured after 20 min. The highest relative activity of 100% denoted 3.3 mmol/l of D-*threo*-*β*-(methylsulfonylphenyl) serine

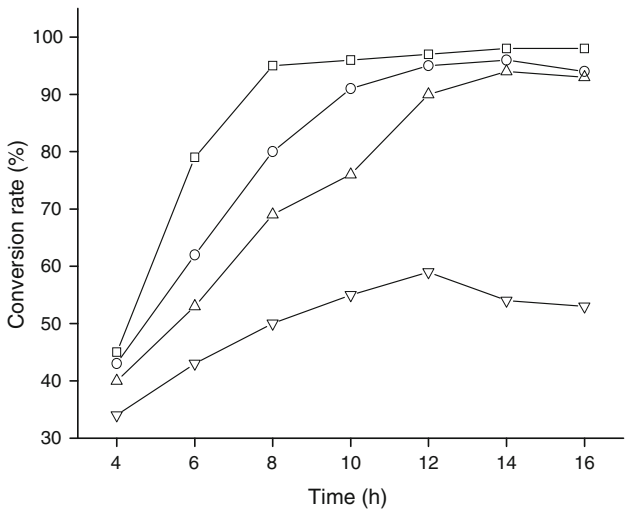


Fig. 3 Conversion rate time course of DL-*threo*-*β*-(methylsulfonylphenyl) serine. The reaction was carried out in a 10 ml mixture containing 1 g immobilized SHMT cells at pH 7.5. The DL-*threo*-*β*-(methylsulfonylphenyl) serine concentrations were 50 mmol/l (□), 100 mmol/l (○), 200 mmol/l (Δ), and 400 mmol/l (▽) respectively. The mixture was kept at 45°C as it was being shaken

Operational stability of immobilized cells

In evaluating immobilized cell stability, the beads were continuously used ten times. As shown in Fig. 4, bead activity did not change significantly during the first five times the beads were used, and the average conversion rate from DL-*threo*-*β*-(methylsulfonylphenyl) serine to D-*threo*-*β*-(methylsulfonylphenyl) serine during these times was

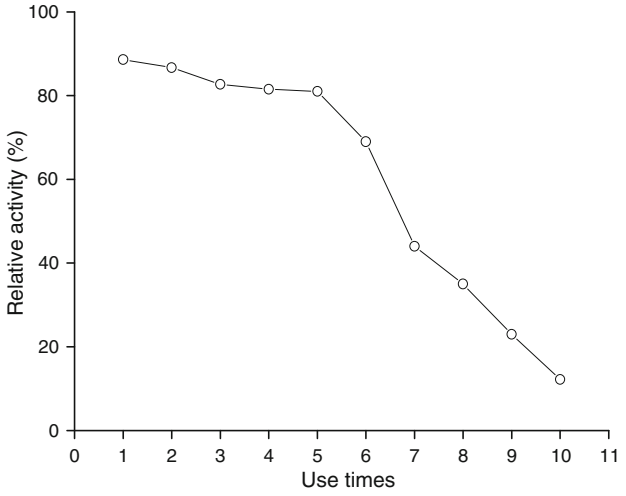


Fig. 4 Operational stability of the immobilized cells. The reaction was carried out in a 10 ml mixture containing 200 mmol/l DL-*threo*-*β*-(methylsulfonylphenyl)serine, and 1 g immobilized cells at pH 7.5. The mixture was kept at 45°C as it was being shaken. D-*threo*-*β*-(methlsulfonylphenyl) serine concentrations were measured after 14 h

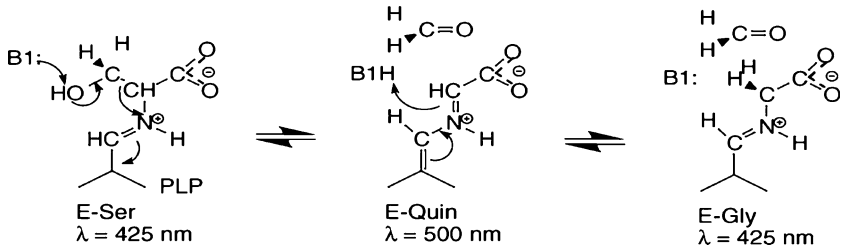
84.1%. When the beads were used more than five times, a significant enzyme activity degradation was observed. Upon tenth usage, the conversion rate reduced to 12.2%. The average conversion rate during the ten-time usage was 60.4%. The immobilized cells exhibited favourable operational stability.

Isolation and identification of D-threo-*β*-(methlsulfonylphenyl) serine

The reaction mixture (100 ml) containing 96.8 mmol/l D-*threo*-*β*-(methlsulfonylphenyl) serine was concentrated, crystallized from 80% methanol and dried in vacuo for 2 h to give 1.98 g (78.9%) of crystals. Above crystals were dissolved with water and applied to a silica gel column (4.5 × 20 cm) (silica 60, Kanto Chemical Co., Tokyo, Japan), which was previously equilibrated with a solution comprising 69% (v/v) butanol, 17% ethanol, and 14% water. Elution was carried out with the same solvent the fractions containing D-*threo*-*β*-(methlsulfonylphenyl) were collected and lyophilized to give 1.35 g (68.1%) of crystals. *mp* 194−197°C (decomp), $[\alpha]_D^{20}$ 56.5° ($c = 0.500$, 5 mol/l HCl). ^{1}H NMR (200 MHz, D_2O): δ 3.18 (s, 3H), 3.85 (d, 1H), 5.32 (d, 1H), 7.59 (m, 2H), 7.90 (m, 2H). *Anal*. Calcd for $C_{10}H_{13}NO_5S$: C, 46.32; H, 5.05; N, 5.40. Found: C, 46.17; H, 5.01; N, 5.38.

Discussion

During the past two decades, the understanding of the evolutionary relationships amongst vitamin B6-dependent



Scheme 1 Proposed retroaldol mechanism for conversion of C^3-OH amino acids to an aldehyde and glycine (serine is used as an example). Proposed mechanisms for reactions catalyzed by SHMT with acid–base groups labeled as B: (base) and BH (acid)

enzymes and of the structural basis of their mechanistic diversity and uniformity have increased markedly (Hayashi 1995; Mehta and Christen 2000). One member of this family, serine hydroxylmethyl transferase (SHMT), whose crystallographic structure has been determined from several sources (Renwick et al. 1998; Scarsdale et al. 1999, 2000; Szebenyi et al. 2000), shows a particularly broad reaction specificity. In vitro and in the absence of tetrahydropteroyl-glutamate (H_4PteGlu), SHMT catalyzes decarboxylation, transamination, retroaldol cleavage and racemization reactions, at rates sometimes approaching and even exceeding those of the physiological reaction (Shostak and Schirch 1988).

SHMT catalyzes the cleavage of several C^3-OH amino acids varying in substituent and stereochemistry at C^3, with most research focusing on threonine and β-phenylserine. None of these reactions requires H_4PteGlu as a co-substrate, and the rates approach or exceed the rate of H4PteGlu- dependent serine cleavage (Matthews and Drummond 1990). The stereochemistry at C^3 is relaxed; cleavage occurs of both *threo* and *erythro* diastereomers, but with the *erythro* isomer being favored over the *threo* (allothreonine over threonine) (Schirch and Gross 1968; Ulevitch and Kallen 1977), which agreed with the results of a this report. How the active site could accommodate large substituents on C^3 in both the *erythro* and *threo* positions was puzzling. The answer came from structural studies showing a hydrophobic cavity at the active site that can accept C^3 substituents in either configuration (Scarsdale et al. 1999; Szebenyi et al. 2004). Binding the phenyl group of *erythro*-β-phenylserine into this cavity makes the C^3-OH group synperiplanar, while fitting the corresponding group of the *threo* species into the cavity requires an antiperiplanar hydroxyl group. The retroaldol mechanism is shown in Scheme 1.

The speed of retroaldol reaction, catalyzed by SHMT is significantly affected by pH. Generally, a high pH inhibits reaction. In our study's result, an acceleration of reaction was observed with pH increase. The SHMT activity reached its maximum at pH 7.5. For improving the yield of D-*threo* configuration, degrade production, glycine plus the corresponding aldehydes, was recycled for synthesis of DL-*threo*-β-hydroxy-α-amino acid.

We characterized *gly* A, a vitamin B6-dependent aldolase from *E. coli* with respect to the application in biocatalysis processes, and immobilized *E. coli* cells with SHMT activity was used as catalysts. The enzyme exhibits excellent diastereoselectivity for the formation of D-*threo*-amino acids under process conditions. The results demonstrate an improvement in enzymatic resolution and display vast potential in industrialization. We only investigated cleavage reaction of five C^3-OH amino acids in this paper, In fact, C^3 substituents, such as methylphenyl, methoxyphenyl, ethoxyphenyl, halogenophenyl, hydroxyphenyl, acetylphenyl, cyanophenyl, biphenylyl and sulfamoylphenyl, tert-butyl or a heteroaromatic mono- to bi-cyclic radical of the group of pyrrolyl, thienyl, furyl, pyridinyl, pyrazinyl, quinolinyl, isoquinolinyl and phthalazinyl, may be also as substrates of SHMT. Some of them have important research and application value.

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