Enzymatic synthesis of chiral amino acid sulfoxides by Fe(II)/α-ketoglutarate-dependent dioxygenase

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Abstract

Asymmetric sulfoxidation of sulfur-containing L-amino acid was successfully achieved through bioconversion using IDO, which is an Fe(II)/α-ketoglutarate-dependent dioxygenase previously found in *Bacillus thuringiensis* strain 2e2. IDO catalyzed sulfoxidation of L-methionine, L-ethionine, *S*-methyl-L-cysteine, *S*-ethyl-L-cysteine, and *S*-allyl-L-cysteine into corresponding (*S*)-configured sulfoxides such as (+)-methiin and (+)-alliin, which are responsible for valuable physiological activities in mammals, and have high stereoselectivity. Here, we established an effective preparative laboratory scale production method to obtain optically pure chiral sulfoxides using an IDO biocatalyst.

1. **Introduction**

Chiral sulfoxides are noteworthy reagents in a wide range of industrial fields. They have been used as chiral building blocks and chiral auxiliaries in the organic synthesis of other optically active compounds.[1] In the medicinal and pharmaceutical industry, chiral sulfoxide derivatives are of special importance because of their high biological activity.[2] Also, chiral sulfinyl groups occur naturally in some functionalized amino acids, including L-methionine (*S*)-sulfoxide [(+)-MetSO] in the methionine metabolism of mammals[3] and L-cysteine-related sulfoxides found in *Allium* and *Brassica* species.[4,5] In particular, *S*-methyl-L-cysteine (*S*)-sulfoxide [(+)-methiin] and *S*-allyl-L-cysteine (*S*)-sulfoxide [(+)-alliin] are promising materials for use in functional foods or drugs since they have antibiotic,[6] antioxidant,[7,8] anti-inflamatory,[9] anti-diabetic,[10,11] anti-Alzheimer's,[12] and anti-cholesterolemic[13] effects. Also, *S*-ethyl-L-cysteine (*S*)-sulfoxide[(+)-ethiin] is known to be a minor flavor precursor in *Allium* species.[14]

To date, many production methods for chiral sulfoxides have been established using chemical and biological catalysts.[15] In particular, biocatalytic oxidation of prochiral sulfides is an advantageous method because of the highly stereoselective and chemoselective properties of oxidizing enzymes such as monooxygenases, dioxygenases, and peroxidases.[16-18] A cyclohexanone monooxygenase of *Acinetobacter calcoaceticus*[19] and flavin-containing monooxygenases (FMOs) in mammalian microsomes[20-22] are known to promote sulfoxidation activity in sulfur-containing amino acids.

Previously, we found that an Fe(II)/α-ketoglutarate-dependent dioxygenase (IDO) from *Bacillus thuringiensis* strain 2e2 catalyzed hydroxylation of L-isoleucine into (2*S*,3*R*,4*S*)-4-hydroxyisoleucine.[23,24] In addition to stereoselective hydroxylation, IDO also catalyzed stereoselective sulfoxidation of L-methionine and L-ethionine.[25]

In this study, we also found that IDO catalyzed asymmetric oxidation of several

other sulfur-containing L-amino acids with high yield. Furthermore, we demonstrated dried microbial cells containing IDO (IDO-catalyst) was useful to apply to the preparative laboratory scale production of valuable chiral sulfoxides such as (+)-MetSO, (+)-methiin, (+)-ethiin, and (+)-alliin.

2. Results and Discussion

2.1. Conversion of sulfur-containing L-amino acids

We investigated the IDO-catalyzed oxidation of multiple sulfur-containing L-amino acids (Table 1). The dried cell powder of IDO-expressing *E. coli* (IDO-catalyst) was used as a biocatalyst for oxidation. IDO-catalyst converted several *S*-alkyl-L-homocysteins like L-methionine (Met) and L-ethionine (Eth) and several *S*-alkyl-L-cysteines, such as *S*-methyl-L-cysteine (SMCys), *S*-ethyl-L-cysteine (SECys), and *S*-allyl-L-cysteine (SACys), of which Met, SMCys, SECys, and SACys were better substrates of IDO-catalyst (complete conversion of 10 mM substrate in most cases). IDO-catalyst conversion of Eth, which had a longer alkyl chain than Met, was somewhat less (67% conversion). IDO-catalyst showed 0.064 μmol/min/mg (U/mg) of specific activity when Met was used as a substrate. On the other hand, the purified IDO enzyme, which was prepared as previously reported,[25] showed 0.30 U/mg of the specific activity to Met. From these results, it was estimated that IDO-catalyst contained approximately 20% (w/w) of active form of IDO enzyme.

The product of IDO-catalyst in the reaction mixture with Met was analyzed with the 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA) method for HPLC analysis and compared with the elution order of (±)-MetSO reported previously.[26] The product was then determined to be L-methionine (*S*)-sulfoxide [(+)-MetSO], as shown in Figure 1a and had >99% diastereomer excess (*de*). Similarly, the product with Eth was analyzed using the FDAA method and determined to have >99% *de*. According to the published data on L-ethionine sulfoxides in optical rotation and NMR measurements,[27] the reaction product was determined to be L-ethionine (*S*)-sulfoxide

[(+)-EthSO], as shown in Figure 1a. In order to identify the products of IDO-catalyst in the reaction mixtures with *S*-alkyl-L-cysteines such as SMCys, SECys, and SACys, they were analyzed with dansylchloride (Dns-Cl) methods for HPLC analysis and compared with the elution orders of (±)-methiin, (±)-ethiin, and (±)-alliin reported previously[28]. IDO-catalyst was thereby explained to convert SMCys, SECys, and SACys into *S*-methyl-L-cysteine (*S*)-sulfoxide [(+)-methiin], *S*-ethyl-L-cysteine (*S*)-sulfoxide [(+)-ethiin], and *S*-allyl-L-cysteine (*S*)-sulfoxide [(+)-alliin], respectively (Figure 1b). Thus, IDO-catalyst catalyzed (*S*)-specific sulfoxidation of various sulfur-containing L-amino acids. The stereoselectivity in the formations of these *S*-alkyl-L-cysteine sulfoxides was 88% *de*, 91% *de*, and 83% *de*, respectively. These *de* values were lower than those of *S*-alkyl-L-homocysteine sulfoxides. These differences in *de* values are caused by the relative position of the sulfur atom in amino acid molecules. IDO-catalyzed oxidation proceeded more stereospecifically using amino acids with a sulfur atom at the δ-position than those at the γ-position. It was inconsistent with our previous observation that IDO catalyzed absolutely stereoselective γ-hydroxylation of some L-amino acids.[25]

2.2. Effects of pH and temperature on activities and stabilities of IDO-catalyst

Thus, IDO-catalyst is a good biocatalyst for asymmetric synthesis of chiral sulfoxides. In order to identify the best conditions for the biocatalysis, the pH and temperature profiles of the IDO-catalyst were determined using the (+)-alliin as a representative product (Figure 2). IDO-catalyst showed a pH optimum between 6.0 and 7.0, and retained over 90% activity after 1 h-incubation at pH 7.0 to 9.0 (Figure 2a). Also, IDO-catalyst showed a temperature optimum at 28°C, and retained over 80%

activity after 1 h-incubation at 20 to 28°C (Figure 2b). From these results, the best conditions for the bioconversion were set at pH 7.0 and 28°C. So, it was found that IDO catalyzed two different oxidations of amino acids, sulfoxidation and hydroxylation, under the similar reaction conditions.[23]

2.3. Preparative laboratory scale productions of chiral amino acid sulfoxides

The preparative laboratory scale productions of (+)-MetSO, (+)-methiin, (+)-ethiin, and (+)-alliin from L-amino acids were carried out under optimal conditions. As shown in Figure 3, each product was formed from 50 mM of substrate for 4 h, resulting in an almost stoichiometric conversion of L-amino acid substrates to products. Among them, Met was the best substrate of IDO-catalyst and 47 mM of (+)-MetSO was produced from 50 mM of Met (94% yield with >99% *de*) in the 4-h reaction (Table 2). Estimated total turnover number of IDO enzyme included in IDO-catalyst used was 1,342 during this conversion. Also, SMCys and SECys were linearly converted into (+)-methiin and (+)-ethiin, and then the product amount reached 32 mM (64% yield with 93% *de*) and 43 mM (86% yield with 95% *de*) in the reaction, respectively. In particular, (+)-ethiin content is very low in the tissue of *Allium* species[29] and its own function has not been defined yet. It was expected that (+)-ethiin also have valuable physiological properties as well as (+)-methiin and (+)-alliin. In contrast, the conversion of SACys proceeded linearly until 2 h but weakened after 2 h, and then only 35 mM of (+)-alliin was produced (70% yield with 81% *de*). A high concentration of (+)-alliin (about >30 mM) may have inhibited the sulfoxidation activity of IDO-catalyst.

3. Conclusion

Asymmetric oxidation of sulfur-containing L-amino acid with IDO-catalyst proved a very efficient way to produce various chiral amino acid sulfoxides, including (+)-methiin and (+)-alliin, which show noteworthy physiological activities in mammals. The dried cell powder of IDO-expressing *E. coli* was a handy biocatalyst because it was sufficiently stable to sustain the oxidation activity for several hours. With our method established in this study, preparative laboratory scale productions of chiral sulfoxides with high optical purity could easily be provided through bioconversion, allowing further to development of their industrial applications.

4. Experimental Section

4.1. General Information

All reagents and solvents were as obtained by commercial source. Reactions were monitored by thin-layer chromatography (TLC) which was performed with Kieselgel 60 F_{254} plates (Merck, NJ, USA) and visualized using ninhydrin staining. ^{1}H NMR and ^{13}C NMR were recorded on an Avance 500 (BrukerBioSpin, MA, USA). D_2O solutions were used for ^{1}H NMR analysis. D_2O solutions containing acetone as an internal standard were used for ^{13}C NMR analysis. Chemical shifts are reported in parts per million (ppm, δ) relative to residual solvent signals (H_2O, δ 4.79) or relative to acetone (δ 30.89). Coupling constants (*J* values) are given in Hz, and peak multiplicities are denoted by s (singlet), d (doublet), dd (doublet of doublets), m (multiplet), and t (triplet). Optical rotation was measured with a JASCO DIP-370 spectrometer.

4.2. Chemicals

(±)-MetSO was purchased from Sigma-Aldrich (St. Louis, USA), and (±)-alliin and (±)-methiin were purchased from LKT Laboratories, Inc. (St. Paul, USA). In order to obtain (±)-EthSO and (±)-ethiin, Eth and SECys were oxidized in 30% (w/w) hydrogen peroxide for 24 h and hand dried under reduced pressure, respectively.

4.3. Recombinant Bacterial Strain and Preparation of IDO-Catalyst

Escherichia coli Rosetta 2 (DE3) carrying pET-IDO (2e2) was used for enzyme expression.[25] Recombinant *E. coli* cells were grown aerobically at 37°C and 300 rpm in 250 mL LB medium with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). At an OD_{600} =1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the cultures were incubated for 16 h at 28°C and 300 rpm. The cells were harvested by centrifugation (8000 × *g*, 30 min, 4˚C) and washed in physiological saline twice. The cell pellet was air-dried, dehydrated using silica gel, and pounded in a mortar to obtain dried cell powder as IDO-catalyst.

4.4. Derivatization and HPLC Assay

Amino acids were analyzed by HPLC after derivatization with 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA)[26] or dansylchloride (Dns-Cl).[28] For the FDAA method, the derivatives were prepared by mixing 30 μl of the sample solution, 50 μl of 1% FDAA in acetone, and 10 μl of 1 M sodium bicarbonate. The reaction mixture was heated at 40°C for 1 h, and then 5 μl of 2 M HCl was added. The derivatized products were analyzed by HPLC. HPLC analysis was performed with a Waters Alliance 2695 HPLC System (Waters, Milford, USA) equipped with a Beckman

Ultrasphere ODS column (5 μm, 250 × 4.6 mm, HiChrom, Reading, UK) and maintained at 40°C. For analysis, 50 mM triethylamine phosphate (solvent A) and acetonitrile (solvent B) were used as the mobile phase, with a flow rate of 1 ml/min, the gradient of solvent B: 0-15 min: 15%; 15-30 min: 15%-22%; 30-33 min: 22-40%; 33-40 min: 40%; 40-43 min: 40-15%; 43-47 min: 15%, and detection wavelength of 360 nm. The retention times of (+)-MetSO, (-)-MetSO, (+)-EthSO, and (-)-EthSO were 17.0, 18.0, 22.1, and 23.4 min, respectively. For the Dns-Cl method, the derivatives were prepared by mixing 100 μl of the sample solution, 250 μl of 10 mM Dns-Cl in acetonitrile, and 0.65 ml of a 20 mM borate buffer (pH 9.2). The mixture was briefly shaken, allowed to stand at room temperature for 15 min, filtered through a 0.45 μm nylon filter, and analyzed by HPLC. HPLC analysis was performed with a Waters Alliance 2695 HPLC System equipped with a Varian Microsorb MV C18 column (5 μm, 250 × 4.6 mm, Agilent Technologies, Santa Clara, USA) and maintained at 40°C. For analysis, 50 mM sodium acetate buffer (pH 5.0, solvent A) and methanol (solvent B) were used as the mobile phase, with a flow rate of 0.9 ml/min. The gradient of solvent B is as follows: 0-35 min, 30%-40%; 35-60 min, 40%-75%; 60-65 min, 75%; 65-70 min, 30%; and a detection wavelength of 250 nm. The retention times of (-)-methiin, (+)-methiin, (-)-ethiin, (+)-ethiin, (-)-alliin, and (+)-alliin were 20.7, 25.5, 25.9, 31.5, 31.5, and 38.9 min, respectively.

4.5. Bioconversion of Sulfur-Containing Amino Acids with IDO-catalyst

IDO-catalyst was used for bioconversion of sulfur-containing amino acids. The standard reaction mixture contained 10 mM substrate, 15 mM α-ketoglutarate, 0.5 mM $FeSO_4 \cdot 7H_2O$, 2 mM ascorbate, 1 mM DTT, and 2 mg/ml (0.13 U/ml) IDO-catalyst

described above in 1 ml of a 50 mM Bis-Tris/HCl buffer (pH 6.0). For the bioconversion of sulfur-containing amino acids, the mixture was slightly shaken at 28°C for 16 h. The effects of pH on the activity were measured in the standard reaction mixture using SACys as a substrate at 28°C for 30 min, except 150 mM GTA buffer (50 mM 3,3-dimethylglutaric acid, 50 mM Tris/HCl, 50 mM 2-amino-2-methyl-1,3-propanediol, pH 4-10) was used instead of Bis-Tris/HCl buffer. The pH stability was examined in the standard reaction mixture by measuring the residual activity after incubation in GTA buffer for 1 h at 28°C. The effects of temperature on the activity were measured in the standard reaction mixture using SACys as a substrate at 20-55°C for 30 min. The temperature stability was examined in the standard reaction mixture by measuring the residual activity after incubation at each temperature for 1 h. For the reparative laboratory scale productions of chiral amino acid sulfoxides, the reaction mixture contained 50 mM substrate, 100 mM α-ketoglutarate, 0.5 mM $FeSO_4 \cdot 7H_2O$, 2 mM ascorbate, 1 mM DTT, and 5 mg/ml (0.32 U/ml) of IDO-catalyst in 1 ml of a 50 mM Tris/HCl buffer (pH 7.0). The mixture was slightly shaken at 28°C. After 0, 1, 2, and 4 h of incubation, 50 μl aliquots were taken and added to an equal volume of 20 mM ethylenediamine-*N*,*N*,*N'*,*N'*-tetraacetic acid (EDTA). The concentrations of amino acid sulfoxides produced were determined with HPLC and ^{1}H-NMR. All measurements were performed at least three times.

4.6. Isolation of Oxidized Amino Acid Products

The reaction mixtures were adjusted to pH 2.0 with 1 M HCl and applied to positive ion exchange resin (DOWEX 50W×8; Dow Chemical, Midland, USA), which was previously equilibrated with distilled water, and were eluted with 1 M NH_3 solution.

The fractions containing oxidized amino acid products were passed through negative ion exchange resin (DOWEX 2×8), which was previously equilibrated with distilled water, and were eluted with 1 M HCl. The fractions containing the products were combined, freeze-dried, and dissolved in D_2O. Isolated yields after purification were 90, 88, 79, 70, and 65% for MetSO, EthSO, methiin, ethiin, and alliin, respectively.

4.7 L-Methionine (*S*)-sulfoxide [(+)-MetSO]

^{1}H NMR (500 MHz, D_2O) δ: 2.30 (dd, *J* = 14.35, 7.75 Hz, 2H), 2.73 (s, 3H), 2.95-3.07 (m, 2H), 3.88 (t, *J* = 6.25 Hz, 1H). ^{13}C NMR (125 MHz, D_2O) δ :24.41, 37.21, 48.95, 54.01, 173.79. $[\alpha]_D^{25}$ +67.3 (c 0.1, H_2O). ESI-MS (m/z) = 166 $[M+H]^+$.

4.8. L-Ethionine (*S*)-sulfoxide [(+)-EthSO]

^{1}H NMR (500 MHz, D_2O) δ: 1.30 (t, *J* = 7.48 Hz, 3H), 2.28-2.32 (m, 2H), 2.86-2.93 (m, 1H), 2.95-3.01 (m, 2H), 3.88 (t, *J* = 6.28 Hz, 1H). ^{13}C NMR (125 MHz, D_2O) δ: 6.58, 24.59, 45.08, 46.25, 54.04, 173.82. $[\alpha]_D^{25}$ +30.9 (c 0.1, H_2O). ESI-MS (m/z) = 180 $[M+H]^+$.

4.9. *S*-Methyl-L-cysteine (*S*)-sulfoxide [(+)-Methiin]

^{1}H NMR (500 MHz, D_2O) δ: 2.82 (s, 3H), 3.22 (dd, *J* = 13.87, 7.73 Hz, 1H), 3.47 (dd, *J* = 13.93, 5.98 Hz, 1H), 4.18 (dd, *J* = 7.70, 6.05 Hz, 1H). ^{13}C NMR (125 MHz, D_2O) δ: 38.79, 51.73, 53.88, 172.34. $[\alpha]_D^{25}$ +96.1 (c 0.1, H_2O). ESI-MS (m/z) = 152 $[M+H]^+$.

4.10. *S*-Ethyl-L-cysteine (*S*)-sulfoxide [(+)-Ethiin]

^{1}H NMR (500 MHz, D_2O) δ: 1.30 (t, *J* = 7.48 Hz, 3H), 2.91-2.97 (m, 1H), 3.02-3.08 (m, 1H), 3.19 (dd, *J* = 13.95, 7.75 Hz, 1H), 3.43 (dd, *J* = 13.93, 5.98 Hz, 1H), 4.20 (dd, *J* = 7.40, 6.35 Hz, 1H). ^{13}C NMR (125 MHz, D_2O) δ: 6.42, 46.52, 51.05, 51.70, 172.31. $[\alpha]_D^{25}$ +40.0 (c 0.1, H_2O). ESI-MS (m/z) = 166 $[M+H]^+$.

4.11. *S*-Allyl-L-cysteine (*S*)-sulfoxide [(+)-Alliin]

^{1}H NMR (500 MHz, D_2O) δ: 3.21 (dd, *J* = 13.95, 7.65 Hz, 1H), 3.42 (dd, *J* = 13.90, 6.30 Hz, 1H), 3.63 (dd, *J* = 13.38, 8.00 Hz, 1H), 3.84 (dd, *J* = 13.45, 6.90 Hz, 1H), 4.18 (dd, *J* = 7.45, 6.38 Hz, 1H), 5.48 (dd, *J* = 17.08, 1.08 Hz, 1H), 5.54 (d, *J* = 10.15 Hz, 1H), 5.87-5.95 (m, 1H). ^{13}C NMR (125 MHz, D_2O) δ: 50.59, 51.56, 55.79, 125.11, 125.94, 172.31. $[\alpha]_D^{25}$ +31.3 (c 0.1, H_2O). ESI-MS (m/z) = 178 $[M+H]^+$.

Acknowledgements

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Figure legends

Figure 1. Asymmetric oxidation of sulfur-containing L-amino acids by IDO-catalyst. Highly selective (*S*)-configured sulfoxidation of *S*-alkyl-L-homocysteins (a) and *S*-alkyl-L-cysteines (b).

Figure 2. Effects of pH and temperature on activity and stability of (+)-alliin formation with IDO-catalyst. For the each reaction, 10 mM SACys and 0.13 U/ml IDO-catalyst was used. (a) The effects of pH on the activity and stability. (b) The effects of temperature on the activity and stability.

Figure 3. Preparative laboratory productions of chiral amino acid sulfoxides. For the each reaction, 50 mM substrate and 0.32 U/ml IDO-catalyst was used. All measurements were performed at least three times. (+)-MetSO (○), (+)-methiin (□), (+)-ethiin (△), (+)-alliin (◇).

Table 1. Bioconversion of sulfur-containing amino acids with IDO-catalyst

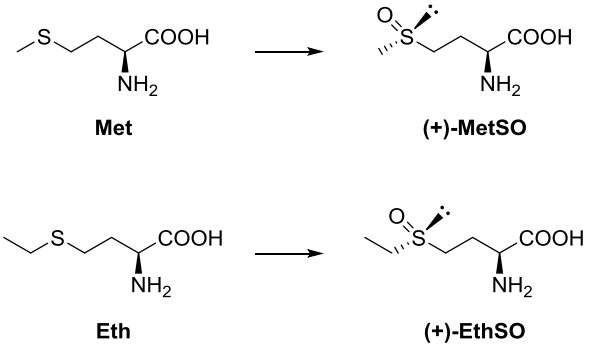
IDO Substrate	Derivatization method for HPLC	Conversion (%)	Product	
			Configuration of sulfinyl group	*de* (%)
Met	FDAA	100	(*S*)	>99
Eth	FDAA	67	(*S*)	>99
SMCys	Dns-Cl	96	(*S*)	88
SECys	Dns-Cl	100	(*S*)	91
SACys	Dns-Cl	100	(*S*)	83

Table 2. Preparative laboratory productions of chiral amino acid sulfoxides with IDO-catalyst

Sulfoxide	Produced (mM)	Yield (%)	*de* (%)
(+)-MetSO	47	94	>99
(+)-Methiin	32	64	93
(+)-Ethiin	43	86	95
(+)-Alliin	35	70	81

Figure 1.

(a)


Met
(+)-MetSO
Eth
(+)-EthSO

(b)

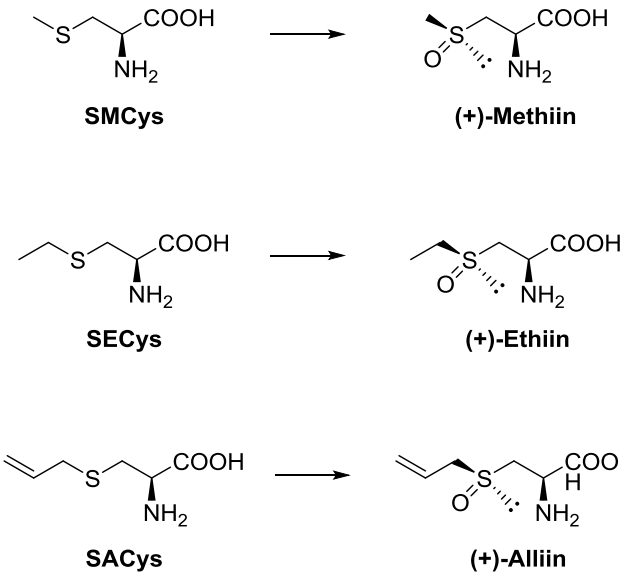

SMCys
(+)-Methiin
SECys
(+)-Ethiin
SACys
(+)-Alliin

Figure2(a)

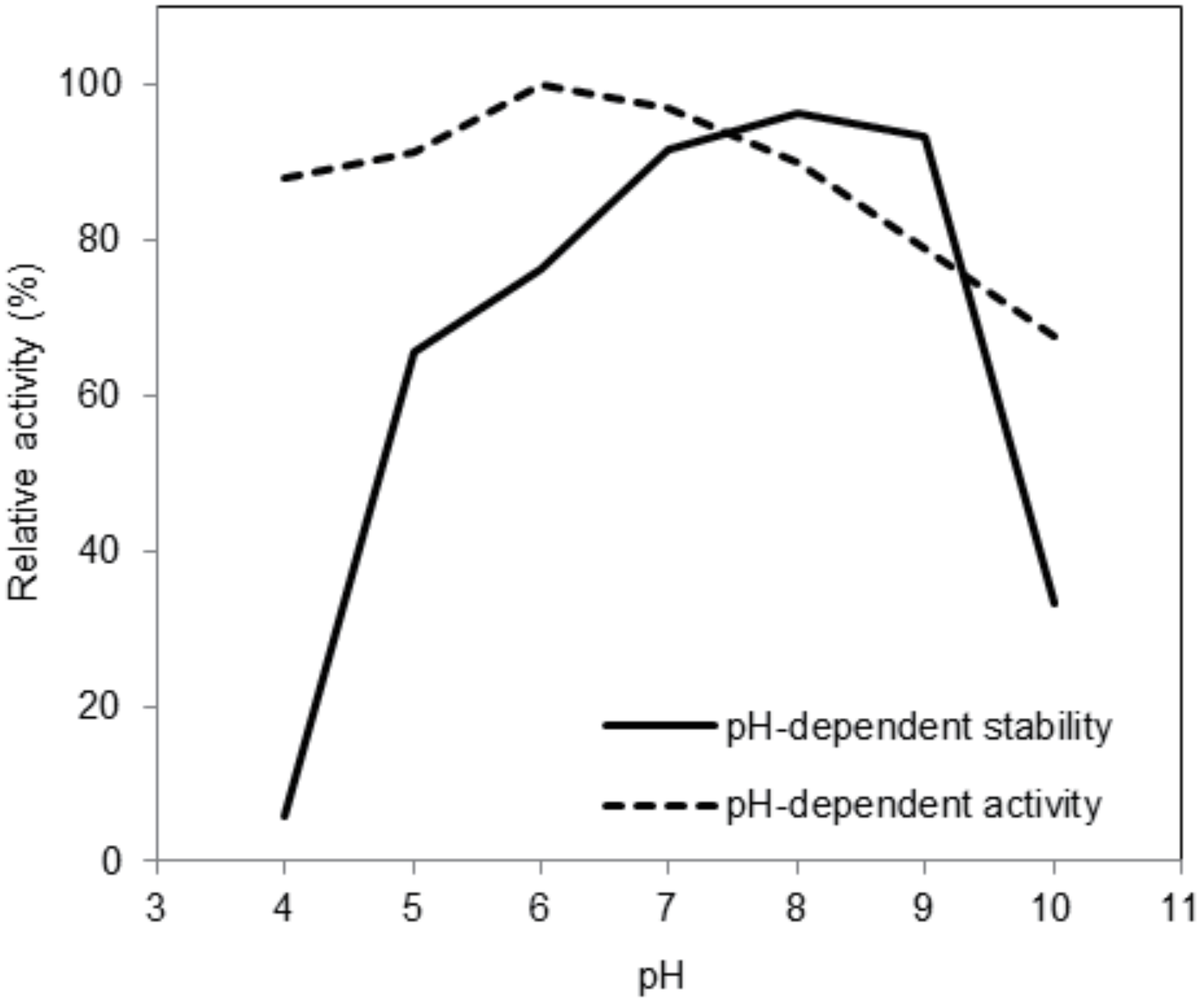

100
80
60
40
20
0
Relative activity (%)
3
4
5
6
7
8
9
10
11
pH
pH-dependent stability
pH-dependent activity

Figure2(b)

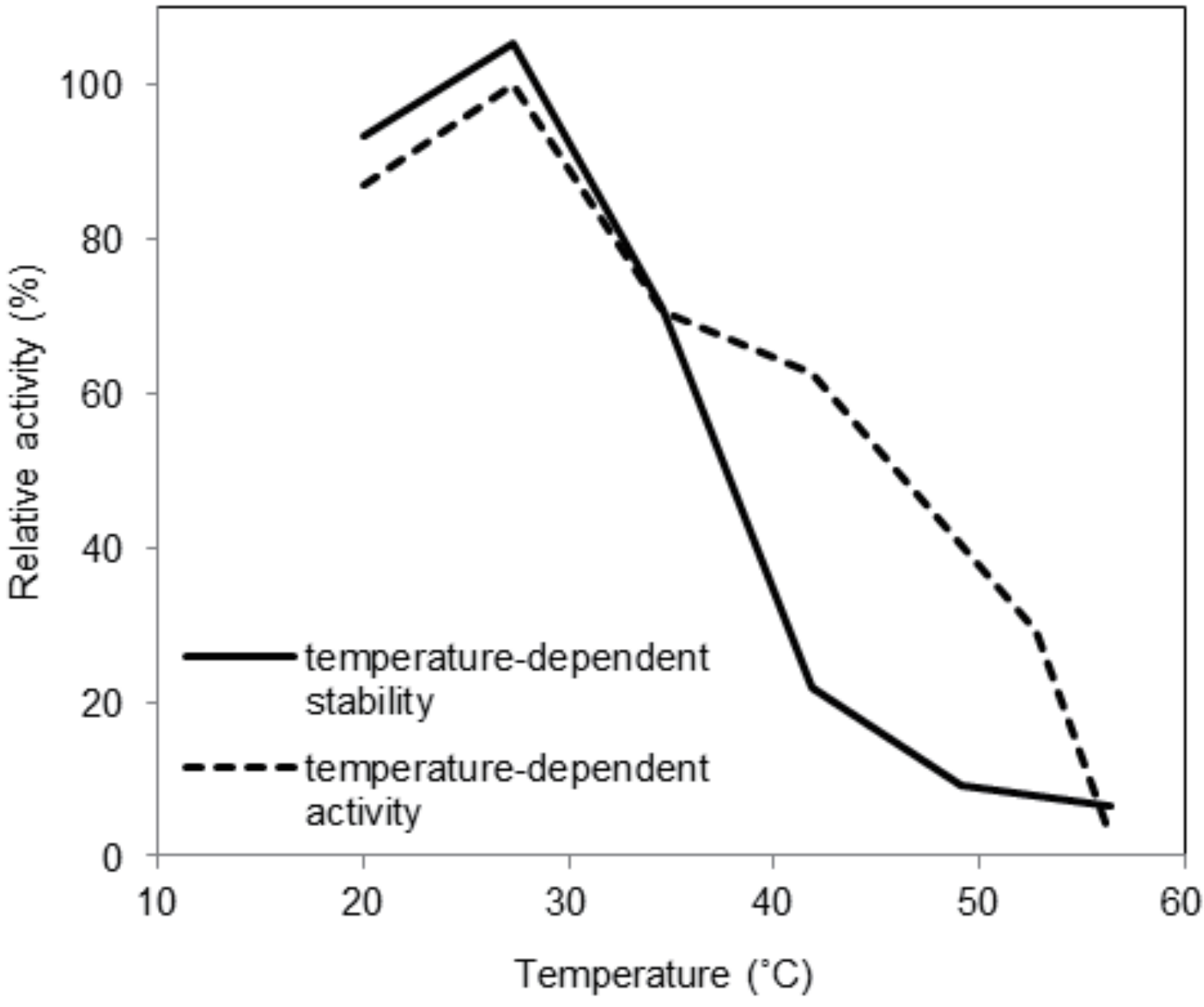
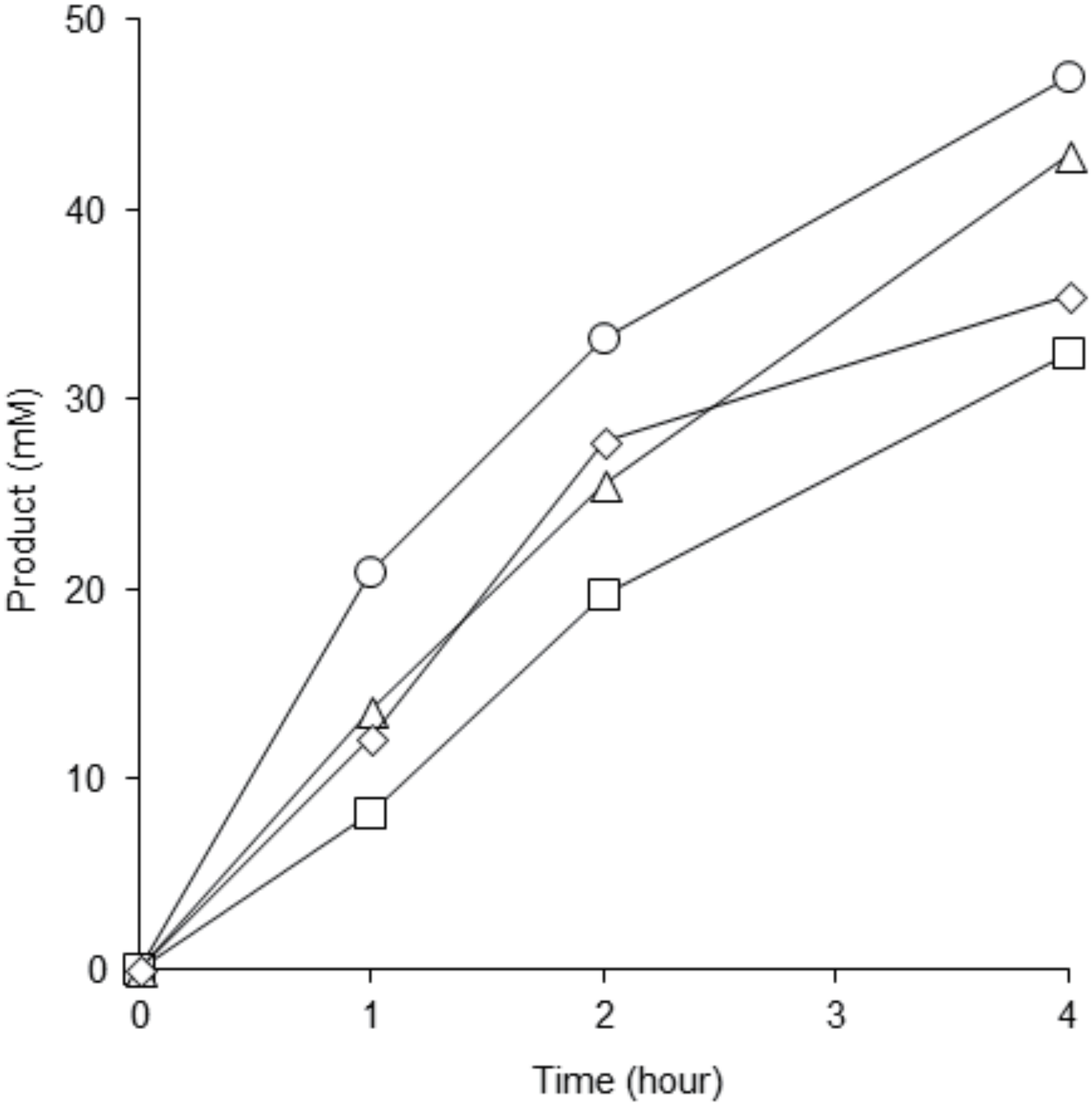
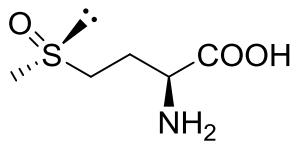

100
80
60
40
20
0
Relative activity (%)
10
20
30
40
50
60
Temperature (°C)
temperature-dependent stability
temperature-dependent activity

Figure3



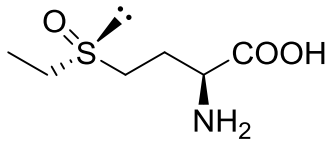


L-Methionine (*S*)-sulfoxide

$[\alpha]_D$ +67.3 (c 0.1, H_2O).

Absolute configuration: (2*S*,(S)*S*)

Source of chirality: L-methionine, enzymatic sulfoxidation

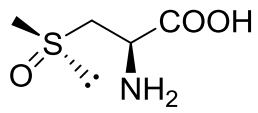


L-Ethionine (*S*)-sulfoxide

$[\alpha]_D$ +30.9 (c 0.1, H_2O).

Absolute configuration: (2*S*,(S)*S*)

Source of chirality: L-ethionine, enzymatic sulfoxidation

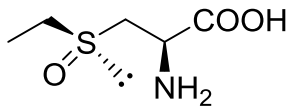


S-Methyl-L-cysteine (*S*)-sulfoxide

$[\alpha]_D$ +96.1 (c 0.1, H_2O).

Absolute configuration: (2*S*,(S)*S*)

Source of chirality: *S*-methyl-L-cysteine, enzymatic sulfoxidation

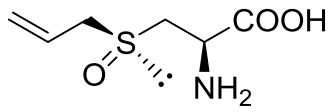


S-Ethyl-L-cysteine (*S*)-sulfoxide

$[\alpha]_D$ +40.0 (c 0.1, H_2O).

Absolute configuration: (2*S*,(S)*S*)

Source of chirality: *S*-ethyl-L-cysteine, enzymatic sulfoxidation



S-Allyl-L-cysteine (*S*)-sulfoxide

$[\alpha]_D$ +31.3 (c 0.1, H_2O).

Absolute configuration: (2*S*,(S)*S*)

Source of chirality: *S*-allyl-L-cysteine, enzymatic sulfoxidation