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Unnatural Amino Acid Synthesis by Thermostable *O*-Phospho-L-serine Sulfhydrylase from Hyperthermophilic Archaeon *Aeropyrum pernix* K1

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O-Acetyl-L-serine sulfhydrylase (OASS) from plants and bacteria synthesizes cysteine and unnatural amino acids that are important building blocks for active pharmaceuticals and agrochemicals. A thermostable *O*-phospho-L-serine sulfhydrylase from hyperthermophilic archaeon *Aeropyrum pernix* K1 (OPSSAp) exhibits a function similar to OASS. In the present study, we examined the synthesis of various unnatural amino acids using OPSSAp and demonstrated OPSSAp could efficiently synthesize various sulfur-containing amino acids. OPSSAp would be useful for industrial production of biologically important unnatural amino acids.

Keywords: *O*-Phospho-L-serine sulfhydrylase | Thermostable | Unnatural amino acid synthesis

Unnatural amino acids are attractive compounds not only for their role as building blocks for active pharmaceuticals and agrochemicals containing chiral centers, but also for their biological activity. As building blocks, unnatural amino acids can be used to stabilize peptide bond against proteolytic attack as well as to mimic natural structural analogues with an altered chemical property.[1] One of the unnatural amino acids, *S*-phenyl-L-cysteine is a building block for a drug to an anti-acquired immune deficiency syndrome, which is one of the leading human immunodeficiency virus-protease inhibitors in the world market. Furthermore, biological activities of unnatural amino acids themselves are also remarkable. For example, β-(3-hydroxy-4-pyridon-1-yl)-L-alanine (L-mimosine) present in *Mimosa* and *Leucaena* spp. (Leguminosae) is a thyrotoxic amino acid and causes loss of hair in growing animals and shows inhibitory activity toward DNA replication in mammalian cells.[2,3] Furthermore, *S*-allyl-L-cysteine is one of the sulfur-containing compounds derived from garlic and is able to attenuate oxidative stress, to exert neuroprotective property in various neurotoxic conditions, and to ameliorate cognitive deficits in streptozotocin-diabetic rats.[4] *S*-(Carboxymethyl)-L-cysteine (carbocysteine) and *S*-(3-hydroxypropyl)-L-cysteine (fudosteine) are used as an expectorant and a mucolytic agent (Figure 1).[5,6] Therefore, large-scale production of bioactive unnatural amino acids using enzymes is very important.

O-Acetyl-L-serine sulfhydrylase (OASS), which is a pyridoxal phosphate (PLP)-dependent enzyme, catalyzes the synthetic reactions of cysteine and various unnatural amino acids from *O*-acetyl-L-serine (OAS) and sodium sulfide or nucleophiles (Scheme 1).[7] The active site of OASS contains PLP linked to lysine residue as an internal Schiff base. Binding with primary substrate OAS displaces the lysine to form an external Schiff base, initiating the first half-reaction that yields an α-aminoacrylate intermediate linked to PLP. The second half-reaction involves the addition of a secondary substrate, such as sulfide or nucleophile, to the α-aminoacrylate intermediate to generate an external Schiff base with cysteine or the corresponding unnatural amino acid. The active-site lysine reacts with this external Schiff base to release cysteine or the unnatural amino acid, then regenerating the internal Schiff base with lysine.

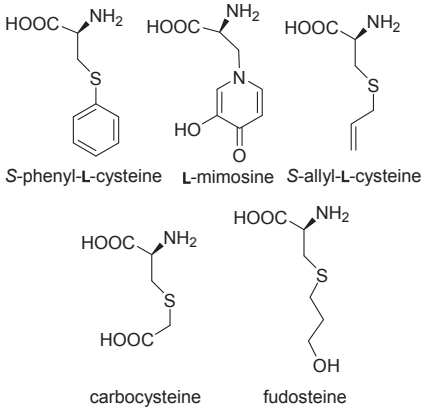

Figure 1. Chemical structures of unnatural amino acids.

It has been well studied that plant OASS produces novel β-substituted L-amino acids when offered unnatural nucleophiles.[8] Furthermore, Maier has reported that two isozymes of OASS from *Escherichia coli*, OASS-A and OASS-B, can produce unnatural amino acids from OAS and thiol compounds such as thiophenol and mercaptoethanol, or *N*-heterocycles such as triazole and tetrazole, and that the unnatural amino acids can be produced efficiently by metabolic engineering of the cysteine-biosynthetic pathway of *E. coli*.[1] Rabeh et al. and Zhao et al. have also reported the synthesis of unnatural amino acids using OASS.[9,10]

Aeropyrum pernix is a hyperthermophilic archaeon that grows optimally within a temperature range between 90 and 95 °C. The putative product of the gene encoding OASS (OASSAp: APE1586) was found in the genome database of *A. pernix* and its physiological role was examined. It exhibits the activity for OASS and cystathionine β-synthase (CBS), and it has therefore been speculated that OASSAp might be an ancestral enzyme of OASS and CBS.[11] About OASS activity of OASSAp, OAS is labile at the optimal growth temperature of *A. pernix*, 90–95 °C and this suggests that *A. pernix* can circumvent the thermal instability of OAS.

Sulfhydrylation reactions of OASSAp using some substrate candidates were then carried out and *O*-phospho-L-serine (OPS)

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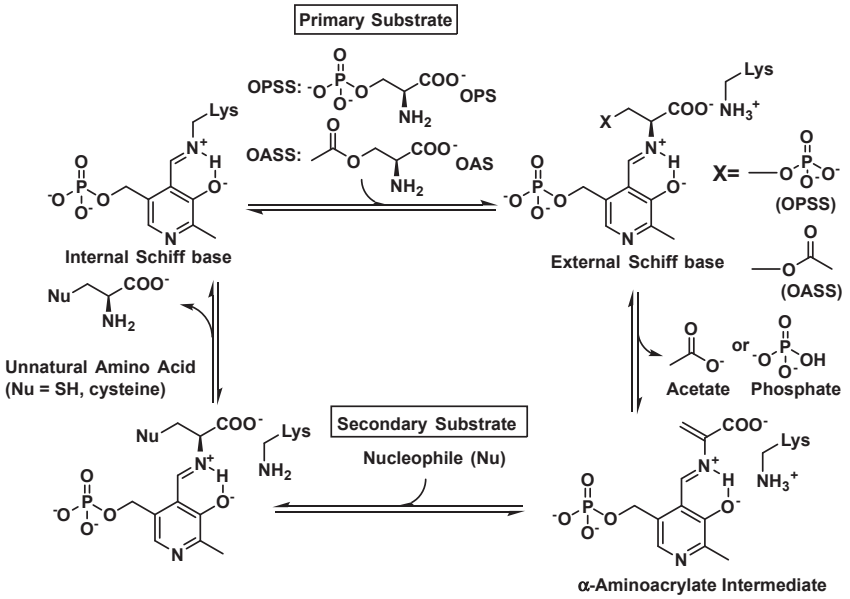

Scheme 1. Mechanism of synthetic reaction of cysteine or unnatural amino acid catalyzed by *O*-acetylserine sulfhydrylase (OASS) and *O*-phosphoserine sulfhydrylase (OPSS).

was found to be the best substrates of OASSAp. This enzymatic reaction of sulfhydrylation using OPS and sulfide was recognized as a novel one and the enzyme was named *O*-phospho-L-serine sulfhydrylase (OPSS; EC2.5.1.65).[12]

If OPSS from *A. pernix* (OPSSAp) can synthesize unnatural amino acids like OASS (Scheme 1), it may improve the yield of some unnatural amino acids compared to that by *E. coli* system, because higher temperature is favorable to chemical and enzymatic reactions, unless enzyme is denatured at the reaction temperature. The activity for the sulfhydrylation reactions of OPSSAp reached the maxima at 90 °C and the rate constant (14000 s^{-1} at 85 °C)[12] is significantly higher than that of *E. coli* OASS-A (2030 s^{-1} at 25 °C).[13] Furthermore, OPSSAp retained ca. 90% of its activity after the enzyme solution had been incubated for 6 h at 100 °C at pH 6.1 and 6.7,[11] while OASS-B from *E. coli* was completely inactivated at 70 °C for 1 h.[10] OPSSAp also showed higher stability toward organic solvents such as *N*,*N*-dimethylformamide (DMF) and 1,4-dioxane than OASS-B from *E. coli* as reported previously.[14] These properties should make them particularly useful as industrial biocatalysis. In this paper, we analyzed the characterization of unnatural amino acid synthesis using OPSSAp and compared with that of OASS-A and OASS-B from *E. coli* and chemical synthesis of *S*-(4-hydroxyphenyl)-L-cysteine.

O-Phospho-L-serine, OPS, and 16 nucleophiles were used as substrates to synthesize unnatural amino acids with OPSSAp. The nucleophiles were selected in such a way that the reaction products are commercially available and comparable to the previous results by OASS from *E. coli* (Figure 2 and Table 1). OPSSAp was expressed and purified from recombinant *E. coli* Rosetta (DE3) cells according to the methods described previously.[11] In enzymatic reaction, 2.5 mM OPS and 2.5 mM nucleophile (1 mM in the case of **7** to **10**) in 50 mM potassium phosphate (Kpi) buffer (pH 7.5) containing 0.2 mM PLP and 1 mM EDTA were incubated with OPSS (0.05–40 μg) at 60 or

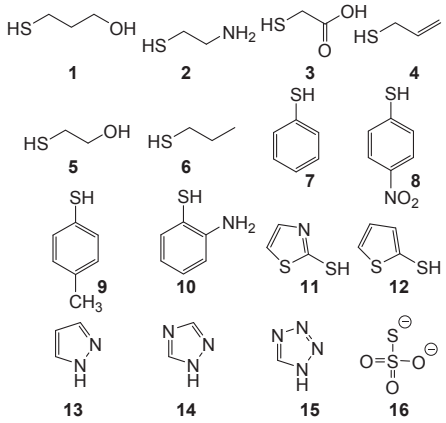

Figure 2. Chemical structures of secondary substrates (nucleophiles) used in this study. The entry numbers (**1** to **16**) correspond to those in Table 1.

80 °C for 1–10 min. Thiol compounds were dissolved in DMF due to their insolubility in water and higher boiling point of DMF, and the reaction mixture contains 5% DMF and 10% DMF in aliphatic thiol and aromatic or heterocyclic thiols, respectively. After the reaction was terminated by 1 M HCl, the reaction mixture was filtered using a 0.1-μm membrane filter (Ultrafree-MC Durapore poly(vinylidene difluoride) 0.1 μm, amicon, Millipore) and was analyzed by amino acid analyzer (L-8800A, Hitachi) or high-performance liquid chromatography system (GILSON) that was used to analyze the production of *S*-(2-aminophenyl)-L-cysteine, *S*-sulfo-L-cysteine, and *S*-4-hydroxyphenyl-L-cysteine when 2-aminothiophenol (**10**), thiosulfate (**16**) and 4-hydroxythiophenol was used as a nucleophile. The detailed reaction conditions and analysis method are described in Table S1. Specific activity of OPSSAp toward

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Table 1. Nucleophiles, products, and specific activity in the synthesis of unnatural amino acids catalyzed by OPSSAp

Entry	Nucleophiles	Products	Specific activity /μmol min^{-1} mg^{-1}-protein[d]
	sodium sulfide[a]	L-cysteine	28 ± 2.4
	sodium sulfide[b]	L-cysteine	48 ± 4.5
1	3-mercapto-1-propanol[b]	*S*-(3-hydroxypropyl)-L-cysteine	13 ± 1.3
2	2-aminoethanethiol[a]	*S*-(2-aminoethyl)-L-cysteine	0.98 ± 0.04
3	mercaptoacetic acid[b]	*S*-(carboxymethyl)-L-cysteine	8.2 ± 0.05
4	allyl mercaptane[b]	*S*-allyl-L-cysteine	71 ± 2.5
5	2-mercaptoethanol[b]	*S*-(2-hydroxyethyl)-L-cysteine	97 ± 4.8
6	1-propanethiol[b]	*S*-propyl-L-cysteine	11 ± 0.7
7	thiophenol[c]	*S*-phenyl-L-cysteine	48 ± 1.2[e]
8	4-nitrothiophenol[c]	*S*-(4-nitrophenyl)-L-cysteine	0.9 ± 0.03
9	4-methylbenzenethiol[c]	*S*-(4-tolyl)-L-cysteine	19 ± 3.1
10	2-aminothiophenol[c]	*S*-(2-aminophenyl)-L-cysteine	29 ± 2.1
11	1,3-thiazole-2-thiol[c]	*S*-(2-thiazolyl)-L-cysteine	0.19 ± 0.005
12	2-mercaptothiophene[c]	*S*-(2-thienyl)-L-cysteine	26.5 ± 1.7
13	pyrazole[a]	3-(1-pyrazolyl)-L-alanine	0.0017 ± 0.0002
14	1,2,4-triazole[a]	3-(1,2,4-triazol-1-yl)-L-alanine	0.15 ± 0.05
15	1,2,3,4-tetazole[a]	3-(2-tetrazolyl)-L-alanine	0.20 ± 0.02
16	sodium thiosulfate[a]	*S*-sulfo-L-cysteine	2.3 ± 0.09

[a]The reaction solvent is 50 mM Kpi buffer (pH 7.5) with 0.2 mM PLP and 1 mM EDTA. [b]The reaction solvent is 50 mM Kpi buffer (pH 7.5) with 5% DMF (v/v), 0.2 mM PLP and 1 mM EDTA. [c]The reaction solvent is 50 mM Kpi buffer (pH 7.5) with 10% DMF (v/v), 0.2 mM PLP, and 1 mM EDTA. [d]Values are expressed as mean ± standard deviation of three experiments. [e]Values are expressed as mean ± standard deviation of two experiments.

cysteine was determined by colorimetric assay with acid ninhydrin reagent.[15,16]

The result showed that specific activity toward some thiol nucleophiles such as allyl mercaptane (**4**) and 2-mercaptoethanol (**5**) was higher than that toward sodium sulfide to produce L-cysteine (Table 1). When **5** was used as a nucleophile, OPSS showed the highest specific activity (97 μmol min^{-1} mg^{-1}-protein) to produce *S*-2-hydroxyethyl-L-cysteine. OPSSAp could also efficiently produce *S*-allyl-L-cysteine, which is a bioactive molecule with anti-diabetic, antioxidant, and anti-inflammatory properties as mentioned above, from OPS and **4**. Therefore, OPSSAp is a very useful biocatalyst for the production of *S*-allyl-L-cysteine. Thiophenol (**7**) and thiophenol with electron-donating groups (**9** and **10**) showed higher activity than that with electron-withdrawing group (**8**) when aromatic thiols were used as nucleophile. Furthermore, OPSSAp could synthesize more *S*-propyl-L-cysteine (from 1-propanethiol, **6**) and *S*-phenyl-L-cysteine (from thiophenol, **7**) than OASSs from *E. coli*, although it cannot be simply compared due to the different substrate concentrations.[10]

On the other hand, specific activity of OPSSAp toward *N*-heterocycles was very low. The specific activity increased corresponding to the number of nitrogen atoms in 5-membered ring, such as two nitrogen atoms in pyrazole (**13**, 0.0017 μmol min^{-1} mg^{-1}-protein) to four nitrogen atoms in 1,2,3,4-tetrazole (**15**, 0.2 μmol min^{-1} mg^{-1}-protein). These specific activities ranged from 1/17000 to 1/140 as compared to that of sodium sulfide in Kpi buffer (28.5 μmol min^{-1} mg^{-1}-protein) and much less than those of OASSs from *E. coli*.

For example, the specific activities of OASS-A and OASS-B from *E. coli* to **15** are 16.5 and 19.7 μmol min^{-1} mg^{-1}-protein, respectively.[10] Furthermore, OPSSAp could use thiosulfate (**16**) to synthesize *S*-sulfo-L-cysteine (2.3 μmol min^{-1} mg^{-1}-protein).

Compared to substrate (nucleophile) specificity with OASS isozymes from *E. coli*, OPSSAp was more similar to OASS-B in that OASS-B can use thiosulfate and thiophenol much better than OASS-A, and pyrazole and triazole less than OASS-A.[1,10] This result was consistent with structure-based amino acid sequence alignment of OASS-B with OASS-A. It showed that OASS-B contains the large residues Arg210, Arg211, and Trp212 and a three-residue insertion at the pocket around the active center, whereas OASS-A contains merely the small residues Gly230, Ala231, and Gly232. In other words, OASS-B has a three-residue insertion bulging toward the outside and thus enlarging the active center pocket in comparison to OASS-A.[17] This additional space enables OASS-B to accept thiosulfate. OPSSAp has two arginine residues in the corresponding region and may have similar activity to OASS-B.

Subsequently, we examined whether OPSSAp can easily synthesize unnatural amino acid, *S*-(4-hydroxyphenyl)-L-cysteine, which is a candidate antitumor agent for melanoma.[18] The specific activity of OPSSAp toward 4-hydroxythiophenol was 17 ± 1.1 μmol min^{-1} mg^{-1}-protein. The value is expressed as mean ± standard deviation of three experiments. This compound was reported to be chemically synthesized by refluxing a mixture of phenol and L-cystine in 47% aqueous HBr for 2 h, followed by separation from a side product, *S*-(2-hydroxyphenyl)-L-cysteine by column chromatography and recrystallization in 37% overall yield (Scheme 2a).[19]

In enzymatic reaction, 148 mg of OPS (80 mM) and 101 mg of 4-hydroxythiophenol (80 mM) were incubated with 125 μg of OPSSAp (0.3 μM) at 80 °C for 3 h in 10 mL of 0.1 M Kpi buffer (pH 7.5) solution (Scheme 2). The pH of the reaction solution was adjusted to 6 by 1 M HCl and crystallized by cooling the solution on ice. The crystal was separated by centrifugation and recrystallized by aqueous ethanol. The identification of the

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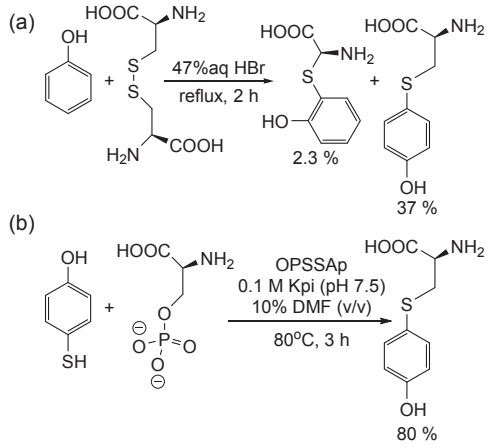

Scheme 2. Synthesis of *S*-(4-hydroxyphenyl)-L-cysteine by (a) chemical reaction[19] and (b) OPSSAp-catalyzed reaction.

product, *S*-(4-hydroxyphenyl)-L-cysteine, was confirmed by ^{1}H NMR (JEOL, JNM-AL400, 2.5 M DCl–D_2O, 3-(trimethylsilyl)propanoic-2,2,3,3-d_4 acid as an internal reference, δ 3.33 (2H, d, $J = 5.1$ Hz, CH_2), 4.10 (1H, t, $J = 5.1$ Hz, CH), 6.75 (2H, d, $J = 7.7$ Hz, C_2 and C_6 H), 7.33 (2H, d, $J = 7.7$ Hz, C_3 and C_5 H), Figure S1)[19] and mass spectrometry (JEOL, JMS-S3000, calcd. for $C_9H_{12}NO_3S$ $[M + H]^+$ 214.05324, found m/z 214.05327). The yield of *S*-4-hydroxyphenyl-L-cysteine was 80% and higher than the reported chemical synthesis yield (37%), as shown in Scheme 2b.

In conclusion, we found that *O*-phospho-L-serine sulfhydrylase from *A. pernix* (OPSSAp), could synthesize unnatural amino acids with a similar substrate specificity to OASS-B from *E. coli*. OPSSAp is very useful to produce sulfur-containing unnatural amino acids such as *S*-allyl-L-cysteine, *S*-2-hydroxyethyl-L-cysteine, *S*-phenyl-L-cysteine, and *S*-4-hydroxyphenyl-L-cysteine. We have already determined three-dimensional structures of OPSSAp, and analyzed detailed reaction and substrate-recognition mechanisms.[16,20,21] The improvement of the yield of various unnatural amino acids may be achieved by genetically modified OPSSAp enzymes using computer modeling and site-directed mutagenesis or random mutagenesis technology. Such protein engineering research in combination with further kinetic study on secondary substrate (nucleophile) specificity is necessary for the further development of industrial process for producing pharmaco-therapeutically important unnatural amino acids by using OPSSAp enzyme.

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Supporting Information is available on http://dx.doi.org/10.1246/cl.170822.

This manuscript is dedicated to the late Professor Yoshihiko Ito.

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