Enzymatic Production of L-Cysteine from DL-2-Amino-4-thiazoline-4-carboxylic Acid by *Pseudomonas thiazolinophilum*: Optimal Conditions for the Enzyme Formation and Enzymatic Reaction

Konosuke SANO and Koji MITSUGI

Ajinomoto Co., Inc., Central Research Laboratories, 1-1 Suzuki-cho,
Kawasaki-ku, Kawasaki 210, Japan

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Cultural conditions of *Ps. thiazolinophilum* AJ 3854 for the production of the enzyme which could form L-cysteine from DL-2-amino-4-thiazoline-4-carboxylic acid (ATC) and the reaction conditions of this enzyme were investigated. This enzyme was perfectly inducible, intracellular and growth-associated type. A remarkable inactivation of enzyme was observed especially in the growing phase but could be prevented by the addition of 1~10 mM Mn^{2+} or by the feeding of ATC as the inducer at the mid-logarithmic phase. Enzymatic degradation of L-cysteine (or L-cystine) formed from ATC could be prevented by the addition of hydroxylamine or semicarbazide. Thus, L-cysteine and L-cystine were quantitatively produced from ATC. Optimal pH and temperature of enzymatic reaction were 8.2 and 42°C (2hr), respectively. A sigmoidal reaction curve was observed when intact cells were used as enzyme source, but sonic treatment of cells made the curve linear.

Some *Pseudomonas* bacteria isolated from soil had potent activities of asymmetrical hydrolysis of DL-2-amino-4-thiazoline-4-carboxylic acid (ATC), from which L-cysteine(CySH) was quantitatively transformed.¹⁾ DL-ATC is an intermediate of a chemical synthetic process of DL-CySH.²⁾ To study the feasibility of commercial production of L-CySH by this new enzymatic method, the optimal conditions of enzymatic process, especially of enzyme production and enzyme reaction, were looked for.

MATERIALS AND METHODS

Microorganisms. *Pseudomonas thiazolinophilum* nov. sp. AJ 3854 isolated from soil¹⁾ was used.

Media. Medium C for enzyme production was a natural medium composed of 0.5% yeast extract, 0.5% peptone, 2% glycerol, 0.25% NaCl, 0.1% KH_2PO_4 , 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0007% $MnSO_4 \cdot 5H_2O$, 0.2% DL-ATC $\cdot 3H_2O$ and deionized water. The pH was adjusted to 7.0 with KOH.

Medium D-1 for enzyme production was a synthetic one composed of 2% glucose, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 , 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0007% $MnSO_4 \cdot 5H_2O$, 0.2% DL-ATC $\cdot 3H_2O$, 2% $CaCO_3$ and deionized water. The pH was adjusted to

7.0 with KOH.

Medium D-2 was almost the same as Medium D-1 except for the enrichment of $MnSO_4 \cdot 5H_2O$ to 0.056%.

Cultivation for enzyme production. Fifty ml of each medium inoculated with bacterial cells grown on a solid bouillon was dispensed in a 500-ml flask and aerobically cultured at 30°C.

Determination of bacterial growth. After dilution of 0.2 ml cultured broth with 5 ml of 0.1 N HCl, optical density at 562 nm was measured by Hiram spectrophotometer.

Enzyme assay. L-CySH-forming activity from DL-ATC was assayed by measuring the amounts of L-CySH formed at 40°C for 1~2 hr in the reaction mixture, pH 8.2, composed of 1% DL-ATC $\cdot 3H_2O$, 0.14% $NH_2OH \cdot HCl$, 1% KH_2PO_4 , and 50% the cultured broth as enzyme source. In some experiments, the intact cells washed and resuspended with equal volume of 0.8% NaCl or the sonicate was used in stead of the cultured broth. The amount of L-CySH and L-cystine (Cys) was determined by microbioassay method using *Pediococcus cereviciae* ATCC 8081. This bacterium can not grow on D-CySH nor D-Cys, but grows on L-CySH and L-Cys (the growth promoting activity of L-Cys is twice of that of L-CySH as molar base). The total enzyme activity was that of 1 ml of cultured broth and the specific activity was that of 1 OD unit of cell in 1 ml.

RESULTS AND DISCUSSION

I. Establishment of the optimal culture conditions

I-1. Inductivity and locality of enzyme. Inductivity of L-CySH-forming enzyme was examined by the cultivation of *Ps. thiazolinophilum* AJ 3854 in Medium C contained varied amounts of DL-ATC. The result shown in Fig. 1 indicates that this enzyme activity is completely inducible. The optimal concentration of DL-ATC·3H₂O as enzyme inducer was 0.2~0.5%.

FIG. 1. Induction of L-CySH-forming Enzyme by ATC.

Ps. thiazolinophilum AJ 3854 was cultured for 15 hr in medium C supplemented with DL-ATC at the indicated concentrations. Enzyme source was the intact cells.

Whether the enzyme is excreted out of cells or not during the growth is very important from the industrial point of view, because the locality of enzyme has much influence on the isolation or immobilization of enzyme. In this case, L-CySH-forming activity from DL-ATC was found absolutely in the precipitate when the fresh broth was centrifuged. This fact suggested that the enzyme existed in bacterial cells or was bound to the cells.

I-2. Time course of cultivation. Bacterial growth and the enzyme activity were followed in medium C as shown in Fig. 2, this enzyme was said to be rather growth-associated type. The maximal activity was obtained at mid-logarithmic growth phase, then the activity began to decrease very quickly. This tendency was more drastic in the synthetic medium D-1.

FIG. 2. Time Course of Enzyme Production.

Ps. thiazolinophilum AJ 3854 was cultured in medium C. The cultured broth was used as enzyme source.

Since the activity decreased before the end of growing phase, the specific activity (activity per unit amount of cells indicated as optical density) fell down more drastically.

The cultivation of this bacterium started with pH 6.4, but the pH of the medium rose gently to 7.4 and fell down to 5.4. In addition, these pH changes were sharply coincided with the decrease in enzyme activity. The prevention of this decrease is one of the most important points to make this process practical.

I-3. Relationship between pH control and the decrease in enzyme activity. It was expected that pH control of culture medium might protect the enzyme from its inactivation, because the decrease in pH seemed to cause the enzyme inactivation. But, unexpectedly, the control of pH to neutral with 5% CaCO₃ or 1% KH₂PO₄ plus KOH accelerated the inactivation of enzyme rather than the prevention (Table I).

I-4. Effect of yeast extract on enzyme production. In order to establish the economical and effective medium for enzyme production, synthetic medium D-1 was employed as basal medium which was at first settled for the minimal medium to isolate auxotrophic mutants, and substances promoting enzyme production or stabilizing enzyme activity were selected. As shown in Table II, yeast extract at first found to have promoting activity of the enzyme much more than the bacterial growth. This effect of yeast extract was substantially the

TABLE I. EFFECT OF pH CONTROL ON THE ENZYME INACTIVATION

Ps. thiazolinophilum AJ 3854 was cultured in medium C supplemented with CaCO_3 or KH_2PO_4 . Total enzyme activity of the broth was shown in the term of mg/ml of L-Cys formed in 2 hr.

Cultivation (hr)	pH		Growth		Total act.	
	15	20	15	20	15	20
Additions (%)						
None	5.2	5.3	0.60	0.68	2.7	2.9
CaCO_3 (2)	6.1	6.2	0.58	0.84	1.7	0.5
(5)	6.2	6.2	0.60	0.80	1.4	0.5
KH_2PO_4 (0.4)	6.4	6.4	0.68	0.88	2.6	1.1
(0.9)	6.4	6.6	0.60	0.89	2.8	1.1

TABLE II. EFFECT OF YEAST EXTRACT ON THE PRODUCTION OF ENZYME

Ps. thiazolinophilum AJ 3854 was cultured for 20 hr in the synthetic medium D-1 supplemented with various amounts of yeast extract. Glycerol instead of glucose was also employed. Other conditions were the same as in Tabel I.

Conc. of yeast ext.	Carbon source	pH	Growth	Total act.
0	Glucose	6.8	0.72	0.48
0.01		6.6	0.74	0.54
0.1		6.5	0.74	0.72
0.5		5.9	0.72	1.31
0		5.9	0.44	0.05
0.01	Glycerol	5.8	0.54	0.18
0.1		5.6	0.64	0.36
0.5		5.4	0.76	0.72

same, either the carbon source was glucose or glycerol. On the production of this enzyme there might be no glucose effect, since the amount of enzyme activity produced in glucose medium was not less but even superior than that in glycerol medium.

I-5. Effect of amino acids, nucleic acids, vitamins and metallic ions on the production of the enzyme. As mentioned above, yeast extract had remarkable promoting effect on the enzyme production. Then, the effective component in yeast extract was examined. Supplement of amino acids, nucleic acids bases, vitamins, or metallic ions in minimal medium D-1 had various effects on the enzyme production as shown in Table III. Among various effective substances, L-glutamic acid, L-leucine, L-

proline, Fe^{2+} , Mn^{2+} , and Mo^{2+} were prominent.

I-6. Effects of Mn^{2+} and Fe^{2+} on enzyme production and enzyme stabilization. As the representative of these effectors above mentioned, the roles of Mn^{2+} and Fe^{2+} were studied in detail. As shown in Fig. 3-A, 5 mM of Mn^{2+} or Fe^{2+} affected scarcely on the growth of *Ps. thiazolinophilum* AJ 3854, while both metallic ions had remarkable promoting effect on enzyme production (Fig. 3-B). The enzyme activity, however, still decreased at growing phase, and the specific activity sharply declined as shown in Fig. 3-C. The optimal concentration of Mn^{2+} for the enzyme production was between 0.1 and 10 mM as shown in Fig. 4. In addition, some stabilizing effect on the enzyme was observed in the presence of 1~10 mM Mn^{2+} . These remarkable effects of Mn^{2+} were observed without any distinguishable effect on the bacterial growth.

I-7. Timely induction of enzyme synthesis. From the data in Figs. 2 and 3, one of the reasons which inactivated the enzyme activity seemed to be ascribed to the fact that the enzyme might be synthesized too early in the growth phase. If the synthesis of enzyme could be delayed and the maximal activity could be attained in late logarithmic phase, the enzyme activity might be substantially stabilized. From this point of view, ATC as the inducer was fed at various phases of growth. A typical result is shown in Fig. 5, in which the enzyme activity was kept stable over 8 hr.

II. Establishment of the optimal reaction-conditions

II-1. Inhibitors of CySH-decomposing enzyme(s). L-CySH was observed to be decomposed in some strains which could form L-CySH from DL-ATC. In the case of AJ 3854, the yield of L-CySH plus L-Cys was lower than 50%, in spite of the complete consumption of the substrate, DL-ATC. When the ATC was hydrolyzed without any inhibitor of L-CySH-decomposition in a sealed test tube, strong smell of H_2S was detected. This finding suggests that L-CySH might be decomposed by

TABLE III. EFFECTS OF AMINO ACIDS, NUCLEIC ACID BASES, VITAMINS AND METALLIC IONS
ON THE PRODUCTION OF ENZYME
Conditions were the same as in Table II.

Supplements	Conc.	pH	Growth	Total act.	Supplements	Conc.	pH	Growth	Total act.
None		7.4	0.70	0.32	Thiamine·HCl	1 mg/liter	7.4	0.65	0.34
L-Alanine	0.02%	7.3	0.70	0.25		10	7.3	0.67	0.33
	0.2	7.2	0.71	0.40	Riboflavin	1	7.5	0.67	0.28
L-Arginine	0.02	6.9	0.66	0.32		10	7.4	0.70	0.35
	0.2	6.7	0.46	0.34	Pyridoxine·HCl	1	7.3	0.74	0.42
L-Aspartic acid	0.02	7.5	0.67	0.40		10	7.2	0.72	0.41
	0.2	7.2	0.73	0.56	Pyridoxal·HCl	1	7.3	0.70	0.38
L-Cysteine·HCl·H ₂ O	0.01	7.1	0.68	0.51		10	7.3	0.71	0.41
	0.1	6.9	0.71	0.36	Pantothenic acid	1	7.3	0.71	0.42
L-Glutamic acid	0.05	7.3	0.70	0.48		10	7.3	0.72	0.43
	0.5	7.3	0.78	0.69	Nicotinic acid	1	7.4	0.68	0.38
Glycine	0.01	7.4	0.68	0.33		10	7.4	0.70	0.47
	0.1	7.0	0.72	0.39	p-Aminobenzoic acid	0.2	7.5	0.69	0.37
L-Histidine·HCl	0.01	7.2	0.71	0.47		2	7.3	0.58	0.36
	0.1	7.1	0.68	0.54	Biotin	0.01	7.5	0.65	0.40
L-Isoleucine	0.01	7.2	0.70	0.35		0.1	7.3	0.72	0.37
	0.1	7.3	0.76	0.39	Folic acid	0.01	7.5	0.66	0.33
L-Leucine	0.01	7.3	0.69	0.36		0.1	7.4	0.68	0.41
	0.1	7.2	0.72	0.70	CuCl ₂ ·2H ₂ O	0.5 mm	6.5	0.69	0.33
L-Lysine·HCl	0.02	5.0	0.24	0.24		5	5.4	0.09	0.04
	0.2	6.6	0.76	0.54	MnSO ₄ ·4H ₂ O	0.5	7.4	0.66	0.29
L-Methionine	0.01	7.2	0.71	0.35		5	6.9	0.69	0.60
	0.1	7.2	0.71	0.40	MgSO ₄ ·7H ₂ O	0.5	7.1	0.73	0.71
L-Phenylalanine	0.01	7.4	0.67	0.37		5	7.4	0.66	0.60
	0.1	7.3	0.77	0.51	ZnSO ₄ ·7H ₂ O	0.5	6.6	0.66	0.32
L-Proline	0.01%	7.2	0.72	0.54		5	5.8	0.44	0.47
	0.1	7.2	0.72	0.61	Na ₂ MoO ₄ ·2H ₂ O	0.5	7.3	0.69	0.60
L-Serine	0.005	7.3	0.68	0.47		5	7.3	0.72	0.56
	0.05	7.1	0.74	0.58	CoSO ₄ ·7H ₂ O	0.5	7.4	0.66	0.37
L-Threonine	0.01	7.2	0.68	0.44		5	7.0	0.04	0.19
	0.1	7.3	0.72	0.55	NiCl ₂ ·6H ₂ O	0.5	7.3	0.58	0.40
L-Tryptophan	0.005	7.3	0.72	0.47		5	5.5	0.08	0.14
	0.05	7.2	0.74	0.51	FeSO ₄ ·7H ₂ O	0.5	7.3	0.67	0.59
L-Tyrosine	0.01	7.5	0.67	0.32		5	7.2	0.70	0.69
	0.1	7.3	0.76	0.42	AlCl ₃ ·6H ₂ O	0.5	7.3	0.62	0.52
L-Valine	0.01	7.4	0.72	0.44		5	7.2	0.62	0.44
	0.1	7.4	0.75	0.45	Fe(SO ₄) ₃	0.5	7.2	0.72	0.44
Adenine	0.001	7.5	0.67	0.36		5	7.1	0.70	0.52
	0.01	7.3	0.72	0.34	SnCl ₂ ·2H ₂ O	0.5	7.0	0.46	0.08
Guanine	0.001	7.5	0.66	0.36		5	7.5	0.06	0.06
	0.01	7.4	0.71	0.43					
Uracil	0.001	7.3	0.73	0.40					
	0.01	7.3	0.72	0.43					
Cytosine	0.001	7.4	0.70	0.42					
	0.01	7.5	0.69	0.35

FIG. 3. Effect of Fe^{2+} and Mn^{2+} on the Production and the Stabilization of Enzyme.

Ps. thiazolinophilum AJ 3854 was cultured in medium D-1 (○) supplemented with 5 mM Mn^{2+} (▲), 5 mM Fe^{2+} (●) and 2.5 mM each of Mn^{2+} and Fe^{2+} (△). (A) is the growth, (B) is the total enzyme activity and (C) is the specific activity. Enzyme source was the intact cells.

FIG. 4. Optimal Supplementary Concentration of Mn^{2+} on the Production and the Stabilization of Enzyme.

Ps. thiazolinophilum AJ 3854 was cultured in medium D-1 supplemented with Mn^{2+} at the indicated concentrations. ○, no addition; ●, 0.1 mM; ■, 0.5 mM; △, 1 mM; ▲, 5 mM; □, 10 mM; and ×, 20 mM. Enzyme source for assay was the intact cells.

FIG. 5. Timely Induction of Enzyme Synthesis.

Ps. thiazolinophilum AJ 3854 was cultured in medium D-2. The inducer, ATC, was added at the mid-growing phase (at 13 hr cultivation). The concentrations of DL-ATC·3H₂O were 0.4% (●), 0.2% (▲) and none (■). Enzyme source was the intact cells.

cysteine desulfhydrase³⁾ which attacked L-CySH and produced H₂S, pyruvic acid and NH₃. This enzyme is known to require pyridoxal-phosphate as cofactor.⁴⁾ It is well known that the pyridoxal-phosphate-trapping agents inhibit the pyridoxal-phosphate-requiring enzyme-activity. Thus, some reagents were examined as the inhibitors of L-CySH-decomposition. Then, hydroxylamine and semicarbazide were found as potent inhibitors. In Fig. 6, the optimal concentration of hydroxylamine is shown. And time course of the reaction in the presence or absence of hydroxylamine is shown in Fig. 7.

FIG. 6. Optimal Concentration of NH_2OH to Prevent the Degradation of L-CySH.

Ps. thiazolinophilum was cultured in medium C for 15 hr and the cells were washed and suspended in equal volume of 0.8% aqueous NaCl. Enzyme reaction was performed at 30°C for 7 hr. Reaction mixture consisted of 1% DL-ATC·3H₂O, 1% KH₂PO₄, 5% cell suspension and indicated amounts of NH_2OH .

FIG. 7. Time Course of Enzyme Reaction with or without NH_2OH .

The concentration of NH_2OH was 20 mM (0.14%). Other conditions were the same as in Fig. 6.

II-2. Optimal pH. The optimal pH of CySH-forming reaction from DL-ATC was examined. The pH of enzyme reaction mixture containing 50% cultured broth (medium C) as enzyme source was adjusted with KOH or HCl, and incubated at 30°C for 2 hr. The optimal pH was 8.2 in 2 hr-incubation (Fig. 8).

Fig. 8. Optimal pH of L-CySH-forming Enzyme from DL-ATC.

Conditions were described in the text.

II-3. Optimal temperature. The optimal temperature of L-CySH-forming enzyme reaction from DL-ATC was 42°C in 2 hr incubation, where the reaction mixture contained 50% cultured broth (medium C) and the pH was 8.2 (Fig. 9).

FIG. 9. Optimal Temperature of L-CySH-forming Enzyme from ATC.

Conditions were described in the text. Reaction time: One hr (Δ), 2 hr (○),

II-4. pH stability. pH stability of L-

FIG. 10. pH Stability of L-CySH-forming Enzyme from ATC.

Conditions were described in the text. Storage: 18 hr at 4°C (○), 18 hr at 30°C (Δ).

CySH-forming enzyme at 4°C and 30°C was examined with 15 hr-cultured broth (medium C) as enzyme source. After 18 hr-incubation of the broth whose pH was adjusted to pH 4~12 with HCl or KOH, the residual enzyme activity was determined at pH 8.2. As shown in Fig. 10, this enzyme activity was fairly stable between pH 6~10. There was an unexpected phenomenon that the incubation at rather high temperature (30°C) resulted in the activation of enzyme activity, almost twice as much activity as that at the starting point.

II-5. Temperature stability. The enzyme activity was stable below 40°C and completely inactivated above 50°C, after the cultured broth as enzyme source being incubated for 1 hr at varied temperature, as shown in Fig. 11.

FIG. 11. Temperature Stability of L-CySH-forming Enzyme from ATC.

Conditions are described in the text.

FIG. 12. Time Course of Enzyme Reaction by Intact Cells or Sonicate of *Ps. thiazolinophilum* AJ 3854.

Cells cultured in medium C were sonicated for 5 min. Other conditions were the same as in Fig. 7.

II-6. Time course of enzyme reaction. The difference of shapes in 1 hr and 2 hr reaction (Fig. 9) suggests an abnormal time course of enzyme reaction. As a matter of fact, the pattern of time course of enzyme of reaction with washed cells was sigmoidal, while that of sonicate was linear (Fig. 12). The difference might be ascribed to the existence of permeability-barrier against ATC which could be broken by sonication. The acceleration of reaction velocity of the washed cells in late reaction phase also occurred in parallel with autolysis of cells.

II-7. Apparent activation of enzyme. The L-CySH-forming activity from DL-ATC of cells at early phase of incubation was accelerated by various treatments. As shown in Table IV, some surfactants such as cetyltrimethyl-ammonium chloride and sodium dodecylsulfate, some cell-lysing factor such as NLF (native cell lytic factor),⁵⁾ and some cell-destructive treatment such as sonication were much effective. These results may confirm the existence of permeability-barrier of ATC in this bacterium. However, such treatments in Table IV or autolysis of cells can break the barrier,

TABLE IV. TREATMENT OF CELLS AND THE ENZYME ACTIVITY

Ps. thiazolinophilum was cultured in medium C for 15 hr. SDS (sodium dodecylsulfate), CTAC (cetyltrimethylammonium chloride), or NLF (native cell lysing factor) was added and incubated for 1 hr at 30°C. Then their enzyme activities in these treated broth were determined.

Treatment	Dose	Enz. act.
Sonic	1 min	100
	2	119
	3	154
	4	107
SDS	0.01 %	104
	0.05	135
	0.1	7
CTAC	0.005	110
	0.01	162
	0.05	152
	0.1	1
NLF	4.8	131
	9.1	159
	16.7	158

thus it might not decrease the possibility of commercial production of L-CySH by this enzymatic method.

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