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Purification and Characterization of Cysteine Desulfhydrase from *Streptomyces albidoflavus* SMF301

Jae Gon Ryu, Sung Gyun Kang, In Seop Kim, Yong Taik Rho, Sang Hee Lee and Kye Joon Lee*

Department of Microbiology, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

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Cysteine desulfhydrase (EC 4.4.1.1) was purified from the culture supernatant of *Streptomyces albidoflavus* SMF301 by hydroxyapatite, gel filtration and Resource Q ion-exchange chromatography with a purification fold of 219. The molecular weight of the native enzyme was estimated to be 240 kDa consisting of six identical subunits. The enzyme was stabilized by dithiothreitol and pyridoxal 5'-phosphate during the purification procedures. The optimum pH and temperature were pH 8.6 and 35°C, respectively. The N-terminal amino acid sequence was identified as A-P-L-P-T-A-D-V-D-R-S-D-P-G-Y-R-E-W-L-G-E-A-V. The purified cysteine desulfhydrase had a high substrate specificity toward cysteine, and exhibited no cystathionine γ-lyase activity. The K_m value for cysteine was determined to be 0.37 mM.

Key words: Cysteine desulfhydrase, Streptomyces, purification, N-terminal sequence

Streptomycetes are important as producers of useful metabolites. They are also of interest because of their unique morphological complexity: they form a substrate mycelium, with an aerial mycelium which differentiates into arthrospores. Aerial growth and spore formation probably reflect nutrient limitation, reduced growth rate, and the accumulation of growth inhibitors (3). Although studies on the mechanisms involved in cell differentiation have been carried out mostly on solid cultures, submerged cultures would give some advantages in the elucidation of relationships between environmental change and spore formation.

A strain of *Streptomyces albidoflavus* producing abundant spores both in submerged cultures and on solid cultures was selected (19). The cellular content of carbon, hydrogen, nitrogen, and phosphorus in submerged and aerial spores was similar, but the contents of metal ions (K, Na, Ca, and Mg) were very different. Glutamic acid, alanine, and glycine, all known to be cell wall components, were the major amino acids in both types of spores. However, the cysteine content in submerged spores was higher than in aerial spores. The major fatty acid in aerial spores was n-C_{18} (61.74%), whereas it was ai-C_{16} (33.68%) in submerged spores. The contents of ai-C_{14}, and ai-C_{17} in submerged spores were also very much higher than in aerial spores. Unsaturated fatty acids were found in both kinds of spores but not in mycelium; they were particularly abundant in submerged spores. The composition of menaquinones in the two kinds of spores also varied. The resistance of aerial spores to lysozyme digestion, mild acid treatment, heating and desiccation was higher than that of submerged spores, but the submerged spores were more resistant to sonication. (14).

Kinetic analysis on the relationship between environmental changes and sporulation of *Streptomyces albidoflavus* SMF301 in submerged cultures was attempted. Specific submerged spore formation rate (q_{spo}) was inversely related to the specific mycelium growth rate (μ). The optimum growth rate for submerged spore formation was 0.05 h^{-1} when the maximum value of q_{spo} was 1.0×10^6 spores g^{-1} h^{-1}. The turnover rate of biomass at maximum growth yield was 0.029 h^{-1} when 5.6×10^6 spores were formed from 1 g of mycelium (20).

The morphological differentiation in both solid and submerged culture was clearly affected by the addition of cysteine to the culture. The redox-potential of the culture was reduced by the addition of cysteine. The redox-potential was returned to normal when the cysteine was hydrolyzed to ammonium ions, hydrogen sulfide, and pyruvate by cysteine desulfhydrase (9). Cysteine is an im-

* To whom correspondence should be addressed

portant sulfur source in microbial growth where cystathionine-lyase was the most responsible enzyme involved in the metabolism, but cysteine desulfhydrase producing H2S, NH3 and pyruvate from cysteine was a prevalent enzyme in microorganisms isolated from soil (15, 18). It was reported that cysteine is one of the major amino acids in submerged spores of *Streptomyces albidoflavus* SMF301 (14). Cysteine is also an important precursor in the β-lactam antibiotic biosynthesis in β-lactam producing actinomycetes where α-aminoadipic acid, cysteine and valine are fused to form ACV (δ-L-α-aminoadipyl-L-cysteinyl-D-valine), the starting molecule for the biosyntheses of penicillin and cephalosporin-type antibiotics (6).

In order to understand the cysteine metabolism in *Streptomyces albidoflavus* SMF301 in relation to the morphological differentiation, we purified cysteine desulfhydrase and its physicochemical properties were analyzed.

Materials and Methods

Microorganism and media

The microorganism used in this study was *Streptomyces albidoflavus* SMF301 (19). Stock culture medium was Bennett's agar consisted of (w/v): yeast extract 0.1%, beef extract 0.1%, casamino acid 0.2%, glucose 1% and agar 18%. The seed medium was YED containing 1% yeast extract and 1% glucose. Chemically defined medium for the main culture consisted of glucose 1%, NH_4Cl 0.1%, KH_2PO_4 0.1%, K_2HPO_4 0.34%, $MgSO_4$ 0.03%, $FeSO_4$ 0.001%, $CaCl_2$ 0.001%, $MnCl_2$ 0.0002%, $ZnCl_2$ 0.0002%, $CuSO_4$ 0.00004% and $CoCl_2$ 0.00004%.

Strain maintenance and culture conditions

Strains were maintained by transfer to slopes of stock culture medium each month, and were stored at 4°C. One loopful of mycelium and spores was used to inoculate 30 ml of the seed culture medium and incubated at 28°c for 2 d. The seed culture was used to inoculate 3 L of the main culture medium contained in a jar fermentor (Model KF-5 L, Korea Fermentor Co.). The culture temperature was maintained at 28 °C and the initial pH was controlled at 7.0. Agitation and aeration rates were 200 rpm and 1 v/v/min, respectively.

Measurement of mycelium growth and sulfide production

To measure mycelium growth, mycelium was harvested by centrifugation (10,000 g, 10 min) and washed twice with physiological saline solution and once with distilled water. The washed mycelium was collected by vacuum filtration (Whatman GF/C paper), dried at 80°C for 24 h, and weighed. Sulfide production rate was measured by Zn-S precipitation method (11). Zn-acetate solution (5 mM) was placed in the gas outlet of a fermentor, and gaseous hydrogen sulfide emitted from culture medium was trapped as a Zn-S precipitates.

Activity assay of cysteine desulfhydrase and cystathionine γ-lyase

Cysteine desulfhydrase (CDSH) activity was assayed by measuring the rate of production of sulfide according to methylene blue method (11). One unit of CDSH was defined as the amount to produce 1 μM of sulfide from cysteine per min. In order to measure cystathionine γ-lyase activity, L-cystathionine was used as a substrate and the amount of L-cysteine formed was determined by the modified ninhydrin method (5, 16). One unit of cystathionine -lyase was defined as the amount to produce 1 μM of cysteine per min.

Purification of cysteine desulfhydrase

Protein concentration was measured by Bradford method (1) using bovine serum albumin (Sigma, Co.) as a reference protein. When CDSH activity reached its maximum in a batch culture, mycelium was harvested by centrifugation at 10,000 g for 30 min and then washed twice with buffer A (1 mM EDTA, 1 mM dithiothreitol, 0.1 mM pyridoxal 5'-phosphate in 5 mM potassium phosphate buffer; pH 7.2). The washed mycelium (133 g in wet weight) was resuspended in buffer A containing PMSF (phenyl-methyl-sulfonyl fluoride) and then disrupted by ultrasonicator (130 w, total 30 min). Cell free enzyme extract was obtained by centrifugation at 40,000 g for 1 h and then concentrated by ultrafiltration.

Concentrated enzyme solution was applied to a column (2.5×40 cm) of hydroxylapatite equilibrated in buffer A. Fractions (8.0 ml) were collected and flow rate was 30 ml/h. The active fraction was not retained by the resin, and was eluted with buffer A. The active fractions obtained from the hydroxylapatite chromatography were concentrated by ultrafiltration and then applied to a column (2.5×65 cm) of Sephadex G-150 equilibrated with 50 mM K-phosphate buffer, pH 7.2 containing 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM pyridoxal 5'-phosphate. The volume of each fraction was 4.5 ml and collected at a flow rate of 0.9 ml/h.

The active fractions obtained by gel filtration were concentrated by ultrafiltration and then applied to a column of Resource Q (bed volume: 1 ml)

equilibrated with 0.05 M Tris-HCl (pH 8.0) containing 1 mM DTT and 1 mM EDTA. After washing with 5 ml of equilibrium buffer, elution was carried out with 20 ml of a linear gradient of NaCl (0 to 1 M). The volume of each fraction was 1 ml and collected at a flow rate of 1 ml/min. CDSH was collected in fractions containing about 0.5 M NaCl.

The active fractions obtained from the Resource Q chromatography were applied to a column of Superose 12HR (bed volume: 25 ml) connected to FPLC system equilibrated with 50 mM potassium phosphate buffer, pH 7.2 containing 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM pyridoxal 5'-phosphate. The volume of each fraction was 0.2 ml and collected at a flow rate of 0.2 ml/min

Molecular mass was determined by sodium dodecyl sulfate-gel electrophoresis in 10% of the polyacrylamide slab gels using the Tris/glycine buffer system described by Laemmli (13). The gels were stained with silver staining method. The relative molecular mass of the enzyme subunit was obtained from the relative mobility of the standard proteins. The molecular weight of native protein was determined by chromatography on a Superose 12 HR 10/30 (Pharmacia, Co.) with standards of molecular weight from 12.5 kDa to 240 kDa.

Characterization of cysteine desulfhydrase

The optimum pH for CDSH toward cysteine was measured over range of 3.0 to 10.5 by the use of following buffers at 0.1 M; citrate buffer (pH 3.0 to 6.0), phosphate buffer (pH 6.0 to 8.0), Tris-HCl buffer (pH 8.0 to 9.0), glycine-NaOH buffer (pH 9.0 to 10.5). The optimum temperature for CDSH toward cysteine was determined within a range of 10 to 60°C at the optimum pH.

The catalytic activity towards various amino acids was measured using L-cysteine, L-cystine, D,L-homocysteine, L-serine, L-homoserine, L-cystathionine, L-methionine, L-phenylalanine and S-2-aminoethyl-L-cysteine as the substrates. Pyruvate production from the substrates was measured by 2,4-dinitrophenylhydrazine method and hydrogen sulfide production was measured by the methylene blue method (11). K_m values of purified CDSH for cysteine was determined from a Lineweaver-Burk of the initial velocities obtained with cysteine concentrations between 0.05 to 0.75 mM.

The N-terminal amino acids of the CDSH were sequenced by the automated Edman degradation method with MilliGen/Biosearch 6600 prosequence protein sequencing system (Millipore, Co.)

Results and Discussion

Induction of cysteine desulfhydrase by the addition of cysteine

The effect of the addition of cysteine (1 mM) on mycelium growth and the induction of cysteine desulfhydrase (CDSH) during a submerged culture of *S. albidoflavus* SMF301 were determined. Although the growth was not greatly affected by the addition of cysteine (Fig. 1A), the activity of CDSH increased remarkably as cysteine was added (Fig. 1B). Since CDSH activity was detected in the culture without the addition of cysteine, it was thought that CDSH was expressed not only as a constitutive mode but also as an inducible mode. Moreover, extracellular hydrogen sulfide concentration rapidly increased immediately after cysteine was added (Fig. 1C). The hydrogen sulfide production was closely related to the change of CDSH activity, indicating that the accumulation of hydrogen sulfide resulted from the metabolism of cysteine by CDSH.

It was reported that high concentration of cysteine was very toxic to bacterial growth (2, 17), a rapid increase of CDSH activity after the addition

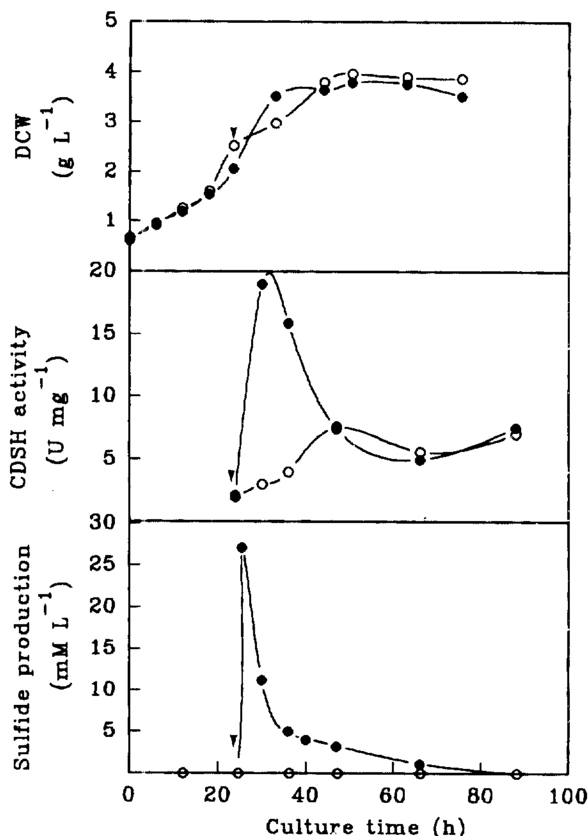

Fig. 1. Effect of addition of cysteine on the induction of cysteine desulfhydrase in *Streptomyces albidoflavus* SMF301. 1 mM of cystein (●) was added to the culture at the time indicated by an arrow. Control culture was added with saline (○).

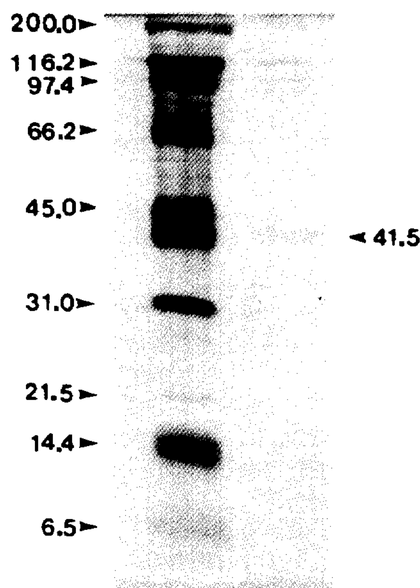

Fig. 2. SDS-PAGE of purified cysteine desulfhydrase. Proteins were stained with silver. Protein standards were (1) myosin (200 kDa), (2) β-galactosidase (116.2 kDa), (3) phosphorylase b (97.4 kDa), (4) serum albumin (66.2 kDa), (5) ovalbumin (45 kDa), (6) carbonic anhydrase (31 kDa), (7) trypsin inhibitor (21.5 kDa), (8) lysozyme (14.4 kDa) and (9) aprotinin (6.5 kDa).

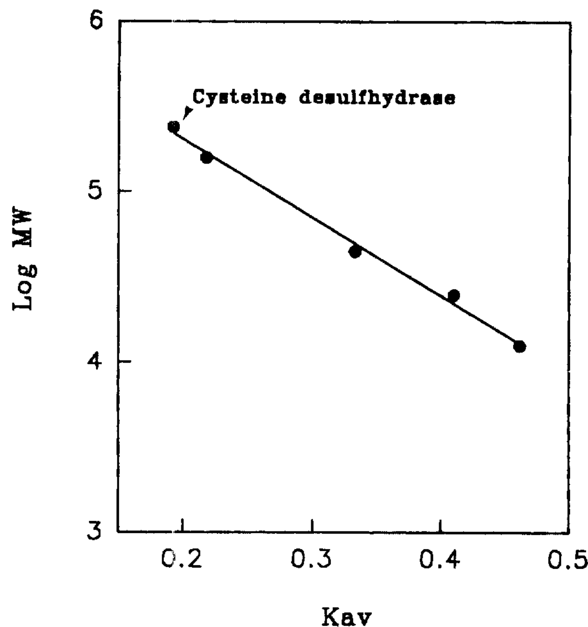

Fig. 3. Estimation of the native molecular mass of cysteine desulfhydrase. The molecular mass estimated by gel filtration on Superose 12 HR (1×30 cm) connected to FPLC system. Protein standards were catalase (240 kDa), aldolase (158 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa) and cytochrome C (12.5 kDa). Void volume was determined with blue dextran (2,000 kDa).

of cysteine would play a role in reducing the cysteine toxicity.

Purification of cysteine desulfhydrase

CDSH was purified 219-fold with a recovery of 0.1% by hydroxylapatite, gel filtration, and Resource Q ion exchange chromatographies (Fig. 2 and Table 1). Since the enzyme was found to be unstable during the purification, 1 mM of dithiothreitol and 0.1 mM of pyridoxal 5'-phosphate were used to increase the stability of the CDSH as reported in the purification of CDSH from *Salmonella typhimurium* (10). Phenyl-methyl-sulfonyl fluoride (PMSF) and EDTA were also found to be useful for preventing actions of proteases produced by *S. albidoflavus* SMF301 (7, 8).

Molecular weight and N-terminal amino acid sequence

The purified enzyme showed a single band in SDS-PAGE (Fig. 2), and the molecular weight of the denatured CDSH was estimated to be 41.5 kDa. The molecular weight of native enzyme obtained from Superose 12 HR FPLC system was about 240 kDa (Fig. 3). From the above results, it was concluded that the purified CDSH was composed of 6 subunits with an identical molecular weight. This result was well matched to the fact that the CDSHs from *Salmonella typhimurium* and *Aerobacter aerogenes* were hexamer (10, 12).

The N-terminal amino acids of the purified CDSH was determined to be A-P-L-P-T-A-D-V-D-R-S-D-P-G-Y-R-E-W-L-G-E-A-V. The sequence was used to search for homologous sequences in the GenBank database and showed no significant

Table 1. Purification of cysteine desulfhydrase from *Streptomyces albidoflavus* SMF301

Purification steps	Total volume (ml)	Protein conc. (mg)		Enz. act. (U)		Specific ativity	fold	Yield(%)
		per ml	total	per ml	total			
Cell free extract	390	5.12	1,996.8	3,245	1,265,550	633.8	1	100
Hydroxylapatite	275	0.03	8.2	319	87,725	10,698	16.9	6.9
Sephadex G-150	80	0.029	2.32	672	53,744	23,165	36.5	4.2
Resource Q	5.6	0.048	0.27	5,671	31,758	117,622	185.6	2.5
Superose 12	0.4	0.027	0.01	3,749	1,499	138,862	219.1	0.1

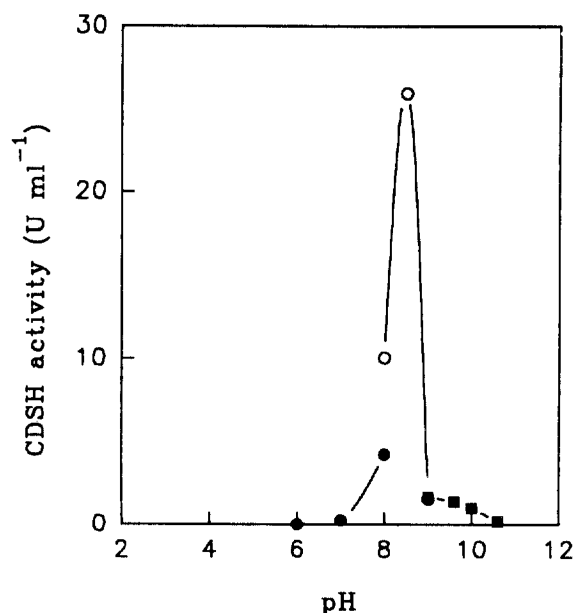

Fig. 4. Optimum pH of cysteine desulfhydrase.

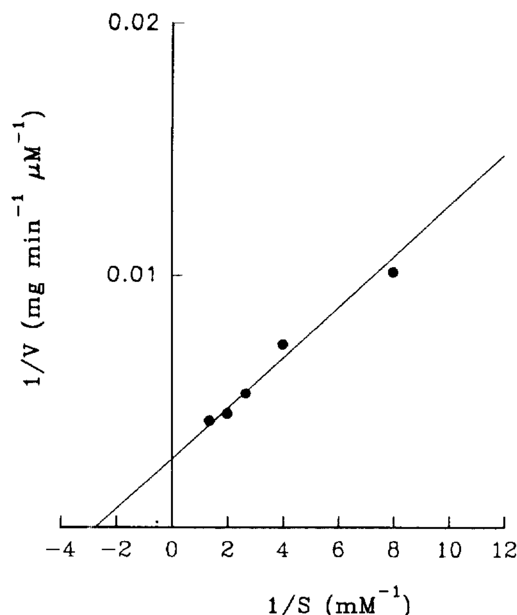

Fig. 5. A Lineweaver-Burk plot for the desulfuration of cysteine. The enzyme was assayed in the presence of different amounts of cysteine at pH 8.5 and 37°C.

homology to any enzyme or protein. This is the first report of the N-terminal amino acid sequence of CDSH.

Optimum reaction conditions and substrate specificity

The optimum pH of the purified CDSH was 8.5 with a sharp decline in activity at pH below and above it (Fig. 4). It is interesting to note that the optimum pH of *Salmonella typhimurium* was also 8.6. The optimum pH of 8.6 was partially due to the pK value of 8.6 for the sulfhydryl group of free cysteine used as the substrate (4). The optimum temperature of CDSH at the optimum pH was 35°C (data not shown).

Table 2. Substrate specificy of cysteine desulfhydrase using various cysteine analogs as a substrate

Substrate	Product formation (μM min^{-1} mg^{-1})
H_2S production from	
L-cysteine	185.9
L-cystine	0
Pyruvate production from	
L-cysteine	20.3
L-cystine	0
D,L-homocysteine	0
L-serine	0
L-cysteinesulfinate	0
L-methionine	0
S-2-aminoethyl-L-cysteine	0
Cysteine production from	
L-cystathionine	0

The catalytic activity of CDSH towards various cysteine analogues were measured (Table 2). The enzyme hydrolyzed L-cysteine to produce hydrogen sulfide, but did not show any activiy against cystine. It produced pyruvate from cysteine, but did not produce pyruvate from L-cystine, D,L-homocysteine, L-serine, L-homoserine, L-methionine, L-phenylalanine and S-2-aminoethyl-L-cysteine. Moreover, CDSH did not show any activity of the elimination reaction of cystathionine, showing no cystathionine γ-lyase activity. In contrast, the CDSH from a mammalian source had been reported to contain cystathionine γ-lyase activity (21). The K_m and V_{max} values obtained with cysteine as a substrate were 0.37 mM and 0.27 μM min^{-1} mg^{-1} (Fig. 5). The K_m of CDSH from *Salmonella typhimurium* was 0.17 to 0.21 mM (10).

From these result, it was clear that the CDSH purified from supernatant of *S. albidoflavus* SMF 301 was a novel enzyme isolated from an actinomycete for the first time.

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References

1. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein u-

tilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

2. **Carlsson J., G.P.D. Granberg, G.K. Nyberg, and M. K. Edlund.** 1979. Bactericidal effect of cysteine exposed to atmospheric oxygen. *Appl. Environ. Microbiol.* **37**, 383-390.
3. **Chater, K.F.** 1984. Morphological and physiological differentiation in *Streptomyces*. In *Microbial Development*, pp. 89-115. Edited by R. Losick & L. Shapiro. Cold Spring Harbor Laboratory.
4. **Collins, J.M. and K.J. Monty.** 1973. The cysteine desulfhydrase of Salmonella typhimurium: Kinetic and catalytic properties. *J. Biol. Chem.* **248**, 5943-5949.
5. **Gaitonde, M.K.** 1967. A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. *Biochem. J.* **104**, 627-633.
6. **Jensen, S.E., W.S. Westlake, and S. Wolfe.** 1982. Cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to penicillins by cell free extracts of *Streptomyces clavuligerus. J. antibiot.* **35**, 483-490.
7. **Kang, S.G., I.S. Kim, Y.T. Rho, and K.J. Lee.** 1995. Production dynamics of extracellular proteases accompanying morphological differentiation of *Streptomyces albidoflavus* SMF301. *Microbiol.* **141**, 3095-3103.
8. **Kang, S.G., I.S. Kim, J.G. Ryu, Y.T. Rho, and K.J. Lee.** 1995. Purification and characterization of trypsin-like protease and metalloprotease in *Streptomyces albidoflavus* SMF301. *J. Microbiol.* **33**, 307-314.
9. **Kim, J.W., S.K. Kang, Y.T. Rho, and K.J. Lee.** 1994. L-cysteine metabolism and the effects on mycelium growth of *Streptomyces albidoflavus* SMF301 in submerged culture. *J. Microbiol. Biotechnol.* **4**, 159-164.
10. **Kredich, N.M., B.S. Keenan, and L.J. Foote.** 1972. The purification and subunit structure of cysteine desulfhydrase from Salmonella typhimurium. *J. Biol. Chem.* **247**, 7157-7162.
11. **Kredich, N.M., L.J. Foote, and B.S. Keenan.** 1973. The stoichiometry and kinetics of the inducible cysteine desulfhydrase from *Salmonella typhimurium. J. Biol. Chem.* **248**, 6187-6196.
12. **Kumagai, H., S. Sejima, Y.J. Choi, H. Tanaka, and H. Yamada.** 1975. Crystallization and properties of cysteine desulfhydrase from *Aerobacter aerogenes. FEBS Lett.* **52**, 304-307.
13. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
14. **Lee, K.J. and Y.T. Rho.** 1993. Characteristics of spores formed by surface and submerged cultures of *Streptomyces albidoflavus* SMF301. *J. Gen. Microbiol.* **139**, 3131-3137.
15. **Morra, M.J. and W.A. Dick.** 1991. Mechanisms of H_2S production from cysteine and cystine by microorganisms isolated from soil by selective enrichment. *Appl. Env. Microbiol.* **57**, 1413-1417.
16. **Nagasawa, T., H. Kanzaki, and H. Yamada.** 1984. Cystathionine γ-lyase of *Streptomyces phaeochromogens*: The occurrence of cystathionine γ-lyase in filamentous bacteria and its purification and characterization. *J. Biol. Chem.* **259**, 10393-10403.
17. **Nyberg, G.K., G.P.D. Granberg, and J. Carlsson.** 1979. Bovine superoxide dismutase and copper ions potentiate the bactericidal effect of autooxidizing cysteine. *Appl. Envion. Microbiol.* **38**, 29-34.
18. **Ohkishi, H., D. Nishikawa, H. Kumagai, and H. Yamada.** 1981. Distribution of cysteine desulfhydrase in microorganisms. *J. Biol. Chem.* **45**, 253-257.
19. **Rho, Y.T., H.T. Kim, K.H. Oh, H.I. Kang, A.C. Ward, M. Goodfellow, Y.C. Hah, and K.J. Lee.** 1992. Numerical identification of a Streptomyces strain producing spores in submerged culture. *Kor J Microbiol.* **30**, 278-285.
20. **Rho, Y.T. and K.J. Lee.** 1994. Kinetic studies on mycelium growth and spore formation in submerged cultures of *Streptomyces albidoflavus SMF301. J. Gen. Microbiol.* **140**, 2061-2065.
21. **Weinstein, C.L., H. Haschemeyer, and O.W. Griffith.** 1988. *In vivo* studies of cysteine metabolism: Use of D-cysteinesulfinate, a novel cysteinesulfinate decarboxylase inhibitor, to probe taurine and pyruvate synthesis. *J. Biol. Chem.* **263**, 16568-16579.