Occurrence of a Novel Enzyme, L-Lysine Oxidase with Antitumor Activity in Culture Extract of *Trichoderma viride*

Hitoshi KUSAKABE, Kenjiro KODAMA, Haruhiko MACHIDA,
Yuichiro MIDORIKAWA, Akira KUNINAKA, Haruo MISONO*
and Kenji SODA*

Research Laboratory, Yamasa Shoyu Co., Ltd., Choshi 288, Japan

*Laboratory of Microbial Biochemistry, Institute for Chemical
Research, Kyoto University, Uji 611, Japan

Received September 18, 1978

An aqueous extract of culture of *Trichoderma viride* Y244-2 on wheat bran showed antitumor activity against L5178Y mouse leukemic cells *in vitro* and L1210 mouse leukemia *in vivo*. The principle of the antitumor activity was proved to be an L-lysine-oxidizing enzyme. Oxygen consumption was accompanied by the formation of α -keto acid, ammonia and hydrogen peroxide in the enzyme reaction with L-lysine. The ratio of amount of oxygen consumed to those of α -keto acid and ammonia formed in the presence of catalase was approximately 1:2:2. This enzyme is an L-amino acid oxidase which is highly specific for L-lysine. Thus, we designated it as L-lysine oxidase. Identity of the antitumor substance with the enzyme was confirmed by DEAE-cellulose column chromatography: the elution pattern of growth-inhibitory activity against L5178Y cells coincided with that of L-lysine oxidase activity.

The clinical use of L-asparaginase¹⁾ has stimulated the search for other enzymatic therapies for neoplastic diseases. Recently, the several enzymes that degrade essential amino acids have been shown to depress tumor growth. Phenylalanine ammonia-lyase from *Rhodotorula glutinis* inhibits both growth of murine and human leukemic cells *in vitro*²⁾ and growth of L5178Y mouse leukemia *in vivo*.³⁾ Kreis *et al.* reported that methionine γ -lyase from *Clostridium sporogenes* causes inhibition of the growth of P815 and L1210 cells *in vitro*.⁴⁾ Recently, threonine deaminase from sheep liver, which catalyzes the irreversible α , β -elimination of L-threonine, was shown to inhibit the growth of mouse leukemic cells.⁵⁾ No L-amino acid oxidases however, have been reported to have antitumor activity. This is probably owing to the re-amination of α -keto acids produced to yield the original amino acids through enzymatic transaminations in cells.

L-Amino acid oxidases have been isolated from various sources including microorganisms,^{6~9)} snake venom,¹⁰⁾ rat kidney,¹¹⁾ bird liver,^{12~16)} and invertebrates.^{17,18)} L-Lysine is

a poor substrate for almost all of the oxidases. The enzyme from turkey liver^{12~15)} catalyzes the oxidation of L-lysine, but is not specific for L-lysine: it oxidizes efficiently also other basic amino acids such as L-arginine and L-histidine.

In the course of search of new antineoplastic agents, we found a heat-labile antitumor substance with high molecular weight in the aqueous extract of culture of *Trichoderma viride* Y244-2 on wheat bran. The present investigation was undertaken to characterize the substance. We describe here evidence for the occurrence of a new antitumor enzyme, L-lysine oxidase.

MATERIALS AND METHODS

Materials. RPMI 1640 medium¹⁹⁾ was purchased from Nissui Co., Ltd. Trypsin, chymotrypsin and ribonuclease A were obtained from Sigma Chemicals Co. Pronase was from Kaken Chemicals Co. Nuclease P₁ was prepared in Yamasa Shoyu Co., Ltd. Amino acids, vitamins and the other chemicals were of analytical grade.

Microorganism and culture. A mold, strain No. Y244-2, was isolated from a soil sample and identified as *Trichoderma viride* (unpublished data). The mold was grown on wheat bran at 37°C for 7 days. The

cultures were extracted with 10 times amount (v/w) of water for 2 hr at room temperature with gentle shaking. The extract is hereafter referred to as the wheat bran culture extract.

Assay of growth-inhibitory activity against L5178Y cells. L5178Y mouse leukemic cells were grown in RPMI 1640 medium containing 10% calf serum at 37°C. The samples appropriately diluted with phosphate-buffered saline were added to 9 volumes of the suspension of cells in a logarithmic phase between 8×10^4 /ml and 1.2×10^5 /ml. After incubation at 37°C for various periods in 5% CO₂, the number of cells was counted with a microcell-counter. Growth-inhibitory activity of the sample against L5178Y cells was shown as the minimum dilution rate required to induce 50% inhibition of the cell growth after incubation for 48 hr.

Evaluation of antitumor activity against L1210 mouse leukemia. L1210 cells (1×10^5 cells) were inoculated *i. p.* in BDF₁ mice on day 0, then 0.2 ml of the culture extract was injected *i. p.* once daily from day 1 to day 5. Increase of life-span (ILS) was calculated as follows:

$$\text{ILS}(\%) = \left(\frac{\text{Survival time of treated mice}}{\text{Survival time of untreated mice}} - 1 \right) \times 100$$

Analytical procedures. Protein concentration was measured by the method of Lowry *et al.*²⁰⁾ Ammonia was determined with Nessler's reagent by the procedure of Johnson.²¹⁾ α -Keto acids were determined by the method of Soda.²²⁾

Assay of oxygen consumption. Oxygen consumption was measured manometrically. The vessel contained a mixture of 20 μ mol of L-lysine, 170 μ mol of potassium phosphate buffer, pH 7.4, 350 units of bovine liver catalase and crude enzyme in a total volume of 2.0 ml. After the reaction mixture was incubated at 37°C for various times and deproteinized by addition of 0.2 ml of 2 N HCl, ammonia and α -keto acid were determined as described above.

Ammonium sulfate-precipitated preparation. The culture extract was brought to 70% saturation with ammonium sulfate. The precipitate collected by centrifugation was dissolved in 0.02 M potassium phosphate buffer, pH 7.4, and dialyzed against the same buffer. The insoluble materials formed during dialysis were removed by centrifugation.

DEAE-cellulose column chromatography. The dialyzed enzyme solution (3.6 g as protein) was applied to a DEAE-cellulose column (4.5 $\times$ 68 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.4. After the column was washed with 3 liters of the same buffer, the enzyme was eluted with a linear gradient of 0 to 0.6 M NaCl.

RESULTS

Antitumor activity of the culture extract of *Trichoderma viride* Y244-2

The wheat bran culture extract of *Trichoderma viride* Y244-2 was found to have an inhibitory effect on growth of L5178Y cells (Fig. 1). The complete inhibition of cell growth was shown at 1:160 dilution and 50% inhibition was at 1:320 dilution. The sensitivity of another type of cultured cells to the

FIG. 1. Effect of the Culture Extract of *Trichoderma viride* Y244-2 on Growth of L5178Y Cells.

○, control; ●, 1:1280 dilution; △, 1:640 dilution; ▲, 1:320 dilution; □, 1:160 dilution; ■, 1:80 dilution.

FIG. 2. Effect of the Culture Extract of *Trichoderma viride* Y244-2 on Growth of Mouse L-Cells.

L-Cells (4×10^5 cells/plate) were grown in minimum essential medium containing 10% calf serum. After 24 hr incubation at 37°C, the medium was discarded and replaced by the medium containing the culture extract at the point indicated by an arrow. The number of trypsinized cells was determined with a microcell-counter.

○, control; ●, 1:1280; △, 1:640; ▲, 1:320.

extract was tested. Figure 2 shows that the culture extract completely inhibited the growth of mouse L-cells at 1:320 dilution. In addition to these *in vitro* activities, the extract exhibited antitumor activity in mice bearing L1210 leukemia. When administered from day 1 to 5, the dose of 0.2 ml/mouse/day increased the life-span by 30.5%.

The growth-inhibitory activity of the extract against L5178Y cells was not affected by heating at 45°C for 30 min in the pH range of 5 to 10, but was lost completely by treatment at 75°C for 30 min. The active fraction was fully precipitated from the extract by addition of ammonium sulfate (70% saturation), and was not dialyzable. This suggests that the antineoplastic substance is macromolecular. The activity was not influenced by the treatments with trypsin, chymotrypsin, pronase, RNase A and nuclease P₁. When the ammonium sulfate-precipitated preparation was added to the culture of L5178Y cells at various growth time, the growth of the cells was immediately inhibited (Fig. 3). Cytotoxic effect of the preparation on L5178Y cells are also shown in Fig. 4. Aliquots of the cell suspension were taken out at 0, 10, 20 and 40 min after addition of the preparation to L5178Y culture. After the cells were washed twice with RPMI

FIG. 3. Effect of Ammonium Sulfate-precipitated Preparation on Growth of L5178Y Cells.

The ammonium sulfate-precipitated preparation was added to the culture of L5178Y cells at 12, 24, 30 and 48 hr as indicated by arrows.

○, control; ●, 0.1 mg protein/ml.

FIG. 4. Effect of Preincubation Time of L5178Y Cells with Ammonium Sulfate-precipitated Preparation on Growth of the Cells.

L5178Y cells (3×10^5 /ml) were incubated with ammonium sulfate-precipitated preparation (0.1 mg protein/ml) for various times. After the cells were washed with RPMI 1640 medium, the cells were grown in the fresh medium containing 10% calf serum. ○, control; ●, 0 min; △, 10 min; ▲, 20 min; □, 40 min.

1640 medium to remove the active substance and resuspended in the fresh medium supplemented with 10% calf serum; the cell growth was measured over 3 days. The cell growth was depressed considerably by the treatment for 10 to 40 min. After 2 days, the growth of these treated cells were restored.

Antitumor activity and L-lysine-degrading activity

Since the antitumor substance was found to be a biopolymer as described above, we examined whether it is an enzyme that degrades a growth factor such as amino acids and vitamins required for the growth of L5178Y cells. When RPMI 1640 medium was preincubated with the ammonium sulfate-precipitated preparation at 37°C for 24 hr, and heated at about 75°C for 30 min, the growth of L5178Y cells in the pretreated medium was significantly depressed. As shown in Table I, the growth of L5178Y cells was almost fully restored by addition of L-lysine at the same concentration as that in RPMI 1640 medium,¹⁹⁾ but by none of other amino acids and vitamins contained in the medium. This finding suggests that the

TABLE I. EFFECTS OF VITAMINS AND AMINO ACIDS ON GROWTH OF L5178Y CELLS IN THE MEDIUM PREINCUBATED WITH THE AMMONIUM SULFATE-PRECIPITATED PREPARATION

RPMI 1640 medium was preincubated with the ammonium sulfate-precipitated preparation (0.1 mg protein/ml) for 24 hr at 37°C, and then heated at 75°C for 30 min. L5178Y cells (2×10^5 /ml) were added to each tube containing fresh or the preincubated medium supplemented by each of the medium components, and incubated at 37°C for 48 hr. The concentration of the components was the same as that of RPMI 1640 medium.

Exp.	Medium	Addition	Cell growth (%)
1	Fresh	—	100.0
	Preincubated	—	30.6
		Riboflavine	33.8
		Biotin	31.7
		Pantothenic acid	32.5
		Thiamine	30.4
		Nicotinamide	31.2
		Inositol	33.2
		Pyridoxine	30.9
		Choline	32.5
		Folic acid	33.1
2	Fresh	—	100.0
	Preincubated	—	41.3
		L-Arginine	26.3
		L-Asparagine	41.1
		L-Asparagic acid	40.7
		L-Cystine	N. T.
		L-Glutamic acid	47.4
		L-Glutamine	39.9
		L-Glycine	41.6
		L-Histidine	38.3
		L-Hydroxyproline	41.4
		L-Isoleucine	39.3
		L-Leucine	35.5
		L-Lysine	96.9
		L-Methionine	39.2
		L-Phenylalanine	39.2
		L-Proline	46.2
		L-Serine	44.1
		L-Threonine	40.0
		L-Tryptophan	44.1
		L-Tyrosine	44.2

N. T., not tested.

antitumor substance in the culture extract is an enzyme which can degrade L-lysine, an essential amino acid for mammals, in the medium.

Reaction of the crude enzyme preparation with L-lysine

The ammonium sulfate-precipitated preparation was tested for its ability to catalyze the oxidation of L-lysine. The rate of oxygen consumption was measured manometrically. The uptake of oxygen proceeded linearly with time as shown in Fig. 5-a. Ammonia and α -keto acid also were formed linearly (Figs. 5-b and 5-c). Heat-treatment of the enzyme completely abolished both oxygen consumption and formation of ammonia and α -keto acid. The ratio of amount of oxygen consumed to those of ammonia and α -keto acid formed in the presence of catalase was approximately 1:2:2. To determine whether hydrogen peroxide is produced in the reaction, oxygen consumption was measured in the presence or

FIG. 5. Oxygen Consumption, and Formation of α -Keto Acid and Ammonia during the Reaction of the Enzyme with L-Lysine.

Oxygen consumption was measured manometrically. The vessels contained 20 μ mol of L-lysine, 170 μ mol of potassium phosphate buffer, pH 7.4, 350 units of catalase and 0.8 mg of the crude enzyme protein in a total volume of 2.0 ml. After 5, 10, 15 and 20 min of incubation at 37°C, the reaction mixture was deproteinized with the addition of 0.2 ml of 2N HCl. Ammonia and α -keto acid were determined as described in MATERIALS AND METHODS.

●, native enzyme; ○, heat-treated enzyme.

TABLE II. EFFECT OF CATALASE ON OXYGEN UPTAKE

Oxygen uptake was measured manometrically. The vessels contained 170 μ mol of potassium phosphate buffer, pH 7.4, 0 or 350 units of catalase, 20 μ mol of L-lysine, and 0.2 ml of culture extract or ammonium sulfate-precipitated preparation (1.7 mg as protein) in a total volume of 2.0 ml. The reaction was carried out at 37°C for 20 min.

Addition	O ₂ Uptake (μ l)	
	Culture extract	Ammonium sulfate-precipitated preparation
None	32.9	238.0
Catalase	17.7	175.8

absence of catalase. Table II shows that oxygen consumption was significantly reduced by addition of catalase. In the experiment with the culture extract, where relatively small amount of oxygen was consumed, the rate of oxygen consumption was decreased about 50% by addition of catalase. This suggests that the oxidation of L-lysine is accompanied with the formation of hydrogen peroxide. When a considerably large amount of oxygen was consumed with the ammonium sulfate-precipitated preparation, the effect of catalase was decreased. This decline of catalase effect is probably due to a decrease of oxygen concentration and a low affinity of the enzyme for oxygen. These results show that the antineoplastic L-lysine-degrading enzyme is a kind of L-amino acid oxidase that catalyzes the oxidative deamination of lysine.

Substrate specificity of L-lysine-degrading enzyme

The crude L-lysine-degrading enzyme preparation was examined for its ability to catalyze the oxidation of various amino acids (Table III). L-Lysine is the preferred substrate. L-Ornithine, L-phenylalanine, L-arginine and L-histidine were also oxidized to a lesser extent. The other L-amino acids tested and D-lysine were not substrates. This enzyme is distinct from L-amino acid oxidases so far studied in the substrate specificity. Therefore,

TABLE III. SUBSTRATE SPECIFICITY OF L-LYSINE OXIDASE

Oxygen uptake was measured manometrically. The vessels contained 170 μ mol of potassium phosphate buffer, pH 7.4, 350 units of catalase, 20 μ mol of L-amino acid and 1.6 mg of protein (ammonium sulfate-precipitated preparation) in a total volume of 2.0 ml. The reaction was carried out at 37°C for 20 min.

Substrate	O ₂ Uptake (μ l)	Relative activity (%)
L-Lysine	156.6	100.0
L-Ornithine	32.2	20.5
L-Phenylalanine	9.3	5.9
L-Arginine	6.4	4.3
L-Histidine	5.7	3.6
L-Glycine	0	0
L-Alanine	0	0
L-Leucine	0	0
L-Isoleucine	0	0
L-Methionine	0	0
L-Serine	0	0
L-Threonine	0	0
L-Asparagine	0	0
L-Asparagic acid	0	0
L-Glutamic acid	0	0
L-Proline	0	0
D-Lysine	0	0

we designated this enzyme as L-lysine oxidase.

Identity of the antitumor substance with L-lysine oxidase

In order to show the identity of the antitumor substance with L-lysine oxidase, elution patterns of antitumor and L-lysine oxidase activities from a DEAE-cellulose column were compared (Fig. 6). The peak of antitumor activity coincided with that of the enzyme activity. This result also shows that the antitumor activity is ascribed to L-lysine oxidase.

DISCUSSION

In the present study, we identified the antitumor substance found in the wheat bran culture extract of *Trichoderma viride* Y244-2 as L-lysine oxidase, a new L-amino acid oxidase which is highly specific for L-lysine.

In the presence of catalase, 0.5 mol of oxygen is consumed per mol of α -keto acid and ammonia formed. This and other stoichiometric

FIG. 6. DEAE-cellulose Column Chromatography of L-Lysine Oxidase.

Oxygen uptake was measured in the absence of catalase. Experimental conditions are described in MATERIALS AND METHODS. ○, O₂ uptake; ●, ammonia formation; △, L5178Y growth inhibition; ----, A₂₈₀; —, NaCl concentration.

studies of the enzymatic oxidation of lysine (Fig 5 and Table II) show that L-lysine is oxidized to equimolar amounts of α -keto acid, ammonia and hydrogen peroxide with consumption of equimolar amount of oxygen. We have not yet investigated which amino group of L-lysine is eliminated by the oxidation. The enzyme catalyzes also the oxidation of L-ornithine, L-phenylalanine, L-arginine and L-histidine. The results suggest on the analogy of the oxidation of α -amino acids that α -amino group of L-lysine is oxidized to yield α -keto- ϵ -aminocaproate as follows:

α -keto- ϵ -aminocaproate is probably in equilibrium with the cyclized form, Δ^1 -piperideine-2-carboxylate.

Most of L-amino acid oxidases from various sources act only slightly on L-lysine. The oxidases from turkey liver^{12~15}) and *Proteus rettgeri*⁹) oxidize effectively L-lysine and several other basic amino acids. Paik *et al.* reported that L-lysine can be oxidized by snake venom L-amino acid oxidase when the pH is favor-

able.²³) However, these oxidases oxidize efficiently various other amino acids as well as L-lysine. The enzyme from *Trichoderma viride* Y244-2 is highly specific for L-lysine as indicated in Table III. The enzyme is unique in this regard, and thus, was designated as L-lysine oxidase. The enzyme plays probably an important role in the lysine metabolism *via* α -keto- ϵ -aminocaproate (Δ^1 -piperideine-2-carboxylate) proposed early by Rothstein.^{24~26})

Since the principle of antitumor activity found in guinea pig serum was identified as asparaginase,^{27,28}) several enzymes that degrade a factor required for tumor growth have been studied, *e.g.* phenylalanine ammonia-lyase,^{2,3}) tyrosine phenol-lyase,²⁹) methionine γ -lyase,⁴) threonine deaminase⁵) and carboxypeptidase G₁.^{30,31}) The antitumor activity of these enzymes except asparaginase was demonstrated with the purified enzyme preparations. In the present study we demonstrated a novel antitumor enzyme, L-lysine oxidase in the wheat bran culture extract of the mold. We have previously shown by the same procedure as shown in Table I that the principle of antitumor activity found in the wheat bran culture extract of *Aspergillus* sp. Y8-5 is a folate-deaminating enzyme.³²) This procedure is, therefore, widely applicable to a screening test for the heat-labile and high-molecular

antineoplastic substances that degrade a growth factor.

The close relationship between L-lysine oxidase activity and the growth-inhibitory activity against L5178Y cells was observed with the culture extract and the ammonium sulfate-precipitated preparation. The identity of the antitumor substance with the enzyme was also confirmed by DEAE-cellulose chromatography (Fig. 6): the fractions containing L-lysine oxidase exclusively showed growth-inhibitory activity against L5178Y cells. In addition, the enzyme purified to homogeneity exhibited the antitumor activity against L5178Y cells *in vitro* and L1210 leukemia *in vivo*.³³⁾ Therefore, the enzyme is responsible for the antitumor activity.

When *Trichoderma viride* Y244-2 was grown on a liquid medium containing potato extract at 28°C for 4 days, the culture filtrate diluted 1:50 showed no significant activity against L5178Y cells (data not presented). No appreciable oxygen uptake was also observed, when the culture filtrate was incubated with L-lysine. These findings suggest that L-lysine oxidase is produced specifically by cultivation on wheat bran. The wheat bran culture is known to be suitable for the production of several extracellular hydrolases such as nuclease P₁.³⁴⁾ The present work provides the first example of the production of extracellular L-amino acid oxidase by wheat bran culture. Further characterization of L-lysine oxidase and the detailed study of the enzyme production by wheat bran culture are currently in progress.

REFERENCES

- 1) R. H. Adamson and S. Fabro, *Cancer Chemother. Rep.*, **52**, 617 (1968).
- 2) C. W. Abell, W. J. Stith and D. S. Hodgins, *Cancer Res.*, **32**, 258 (1972).
- 3) C. W. Abell, D. S. Hodgins and W. J. Stith, *ibid.*, **33**, 2529 (1973).
- 4) W. Kreis and C. Hession, *ibid.*, **33**, 1866 (1973).
- 5) R. S. Greenfield and D. Wellner, *ibid.*, **37**, 2523 (1977).
- 6) P. K. Stumpf and D. E. Green, *J. Biol. Chem.*, **153**, 387 (1944).
- 7) S. G. Knight, *J. Bacteriol.*, **55**, 401 (1948).
- 8) K. Burton, *Biochem. J.*, **50**, 258 (1952).
- 9) J. A. Duerre and S. Chakrabarty, *J. Bacteriol.*, **121**, 656 (1975).
- 10) D. Wellner, "Methods in Enzymology," Vol. XVIIIB, ed. by H. Tabor and C. W. Tabor, Academic Press Inc., New York and London, 1971, p. 597.
- 11) M. Nakano and T. S. Danowski, *ibid.*, p. 601.
- 12) P. Boulanger and R. Osteux, *Compt. Rend. Acad. Sci.*, **241**, 125 (1955).
- 13) P. Boulanger, J. Coursaget, J. Bertrand and R. Osteux, *ibid.*, **244**, 2255 (1957).
- 14) P. Boulanger, J. Bertrand and R. Osteux, *Biochim. Biophys. Acta*, **26**, 143 (1957).
- 15) P. Boulanger and R. Osteux, *ibid.*, **21**, 552 (1956).
- 16) J. Struck and I. W. Sizer, *Arch. Biochem. Biophys.*, **90**, 22 (1960).
- 17) H. Blaschko and D. B. Hope, *Biochem. J.*, **62**, 335 (1956).
- 18) J. Roche, P. Glahn, P. Manchon and N. Thoai, *Biochim. Biophys. Acta*, **35**, 111 (1959).
- 19) G. Moore, D. Mount, G. Tara and N. Schwartz, *Cancer Res.*, **23**, 1735 (1963).
- 20) O. H. Lowry, N. J. Rosebrough, A. L. Faar and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 21) M. J. Johnson, *ibid.*, **137**, 575 (1941).
- 22) K. Soda, *Anal. Biochem.*, **25**, 228 (1968).
- 23) W. K. Paik and S. Kim, *Biochim. Biophys. Acta*, **96**, 66 (1965).
- 24) M. Rothstein and L. L. Miller, *J. Biol. Chem.*, **211**, 851 (1954).
- 25) M. Rothstein and L. L. Miller, *J. Am. Chem. Soc.*, **76**, 1459 (1954).
- 26) M. Rothstein and D. M. Greenberg, *J. Biol. Chem.*, **235**, 714 (1960).
- 27) J. D. Broom, *Nature*, **191**, 1114 (1961).
- 28) J. D. Broom, *J. Exptl. Med.*, **118**, 99 (1963).
- 29) G. G. Meadows, J. DiGiovanni, L. Minor and G. W. Elmer, *Cancer Res.*, **36**, 167 (1976).
- 30) B. A. Chabner, P. L. Chello and J. R. Bertino, *ibid.*, **32**, 2114 (1972).
- 31) B. A. Chabner, D. G. Johns and J. R. Bertino, *Nature*, **239**, 395 (1972).
- 32) H. Kusakabe, K. Kodama, Y. Midorikawa, H. Machida, A. Kuninaka and H. Yoshino, *Agric. Biol. Chem.*, **38**, 1753 (1974).
- 33) H. Kusakabe, K. Kodama, A. Kuninaka and K. Soda, in preparation.
- 34) M. Fujimoto, A. Kuninaka and H. Yoshino, *Agric. Biol. Chem.*, **38**, 777 (1974).