Jpn. J. Cancer Res. 84, 1195–1200, November 1993

# Chemical Modification by Polyethylene Glycol of the Anti-tumor Enzyme Arginine Deiminase from *Mycoplasma arginini*

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**Amino acid-degrading enzymes are known to inhibit the growth of tumor cells in culture by depleting amino acids in the medium. Here we demonstrate that arginine deiminase (EC 3.5.3.6) from *Mycoplasma arginini* had stronger growth-inhibitory activity against all 4 kinds of tumor cell lines tested than L-asparaginase and arginase, which are well-known anti-tumor enzymes. Next, chemical modification of the arginine deiminase molecule with polyethylene glycol was shown to enhance its potency as an anti-tumor enzyme. The percentage of modified amino groups per molecule was estimated to be 51% of the total amino groups, and the average molecular weight was estimated to be about 400,000 by gel-filtration HPLC. The enzymic activity of the modified enzyme was 25.5 units/mg protein, which was equivalent to 57% of that of the native enzyme. The modified enzyme strongly inhibited growth of a mouse hepatoma cell line, MH134, at a concentration of more than 10 ng/ml, showing almost the same dose-response curve as the native enzyme. When a bolus of 5 units of the modified enzyme was intravenously injected into male $BDF_1$ mice, L-arginine in the blood completely disappeared within 5 min, and remained undetectable for more than 8 days. On the other hand, in the case of bolus injection of the same number of units of native enzyme, the plasma L-arginine level recovered up to 66% of the control level at 8 days. These results suggest that this modified enzyme has a longer plasma clearance time and may be more effective as a new anti-tumor agent than the native enzyme.**

Key words: Arginine deiminase — Chemical modification — Polyethylene glycol — Growth inhibition — Anti-tumor agent

Potency of anti-tumor activities of amino acid-degrading enzymes depends on various enzymic properties such as the Michaelis constant ($K_m$), specific activity, optimum pH, and stability, as well as susceptibility of target tumor cells to the lack of specific amino acids. For example, L-asparaginase (EC 3.5.1.1),[1,2] arginase (EC 3.5.1.1),[3,4] phenylalanine ammonialyase (EC 4.3.1.5),[5] and tryptophanase (EC 4.1.99.1)[6] are known to inhibit growth of tumor cells by depleting the respective amino acids in culture. However, only L-asparaginase from *Escherichia coli* has been successfully used for clinical tumor therapy, and its use has been limited to leukemia and lymphosarcoma.[7] Recently, we reported that arginine deiminase (EC 3.5.3.6) purified from *Mycoplasma arginini* strongly inhibited the growth of various kinds of human tumor cells *in vitro*,[8] and prolonged the survival time of mice implanted with four kinds of tumors,[9] hepatoma (MH134), colon carcinoma (Colon 26), sarcoma (S-180), and melanoma (B16). No toxic effect of the enzyme was evident in the mice at doses up to about 100 times higher than the minimum effective dose *in vivo*.[9] These characteristics of *M. arginini*-derived arginine deiminase mean that it is a promising candidate for use as a new anti-tumor agent.

There are two general problems involved in the therapeutic use of such microbial enzymes, that is, antigenicity and rapid plasma clearance. To overcome these problems, many attempts have been made to alter the properties of the enzymes by chemical modification with various kinds of polymers such as polysaccharides,[10] polyethers,[11] and amino acid polymers.[12] However, the retention of catalytic activity following modification has been poor in many cases. Polyethylene glycol (PEG) is well known to have convenient properties as a chemical modifier, such as low toxicity, low antigenicity, and increased bioavailability of the modified protein.[13] So modification of enzymes, hormones, lymphokines, and other proteins with PEG has been carried out in many laboratories. For example, the modification of L-asparaginase with PEG decreased antigenicity[14] and plasma clearance rate[15,16] of the native enzyme, and enhanced its anti-tumor potency in clinical therapy.[17]

In the present study, four kinds of mouse tumor cell lines were used to compare the *in vitro* growth-inhibitory activity of arginine deiminase from *M. arginini* with that of L-asparaginase and arginase. Next, we synthesized and purified PEG-modified arginine deiminase, and defined

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its physical and enzymic properties. Moreover, to test the possible usefulness of this modified enzyme as a new anti-tumor drug, its *in vitro* growth-inhibitory activity against tumor cells and the L-arginine level in the blood after its i.v. injection into mice were examined.

## MATERIALS AND METHODS

**Mouse tumor cell lines** Hepatoma cell line MH134 was a kind gift from Dr. H. Taguchi, SRL Company, Tokyo; colon carcinoma cell line Colon 26 was generously donated by Dr. T. Tashiro, Japanese Foundation for Cancer Research, Tokyo; and sarcoma cell line S-180 and leukemia cell line L1210 were purchased from Dainippon Seiyaku, Tokyo.

**Materials** Monomethoxypolyethylene glycol with average molecular weight of 5,000 (Aldrich) was used without further purification. Cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) was obtained from Wako Pure Chemical Industries (Osaka). Activated $PEG_2$ (2-methoxy-polyethylene glycol-4,6-dichloro-1,3,5-triazine) was prepared as described by Kamisaki *et al.*[15] Arginine deiminase was purified to homogeneity from the cell extract of *M. arginini* as previously reported.[9] L-Asparaginase from *E. coli* (more than 200 units/mg protein) and arginase from bovine liver (215 units/mg protein) were purchased from Seikagaku Corporation (Tokyo) and Sigma Chemical Corporation (St. Louis, MO), respectively.

**Preparation of PEG-arginine deiminase** The chemical modification of arginine deiminase with activated $PEG_2$ was carried out by the method of Abuchowski *et al.*[11] Activated $PEG_2$ (1.6 mg) was added to 20 ml of enzyme solution (2.5 mg/ml) in 0.1 *M* sodium carbonate buffer (pH 9.0), and the mixture was stirred at 37°C for 30 min. Then 180 ml of cold 1 *M* potassium phosphate buffer (pH 7.0) was added to the sample solution to stop the reaction. The reaction mixture was concentrated to 30 ml with an ultrafiltration membrane (Amicon Diaflo YM-30). Next, the PEG-arginine deiminase was purified by gel-filtration chromatography on a Sephacryl S-300 HR (Pharmacia) column (4.4×90 cm) with 10 m*M* potassium phosphate buffer (pH 7.0) used for elution.

**Characterization of PEG-arginine deiminase** The degree of modification at amino groups in the arginine deiminase molecule was determined by measuring the amount of free amino groups by the calorimetric method using trinitrobenzene sulfonate.[18] The protein concentration of native- and PEG-arginine deminase was determined by the Lowry method with bovine serum albumin as the standard. The enzymic activity of arginine deiminase was assayed as previously described according to the method of Oginski.[19] One unit of enzymic activity was defined as the amount of enzyme that converted 1 $\mu$mol of L-arginine to L-citrulline per minute under the assay conditions employed. The molecular weight of the enzymes was analyzed by gel-filtration HPLC on a TSKgel G3000 $SW_{XL}$ column (Toso, Tokyo).

**Assay of *in vitro* growth-inhibitory activity** The growth-inhibitory activities of three amino acid-degrading enzymes, arginine deiminase, L-asparaginase, and arginase, were assessed in 4 kinds of mouse tumor cell lines. The test cells ($1.0\times10^4$ cells/well) were inoculated in triplicate into the wells of 24-well microplates, each well containing 1.0 ml of basal medium (MEM medium was used for S-180, Colon 26, and L1210 cells; and PRMI 1640 medium, for MH134 cells) supplemented with 10% fetal bovine serum (Irvine Scientific, Santana, CA). Twenty microliters of sterilized enzyme solution was then added to each well to a final concentration of 1 to 1000 ng/ml, and the plates were incubated at 37°C in a humidified 5% $CO_2$-95% air atmosphere. After 3 days in culture, the cell number was determined with an automatic cell counter (Coulter, Hialeah, FL). MH134 cells were used for the comparison of growth-inhibitory activities between the native enzyme and PEG-arginine deiminase at final enzyme concentrations of 2.5 to 40 ng/ml.

**Analyses of amino acids in plasma** The same number of units (5 units/mouse) of native enzyme or PEG-arginine deiminase was singly injected into the tail vein of male $BDF_1$ mice (N=3 for each enzyme). Blood samples were obtained periodically from the tail vein, and amino acids in the blood were modified by use of the fluorescent reagent NBD-F (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole) according to the method of Imai and Watanabe.[20] Modified L-arginine and L-citrulline were analyzed by reverse-phase high-performance liquid chromatography (HPLC) on a $\mu$-Bondapack $C_{18}$ (Waters, Milford, MA) column (10 $\mu$m, 3.9×300 mm).

## RESULTS

***In vitro* growth-inhibitory activities of amino acid-degrading enzymes** The *in vitro* growth-inhibitory activities of arginine deiminase, L-asparaginase, and arginase against 4 kinds of mouse tumor cell lines are shown in Fig. 1. Among the three enzymes, arginine deiminase most strongly inhibited the growth of all tumor cells tested at concentrations of 10 or 100 ng/ml. When the enzyme was added to the culture medium at a concentration of 100 ng/ml or higher, all tumor cells were killed during the 3-day culture by the depletion of L-arginine owing to the catalytic reaction of the enzyme. The potency of growth-inhibitory activity of L-asparaginase on L1210 cells was almost the same as that of arginine deiminase. However, L-asparaginase showed about 10-fold weaker growth-inhibitory activity than arginine deiminase against the other tumor cell lines. In the case of arginase,

the growth of MH134 cells was not inhibited until a concentration of 1 $\mu$g/ml was used, and growth of the other cell lines was hardly affected even at this concentration. These results demonstrate that the *in vitro* growth-inhibitory activity of arginase was more than 100 times weaker than that of arginine deiminase against these tumor cell lines.

**Preparation and properties of PEG-arginine deiminase**

The native arginine deiminase was modified with monomethoxypolyethylene glycol by use of cyanuric chloride as a coupler. The synthesized PEG-arginine deiminase was purified by gel-filtration chromatography on a Sephacryl S-300 column. The eluted fractions corresponding to an apparent molecular weight of more than about 200,000 were pooled as the PEG-arginine deiminase fraction (data not shown). The purity and molecular weight of both native and modified enzymes were analyzed by gel-filtration HPLC on a TSKgel G3000 $SW_{XL}$ column.

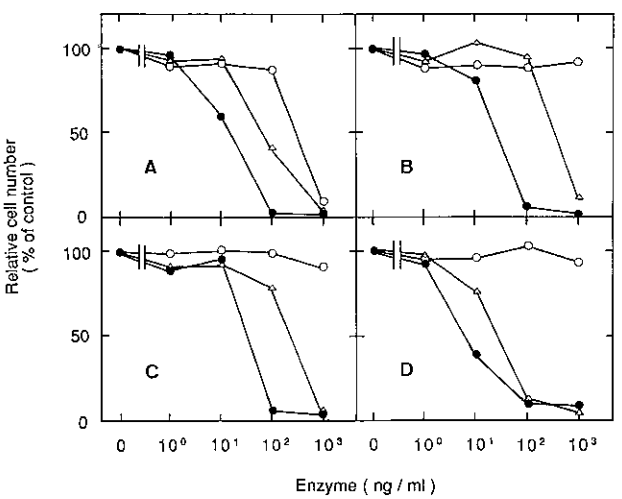


Fig. 1. Effects of arginine deiminase (●), arginase (○), and L-asparaginase (△) on growth of MH134 (A), S-180 (B), Colon 26 (C), and L1210 (D) cells. Each point represents the mean obtained from triplicate wells. The average cell number per well ($\times 10^{-5}$) in control cultures after 3 days was 13.0 (MH134), 4.97 (S-180), 8.47 (Colon 26), and 22.6 (L1210). Other experimental conditions are described "Materials and Methods."

The native enzyme was eluted at a retention time corresponding to a molecular weight of 90,000 and showed a single sharp peak (Fig. 2A). On the other hand, the modified enzyme showed a broad peak corresponding to a molecular weight range of 300,000 to 500,000 (Fig. 2B). The average molecular weight of PEG-arginine deiminase was estimated to be about 400,000. The enzyme activities of native and modified arginine deiminase were 44.5 and 25.5 units/mg protein, respectively;

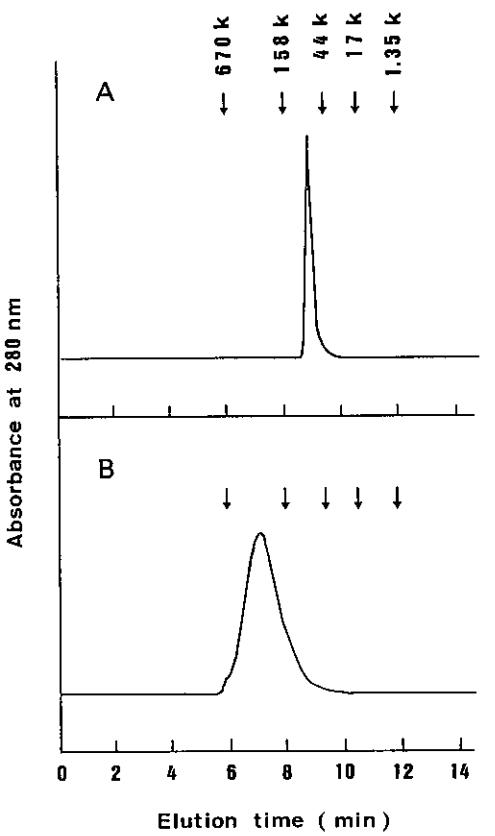


Fig. 2. Gel-filtration HPLC of native- (A) and PEG-arginine deiminase (B) on a TSK G3000 $SW_{XL}$ column. The protein standards used were thyroglobulin (Mr 670,000), gamma globulin (Mr 158,000), ovalbumin (Mr 44,000), myoglobin (Mr 17,000), and vitamin B12 (Mr 1,350).

Table I. Molecular Weight, Enzyme Activity, and Degree of PEG-modification of Native Enzyme and PEG-arginine Deiminase

| Enzyme | Molecular weight | Enzymic activity (U/mg protein) | PEG-modification (%) |
|---|---|---|---|
| Native arginine deiminase | 90,000 | 44.5 (100%) | 0 |
| PEG-arginine deiminase | 400,000 | 25.5 (57.3%) | 51 |

i.e., 43% of the enzymic activity was lost by the modification. The degree of modification of the amino groups per molecule was determined to be 51% by measuring the amount of free amino groups in both native and modified enzyme molecules (Table I).

***In vitro* growth-inhibitory activity** The *in vitro* growth-inhibitory activity of native enzyme and PEG-arginine deiminase against mouse hepatoma cell line MH134 is shown in Fig. 3. Both enzymes strongly inhibited the growth of the tumor cells, showing almost the same sigmoidal dose-response curves. The concentration required for 50% growth inhibition ($IC_{50}$) was estimated to be about 8 ng protein/ml for the native enzyme and about 9 ng protein/ml for the modified one. When the enzymes were added to the culture medium at concentrations of more than 20 ng/ml, the tumor cells were almost completely killed during the 3-day incubation. In another *in vitro* experiment, mouse colon carcinoma cell line Colon 26 was also inhibited by these enzymes with almost the same dose-response curves as for MH134 cells (data not shown).

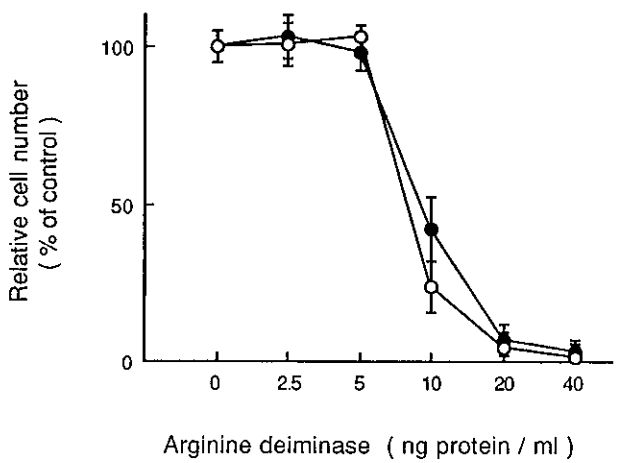


Fig. 3. Effects of various concentrations of native enzyme (○), and PEG-arginine deiminase (●) on growth of MH134 cells. Each point represents the mean±SD (bar) from triplicate wells. The average cell number per well in control cultures after 3 days was $8.93\times10^5$.

**Analyses of L-arginine and L-citrulline in plasma** To study the potency of PEG-arginine deiminase as an anti-tumor enzyme, the concentration of L-arginine and L-citrulline in mouse plasma was analyzed periodically after a single i.v. injection of native or modified enzyme (Table II). L-Arginine was completely cleared from the plasma within 5 min after injection of either enzyme, with a reciprocal increase in L-citrulline. In the case of the modified enzyme, L-arginine remained undetectable for at least 8 days, and then recovered to 15% of the control level by 15 days, while L-citrulline was increased 16-fold over the control level at 3 days, and the level decreased gradually to 142% of the control level by 15 days. In the case of the native enzyme, L-arginine remained undetectable for 3 days and then its level recovered to 66% of the control level by 8 days. The maximum citrulline level was about 4 times that of the control at 3 days. These results indicate that the plasma clearance time of native arginine deiminase was prolonged by the modification with PEG. No toxic effect of PEG-arginine deiminase was observed in any mouse during the experiments (data not shown).

Table II. Time Course of Plasma Levels of L-Arginine and L-Citrulline in Mice after Intravenous Injection (5 units/mouse) of Native- or PEG-arginine Deiminase

| Time after injection | Plasma concentration±SD ($\mu M$) (% of control) | | | |
|---|---|---|---|---|
| | Native arginine deiminase | | PEG-arginine deiminase | |
| | L-Arginine | L-Citrulline | L-Arginine | L-Citrulline |
| Control | 177.0±23.3 (100) | 68.4±14.9 (100) | 242.0±31.2 (100) | 77.6±2.0 (100) |
| 5 min | <5.0[a] (<3) | 233.1±31.0 (341) | <5.0[a] (<3) | 287.0±34.7 (370) |
| 3 days | <5.0[a] (<3) | 289.6±27.5 (423) | <5.0[a] (<3) | 1245.9±318.9 (1606) |
| 8 days | 117.3±18.7 (66) | 65.8±19.9 (96) | <5.0[a] (<3) | 340.6±79.2 (439) |
| 15 days | 91.4±5.4 (52) | 135.5±2.7 (198) | 36.8±40.3 (15) | 109.9±13.2 (142) |

*a*) The detection limit for plasma L-arginine level was 5.0 $\mu M$.

## DISCUSSION

Recently we studied the growth-inhibitory activity of arginine deiminases derived from various kinds of microorganisms, such as *Mycoplasma, Pseudomonas,* and *Streptococcus.* As a result, arginine deiminase purified from *M. arginini* showed potent anti-tumor activities against various kinds of tumor cells *in vitro* and *in vivo*,[8, 9] whereas that from *M. hominis* showed low growth-inhibitory activity, presumably because of its acidic optimum pH (data not shown). In the present study, the *in vitro* growth-inhibitory activity of arginine deiminase from *M. arginini* was compared with that of L-asparaginase from *E. coli* and with that of arginase from bovine liver, both of which are well-known anti-tumor amino acid-degrading enzymes. Among the three enzymes, arginine deiminase most strongly inhibited the growth of all four cell lines tested. Both L-asparaginase and arginase are known to inhibit the growth of leukemia cells.[1, 4] In accord with these previous findings, the former enzyme showed potent growth-inhibitory activity against leukemia cell line L1210 at a concentration of more than 10 ng/ml. However, the latter enzyme did not inhibit the growth of L1210 cells even at 1 $\mu$g/ml, whereas arginine deiminase inhibited it at 10 ng/ml. This great difference between two arginine-degrading enzymes in their growth-inhibitory activities seems to originate largely from the difference in their $K_m$ values for L-arginine: 0.2 m*M* with arginine deiminase[9] and 10.5 m*M* with arginase.[21] These results suggest that arginine deiminase from *M. arginini* is a promising candidate as an anti-tumor drug, so we selected this enzyme as a new target protein for chemical modification.

Tumor therapy using amino acid-degrading enzymes is dependent on the plasma concentration of the amino acid that is the substrate for the enzyme, so plasma clearance time of the enzyme is a very important factor determining the efficacy of an enzyme as an anti-tumor drug. For example, it is known that L-asparaginase from *E. coli,* which has a half-life time of 2.5–7.3 h in mouse blood, shows anti-tumor activity *in vivo,* whereas the enzyme from yeast, which is completely eliminated from the blood within 1 h, does not.[22] In a previous study, we reported that arginine deiminase purified from *M. arginini* had a half-life time of 4 h, and showed anti-tumor activity in mice implanted with various kinds of tumor cells.[9] However, the *in vivo* minimum effective dose of this enzyme (10 mg/kg for single i.v. injection, and 0.2 mg/kg for 10 daily i.v. injections against mouse bearing hepatoma cell line MH134) as a biological anti-tumor reagent seems a little higher. These previous results suggest that if the stability of this enzyme in the blood stream can be increased, the potency of its anti-tumor activity would be enhanced.

In the present study, we prepared PEG-arginine deiminase with almost the same *in vitro* growth-inhibitory activity as the native enzyme ($IC_{50}$ values were 8 ng protein/ml for native enzyme and 9 ng protein/ml for modified enzyme), whereas the enzymic activity of PEG-arginine deiminase was significantly lower than that of native enzyme (57.3%). These results suggest that PEG-arginine deiminase in the culture medium may be more stable than the native enzyme to proteases present in the fetal bovine serum added to the culture medium.

We previously reported that arginine deiminase from *M. arginini* is a dimeric protein consisting of two identical subunits.[9] Recently, our laboratory determined its complete nucleotide sequence of 1,230 bp, coding 410 amino acids (nucleotide sequence accession number in the EMBL Data Library is X54141). One subunit of this enzyme has 29 primary amino groups, i.e., 28 lysine side chains and one N-terminal residue, at which modification with activated $PEG_2$ is most likely to occur. Therefore when 51% of the primary amino groups in the dimeric form of this enzyme was modified by activated $PEG_2$, the number of covalently bound activated $PEG_2$ molecules (average molecular weight about 10,000) was calculated at about 30. Consequently the molecular weight of PEG-arginine deiminase was calculated at about 390,000, because the molecular weights of native enzyme and 30 equivalents of activated $PEG_2$ were 90,000 and 300,000, respectively. Actually the average molecular weight of modified enzyme was estimated to be about 400,000, ranging from 300,000 to 500,000, by gel-filtration HPLC in this study. This experimental result is in good accord with the calculated value.

The anti-tumor potency of arginine deiminase depends on the disappearance time of L-arginine from the blood after its administration. So we examined the L-arginine level in mouse plasma after a single i.v. injection of native enzyme or PEG-arginine deiminase. The modified enzyme significantly prolonged the L-arginine disappearance time in mouse plasma as compared with the native enzyme. When a single i.v. injection of 5 units (about 0.2 mg protein) of PEG-arginine deiminase was administered to mice, L-arginine remained undetectable in the blood stream for at least 8 days. This result suggests that an injection of PEG-arginine deiminase at a dose of 5 units/mouse (about 10 mg protein/kg) only once a week may be sufficient to exhibit maximum *in vivo* anti-tumor activity, whereas 10 daily injections of native enzyme at a dose of 5 units/mouse (about 5 mg protein/kg) were needed to achieve the same result in the previous study.[9] However, further therapeutic, toxicological, and immunological studies on PEG-arginine deiminase are required prior to its clinical trial as a new anti-tumor agent.

*(Received June 14, 1993/Accepted August 16, 1993)*

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