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Antitumor Potentiality of Enzyme Preparations of Pumpkin Ascorbate Oxidase and Shiitake Mushroom Polyphenol Oxidase

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A crude preparation of ascorbate oxidase was prepared from Japanese pumpkin by treating extract successively with barium acetate, ammonium sulfate, magnesium sulfate and alumina. On the other hand, a purified preparation was obtained by barium acetate treatment, ammonium sulfate fractionation, acetone precipitation, twice chromatography on DEAE cellulose column and gel filtration with sephadex G 100. Both preparations contained antitumor potentiality against sarcoma-180, even though ascorbate oxidase activity had been decreased after long storage in frozen state or had been completely lost by carboxymethylation of ascorbate oxidase preparation. In addition, antitumor potentiality was maintained more or less when ascorbate oxidase had been inactivated by heating or splitting off with trypsin. Thus, it was indicated that the antitumor potentiality of ascorbate oxidase preparation was attributed to the protein itself, but has no intimate relationship with the enzymatic activity.

On the other hand, a crude preparation of polyphenol oxidase, a similar copper enzyme to ascorbate oxidase, was prepared from shiitake mushroom by treatments with acetone, calcium acetate and ammonium sulfate. Its antitumor potentiality was also confirmed for sarcoma-180 and MH-134.

INTRODUCTION

In connection with the antitumor potentiality of ascorbic acid (AA) and its derivatives (Nakamura and Yamafuji, 1968; Yamafuji *et al.*, 1971b; Omura *et al.*, 1974), it was found that the crude preparation of ascorbate oxidase (AAO; E.C. 1. 10. 3. 3) obtained from pumpkin (*Cucurbita moschata* Duchesne) repressed the growth of implanted sarcoma-180 (Omura *et al.*, 1973). In addition, it was presumed that the antitumor potentiality was attributed to AAO protein itself rather than the enzymatic activity. Therefore, study was further carried out to confirm the above presumption with the much purified AAO preparation.

On the other hand, the antitumor potentiality of AAO protein is interesting, because AAO is copper-containing enzyme and Cu^{2+} plays an important role in the repression of the growth of tumor by reductones (Yamafuji and Mura-

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kami, 1968; Yamafuji et *al.*, 1970; Murakami and Yamafuji, 1970). Since the other copper-containing enzymes were expected to possess the similar potentiality, polyphenol oxidase (PPO; E.C. 1. 10. 3. 1) from shiitake mushroom (*Lentinus edodes* (Berk.) Sing.) was also examined.

EXPERIMENTAL

Assay of antitumor potentiality

The antitumor potentiality was assayed by the usual procedure (Omura et *al.*, 1974) described in the preceding paper.

Assay of AAO activity

According to Shen *et al.* (1945), AAO activity was estimated as follows, because the procedure was more convenient than the manometrical method of Dawson and Magee (1955), while with less accuracy.

One ml of AA (2 mg/ml) was added to 5 ml of enzyme solution (0.2 mg/ml 0.1 M phosphate buffer, pH 5.7) and incubated at 15°C for 10 minutes. Reaction was stopped by adding 2 drops of glacial acetic acid and filled up to 50 ml with water. The solution was titrated with 0.3 % 2,6-dichlorophenol-indophenol solution and the activity was obtained according to the following equation.

$$\text{Activity unit} = \left(1 - \frac{A - B_1}{C - B_2}\right) \times 100$$

where A : titration value against to the mixture after reaction

B₁ : titration value against to the enzyme solution

B₂ : titration value against to 50 ml of aqueous acetic acid solution

C : titration value of 1 ml of AA solution.

Specific activity was also expressed by AAO unit per mg protein which had been estimated by the Folin's method or the absorption at 280nm.

Assay of PPO activity

PPO is generally estimated by the manometrical method. However, for convenience, the activity was assayed by estimating the increase of absorbancy due to browning of the reaction mixture accompanied with oxidation of catechol as substrate. The reaction mixture containing 2 ml of 0.03 M catechol, 2 ml of 0.05 M phosphate buffer (pH 6.8) and 1 ml of the enzyme solution, was incubated at 37°C for 2 hours, and the increase in absorbancy at 470nm (E₄₇₀) was estimated. In the control, the enzyme solution inactivated by heating at 100°C for 10 minutes was employed. Protein content in the enzyme solution was indicated as the absorbancy at 280 nm (E₂₈₀). Specific activity was expressed as E₄₇₀/E₂₈₀.

Estimation of viscosity of DNA

The time course of viscosity of the mixture was measured by a Ostwald's viscometer at 37°C. The mixture contained calf thymus DNA (prepared by SDS-phenol method), AAO, AA, sodium citrate and phosphate buffer (pH 7.0) to give the final concentrations of 100 µg/ml, 200 µg/ml, 1 mM, 0.1 M, and 0.01 M, respectively. Preliminary experiment indicated that the action of DNase I

was inhibited by 0.1 M sodium citrate.

RESULTS AND DISCUSSION

Crude AAO preparation from pumpkin

Prior to the preparation of the enzyme, AAO activity in peel, epicarp, mesocarp and endocarp of pumpkin was estimated. Concerning each part, 10 g pieces were homogenized with 20 ml of water in a mortar and centrifuged at 3,000 r.p.m. for 10 minutes. The activity of the supernatant was assayed and shown in Fig. 1. The highest activity was observed in peel and a considerable one was detected in both epicarp and endocarp, but almost no one was shown in mesocarp.

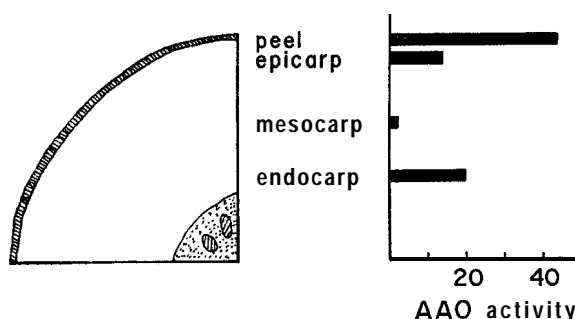


Fig. 1. AAO activity of peel and pericarp of Japanese pumpkin.

Crude AAO was prepared according to Powers *et al.* (1944). For preparation on a large scale, pumpkin was homogenized preferably with an electric meat-grinder and filtered through a nylon filter-bag resistant to high pressure using an oilpressor. In this case, peel was excluded, since it is very hard and disturbs filtration, although it contains the high activity of AAO. About 6.5 liter juice were obtained from 50 kg pumpkin. The pH of the juice was adjusted to 7.6 with sodium tetraborate. It was mixed with 0.01 volume of 1 M barium acetate and left over night. The supernatant was carefully siphoned and ammonium sulfate was dissolved to 0.3 saturation. After filtering by suction through a bed of Hyflo Super-Gel, ammonium sulfate was further dissolved to 0.6 saturation and filtered. The precipitate collected was dissolved in 0.067 M disodium phosphate by dispersion. Undissolved proteins were discarded by filtration. Magnesium sulfate was then dissolved in the filtrate in a ratio of 500 g per 1 liter and centrifuged. Magnesium sulfate was further dissolved in a ratio of 400g to 1 liter of the supernatant. The precipitate collected by centrifugation was dissolved in 0.067 M disodium phosphate and dialysed against water for 3 days in cold with several changes. In the preceding study (Omura *et al.*, 1973), AAO preparation was employed after centrifuging insoluble matter. However, the enzyme was adsorbed on alumina gel and eluted with 0.067 M disodium phosphate. The extract with 92.4 specific activity was dialysed against water and the preparation having 85.8 specific activity was kept in frozen state. A

freeze drying procedure was not preferred owing to the inactivation of AAO and the decrease of the solubility in water.

The antitumoric potentiality of the crude AAO preparation thus obtained was then estimated. The preparation was dissolved in Ringer's solution and 0.2 ml (0.2 mg preparation ; 10 mg/kg) were subcutaneously injected 10 times (once a day from the next day after implanting sarcoma-180 except on the 3rd day) and killed on the 15th day for the evaluation. The result is shown in Table 1.

Table 1. Antitumoric potentiality of a crude AAO preparation.

| Experiment | Control | AAO preparation |
|-------------------------------------|----------------|-----------------|
| AAO activity | — | 85.8 |
| Body weight, average \pm S.E., g | 28.6 \pm 0.8 | 28.3 \pm 1.0 |
| Tumor weight, average \pm S.E., g | 2.7 \pm 0.7 | 0.6 \pm 0.3 |
| Repression, % | | 78.7 |

On the other hand, mice were administered with 0.1 mg (5 mg/kg) AAO preparation 9 times (once a day from the next day after implanting sarcoma-180) and sacrificed for the assay on the 34th day after normal breeding. For an old preparation which had been kept for about a year and its specific activity had been reduced to 15.1, the antitumoric potentiality was also similarly estimated and indicated in Table 2. During the experiment, variations in average of body weight of mice and in average size of tumor were determined and shown in Fig. 2.

Table 2. Antitumoric potentiality of crude AAO preparations with different enzymatic activity.

| Experiment | Control | Fresh preparation | Old preparation |
|-------------------------------------|----------------|-------------------|-----------------|
| AAO activity | | 85.8 | 15.1 |
| Body weight, average \pm S.E., g | 34.4 \pm 2.2 | 31.0 \pm 1.4 | 30.4 \pm 1.7 |
| Tumor weight, average \pm S.E., g | 5.0 \pm 1.7 | 2.1 \pm 1.4 | 2.4 \pm 1.3 |
| Repression, % | | 58.1 | 52.8 |

From Tables 1 and 2 and Fig. 2, it is established that the crude AAO preparation possesses some antitumoric potentiality. In particular, the potentiality was observed in the assay of longer period too, even with a little less efficiency, when doses had been reduced by half and administered only in the first stage of the experiment. In addition, the result suggests that the antitumoric potentiality is not attributed to the enzymatic activity of AAO. Although administration of the AAO preparation seemed to have some repressing effect on the growth of mice, there was no difference between the effect of the fresh and old preparations.

Purified AAO preparation from pumpkin and its modified enzymes

According to the procedure of Tokuyama *et al.* (1965), AAO was further purified and estimated the antitumoric potentiality. As in the case of the crude AAO preparation, precipitate between 0.3 and 0.6 saturation with ammonium

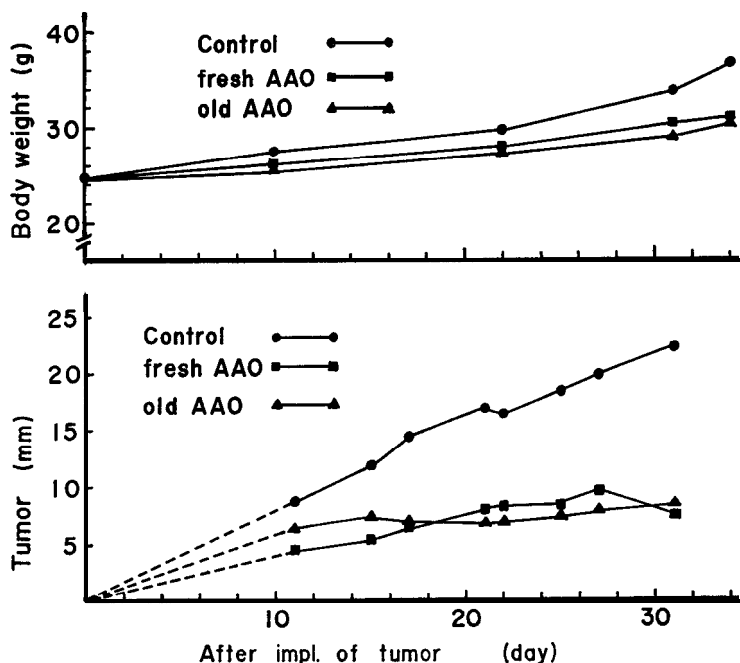


Fig. 2. Growth of tumor and variation of body weight of mice.

sulfate was obtained, dissolved in water and filtered with 1.5 % celite (Johns-Manville No. 535) by suction to remove some insoluble material. Sodium chloride was dissolved in the filtrate to give a final concentration of 1.3 %. Under vigorous agitation, 0.9 volume of acetone cooled with dry ice was added and the precipitate formed was collected. It was then dissolved in ice cold water (0.1 volume of the filtrate in the preceding step), filtered and dialysed against cold water for 5 to 6 hours. Undissolved proteins were removed by filtration and the filtrate was dialysed against 0.01 M Tris buffer (pH 7.6) over night. AAO was adsorbed on DEAE cellulose column, 3 cm x 22 cm, saturated with the same buffer. The column was washed with buffer and then eluted with 0.06 M sodium chloride-0.01 M Tris buffer at a flow rate of 100 ml per hour. The pale green part was again adsorbed on DEAE cellulose column and eluted stepwisely with 0.03 M sodium chloride-0.01 M Tris buffer and 0.06 M sodium chloride-0.01 M Tris buffer. The latter was dialysed against 0.02 M phosphate buffer (pH 7.0). By these chromatographic processes, specific activity was increased from 438 to 1380, but total activity was reduced from 334,000 to 92,000. The AAO solution was then passed through a column of sephadex G-100 developed with 0.02 M phosphate buffer (pH 7.0), 1.7 cm x 40 cm, at a flow rate of 20 ml per hour using the same buffer. As depicted in Fig. 3, the active fractions No. 6 -No. 12 (each 5 ml) were pooled and concentrated by ultrafiltration in a colodion bag to a final concentration of 1 mg/ml and kept in a frozen state. By gel filtration, 20 mg of protein was recovered from 35 mg. The AAO preparation was greenish blue and showed a single band by DEAE cellulose chromato-

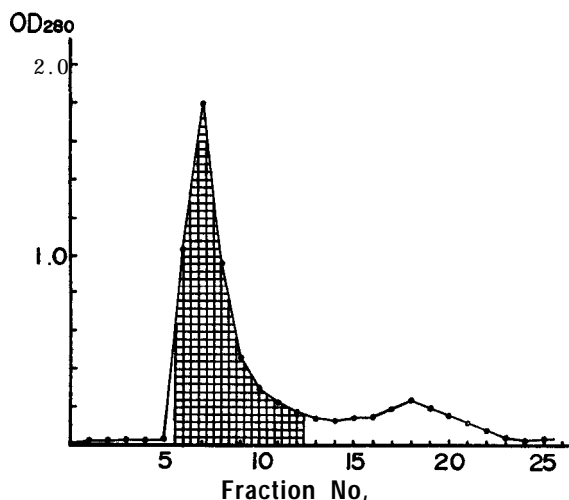


Fig. 3, Gel filtration of AAO in preparing a purified preparation.

graphy, gel filtration with sephadex G-100 and paper electrophoresis. Specific activity was 1,400 and protein content by Cu-Folin method was shown to be above 98 % and copper content 0.065 % by a diethylthiocarbamic acid method.

In order to confirm the result denying the relationship between the antitumor potentiality and the enzymatic activity, AAO preparation was modified and estimated its antitumor potentiality. According to Crestfield *et al.* (1963), AAO was carboxymethylated. Since Starks and Dawson (1962) reported that AAO contains 17.8 cysteine, but not free SH group in a molecule, S-S bond was reduced to SH with mercaptoethanol before carboxymethylation. AAO (5 mg) was mixed with 2.885 mg of crystalline urea, 0.05 mg EDTA, 0.05 ml of mercaptoethanol and 1.5 ml of 1.5 M Tris buffer (pH 8.6) and the solution made to 7 ml with water was incubated at 22° to 25°C for 4 hours. Into the mixture, 0.134 g of recrystallized iodoacetic acid dissolved in 1 N sodium hydroxide (0.5 ml) were added and kept at the same temperature for 15 minutes. To separate the enzyme from the reagents, the reaction mixture was filtered through a column of sephadex G-100 covered with aluminium leaf.

On the other hand, AAO was reported to contain 54.6 of arginine and 37.4 of lysine per molecule (Stark and Dawson, 1962). Therefore, AAO was partially decomposed by limited digestion with trypsin. Five ml AAO solution (containing 5mg) were heated at 100°C for 3 minutes and incubated at 37°C for 6 hours with 50 μ g trypsin (Merck). The reaction mixture was heated at 100°C for 5 minutes to stop the reaction. By gel filtration with sephadex G-100, the product was recovered from the effluent and the decomposition of AAO was certified as shown in Fig. 4.

To prepare heat-denatured AAO, the enzyme solution was mixed with sodium hydroxide to raise its pH to 10.8, heated at 100°C for 30 minutes and neutralized with hydrochloric acid.

The carboxymethylated, trypsinized or heat-denatured AAO preparation thus

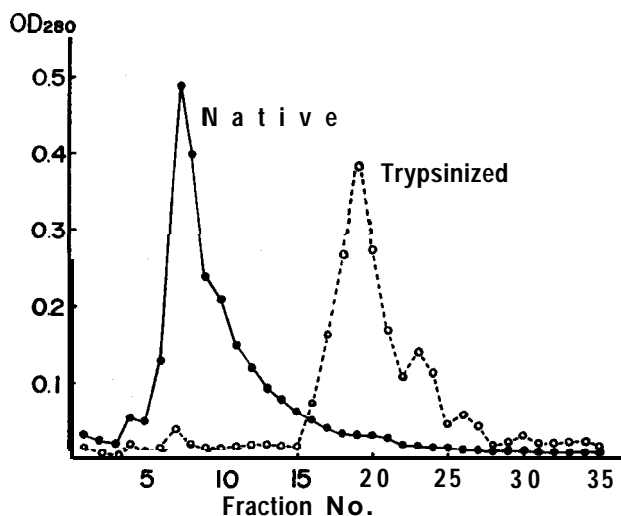


Fig. 4. Trypsin treatment of the the purified AAO preparation.

Table 3. Antitumoric potentiality of purified and its modified preparations.

| Experiment | Contr | Native prep. | Trypsinized prep. | C M - p r e p . | Heated prep. | T r y p s i n |
|----------------------------------------|----------------|-----------------|----------------------|-----------------|-----------------|----------------|
| AAO activity | — | 1400 | 0 | 0 | 0 | — |
| Body weight, average \pm S.E., g | 30.8 \pm 1.5 | 27.6 \pm 0.8 | 27.7 \pm 1.0 | 28.1 \pm 1.0 | 26.9 \pm 0.9 | 30.0 \pm 1.8 |
| Tumor weight, average \pm S.E., g | 4.7 \pm 0.5 | 1.0 \pm 0.1 | 2.5 \pm 0.2 | 1.2 \pm 0.1 | 1.9 \pm 0.4 | 4.9 \pm 9.5 |
| Repression, % | — | 78.9 | 47.0 | 73.7 | 59.8 | +4.3 |

modified was established to have completely lost the enzymatic activity. The antitumoric potentiality of the native and modified preparations was assayed. For comparison, effect of trypsin itself was also estimated. Every 0.1 mg of the preparations (5 mg/kg) was administered on the next, 2nd, 3rd, 5th and 7th day after implanting the tumor and the weight of tumor was estimated on the 15th day.

Their repression ratios are summarized in Table 3. The native and the carboxymethylated AAO preparations showed the relatively high potentiality, while it was reduced to some extent by heat denaturation. However, digestion of the AAO protein brought about the reduction of the antitumoric potentiality, although it was still remained. On the contrary, trypsin itself has not the potentiality at all. The results support the preceding presumption that the antitumoric potentiality attributed to AAO protein itself, but did not depend on the enzymatic activity of AAO. In addition, it suggests that high molecular state of the AAO protein is required for appearance of the antitumoric potentiality.

In the preceding paper (Omura et al., 1973), it was observed that DNA was depolymerized by the action of the crude AAO preparation in connection

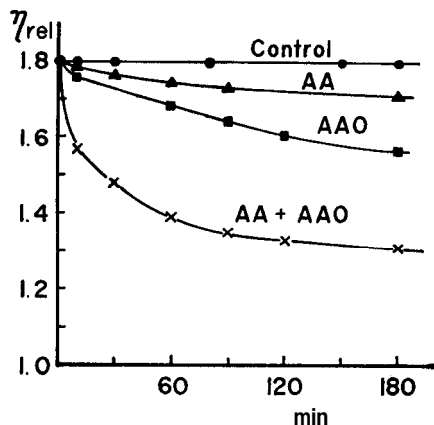


Fig. 5. Effect of a purified AAO preparation on viscosity of DNA solution.

with its antitumor potentiality. Therefore, the effect of the purified AAO preparation on the viscosity of DNA solution was examined and indicated in Fig. 5. With the purified preparation too, some decrease of the viscosity was observed. Since it was established that 0.1 M sodium citrate completely inhibits DNase action in the preliminary experiment, the result in Fig. 5 suggests some depolymerization of DNA by AAO protein. Addition of the substrate AA caused the remarkable decrease in the viscosity. So far as the action of AA and DAA is concerned (Yamafuji *et al.*, 1971b), it seems to involve participation of AA free radical which may be produced in the AAO action.

Crude PPO preparation from shiitake mushroom

Since PPO is also a copper-containing enzyme as AAO, the similar antitumor potentiality was expected. Therefore, crude PPO preparation was prepared from shiitake mushroom according to the procedure of Bouchilloux *et al.* (1963) and its potentiality was examined.

Fresh shiitake mushrooms from a city market were kept at -16° to -20°C for at least a day in a frozen state. They were homogenized with 1.4 volumes of cold acetone in a Waring blender for 1 minute and filtered quickly on a Buchner's funnel under covering with nylon cloth to protect from oxygen. The semi-frozen pulps were homogenized again with acetone. Acetone treatment was repeated for another few times to be dehydrated completely. The powder was homogenized for 2 to 3 minutes into equal volume of 30 % acetone under cooling with ice and centrifuged at 6,000 r.p.m. for 10 minutes. Into the supernatant, 1.5 volumes of cooled acetone were added and kept at -16°C . Precipitate was collected by centrifugation and dissolved immediately in 0.075 volume ice-cold water. 10 % calcium acetate was gradually added to a final concentration of 1% and centrifuged to remove the precipitate formed. Saturated solution of ammonium sulfate was added to the supernatant to 0.73 saturation and kept at 0°C for 2 hours. The resulting precipitate was dissolved in small volume of ice-cold water and then dialysed against 0.005 M phosphate buffer (pH 6.8) in cold. About 20 g of the crude preparation were obtained from 1 kg of shiitake

mushroom. The specific activity was increased by ammonium sulfate fractionation of calcium acetate fraction from 0.118 to 4.40.

The crude PPO preparation, was administered once a day for 7 times after implantation of the tumor except on the 5th day in a dose of 0.2 mg (10 mg per kg body weight). Mice were sacrificed on the 15th day after implantation and the result is shown in Table 4.

Table 4. Antitumoric potentiality of a crude PPO preparation of shiitake mushroom to sarcoma-180.

| Experiment | Control | PPO preparation |
|-------------------------------------|----------------|-----------------|
| PPO activity | — | 4.4 |
| Body weight, average \pm S.E., g | 30.5 \pm 1.3 | 22.7 \pm 0.8 |
| Tumor weight, average \pm S.E., g | 3.4 \pm 0.4 | 0.4 \pm 0.1 |
| Repression, % | | 88.7 |

The antitumoric potentiality had been usually assayed on sarcoma-180. In order to confirm a possibility to possess certain effect on the other tumors, the antitumoric potentiality was examined using MH-134. This clone of the tumor was originated by administration with carbon tetrachloride and transplanted in the National Institute of Genetics. To male C3H mice aged 4 weeks, about 5×10^6 cells of MH-134 were inoculated subcutaneously. The crude PPO preparation was administered by injection into abdominal cavity for 10 times (once a day from the next day after implanting the tumor to the 10th day) with a dose of each 0.2 mg (10 mg/kg). Tumor weight was measured on the 14th day and indicated in Table 5.

Table 5. Antitumoric potentiality of a crude PPO preparation of shiitake mushroom to sarcoma MH-134.

| Experiment | Control | PPO preparation |
|-------------------------------------|----------------|-----------------|
| PPO activity | — | 4.4 |
| Body weight, average \pm S.E., g | 20.8 \pm 1.8 | 19.5 \pm 2.0 |
| Tumor weight, average \pm S.E., g | 1.2 \pm 0.2 | 0.5 \pm 0.1 |
| Repression, % | | 68.3 |

These results indicated that the crude PPO preparation possesses the antitumoric potentiality on sarcoma-180 as well as on MH-134 too.

Recently, the antitumoric potentiality of proteins has been reported. For example, polyhedral protein in silkworm was shown to be effective on sarcoma-180 by Yamafuji *et al.* (1971a) in our institute. On the other hand, the transference of Ehrlich ascites was inhibited by cucumber AAO (Takamiya and Tsuda, 1973) and the accumulation of Ehrlich ascites was repressed and the life span of the host was elongated by administration of glutaminase A and L-leucine dehydrogenase (Oki *et al.*, 1973). The present report indicates that the antitumoric potentiality is attributed to protein itself, but not to the enzymatic activity. In addition, it is particularly interesting that the effective proteins of AAO and PPO contain copper, in connection with the finding that Cu^{2+} enhances

the action of reductones in repression of the growth of tumor and in degradation of DNA.

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