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# Antitumour Action of an Ascorbate Oxidase Preparation and its Interaction with Deoxyribonucleic Acid

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Ascorbic acid oxidase isolated from pumpkin, *Cucurbita moschata*, inhibits the growth of sarcoma-180. The enzymatic protein freed from copper by KCN-treatment and the protein denatured by heating have also the sarcoma-inhibiting activity. These three kinds of proteins are able to combine with DNA ; the combination enhances the breakability of the latter.

## INTRODUCTION

In the course of studies on antitumouric potentiality of ascorbic acid, we have found that the potency is strengthened by mixing ascorbate oxidase preparation. Pursuing this problem, it has now been clarified that the oxidase protein itself possesses the capability to repress transplanted tumour and to interact with DNA.

## MATERIALS AND METHODS

### Preparation of Ascorbic Acid Oxidase

Plants are usually used for isolating ascorbate-oxidizing enzyme and a highly purified one has been obtained from ***Cucurbita moschata*** (Powers *et al.*, 1944). Endocarp was taken from 40 kg pumpkins and ground with electric meat grinder. The oxidase was prepared with ammonium- and Mg-sulfate according to the procedure proposed by Powers. The amount of enzymic protein was about 1 g after freeze-drying.

Measurement of oxidase action was performed with 2,6-dichlorophenol-indophenol as described by Shen *et al.* (1945). The activity of enzyme from Japanese pumpkin was lower than that reported by Powers. In addition, as the oxidase contains copper as essential constituent, its content was estimated by the method of Poillon and Dawson (1963). The quantity of Cu in our preparation was 0.008 %.

### Antitumouric Capability of Ascorbate Oxidase Protein

The tests were conducted principally in accordance with the descriptions in our previous papers (Yamafuji ***et al.***, 1971a, 1971b). The oxidase in Ringer's

solution was injected into male ddN-mice every day from 24 hours after implanting sarcoma-180. The injection was repeated 7 to 9 times and animals were killed on the 20th day.

The investigation was further carried out with Cu-freed protein. For this, the enzyme was dissolved in 0.01 M KCN; the solution was dialyzed at 5°C first against K-cyanide of the same molarity for 24 hours and then against water for 2 days. Applying the methods mentioned above, it was confirmed that copper is not detected in the protein obtained and its enzymatic activity has been lost completely.

The animal test was also performed with heat-denatured protein. The oxidase solution was mixed with NaOH to raise its pH to 10.8, kept at 100°C for 30 min. and neutralized with HCl. These KCN-treated and heated proteins were appropriately diluted with Ringer's solution and their anticancer potency was examined as in the case of active enzyme. In all tests, 0.2 ml of Ringer's solution were injected in the control.

### Interaction of Ascorbate Oxidase Protein with DNA

For proving the complex formation, the native or KCN-treated protein was dissolved in water and mixed with HCl or NaOH to regulate its pH. One ml of calf thymus DNA (0.45 mg/ml) and 4 ml of $10^{-3}$ M buffer were added to 2 ml of protein solution (3.7 mg/ml). The buffers used were acetate, phosphate and borate + NaOH according to pH-values. DNA was dissolved in respective buffer and in the control buffer was mixed instead of DNA solution. The mixtures were maintained at 0°C for **20** min. and centrifuged for 1 hour at **40,000** r. p. m. by Beckman L-ultracentrifuge with 50-rotor. The amount of protein in the supernatant was then determined with Folin-reagent.

To examine whether the complex formation accompanies a DNA-breakage, a mixture of 4 ml of protein (5 mg/ml), 3 ml of thymus DNA (0.67 mg/ml) and 3 ml of 0.05 M buffer was incubated at 37°C. After 1 hour, the mixture was treated with 90 % phenol containing 1 % Na-dodecylsulfate and centrifuged to remove proteinous portion. The solution was dialyzed against 0.15 M NaCl+0.015 M Na-citrate (SSC) and fractionated by sucrose gradient centrifugation at pH 7. In the control, the incubation, treatment and fractionation were applied in the same way to a DNA-solution without protein.

In order to confirm that the DNA-cleavage accompanied with the complex formation was not due to contaminated DNase, 0.8 ml of DNA (0.64 mg/ml) were mixed with 3.2 ml of 0.02 M phosphate buffer (pH 7), 0.5 ml of various inhibitors for this enzyme and 0.5 ml of protein (2 mg/ml), and the measurement of the viscosity of mixture was performed immediately.

With the aim of investigating the interaction between DNA and heat-denatured protein, ascorbate oxidase was dissolved in 0.002 M $Na_2CO_3$, kept at 100°C for 2 min., neutralized with HCl and centrifuged at 9,000 r.p.m. for 15 min. The neutralization caused no precipitation. The clear supernatant was employed for the investigation of complex formation by applying the procedure described above. To prove the DNA-splitting, a mixture of 0.6 ml of supernatant containing heated protein (1 mg/ml), 2 ml of DNA in 0.01 SSC and 0.4 ml of 0.1 M phos-

phate buffer (pH 7) was incubated for 6 hours at 37°C. The mixture was then dialyzed against 0.2 M KC1 + 0.01 M $Na_2HPO_4$ at 5°C for 24 hours and gradient-centrifuged using 5 — 20 % sucrose containing KC1 + $Na_2HPO_4$ of the same concentration.

For the purpose of verifying further the enhancement of the breakability of DNA by the complex formation, oxidase protein had been reacted with thymus DNA which was later removed from the protein with phenol as mentioned in the section of 'DNA-breakage-test'. Two ml of DNA in SSC (0.2 mg/ml) were mixed with 0.4 ml of 0.1 M Tris (pH 8), 1.2 ml of 0.007 M $CuSO_4$ and 0.4 ml of 0.02 M hydroxylamine sulfate, and the solution was kept at 37°C. At regular intervals, 0.2 ml of 12 % perchloric acid were added to 1 ml of reaction mixture. After centrifuging, the absorbancy of the supernatant was estimated at 260 mμ. In the control, the DNA which had been treated without protein as in the case of 'DNA-breakage-control' was used simultaneously.

## RESULTS

### Antitumouric Capability of Ascorbate Oxidase Protein

The data of animal tests are indicated in Table 1; it has thus been found that untreated, Cu-freed and heat-denatured proteins inhibit the growth of implanted sarcoma to almost the same degree. In view of the fact that the enzymatically inactivated proteins display the inhibiting potentiality and the protein-content of our enzyme preparation is over 99 %, it seems reasonable to assume that the inhibition is to be attributed to the nature of ascorbate oxidase protein itself. A supplemental experiment disclosed that bovine blood albumin (Armour Co., Fract.-V, powder) shows no tumour-suppressing action under the present experimental conditions. The body weight of animal group injected with oxidase proteins was lower by ca. 10 % than that of the control at the end of each test.

**Table 1.** Antitumour activity of oxidase protein.

| Agent | No. of mice | Place of injection | Dose in each injection (mg/kg) | Times of injection | Average tumour weight (g) | Inhibition ratio (%) |
|---|---|---|---|---|---|---|
| Native protein | 10 | Subcutaneous | 10 | 7 | *1.3* | *71.7* |
| Control | 10 | Subcutaneous | | 7 | *4.6* | |
| KCN-treated protein | 10 | Subcutaneous | 10 | 9 | 1.1 | *70.3* |
| Control | 10 | Subcutaneous | | 9 | *3.7* | |
| Heated protein | 10 | Subcutaneous | 10 | 7 | *1.2* | *72.7* |
| Control | 10 | Subcutaneous | | 7 | *4.4* | -- |

### Interaction of Ascorbate Oxidase Protein with DNA

As can be seen from Fig. 1, native, untreated oxidase combines with thymus DNA. Although this protein has its isoelectric point at weak acidic reaction, a considerable amount of it bound with DNA in alkaline solutions. At pH 7, the ratio of DNA to protein in the complex was about 1: 20. Similar relations were also observed with KCN-treated protein.

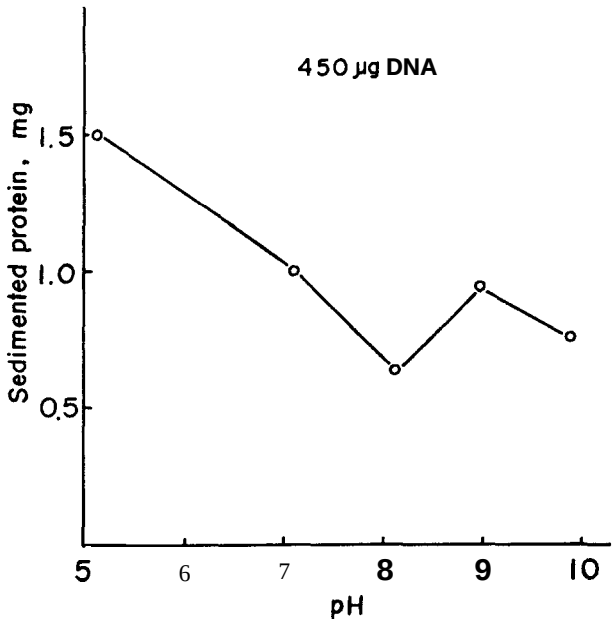


**Fig. 1.** Complex formation of DNA with native oxidase protein.

As is obvious from Fig. 2, DNA is broken in binding with oxidase protein at neutral reaction. The breakage of native protein was stronger than that of KCN-treated one.

It was further observed that DNA-cleavage, as illustrated in Fig. 3 occurs in the complex formation at pH 5.5, and that the cleaving degree is higher than in the case of pH. 7.

Experiments with inhibitors for DNase revealed that all reagents applied, as depicted in Fig. 4, have no influence upon the viscosity-lowering, suggesting that the oxidase protein used contains no DNA-depolymerizing enzyme.

In the investigation on the complex formation with heat-denatured protein, it was first corroborated that the ratios of heated protein combined with DNA are almost the same as those of native one (cf. Fig. 1). Furthermore, we ascertained that a DNA-splitting, as is clear from Fig. 5, takes place in the combining process. In one control, it had beforehand been proved that the complex can be completely dissociated in 0.2 M KCl, and that the gradient centrifugation of DNA is not affected by the salt of this molarity.

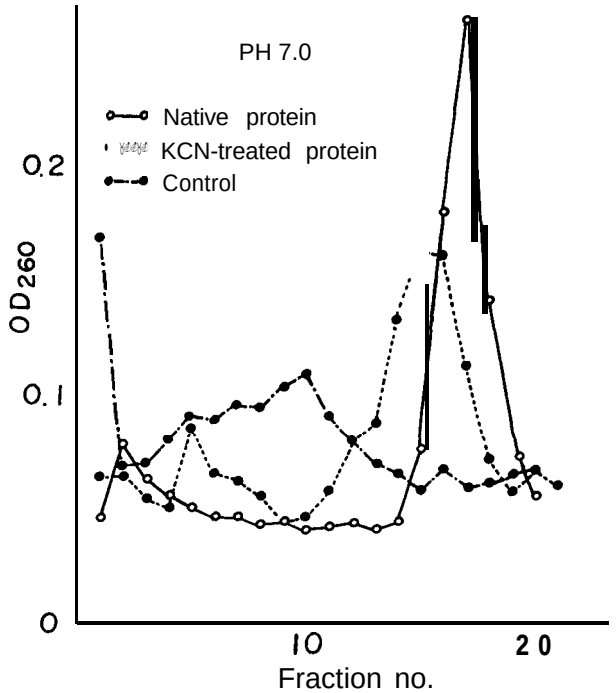


*Fig. 2.* Sedimentation pattern of DNA freed from complex formed with oxidase protein at pH 7.

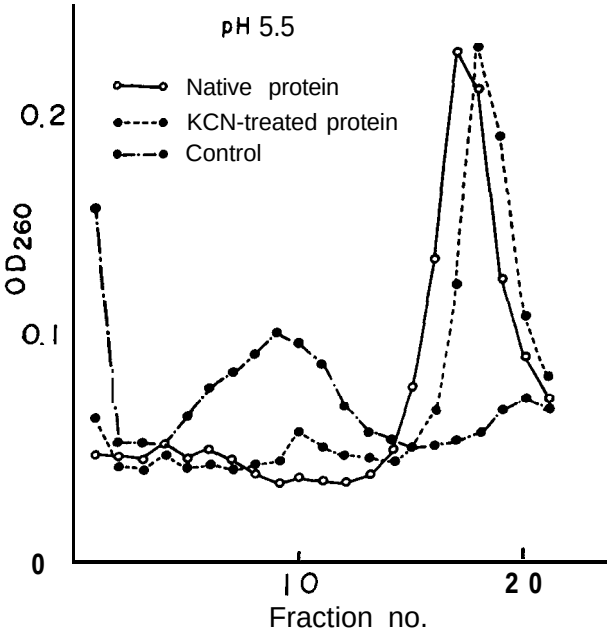


Fig. 3. Sedimentation pattern of DNA freed from complex formed with oxidase protein at pH 5.5.

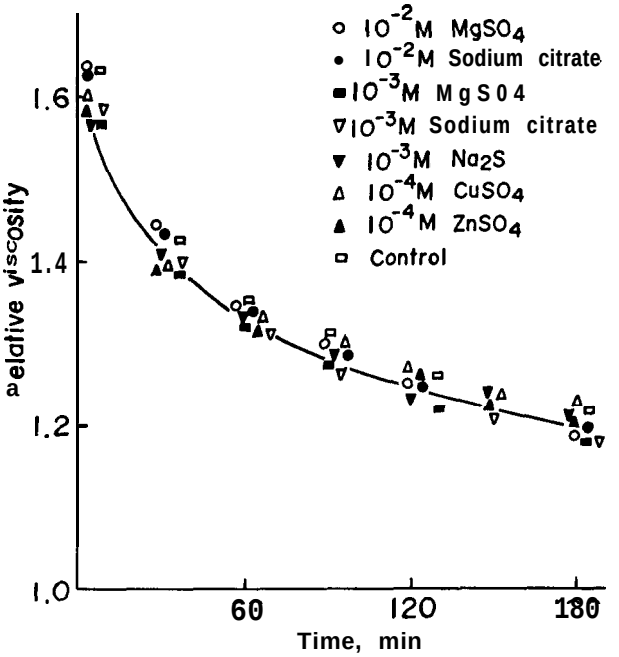


**Fig. 4.** Viscosity of DNA-solutions containing DNase-inhibitors.

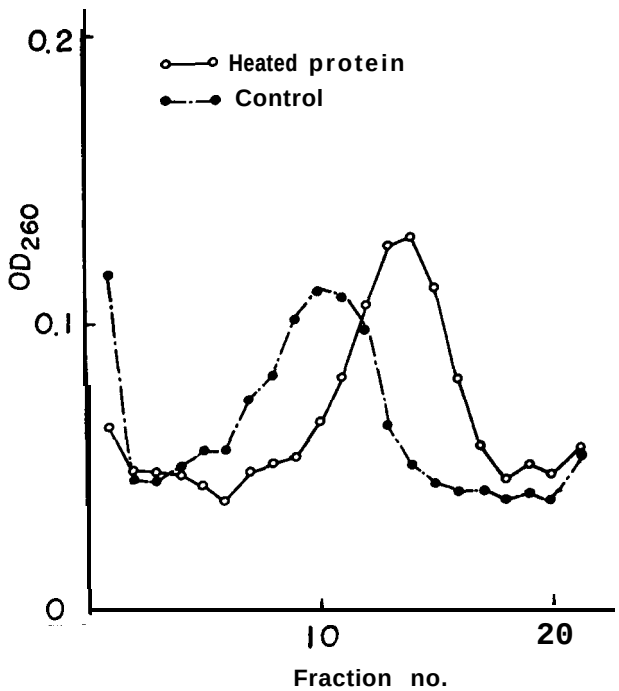


**Fig. 5.** Sedimentation pattern of DNA dissociated from complex formed with heated oxidase protein.

Previously, we found that the cleavage of nucleic acids by $NH_2OH$ is increased by cupric ions (Yamafuji *et al.*, 1971c). Applying this method, it was further demonstrated that the breakability of DNA, as is evident from Fig. 6, can be enhanced by forming a complex with oxidase protein.

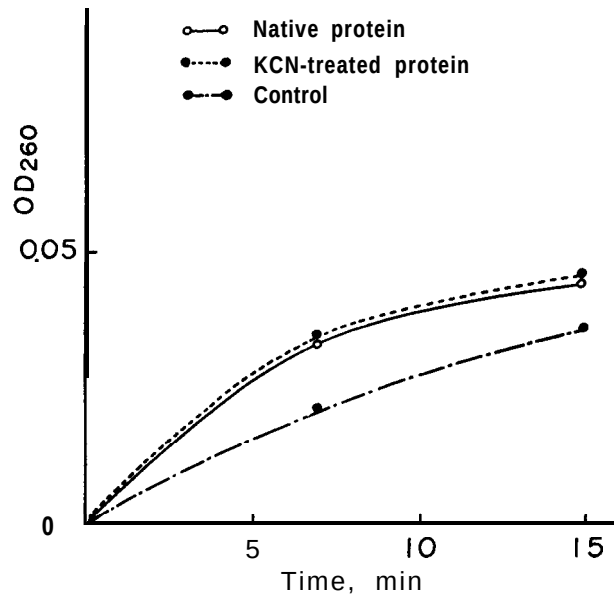


Fig. 6. Production of acid-soluble substances by hydroxylamine + copper from DNA freed from complex formed with oxidase protein.

## DISCUSSION

It has been reported in our preceding paper (Yamafuji *et al.*, 1971a) that viral polyhedral protein of silkworms binds with DNA and suppresses tumour. In the present study, we have been able to obtain similar observations using a plant protein preparation catalyzing the oxidation of ascorbic acid which possesses the ability to break DNA (Yamafuji *et al.*, 1971b). On the other hand, it has been proved that the hen egg albumin, soya bean protein (Murakami and Yamafuji, 1968) and bovine blood albumin exhibit no sarcoma-repressing potentiality. It is surmised that enzymes attacking substances which can react with nucleic acids have special conformation for interacting with DNA. Such sorts of enzymic proteins may also participate in cytodifferentiation.

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