Note

Partial Purification and Some Properties of Mitomycin C Inactivating Factors of *Pseudomonas convexa* 4-87

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The development of short-term mutagenicity tests has aided the remarkable progress in screening chemical and natural mutagens and carcinogens from our environment. In the course of detecting mutagens, some factors which suppressed mutagenicity were discovered.^{1,2)}

In the past decade, many more desmutagens have been found, most of which were of chemical and plant origins. Their modes of action have been thoroughly investigated. We have screened desmutagenic factors against such mutagens as mitomycin C (MMC), sterigmatocystin, tobacco tar neutral fraction, and Trp-P1 from cultured broths and cell extracts of 160 strains of bacteria, including about 60 aerobes with the rest being mostly anaerobic intestinal bacteria. Desmutagenic activity was assayed by the Rec-assay reported by Kada *et al.*³⁾ using spores of the recombination-deficient strain M45 of *Bacillus subtilis*. A certain amount of mutagen, which caused a 5 to 10 mm growth inhibition zone around the paper disc with strain M45 and no inhibition zone with its wild type, H15, was incubated with cultured broth or cell extract at 37°C for 1 hr. The mutagenicity after incubation was checked by the spore Rec-assay. When the sample contained desmutagenic activity, the growth inhibition zone diminished or decreased. Eight strains out of 62, 105 out of 162, 5 out of 62, and 83 out of 100 possessed desmutagenic activity against sterigmatocystin, MMC, tobacco tar neutral fraction, and Trp-P1, respectively. But the desmutagenic activity except for MMC was rather weak and not further studied. We focused on the desmutagenic factor against MMC, because such a factor may be applicable for the suppressor of the side-effects of this highly toxic anticancer agent. Among effective strains obtained, we chose *Pseudomonas convexa* 4-87 to investigate because of its strong desmutagenic activity in the cell extract. The difference of growth inhibition zones with strains M45 and H15

was decreased after treating with MMC of several doses and with this cell extract, confirming the existence of desmutagenic activity. So, only experiments with strain M45 were done for the investigation of the activity hereafter.

Cells were obtained by cultivating *P. convexa* 4-87 at 37°C for 20 hr in bouillon supplemented with 1% glucose (w/v). The cell extract was prepared by disrupting cells with sonic oscillation. Desmutagenic activity was recovered in the supernatant fraction of ultracentrifugation (100,200 × *g* for 1 hr). To investigate its activity, the following experiments were done as described in the legends of Table I. After treatment, 60 μl of the sample was mixed with 10 ng of MMC in 5 μl of dimethylsulfoxide (DMSO), incubated at 37°C for 1 hr, transferred onto a paper disc, and the mutagenicity was checked by measuring the growth inhibition zone with strain M45 (Table I). The activity was lost by heat treatment and Pronase P digestion. The activity was also lost by dialysis against deionized water but was regained by the addition of NAD(P)H; the effect of NAD(P)H was enhanced by Ca²⁺ and Mg²⁺, but not by Zn²⁺, Fe²⁺, Cu²⁺, or Mn²⁺. These results suggest that the desmutagenic factor against MMC in the cell extract of *P. convexa* 4-87 was proteinous and

TABLE I. EFFECTS OF TREATMENTS
ON THE DESMUTAGENIC ACTIVITY
OF *P. convexa* 4-87

Treatment	Inhibition zone (mm) to strain M45
a) Control	6.1
b) No treatment	0
c) Heat treated	6.0
d) Pronase P treated	6.1
e) Dialyzate	6.1
f) Dialyzate + NADH	1.0
f) Dialyzate + NADPH	1.0
g) Dialyzate + NADH + Ca ²⁺	0
g) Dialyzate + NADH + Mg ²⁺	0
g) Dialyzate + NADH + Zn ²⁺	5.8
g) Dialyzate + NADH + Fe ²⁺	5.0
g) Dialyzate + NADH + Ca ²⁺	6.0
g) Dialyzate + NADH + Mn ²⁺	3.0

a) Ten ng of MMC in 5 μl of DMSO was mixed with 60 μl of 50 mM Tris buffer (pH 7.0).

b) Supernatant fraction of ultracentrifugation of cell extract.

c) Heated in boiling water for 5 min, precipitate removed by centrifugation.

d) Pronase P (750,000 tyrosine units/g) was added to the concentration of 100 μg/ml and treated at 37°C for 30 min.

e) Dialyzed thoroughly against deionized water.

f) NAD(P)H was added to dialyzate to 200 μg/ml.

g) Metal ion was added to 5 mM.

required NAD(P)H for activity. By ammonium sulfate precipitation, activity was recovered between the 40 to 60% saturated fraction.

According to the results of gel filtration, desmutagenic activity was in two fractions. The major fraction, eluted from the column more slowly, was named fraction I and the minor one, which eluted faster, was named fraction II. We tried to purify both fractions I and II by column chromatography as usual, but complete purification was unsuccessful, because one minor band was observed on disc gel electrophoresis.

Some properties were investigated using the two partially purified fractions obtained above. Molecular weight estimation of the major fraction I was done by gel filtration. According to its elution volume, the molecular weight was estimated as 27,000~28,000. Requirements for cofactors were investigated by the Rec-assay and spectrophotometric assay. We found that both major fraction I and minor fraction II required a reduced form pyridine-dinucleotide, NAD(P)H, for the expression of their activity in the Rec-assay. The results of the spectrophotometric assay coincided with the above only for fraction I; the decrease in absorbance at 340 nm was observed only when fraction I, MMC, and NAD(P)H were incubated together, but with fraction II, no absorbance change was observed even when Ca^{2+} and Mg^{2+} were added to the concentration of 2 mM, although preliminary experiments had shown their effect on the desmutagenic activity of *P. convexa* 4-87. This decrease in absorbance seems mainly dependent on the oxidation of NAD(P)H because at this wavelength, its molecular extinction coefficient is much larger than that of MMC. It is not clear why fraction II did

not show the decrease in absorbance at 340 nm although it showed desmutagenic activity in the Rec-assay only when NAD(P)H was added to the incubation mixture. It may require another factor to express desmutagenic activity.

Kada *et al.* reported that the desmutagenic factor discovered in cabbage juice was a peroxidase with NADPH-oxidase activity. But the factor did not lose activity through dialysis against a buffer, indicating that cofactor is not directly involved in inactivating mutagenicity.²⁾ So the factor we discovered, for which NAD(P)H is essential for activity, differs from that Kada *et al.* reported.

The fact that fraction I requires NAD(P)H suggests that a reductive reaction happened with MMC. The mode of action for fraction I and how the mutagenicity of MMC was suppressed will be published elsewhere.

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REFERENCES

- 1) T. Osawa, H. Ishibashi, M. Namiki and T. Kada, *Biochem. Biophys. Res. Commun.*, **95**, 835 (1980).
- 2) K. Morita, M. Hara and T. Kada, *Agric. Biol. Chem.*, **42**, 1235 (1978); T. Inoue, K. Morita and T. Kada, *Agric. Biol. Chem.*, **45**, 345 (1981).
- 3) K. Hirano, T. Hagiwara, Y. Ohta, H. Matsumoto and T. Kada, *Mutation Res.*, **97**, 339 (1982).