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INTERACTION BETWEEN ORGANOPHOSPHORUS COMPOUNDS AND DNA ASSAYED BY THE RESTRICTION ENDONUCLEASE *Eco*RI

Restriction endonucleases due to the nature of their action may provide information on the location or the sequence specificity of a compound that binds to DNA. On the other hand, the action of the enzymes may be disturbed by compounds that have an ability to methylate DNA bases. The latter feature can be considered as a simple method for primary selection of potentially genotoxic compounds. In the present work we investigated the action of restriction endonuclease *Eco*RI on DNA which had been incubated with some organophosphorus agents. pUC19 plasmid DNA at a concentration of 78 μg/ml was incubated for 72 h with organophosphorus insecticides parathion, methylparathion and their main metabolites: paraoxon and methylparaoxon, respectively, at a concentration of 300 μM. After incubation nonbound insecticides were removed and DNA was subjected to 1 h incubation with the restriction endonunclease *Eco*RI and electrophoresed in 0.8% agarose gel. Organophosphorus compound methylparaoxon evoked unwinding of supercoiled DNA and the action of *Eco*RI on the DNA was disturbed that was displayed in changes in restriction pattern.

1. INTRODUCTION

Restriction enzymes depend for their action on precise recognition of specific palindromic sequences in DNA and precise cleavage at defined sites [1]. The recognition cleavage pattern can be altered by a variety of perturbations to DNA sequences, including the incorporation of base analogues [2–4], specific or random methylathions [1, 5, 6], radiation damage [7–9] and anticancer drug action [10–12]. Therefore, restriction enzymes can provide information on sequence specificity of binding a chemical to DNA and its ability to methylate DNA bases. This feature makes restriction enzymes useful tool for primary selection for potential genotoxicity of drugs, environmental pollutants and other chemicals.

The results of studies on inhibitory effect of chemicals on the cleavage effectiveness of restriction endonucleases can contribute to further understanding of how DNA can be protected from the enzyme-catalyzed hydrolysis.

In addition, restriction enzyme inhibition studies can be employed to map the location of binding sites of drugs on DNA [13–15].
In the present work the action of the restriction endonuclease *Eco*RI on DNA modified by an organophosphorus insecticide or its metabolite was investigated. Organophosphorus insecticides and their analogues are compounds that contain alkyl groups, so they can methylate DNA bases.

2. MATERIALS AND METHODS

DNA and restriction enzyme

pUC19 plasmid DNA and restriction endonuclease *Eco*RI were obtained from Peterfarm (Sieradz, Poland). The concentration of DNA was estimated spectrophotometrically. Plasmid DNA typically contained about 80% double stranded covalently closed circular supercoiled molecules, 20% open relaxed circular molecules and no linear molecules.

Chemicals

Organophosphorus insecticides parathion (*O,O*-diethyl *O*-4 nitrophenyl phosphorothioate) and methylparathion (*O,O*-dimethyl *O*-4 nitrophenyl phosphorothioate) as well as their main metabolites paraoxon (*O,O*-diethyl *O*-4 nitrophenyl phosphate) methylparaoxon (*O,O*- dimethyl *O*-4 nitrophenyl phosphate) at purity of 95–99% were supplied by Instytut Przemysłu Organicznego (Warsaw, Poland) and Dr. Ehrenstorfer GmbH (Augsburg, Germany). Chemical structures of these agents are displayed in Fig. 1.

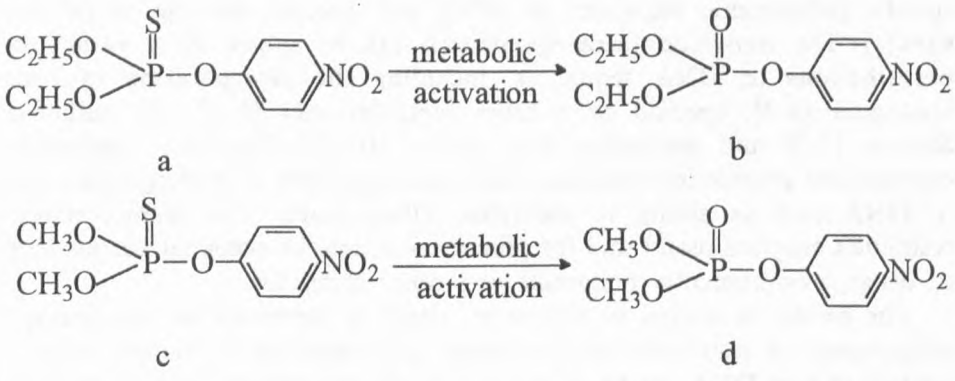

Fig. 1. Chemical structure of parathion (a), paraoxon (b), methylparathion (c) and methylparaoxon (d)

Chemicals treatment

An insecticide or its metabolite were derived from stock (50 mM) ethanolic solutions to give a final concentration of 300 μM. The DNA and ethanol concentration in all samples were 78 μg/ml and 0.384%, respectively. The control received, instead of an insecticide, ethanol, the concentration of which did not affect the processes under study. The samples were incubated in the dark for 72 h at 37° C in a buffer comprising 45 mM Tris-borate, 1 mM EDTA, pH 8.0. Non-bound organophosphorus compound were removed by ultrafiltration.

After incubation with organophosphorus compounds DNA was subjected to incubation for 2 h at 37° C with the restriction endonuclease *Eco*RI at a concentration of 5 U/μg DNA.

Sample analysis

Covalently closed circular DNA (CCC), such as the pUC19 plasmid used in this experiment, is a sensitive probe for detecting strand scission [16]. Such plasmid can be cleaved at any of phosphodiester linkages which permits the DNA strands to unwind, resulting in relaxed form OC DNA [17]. Introduction of another nick adjacent to the first but on the opposite strand gives linear form DNA. Some chemicals may not only cause DNA strand breakage but also unwinding of negatively supercoiled DNA by intercalative covalent modification [18–20]. Both processes lead to a formation of a relaxed circle which agarose gel electrophoretic mobility is about half that of the supercoil. The samples were ran in 0.8% agarose, stained with ethidium bromide, placed in a UV transilluminator and photographed with a Polaroid camera using a black and white Polaroid film type 665. The negatives were scanned with a Desaga densitometr, model CD 60.

3. RESULTS

Electrophoresis pattern of pUC19 plasmid DNA after 72 h incubation with organophosphorus insecticides and their metabolites with subsequent cleavage with restriction endonuclease *Eco*RI is displayed in Fig. 2. It can be seen that parathion and paraoxon as well as methylparathion (lanes 3, 5 and 7, respectively) did not affect plasmid pUC19 DNA as compared with the control sample (lane 1). The action of the restriction endonuclease *Eco*RI on DNA following incubation with these compounds (lanes 4, 6 and 8, respectively) was unchanged as compared with the corresponding control sample (lane 2).

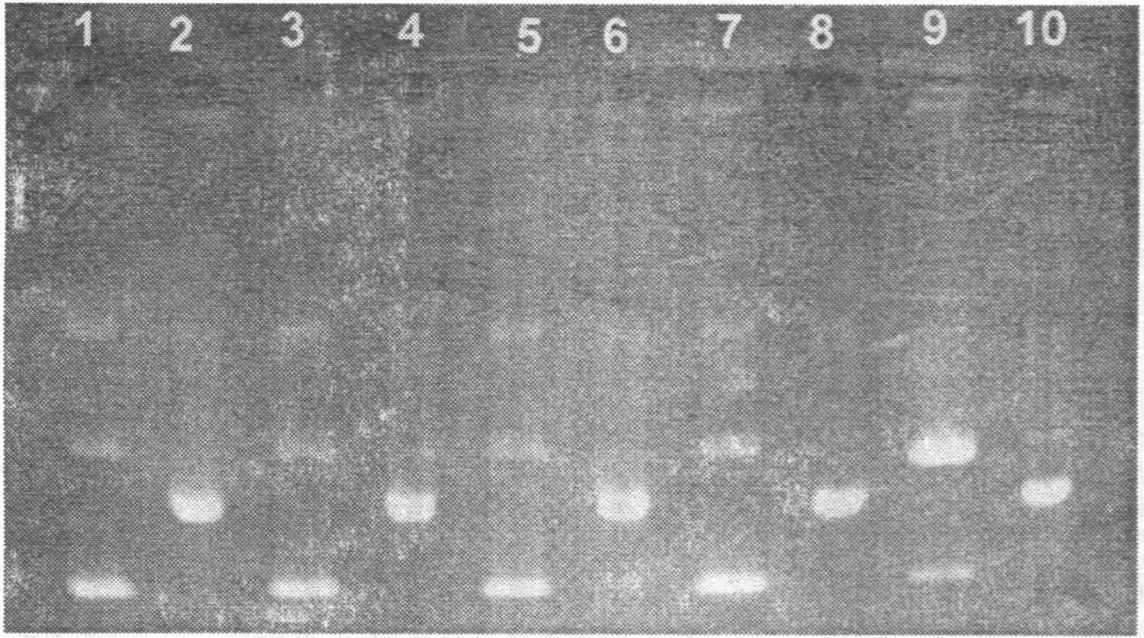

Fig. 2. Agarose gel electrophoresis of plasmid pUC19 DNA digested with *Eco*RI after incubation with organophosphorus compounds. Lane 1 and 2 show pUC19 DNA (78 μg/ml) alone and digested with *Eco*RI (2 U/μg). Lanes 3, 5, 7, 9 show DNA after 72 h incubation at 37°C with parathion, paraoxon, methylparathion and methylparaoxon, respectively; lanes 4, 6, 8, 10 DNA incubated with the same compounds and digested with *Eco*RI. The organophosphorus compounds were applied at a concentration of 300 μM

The main metabolite of methylparathion – methylparaoxon evoked unwinding of supercoiled DNA (lane 9) which can be observed as the increase of the optical density of the band of OC form and decrease of optical density of CCC form.

Comparing to the control sample, no differences were obserwed in electrophoretic pattern after incubation of plasmid pUC19 DNA with methylparathion (lane 2). The increase of optical density of the band of OC form and the decrease of optical density of CCC form band followed after incubation of plasmid DNA with methylparaoxon. The action of *Eco*RI on methylparaoxon-treated DNA (lane 10) was disturbed because the DNA cleaving by the enzyme was incomplete compared to the control (lane 2), which can be seen as appearance of OC form corresponding to uncut plasmid and the decrease of L form corresponding to cut plasmid compared to the control.

4. DISCUSSION

The results obtained in this experiment indicate that the restriction endonuclease *Eco*RI can be used to investigate DNA damage activity of methylparaoxon, the main metabolite of commonly used organophosphorus insecticide methylparathion.

The action of the enzyme on the methylparaoxon-modified DNA was partially inhibited, which was displayed in the appearance of band corresponding to uncut DNA, absent in the control sample. Incomplete cleavage may be due to methylathion of DNA bases by methylparaoxon. Organophosphorus compounds contains alkyl groups [21] that can be transferred to DNA bases causing their methylathion and inhibition of the action of restriction enzymes if methylathion occurs in the sequence that is recognised by these enzymes. On the other hand methylparaoxon, as well as many organophosphorus compounds used as insecticides, contains phosphorothioate residue. It was shown, that such residues located within one strand of doublestranded DNA exerted the inhibitory effect on the hydrolytic activity of the restriction endonuclease *Eco*RV [22]. Specific incorporation of a phosphorothioate group at the site of cleavage can yield a sequence that can be cleaved at a lower rate compared to the unmodified substrate [23]. It is important and in agreement with the performed study, that the presence of a phosphorothioate at the potential site of cleavage is not always sufficient for complete inhibition of a restriction enzyme [24, 25]. In many cases phosphorothioate groups exhibit specific inhibitory effect upon other hydrolytic enzymes. For example, detailed studies of the

3′-5′-exonuclease activity of the Klenov fragment, snake venom phosphodiesterase, and exonuclease III showed that these enzymes cleaved phosphorothioate linkages approximately 100 times more slowly than phosphate linkages [26, 27].

Methylparaoxon unwinded supercoiled plasmid DNA. This process can result from either strand nicking activity of methylparaoxon or covalently intercalation of this compound between base pair in DNA. If the nick caused by methylparaoxon was in the sequence recognized by the endonuclease, the enzyme could not cleave DNA because the restriction pattern would be altered.

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ODDZIAŁYWANIE POMIĘDZY ZWIĄZKAMI FOSFOROORGANICZNYMI I DNA OCENIANE PRZY UŻYCIU ENDONUKLEAZY RESTRYKCYJNEJ *Eco*RI

Enzymy restrykcyjne ze względu na istotę swojego działania mogą być źródłem informacji na temat miejsca lub specyficzności sekwencyjnej wiązania substancji do DNA. Z drugiej strony, działanie enzymów może być zaburzone przez związki mające zdolność do metylacji zasad DNA. Cecha ta może być wykorzystywana do pierwotnej selekcji związków potencjalnie genotoksycznych. W pracy badano działanie enzymu restrykcyjnego *Eco*RI na DNA, które było uprzednio inkubowane ze związkami fosforoorganicznymi. DNA plazmidu pUC19 o stężeniu 78 μg/ml był inkubowany przez 72 h z insektycydami fosforoorganicznymi parationem i metyloparationem oraz z ich głównymi metabolitami, odpowiednio paraoksonem i metyloparaoksonem o stężeniu 300 μM. Po inkubacji nie związane insektycydy były usuwane przez ultrafiltrację, a DNA poddawano 1 h inkubacji z endonukleazą restrykcyjną *Eco*RI i analizowano przez elektroforezę w 0.8% żelu agarozowym. Związek fosforoorganiczny metyloparaokson powodował rozwinięcie superskręconego DNA plazmidu pUC19, a działanie *Eco*RI na ten DNA było zaburzone, co znalazło swe odbicie w zmienionym obrazie elektroforetycznym.