IN VITRO CLEAVAGE OF SUPERCOILED DOUBLE STRANDED DNA BY CRUDE EXTRACT OF Annona squamosa L.*

PEMOTONGAN DNA SUPERKOIL UNTAI GANDA SECARA IN VITRO OLEH EKSTRAK GUBAL Annona squamosa L.

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ABSTRACT

The ability of cleaving supercoiled double stranded DNA has recently been found in several ribosome-inactivating proteins (RIP), a group of toxic proteins produced in plants, such as trichosanthin from Trichosanthes kirilowii, ricin from Ricinus communis and pokeweed antiviral protein (PAP) from Phytolacca americana. This potent activity makes them excellent candidates as the toxic part of immunotoxins for cancer therapy. The supercoiled DNA cleaving activity was used to identify the presence of RIP in Annona squamosa, a plant which has been traditionally used to prevent pregnancy.

Results showed that the crude extract of A. squamosa seeds expressed enzymatic activity to cleave supercoiled double stranded DNA into a nick circular conformation at low concentrations. Incubation at high concentration indicated that supercoiled DNA was cleaved into a linear form. However, it had no effect on a linear DNA. It can be concluded that A. squamosa seeds contain RIP-like protein.

Key word: Ribosome-inactivating protein (RIP), Annona squamosa L.

ABSTRAK

Kemampuan untuk memotong DNA superkoil untai ganda secara in-vitro akhir-akhir ini diterima dianggap oleh beberapa ribonulease-inactivating protein (RIP), yakni askeplopok protein asam yang dihasilkan oleh beberapa tanaman, seperti trichosanthin yang berasal dari tanaman Trichosanthes kirilowii, ricin dari Ricinus communis dan pokeweed antiviral protein (PAP) dari Phytolacca americana. Aktivitas ini dapat menghasilkan RIP sebagai ujung toksik dari satu immunotoxin untuk pengobatan penyakit kanker.

Aktivitas memotong DNA superkoil diuji dengan adanya kandungan RIP dalam Annona squamosa, yakni tanaman yang secara tradisional telah digunakan sebagai pencegah kehamilan.

*) This paper was presented at Indonesian Biotechnology Conference, Jakarta June 17th 1997
HASIL yang diporeleh menunjukkan bahwa ekstrak gabal A.squamosa mempunyai aktivitas menghambat DNA. Hal ini menunjukkan bahwa A.squamosa memiliki potensi untuk menjadi agen anti-penuaan.

Kata kunci: Ribosome-inactivating protein (RIP), Annona squamosa.

INTRODUCTION

Many plants tissue are known to produce substances which are toxic to other organisms, and are produced primarily as part of the cell cycle. This process is known as ribosome-inactivating protein (RIP). According to their structure, RIPs can be classified into two major types. Type I consists of a single chain with a molecular weight around 30 kDa, while type 2, with a molecular weight around 60 kDa, usually consists of two chain (A and B) connected by disulfide bond. The A chain is homologous to type I RIP and is responsible for the toxicity of the molecule. The B chain is a lectin which binds the toxin to the cell surface and facilitates the entry of A chain into the cell (Barbieri et al., 1993).

Besides the activities of RIPs on ribosomal RNA, several RIPs demonstrate to exhibit a unique enzyptic activity on cleaving supercoiled double stranded DNA into the nicked circular or linear form. RIPs only act on supercoiled and nick-circular DNA and do not cleave the linear form of the same molecule (Ling et al., 1994). This phenomenon was first reported with trichosanthin, an abortifacient, immunosuppressive and anti-tumor protease purified from the traditional Chinese herb medicine Tian Hua Fen (Li et al., 1991).

Interest in RIPs is growing due to several discoveries, such as the anti viral activity of zirabilis antiviral protein (MAP), a type I RIP which has successfully focused attention on its use as potential anti-HIV (Lee-Jhang et al., 1994). The potent cytotoxicity also makes them excellent candidates as the toxic part of immunotoxin for cancer therapy (Goldman et al., 1994).

Annona squamosa (srikaya) seeds were obtained from local market, and Virolilites velutina leaves were collected from garden. pUC19, pBR322 were obtained from laboratory stock of UFC for Biotechnology GMU. Ricin, abrin, pokeweed antiviral protein (PAV), gelatin, dephospho casein (DT) were kindly obtained from Prof J M Lord, Warwick University.

Preparation of A. squamosa seeds extract

A.squamosa seeds extract were prepared by grinding in 0.14 NaCl in 5mM sodium phosphate buffer pH 7.2 (5 ml per g). Following overnight stirring at 4°C the extract were strained and centrifuged (9000 g, 40 minutes). The supernatant was separated from the sediment and from floating fat (Reip et al., 1983).
Preparation of supercoiled DNA

**Escherichia coli** DH5α harboring pUC19 or pBR322 was cultured in LB medium containing ampicillin 150 mg/ml at 37°C. After reaching the stationary growth phase, total plasmid DNA was purified by the modified alkaline lysis procedure (Epper et al., 1989).

**Cleavage of supercoiled DNA with R1Ps**

One μg of plasmid DNA (pUC19) was incubated with various amounts of extract/R1Ps to volumes of 20 μl containing 50 mM Tris-Cl, 10 mM MgCl₂, 100 mM NaCl, pH 8.0, at room temperature for 1 hour. At the end of the reaction, 10 μl of loading buffer (30% glycerol, 200 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF) were added. Electrophoresis was carried out in 0.5xTBE buffer in a 1% agarose gel. DNA bands were visualized by staining with ethidium bromide. Incubation of linear DNA (EcoRI-linearized pUC19) with extract/R1Ps was carried out as described above.

**RESULTS AND DISCUSSION**

**Cleavage supercoiled double stranded DNA by several R1Ps**

Several R1Ps were used in this experiment, including ricin, one of the most intensively type 2 R1Ps and ricinostatin (type 1 R1Ps) which were known to possess the activity of cleaving supercoiled DNA (Ling et al., 1994). To provide more evidence, other R1Ps, gelolin, PAP and abrin were also used in this experiment. When 1 μg pUC19 was incubated with 2 μg of each R1P, it was shown that supercoiled DNA (Figure 1a) was cleaved to give a nicked circular form which moved significantly slower than the supercoiled DNA (Figure 1c) and a linear form (Figure 1b) which moved in between supercoiled DNA and nicked circular DNA. Similar result was also observed when pBR322 was used as a substrate (data not shown). All these results were obtained from R1Ps bearing specific RNA-N-glycosylase. When pUC19 was treated with dephosphorylation toxin (D1Ts) (Figure 1, lane 10), another kind of toxin which exhibits protein synthesis inhibition effect via adenosine diphosphate (ADP)-ribosylation of elongation factor 2 (Chang, 1989), similar result was also obtained. At a concentration of 2 μg D1Ts exhibited apparent activity on supercoiled DNA in a fashion similar to that of type 1 (PAP, trichostatin, gelolin) as shown in Figure 1 line 6, 8, and 11 respectively, or type 2 R1Ps (abrin, ricin) as indicated in Figure 1, lane 9, and 3 respectively. It can be seen from Figure 1 that at the same concentration, gelolin was able to cleavage supercoiled DNA more extensively than PAP, D1Ts and ricin, indicating that gelolin was more active than D1Ts, PAP and ricin, respectively. All these results demonstrated that most R1Ps (type 1 and type 2) and D1Ts have similar activity on supercoiled DNA. This result may be a reflection of the intensive identity of their quaternary structure, however the exact mechanism is yet known.

**Cleavage double stranded DNA by A. squamosa seed extract**

Searching the presence of R1Ps in several pharmaceutical plants grown in Java using R1P activity on supercoiled DNA has found that one of the plant is A. squamosa. When pUC19 was incubated with increasing amount of seed extract at room temperature (25°C), the supercoiled DNA band in the agarose gel became gradually fainted, whilst nicked and linear bands began to appear (Figure 2). The supercoiled DNA completely disappeared at concentration of 25 μg of total protein (Figure 2, line 7).

Mujahid Fawzi Sami Indrasena 9(6), 1994
Figure 1. Cleavage of supercoiled pUC19 by several RIPs:

Figure 2. Cleavage of supercoiled pUC19 by A. squamata seeds extract at 25°C
(1): λ
The same result is also observed using *M. pulpa* extract. *M. pulpa* is known to contain *M. pulpa* antiviral protein (MAP), a kind of type I RIP (Figure 1 lane 2). However this extract has little effect at 20°C, as indicated by a slight increase in the intensity of nicked circular form (Figure 3 lane 3) and no effect at 15°C (Figure 3, lane 3, and 4). The assay was conducted under normal enzymatic digestion conditions with Mg²⁺ present in the reaction buffer, since Mg²⁺ is an essential cofactor for all restriction endonucleases.

Figure 3. Thermal effect on the DNA cleaving.
(1) pUC19, (2) cleaving pUC19 by *A. squamosa* seed extract at 20°C, (3), (4) cleaving pUC19 by *A. squamosa* seed extract at 15°C.

Figure 4. Cleaving of supercoiled DNA by *A. squamosa* seed extract in the absence of Mg²⁺.
(1) pUC19, cleaving pUC19 using (2) 5μg extract, (3) 10μg extract, (4) 15μg extract, (5) 20μg extract, (6) 25μg extract.
To prove that the DNA cleaving activity is due to seed extract containing R1P-like protein, and not to some endonucleases contamination, the assay was repeated in the absence of Mg²⁺ (Figure 4). The data show that A. squamosa seed extract exhibits the DNA cleaving activity in the absence of Mg²⁺. In addition, in agreement with the result produced by several R1P, once the circular DNA has been converted into a linear one by treatment with EcoR1, this seed extract shows no further effect (Figure 5), even when the concentration of the extract was increased up to 25 μg of total protein (Figure 5 line 6). This result suggests that A. squamosa seeds contain R1P which is responsible for its abortifacient activity. However, to prove this evidence, further studies have to be carried out to observe its α-glucosidase activity and inhibition of protein synthesis.

Figure 5. The effect of A. squamosa seed extract on linear DNA (1): linearised pUC19; linearised pUC19 incubated with (2): 7.5 μg extract; (3): 10 μg extract; (4): 15 μg extract; (5): 20 μg extract; (6): 25 μg extract.

CONCLUSION

Abortifacient activity of psophus (type 2 R1P), gynost and PAP (type 1 R1P) and M. vulgaris leaves extract (plant containing type 1 R1P) have similar activity to ephedrine and trichostatin on their ability to cleave supercoiled DNA. A. squamosa seed extract also demonstrate to cleave supercoiled DNA, which indicates that A. squamosa may contain R1P that is responsible for its abortifacient activity.

REFERENCES


Eperon,E., 1989, Biochemistry Department Unv. of Leicester UK (Personal communication).


