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: trichosantin from *Trichosanthes kirilowii*, ricin from *Ricinus communis* and pokeweed antiviral protein (PAP) from *Phytalocca americana*. This potent activity makes them excellent candidates as the toxic part of immunotoxin 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 contains 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 diketahui dimiliki oleh beberapa ribosome-inactivating protein (RIP), yakni sekelompok protein toksik yang dihasilkan oleh beberapa tanaman, seperti: trichosantin yang berasal dari tanaman *Trichosanthes kirilowii*, ricin dari *Ricinus communis* dan pokeweed antiviral protein (PAP) dari *Phytalocca americana*. Aktivitas ini akan dapat menjadikan RIP sebagai ujung toksik dari suatu immunotoksin untuk pengobatan penyakit kanker

Aktivitas memotong DNA superkoil ini digunakan untuk menguji adanya kandungan RIP dalam *Annona squamosa*, yakni tanaman yang secara tradisional telah digunakan sebagai pencegah kehamilan.

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Hasil yang diperoleh menunjukkan bahwa ekstrak gubal *A.squamosa* mempunyai aktivitas memotong DNA superkoil memjadi bentuk nik sirkuler pada kadar rendah. Inkubasi pada kadar yang tinggi menunjukkan bahwa, DNA superkoil terpotong menjadi bentuk liniernya. Akan tetapi hal ini tidak berefek terhadap DNA linier. Sehingga dapat disimpulkan bahwa *A.squamosa* mengandung suatu protein sejenis RIP.

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

INTRODUCTION

Many plants tissue are known to produce substances which are toxic to other organism, and irreversibly inactivate eukaryotic ribosome by cleaving the N-glycosidic bond in the A_{4324} position of 28S RNA fraction so that they are no longer able to function in protein synthesis. These plant proteins are known as ribosome-inactivating protein (RIP). According to their structure, RIPs can be classified into two major types. Type 1 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 1 RIP and is responsible for the toxicity of the molecule. The B chain is a lectin which binds the toxic 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 enzymatic activity on cleaving supercoiled double stranded DNA into the nicked circular or linear form. RIPs only act on supercoiled and nick-circular DNA and seldom cleave the linear form of the same molecule (Ling *et al*, 1994). This fenomenon was first reported with trichosantin, an abortifacient, immunosupressive and anti tumor protein 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 mirabilis antiviral protein (MAP), a type 1 RIP which has successfully focused attention on its use as potential anti-HIV (Lee-Huang *et al*, 1994). The potent cytotoxicity also makes them excellent candidates as the toxic part of immunotoxin for cancer therapy (Goldmacher *et al*, 1994)

Annona squamosa is a tropical plant that is traditionally used as abortifacient (Glauce *et al*, 1990; Nurlaila, 1995). It is therefore worthwhile to identify whether this active substance is also RIP. In this communication we discover that *A.squamosa* extract posseses the ability of cleaving supercoiled DNA.

METHODOLOGY

Materials and methods

Annona squamosa (srikaya) seeds were obtained from local market, and *Mirabilis yalapa* leaves were collected from garden. pUC19, pBR322 were obtained from laboratory stock of IUC for Biotechnology GMU. Ricin, abrin, pokeweed antiviral protein (PAP), gelonin, diphtheria toxin (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 (10 ml per g). Following overnight stirring at 4^0C the extract were strained and centrifuged (28000 g, 30 minutes). The supernatant was separated from the sediment and from floating fat (Stirpe *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 (Eperon, 1989).

Cleavage of supercoiled DNA with RIPs

One μg of plasmid DNA (pUC19) was incubated with various amounts of extract/RIPs to volume of 20 μl containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 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 visualised by staining with ethidium bromide. Incubation of linear DNA (*Eco*RI-linearised pUC19) with extract/RIPs was carried out as described above.

RESULTS AND DISCUSSION

Cleavage supercoiled double stranded DNA by several RIPs

Several RIPs were used in this experiment, including ricin, one of the most intensively type 2 RIPs and trichosantin (type 1 RIPs) which were known to possess the activity of cleaving supercoiled DNA (Ling *et al*, 1994). To provide more evidence, other RIPs, gelonin, PAP and abrin were also used in this experiment. When 1 μg pUC19 was incubated with 2μg of each RIPs. it was shown that supercoiled DNA (Figure 1 a) was cleaved to give a nicked circular form which moved significantly slower than the supercoiled DNA (Figure 1 c), and a linear form (Figure 1 b) which moved in between supercoiled DNA and nicked circular DNA. Similar result was also observed when pBR322 was used as a substrats (data not shown). All these results were obtained from RIPs bearing specific RNA-N-glycosidase. When pUC19 was treated with diphtheria toxin (DTs) (Figure 1, line 10), another kind of toxin which exhibits protein synthesis inhibitory effect via adenosine diphosphate (ADP)-ribosylation of elongation factor 2 (Chang, 1989), similar result was also obtained. At a concentration of 2 μg DTs exhibited apparent activity on supercoiled DNA in a fashion similar to that of type 1 (PAP, trichosanthin, gelonin) as shown in Figure 1 line 6, 8, and 11 respectively, or type 2 RIPs (abrin, ricin) as indicated in Figure1, line 9, and 3 respectively. It can be seen from Figure 1 that at the same concentration, gelonin was able to cleavege supercoiled DNA more extensively than PAP, DTs and ricin, indicating that gelonin was more active than DTs, PAP and ricin, respectivelly.

All these results demonstrated that most RIPs (type 1 and type 2) and DTs have similar activity on supercoiled DNA. This result may be a reflection of the intensive identity of their quarternary structure, however the exact mechanism is yet known.

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

Searching the presence of RIPs in several pharmaceutical plants grown in Java using RIPs 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).

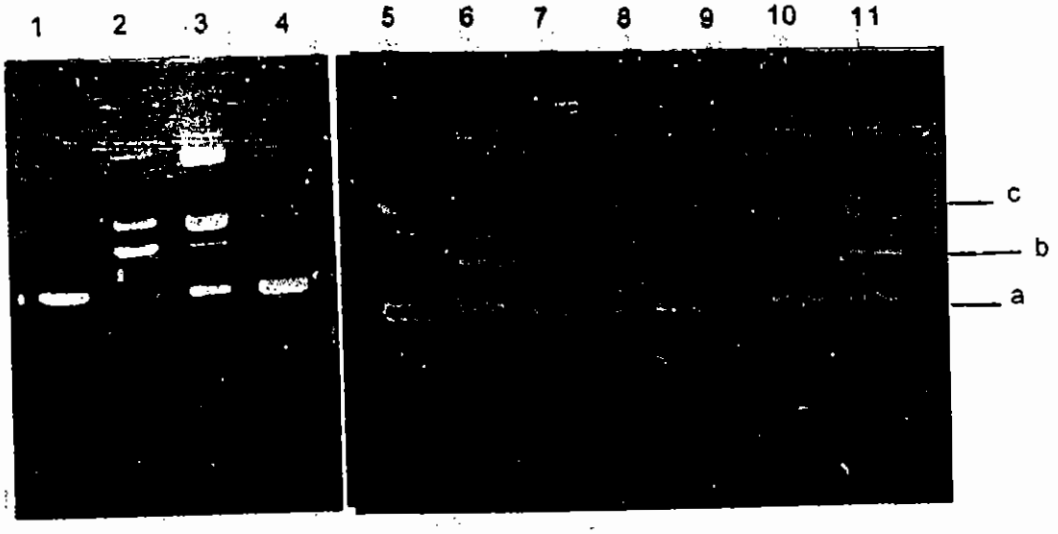

Figure 1 Cleaving of supercoiled pUC19 by several RIPs
(a): supercoiled DNA, (b): linear DNA, (c): nicked circular DNA (1): pUC19 (negative control), (2): *M.yalapa* leaves extract, (3): ricin, (4): ricin B-chain, (5):control- (6): PAP, (7): ricin B-chain, (8): trichosantin, (9): abrin, (10): gelonin, (11): DT

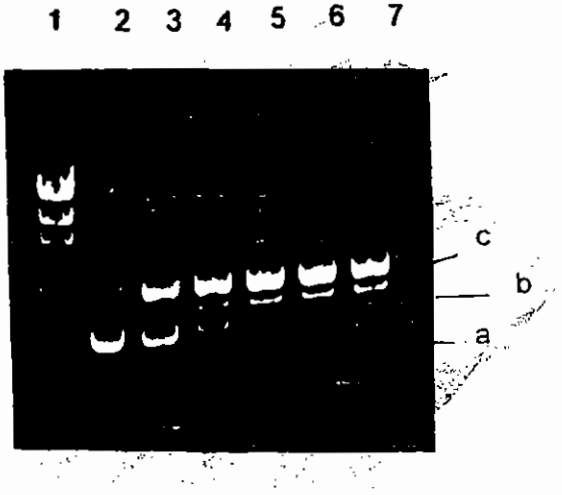

Figure 2 Cleaving of supercoiled pUC19 by *A.squamosa* seeds extract at 25°C
(1): $\lambda_{Hind\ III}$, (2): pUC19, (3): 5μg extract, (4): 10μg extract, (5): 15μg extract, (6): 20μg extract, (7): 25μg extract. (a): supercoiled DNA, (b): linear DNA, (c): nicked circular DNA

The same result is also observed using *Mirabilis yalapa* extract. *M. yalapa* is known to contain Mirabilis antiviral protein (MAP), a kind of type 1 RIP (Figure 1 line 2). However this extract has little effect at 20^0C, as indicated by slightly increase in the intensity of nicked circular form (Figure 3 line 2) and no effect at 15^0C (Figure 3, line 3, and 4). The assay was conducted under normal enzymatic digestion conditions with Mg^{2+} present in the reaction buffer, since Mg^{2+} is an essential cofactor for all restriction endonucleases.

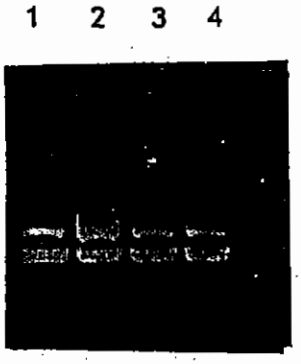

Figure 3 Thermal effect on the DNA cleaving
(1): pUC19, (2): cleaving pUC19 by *A.squamosa* seed extract at 20°C, (3, 4): cleaving pUC19 by *A.squamosa* seed extract at 15°C.

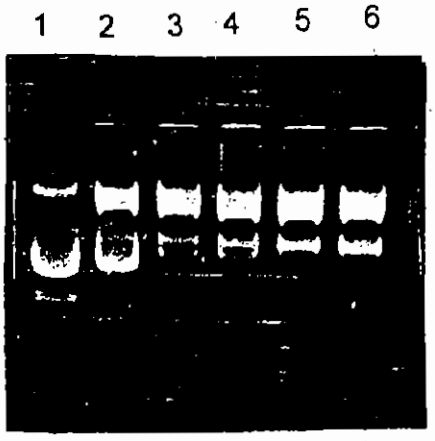

Figure 4. Cleaving of supercoiled DNA by *A.squamosa* seed extract in the absence of Mg^{2+}
(1) : pUC19; cleaving pUC19 using: (2) : 5μg extract, (3) : 10μg extract, (4) : 15μg extract, (5) : 20μg extract, (6) : 25μg extract.

To prove that the DNA cleaving activity is due to seed extract containing RIP-like protein, and not from some endonucleases contamination, the assay was repeated in the absence of Mg^{2+} (Figure 4). The data show that *A.squamosa* seed extract exhibits the DNA cleaving activity in the absence of Mg^{2+}. In addition, in agreement with the result produced by several RIPs, once the circular DNA has been converted into a linear one by treatment with *Eco*RI, 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 RIP which is responsible for its abortifacient activity. However to prove this evidence, further studies have to be carried out to observe its N-glycosidase 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): 5μg extract, (3): 10μg extract, (4): 15μg extract, (5): 20μg extract, (6): 25μg extract.

CONCLUSION

Abrin (type 2 RIP), gelonin and PAP (type1 RIP) and *M. yalapa* leaves extract (plant containing type1 RIP) have similar activity to ricin and trichosantin on their ability to cleave supercoiled DNA. *A.squamosa* seed extract also demonstrate to cleave supercoiled DNA, which indicates that *A.squamosa* may contain RIP that is responsible for its abortifacient activity.

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