Identification of ribosome-inactivating proteins (RIPs) from *Phaleria macrocarpa (Scheff) Boerl.*, a posssible active compound

Identifikasi ribosome-inactivating proteins (RIPs) dari *Phaleria macrocarpa*, kemungkinannya sebagai senyawa aktif

Sismindari
Fakultas Farmasi Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Ribosome-inactivating proteins (RIP), a group of toxic proteins produced in plants has RNA-glycosidase activity that is capable to inhibit mammalian protein synthesis. RIPs also is capable to cleave supercoiled double stranded DNA become nick circular and linear form. These potent activities make them an excellent candidate for cancer therapy. Aqueous extract of *Phaleria macrocarpa* fruits has been traditionally used to treat cancer. This research, therefore, was aimed to identify the presence of RIP in *P. macrocarpa,* which might be responsible for its activity.

The fruit pulp and seed of *P. macrocarpa* were extracted using 5 mM sodium phosphate buffer pH 7 containing 0.14 M sodium chloride. Protein was precipitated from crude extract using ammonium sulphate. RIPs activity was determined using the supercoiled DNA (pUC19) cleavage method.

Results showed that the crude extract of *P. macrocarpa* seeds expressed enzymatic activity to cleave supercoiled double stranded DNA into a nick circular conformation. This activity was seemed to be concentration dependence. It was demonstrated that the activity of protein fraction of seeds extract was higher than that of the crude extract, as indicated by the appearance of linear form at the concentration of 0.3µg/ml. However, no such activity was found on the crude extract of the fruits. It can be concluded that *P. macrocarpa* seeds might contains RIP-like protein.

Key words: Ribosome-inactivating protein (RIPs), *Phaleria macrocarpa*, supercoiled DNA

Abstrak

Ribosome-inactivating protein (RIPs), merupakan sekelompok protein toksik dalam tanaman yang mempunyai anktivitas *RNA-glycosidase* yang mampu menghambat sintesis protein pada mamalia. RIP mempunyai kemampuan memotong DNA superkoil. Adanya aktivitas tersebut, menjadikan RIP sebagai kandidat yang potensial dalam terapi kanker. Secara tradisional, ekstrak air dari buah *P. macrocarpa* telah digunakan dalam pengobatan kanker. Untuk itu, tujuan dari penelitian ini adalah untuk mengidentifikasi keberadaan RIP dalam *P. macrocarpa*, yang kemungkinan merupakan senyawa yang bertanggung jawab dalam aktivitasnya tersebut.

Daging buah dan biji *P. macrocarpa* diektraksi dengan buffer natrium fosfat 5mM pH 7.2 yang mengandung sodium klorid 0,14M. Fraksi protein diperoleh dengan pengendapan menggunakan ammonium sulfat. Aktivitas RIP ditentukan menggunakan metode pemotongan DNA superkoil.

Hasil menunjukkan bahwa ekstrak gubal biji *P. macrocarpa* mempunyai kemampuan memotong DNA superkoil menjadi bentuk nik

sirkularnya. Aktivitas tersebut nampak tergantung pada konsentrasi. Aktivitas fraksi protein dari biji terlihat lebih tinggi dibandingkan dengan ekstrak gubal, seperti ditunjukkan dengan munculnya bentuk linier pada konsentrasi protein sebesar 0.3µg/ml. Aktivitas memotong DNA superkoil tersebut tidak muncul pada *crude extract* dari daging buah. Sehingga dapat disimpulkan bahwa biji *P. macrocarpa* mengandung protein sejenis RIP.
Kata kunci: *Ribosome-inactivating protein* (RIPs), *Phaleria macrocarpa*, DNA superkoil

Introduction

Ribosome-inactivating proteins (RIPs) are a toxic protein present in plants. RIPs, as indicated by the name, inactivate eukaryotic ribosome by cleaving the N-glycosidic bond at the A_{4324} position of 28S RNA fraction so that they are no longer to function in protein synthesis. RIPs can be classified into two major types according to their structure; type 1 consists of a single chain with a molecular weight of around 30 kDa, while type 2, with a molecular weight of around 60 kDa, usually consists of two chains (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 to the cell surface and facilitates the entry of A chain into the cell (Barbieri *et al*, 1993). Besides the N-glycosidic activity, RIP was able to cleave supercoiled double stranded DNA into the nicked circular or linear forms. RIPs only act on supercoiled and nick-circular DNA and seldom cleave the linear form of the same molecule (Ling *et al*, 1994; Sismindari *et al*., 1998). The cleavage activity on supercoiled double stranded DNA 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 been successfully focused on its use as potential anti-HIV (Lee-Huang *et al*, 1994; Wang *et al*, 1999). The potent cytotoxicity makes them an excellent molecules for cancer therapy (Goldmacher *et al*, 1994; Tang *et al*, 2003).

Phaleria macrocarpa is a tropical plant traditionally used as anticancer. People normally use the fruits (pulp and seeds) for routine treatment (Figure 1). In order to develop this plant as a medicine, it is therefore worthwhile to identify whether this active substance in pulp or seeds is RIPs.

Methodology

Figure 1 : *Phaleria macrocarpa* raped fruits

Plant material

Phaleria macrocarpa (Thymelaeceae) fruits were collected from Yogyakarta area. A voucher specimen is deposited in the Laboratory of Life Science Gadjah Mada University. pUC19 were obtained from laboratory stock of Life Sciences Laboratory Gadjah Mada University.

Preparation of P. macrocarpa fruits and seeds extract

P. macrocarpa fruits pulp and seeds extract were prepared by grinding in 0.14 M 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 (crude extract) was separated from the sediment and from floating fat (Stirpe *et al*, 1983, Sismindari *et al*, 1998), followed by protein precipitation using ammonium sulphate at 100% saturation. The precipitated proteins were then dissolved in sodium phosphate buffer pH 6.5 and dialysed to removed the salt.

Preparation of supercoiled DNA

Escherichia coli DH5α harboering pUC19 was cultured in LB medium containing ampicillin 150 µg/ml at 37^0C. After reaching the stationary growth phase, total plasmid DNA was purified by the modified alkaline lysis procedure (Eperon, 1989).

Cleavage of supercoiled DNA by the protein extract

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 30°C for 1 hour. At the end of the reaction, 5 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 (Sismindari *et al*, 1998).

Results And Discussions

Fruits pulp and seeds were used in this study. It was indicated that protein content in either fruits pulp and seeds crude extract were approximately 0.07% and 0.1% w/w of raw materials, respectively. These crude extracts were then analyzed for their ability on cleaving supercoiled double stranded DNA (pUC19). The results indicated that double stranded supercoiled pUC18 DNA was cleaved by the crude extract of *P.macrocarpa* seeds at the concentration of 0.1μg/ml. The cleavage of supercoiled DNA activity seemed to be concentration-dependent, as shown by the decrease of the supercoiled form followed by the increase of nicked circular one (Figure 2). The cleavage activity of this extract was not due to the endonucleases contamination, since consistent with the result produced by several RIPs (Sismindari, *et al*, 1998), the activity was still presence even in the absence of Mg^{2+} (data not shown). In the other hand, no activity was found when pUC18 DNA was treated with crude extract isolated from the fruits pulp, even when the concentration of the extract was increased up to 0.35μg/ml of total protein (Figure 3, lane 2-8). It was indicated that crude extract isolated from *P macrocarpa* fruits pulp not contain RIPs-like proteins.

In order to provide more evidence on seeds extract activity, total protein was then isolated from seeds crude extract. The isolated protein was found to be more active compared to the crude extract. At the concentration of 0.3μg/ml the linear bands began to appear (Figure 4 lane 4). As the cleavage of supercoiled DNA is one of the properties of RIPs both type 1 and type 2, beside the N-glycosidase activity (Li *et al*, 1991; Ling *et al*, 1994), these results suggested that *P.macrocarpa* seeds contain RIPs. These data was supported by the finding that several protein extracts isolated from plants which cleaved supercoiled DNA were known to possess specific RNA-N-glycosidase the activity (Sismindari and Lord, 2000; Rumiyati *et al*, 2000; Sulistiyani *et al*, 2002). Therefore, cleavage of supercoiled DNA is then used as a routinely method for identifying RIPs from plants.

Several lines of evidence suggest that the anti-tumor, anti-viral, and anti-parasitic effects of the plant proteins, such as gelonin or pokeweed antiviral protein (PAP) which are well known as ribosome inactivating proteins (RIPs), are not solely due to the N-glycosidase activity, which is remove an invariant adenine in a conserved loop in the 28 S rRNA (Tumer *et al*, 1997; Peumans *et al*, 2001). There is an alternative effect of RIPs which possess a single-stranded adenine DNA glycosylase activity (Barbieri *et al*,1997). Therefore this protein fraction containing RIPs-like activity of *P.macrocarpa* seeds might responsible for the anticancer activity. While there is still no direct evidence that this activity contributes to cytotoxicity, the ability of RIPs to damage DNA by removal of normal, non-mispaired bases *in vitro* distinguished them from the other members of the DNA glycosylase family, which might also contribute on the activity (Nicholas, *et al*, 1998).

Many fundamental questions about the DNA glycosylase activity of the RIPs also require further investigation. These include whether the removal of adenines is the primary event that leads to DNA breakage, or the DNA breakage is RIP-mediated (Wang and Tumer, 1999), and the activity is limited to the single-stranded regions of supercoiled DNA or also affects double-stranded DNA (Nicolas *et a*l, 2000). It was demonstrated that RIPs able to cleave supercoiled double stranded DNA at the same site of rRNA (Wang and Tumer, 1999). This evidence was supported by He and Liu (2004) which demonstrated that mutants of recombinant cinnamomin A-chain devoid of N-terminal 52 or/and C-terminal 51 amino acid residues lost both the activity of RNA N-glycosidase and the ability to release adenines

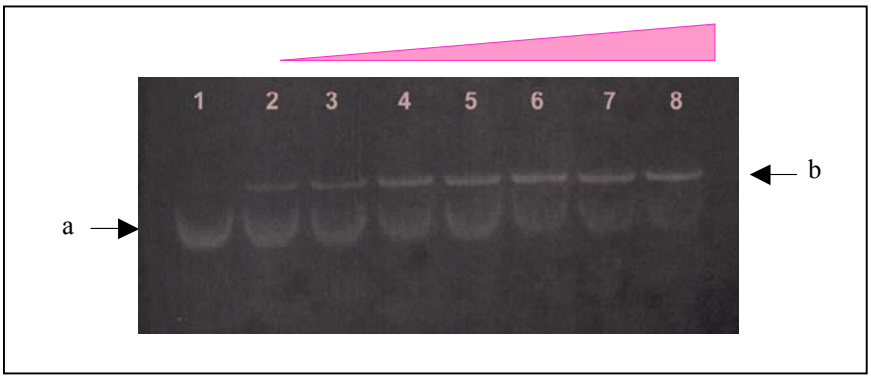

Figure 2 Cleaving of supercoiled pUC19 by seeds crude extract of *Phaleria macrocarpa Lane 1. Untreated pUC19, 2 – 8. Treated pUC19 with* P. macrocarpa *seeds crude extract at a concentration of 0.05 µg/ml to 0.35 µg/ml.*
(a) . supercoiled DNA, (b) . nicked-circular DNA

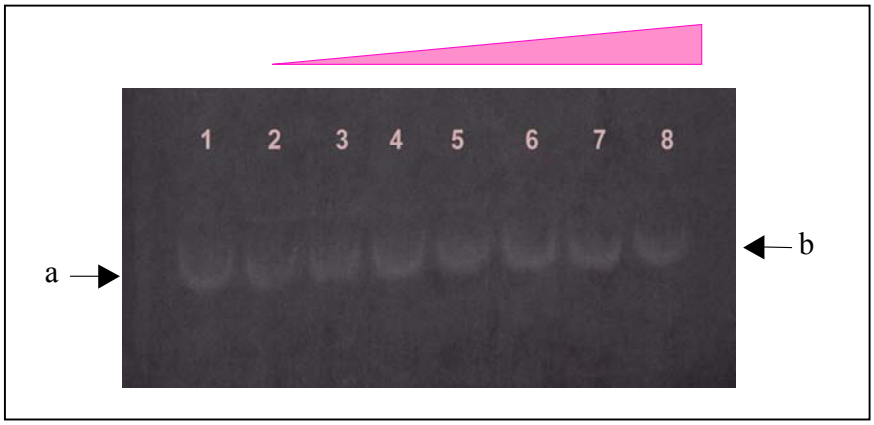

Figure 3 Cleaving of supercoiled pUC19 by *Phaleria macrocarpa* fruits pulp crude extract Lane 1. Untreated pUC19, 2 – 8. treated pUC19 with *P. macrocarpa* fruits pulp crude extract at a concentration of 0.05 µg/ml to 0.35 µg/ml.
(a). supercoiled DNA, (b). nicked-circular DNA

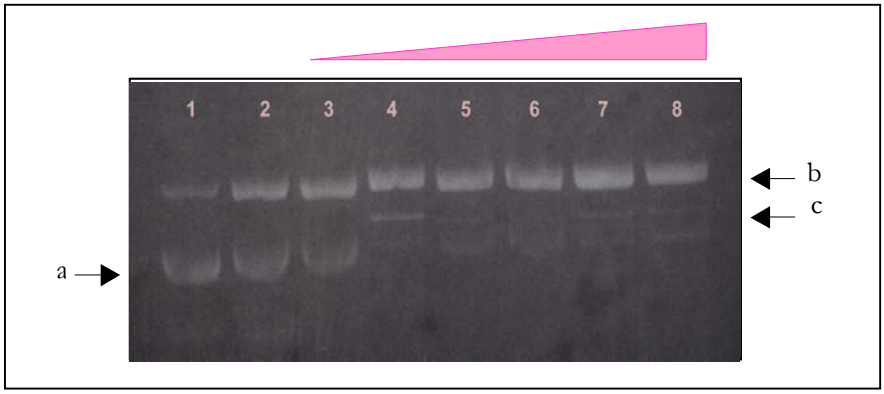

Figure 4. Cleaving of supercoiled pUC19 by *seeds* protein fraction of *Phaleria macrocarpa* Lane 1. Untreated pUC19, 2 – 8. treated pUC19 with *P. macrocarpa* seeds protein fraction at a concentration of 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; and 0.7 µg/ml respectivelly.
(a). supercoiled DNA, (b). nicked-circular DNA, (c). linear DNA

from supercoiled DNA, as well as the ability to cleave supercoiled DNA into nicked and linear forms. It suggested that phosphodiester bonds in the extensively deadenylated region of supercoiled DNA would become fragile and liable to be broken spontaneously owing to the existence of tension in the supercoiled DNA (Barbieri *et al*, 2000; He and Liu, 2004).

Conclusion

The results indicated that in *P. macrocarpa* fruits (pulps and seeds), only seeds contain RIPs-like proteins, which was suggested as a compound responsible for the activity.

Acknowledgements

We are grateful to Drs. Sudjadi Apt,MS.,PhD., Dr.Zullies Ikawati Apt., Faculty of Pharmacy Gadjah Mada University, for the critical reading of this manuscript, and to Ms. Rumbiwati, Life Science Laboratory Gadjah Mada University, as a technician.

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