Jatropha curcas latex inhibits the release of collagenase by gingival fibroblast

Fazwishni Siregar¹, S.M. Soerono Akbar²

Abstract

Folkloric use of J. curcas latex among others are to cure tooth pain, bleeding gum and as anti-inflammatory drug. Collagenase is a neutral protease released by activated macrophage and also by fibroblasts in small amounts. The aim of this study was to evaluate the effect of J. curcas latex on collagenase released by fibroblasts. Four doses of J. curcas latex from 37.5-300 µg/ml were added to 3 human gingival primary fibroblast cell culture. After 1 to 4 days of incubation, collagenase in the supernatant was assayed with collagen. The degradation products were then separated by SDS-PAGE and the density of ¾ αA bands were measured semi-quantitatively by Adobe Photo computer program. Result showed that J. curcas latex decreased collagenase released by human gingival fibroblast, and increasing dose inhibits more. It may be concluded that the latex of J. curcas inhibits the release of collagenase by human gingival fibroblast. (Med J Indones 2007; 16:219-23)

Abstrak


Keywords: Jatropha curcas, collagenase, human gingival fibroblast, collagenase assay, SDS-PAGE

Jatropha curcas, Euphorbiaceae, is a shrub or tree found in tropical areas. J. curcas latex contains tannins, saponins, wax, and resin.¹ Phytochemical screening by Ciulei method identified the main chemical compounds in J. curcas latex which were sterols, flavone aglycones in the ether extract, tannins, reducing compounds, sterol glycosides in the ethyl acetate extract, and tannins, reducing compounds, poliose, saponins in the aqueous extract.² A proteolytic enzyme called curcain can be obtained from the latex by alcohol and acetone precipitation. Curcain is a phytotoxin, which is a toxic protein molecule.³,⁴ In tropical Africa and Southeast Asia the latex is used as hemostatic and a wound dressing and is said to be efficacious in treating scabies, eczema, and ringworm. The latex is applied topically to wasp and bee stings. Furthermore, it is used as a mouth rinse to treat bleeding gums and to soothe a baby’s inflamed tongue. In Indonesia and the Philippines, a little latex on absorbent cotton is commonly used to cure toothache.¹,⁵,⁶ The latex is used as anti-inflammatory by massaging the latex on the traumatic area.⁷

Collagenase is a protease secreted by macrophage, neutrophyl, and also by activated fibroblast.⁸⁻¹¹ It has the unique ability to degrade collagen generating ¾ αA fragment, which is characteristic to collagen degraded by collagenase.¹² This enzyme is important in connective tissue remodeling occurring in normal processes or wound healing, for degradation and resolution of inflammation, however, in certain disease it may result in articular destruction. If collagenase is secreted into extra cellular tissues, it may degrade collagen, basal membrane, and other tissues components.

¹ Graduate from Doctorate Program, Faculty of Dentistry, University of Indonesia, Jakarta, Indonesia
² Faculty of Dentistry, University of Indonesia, Jakarta, Indonesia
Collagen products by collagenase degradation are chemotactic to macrophage, and macrophage accumulation will happen and inflammation will increase. In articular disease it may bring an extensive damage.\textsuperscript{9} Enzyme secretion can be induced by several agents as endotoxin, immune complex, and IL-1$\beta$.\textsuperscript{8} In periodontal disease, collagenase increase with the severity of the disease.\textsuperscript{13}

Several methods are known to detect collagenase products among others is collagenase activity assay with collagen as substrate which products are then separated by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE).\textsuperscript{8,10} The purpose of this study was to evaluate the effect of Jatropha curcas latex on the release of collagenase by fibroblast cells.

**METHODS**

Materials used for cell culture were: Dulbecco’s modified Eagle medium (DMEM), bovine serum albumin (BSA), penicillin G and gentamycin sulphate (Sigma), fetal bovine serum (FBS, Gibco), IL-1$\beta$. Three gingival fibroblast primary cell cultures were donated from the lab of Cell Biology and Histology, Amsterdam Medical Center.

Latex of J. curcas was obtained from trees grown in Research Institute for Plant Medicine and Spices, Bogor. The latex was lyophilized 50 hours and stored at −20°C. Fresh latex was equivalent with 15% solution of freeze-dried latex.

Materials for collagenase assay: APMA buffer: 7 mg APMA (4-aminophenyl mercuric acetate) was weighed in a glass test tube. Then 100 $\mu$L 1N NaOH solution and 1000 $\mu$L distilled water was added and shaken vigorously. About 10 $\mu$L 2N NaOH solution was added and shaken well, followed by 900 $\mu$L distilled water addition. To make APMA buffer, 1 ml APMA solution was added to 9 ml collagenase buffer (according to Ohshima).\textsuperscript{8} Collagenase assay buffer: Tris 50 mM pH 7.5, CaCl$_2$ 10 mM, NaCl 150 mM. Collagen (ICN).

Materials for SDS-PAGE: acrylamide 30% (Biorad), sodium dodecyl sulphate (SDS), Tris, ammonium persulfat (Biorad), TEMED (Biorad), mercaptoethanol, bromophenol blue (BPB), glycerol. SDS 20%: 200 g SDS and 800 ml deionized water was heated to 68°C, and pH was adjusted to 7.2 with HCl. Solution was stored at room temperature. 1.5 M Tris HCl pH 8.8: 18.171 g Tris was dissolved in 100 ml distilled water and pH was adjusted to 8.8 with HCl, and was stored at room temperature. 1 M Tris HCl pH 6.8: 12.114 g Tris was dissolved in 100 ml distilled water and pH was adjusted to 6.8 with HCl and was stored at room temperature. Tris glycine electrophoresis buffer, stock 5X: 15.1 g or 25 mM Tris base, 94 g or 250 mM glycine, were dissolved in 900 ml deionized water. Then, 50 ml 10% SDS was added and pH was adjusted to 8.3. Distilled water was added until the volume of 1.000 ml was reached and was stored at room temperature. Before used, it was diluted 5 times with deionized water. Ammonium persulfat (APS) stock 10% w/v: 1 g APS, 10 ml deionized water, and then was aliquot to 0.3 ml, and freeze. SDS loading buffer 1X: 50 ml 100 mM Tris HCl, pH 6.8, mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol, stored at -20°C. Coomassie Brilliant Blue (CBB): 0.25 g CBB R250, 45 ml methanol, 45 ml distilled water, 10 ml glacial acetic acid, filtered with Whatman no 1 filter paper. Destaining: 30% methanol, 10% acetic acid, 60% distilled water. 8% resolving gel: 4.6 ml distilled water, 2.7 ml 30% acrylamide, 22.5 ml 1.5 M Tris pH 8.8, 0.1 ml ammonium persulfat, 0.006 ml TEMED. Stacking gel 5%: 3.4 ml distilled water, 0.83 ml 30% acrylamide, 0.63 ml 1.0 M Tris pH 6.8, 0.05 ml 10% SDS, 0.05 ml ammonium persulfat, 0.005 ml TEMED.

**Procedure**

Human gingival fibroblast primary cells were cultured with DMEM-FBS 10% in 6-well plates. At confluency, the medium was changed with DMEM-BSA 0.1% added by 4 concentrations of latex from 37.5-300 µg/ml, and 10 ng/ml IL-1$\beta$ to activate collagenase production. Following 1 to 4 days of incubation, supernatants were taken and stored at −20°C. Twenty-microlitre sample was incubated in collagenase assay buffer with APMA to activate latent collagenase at 37°C for 30 minutes. Enzyme assay was carried out by adding 10 µl collagen (2.8 µg/ml) and incubated overnight at 22°C. Enzyme reaction was stopped by adding SDS loading buffer and heating for 5 minutes at 95°C. Reaction products were separated by SDS-PAGE. After overnight CBB staining, the gel was destained for 30 minutes. The gel with blue bands was then photographed, copied into computer disc, and the ¾ $\alpha$A bands density were measured semiquantitatively by Adobe Photo computer programme. Evaluation was based on comparing the density of ¾ $\alpha$A, which is characteristic of collagen degraded by collagenase.
RESULTS

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
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<th>11</th>
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<tr>
<td>Collagen</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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Lane 14, molecular weight marker (MWM). Arrow, ¾ αA band. Longer culture time produced more collagenase. Increasing latex decreased collagenase released.

Table 1. Semiquantitative measurement of ¾ αA band from day 1 and 2

<table>
<thead>
<tr>
<th>J. curcas</th>
<th>Day 1</th>
<th>Day 2</th>
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<tbody>
<tr>
<td>None</td>
<td>103</td>
<td>100</td>
</tr>
<tr>
<td>0 µg/ml</td>
<td>77</td>
<td>127</td>
</tr>
<tr>
<td>37.5 µg/ml</td>
<td>60</td>
<td>94</td>
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<td>75 µg/ml</td>
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<td>39</td>
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<tr>
<td>150 µg/ml</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>300 µg/ml</td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>

Band measurement by Adobe Photo program. At Table 1, the standard 0 was collagen without supernatan of cell culture (Fig. 1 lane 1), standard 100 was collagen with supernatan of day 2 (Fig. 1 lane 13). Increasing latex decreased the density of ¾ αA band.

Table 2. Semiquantitative measurement of ¾ αA band from day 3 and 4

<table>
<thead>
<tr>
<th>J. curcas</th>
<th>Day 3</th>
<th>Day 4</th>
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<tr>
<td>None</td>
<td>99</td>
<td>90</td>
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<tr>
<td>0 µg/ml</td>
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<td>37.5 µg/ml</td>
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<td>100</td>
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<td>75 µg/ml</td>
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<td>70</td>
</tr>
<tr>
<td>150 µg/ml</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>300 µg/ml</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

At Table 2, the standard 0 was collagen without supernatan of cell culture (Fig. 2 lane 1), standard 100 was collagen with supernatan activated by IL-1β and latex J. curcas 37.5 µg/ml of day 4 (Fig. 1 lane 13). Increasing latex decreased the density of ¾ αA band.

Longer culture time released more collagenase proved by the increasing density or thicker αA bands in control cells from day 1 (Fig.1, lane 6), day 2 (Fig.1, lane 13), day 3 (Fig. 2, lane 6), and thickest at day 4 (Fig. 2, lane 12). The αA bands from the IL-1β activated groups were also increased from day 1 (Fig. 1, lane 2) and thicker on day 4 (Fig. 2, lane 8). Collagenase was also released in sample not activated by IL-1β that were appeared as αA band at lane 6 Fig. 1.

Addition of increasing dose of J. curcas latex narrowed the bands that were no longer observed at 300 µg/ml latex. At lane 7 and 14 Fig. 2 treated with 37.5 µg/ml latex, the ¾ αA bands were appeared clearly. These bands narrowed at lane 4 and 10 Fig. 2 which treated with 75 µg/ml latex and more narrowed if treated with 150 µg/ml latex as appeared at lane 5 and 11 Fig. 2.

Semiquantitative measurement of ¾ αA band (Table 1) on Figure 1 also showed that increasing dose of J. curcas
latex decreased the density of 3/4 αA band from 77 on day 1 and 127 on day 2 at 0 μg/ml to 22 on day 1 and 0 on day 2 at concentration 300 μg/ml latex. Similar result was observed on semiquantitative measurement Table 2 taken from Figure 2. The density of 3/4 αA bands were decreased from 83 at 0 μl/ml to 7 at 300 μl/ml on day 3 and from 111 at 0 μl/ml to 0 at 300 μl/ml on day 4.

The result showed that J. curcas latex inhibits the release of collagenase, and increasing dose inhibits more.

DISCUSSION

The purpose of this study was to investigate the effect of latex on the collagenase release by human fibroblast. Collagenase is release mainly by activated macrophage, but it can also be produced and released by activated fibroblast though in small amount. In this study, collagenase was also released in sample not activated by IL-1β that were appeared as ¾ αA band at lane 6 Fig. 1. For control was supernatant of cell culture that neither added with latex nor activated by IL-1.

The narrowing of bands observed in the addition of J. curcas latex was interpreted as inhibition of collagenase released. These narrowing bands were already seen in very low latex concentration (37.5 μl/ml) and at 300 μg/ml no more bands were seen. Preliminary study showed that this latex did not eliminate collagenase activity. Addition of increasing dose of J. curcas latex lowered the band density observed at Table 1 and 2.

The inhibition of collagenase release is probably because of the cytotoxic effect of the latex that influenced the productivity of cells. In this study, the latex concentration was not that killed all the cells, but those that reduced the cells into nearly half compared to the control. This was acquired from cytotoxicity study with MTT assay.

In tissues, it is possible that absorption of latex in small amount may inhibit collagenase production. This enzyme production inhibition may decrease the severity of inflammation. In Peru, the latex is used as anti-inflammatory by massaging in traumatic area. In South East Asia the latex is used to cure toothache. It is also used as gargoyle in bleeding gum that may be interpreted as gingival inflammation or marginal periodontitis. Beside its tannin content, its hemostatic and cytotoxic activity, it is also possible that the inhibition of collagenase release, which increases in inflammation, take place. Inhibition of collagenase release will consequently reduce its effects in tissues.

From this study, it may be concluded that J. curcas latex inhibits the release of collagenase by human gingival fibroblast primary cell culture.

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REFERENCE

