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Original Research Paper

Enhancing indigenous lipase activity of germinated *Jatropha curcas* L. Seeds for the enzymatic degradation of phorbol ester

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ABSTRACT

Both the unique substrate specificity of plant lipases and the enzymatic degradation of phorbol esters (PE) have not been studied extensively. The objectives of the research were to improve the indigenous lipase activity of germinated *Jatropha* seeds and its specificity in the enzymatic PE degradation. Factors that influence the lipase activity of germinated seeds, such as, the effect of the soaking buffer pH, soaking time, growing stage, and aeration during soaking were evaluated. Furthermore, the effects of the growing stage, reaction time, and the amount of enzymes on PE degradation were also evaluated. Results show that stress conditions by changing the soaking buffer pH from neutral into slightly acidic or alkaline conditions inhibited seed germination but induced the lipase production. The highest lipase activity was obtained from growing stage 4, in which the seeds were soaked at pH 6 for 12 h without aeration. Aeration reduced lipase activity about 19.5 times. On the other hand, PE was degraded during germination. The highest activity of germinated seed lipase for PE degradation was also obtained from growing stage 4. Degradation of PE in the *Jatropha* cake using 10 U for 12 h was enough to fulfil the requirement of a non-toxic *Jatropha* seed cake.

1. Introduction

Defatted pressed *Jatropha curcas* L. seed cake (DPJSC) has high protein content and is rich in essential amino acids (Makkar et al., 1998; Saetae et al., 2011). DPJSC has not been utilized as food or feed due to the content of toxic phorbol esters (PE) (Makkar et al., 1997, 1998, 2009). PE and its derivatives were also reported to potentially cause tumours (Aitken, 1986). Therefore, PE must be reduced to a safe level. Various chemical and physical methods have been performed to reduce PE content (Devappa et al., 2008; Martınez-Herrera et al., 2006; Aregheore et al., 2003). Most of the treatments use a combination of alkaline, high temperatures, and solvent extraction, none of which are environmentally friendly.

PE has an ester bond between phorbol moiety and its fatty acid, which is an analogue to diacyl glycerol (Goel et al., 2007; Li et al., 2010; Mentlein, 1986; Cabot, 1985). Ester bonds of PE may be hydrolysed by lipase (triacylglycerol acyl hydrolase, E.C. 3.1.1.3) (Bose and Keharia, 2013). However, data on the enzymatic PE hydrolysis are very limited.

On the other hand, most lipases were obtained from microbes and animals (Ganesan et al., 2009; Treichel et al., 2010; Salis et al., 2007; Mateos Diaz et al., 2006). However, the unique substrate specificity of plant lipases may still have low consideration for industrial application since the optimal production of plant lipases has not yet been studied intensively. In general, lipase activity is absent in un-germinated seeds. It may be produced during germination of oil seeds (Abigor et al., 2002; Haas et al., 2001; Wanasundara et al., 1999). Research on seed germination has mostly focused on optimizing the amount of germinated seeds and shortening the germination time (Abigor et al., 2002; Hu et al., 2009; Olvera-Carrillo et al., 2003; Tanaka-Oda et al., 2009; Agyili et al., 2007; Schelin et al., 2004; Ko et al., 2004; Zhu et al., 2006). However, the specific factors that affected the optimum condition for enhancing lipase activities during germination were not studied intensively yet. The objectives of this research were to evaluate both the specific factors which improve indigenous lipase activities of germinated *Jatropha* seeds during germination and its ability for enzymatic PE degradation. Factors such as, the effect of soaking buffer pH, time of soaking and germination, and aeration during soaking on the lipase activities of germinated seeds were evaluated. The effects of the growing stage, reaction time, and the amount of enzymes on the degradation of PE were also evaluated.

2. Materials and methods

2.1. Materials

Fresh *J. curcas* L. seeds were obtained from farmers around the Gunung Kidul District in the Province of Yogyakarta. Olive oil and

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Phorbol-12-myristate-13-acetate (PMA) standard were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol, hexane, EDTA, KCl, $MgCl_2$, pyridine, oleic acid, acetone, isooctane, NaH_2PO_4, Na_2HPO_4, cupric-acetate, and sucrose were obtained from Merck KGaA (Darmstadt, Germany). Petroleum ether was obtained from J.T. Baker (USA).

2.2. Effect of aeration during seeds soaking on lipase activity

Seeds were soaked in water containing Daconil fungicide (1.5 g/L) at room temperature for 12, 18, and 24 h. Seeds were aerated during the soaking process. The other seeds were not aerated as a control. Subsequently, water was drained and seeds were dried at room temperature for 1 h. Seeds were incubated in the incubator (RH > 90%) for 24 h at 30 °C. The seeds were removed from the incubator, spread onto paper towels, and allowed to germinate at ambient temperature. The amount of germinated seeds was determined every day for 7 days of observation. Germinated seeds were stored at −18 °C until ready for an enzyme assay.

2.3. Effect of seed soaking in various buffers of pH on lipase activity

Seeds were soaked in various pH of 0.1 M buffers (5, 6, 7 and 8) containing fungicide (1.5 g/L) for 12 h at room temperature. Germination was further performed according to Section 2.2 except when otherwise stated. Germinated seeds were stored at −18 °C until ready for an enzyme assay.

2.4. Effect of seed soaking times on lipase activity

Seeds were soaked in a buffer of pH 6 containing fungicide (1.5 g/L) at room temperature for 8, 12, 18, and 24 h. Germination was further performed according to Section 2.2 except when otherwise stated. Germinated seeds were stored at −18 °C until ready for an enzyme assay.

2.5. Effect of the growing stage on lipase activity

Seeds were soaked in a buffer of pH 6 containing fungicide (1.5 g/L) at room temperature for 12 h. Germination was further performed according to Section 2.2 except when otherwise stated. Germinated seeds were stored at −18 °C until ready for an enzyme assay. Growing at stage 0, 1, 2, 3, and 4 were analysed for lipase activities, in which sprout lengths were 0, less than 0.5 cm, 0.5–1 cm, 1–2 cm, and 2–2.5 cm, respectively.

2.6. Enzyme preparation

Extraction of germinated seed lipases were performed according to (18) with some modifications. The seed cotyledons were added into a 0.1 M buffer of pH 7.5 containing of 0.6 M sucrose, 1 mM EDTA, 10 mM KCL, and 1 mM $MgCl_2$ (ratio 1:5 (w/v)) and were further homogenized using a homogenizer at 5200 rpm for 10 min. Suspension was centrifuged for 30 min at 4800 rpm. Supernatant was used as a crude extracted germinated *Jatropha* seed lipase using buffer (BEG) and analysed for lipase activity.

The acetone dried germinated seed powder (ADSP) lipase was prepared according to Enujiugha et al. (2004) with some modifications. Twenty five grams of germinated seeds were peeled and the shells were removed. The seed cotyledons were added into 35 ml of cold acetone (−18 °C) and further homogenized using a homogenizer at 5200 rpm for 10 min. Supernatant was transferred into the column and washed with cold acetone to separate the germinated seed oil (4 °C). The residue was air dried at room temperature to obtain ADSP lipase, which was kept at −18 °C until ready for an assay.

2.7. Production of J. curcas L. seed cake

The seed cotyledons were milled and further dried at 50 °C for 24 h. Seed powder was pressed by a hydraulic press. The seed cake was further milled and sieved. The defatted pressed *Jatropha* seed cake (DPJSC) was obtained by extracting the oil content of cake powder using n-hexane.

2.8. Hydrolysis of phorbol esters

Hydrolysis of PE was conducted by adding ADSP lipase into the suspension of DPJSC in a phosphate buffer of pH 7, followed by incubation at 30 °C in a shaker water bath (Julabo SW23). Samples were taken at certain time periods and cooled in an ice bath. They were then filtered. PE was extracted, and further analysed using a method as described in Section 2.10. The percentage of PE degradation was calculated as follows:

$$\text{PE degradation } (\%) = \frac{\text{PE at time } t}{\text{Initial } PE} \times 100\%$$

2.9. Extraction of phorbol esters

PE were extracted according to Saetae and Suntornsuk (2010) with some modifications. *J. curcas* L. seed cake (5 g) was added into flasks containing 20 ml of methanol. Suspension was stirred at 150 rpm for 5 min. It was then filtered using filter paper. The PE extraction was repeated four times. The extract fractions of all five extractions were pooled together and were rotary-evaporated at 50 °C (IKA-WERKE) to obtain the concentrated PE fractions.

2.10. Analysis of phorbol esters

The extracted PE was analysed by Thin Layer Chromatography (TLC) according to Demissie and Lele (2010). The quantification of PE was performed by CAMAG TLC scanner 3 "dummy" S/N (1.14.16) controlled using CAMAG winCATS planar chromatography software at 263 nm. A standard curve was performed using phorbol-12-myristate-13-acetate. The results were expressed as an equivalent to phorbol-12-myristate-13-acetate.

2.11. Enzyme activity determination

Lipase hydrolytic activity assay was performed according to Marseno et al. (1988) with some modifications. For BEG lipase, hydrolytic reaction was initiated by adding 400 μl BEG lipase into a flask containing 2 ml of olive oil in isooctane (60:100 v/v). The mixture was incubated at 30 °C for 20 min. For ADSP lipase, the reaction was initiated by adding 0.1 g ADSP lipase into a flask containing of a suspension of 2 ml phosphate buffer of pH 7 and 2 ml olive oil in isooctane (60:100 v/v). The mixture was incubated at 30 °C for 20 min. The reactions were stopped by placing the flasks in an ice bath. The forming of free fatty acid was further determined. Unit activity was expressed as the amount of the hydrolysed free fatty acid (μmol) per min.

2.12. Free fatty acid determination

A sample (200 μL) was added into the mixture of isooctane (1.8 ml) and cupric-acetate pyridine (0.4 mL). The mixture was incubated at 30 °C for 10 min, and the absorbance was determined at 715 nm. Free fatty acid (FFA) was determined by difference.