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Characterization of an extracellular lipase from the biocontrol fungus, *Nomuraea rileyi* MJ, and its toxicity toward *Spodoptera litura*

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ABSTRACT

An extracellular lipase from *Nomuraea rileyi* MJ was purified 23.9-fold with 1.69% yield by ammonium sulfate precipitation followed by Sephacryl S-100 HR column chromatography. By mass spectrometry and SDS–polyacrylamide gel electrophoresis, the molecular weight of the homogenous lipase was 81 kDa. The N-terminal sequence was determined as LeuSerValGluGlnThrLysLeuSerLysLeuAlaTyrAsnAsp and it showed no homology to sequences of known lipases. The optimum pH and temperature for activity were 8.0 and 35 °C, respectively. The enzyme was stable in the pH range 7.0–9.0 and at 15–35 °C for 1 h. Higher activity was observed in the presence of surfactants, Na^+, NH_4^+ ions, NaN_3 and ethylenediaminetetraacetic acid (EDTA), while Co^{2+} and Cu^{2+} ions, cysteine and dithiothreitol (DTT) strongly inhibited activity. The purified lipase hydrolyzed both synthetic and natural triglycerides with maximum activity for trilaurin and coconut oil, respectively. It also hydrolyzed esters of *p*-nitrophenol (*p*NP) with highest activity for *p*-nitrophenyl caprate (*p*NPCA). The purified lipase was found to promote *N. rileyi* spore germination *in vitro* in that germination reached 98% in conidial suspensions containing purified lipase at 2.75 U. Moreover, it enhanced toxicity of *N. rileyi* toward *Spodoptera litura* larvae with mortality via topical application reaching 63.3% at 4–10 days post-treatment which calculated to be 2.7 times higher than the mortality obtained using conidial suspensions alone.

1. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are hydrolytic enzymes that catalyze the hydrolysis of fats and oils to glycerol and free fatty acids at the oil–water interface. An important characteristic of lipases is their ability to not only hydrolyze ester bonds, to transesterify triglycerides and to resolve racemic mixtures but also to synthesize ester bonds in nonaqueous media (Kirchner et al., 1985; Macrae and Hammond, 1985). Consequently, lipases have been widely used in various industries to produce foods, dairy products, pharmaceuticals, detergents, textiles, biodiesel, cosmetics, fine chemicals, agrochemicals and new polymeric materials (Jaeger and Eggert, 2002). Further, they can have broad substrate specificity but can also be very selective in the reactions catalyzed, especially regarding high enantioselectivity (Jaeger et al., 1999). A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin (Sharma et al., 2001).

Although lipases are produced by animals, plants and microorganisms, the majority of lipases used for biotechnological purposes have been isolated from bacteria and fungi (Lin et al., 2006). Filamentous fungi are preferred sources since they produce extracellular enzymes. A large number of lipase producing filamentous fungi have been characterized, including those from *Mucor hiemalis f. hiemalis* (Hiol et al., 1999), *Rhizopus oryzae* (Hiol et al., 2000), *Aspergillus carneus* (Saxena et al., 2003), *Penicillium aurantiogriseum* (Lima et al., 2004), *P. camembertii Thom* PG-3 (Tan et al., 2004), *Metarhizium anisopliae* (Silva et al., 2005) and *Yarrowia lipolytica* (Yu et al., 2007). For filamentous fungi, much information is already available concerning the structure and regulation of lipase synthesis at the genetic level and on environmental factors affecting production and activity (Jaeger et al., 1994). With the exception of *M. anisopliae*, little information is available concerning lipases from fungal entomopathogens.

The potential use of entomopathogenic fungi for controlling insect pests is widely recognized. There are several products currently commercially available, including products based on *Beauveria bassiana*, *M. anisopliae*, *Paecilomyces farinosus*, *Lecanicillium lecanii* and *Nomuraea rileyi*. The first attempt to use *N. rileyi* as biological control agent took place in 1955. It is now a well-known mycopathogen of many noctuid pest defoliators, targeting polyphagous species of the genera *Heliothis*, *Spodoptera*, *Pseudoplusia*, *Trichoplusia*, *Plutella* and *Rachiplusia* (Boucias et al., 1984a).

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The life cycle of *N. rileyi* has been reported in a few insect hosts (Boucias and Penland, 1982; Thorvilson et al., 1985; Srisukchayakul et al., 2005). Infection begins with attachment of conidia to the insect integument followed by cuticle penetration. For the latter, synergistic activity of hydrolytic enzymes including lipases, chitinases and proteases is required (Clarkson and Charnley, 1996), since the insect cuticle presents a structurally and chemically complex barrier. The epicuticle contains phenol-stabilized protein covered by a waxy layer containing fatty acids, lipids and sterols (Anderson, 1979). It is a potential target for the action of specific lipases that are important infection enzymes for entomopathogenic fungi. Clarkson and Charnley (1996) reported that cuticle-degrading enzymes, proteases, chitinases and lipases in entomopathogenic fungi participate in penetration of hyphae into the haemocoel. It has been found that *N. rileyi* conidia produce cuticle-degrading enzymes, chitinase, protease and lipase during this process (Boucias et al., 1982), and *M. anisopliae* secretes at least four different classes of protease (St. Leger et al., 1994; Nahar et al., 2004). In *B. bassiana*, virulent and less virulent strains showed differences in total activity of esterase, esterase-lipase, β-galactosidase, chitinase and protease (Kosir et al., 1991). The relationship between lipase production and virulence in *B. bassiana* isolates has been studied. A clear relationship between lipase production and virulence was not found because no correlation was found between the two variables (Varela and Morales, 1995).

Consequently, an in-depth study of *N. rileyi* lipase and its regulation may be considered an important step toward understanding its role in pathogenesis and for the development of strains with improved entomopathogenicity. The objective of this study was to purify and characterize the enzyme and examine its influence on fungal spore germination and insect toxicity.

2. Materials and methods

2.1. Microorganism and culture conditions

N. rileyi MJ strain was isolated from infected *Spodoptera* larva collected in a cabbage field at Maechaem district, Chiang Mai province, Thailand, in the winter of 2002. It was propagated on fish lysate agar [3% (w/v) fish lysate, 1% (w/v) maltose, 1% (w/v) yeast extract and 1.5% (w/v) agar] (Srisukchayakul et al., 2005) for 7–14 days at 25 °C. Conidial suspensions were prepared by extracting conidia from fish lysate slants with 0.01% (v/v) Tween 80 in sterile distilled water. The concentration of conidia was determined with a hemocytometer using the light microscope (Nikon Eclipse E600 microscope, Nikon Instruments, Inc., USA). Conidia (10^6 spores/ml) were inoculated into 1000 ml of basal medium [0.2% (w/v) glucose, 0.5% (w/v) tryptone, 0.01% (w/v) $MgSO_4.7H_2O$, 0.1% (w/v) K_2HPO_4] supplemented with 4% (v/v) castor oil with an initial pH value of 8.0 in a 2000-ml Erlenmeyer flask incubated on rotary shaker at 150 rpm for 8 days at 25 °C. At the fourth day of *N. rileyi* growth, cultures were supplemented with 0.25% (v/v) Tween 80. Cultures were filtered through Whatman filter no. 1 and centrifuged at 10,000*g* for 30 min at 25 °C. The supernatant was considered as crude enzyme and in this preparation, protein concentration and lipase activity were assayed.

2.2. Rearing of larvae

Spodoptera litura (Lepidoptera: Noctuidae) larvae were field collected and reared on fresh castor foliage at 25–27 °C. Adult males and females were mated and resulting F_1 eggs were incubated at 25 °C. First instar larvae were fed on castor foliage until they reached the third instar stage.

2.3. Determination of lipase activity

Yield of *p*-nitrophenol was used to measure lipase activity with *p*-nitrophenylpalmitate (*p*NPP) (Sigma, USA) as the substrate (Maia et al., 2001). The assay mixture consisted of 100 μl of sample and 900 μl of substrate solution containing 10 mg of *p*NPP dissolved in 1 ml of propan-2-ol diluted in 9 ml of 50 mM Tris–HCl pH 8.0 containing 40 mg of Triton X-100 and 10 mg of gum arabic. The assay mixture was incubated at 37 °C for 30 min and the *p*-nitrophenol released was measured at 410 nm. One unit of activity was defined as the amount of enzyme that liberated 1 nmol of *p*-nitrophenol per min under the assay conditions. This method was the standard assay for lipase activity. Alternatively, lipase activity was measured by using palm oil as the substrate (Know and Rhee, 1986). One unit of activity was defined as the amount of enzyme that liberated 1 nmol of free fatty acid per min under the assay conditions. The protein concentration was measured using the method described by Bradford (1976) with bovine serum albumin as the standard.

2.4. Purification of lipase

In the first step of purification, ammonium sulfate precipitation was carried out overnight at 4 °C at a final concentration of 80% saturation. Following centrifugation at 13,200*g* for 40 min at 4 °C, the pellet was dissolved in 50 mM sodium phosphate with 0.15 M NaCl buffer pH 7.0 and dialyzed against distilled water containing 1 mM EDTA. The dialyzed enzyme preparation was analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) to evaluate purity of the lipase present. Gel filtration was used for a subsequent purification step by applying the dialyzed enzyme preparation to a pre-equilibrated HiPrep 16/60 Sephacryl S-100 column (Pharmacia, Sweden) followed by elution at a flow rate of 0.1 ml/min. Fractions (0.5 ml) were collected automatically using an AKTA FPLC collector (Amersham Biosciences, Germany). Each fraction was dialyzed extensively and run on SDS–PAGE to evaluate lipase purity. Protein concentration and lipase activity were also assayed.

2.5. Gel electrophoresis and activity detection

SDS–PAGE was carried out according to Laemmli (1970). After electrophoresis, the method of Valeria et al. (2004) was used to detect lipase activity. Briefly, the gel was washed for 30 min in 2.5% (v/v) Triton X-100 and then washed rapidly with 50 mM sodium phosphate buffer pH 7.0 before it was immersed in a 100 μM solution of methylumbelliferyl butyrate (MUF-butyrate) (Fluka, Switzerland). Blue fluorescent bands indicating lipase activity were visualized using a UV transilluminator at 365 nm.

2.6. Characterization of purified lipase

2.6.1. Effect of pH on the activity and stability of purified lipase

Activity assays were done using the *p*NPP method at 37 °C in assay mixtures buffered to various pH values. The following buffers were used to study the effect of pH and type of buffer on activity and stability of purified lipase: citrate–phosphate pH 6.0, 6.5, 7.0 and 7.5; phosphate pH 7.0, 7.5, 8.0 and 8.5; Tris–HCl pH 8.0, 8.5, 9.0 and 9.5; glycine–NaOH pH 9.0, 9.5 and 10.0, all at 50 mM. Stability assays were done by incubating the purified lipase at 37 °C for 30 and 60 min in buffers of different pH values (glycine–HCl pH 2.0 and 3.0; citrate pH 3.0–6.0; citrate–phosphate pH 5.0–7.0; phosphate pH 7.0 and 8.0; Tris–HCl pH 8.0 and 9.0; glycine–NaOH pH 9.0 and 10.0, all at 50 mM). The residual lipase activity was measured at 37 °C using 50 mM Tris–HCl buffer pH 8.0 by the *p*NPP method.