Mycoscience (2010) 51:405–410
DOI 10.1007/s10267-010-0054-7

FULL PAPER

Purification and characterization of a high-thermostable β-xylanase from newly isolated *Thermomyces lanuginosus* THKU-49

Khwanchai Khucharoenphaisan · Shinji Tokuyama · Vichien Kitpreechavanich

Received: 28 December 2009 / Accepted: 2 April 2010 / Published online: 28 April 2010

Abstract Highly thermostable β-xylanase produced by newly isolated *Thermomyces lanuginosus* THKU-49 strain was purified in a four-step procedure involving ammonium sulfate precipitation and subsequent separation on a DEAE-Sepharose fast flow column, hydroxylapatite column, and Sephadex G-100 column, respectively. The enzyme purified to homogeneity had a specific activity of 552 U/mg protein and a molecular weight of 24.9 kDa. The optimal temperature of the purified xylanase was 70°C, and it was stable at temperatures up to 60°C at pH 6.0; the optimal pH was 5.0–7.0, and it was stable in the pH range 3.5–8.0 at 4°C. Xylanase activity was inhibited by Mn^{2+}, Sn^{2+}, and ethylenediaminetetraacetic acid. The xylanase showed a high activity towards soluble oat spelt xylan, but it exhibited low activity towards insoluble oat spelt xylan; no activity was found to carboxymethylcellulose, avicel, filter paper, locust bean gum, cassava starch, and *p*-nitrophenyl β-D-xylopyranoside. The apparent K_m value of the xylanase on soluble oat spelt xylan and insoluble oat spelt xylan was 7.3 ± 0.236 and 60.2 ± 6.788 mg/ml, respectively. Thin-layer chromatography analysis showed that the xylanase hydrolyzed oat spelt xylan to yield mainly xylobiose and xylose as end products, but that it could not release xylose from the substrate xylobiose, suggesting that it is an endo-xylanase.

Keywords Biotechnological industry · Catalytic properties · Enzyme · Hydrolysis of oat spelt xylan

K. Khucharoenphaisan · V. Kitpreechavanich (✉)
Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
e-mail: fsciwck@ku.ac.th

S. Tokuyama
Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan

Introduction

Thermomyces lanuginosus (formerly known as *Humicola lanuginosa*) is a thermophilic ascomycetes fungus that has attracted considerable interest due to its production of stable enzymes, especially xylanase, which belongs to glycosyl hydrolase family 11. Several *T. lanuginosus* xylanases have been characterized to date. The purified xylanases isolated from *T. lanuginosus* strains SSBP, DSM 5826, ATCC 1645, ATCC 22083, ATCC 26909, ATCC 34626, ATCC 36350, ATCC 58160, and DSM 10635 have been reported to have half-life values of 232, 201, 54, 81, 79, 126, 121, 116, and 40 min, respectively, in 50 mM of buffer at pH 6.5 and 70°C (Singh et al. 2000a; Xiong et al. 2004). The isolation of *T. lanuginosus* from soil samples in Thailand has been intensively pursued in the search for high-stable xylanase, which is regarded as an extremely useful enzyme in the biotechnological industry, such as in the biobleaching process and in animal-feed production. Among the 88 strains of *T. lanuginosus* isolated to date, the crude xylanase obtained from *T. lanuginosus* THKU-49 has shown the highest thermal stability, with a half-life of 266 min at 70°C (Khucharoenphaisan and Kitpreechavanich 2004). To characterize the enzyme and compare it to those reported previously, we have investigated the purification process of the xylanase isolated from *T. lanuginosus*

THKU-49, characterized its bio-chemicals, and examined the catalytic properties of the pure xylanase.

Materials and methods

Fungus strain and culture medium

The *T. lanuginosus* THKU-49 strain used in this study was isolated from soil (Khucharoenphaisan and Kitpreechavanich 2004) and preserved at Bangkok MIRCEN. It was grown on yeast glucose agar medium for stock culture and kept at 4°C.

Two pieces of a 0.5-mm agar block of fungal mycelium grown on yeast glucose medium for 3 days were inoculated into a 50-ml Erlenmeyer flask containing 15 ml of synthetic medium (per liter of water: 2 g KH_2PO_4, 0.3 g $CaCl_2$, 0.3 g $MgSO_4$, 5 g peptone, 3 g yeast extract, 3 g malt extract, 10 g oat spelt). The initial pH of the medium was adjusted to 6.0 and then autoclaved for 20 min at 121°C. The flasks were incubated at 45°C on a reciprocal shaker (model BS-30; JEIO TECH, Seoul, Korea) at a shaking speed of 120 strokes/min for 1–7 days. The clear culture broth that was obtained by filtering the culture medium through Whatman No.1 filter paper (Whatman, New York, NY) was used for the assay of β-1,4-xylanase activity.

Determination of β-xylanase activity

β-Xylanase activity was assayed by determining the concentration of reducing sugars liberated from oat spelt xylan incubated in the diluted enzyme solutions at pH 6.0 and 50°C for 10 min. The reducing sugars were measured using the DNS reagent (3, 5-dinitrosalisylic acid). One unit of β-xylanase activity was defined as the amount of enzyme that produced 1 μM of xylose in 1 min.

Purification of xylanase

All purification steps were performed at 4°C. The filtered culture broth was first subjected to 80% $(NH_4)_2SO_4$ saturation. The precipitated protein was collected by centrifugation at 10,000 *g* for 5 min and dissolved in 20 mM phosphate buffer (pH 6.0). A dialysis bag with a cut-off of 10,000 Da was used to remove salt from the sample, and the dialyzed sample was applied to a DEAE–Sepharose fast flow column (GE Healthcare, Little Chalfont, UK). The bound xylanase was eluted using a 0–400 mM NaCl gradient, and the active fractions were pooled. A large volume of pooled eluted sample was then loaded to a hydroxylapatite column and the bound xylanase eluted using a 20–500 mM phosphate buffer (pH 6.0) gradient. The active fractions were pooled and precipitated using 80% $(NH_4)_2SO_4$ saturation. The precipitated protein collected by centrifugation was dissolved in 20 mM phosphate buffer (pH 6.0), and the sample was applied to a Sephadex G-100 column (GE Healthcare). The enzyme was eluted using 3 mM sodium phosphate buffer (pH 6.0), and the active fractions were combined. The purity of the protein was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli 1970).

Determination of molecular weight

The molecular weight of the proteins was determined using SDS–PAGE following the method of Laemmli (1970). The sample was dissolved in a sample-applicable buffer containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 12.5% (v/v) glycerol, and 15 mM Tris-HCl buffer (pH 6.8) and heated in boiling water bath for 5 min. Each lane was loaded with 50 μg of protein. The stacking and separating gels consisted of 5 and 12.5% polyacrylamide, respectively. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. A low-molecular-weight calibration kit (model SM0431; Fermentas, Burlington, ON, Canada) was used as the molecular weight standards.

Zymogram analysis

The zymogram analysis was performed as described by Ratanakhanokchai et al. (1999) with modifications. The pure enzyme in the sample application buffer was boiled for 2 min and subjected to electrophoresis on an SDS–12.5% polyacrylamide gel as described above. Following the electrophoresis, the gel was first soaked in 25% (v/v) isopropanol with gentle shaking to remove the SDS and renature the proteins in the gel, then washed four times for 30 min each time at 4°C in 50 mM phosphate buffer (pH 6.0). The washed gel was incubated with 1% soluble oat spelt xylan in 50 mM phosphate buffer pH 6 at 50°C for 30 min, soaked in 0.1% Congo red solution for 30 min at room temperature, and washed with 1 M NaCl until the excess dye was removed from the active band. The gel was then submerged in 0.5% acetic acid, which resulted in the background turning dark blue and the activity bands becoming distinct as clear colorless areas.

Effect of temperature and pH on β-xylanase activity and stability

The optimal temperature for xylanase activity was determined by incubating the enzyme in 50 mM sodium phosphate buffer (pH 6.0) at various temperatures (30–100°C) for 10 min and then measuring the amount of xylanase

activity. To ensure stability, we pre-incubated the reactions at the various temperatures for 10 min. The remaining xylanase activity was measured using a standard assay procedure.

The optimal pH for xylanase activity was determined by incubating the enzyme in volumes of 50 mM buffer at various pH values (3.5–8.0) at 50°C for 10 min and then measuring the amount of xylanase activity. To determine the pH stability of the enzyme, we incubated the enzyme at the different pHs mentioned above at 4°C for 60 min. The remaining xylanase activity was measured using a standard assay procedure.

Effect of various additives on β-xylanase activity

The effect of various additives on xylanase activity was analyzed by incubating diluted pure xylanase in the presence of 1 mM solution of K^+, Li^+, Na^+, Sn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, EDTA, and β-mercaptoethanol for 15 min at 50°C prior to the reaction with substrate (Heck et al. 2006). The xylanase activity assayed in the absence of additive was taken as the control value.

Substrate specificity and K_m value

The substrate specificity of the enzyme was determined by incubating the xylanase with 5 mg/ml of each of the following substrates: soluble oat spelt xylan, insoluble oat spelt xylan, carboxymethylcellulose, avicel, filter paper, locust bean gum, cassava starch, and *p*-nitrophenyl β-D-xylopyranoside. The analysis occurred in 50 mM sodium phosphate buffer (pH 6.0) at 50°C for 10 min. The amount of reducing sugars produced in enzyme reaction was estimated using the DNS method.

To calculate the K_m value on the hydrolysis of soluble oat spelt xylan and insoluble oat spelt xylan, 2–10 g/l of each substrate in 50 mM sodium phosphate buffer (pH 6.0) was incubated at 50°C for 2–20 min. The K_m value was calculated from the initial rate based on a Lineweaver–Burk reciprocal plot (Lineweaver and Burk 1934).

Action of xylanase on oat spelt xylan and xylobiose

To determine mode of action of xylanase, 10 mg of oat spelt xylan and xylobiose was separately incubated at 50°C for 24 h with 1.08 ± 0.039 units of xylanase in reaction volume of 1.0 ml containing 50 mM sodium phosphate buffer (pH 6.0). The aliquots were analyzed at differently time intervals for hydrolyzed products on thin-layer chromatography (TLC) plates. Reducing sugars were detected using aniline–hydrogen phthalate reagent (Bennett et al. 1998).

Chemicals

All used chemicals were analytical grade. Oat spelt xylan was purchased from Sigma (St. Louis, MO). Insoluble oat spelt xylan was prepared by suspending 10 g of xylan in 1 l of water. The suspension was boiled for 10 min and then centrifuged at 10,000 rpm for 5 min. The xylan pellet was then collected, washed four times with distilled water, and dried using a lyophilization method. The xylobiose (Lot No. 9-M; Suntory Limited, Japan) contains 99.5% xylobiose and 0.5% xylose.

Results and discussion

Xylanase production by *T. lanuginosus* THKU-49

The production of xylanase by *T. lanuginosus* THKU-49 was studied in shaking culture at 45°C for 7 days using 1% oat spelt xylan as a carbon source. Xylanase production rapidly increased during the first 4 days of cultivation, yielding 45.7 U/ml, and then slowly increased until it reached 62.7 U/ml after 7 days (Fig. 1). The pH of the filtered culture broth rose to 8.14 at 4 days of cultivation, following which it slowly decreased. There was only a slight increase in the level of soluble protein in the culture broth.

Purification of xylanase

Extracellular xylanase produced by *T. lanuginosus* THKU-49 was purified, and the results are shown in Table 1. The enzyme was purified to 7.7-fold purity with a specific activity of 552 U/mg protein. The purity of xylanase was checked using a 12% SDS–PAGE gel. The pure xylanase had a molecular weight of 24.9 kDa (Fig. 2a), and the enzyme activity was found to correspond with a protein

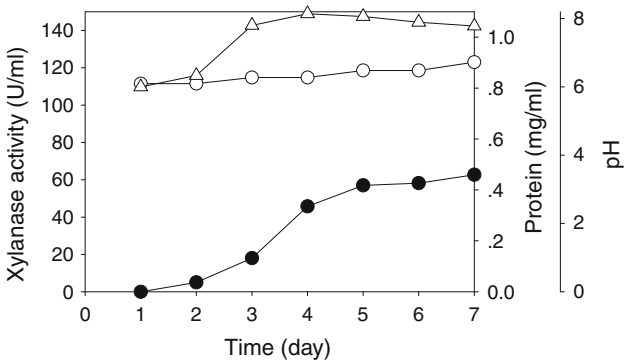

Fig. 1 Time course of xylanase production by the *Thermomyces lanuginosus* THKU-49 strain cultured at 45°C in a medium containing oat spelt xylan as the carbon source. *Filled circles* Xylanase activity, *open circles* soluble protein, *triangles* pH

Table 1 Summary of the purification of xylanase produced by *Thermomyces lanuginosus* THKU-49

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity (folds)
Crude	1,198	81,640	68	100	1
$(NH_4)_2SO_4$ precipitation	211	70,974	336	87	4.9
DEAE-Sepharose column	78	37,400	480	46	7.1
Hydroxylapatite	49	25,090	512	31	7.5
$(NH_4)_2SO_4$ precipitation	41	20,750	512	26	7.5
Sephadex G-100 column	29	14,895	522	18	7.7

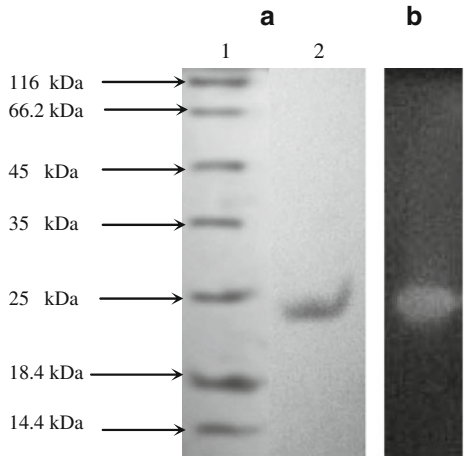

Fig. 2 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of purified *T. lanuginosus* THKU-49 xylanase. **a** Coomassie brilliant blue R-250 straining of the gel: *lane 1* molecular weight marker, *2* purified xylanase. **b** Zymogram for detecting xylanase activity

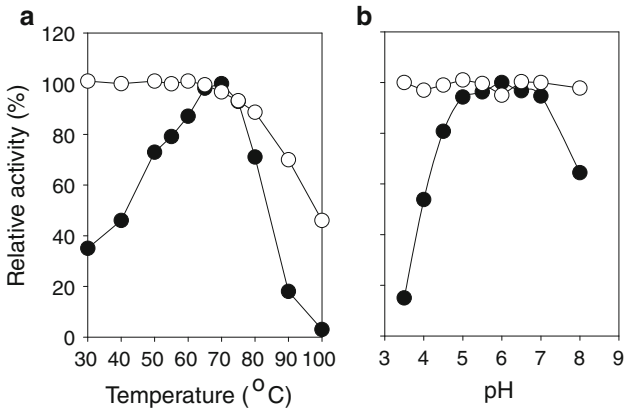

Fig. 3 Effect of temperature (**a**) and pH (**b**) on xylanase activity (*filled circles*) and stability (*open circles*) at each temperature for 60 min in 50 mM sodium phosphate buffer (pH 6.0) of pure xylanases of *T. lanuginosus* THKU-49. The reaction time was 10 min at pH 6.0

band (Fig. 2b) that was similar to bands ranging in size from 21.5 to 26.2 kDa from xylanase isolated from *T. lanuginosus* strains CBS 288.54, DSM 10635, DSM 5826, IOC-4145, SSBP, and Griffon and Maublanc (Bunce), respectively (Kitpreechavanich et al. 1984; Cesar and Mrsa 1996; Schlacher et al. 1996; Singh et al. 2000b; Mônica et al. 2003; Xiong et al. 2004; Li et al. 2005).

Optimization of temperature and pH on β-xylanase activity and stability

The activity of the pure xylanase of *T. lanuginosus* THKU-49 was optimum at 70°C (Fig. 3a) but was low at temperatures above 70°C. At 75°C, the xylanase of THKU-49 exhibited only 93% of its optimum activity, at 80°C, 71%, and at 90°C, only 18%. This enzyme was stable up to temperature of 60°C (Fig. 3a). The enzyme of strain THKU-49 were more stable in phosphate buffer than in citrate buffer. Khucharoenphaisan et al. (2008a) reported that when the buffer concentration increased, the half-life of this enzyme decreased significantly. The half-life of the purified xylanase from *T. lanuginosus* THKU-49 was 336 min at 70°C in 50 mM phosphate buffer (pH 6.0). Pure xylanase obtained from this fungus showed the highest thermostability in this earlier study. When the enzyme was kept at 80°C, the purified enzyme denatured rapidly, and at 90 and 100°C, its activity remained in the range of 1–3% of its original activity.

The optimum pH of the xylanase of *T. lanuginosus* THKU-49 was pH 6.0 and its activity gradually decreased with increasing pH (Fig. 3b). The relative activity of this xylanase at pH 5.0, 5.5, 6.5 and 7.0 was similar to that near pH 6.0 (Fig. 3b), possibly implying that this enzyme has a wide optimum pH. At pH 8.0, the enzyme from THKU-49 still exhibited 64% activity and showed full activity after being stored at 4°C for 60 min in buffers varying in pH from 3.5 to 8.0, respectively, as shown in Fig. 3b. It would therefore be possible to store the enzyme without any loss of activity at a low pH, which would protect it from bacteria. Based on the central composite design (CCD), the optimal temperature and pH of this xylanase was 66°C and pH 6.3, respectively (Khucharoenphaisan et al. 2008b); which is close to those of other xylanases produced by strains CBS 288.54, CAU44, DSM 10635, SSBP, ATCC 46882, DSM 5826, and RT9 (temperature range 60–75°C;

Table 2 Effect of addition of chemicals upon β-xylanase activity obtained from *T. lanuginosus* THKU-49

Metal ions (1 mM)	Xylanase activity (U/ml)
None	1.08 ± 0.039
K^+	0.99 ± 0.002
Li^+	1.08 ± 0.059
Na^+	1.06 ± 0.016
Ca^{2+}	1.05 ± 0.053
Co^{2+}	1.09 ± 0.050
Mn^{2+}	0.93 ± 0.011
Sn^{2+}	0.90 ± 0.015
β-Mercaptoethanol	1.03 ± 0.021
Ethylenediaminetetraacetic acid	0.83 ± 0.106

Values are given as the mean activity ± standard deviation

pH range 6.0–6.5; (Alam et al. 1994; Cesar and Mrsa 1996; Bennett et al. 1998; Singh et al. 2000b; Xiong et al. 2004; Jiang et al. 2005; Li et al. 2005). In our study, the enzymatic activity dropped notably at a high temperature (80°C) and high pH (alkali, pH 8.0).

Additional effect of chemicals upon β-xylanase activity

β-Xylanase activity was assayed in the presence of 1 mM solution of several cations, EDTA, and mercaptoethanol. The β-xylanase activity was observed to decrease considerably in the presence of Mn^{2+}, Sn^{2+}, and EDTA while K^+, Li^+, Na^+, Co^{2+}, Ca^{2+}, and β-mercaptoethanol had no effect on its activity (Table 2). These effects may preclude the use of this xylanase in industrial processes where such chemicals are presented at relevant concentrations. β-Mercaptoethanol did not affect the activity of this xylanase because the latter has no S–S linkage of cysteine residues at the active site.

Most inorganic salts, with the exception Pb^{2+} and Hg^{2+}, have no significant effect on the activity of xylanase isolated from *T. lanuginosus* SSBP (Lin et al. 1999). However, the activity of xylanase produced by *T. lanuginosus* DSM 5826 was observed to be stimulated by 1 mM of Mn^{2+}, Fe^{2+}, and β-mercaptoethanol and inhibited by Hg^{2+} (Cesar and Mrsa 1996).

Substrate specificity and K_m value

Pure xylanase was assayed for its hydrolytic activity against a variety of substances (Table 3). It was found to show a high level of activity towards the soluble oat spelt xylan (1.06 ± 0.009 U/ml) but low activity towards insoluble oat spelt xylan (0.26 ± 0.030 U/ml). The enzyme did not act on carboxymethylcellulose, avicel, filter paper, locust bean gum, cassava starch, and

Table 3 Substrate specificities of the pure enzyme of *T. lanuginosus* THKU-49

Substrates	Xylanase activity (U/ml)
Soluble oat spelt xylan	1.06 ± 0.009
Insoluble oat spelt xylan	0.26 ± 0.030
Carboxymethylcellulose	0.00
Cassava starch	0.00
Avicel	0.00
Filter paper	0.00
Locust bean gum	0.00
p-Nitrophenyl β-D-xylopyranoside	0.00

Values are given as the mean activity ± standard deviation

Table 4 K_m values of the pure xylanases of *T. lanuginosus* THKU-49

Substrates	K_m value (mg/ml)
Soluble oat spelt xylan	7.3 ± 0.236
Insoluble oat spelt xylan	60.2 ± 6.788

p-Nitrophenyl β-D-xylopyranoside. These results indicate that this xylanase is free from all other enzymatic activities, including cellulase, mannanase, amylase, and β-xylosidase.

The K_m value of the enzyme to soluble oat spelt xylan and insoluble xylan was 7.3 ± 0.236 and 60.2 ± 6.788 mg/ml, respectively (Table 4). K_m value such as 3.85, 2.00, and 3.26 mg/ml were found for xylanases produced by *T. lanuginosus* strains DSM 10635, CBS 288.54 and SSBP, respectively (Lin et al. 1999; Xiong et al. 2004; Li et al. 2005).

Action of pure xylanase on oat spelt xylan and xylobiose

To investigate the mode of action of the xylanase, we used oat spelt xylan and xylobiose as the substrates. The results from TLC chromatograms indicated that xylobiose and xylose were the main products after the hydrolysis of oat spelt xylan, especially after a long time incubation (24 h), as shown in Fig. 4. This result indicates that this xylanase may have practical utility in the production of xylobiose and xylose. The enzyme was unable to hydrolyze xylobiose, possibly implying that an oligmer sequence at least three xylose residues long is required for the active binding site of the enzyme. It also suggests that this xylanase could be categorized as an endo-xylanase. Our results are similar to those reported previously by Kitpreechavanich et al. (1984) and Li et al. (2005). The xylanases produced by *T. lanuginosus* strains ATCC 46882 and SSBP liberated mainly xylose and xylobiose from beechwood xylan

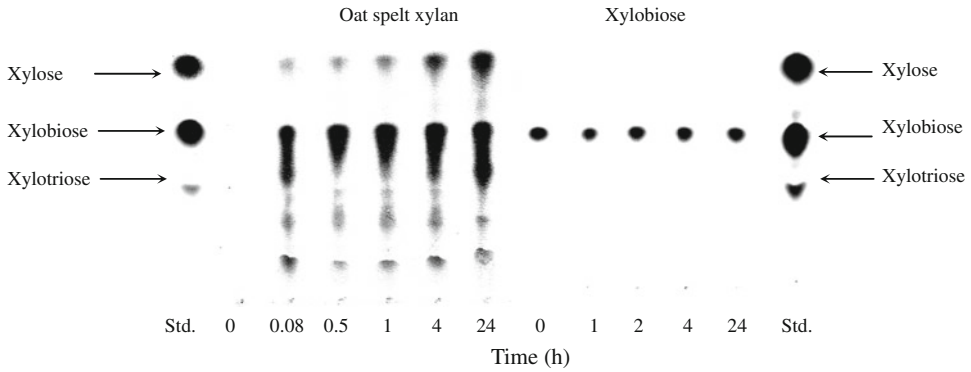

Fig. 4 Thin-layer chromatography analysis of hydrolyzates of oat spelt xylan and xylobiose with *T. lanuginosus* THKU-49 xylanase [reaction mixture (100 µl), containing 1 mg of each substrate with 3 U enzyme in 50 mM sodium phosphate buffer (pH 6.0), was incubated at 50°C for various lengths of time]

(Bennett et al. 1998; Puchart et al. 1999); in contrast, the major products of the hydrolysis of beechwood xylan and oat spelt xylan by xylanase from *T. lanuginosus* ATCC 44008 were found to be xylobiose and xylotriose (Bakalova et al. 2002).

Acknowledgments This work was financially supported from the Thailand Research Fund through the Royal Golden Jubilee PhD Program.

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