รายงานวิจัยฉบับสมบูรณ์

เรื่อง

เอนไซม์สลายไฟบรินจากเพรียงทราย *Perinereis nuntia*

(Fibrinolytic Enzyme from Sand Warm *Perinereis nuntia*)

นำเสนอโดย

ผู้ช่วยศาสตราจารย์ ดร. อภิชาติ กาญจนทัต

สถาบันวิจัยเทคโนโลยีชีวภาพและวิศวกรรมพันธุศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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ABSTRACT

A protease from sandworms (*Perinereis nuntia*) was purified by using a combination of ammonium sulfate precipitation, DEAE cellulose and Superdex-200, respectively. The enriched preparation had a specific activity of 355.74 U/mg proteins and a yield of 18.5% total protein. The molecular weight of this protease was estimated to be 37.4 kDa by SDS-15% (w/v) PAGE. The pH stability of this protease is between pH 7-8, and it is stable up to 40 °C. The activity of the enzyme was inhibited by Cu^{2+} and Co^{2+}, but was enhanced by Ca^{2+} and Mg^{2+} ions. Furthermore, protease activity was potently inhibited by EDTA.

Keywords: protease, sandworms, *Perinereis nuntia*

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LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
μg	microgram
μL	microlitre
A	absorbance
APMSF	4-amidino phenyl-methane sulfonyl fluoride
BSA	bovine serum albumin
Da	dalton
DEAE-cellulose	diethylaminoethyl-cellulose
EDTA	ethylenediamine tetraacetic acid
g	gram
hr	hour
kDa	kilodaton
L	litre
M	molar
μg	microgram
μL	microliter
mA	miliampere
mg	miligram
min	minute
mL	mililiter
mM	milimolar
MW	molecular weight
NaCl	sodium chloride
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
rpm	revolution per minute
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEMED	Tetramethylethylenediamine

TPCK	*N*-α-tosyl-Lphenylalanine chloromethyl ketone
TLCK	1-chloro-3-tosylamido-7-amino-L-s-heptanone
Tris	tris(hydroxymethyl)aminomethane
U	unit activity
v/v	volume by volume
w/v	weight by volume

FULL TEXT

1. Introduction

Blood clots are formed by the conversion of fibrinogen into fibrin via the proteolytic action of thrombin [1] and subsequently, the formation of insoluble fibrin clots. These fibrin clots are dissolved by the hydrolysis of plasmin, which is activated from plasminogen by tissue plasminogen activator [2]. The hydrolysis of fibrin is also known as fibrinolysis. Fibrin clot formation and fibrinolysis are normally well balanced in biological systems. However, when fibrin is not hydrolyzed due to some disorder, thromboses can occur. Myocardial infarction is the most common of these thromboses. The fibrinolytic agents available today for clinical use are mostly plasminogen activators such as tissue type plasminogen activator (t-PA), 1 a urokinase-type plasminogen activator, and the bacterial plasminogen activator streptokinase. Despite their widespread use, all these agents have undesired side effects, exhibit low specificity for fibrin, and are also relatively expensive. Therefore, the searches for other fibrinolytic enzymes from various sources are being continued. Recently, an investigation was conducted involving the isolation of fibrinolytic enzyme from natural extracts, as the fibrinolytic proteases used in thrombolytic therapy exhibit high specificity for fibrin, and are relatively inexpensive.

Many organisms are important sources of thrombolytic agents. Effective thrombolytic agents have been identified and characterized from snake venoms [3-6], vampire bat [7-9], insects [10-14], earthworm [15-19], leech [20], marine green alga [21-23], and microorganisms [24-27]. These developments started a new era in the early treatment of heart attack. Sandworms (*Perinereis nuntia*) of the class Polychaeta play an important role in nutrient cycling and in maintaining and sustaining the benthic environment [28, 29]. The head-down deep deposit-feeding polychaetes are known for having strong effects on bioturbation and nutrient mineralization both by sediment reworking during non-selective feeding and by burrow irrigation [30]. In addition, it is a natural phenomenon that sandworm releases its coelomic fluid when being cut. Therefore, we hypothesized that there is a balance between the cuticle collagen and its coelomic fluid, and the latter is likely to have an activity of proteolysis. To verify our hypothesis, we successfully identified a novel protease from the coelomic fluid of sandworms.

2. Material and method

2.1 Animals

Sandworms (*P. nuntia*) were obtained from Bangphra beach, Chonburi province, Thailand.

2.2 Chemicals

4-amidino phenyl-methane sulfonyl fluoride (APMSF), ammonium sulfate, acrylamide, bis-acrylamide, 1-chloro-3-tosylamido-7-amino-L-s-heptanone (TLCK), hydrochloric acid, mercaptoethanol, sodium acetate, Tetramethylethylenediamine (TEMED), Tris (hydroxymethyl) aminomethane, the divalent metal salts and Ethylenediaminetetraacetic acid (EDTA) were purchased from Merck group, Germany. Ammonium persulfate, coomassie Blue G-250, glacial acetic acid, methanol, sodium chloride (NaCl), sodium hydroxide and sodium dodecylsulfate (SDS) were from BDH. Azocasein, diethylaminoethyl-cellulose (DEAE-cellulose), phenylmethylsulfonyl fluoride (PMSF), Superdex-200, *N*-α-tosyl-Lphenylalanine chloromethyl ketone, (TPCK) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Co. Ltd, USA. All chemicals used were of analytical grade.

2.3 Protease assay

Protease activity was determined by measuring the release of acid-soluble material from azocasein. The enzyme sample (100 μl) was mixed with 400 μl of 1% (w/v) azocasein in 20 mM sodium phosphate buffer (pH 7.2). Following incubation at 37 ^{o}C for 30 min, 500 μl of ice-cold 10% (w/v) TCA was added to the mixture and immediately vortexed. The mixture was placed on ice for 10 min and centrifuged at 15,000×g for 10 min. Absorbance of the supernatant was measured at 340 nm. One unit (U) of protease activity was defined as the amount of the enzyme that causes an increase in absorbance of 0.001 /min.

2.4 Purification procedure

Step 1 Ammonium sulfate precipitation and filtration 1.5 kg of washed sandworms were homogenized in 5 l of 20 mM sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl in a blender and then was left at 4 ^{o}C overnight with continuously stirring. The suspension was then filtered through double-layered cheese cloth followed by centrifugation at 15,000×g for 30 min. Clear supernatant (crude homogenate) was harvested and proper amount of ammonium sulfate was added, with stirring, to 80% saturation followed by continuous stirring at 4 ^{o}C overnight. The precipitate was collected from the suspension by centrifugation at 15,000×g for 30

min. The pellet obtained was then re-dissolved in 20 mM sodium phosphate buffer (pH 7.2), dialyzed (using 3,500 MWCO dialysis tube) against 3 changes of 5 l of water at 4 ^{o}C and then freeze dried. This will be referred to as the ammonium sulphate cut fraction.

Step 2 Ion exchange chromatography The ammonium sulphate cut fraction was resolvated in 20 mM phosphate buffer (pH 7.2) and loaded (10 ml at 25 mg/ml total protein) into a DEAE-cellulose column (1.6 cm i.d.×15 cm length) pre-equilibrated with at least five column-volumes of 20 mM phosphate buffer (pH 7.2), and then eluted from the column using 20 mM phosphate buffer (pH 7.2) with a linear gradient of 0-1.0 M NaCl at a flow rate of 1.0 ml/min. Each collected fraction (10 ml) was screened for protein content as well as protease activities. The fractions containing corresponding activity were pooled, dialyzed, and concentrated by freeze dry to 50 mg/ml for further purification by gel filtration chromatography and analysis. This fraction is subsequently referred to as the "*post-DEAE-cellulose protease fraction*".

Step 3 Gel filtration chromatography The post-DEAE-cellulose protease fraction was applied (2 ml at 50 mg/ml) to a pre-equilibrated (20 mM sodium phosphate buffer (pH 7.2)/100 mM NaCl) Superdex-200 column (1.6 cm×60 cm) and then eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 5.0 ml were collected and assayed for protease activity, and contiguous protease positive fractions (from the same peak) were pooled and dialyzed against an excess of same buffer prior to further analysis. This final preparation is referred to as the "*enriched protease protein fraction*".

2.5 Determination of the protein content

The protein concentration was determined by the standard Bradford assay [31], with dilutions of a known concentration of bovine serum albumin as the standard. The absorbance at 595 nm was monitored with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). During the column chromatographic separations, proteins of the elution profiles were determined by measuring the absorbance at 280 nm.

2.6 Determination of the protein pattern by native-PAGE

The protein from each step of the purification procedure was analyzed for its native protein pattern according to the method of Bollag [32], using a 7.5% (w/v)

acrylamide separating gel and a 5.0% (w/v) acrylamide stacking gel. Tris-glycine buffer pH 8.3 was used as the electrode buffer, and gels were run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. After electrophoresis, proteins in the gel were stained by coomassie Blue R-250 staining (0.1% (w/v) coomassie Blue R-250 in 10% (v/v) acetic acid and 45% (v/v) methanol) followed by several changes of destaining solution (10% (v/v) acetic acid and 45% (v/v) methanol) until the background was clear.

2.7 Molecular weight determination by SDS-PAGE

Discontinuous reducing SDS-PAGE gels were prepared with 0.1% (w/v) SDS in 15% and 5% (w/v) acrylamide separating and stacking gels, respectively, with Tris-glycine buffer pH 8.3 containing 0.1% (w/v) SDS as the electrode buffer, according to the procedure of Laemmli [33]. Samples to be analyzed were treated with reducing sample buffer and boiled for five min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standards were co-resolved in each gel alongside the samples to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were stained with Coomassie blue R-250 staining.

2.8 Effect of temperature on the protease activity

The effect of temperature on the protease activity was determined by incubating the enriched protease containing fractions in 20 mM sodium phosphate buffer (pH 7.2) at various temperatures (4-90 °C at 5 ^{o}C intervals) for 30 min. The samples were cooled to 4 °C and then assayed for residual protease activity with 100% and 0% activity controls.

2.9 The pH-dependence of the protease activity

Incubation of the enriched protease fractions in buffers of broadly similar salinity levels, but varying pH from 2-14 was performed to assess the pH stability of the protease. The buffers used were 20 mM glycine-HCl (pH 2-4), 20 mM sodium acetate (pH 4-6), 20 mM potassium phosphate (pH 6-8), 20 mM Tris-HCl (pH 8-10) and 20 mM glycine-NaOH (pH 10-12). The enriched protease fraction was mixed in each of the different buffer-pH compositions, or 20 mM sodium phosphate buffer (pH 7.2) for the control, and then left for 1 h at room temperature. Next, the samples were adjusted back to 20 mM sodium phosphate buffer (pH 7.2), and assayed for protease

activity. The activities attained were compared with the control which was set as 100% activity**.**

2.10 The effects of metal ions and protease inhibitors on the enzyme activity

The effects of metal ions on enzyme activity were investigated using $MgCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_2$, $CaCl_2$, and $CuSO_2$. The purified enzymes were pre-incubated both in the absence and the presence of bivalent cations including Mg^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+} with a final concentration of 1.0 mM in 20 mM sodium phosphate buffer (pH 7.2) for 1 h at 37 °C. After 1 h of incubation at room temperature, the residual protease activity was measured with 0.1% azocasein. The effects of the protease inhibitors were also assessed using 5.0 mM EDTA, 2.0 mM PMSF, 0.5 mM TLCK, 0.5 mM TPCK, and 0.5 mM APMSF. The enzyme was pre-incubated with the protease inhibitors for 1 h at 37 °C, and the effects were assessed with 0.1% azocasein.

3. Results and discussion

3.1 Enrichment of the protease from sandworms

The occurrence of a protease in the crude extracts of sandworms is reported for the first time in this work. The initial ammonium sulfate precipitation reduced the total protein amount by some 55.7%, compared to 30% loss of protease activity for a 1.58-fold purification (Table 1). This ammonium sulfate cut fraction was then further fractionated by DEAE-cellulose ion exchange chromatography with monitoring of all eluted fractions for protease activity. The protease activity remained in the bound fraction, and eluted from the column as a main peak corresponding to ~530-580 mM NaCl (Figure 1), just after a minor peak (shoulder in Figure 1A) and before the other main protein peak (~650-730 mM NaCl). The post-DEAE-cellulose protease fraction, after dialysis, had a specific protease activity of 276.2 U/mg protein, representing a 43.3-fold purification (Table 2). Note that as well as the loss of ~98.8% of the total protein from the ammonium sulfate cut fraction, this ion exchange chromatography step was also very effective in eliminating pigments, because most of them did not bind to the cellulose matrix. However, other proteins were clearly still abundantly visible on the native PAGE (Figure 2A), and so further enrichment was required.

Based upon the fact that the contaminating proteins may differ in size, and especially may be larger, then Superdex-200 gel filtration chromatography was utilized after the DEAE-cellulose ion exchange chromatography. The post-DEAE-

cellulose protease fraction was concentrated to 50 mg/ml and subjected (5 ml loading at a time) to Superdex-200 gel filtration chromatography. *The proteins were then eluted from the column, revealing* one major peak (Figure 1B). Harvesting the fractions with positive protease activity from the first peak leads to an apparently almost homogenous protein preparation (Figure 2), with purification fold of 55.75 at a recovery yield of 18.5% and a specific protease activity of 355.71 U/mg of protein (Table 1).

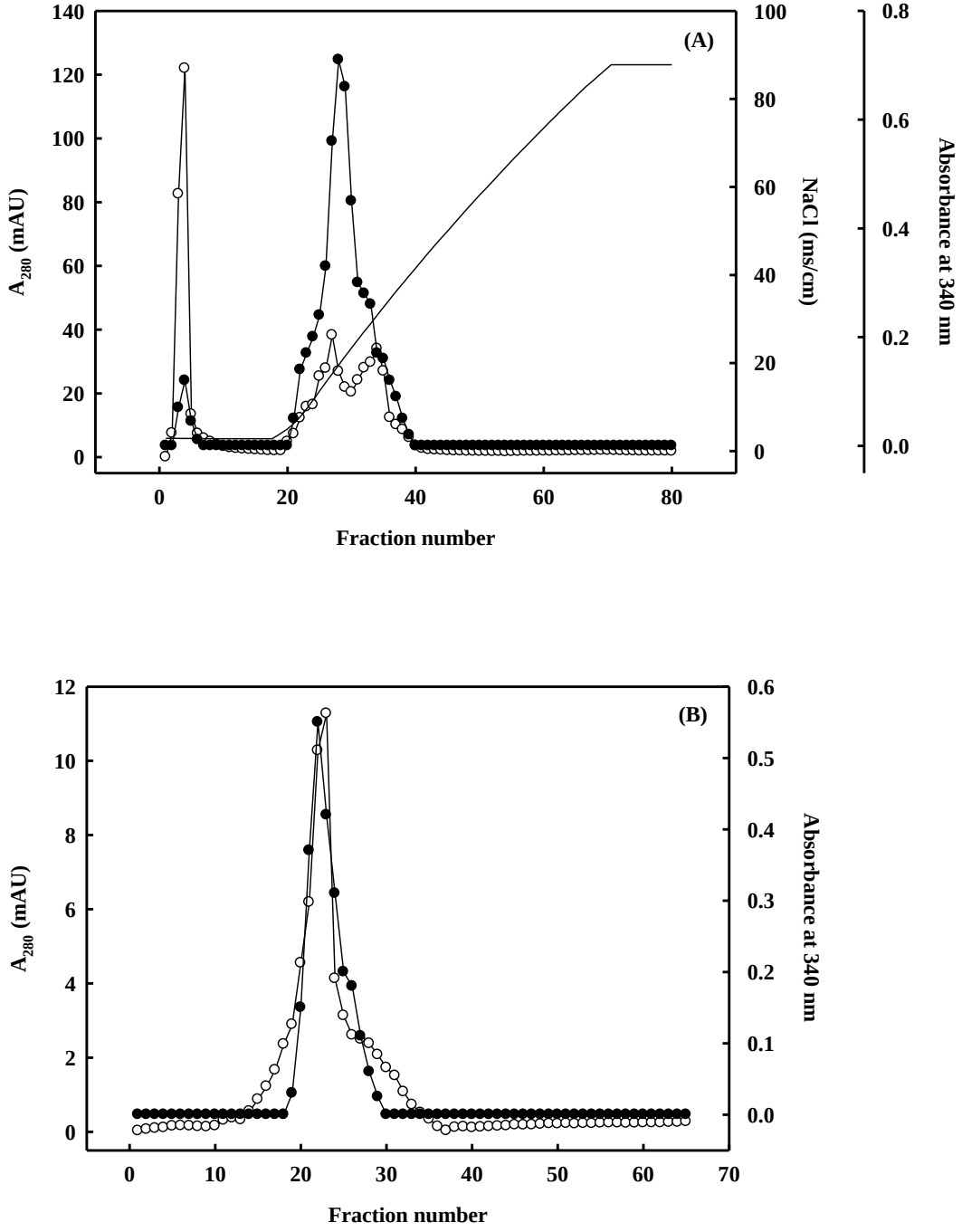

Figure 1 (A) DEAE-cellulose ion exchange chromatography of the ammonium sulfate cut fraction solubilized in 20 mM sodium phosphate buffer (pH 7.2), and eluted in the same buffer but with a linear gradient of 0-1.0 M NaCl at a flow rate of 1 ml/min. (○) Absorbance at 280 nm, (●) protease activity.(B) Superdex-200 gel chromatography of the post-DEAE-cellulose protease fraction. Fractions (5 ml) were eluted with 20 mM sodium phosphate buffer (pH 7.2) / 100 mM NaCl at a flow rate of 0.5 ml/min. (○) absorbance at 280 nm, (●) protease activity.

Table 1 Purified protease enzyme activity yields from sandworms

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U / mg)	Yield (%)	Purification (fold)
Crude extract	295.40	1,885.00	6.38	100.00	1.00
80% $(NH_4)_2SO_4$ cut	130.60	1,320.00	10.11	70.03	1.58
DEAE-cellulose	1.64	452.60	275.98	24.01	43.26
Superdex-200	0.98	348.60	355.71	18.49	55.75

Note. Protease activity was measured by using the azocasein assay, as described under Materials and methods. The enzyme assay for protease was carried out with 1% azocasein solution at 37 ^{o}C for 20 min. The reaction was discontinued by mixing with 10% trichloroacetic acid, and being allowed to stand for 10 min in ice, followed by centrifugation at 15,000 × g for 10 min. The absorbance of the reaction mixture was measured at 340 nm. An enzyme unit was defined as the amount of enzyme-releasing acid-soluble material from azocasein, to yield an absorbance at 340 nm.

3.2 Verification of the protease purity and molecular weight determination

The selected fractions from each step of the protease enrichment process were analyzed for their apparent purity and protein pattern by native-PAGE, where the enriched protease fraction preparation (after Superdex-200 gel filtration chromatography) revealed a single main protein band on the native-PAGE gel (Figure 2A), but only a single band on the denaturing SDS-PAGE gel (Figure 2B). Thus, the enriched protease fraction obtained after Superdex-200 column chromatography should be relatively pure. SDS-PAGE resolution of the purified protease preparations under discontinuous and reducing conditions, revealed an apparent MW of a single protein band of 37.4 kDa (Figure 2B).

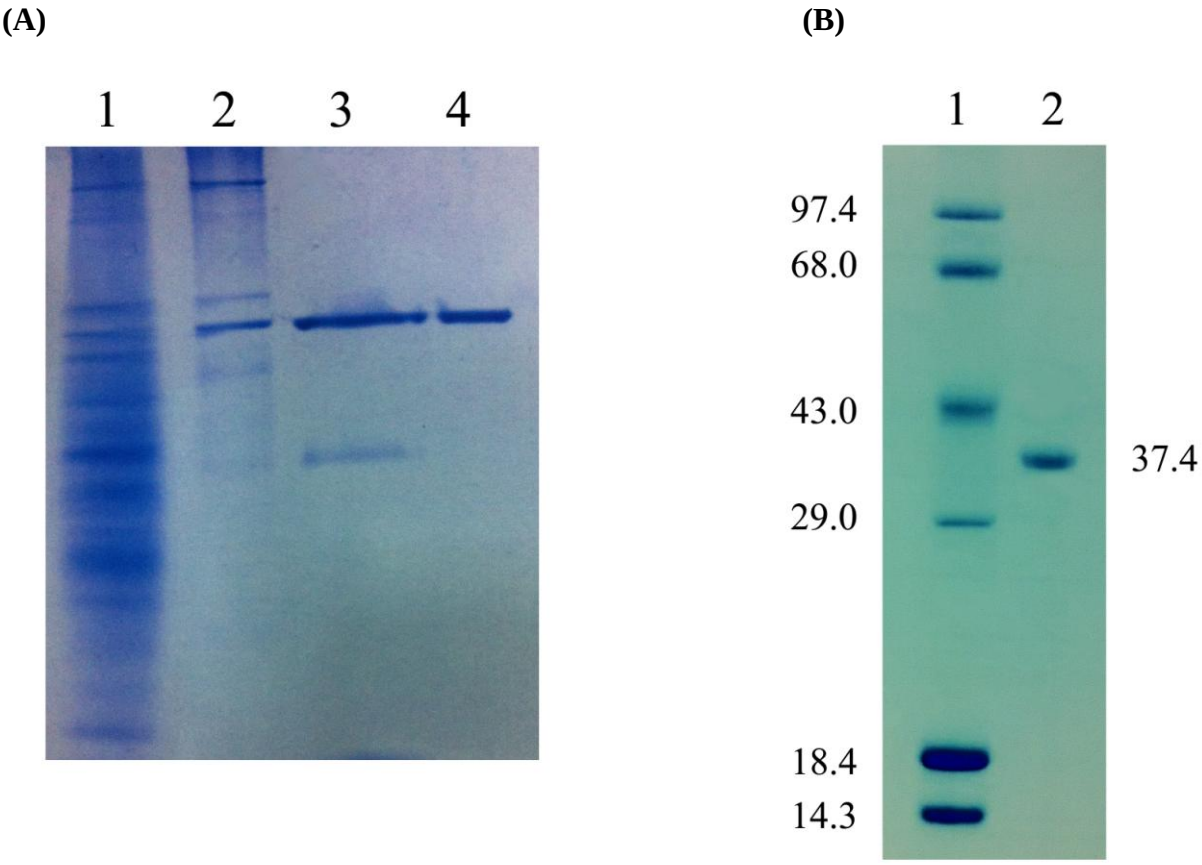

Figure 2 (A) Coomassie blue stained native-PAGE analysis of the sandworms protease fractions from each step of the enrichment and stained for protein by coomassie blue (Lanes 1-4). Lane 1, crude enzyme (20 μg of protein); Lane 2, ammonium sulfate cut fraction (20 μg of protein); Lane 3, post-DEAE-cellulose protease fraction (15 μg of protein); Lanes 4 and 5, enriched protease fraction (post-Superdex-200) (10 μg of protein). (B) Reducing SDS-PAGE analysis, after coomassie blue staining, of the enriched protease fraction (post-Superdex-200) from sandworms: Lane 1, Low molecular weight protein markers; Lane 2, enriched protease fraction (5 μg of protein). Gels shown in (A) and (B) are representative of 3 separate enrichments.

3.3 pH and temperature resistance determination

The pH sensitivity profile of the protease activity of the enriched protease protein fraction exhibited pH stability between pH 7.0-8.0 following a 60 min pretreatment at each pH. The activity, however, declined rapidly with increasing acidity, and less markedly with increasing alkalinity, such that less than 40% protease activity remained at pH 6 and 10, respectively, and no detectable protease activity at pH 2.0-4.0 and 11.0-12.0 (Figure 3A). Note that where different buffers overlapped in the pH only a minor buffer-dependent effect was noted, suggesting that the variations seen are indeed largely due to the different pH values and not the effects from using different buffer. No significant changes in the inhibition activity of the enriched protease fraction was seen when pretreated for 30 min within the temperature range of 4-40 °C, onwards protease activity was decreased with increasing incubation temperature, with essentially no activity being detected after pretreatment at 60 ^{o}C or higher (Figure 3B).

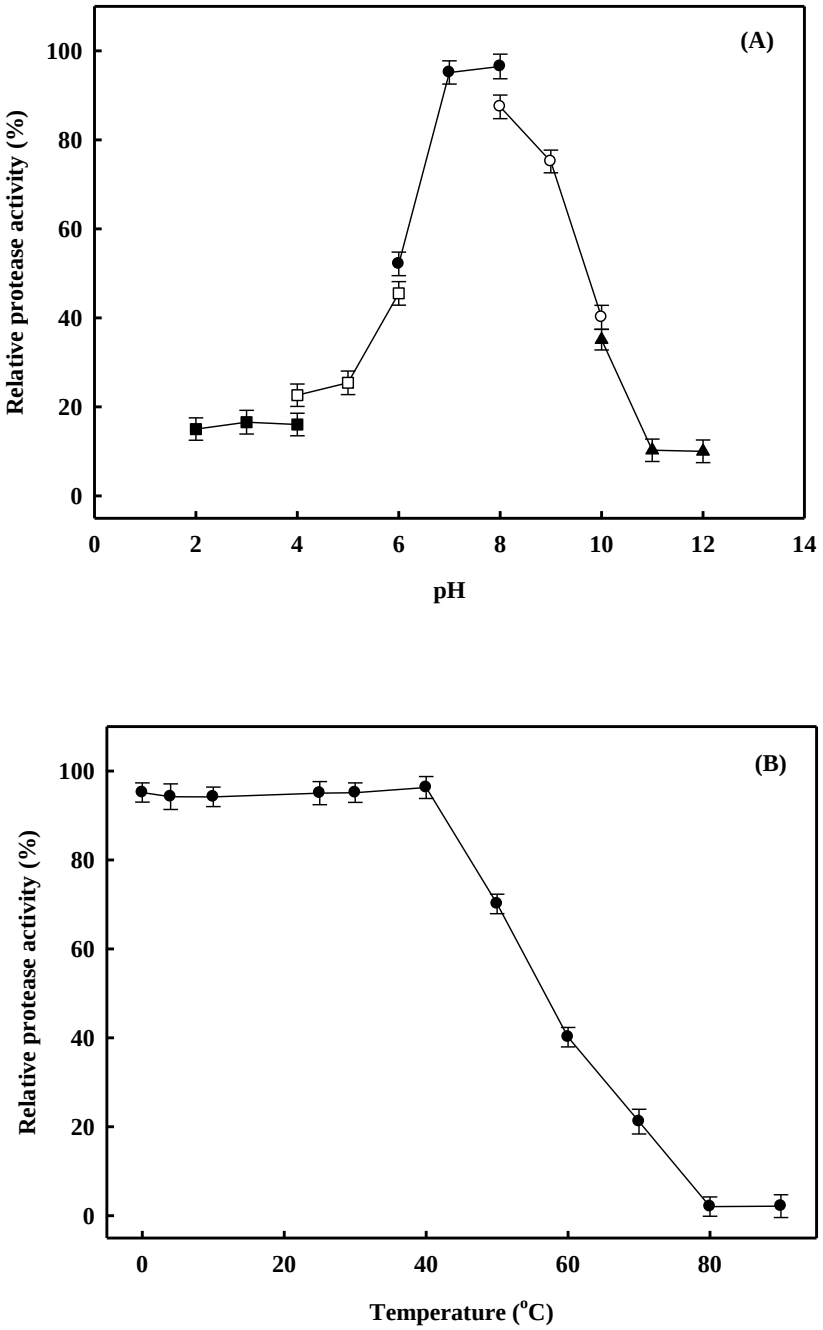

Figure 3 (A) The effect of pH pretreatment on the protease activity of the enriched protease fraction. The following buffer systems were used: (■) 20 mM glycine-HCl (pH 2.0-4.0), (□) 20 mM sodium acetate (pH 4.0 - 6.0), (●) 20 mM potassium phosphate (pH 6.0 - 8.0), (○) 20 mM Tris-HCl (pH 8.0 - 1 0.0) and (▲) 20 mM glycine-NaOH (pH 10.0 - 12.0). (B) Effect of pretreatment temperature on the protease activity of the enriched protease. For both panels the data are shown as the mean $\pm$1 SD and are derived from three experiments.

3.4 Effect *of metal ions and protease inhibitors on the protease activity*

Enzyme activity was inhibited by Cu^{2+} and Co^{2+}, but was enhanced by the addition of Ca^{2+} and Mg^{2+} ions. Furthermore, protease activity was remarkably inhibited by EDTA, thus EDTA functions as an inhibitor of the activity. These results indicate that sandworm protease is a metalloprotease.

Table 2 Effect of metal ions and protease inhibitors on the activity of the protease enzyme was from sandworm

Metal ions and protease inhibitors	Residual activity (%)
Control	100
Cu^{2+}	80.16 ± 0.88
Co^{2+}	70.95 ± 0.55
Ca^{2+}	118.35 ± 0.48
Zn^{2+}	85.16 ± 0.84
Mg^{2+}	102.56 ± 0.66
Fe^{2+}	87.95 ± 0.27
PMSF	80.18 ± 0.95
APMSF	90.14 ± 0.11
EDTA	48.56 ± 0.85
TLCK	80.58 ± 0.48
TPCK	75.82 ± 0.12

4. Conclusion

The potent protease activity purified from the sandworms (*P. nuntia*) was described. Therefore, it is strongly suggested that the *P. nuntia* protease enzyme can be a potential source for developing therapeutic agents for thrombosis. For medicinal application, further studies to elucidate molecular biological characteristics are ongoing.

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APPENDICES

APPENDIX A

Preparation for non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

1. Stock solutions

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

1% Bromophenol blue (w/v)

Bromophenol blue 100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

Acrylamide 29.2 g

N,N,-methylene-bis-acrylamide 0.8 g

Adjust volume to 100 ml with distilled water

Solution B (1.5 M Tris-HCl pH 8.8)

2 M Tris-HCl (pH 8.8) 75 mL

Distilled water 25 mL

Solution C (0.5 M Tris-HCl pH 6.8)

1 M Tris-HCl (pH 6.8) 50 mL

Distilled water 50 mL

10% Ammonium persulfate

Ammonium persulfate 0.5 g

Distilled water 50 mL

Electrophoresis buffer (25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3	g
Glycine	14.4	g
Dissolved in distilled water to 1 litre without pH adjustment		
(final pH should be 8.3)		

5x sample buffer

(312.5 mM Tris-HCl pH 6.8, 50% glycerol, 1% bromophenol blue)

1 M Tris-HCl (pH 6.8)	0.6	mL
Glycerol	5	mL
1% Bromophenol blue	0.5	mL
Distilled water	1.4	mL

3. Native-PAGE

7.5% Separating gel

Solution A	2.5	mL
Solution B	2.5	mL
Distilled water	5	mL
10% Ammonium persulfate	50	μL
TEMED	5	μL

5.0% Stacking gel

Solution A	0.67	mL
Solution B	1	mL
Distilled water	2.3	mL
10% Ammonium persulfate	30	μL
TEMED	5	μL

APPENDIX B

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solutions

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane	24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane	12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

10% SDS (w/v)

Sodium dodecyl sulfate (SDS)	10 g

50% Glycerol (w/v)

100% Glycerol	50 ml

Added 50 ml of distilled water

1% Bromophenol blue (w/v)

Bromophenol blue	100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

Acrylamide	29.2 g
N,N,-methylene-bis-acrylamide	0.8 g

Adjust volume to 100 ml with distilled water

Solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS)

2 M Tris-HCl (pH 8.8)	75 mL
10% SDS	4 mL
Distilled water	21 mL

Solution C (0.5 M Tris-HCl pH 6.8, 0.4% SDS)

1 M Tris-HCl (pH 6.8)	50 mL
10% SDS	4 mL

Distilled water	46	mL

10% Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5	mL

Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)

Tris (hydroxymethyl)-aminomethane	3	g
Glycine	14.4	g
SDS	1	g

Dissolved in distilled water to 1 litre without pH adjustment

(final pH should be 8.3)

5x sample buffer

(60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 14.4 mM 2-mercaptoethanol)

1 M Tris-HCl (pH 6.8)	0.6	mL
Glycerol	5	mL
10% SDS	2	mL
1% Bromophenol blue	1	mL
2-mercaptoethanol	0.5	mL
Distilled water	0.9	mL

3. SDS-PAGE

12.5% Separating gel

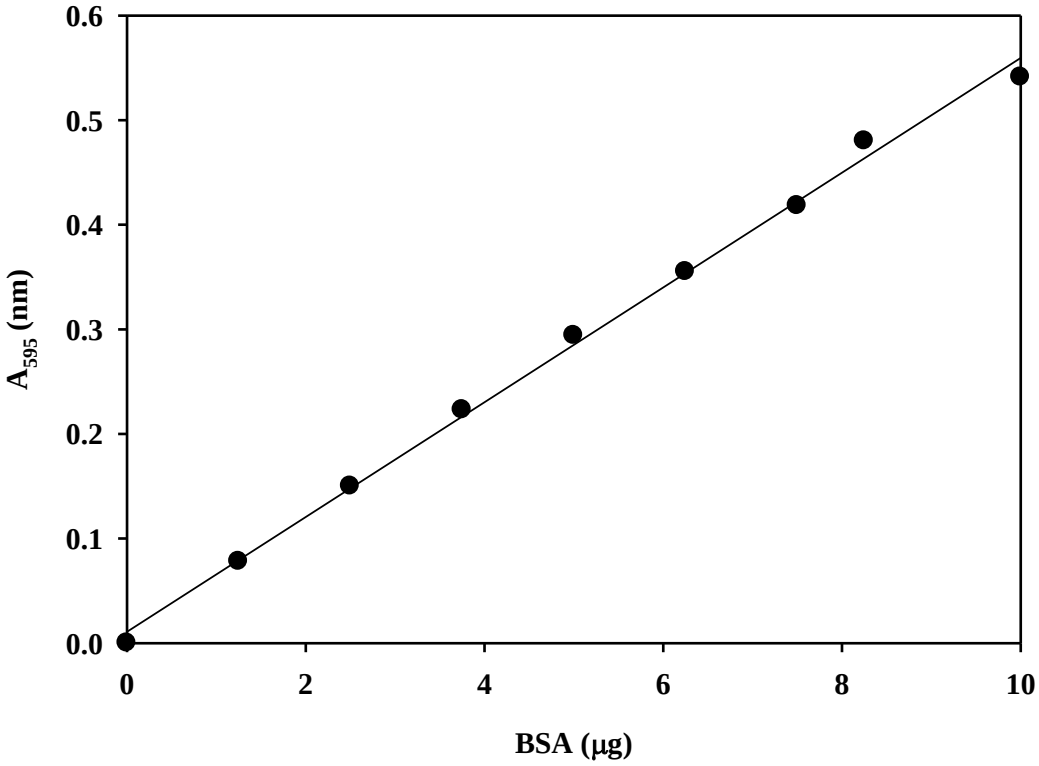
Solution A	4.2	mL
Solution B	2.5	mL
Distilled water	3.3	mL
10% Ammonium persulfate	50	μL
TEMED	5	μL

5.0% Stacking gel

Solution A	0.67	mL
Solution B	1	mL
Distilled water	2.3	mL
10% Ammonium persulfate	30	μL
TEMED	5	μL

APPENDIX C

Calibration curve for protein determination Bradford method



CURRICULUM VITAE

1. Name Dr. Aphichart Karnchnatat
2. Position Assistance Director
3. Address The Institute of Biotechnology and Genetic Engineering,
Chulalongkorn University, Institute Building 3, Phayathai Road,
Pathumwan, Bangkok, 10330, Thailand
Tel: +662-218-8052-6 Fax: +662-253-3543
E-mail: i_am_top@hotmail.com
4. Education

University	Degree	Field	Year
Chulalongkorn University	Ph.D.	Biotechnology	2006
Chulalongkorn University	M.Sc.	Biochemistry	2001
Ramkhamhaeng University	B.Sc.	Chemistry	1998

5. Research interest
   5.1 Enzyme biotechnology
   5.2 Protein and peptide chemistry: Structure and function
   5.3 Chemical natural products
   5.4 Fungal bioremediation
6. Award and honors
   6.1 Office of the National Research Council of Thailand, Uptake of inorganic and organic nitrogen compounds in the cyanobacterium *Aphanothece halophytica* under osmotic stress, 2008.
   6.2 Thailand Toray Science Foundation 2011. Fibrinolytic enzyme from *Perinereis nuntia*, 2011.
   6.3 The third prize for poster presentation, 14th Food Innovation Asia Conference 2012; “Green and Sustainable Food Technology for All”, Antioxidation of polysaccharide-protein complex extracted from *Phaeogyroporus portentosus* (Berk. & Broome) McNabb, 2012
7. Grants and fellowships

7.1 Production, purification and biochemical characterization of lignin degrading enzymes from Psilocybe mushroom and its application in decolorization of synthetic dyes, Ratchadaphiseksomphot Endowment Fund, 2008-2009

7.2 Structure analysis and antitumor activity of polysaccharide from *Phaeogyroporus portentosus* (Berk. & Broome McNabb), The Thailand Research Fund, 2008-2010

7.3 Purification and characterization of lectin from rhizomes of *Curcuma amarissima* Roscoe. TRF-MAG Window II Co-funding, 2008-2010

7.4 L-Asparaginase from xylariaceous fungi and application in antitumor activity, Office of the National Research Council of Thailand, 2009-2011

7.5 Amino acid sequences and biological activities of proteins from xylariaceous fungi, The Institute of Biotechnology and Genetic Engineering, 2009-2010

7.6 Amino acid sequences and biological activities of proteins from *Sterculia monosperma* Vent., The Institute of Biotechnology and Genetic Engineering, 2009-2010

7.7 Purification and characterization of xylanase from endophytic fungi isolated from thai medicinal plants, TRF-MAG Window II Co-funding, 2009-2011

7.8 Purification and characterization of lipase from endophytic fungi isolated from thai medicinal plants, TRF-MAG Window II Co-funding, 2008-2010

7.9 Alpha-glucosidase inhibitor from *Archidendron jiringa* Nielsen. and Parkia speciosa *Hassk*. seeds, TRF-MAG Window II Co-funding, 2008-2010

7.10 Fibrinolytic enzyme from sand warm *Perinereis nuntia*, Thailand Toray Science Foundation 2010.

7.11 Protein and peptide with *acetylcholinesterase* inhibitory activity from the rhizomes of Zingiberaceae plants, TRF-MAG Window II Co-funding, 2010-2012

7.12 Protein and peptide with *antiproliferative* activity of *macrophage RAW 264.7* from the rhizomes of Zingiberaceae plants, TRF-MAG Window II Co-funding, 2010-2012

7.13 Smart biopolymer from Thai medicinal plants for therapeutic use, National Research University, 2010-2012

7.14 Fibrinolytic enzyme from sand warm *Perinereis nuntia*, National Research Council, 2011-2012

7.15 *Tyrosinase* inhibitory activity of the protein hydrolysate from the seeds of Thai fruits, TRF-MAG Window II Co-funding, 2011-2013

8. Publications

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8.2 **Karnchanatat, A.,** Petsom, A., Sangvanich, P., Piaphukiew, J., Whalley, A.J.S., Reynolds, C.D., and Sihanonth, P.***** (2007) Purification and biochemical characterization of an extracellular β-glucosidase from wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. *FEMS Microbiology Letters* 270:162-170.

8.3 **Karnchanatat, A.,** Petsom, A., Sangvanich, P., Piapukiew, J., Whalley, A.J.S., Reynolds, C.D., and Sihanonth, P.* (2008) A novel thermostable endoglucanase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. *Enzyme and Microbial Technology* 2008; 42: 404-413.

8.4 Kheeree, N., Sangvanich, P., Puthong, S., and **Karnchanatat, A.*** (2010) Antifungal and antiproliferative activities of lectin from the rhizomes of *Curcuma amarissima* Roscoe. *Applied Biochemistry and Biotechnology* 162: 912-925.

8.5 Niyomploy, P., Thunyakitpisal, P., **Karnchanatat, A.**, and Sangvanich, P.* (2010) Cell proliferative effect of polyxyloses extracted from the rhizomes of wild tumeric, *Curcuma aromatic* Salisb. *Pharmaceutical Biology* 48: 932-937.

8.6 Konkumnerd, W., **Karnchanatat, A.**, and Sangvanich, P.* (2010) A thermostable lectin from the rhizomes of *Kaempferia parviflora*. *Journal of the Science of Food and Agriculture* 90: 1920-1925.

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Zingiber ottensii Valeton. *Applied Biochemistry and Biotechnology* 2010; 2010; 162: 1938-1951.

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8.10 Charungchitrak, S., Petsom, A., Sangvanich, P., and **Karnchanatat, A.*** (2011) Antifungal and antibacterial activities of lectin from the seeds of *Archidendron jiringa* Neilson. *Food Chemistry* 126: 1025-1032.

8.11 **Karnchanatat, A.***, Tiengburanatam, N., Boonmee, A., Puthong, S., and Sangvanich, P. (2011) Zingipain, A cysteine protease from *Zingiber ottensii* Valeton rhizomes with antiproliferative activities against fungi and human malignant cell lines. *Preparative biochemistry and biotechnology* 41: 201-217.

8.12 Tangngamsakul, P., **Karnchanatat, A.**, Sihanonth, P. and Sangvanich, P.* (2011) An extracellular glucoamylase produced by endophytic fungus EF6. *Applied Biochemistry and Microbiology* 47: 412-418.

8.13 Sawaengsak, W., Saisavoey, T., Chuntaratin, P., and **Karnchanatat, A.*** (2011) Micropropagation of the medicinal herb *Glycyrrhiza glabra* L., through shoot tip explant culture and glycyrrhizin detection. *International Research Journal of Plant Science* 2:129-136.

8.14 Baebprasert, W., **Karnchanatat, A.**, Linblad, P., and Incharoensakdi A.* (2011) Na^+-stimulated nitrate uptake with increased activity under osmotic upshift in *Synechocystis* sp. strain PCC 6803. *World Journal of Microbiology and Biotechnology* 27: 2467-2473.

8.15 Kilaso, M., Kaewmuangmoon, J., **Karnchanatat, A.**, Sangvanich P., and Chanchao, C.* (2011) Expression and characterization of *Apis dorsata* α-glucosidase III. *Journal of Asia-Pacific Entomology* 14: 479-488.

8.16 Boonmee, A., Srisomsap, C., Chokchaichamnankit, D., **Karnchanatat, A.**, and Sangvanich P.* (2011) A proteomic analysis of *Curcuma comosa* Roxb. rhizomes. *Proteome Science* 9: 43.

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8.18 Wipusaree, N., Sihanonth, P., Piapukiew, J., Sangvanich, P., and **Karnchanatat, A.*** (2011) Purification and characterization of a xylanase from the endophytic fungus *Alternaria alternata* isolated from the Thai medicinal plant, *Croton oblongifolius* Roxb. *African Journal of Microbiology Research* 5: 5697-5712.

8.19 Intrama, V., **Karnchanatat, A.**, Bunaprasert, T., and Vadhanasindhu, P.* Critical effects of regulation on Thailand's cosmeceutical development process: human placenta extract *International Journal of Management and Business and Studies* 1: 96-99.

8.20 Songserm, P., Sihanonth, P., Sangvanich, P., and **Karnchanatat, A.*** (2012) Decolorization of textile dyes by *Polyporus seudobetulinus* and extracellular laccase. *African Journal of Microbiology Research* 6: 779-792.

8.21 Panuthai, T., Sihanonth, P., Piapukiew, J., Sooksai, S., Sangvanich, P., and **Karnchanatat, A.*** (2012) An extracellular lipase from the endophytic fungi *Fusarium oxysporum* isolated from the Thai medicinal plant, *Croton oblongifolius* Roxb. *African Journal of Microbiology Research* 6: 2622-2638.

8.22 Moon-ai, W., Niyomploy, P., Boonsombat, R., Sangvanich, P., and **Karnchanatat, A.*** (2012) A Superoxide dismutase purified from the rhizome of *Curcuma aeruginosa* Roxb. as inhibitor of nitric oxide production in the Macrophage-like RAW 264.7 cell line. *Applied Biochemistry and Biotechnology* 166: 2138-2155.

8.23 Yodjun, M., **Karnchanatat, A.,** and Sangvanich, P.* (2012) Angiotensin I-converting enzyme inhibitory proteins and peptides from the rhizomes of Zingiberaceae plants. *Applied Biochemistry and Biotechnology* 166: 2037-2050.

8.24 Virounbounyapat, P., **Karnchanatat, A.** and Sangvanich, P.* (2012) An alpha-glucosidase inhibitory activity of thermostable lectin protein from *Archidendron jiringa* Nielsen seeds. *African Journal of Biotechnology* 11: 10026-10040.

8.25 **Karnchanatat, A.*** and Sangvanich, P. A chitinase-like protein with α-amylase inhibitory activity from Kluai Hom Thong banana Fruit: Musa (AAA group)**.** *Food Biotechnology* 26: 218-238.

8.26 Chantaranothai, C., Palaga, T., **Karnchanatat, A.**, and Sangvanich, P.* (2013) Inhibition of nitric oxide production in the Macrophage-like Raw 264.7 cell line by protein from the rhizomes of Zingberaceae plants. *Preparative biochemistry and biotechnology* 43: 60-78.

8.27 Rungsaeng, P., Sangvanich, P., and **Karnchanatat, A.*** (2013) Zingipain, a ginger protease with acetylcholinesterase inhibitory activity. *Applied Biochemistry and Biotechnology* 170: 934-950.

8.28 **Karnchanatat, A.***, Sihanonth, P., Piapukiew, J., and Sangvanich, P. (2013) An antioxidation and antiproliferation of polysaccharide-protein complex extracted from *Phaeogyroporus portentosus* (Berk. & Broome) McNabb. *African Journal of Microbiology Research* 7: 1668-1680.

8.29 Niyomploy, P., Boonsombat, R., Karnchanatat, A., Sangvanich P.* (2013) A superoxide dismutase purified from the roots from *Stemona tuberose*. *Preparative biochemistry and biotechnology* 170: 525-540.

8.30 Niyomploy, P., Srisomsap, C., Chokchaichamnankit, D., Vinayavekhin, N., Karnchanatat, A., Sangvanich, P. (2014) Superoxide dismutase isozyme detection using two-dimensional gel electrophoresis zymograms. *Journal of Pharmaceutical and Biomedical Analysis* 90: 72-77.

9. Books and research articles

9.1 **Karnchanatat, A.*** and Tiengburanatam, N. (2010) Antimicrobial peptides. *Thaksin University Journal* 13: 101-108.

9.2 **Karnchanatat, A.*** (2012) Antimicrobial activity of lectins from plants**,** Antibacterial Agents / Book 1, ISBN 979-953-307-281-3. (in press)

10. Research conferences

10.1 Incharoensakdi*, A., **Karnchanatat, A.** Effect of salinity on the uptake of choline by *Aphanothece halophytica*. In "American Society of Plant Biologists Annual Meeting 2003". University of Hawaii, Honolulu, Hawaii, USA. (Abstract book)

10.2 Incharoensakdi*, A., Wangsupa, J., Laloknum, S., **Karnchanatat, A.,** Jantaro, S., and Maenpaa, P. Biochemical adaptation of cyanobacteria to high salinity environments: changes in nitrogen metabolism. In "17th FAOBMB Symposium/2nd IUBMB Special Meeting/7thA-IMBN Conference 2004". Chulalongkorn University, Bangkok, Thailand.

10.3 **Karnchanatat, A.**, Petsom, A., Sangvanich, P., Piaphukiew, J., Whalley, A.J.S., Reynolds, C.D., and Sihanonth, P*. Purification and biochemical characterization of an extracellular β-glucosidase from wood-decaying fungus *Daldinia eschscholzii*. In "50th Anniversary of Annual Meeting of the Mycological Society of Japan". 3-4 June, 2006, Aoba-no-mori Park Arts and Culture Hall, Chiba, Japan. (Abstract book)

10.4 **Karnchanatat, A.***, Petsom, A., Sangvanich, P., Piaphukiew, J., Whalley, A.J.S., Reynolds, C.D., and Sihanonth, P. Purification and biochemical characterization of an extracellular β-glucosidase from wood-decaying fungus *Daldinia eschscholzii*. In "II International Conference on Environmental, Industrail and Applied Microbiology (BioMicroWorld 2007)". 28 November-1 December 2007, Seville, Spain. (Proceeding book)

10.5 Kheeree, N., Sangvanich, P., Puthong, S., and **Karnchanatat, A.*** A Lectin from the rhizomes of *Curcuma amarissima* Roscoe and its role as anticancer activity. In "The 2nd Biochemistry and Molecular Biology Conference: Biochemistry and Molecular Biology for Sustainable Development" 7-8 May 2009, Khon Kaen University, Khon Kaen, THAILAND, p. 81-85. (Proceeding book)

10.6 Petnual, P., **Karnchanatat, A.** and Sangvanich, P.* Isolation of lectin from rhizomes of *Cucuma longa* L. with antifungal activity. In "The 2nd Biochemistry and Molecular Biology Conference: Biochemistry and Molecular Biology for Sustainable Development" 7-8 May 2009, Khon Kaen University, Khon Kaen, THAILAND, p. 91-95. (Proceeding book)

10.7 Konkumnerd, W., **Karnchanatat, A.** and Sangvanich, P.* A newly thermostable lectin from *Keampferia parviflora* Wall. Ex Baker. In "The 2nd Biochemistry and Molecular Biology Conference: Biochemistry and Molecular Biology for Sustainable Development" 7-8 May 2009, Khon Kaen University, Khon Kaen, THAILAND, p. 182-186. (Proceeding book)

10.8 Charungchitrak, S., **Karnchanatat, A.**, and Petsom, A.* Purification and characterization of lectin from *Archidendron jiringa* Neilson seeds. In "4th BUU Grad. Research Conference" 13 March 2009, Burapha University, Chonburi, THAILAND, F-P004 (1-8). (Proceeding book)

10.9 Konkumnerd, W., **Karnchanatat, A.** and Sangvanich, P.* A newly thermostable lectin from *Keampferia parviflora* Wall. Ex Baker. In "4th Annual Symposium of Protein Society of Thailand: Protein Research: from basic studies to applications in health sciences" 26-28 August 2009, Chulabhorn Research Institute Conference Center, THAILAND. p. 127-130. (Proceeding book)

10.10 Petnual, P., **Karnchanatat, A.** and Sangvanich, P.* Isolation of lectin from rhizomes of *Cucuma longa* L. with antifungal activity. In "4th Annual Symposium of Protein Society of Thailand: Protein Research: from basic studies to applications in health sciences" 26-28 August 2009, Chulabhorn Research Institute Conference Center, THAILAND. p. 132-136. (Proceeding book)

10.11 Kheeree, N., Sangvanich, P., Puthong, S., and **Karnchanatat, A.*** A Lectin from the rhizomes of *Curcuma amarissima* Roscoe and its role as anticancer activity. In "4th Annual Symposium of Protein Society of Thailand: Protein Research: from basic studies to applications in health sciences" 26-28 August 2009, Chulabhorn Research Institute Conference Center, THAILAND. p. 137-142. (Proceeding book)

10.12 Sawangsak, W., **Karnchanatat, A.**, and Chuntaratin, P.* Micropropagation of *Glycyrrhiza glabra* Linn. And medicinal herb through shoot tips culture. In "*Graduate Research Conference* King Mongkut's Institute of Technology Ladkrabang 2009" 31 August-2 September 2009, King Mongkut's Institute of Technology Ladkrabang, Bangkok, THAILAND, p. 441-446. (Proceeding book)

10.13 Tiengburanatam, N., Sangvanich, P., and **Karnchanatat, A.*** A novel α-glucosidase inhibitor protein from the rhizomes of *Zingiber ottensii* Valeton. In "The 3rd Technology and Innovation for Sustainable Development Conference (TISD2010)" 4-6 March 2010, Nong Khai, THAILAND. p. 355-360. (Proceeding book)

10.14 **Karnchanatat, A.***, Tiengburanatam, N., and Sangvanich, P. A cysteine protease with antifungal activity from *Zingiber ottensii* Valeton rhizomes. In "The 5rd Annual Symposium of Protein Society of Thailand: From Basic

Approaches to Modern Technologies". 23-25 June 2010, Bangkok, THAILAND, p. 213-218. (Proceeding book)

10.15 Wipusaree, N., Sihanonth, P., Piapukiew, J., Sangvanich, P., and **Karnchanatat, A.*** Screening and production of xylanase from endophytic fungi. In "The 22nd Annual Meeting of the Thai Society for Biotechnology, International Conference on Biotechnology for Healthy Living" 20-22 October 2010, Prince of Songkla University, Trang Campus, THAILAND, p. 485-491. (Proceeding book)

10.16 Panuthai, T., Sihanonth, P., Piapukiew, J., Sangvanich, P., and **Karnchanatat, A.*** Screening and production of lipase from endophytic fungi. In "The 22nd Annual Meeting of the Thai Society for Biotechnology, International Conference on Biotechnology for Healthy Living" 20-22 October 2010, Prince of Songkla University, Trang Campus, THAILAND,. p. 619-626. (Proceeding book)

10.17 Songserm, P., Sihanonth, P., Piapukiew, J., Sangvanich, P., and **Karnchanatat, A.*** Decolorization of synthetic dyes by selected white-rot fungi. In "The 22nd Annual Meeting of the Thai Society for Biotechnology, International Conference on Biotechnology for Healthy Living" 20-22 October 2010, Prince of Songkla University, Trang Campus, THAILAND, p. 758-762. (Proceeding book)

10.18 Saisavoey, T., **Karnchanatat, A.**, Thongchul, N., and Chuntaratin, P.* Enhancement of puerarin accumulation in *Pueraria mirifica* cell suspension culture using methyl jasmonate. In "The 22nd Annual Meeting of the Thai Society for Biotechnology, International Conference on Biotechnology for Healthy Living" 20-22 October 2010, Prince of Songkla University, Trang Campus, THAILAND, p. 1076-1083. (Proceeding book)

10.19 Virounbounyapat, P. **Karnchanatat, A.** and Sangvanich, P.* Protein from seeds of Djenkol Bean *Archidendron Jiringa* Nielsen. with alpha-glucosidase inhibitory activity. In "The 22nd Annual Meeting of the Thai Society for Biotechnology, International Conference on Biotechnology for Healthy Living" 20-22 October 2010, Prince of Songkla University, Trang Campus, THAILAND, p. 1200-1205. (Proceeding book)

10.20 Panuthai, T., Sihanonth, P., Piapukiew, J., Sangvanich, P., and **Karnchanatat, A.*** Screening and production of lipase from endophytic

fungi. In "The 11th *Graduate Research Conference Khon Kaen University* 2011" 28 January 2011, *Khon Kaen,* THAILAND, p. 635-642. (Proceeding book)

10.21 Wipusaree, N., Sihanonth, P., Piapukiew, J., Sangvanich, P., and **Karnchanatat, A.*** Screening and production of xylanase from endophytic fungi. In "The 11th *Graduate Research Conference Khon Kaen University* 2011" 28 January 2011, *Khon Kaen,* THAILAND,p. 643-648. (Proceeding book)

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10.23 Rungsaeng, P., Sangvanich, P., and **Karnchanatat, A.*** Protein with acetylcholinesterase inhibitory activity from the rhizomes of Zingiberaceae plants. In "The 11th *Graduate Research Conference Khon Kaen University* 2011" 28 January 2011, *Khon Kaen,* THAILAND, p. 654-658. (Proceeding book)

10.24 Moon-ai, W., **Karnchanatat, A.***, and Sangvanich, P. Purification and characterization of superoxide dismutase from the rhizome of *Curcuma aeruginosa* Roxb. In "The 11th *Graduate Research Conference Khon Kaen University* 2011" 28 January 2011, *Khon Kaen,* THAILAND, p. 659-665. (Proceeding book)

10.25 Chantaranothai, C., Palaga, T., **Karnchanatat, A.*,** and Sangvanich, P. Inhibitory activity against nitric oxide production in Macrophage RAW 264.7 from the protein of the rhizomes of Zingiberaceae plants. In "The 11th *Graduate Research Conference Khon Kaen University* 2011" 28 January 2011, *Khon Kaen,* THAILAND, p. 666-672. (Proceeding book)

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***** Corresponding author