รายงานวิจัยฉบับสมบูรณ์

เรื่อง

แอล-แอสพาราจิเนสจากเชื้อราสกุลไซลาเรีย และการประยุกต์ใช้ในการยับยั้งเซลล์มะเร็ง

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ABSTRACT

From 30 xylariaceus fungi isolates, ten were found to produce extracellular L-Asparaginase (ASNase) activity, with *Xylaria feejeensis* isolate XL001, yielding the highest level. The ASNase activity in the Czapek Dox medium of XL001 was highest with 2.0 g/L glucose and 10 g/L L-asparagine as the carbon, nitrogen sources, respectively. A 42.5 kDa ASNase was enriched 41.4-fold to apparent homogeneity from XL001 culture media using 80% saturation ammonium sulfate precipitation, DEAE-cellulose anion exchange and Superdex-75 gel filtration chromatography, but at a final yield of only 2.21%, an optimal temperature of 45 ^{o}C, with >90% activity from -20 to 45 ^{o}C, a broad pH range of 3.0-11.0 (optimal at pH 5.0), and was sensitive to most divalent cations but especially by Hg^{2+}, Cu^{2+} and EDTA. Moreover, relatively strong anti-proliferative activities were found against the five human cell lines with IC_{50} values ranging from 2.178 ± 0.013 μg/mL (breast cancer; BT474) to 7.145 ± 0.009 μg/mL (hepatoma cancer; HEP-G2).

Keywords: *Xylaria feejeensis*, L-asparaginase, anti-proliferative activity

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LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
µg	microgram
µL	microlitre
A	absorbance
AS	asparagine synthetase
ASN	L-asparagine
ASNase	L-asparaginase
BSA	bovine serum albumin
Da	dalton
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
rpm	revolution per minute
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane
U	unit activity

v/v	volume by volume
w/v	weight by volume

FULL TEXT

1. Introduction

Enzyme supplementation has been known to help all of these conditions. Therapeutic use of enzymes has been studied and used extensively in Europe, Asia and to a lesser extent the United States for at over 40 years. Enzymes as drugs have two important features that distinguish them from all other types of drugs. First, enzymes often bind and act on their targets with great affinity and specificity. Second, enzymes are catalytic and convert multiple target molecules to the desired products. These two features make enzymes specific and potent drugs that can accomplish therapeutic biochemistry in the body those small molecules cannot. These features render possible the production of potent drugs, which could carry out therapeutic biochemistry *in vivo*. Biotechnological advancements have enabled for enhanced potency and specificity among enzymes with a production at a lower cost (Vellard, 2003).

Cancer, particularly leukemia, is a global problem and in spite of sincere efforts paid in the past, search for efficient drugs to solve this problem is being continued worldwide. Acute lymphoblastic leukemia (ALL) is cancer of the white blood cells, the cells that normally fight infections. As the number of lymphocytes increase in the blood and bone marrow, there is also less room for healthy white blood cells, red blood cells, and platelets. As a consequence ALL patients often suffer infections, anemia, and easy bleeding. One of the primary drugs used in the treatment of ALL is L-asparaginase (ASNase).ASNase is an enzyme that hydrolyzes amino acid L-asparagine (ASN) to L-aspartic acid (ASP) and ammonia. Most human tissues can self-synthesize ASN from L-glutamine by the action of asparagine synthetase (AS). Certain neoplastic tissues, including ALL cells, however, express significantly lower levels of AS and thus have to rely solely on extracellular source of ASN to maintain protein synthesis. Systemic depletion of ASN by ASNase would therefore impair protein biosynthesis in these cells, leading to their deaths through cellular dysfunction.

Escherichia coli ASNase anti-tumour activity was previously demonstrated by Broome (1961) and Mashburn and Wriston (1964). Its production using microbial systems has attracted considerable attention owing to their cost effective and eco-friendly nature. For treatment with ASNase, current treatment protocols of ALL and

lymphosarcoma do not employ ASNase as a single agent because it is highly toxic drug with a low therapeutic index, and its therapeutic response rarely occurs without some evidence of toxicity. The therapeutic effect and/or toxicity of drugs often correlates with their dosage and concentration in body fluids, but no simple method for measuring ASNase activity in biological samples or monitoring its activity in serum of patients during ASNase therapy is currently available. In fact, it is always a part of multiple agent regimens and combined with drugs having definitive immunosuppressive effects (Ylikangas and Mononen, 2000). ELSPAR, ONCASPAR, ERWINASE and KIDROLASE are the brand names of ASNase. The FDA has approved ASNase for effective treatment of ALL and lymphosarcoma.

Filamentous fungi also serve as good source for ASNase for example; *Aspergillus*, *Penicillium* and *Fusarium* (Nakahama *et al.*, 1973; Gulati *et al.*, 1997; Sarquis *et al.*, 2004; Elzainy and Ali 2006) have more potential for asparaginase production (Wiame *et al.*, 1985). Furthermore, ectomycorrhizal fungi associated with *Pinus pinaster* and *Pinus radiate* in Western Australia were found to produce asparaginase (Bell and Adams, 2004). Consequently, the aim of this study was to production of ASNase from the culture of selected xylariaceus fungi, as a prerequisite to further study this ASNase with respect to cytotoxicity assay for human malignant cell lines activities.

2. Material and method

2.1. Chemical

L-asparagine, Nessler's reagent, and Trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (USA). The reagents used in polyacrylamide gel electrophoresis (PAGE) were obtained from Plusone Pharmacia Biotech (Sweden), except the low molecular weight calibration kit, used as standard molecular weight marker proteins, which was purchased from Amersham Pharmacia Biotech (UK). All other biochemical reagents and general chemicals used in the investigation were of analytical grade.

2.2 Organisms

All 30 strains of xylariaceous fungi used in this study were obtained from the culture collection of the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Thailand. Each fungus was grown on potato dextrose agar (PDA) at 25 °C for about seven days and maintained at 4 °C until use. Each stocked fungus was sub-cultured every two or three months.

2.3 Screening of xylariaceous fungi for extracellular ASNase production

All isolates of xylariaceus fungi were cultured on PDA for 7 days. The 5 mm disc of mycelium was transferred to the tested agar media. The agar plate assay was routinely used for the screening of ASNase production. Modified Czapex Dox's (MCD) agar contains the following ingredients (g/L of distilled water). glucose 2 g, L-asparagine 10 g, KH_2PO_4 1.52 g. KCl 0.52 g, $MgSO_4.7H_2O$ 0.52 g, $FeSO_4.7H_2O$ 0.01 g, agar 20 g, distilled water 1000 mL, pH 5.5) was used for plate assay. A 2.5% stock solution of phenol red was prepared in ethanol (pH 6.2) and 3 mL of this was added to 1000 mL of Czapek Dox medium. Control plates were MCD agar without asparagine. After five days of incubation at 25 °C, the appearance of a pink zone around the fungal colony in an otherwise yellow medium indicated ASNase activity (Gulati et al. 1997).

2.4 Identification of the xylariaceous fungi isolate

The xylariaceous fungi strain which showed the highest level of ASNase production was then identified to species using morphological and molecular systematic approaches. Morphological identification used both macroscopic and microscopic characters, whilst the molecular identification was based upon the DNA sequence similarity of the internal transcribed spacer (ITS) regions of the rDNA, comparing this isolate to those in the NCBI GenBank database. Genomic DNA was prepared from fresh mycelial cultures of the selected endophytic fungal isolate and extracted with cetyltrimethylammonium bromide (CTAB), as described in Zhou *et al.* (Zhou et al., 1999). PCR amplification of the internal transcribed spacer (ITS) was performed in a total volume of 35 µl which was comprised of approx. 100 ng genomic DNA, 1 × PCR master Mix (Fermentas, Califonia, USA), and 100 *nM* of *ITS1F* primer, and 500 *nM ITS4 primer.* The amplification was performed in a thermocycler with a PCR profile of 94 °C for 5 min, followed by 38 cycles of 94 °C for 1 min, 51 °C for 1 min and 72 °C for 1 min, plus a final extension of 72 °C for 5 min. The PCR reactions were purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and were direct sequenced on both the leading and lagging strands (using the ITSF1 and ITS4 primers, respectively) commercially by Macrogen (Seoul, Korea). The complete consensus sequence was then used to BLASTn search the NCBI GenBank database using the default settings, with the top 100 highest sequence similarity hits being recorded and compared. Species annotation of the deposited ITS sequences in

the GenBank database were taken on trust and used to convert the molecular operational taxonomic unit (MOTU) designation of the fungal isolate to a likely species designation where the % sequence similarity was high enough (>97%).

2.5 Spectrophotometric assay of ASNase enzyme

ASNase activity was measured by the modified method of Imada et al. (1973). A reaction mixture containing 0.5 mL of 0.5 M Tris-HCl buffer (pH 8.2), 0.1 mL of 40 mM L-asparagine, 1.0 mL of suitably diluted enzyme source (culture filtrate of an endophyte) and 0.4 mL of distilled water (total volume of 2.0 mL) was incubated at 37 °C for 30 min. The reaction was terminated by adding 0.5 mL of 1.5 M tricholoroacetic acid (TCA). Blank tubes were prepared by adding the enzyme source after the addition of TCA. After termination of the reaction, 3.7 mL volume of distilled water and 0.2 mL of Nessler's reagent were added to 0.1 mL of the above reaction mixture and incubated for 20 min. The amount of ammonia released during the reaction was determined by measuring the absorbance at 450 nm. One international unit (IU) of ASNase is the amount of enzyme needed to liberate 1 µmole of ammonia in 1 min at 37 °C (Imada et al. 1973).

$$\text{Units/mL enzyme} = \frac{(\mu\text{mole of } NH_3 \text{ liberated})\ (2.5)}{(0.1)\ (30)\ (1)}$$

2.5 = Initial volume of enzyme mixture (mL)

0.1 = Volume of enzyme mixture used in final reaction (mL)

30.0 = Incubation time (minutes)

1.0 = Volume of enzyme used (mL)

2.6 Effect of carbon and nitrogen concentration on enzyme activity

The fungus was inoculated in 100 mL MCD broth with different concentrations of glucose (0.2, 0.5, 1.0, 2.0 or 4.0 g/L) or L-asparagine (1.2, 2.5, 5.0, 10.0 or 20.0 g/L) and incubated as mentioned above, and then incubated at room temperature (30 °C) for 7 days. The culture filtrate was assayed for enzyme activity on the end of incubation. All experiments were done with triplicate flasks, with the results reported as the mean ± 1SE.

2.7. Protein content determination

Protein contents were determined by the Bradford assay (Bradford, 1976), using 5, 10, 15 and 20 µg/ml of bovine serum albumin (BSA) as the standard to construct the calibration curve. For each serial two-fold dilution of the sample in

deionized water, 50 µl aliquots were transferred into each of three wells of a microtiter plate and 50 µl of Bradford's reagent (100 ml contains: 10 mg Coomassie Brilliant Blue G-250 and 10 ml of 85% (v/v) phosphoric acid, dissolved in 95% (v/v) ethanol) was added to each well. The plate was shaken (Biosan, OS-10, Latvia) for 5 min and then left for 10 min before reading the absorbance at 595 nm using an ELISA plate reader (Biotek Synergy HT, Biotek instrument, USA). The obtained OD was converted to the protein concentration using the linear equation computed from the standard curve. During the column chromatographic separations, the elution peak profiles of proteins were determined by measuring the absorbance at 280 nm.

2.8 Purification of ASNase

The purification protocol includes different steps sequentially like ammonium sulphate precipitation, ion exchange and gel filtration chromatographies. After each step, the ASNas activity and total protein content were determined. PAGE was also carried after each step.

2.8.1 $(NH_4)_2SO_4$ Precipitation

To 5 liters of culture supernatant, $(NH_4)_2SO_4$ was slowly added with stirring to a final 80% saturation and then left to stand overnight at 4 °C. The precipitate was collected by centrifugation at 15,000 × g for 30 min (Beckman Coulter, USA), and dissolved in 50-75 mL of distilled water, dialyzed (3,500 MWCO) against 3 changes of 5 L distilled water at 4 °C and then concentrated by lyophilization (Labconco, USA) to ~50 mg/mL, which is referred to hereafter as the "*ammonium sulfate cut fraction*".

2.8.2 DEAE-cellulose ion exchange chromatography

DEAE-cellulose ion exchange chromatography was performed with a 1.6 cm × 15 cm column using an automatic liquid chromatography system (AKTA prime, Amersham Pharmacia Biotech, Sweden). The column was equilibrated with 5 column-volumes of 20 mM Tris-HCl (pH 7.0). Thereafter, 5 ml samples (400 mg protein) of the ammonium sulfate cut fraction were injected into the column and eluted with the same buffer at a flow rate of 1.0 mL/min, collecting 10-ml fractions before a linear 0-1.0 M NaCl gradient in the same buffer was applied over the next 55 fractions. The eluted fractions were monitored for protein content with a UV detector at 280 nm and for ASNase activity as described in above. The fractions containing ASNase activity from the column were pooled, dialyzed against 3 changes of 5 L of

distilled water and concentrated to ~50 mg/mL, and is referred to as the "*post-DEAE-cellulose ASNase fraction*".

2.8.3 Superdex-75 gel filtration chromatography

The post-DEAE-cellulose ASNase fraction was then further enriched by preparative Superdex-75 column (1.6 cm × 60 cm) chromatography. The column was equilibrated with two column-volumes of 100 mM NaCl / 20 mM Tris-HCl (pH 7.0), and then 2 ml of the post-DEAE-cellulose ASNase fraction solution (50 mg protein) was injected and eluted in the same buffer at a flow rate of 0.5 mL/min and collecting 5 ml fractions. Fractions were monitored for protein with a UV detector at 280 nm and for ASNase activity as described in above. ASNase active fractions were pooled, dialyzed against 3 changes of 5 L of distilled water and concentrated to ~5 mg/mL, and is referred to as the "*enriched ASNase fraction*".

2.9 Determination of enzyme purity by native-PAGE and ASNase activity staining

The enzyme from each step of purification was analyzed by its native protein pattern and its purity according to the method from literature (Bollag and Rozycki, 1996). Electrophoresis conditions, protein and activity staining are described below.

2.9.1 Non-denaturating gel electrophoresis

Native PAGE was performed with 10% and 5% (w/v) acrylamide separating and stacking gels, respectively, with 25 mM Tris-glycine (pH 8.3) as the electrode buffer. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Hoefer model miniVE, Pharmacia Biotech, UK). After electrophoresis, proteins in the gel were visualized by Coomassie blue R-250 (Sigma) staining.

2.9.2 Coomassie blue staining

Native and reducing SDS-PAGE gels were stained by immersion in 0.1% (w/v) Coomassie blue R-250 inl 0% (v/v) acetic acid / 45% (v/v) methanol for 45 min. Destaining was performed by immersing the gel in 10% (v/v) acetic acid / 45% (v/v) methanol, with several changes of this destaining solution until the background was clear.

2.9.3 Molecular weight determination by SDS PAGE

Discontinuous reducing 0.1% (w/v) SDS-PAGE was performed according to the procedure of Laemmli (Laemmli, 1970) using 12.5% and 5% (w/v) acrylamide resolving and stacking gels, respectively. Samples were treated with reducing (2-

mercaptoethanol containing) sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. High and low molecular weight standards were coresolved on each gel and used to determine the subunit molecular weight of the enriched ASNase enzyme. After electrophoresis, proteins in the gel were visualized by staining with Coomassie blue R-250.

2.10 Effect of temperature on the ASNase activity and thermostability

The effect of temperature on the ASNase activity was determined by incubating the enriched ASNase fraction in 0.5 M Tris-HCl buffer (pH 8.2) at various temperatures (-20-90 °C at 10 °C intervals) for 30 min assaying the ASNase activity. The thermostability of the ASNase was investigated by preincubating the enriched ASNase fraction at various temperatures (-30-60 °C in 10 °C intervals) in the same buffer for the indicated fixed time intervals (10-120 min), cooling to 4 °C and then assaying the residual ASNase activity.

2.11 pH-dependence of the ASNase activity

Incubating the enriched ASNase fraction in buffers of broadly similar salinity levels, but varying in pH from 2-14, was used to assess the pretreatment pH stability and the pH optima of the ASNase. The buffers used were (all 20 mM) glycine-HCl (pH 2.0-4.0), sodium acetate (pH 4.0-6.0), potassium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0-10.0) and glycine-NaOH (pH 10.0-12.0). The enriched ASNase fraction was mixed in each of the different pH-buffer compositions, plus the control (20 0.5 M Tris-HCl buffer (pH 8.2)). For pH stability, the above ASNase -buffer mixtures were left for 30 min at room temperature and then adjusted back to 0.5 M Tris-HCl buffer (pH 8.2) and assayed for ASNase activity. The control incubation was set at 100% activity and the activity of the samples from the different pH buffers were expressed as the % activity relative to that of the control (set at 100%). For evaluation of the pH optima of the enriched ASNase, the different pH buffer-enzyme mixtures were adjusted in substrate concentration, as per section ASNase activity assay, and performed over 30 minutes. The activity of the enzyme in each pH was then related to that of the control, set to 100%.

2.12 Effect of metal ions on the ASNase activity

The effect of preculture with different divalent metal cation salts (mostly chloride anions but also two sulfate anions) and the chelating agent ethylenediamine tetraacetic acid (EDTA), on the ASNase activity of the enriched ASNase fraction was

evaluated. The enriched ASNase fraction was incubated for 30 min with one of Ca^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} (all as chlorides), Cu^{2+} or Zn^{2+} (as sulfates) or EDTA, at one of three concentrations (1, 5 and 10 mM) with continuous shaking. The residual ASNase activity was then evaluated, and from this the relative ASNase activity (%) was calculated taking the residual ASNase activity found in the control samples (without the addition of metal salts or EDTA) as 100%.

2.13 Assay of antiproliferative activity

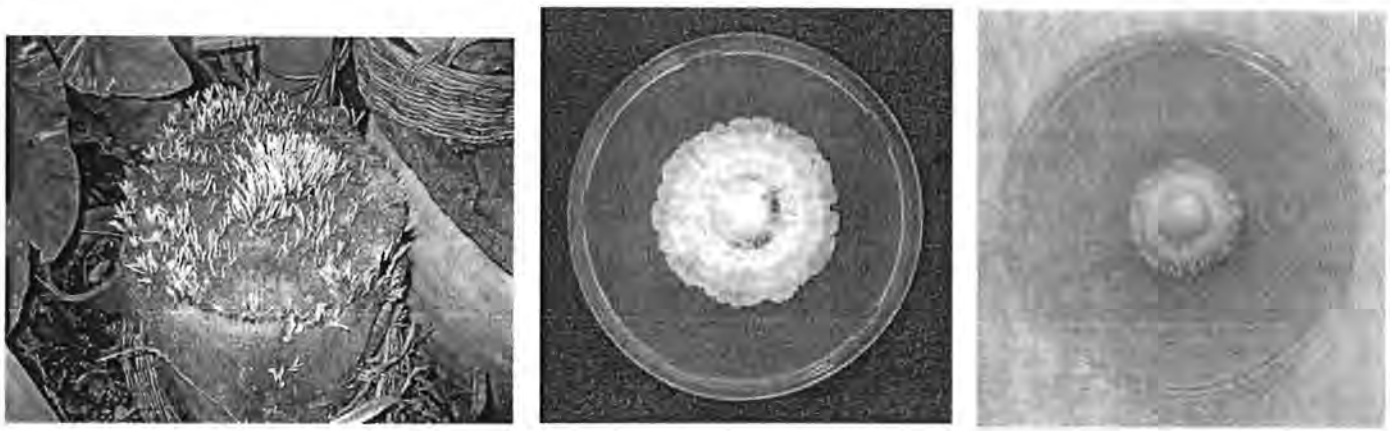
The bioassay for *in vitro* antiproliferative activity towards five human cell lines, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon), was performed *in vitro* in tissue culture. Cells were routinely maintained in complete media, comprised of RPMI, at 37 °C in an atmosphere of 5% (v/v) CO_2. Cells were seeded at 8×10^4 cells/cm^2 in a total of 1 mL complete media. Prior to the assay cells at confluence were trypsinized aspirated and washed before seeding at a final density of 5 x10^3 cells/μL in 200 μL of complete media in 96 well plates and cultured for 24 h as above. Then serial dilutions of the purified lectin were added (0-35 μg/mL final concentration) in a total volume of 200 μL complete media to the cell cultures and incubated for a further 72 h. Next, 10 μL of MTT (3-[5, 5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium bromide) solution (5 mg/mL) was then added to each well, incubated for 4 h, the media aspirated off and the cells gently washed to remove all remaining media prior to the addition of 150 μL DMSO per well for 30 min. The cell remnants and solution were then aspirated to ensure all the cells were lysed and the crystals dissolved, and the absorbance at 540 nm was measured using a microtiter reader. Controls included the absence of cells or the crude ASNase enzyme.

3. Results and discussion

3.1 Screening of ASNas positive cultures by rapid-plate assay method

To select the best laccase-producing strain from total 30 strain xylariaceous fungi, each strain was cultured on L-asparagine media supplemented with a dye indicator (phenol red). The indicator is pH sensitive. Normally it gives yellow color to media (in acidic and neutral conditions), it gives the pink color to the media when the pH changes from acidic to alkaline condition. The pink zone around fungal colony indicates the pH alteration which originated from ammonia accumulation in the medium. Five days before the green zone around the colony were recognized and

measured. Although all 30 isolates were found to secrete ASNas, the isolate XL001 showed the highest ratio, and therefore it was selected for further study. For genotypic identification, the ITS region of isolate XL001 was amplified, directly sequenced and compared to those in the NCBI GenBank database using the BLASTn algorithm. The PCR amplicon containing the ITS sequence of the isolate XL001 is 532 bp long and exhibited highest sequence identity (97%) to *Xylaria feejeensis*. Thus, both the morphological, cultural and molecular characteristics are consistent with isolate XL001 belonging to *Xylaria* and being related to *X. feejeensis*.



(A) (B) (C)

Figure 1 (A) Mature teleomorphic stroma of *X. feejeensis* XL001 on mango twig, (B) cultured on PDA, and (C) color change in the medium (yellow to pink) around colony indicates production of enzyme.

3.2 Effect of carbon source on ASNas production

The glucose concentration was varied from 0.2 to 4.0 g/L. The ASNas activity obtained under various glucose concentrations is given in Figure 2. As the glucose concentration was varied from 0.2 to 4.0 g/L the ASNas activity was found to increase. The maximum enzyme activity of 32.77 U/mL was obtained for 2.0 mg/mL glucose carbon source at 7 days of fermentation time. The low enzyme activity was observed when glucose concentration was increased from 2.0 to 4.0. The decrease in enzyme activity might be due to glucose inhibition on growth of *X. feejeensis* XL001 and catabolic repression of ASNas production. Hence the 2.0 g/L glucose was used for further optimization studies.

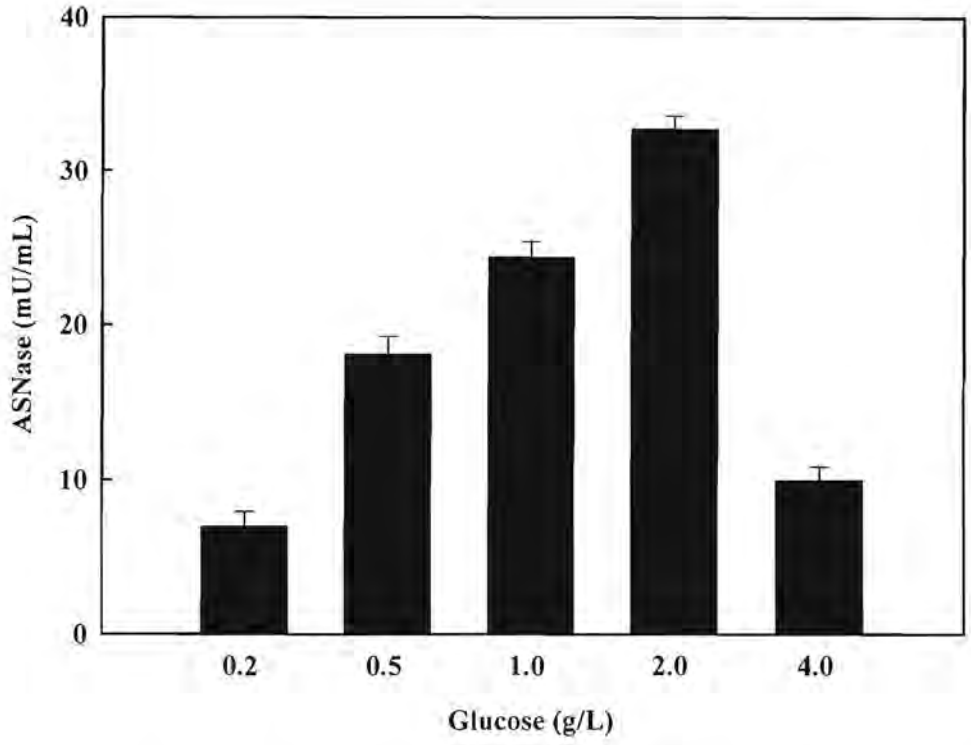


Figure 2 Effect of glucose (carbon source) concentration of growth medium on ASNase enzyme activity and amount of glucose in the medium of *X. feejeensis* isolate XL001 (Bars represent standard error).

3.3 Effect of nitrogen source on ASNas production

ASNas as a nitrogen source in the medium is known to stimulate the production of enzyme activity (Lapmak et al. 2010). In *X. feejeensis* isolate XL001, enzyme activity increased with increasing concentration of L-asparagine in the medium (Figure 3), suggesting that in this fungus also a high concentration of L-asparagine at 10 g/L is suitable for ASNas production.

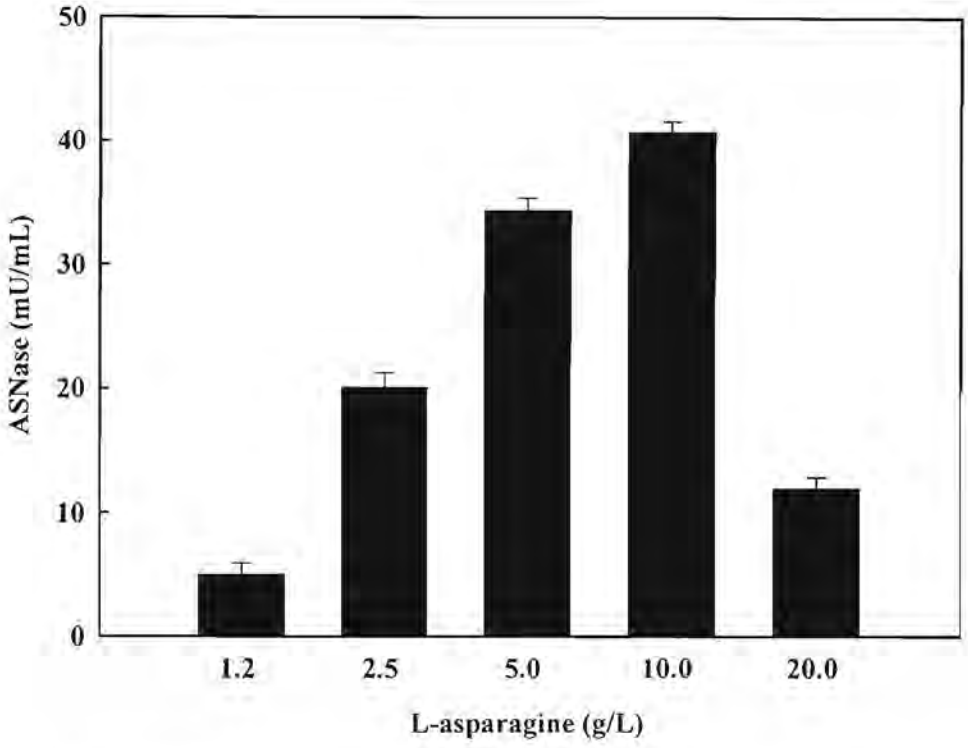


Figure 3 Effect of substrate (nitrogen source) concentration of growth medium on ASNase enzyme activity and amount of glucose in the medium of *X. feejeensis* isolate XL001 (Bars represent standard error).

3.4 Purification of ASNase

At the end of the cultivation period, mycelia were removed by filtration through Whatman 3M chromatography paper. This ammonium sulfate cut fraction was then subjected to DEAE-cellulose anion exchange chromatography. The ASNase active fraction was adsorbed onto the DEAE-cellulose column, allowing separation from the unbound proteins, and eluted from the column at 200-375 mM NaCl, whereas the non-ASNase active bound protein eluted as a double peak at lower and equal salt levels (Figure 4A). Thus, the elution pattern showed a single ASNase activity peak which was harvested and pooled. Compared to the ammonium sulfate cut fraction, the post-DEAE-cellulose ASNase fraction showed a 63% reduction in the total protein content for only a loss of 16% ASNase activity (Table 1), but the preparation was still not homogenous (Figure 5A).

Thus, the post-DEAE-cellulose ASNase fraction was further fractionated using Superdex-75 gel column chromatography, where a sharp peak was eluted free of most of the other ASNase activity negative proteins (Figure 4B). Compared to the post-DEAE-cellulose ASNase fraction, although the post-Superdex-75 fraction (enriched ASNase fraction) showed a 99.4% reduction in the total protein content this was achieved at the cost of a 93.4% loss of ASNase activity, resulting in a 10.9-fold activity enrichment (Table 1). Overall, a 41.4-fold enrichment for a 2.21% yield was obtined after the three enrichmnt stages, compared to the crude culture filtrate (Table 1). The enriched ASNase fraction (post-Superdex-75), with a specific activity of 156.3 U/mg of protein (Table 1) and was enrinched to or near to apparent homogeneity (Figure 5A),

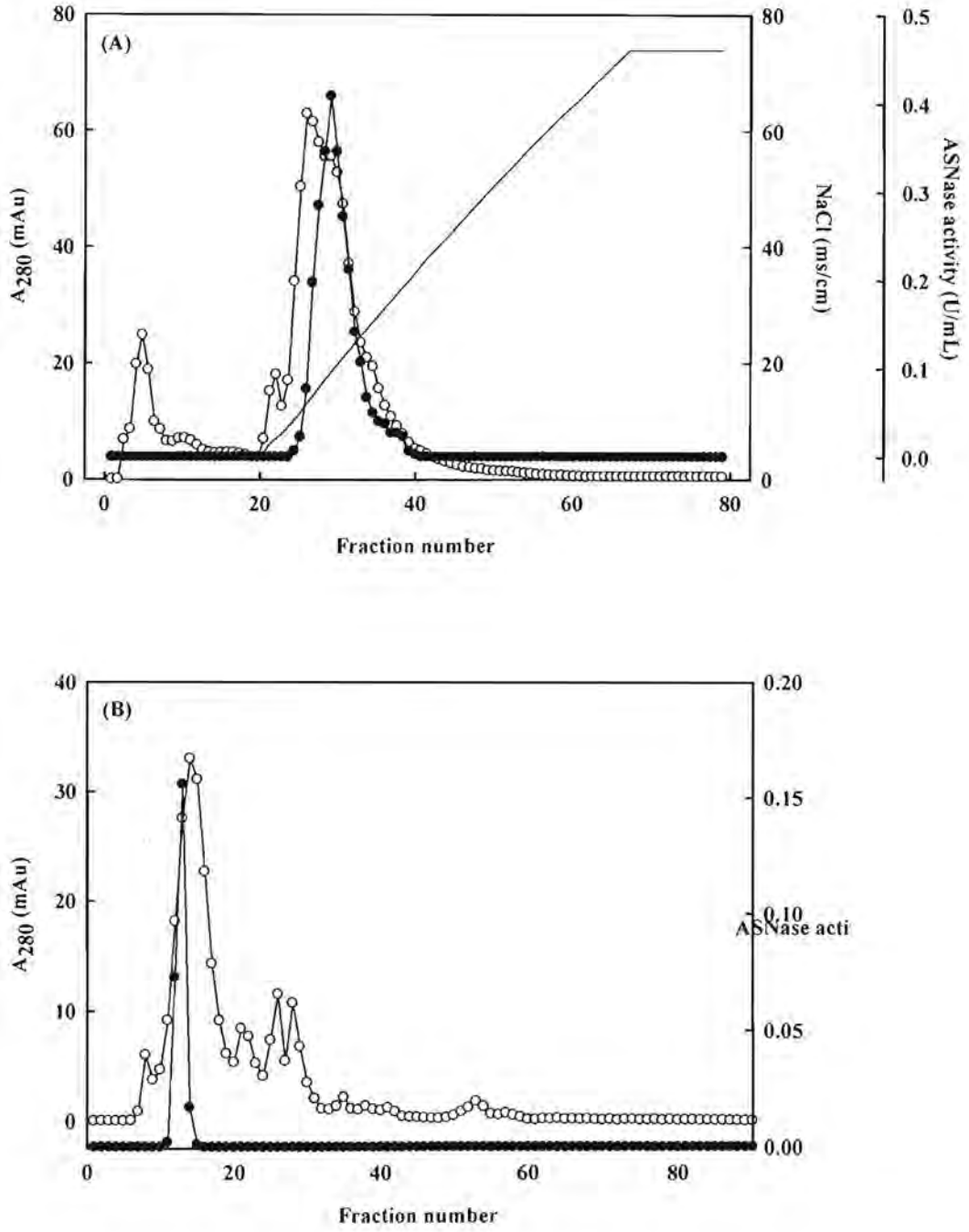


Figure 4. Profile of the enrichment of the *X. feejeensis* isolate XL001 extracellular ASNase extract by; (A) DEAE-cellulose ion-exchange chromatography of the ammonium sulfate cut fraction (400 mg protein) eluted in 20 mM Tris-HCl (pH 7.0) with a 0-1 M NaCl linear gradient; and (B) Superdex-75 gel chromatography of the post-DEAE-cellulose ASN fraction (50 mg) eluted in 100 mM NaCl / 20 mM Tris-HCl (pH 7.0). For both panels A and B; absorbance at 280 nm (○), ASNase activity (●). Profiles shown are representative of 3 different enrichments.

Table 1. Enrichment summary for the ASNase from *X. feejeensis* isolate XL001

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	2,996.0	11,315	3.78	100.0	1.00
80% $(NH_4)_2SO_4$ cut	765.0	4,740	6.20	41.9	1.64
DEAE-cellulose	280.0	4,000	14.30	35.4	3.78
Sephadex-75	1.6	250	156.30	2.2	41.4

3.5 Determination of enzyme purity and protein pattern on native-PAGE

The ASNase from each step of enrichment was analyzed for purity and protein pattern by native-PAGE, with protein and enzyme activity staining (Figure 5A). Whilst the post-DEAE-cellulose ASNase fraction still showed multiple components, the enriched ASNase fraction (post-Superdex-75 ASNase fraction) showed a single protein band on native-PAGE, suggesting a high degree of purity, with only a enzyme (fluorescence) band seen when using methylumbelliferyl butyrate as the substrate, and at the same position (R_f), supporting that the enriched ASNase fraction was a pure or near pure enzyme.

3.6 Molecular weight determination

Discontinuous reducing SDS-PAGE, a relatively sensitive technique for ASNase separation, revealed a single strong band with an apparent molecular weight of 42.5 kDa after Coomassie blue R250 staining (Figure 5B). This supports enrichment to near homogeneity and suggests that the purified ASNase could be a monomeric protein, or at least if a multimeric one that dissociates into subunits under these enrichment conditions, that this 37.4 kDa subunit has ASNase activity alone.

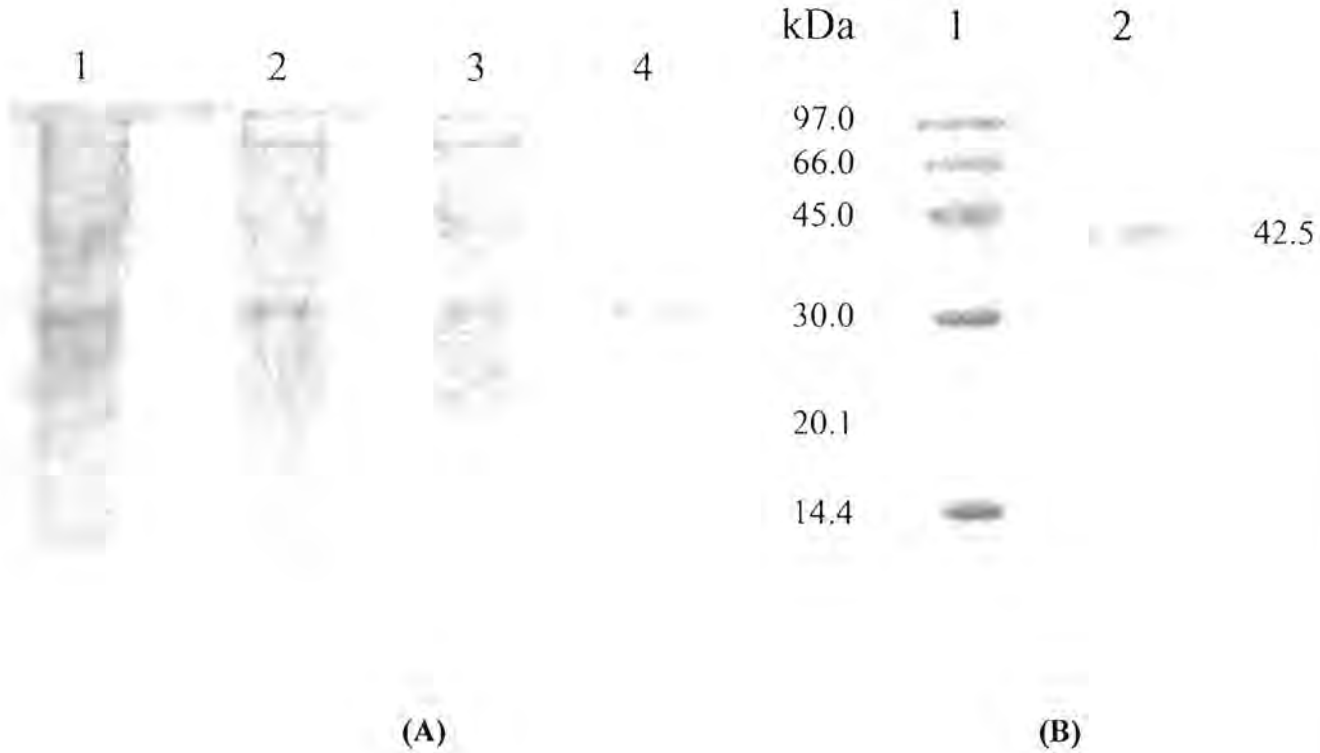


Figure 5. (A) Coomassie blue stained native-PAGE analysis of the from *X. feejeensis* isolate XL001 ASNase fractions from each step of the enrichment and stained for protein by coomassie blue (Lanes 1 - 4). Lane 1, crude enzyme (20 μg of protein); Lane 2, ammonium sulfate cut fraction (20 μg of protein); Lane 3, post-DEAE-cellulose ASNase fraction (15 μg of protein); Lanes 4 and 5, enriched ASNase fraction (post-Superdex-75) (10 μg of protein). (B) Reducing SDS-PAGE analysis, after coomassie blue staining, of the enriched ASNase fraction (post-Superdex-75) from *X. feejeensis* isolate XL001: Lane 1, Low molecular weight protein markers; Lane 2, enriched ASNase fraction (5 μg of protein). Gels shown in (A) and (B) are representative of 3 separate enrichments.

3.7. Effect of temperature on the ASNase activity and thermostability

Figure 6(A) depicts the effect of different temperatures on the relative activity of the enriched ASNase from *X. feejeensis* isolate XL001. The ASNase activity was relatively unchanged from -20 °C to 30 °C and then slightly increased with an increase in the temperature up to 40 °C, its maximum activity, before declining sharply at 50 °C with no activity at 60 °C or higher. The thermal stability of the ASNase from *X. feejeensis* isolate XL001 was determined by maintaining the enzyme at various temperatures ranging from 30-60 °C for 120 minutes in 20 mM sodium acetate buffer pH 5.0. The enzyme retained more than 90% of its original activity between 50-60 °C, whilst at 70 °C and above the enzyme retained less than 50% of its original activity (data not shown). The thermostability of the enzyme was also determined by incubating the enzyme for one hour at 30 °C, 40 °C and 60 °C at pH 5.0. The enzyme showed essentially full activity after 120 min at 30 or 40 °C with, interestingly, a slightly higher activity after all pre-incubation time points at 40 than at 30 °C (Figure 6B). At 50 °C the activity decreased after 20 min preincubation but was still at > 45% activity after 120 min. In contrast, at 60 °C the enzyme rapidly lost activity falling to 50% and no activity after <5 and 120 min, respectively.

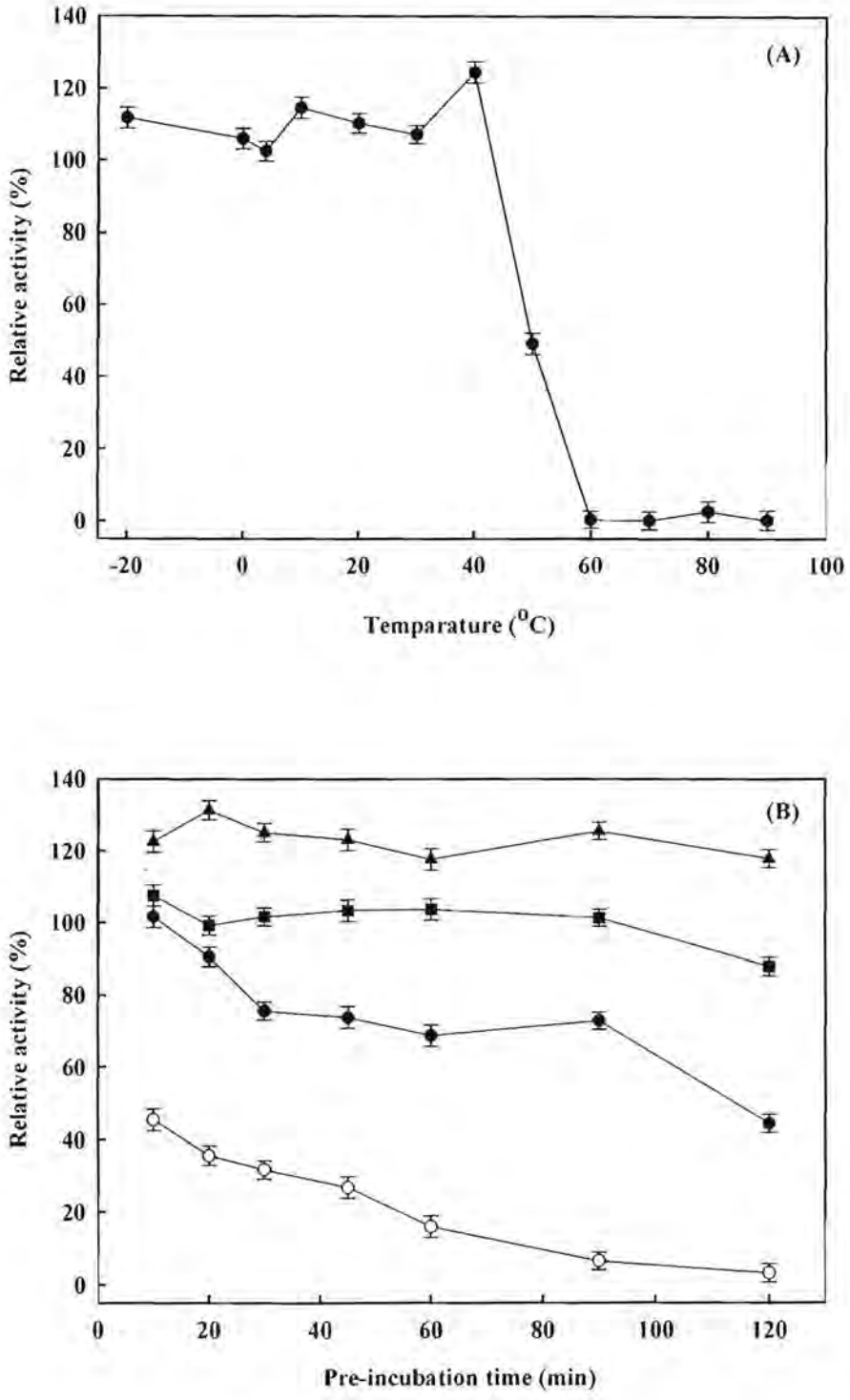


Figure 6. The (A) optimal reaction (enzyme) temperature and (B) thermostability of the enriched ASNase fraction from *X. feejeensis* isolate XL001, assayed in 0.5 M Tris-HCl buffer (pH 8.2) at (■) 30 °C, (▲) 40 °C, (●) 50 °C and (○) 60 °C. For both panels A and B the data are shown as the mean ± 1 SEM and are derived from three repeats.

3.8. Effect of pH on ASNase activity and stability

The optimum pH for ASNase activity was 5.0 when assayed at room temperature for 60 min, giving a relative ASNase activity of 112.8%, but the activity level was maintained at over 90% across the broad pH range of 3.0-11.0 for 60 min, with less than 20% and 40% residual activity at pH 2.0 and 12.0, respectively (Figure 7). The considerable stability at acid-alkaline pH values of this ASNase from *X. feejeensis* isolate XL001 makes it potentially effective for use in industry.

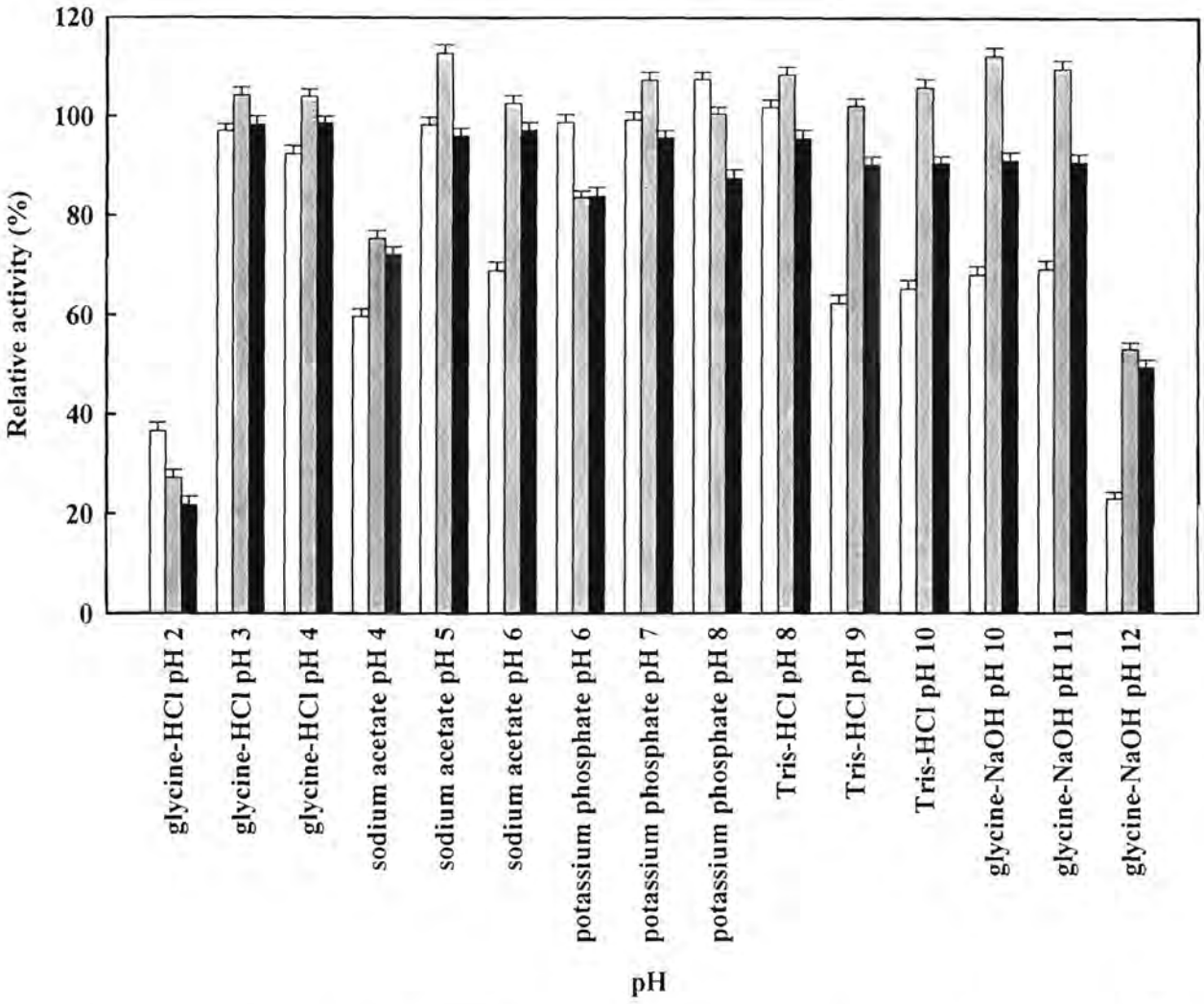


Figure 7. Effect of pH on the activity of the enriched ASNase fraction from *X. feejeensis* isolate XL001. The effect of pH on ASNase activity was evaluated in (all 20 mM) glycine-HCl buffer for pH 2.0-4.0, sodium acetate buffer for pH 4.0-6.0, potassium phosphate buffer for pH 6.0-8.0, Tris-HCl buffer for pH 8.0-10.0, and glycine-NaOH buffer for pH 10.0-12.0 at various time for (white) 30, (grey) 60, and (dark) 90 min The data are shown as the mean $\pm$ 1 SEM and are derived from three repeats.

3.9. Effect of metals and reagents

ASNase activity was strongly inhibited by Hg^{2+} in a dose-dependent manner (Table 2). EDTA and Cu^{2+} were also found to be inhibitory causing up to 57% and 80% inhibition of the enzyme activity at 10 mM (Table 2). Some other metal ions like Mg^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} decreased the enzyme activity in a dose-dependent manner but to a much lower extent, whilst Ca^{2+} caused a low level of inhibition at 1 mM but this was negated at higher ion concentrations to no inhibition at 10 mM. The inhibition of the enzyme activity by Hg^{2+} ions may be due to its interaction with sulphydril groups, suggesting that there is an important cysteine residue in or close to the active site of the enzyme.

Table 2. The effect of divalent cation salts and the chelating agent EDTA on the ASNase activity of the enriched ASNase fraction from *X. feejeensis* isolate XL001.

Reagent	Relative ASNase activity (%)[a]		
	1 mM	5 mM	10 mM
Control[b]	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00
$MgCl_2$	89.8 ± 0.42	75.5 ± 0.80	64.7 ± 0.15
$MnCl_2$	79.4 ± 0.14	78.7 ± 0.11	73.7 ± 0.53
$CuSO_4$	65.7 ± 0.46	22.8 ± 0.40	19.5 ± 0.14
$CaCl_2$	86.4 ± 0.31	90.7 ± 0.02	100.2 ± 0.02
$ZnSO_4$	94.7 ± 0.04	84.6 ± 0.05	57.9 ± 0.13
$FeCl_2$	66.7 ± 0.25	55.7 ± 0.76	55.0 ± 0.61
$HgCl_2$	50.3 ± 0.06	21.0 ± 0.94	6.78 ± 0.02
EDTA	57.0 ± 0.15	46.6 ± 0.05	43.3 ± 0.09

[a]The relative activity was determined by measuring the ASNase activity at 30 min at 37 °C in 0.5 M Tris-HCl buffer (pH 8.2) after pre-incubation at 30 °C for 30 min with the indicated reagents and concentrations, [b]using the activity seen in the absence of such reagents in 0.5 M Tris-HCl buffer (pH 8.2) alone as 100%. Results are shown as the average ± 1 SEM from a representative assay performed in triplicate. Means within a column or across a row that are followed by a different lower case letter are significantly different.

3.7 Anti-proliferation /cytotoxicity assay for human malignant cell lines

The anti-proliferative or cytotoxic effect of crude ASNase highest efficiency against BT474 (breast) with an IC_{50} value of 2.178 ± 0.013 μg/mL down to the lowest for HEP-G2 (hepatoma) with an IC_{50} of 7.145 ± 0.009 μg/mL. However, the dose-dependent effect (inhibition of proliferation and or cytotoxicity) was different between the two cell lines. For the HEP-G2 cell line, although a larger IC_{50} was evident, a greater degree of inhibition over a narrower dose range was obtained than that seen with the BT474 cell line which displayed a 4.4 fold lower IC_{50} value but a lower maximal inhibition level spread over a larger dose range. This could suggest

different mechanisms, be that receptors, with different K_a values, or differences in the number and duration of receptor crosslinking or in internalization pathways etc.

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APPENDICES

APPENDIX A

MEDIA

The media were prepared by sterilization in the autoclave at 121 °C for 15 minutes.

1. Potato dextrose agar (PDA)

Potato, peeled and diced	200	g
Glucose	20.0	g
Agar	15.0	g
Distilled water	1000	ml

Boil 200 g of peels, dried potato for 1 hr in 1000 ml. of distilled water. Filter, and make up the filtrate to one liter. Add the glucose and agar and dissolve by streaming and sterilize by autoclaving at 121 °C for 15 minutes.

2. Modified Czapex Dox's (MCD) agar (per liter)

glucose	2.0	g
L-asparagine	10.1	g
KH_2PO_4	1.52	g
KCl	0.52	g
$MgSO_4 \cdot 7H_2O$	0.52	g
$FeSO_4.7H_2O$	0.01	g
agar	20	g
pH 5.5		

APPENDIX B

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 C

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

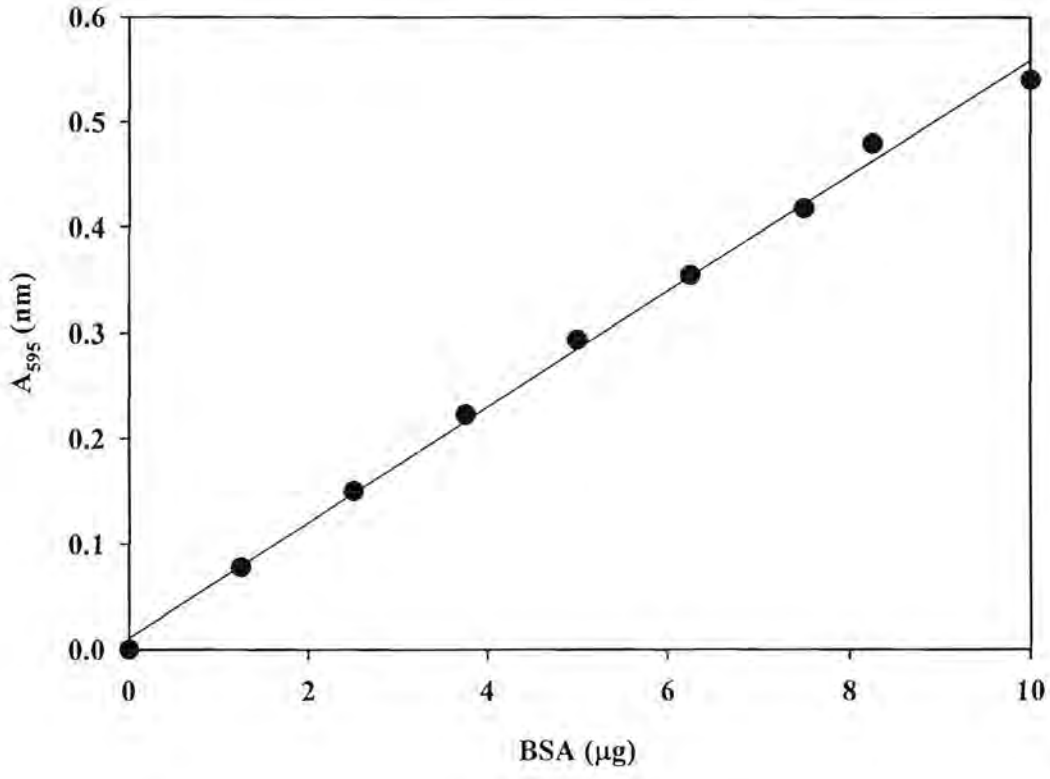
Solution A	4.2 mL
Solution B	2.5 mL
Distilled water	3.3 mL
10% Ammonium persulfate	50 µL
TEMED	5 µL

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 D

Calibration curve for protein determination Bradford method



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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.
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 Protein and peptide with *antiproliferative* activity of *macrophage RAW 264.7* from the rhizomes of Zingiberaceae plants, TRF-MAG Window II Co-funding, 2011-2012

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

7.12 Fibrinolytic enzyme from sand warm *Perinereis nuntia*, National Research Council, 2012-2013

8. Publications

8.1 Incharoensakdi, A.* and **Karnchanatat, A.** (2003) Salt stress enhances choline uptake in the halotolelant cyanobacterium *Aphanothece halophytica*. *Biochimica et Biophysica Acta* 1621: 102-109.

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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8.8 Tiengburanatam, N., Sangvanich, P., Boonmee, A and **Karnchanatat, A.*** (2010) A novel α-glucosidase inhibitor protein from the rhizomes of *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.

8.17 Boonmee, A., Srisomsap, C., **Karnchanatat, A.**, and Sangvanich P.* Biologically active proteins from *Curcuma comosa* Roxb. Rhizomes. *Journal of Medicinal Plants Research* 5: 5208-5215.

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.* (2012) 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* (in press)

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

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