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THESIS

PURIFICATION AND CHARACTERIZATION OF A RIBOSOME-INACTIVATING PROTEIN FROM SEED COAT OF *JATROPHA CURCAS*

CHANTHAKAN NUCHSUK

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Chanthakan Nuchsuk 2012: Purification and Characterization of a Ribosome-Inactivating Protein from Seed Coat of *Jatropha curcas*. Doctor of Philosophy (Biochemistry), Major Field: Biochemistry, Department of Biochemistry. Thesis Advisor: Associate Professor Sunanta Ratanapo, Ph.D. 172 pages.

A new type 1 ribosome-inactivating protein, which demonstrated lectin-like hemagglutination activity, was first purified from the seed coat of *J. curcas* Linn. by ammonium sulfate precipitation and chromatography on DEAE-Sephacel™ and CM-cellulose columns. It was designated as "Jc-SCRIP". Purification fold of Jc-SCRIP increased 113.5 times in final step with 1.12% yield of the total protein. It was a monomeric glycoprotein of a molecular mass of 38,938 Da, as determined by MALDI-TOF/MS. Structural analysis of Jc-SCRIP indicated that its major structure was β-sheet with N-terminal amino acid sequence: AINGGVA. The neutral sugar content of Jc-SCRIP was about 4.80% (w/w). It possessed strong *N*-glycosidase activity that released an RNA fragment of approximately 560 nucleotides from the rabbit reticulocyte rRNA after acidic aniline treatment. The positive antimicrobial effect of Jc-SCRIP was tested by agar dilution technique against 9 human-pathogenic bacteria and 1 fungus. Its most potent inhibitory activity was against *Staphylococcus epidermidis* ATCC 12228, with MIC value of 7.81 μg/ml. Jc-SCRIP showed the cytotoxic effects to cell lines of a human breast adenocarcinoma (MCF-7), a colon adenocarcinoma (SW620), and a liver carcinoma (HepG2), with IC_{50} values of 0.15, 0.25 and 0.40 μM, respectively. Jc-SCRIP had the larvicidal effects to the third instars larvae of mosquitoes, *Aedes aegypti* Linn. and *Culex quinquefasciatus* Say with LC_{50} values of 1.44 and 0.0303 mg protein/ml, respectively. It also showed the larvicidal effects to the second instars larvae of *Spodoptera litura* and *Spodoptera exigua* with LC_{50} values of 0.0525 and 0.0629 mg protein/ml, respectively. The potent larvicidal activity of Jc-SCRIP suggests that it may be used as a low cost natural agent to control both mosquitoes and crop pests. Further evaluation of biosafety to human and its toxic stability should be done before the application use.

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Chanthakan Nuchsuk

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
DMST	Culture Collection for Medical Microorganism, Department of Medical Sciences Thailand
CD	Circular Dichroism
Gal	Galactose
GalNAc	*N*-acetyl galactosamine
NANA	*N*-acetyl neuramic acid
HepG2	Human hepatoma cell lines
MCF-7	Breast cancer cell lines
SW620	Human colon adenocarcinoma cell lines
IC_{50}	The half maximal inhibitory concentration
ID_{50}	The median infective dose
LC_{50}	The median lethal concentration
LD_{50}	The median infective dose
RIPs	Ribosome-inactivating proteins
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate

PURIFICATION AND CHARACTERIZATION OF A RIBOSOME-INACTIVATING PROTEIN FROM SEED COAT OF *JATROPHA CURCAS*

INTRODUCTION

Due to the price of oil and the demand of energy consumption has been increasing; interest in alternative energy is also rising. Alternative energy is an energy used for fuel substitution, divided into two types depends on original resource; alternative energy from depleted resources such as coal and the other one from non-depleted resources that can be renewable such as solar, wind, biomass, and hydro.

Biomass from agricultural and industrial by-product is interesting to use as fuel substitution. Biodiesel is a renewable fuel produced from agricultural resources such as vegetable oils which made through a chemical process called transesterification. It does not contain petroleum, but it can be mixed at any level with petroleum diesel to create a biodiesel mixed. Biodiesel is simple to use, biodegradable, nontoxic, and essentially free of sulfur aromatics thus it is better for the environment. Moreover, it can be used in diesel engines with little modifications.

J. curcas is one of the important target plants for energy source. This plant has potential as a renewable energy crop because its oil may be used directly with slow speed diesel engine. *J. curcas* Linn, commonly known as physic nut or purging nut, belongs to the family Euphorbiaceae, which is widely distributed in tropical area. They are used as medicine and in several important manufactures. All parts of the tree can be used for multipurposes. The tree itself has been used for erosion control. The bark is rich in tannin. The leaves and roots have been used as a remedy for cancer, antiseptic, diuretic and haemostatic (Osoniyi and Onajobi, 2003). The fruits contain viscous oil that can be used as a diesel substitute, for soap making and in cosmetics industry. Latex has great medical potentials because possesses both procoagulant and anticoagulant activities (Osoniyi and Onajobi, 2003). The seed coat is considered

toxic and can cause acute abdominal pain and a burning sensation in throat followed by vomiting and diarrhea.

Many plants contain proteins identified as ribosome-inactivating proteins or RIPs that are a group of toxic proteins and can irreversibly inactivate ribosome (He and Liu, 2003) leading to interrupt protein synthesis because they can alter the conformation of the RNA and inhibit protein translation by their *N*-glycosidase activity (EC 3.2.2.22), selectively cleaving the N-glycosyl bond of a specific adenine residue at a highly conserved site known as "Sarcin/Ricin Domain" in ribosome. RIPs have been interested especially type 1 RIP because they are used as components of immunotoxin. They will be used to eliminate such targets as harmful cell, neoplastic, immunocompetent and parasitic cells (Lin *et al*., 2003).

In 1914, a toxic protein was isolated from the seed kernel of *J. curcas* by Felke, and was designated as "curcin" (Lin *et al*., 2003), a toxalbumin which is highly irritant and produces deleterious effects on blood (Osoniyi and Onajobi, 2003). Curcin has the biological activities and consisting of type 1 RIP, a single chain protein which can cleave the N-glycosidic bond of A_{4324} of rat liver 28s rRNA causing the inhibition of protein synthesis (Stripe *et al*., 1976; Endo and Tsurugi, 1987).

The biodiesel production from physic nut produces a large amount of seed coat which becomes agricultural waste. The value-added applications of them have been of interested. Several biochemical techniques will be used to isolate, characterize, and study the biological functions of the toxic protein from the seed coat of physic nut and thus leading to further applications.

OBJECTIVES

1. To purify and characterize a ribosome-inactivating protein (RIP) from the seed coat of *J. curcas*.
2. Application use of the purified RIP.

LITERATURE REVIEW

1. Introduction to *Jatropha curcas* Linn

1.1 Botanical description of *J. curcas*

Jatropha curcas Linn., commonly known as physic nut or purging nut, belongs to the family Euphorbiaceae, which is widely distributed in tropical area, Mexico, Nicaragua, North East of Thailand and in some parts of India (Openshaw, 2000). It is a drought resistant shrub or small tree with a grey bark which can reach a height of up to 5 m (Figure 1A). Sometimes it is grown as an ornamental plant. The flowers of *J. curcas* are small, green yellow to yellow white in color. Flowering season is spring to summer. The pollination is done by insects such as bees. The leaves are heart-shaped, up to 15 cm wide and they contain long stalks. The leaf margins are indented about 3 to 5 lobed with 3 to 5 veins radiating out from the leaf base. The fruits are 2.5 to 4 cm long, oval-shaped; when immature the fruits are green and fleshy. They become dark brown when ripening. The ripe fruits split to release 2 to 3 black seeds (Figure 1B).

(A) (B)

Figure 1 *Jatropha curcas* or physic nut is a poisonous small tree belonging to the family of Euphorbiaceae and grown in a drought-resistant area. (A) The tree has a grey bark and grows up to 5 to 7 m in height. (B) The immature seeds are fleshy green before ripening to dark brown and separate to 2 to 3 seeds.

1.2 Multipurpose usages of *J. curcas*

All parts of *J. curcas* tree can be used for numerous purposes. It is widely cultivated in tropical areas as a living fence in the fields. The tree itself has been used for erosion control. The flower attacks bees for honey production (Sirisomboon *et al*., 2007). The bark is rich in tannin. The leaves contain antiflammatory substances such as the flavonoids apigenin and its glycosides vitexin and isovitexin, the sterols stigmasterol, beta-D-sitosterol and its beta-D-glucoside (Makkar and Becker, 2009) and are used for rearing silkworm (Sirisomboon *et al*., 2007). Roots have been used for cancer, antiseptic, diuretic and haemostatic treatments (Osoniyi and Onajobi, 2003). The fruits contain viscous oil that can be used as a diesel substitute, for soap making, cosmetics industry, lubricant, insecticide, varnish (when mixed with iron oxide) (Sirisomboon *et al*., 2007), wool spinning and also has a strong purgative action and widely used for curing skin diseases and pain such as rheumatism (Heller, 1996). Extracts from fruits showed pregnancy-terminating in rats (Goonasekera, 1995). Latex has great medical potentials because it possesses both procoagulant and anticoagulant activities (Osoniyi and Onajobi, 2003). The latex contains pesticide and mollusc control properties (Sirisomboon *et al*., 2007). In addition, it contains an alkaloid known as Jatrophine which is believed to be having anticancer property and also has a proteolytic enzyme named as curcain. It has antimicrobial properties against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Streptococcus pyogenes* and *Candida albicans* (Heller, 1996; Thomas, 1989). Seeds are good source for phytate which was used for cancer prevention, reduction in iron-induced oxidative injury and prevention of lipid peroxidation (Singh *et al*., 2003; Makkar and Becker, 2009). The black thin shell seeds are considered toxic and can cause acute abdominal pain and a burning sensation in throat followed by vomiting and diarrhea.

1.3 General toxicity of *J. curcas*

All cases of systemic poisoning have resulted from ingestion of plant material. Only 1-3 seeds of *J. curcas* may produce toxicity to human. The symptoms of poisoning in human are largely associated with gastro-intestinal irritation. There is an acute abdominal pain and a burning sensation in the throat about half an hour after ingestion of the seeds followed by nausea, vomiting and diarrhea. In severe poisoning, theses symptoms progress to haemorrhagic gastroenteritis and dehydration. Children are more susceptible than adult.

Toxicity of *J. curcas* seeds could be caused by several components. Phorbol esters have been identified as the main toxic agent responsible for toxicity. They are a plant-derived organic compound. They occur in many plants of Euphorbiacaeae and Thymelaeaceae especially in Jatropha seed and oil (Goel *et al*., 2007; Li *et al*., 2010). Structures of six phorbol esters in *J. curcas* have now been determined using NMR. Phorbol esters themselves do not induce tumor but they promote growth of tumor. Thus, they are considered as co-carcinogen (Goel *et al*., 2007; Li *et al*., 2010). The concentration of phorbol esters varies from 2 to 3 mg/g in seed kernel and 2 to 4 mg/g in oil depending on varieties of *J. curcas* (Li *et al*., 2010; Makkar *et al*., 2007). Various organic and aqueous extracts of phorbol esters from seeds of *J. curcas* exhibited different toxic symptoms depending on dose, mode of administration and sensitivity of the animals being tested (Li *et al*., 2010; Trebien *et al*., 1988). Acute toxicity of purified phorbol ester isolated from Jatropha seed oil was accessed in male Swiss Hauschka mice by intragastric administration and the LD_{50} was determined. The LD_{50} for mice was 27.34 mg/ml and histopathological studies on the organs from the dead mice showed prominent lesions mainly found in lung and kidney (Li *et al*., 2010). The fertility regulatory effect of the fruit and seeds extracts of *J. curcas* were investigated by oral administration of the extracts to pregnant rats for varying period of time. The results revealed that the interruption of pregnancy occurred at an early stage after implantation (Goonasekera *et al*., 1995).

Toxicity of *J. curcas* seeds is also due to the presence several anti-nutritional factors such as trypsin inhibitor, phytate, saponin at high levels.

Trypsin inhibitor activity content in seeds of *J. curcas* is high (Azzaz *et al*., 2011). It ranged between 18.4-26.85 mg/ml (Makkar *et al*., 1998). Trypsin inhibitors interfere with the physiological process of digestion through interference with the normal functioning of pancreatic proteolytic enzymes in non-ruminants, leading to severe growth depression (White *et al*., 1989; Azzaz *et al*., 2011). It is possible that the anti-nutrient effect of trypsin inhibitors is due to their direct interaction with pancreatic proteolytic enzymes and a corresponding reduction in the digestibility of the proteins of the diet (Hajos *et al*., 1995; Azzaz *et al*., 2011).

Phytates may decrease the bioavailability of minerals especially Ca^{2+} and Fe^{2+}. They have also been implicated in decreasing protein digestibility by forming complexes and also by interacting with enzymes such as trypsin and pepsin (Martinez-Herrera *et al*., 2006).

Saponins, natural triterpene plant glycosides, are found in many plants. They have been of interested because of their physiological activities. They may serve as an anti-nutritional factor and antimicrobial agent protecting the plant against microbes and fungi (Makkar *et al*., 1998; Abou-Arab *et al*., 2010).

2. Plant ribosome-inactivating proteins (RIPs)

2.1 Classification of plant RIPs

Plant RIPs are classified into three groups based on their physical properties.

Type 1 RIPs such as saporin (from seeds of *Saponaria officinalis*), trichosanthin (from root tubers of *Trichosanthes kirilowii* Maxim) and pokeweed antiviral protein (from leaves of *Phytolacca americana*) consist of a single polypeptide chain of about 30 kDa. The proteins exhibit specific RNA *N*-glycosidase activity and are strongly basic proteins (pH 8-10) (Lin *et al.,* 2003). They are about 10^6-fold less toxic than type 2 RIPs on animal cells. The type 1 RIPs do not bind to cells and do not enter easily into the cytoplasm consequently they have a relatively low toxicity to cells and animals (Stirpe *et al.,* 2007). But they become highly toxic if they are introduced into cells by linkage to an appropriate carrier for binding to cells. For example, gelonin conjugated with Con A was the first experiment revealing that the conjugated form is more toxic to cells than the free form (Stirpe *et al*., 1980). Type 1 RIPs are frequently found more than type 2 RIPs and appeared to be preferentially distributed among plants belonging to some families such as Caryophyllaceae, Cucurbitaceae, and Euphorbiaceae (Stirpe, 2004). Examples of type 1 RIPs are shown in Table 1.

Table 1 Examples of type 1 RIPs

Species	RIPs	Organ
Phytolacca americana	PAP	spring leaf
(pokeweed)	PAP II	summer leaf
	PAP-S	seed
Dianthus carryophylus	dianthin 30	leaf
(carnation)	dianthin 32	leaf
Mirabilis jalapa	MAP	root
(four o'clock)		
Trichosanthes kirlowii	trichosanthin	root
(Chinese cucumber)		

Note: PAP, pokeweed antiviral protein; MAP, Mirabilis antiviral protein

Source: Hartley and Lord (2004)

Type 2 RIPs are highly toxic heterodimeric proteins with enzymatic and lectin properties in separating polypeptide subunits. Each subunit has molecular weight approximately of 30 kDa (Nielsen and Boston, 2001). One polypeptide with RIPs activity (A-chain), pI between 4.8 and 8, is linked to a galactose binding lectin (B-chain) through a disulfide bond (Stirpe and Barbieri, 1986). The A-chain shows specific RNA *N*-glycosidase activity which removes a single adenine from rRNA (A_{4324} from rat liver rRNA or A_{2660} from *E.coli* rRNA) and also from a variety of other polynucleotides e.g. poly (A) and DNA (Pelosi *et al*., 2005; Battelli *et al*., 1997). Type 2 RIPs are considered to be very potent toxin such as ricin. It can bind to the cell-surface receptors containing terminal galactose through their B subunit and enter inside the cell by receptor-mediated endocytosis and are transported to ER by retrograde pathway (Narayanan *et al*., 2004). The intersubunit disulfide bond which is essential for their toxicities is reduced in the ER followed by translocation of the A-chain to cytosol by an ER-associated degradation pathway, thus causing cell death (Nielsen and Boston, 2001; Battelli *et al.,* 1997; Pelosi *et al.,* 2005; Narayanan *et al*.,

2004). Ricin A chain has a turnover number (K_{cat}) of 1500 ribosomes per min and a Michaelis constant (K_m) of 0.1 μM (Ippoliti *et al*., 1992; Frankel *et al*., 1990). Type 2 RIPs are thought to have evolved from a gene fusion between an ancestral type 1 RIP gene, and a lectin gene (Hartley and Lord, 2004). A few plants such as *Sambucus nigra* and Dutch iris (*Iris hollandica*) produce type 1 and type 2 RIPs simultaneously in the same tissue. In some plant tissues, type 2 RIPs are highly abundant such as in mature *Ricinus communis* seeds ricin accounts for about 5% of the soluble protein and elderberry bark (*Sambucus nigra*) produce three type 2 RIPs each representing higher than 20% of the total protein (Hartley and Lord, 2004). Examples of type 2 RIPs are shown in Table 2.

Table 2 Examples of type 2 RIPs

Species	RIPs	Organ	Sugar specificity
Ricinus communis	ricin	seed	Gal/GalNAc
(castor oil plant)	*R. communis* agglutinin	seed	Gal>GalNAc
Abrus precatorius	abrin	seed	Gal/GalNAc
	A. precatorius agglutinin	seed	Gal
Adenia volkensii	volkensin	root	Gal
Momordica charantia	momordin	seed	Gal>GalNAc
Viscum album	viscumin	leaf	Gal/GalNAc
(mistletoe)			
Sambucus ebulus	ebulin b	bark	NANA
(dwarf elder)			
Sambucus nigra	SNAI	bark	NANA
(elderberry)	SNAV (nigrin b)	bark	GalNAc>Gal
	SNAIf	fruit	NANA

Note: Gal, galactose; GalNAc, *N*-acetylgalactosamine; NANA, *N*-acetylneuramic acid

Source: Hartley and Lord (2004)

Type 3 RIPs are synthesized as inactive precursor (proRIPs) that require proteolytic processing events. These RIPs are much less widespread than type 1 and type 2 RIPs. Type 3 RIPs are composed of a type 1 RIP-like N-terminal domain covalently linked to a C-terminal domain with unknown function (Reinbothe *et al*., 1994; He and Liu, 2003). At present, type 3 RIPs have been characterized only from maize and barley. The RIP from maize (*Zea mays* L.) is produced in the endosperm and accumulated in seed as an inactive 34 kDa precursor (proRIP), which is converted into an active form by proteolytic cleavage. This involves removal of 16 amino acids from the N terminus, several amino acids from the C terminus, and 25 amino acids from the center of the RIP polypeptide resulting in a two-chain, activated form of RIP that is called "αβ RIP" or also known as "b-32". These chains are tightly associated but are not covalently linked (Hey *et al.,* 1995). The RIP from barley has one chain also known as "JIP60". In barley leaf segments treatment with jasmonic acid (JA) or methyl jasmonate (MJ) lead to *de novo* synthesis of proteins, so-called jasmonate-induced proteins (JIPs) with a molecular mass of 66 kDa (JIP60) (Dunaeva *et al.,* 1999). JIP60 is induced by jasmonic acid and activated after proteolytic cleavage to remove internal and COOH-terminal domains (Chaudhry *et al.,* 1994; Nielsen *et al.,* 2001). The function of the extra domains in this type is unknown. The activated protein is similar in enzymatic activity to type 1 RIPs when internal and COOH-terminal domains are removed (Nielsen *et al*., 2001). Comparison of three types RIPs are shown in Figure 2 and Table 3. Molecular properties of various plant RIPs are summarized in Table 4.

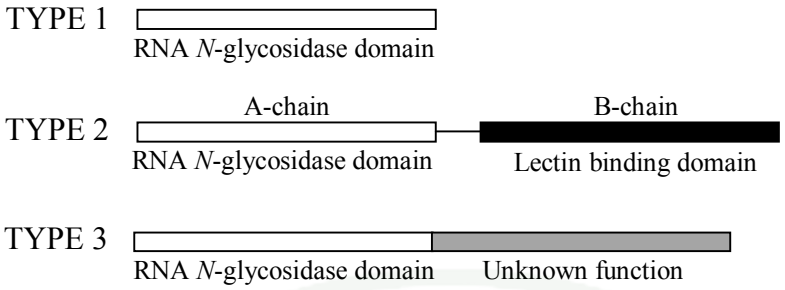

Figure 2 Comparison of the primary structures of the three types of plant RIPs.

Table 3 Classification of plant RIPs and their properties

RIPs	Structure	Molecular mass (kDa)	Inhibition of protein synthesis (IC_{50}, nM)		Toxicity to mice (LD_{50}, mg/kg)
			[a]Cell-free	HeLa cells	
Type 1	one chain	26-32	<0.01-4.0	170->3300	0.95-44
Type 2	two chains	60-65	43-88	0.0003-1.7	0.0017-0.008
Type 3					
-maize RIP (b-32)	two chains	16.5 and 8.5	0.07	-	-
-barley RIP (JIP60)	one chain	66	-	-	-

Note: IC_{50}, concentration causing 50% inhibition of protein synthesis

[a]Cell-free in a rabbit reticulocyte lysate system

Source: Stirpe (2004); Dunaeva *et al.* (1999); Hey *et al.* (1995); Chaudhry *et al.* (1994)

Table 4 Summary of molecular properties of various plant RIPs

RIPs	Tissue	Plant	M.W. (kDa)	pI	Cell-free translation inhibitory activity (IC_{50})	Glycosylation	References
Curcin	Seeds	*Jatropha curcas* L.	28	8.5	0.19 nM	Glycoprotein	Lin *et al*., 2010
Luffin P1	Seeds	*Luffa cylindrica*	5.2	-	0.88 nM	-	Li *et al*., 2003
Luffacylin	Seeds	*Luffa cylindrica*	7.9	-	140 pM	-	Parkash *et al*., 2002
Luffin- S	Seeds	*Luffa cylindrica*	10	-	0.34 nM	Glycoprotein	Gao *et al*., 1994
Luffangulin	Seeds	*Luffa acutangula*	5.6	-	3.5 nM	-	Wang and Ng, 2002
Luffaculin 1	Seeds	*Luffa acutangula*	28	8.86	-	Glycoprotein	Lin *et al*., 2002
Luffaculin 2	Seeds	*Luffa acutangula*	28	9.05	-	Glycoprotein	Lin *et al*., 2002
Trichosanthin	Root tubers	*Trichosanthes kirilowii*	25.7	9.4	31.2 pM	Non-glycoprotein	Li *et al*., 2003; Wang *et al*., 1986
Trichokirin	Seeds	*Trichosanthes kirilowii*	27	>9	0.06-0.113 nM	Glycoprotein	Casellas *et al*., 1988
Trichosanthrip	Seeds	*Trichosanthes kirilowii*	10.9	-	1.6 ng/ml	-	Shu *et al*., 2009
Trichobitacin	Root tubers	*Trichosanthes kirilowii*	27.2	9.6	-	Glycoprotein	Jin *et al*., 1997
α-Kirilowin	Seeds	*Trichosanthes kirilowii*	28.8	-	-	-	Wong *et al*., 1996
β-Kirilowin	Seeds	*Trichosanthes kirilowii*	27.5	-	(LD_{50}) 1.8 ng/ml	-	Dong *et al*., 1994
Karasurin-A	Root tubers	*Trichosanthes kirilowii*	28	10.1	-	-	Toyokawa *et al*., 1991a,b
Karasurin-B	Root tubers	*Trichosanthes kirilowii*	28	10.2	-	-	Toyokawa *et al*., 1991b
Trichomaglin	Root tubers	*Trichosanthes lepiniate*	24.7	5.8	(LD_{50}) 10.1 nM	-	Chen *et al*., 1999
Trichoanguin	Seeds	*Trichosanthes anguina*	35	9.1	0.08 nM	Glycoprotein	Chow *et al*., 1999
Hypsin	Fruiting bodies	*Hypsizigus marmoreus*	20	-	7 nM	-	Lam and Ng, 2001
Marmorin	Fruiting bodies	*Hypsizigus marmoreus*	10	-	-	-	Wong *et al*., 2008
Bryodin	Roots	*Bryonia dioica*	30	≥9.5	(LD_{50}) 0.12 nM	Glycoprotein	Stirpe *et al*., 1986
ME1	Roots	*Mirabilis expansa*	27	>10	-	-	Vivanco *et al*., 1999
ME2	Roots	*Mirabilis expansa*	27.5	>10	-	-	Vivanco *et al*., 1999

Table 4 (Continued)

RIPs	Tissue	Plant	M.W. (kDa)	pI	Cell-free translation inhibitory activity (IC_{50})	Glycosylation	References
Cochinin B	Seeds	*Momordica cochichinensis*	28	>9	0.36 nM	Glycoprotein	Chuethong *et al*., 2007
Velin	Fruiting bodies	*Flammulina velutipes*	19	-	2.5 nM	-	Ng and Wang, 2004
Velutin	Fruiting bodies	*Flammulina velutipes*	13.8	-	0.29 nM	-	Wang and Ng, 2001
Flammin	Fruiting bodies	*Flammulina velutipes*	30	-	1.4 nM	-	Ng and Wang, 2004
Flammulin	Fruiting bodies	*Flammulina velutipes*	40	-	0.25 nM	-	Wang and Ng, 2000
Gypsophilin	Leaves	*Gypsophila elegans*	28	10.1	33 pM	-	Yoshinari *et al*., 1997
Hispin	Seeds	*Benincasa hispada*	21	-	165 pM	-	Ng and Parkash, 2002
Amaranthin	Leaves	*Amaranthus viridis*	30	9.8	25 pM	-	Kwon *et al*., 1997
PAP	Leaves	*Phytolacca americana*	29	-	-	-	Hur *et al*., 1995
PAP-H	Hairy root	*Phytolacca americana*	29.5	7.8	-	-	Park *et al*., 2002
PD-S2	Seeds	*Phytolacca dioica* L.	29.6	-	-	Glycoprotein	Blanco *et al*., 1997
PD-Ls	Leaves	*Phytolacca dioica* L.	28-32	≥8.5	-	Glycoprotein	Di Maro *et al*., 1999, 2009
Dodecandrin	Leaves	*Phytolacca dodecandra*	29	-	-	-	Ready *et al*., 1984
Heterotepalins	Leaves	*Phytolacca heterotepala*	28-36	8.5-9.5	-	Glycoprotein	Di maro *et al*., 2007
Volvarin	Fruiting bodies	*Volvariella volvacea*	29	-	0.5 nM	-	Yao *et al*., 1998
α-Mormocharin (α-MMC)	Seeds	*Momordica charantia*	30	9	0.12 nM	Glycoprotein	Puri *et al*., 2009
β-Mormocharin (β-MMC)	Seeds	*Momordica charantia*	29	9	0.11 nM	Glycoprotein	Puri *et al*., 2009
γ-Momorcharin	Seeds	*Momordica charantia*	11.5	-	55 nM	Non-glycoprotein	Puri *et al*., 2009; Pu *et al*., 1996

Table 4 (Continued)

RIPs	Tissue	Plant	M.W. (kDa)	pI	Cell-free translation inhibitory activity (IC_{50})	Glycosylation	References
Momordin a	Seeds	*Momordica charantia*	29.4	>10	-	Glycoprotein	Minami *et al*., 1992
Momordin b	Seeds	*Momordin charantia*	29.4	>10	-	Glycoprotein	Minami *et al*., 1992
MAP 30	Seeds	*Momordica charantia*	30	-	3.3 nM	Glycoprotein	Puri *et al*., 2009
Momorcochin	Root tubers	*Momordica cochinchinensis*	32	-	-	Glycoprotein	Yeung *et al*., 1987
Moschatin	Seeds	*Cucurbita moschata*	29	9.4	0.26 nM	-	Xia *et al*., 2003
Cucurmosin	Sarcocarp	*Cucurbita moschata*	-	-	-	Glycoprotein	Hou *et al*., 2008
Pepocin	Fruit	*Cucurbita pepo*	26	9.9	15.4 pM	Non-glycoprotein	Yoshinari *et al*., 1996
Dianthin 30	Leaves	*Dianthus caryophyllus*	29.5	8.65	-	Glycoprotein	Falasca *et al*. 1982
Dianthin 32	Leaves	*Dianthus caryophyllus*	31.7	8.55	-	Glycoprotein	Falasca *et al*. 1982
Gelonin	Seeds	*Gelonium multiflorum*	30	8.15	-	Glycoprotein	Falasca *et al*. 1982
Abelesculin	Seeds	*Abelmoschus esculentus*	30	>10.1	-	-	Kondo *et al*. 2007
-	Seeds	*Sponaria* ocymoides	30.2	>9.5	46 pM		Bolognesi *et al*. 1995
-	Seeds	*Vaccaria pyramidata*	28	>9.5	89 pM		Bolognesi *et al*. 1995
-	Seeds	*Saponaria officinalis*	29.5	≥9.5	-	-	Stirpe *et al*. 1983
-	Seeds	*Saponaria officinalis*	29.5	≥9.5	-	-	Stirpe *et al*. 1983
-	Seeds	*Agrostemma githago*	30.6	7.7	-	Glycoprotein	Stirpe *et al*. 1983
-	Seeds	*Agrostemma githago*	29.5	8.7	-	Glycoprotein	Stirpe *et al*. 1983
-	Seeds	*Agrostemma githago*	29.6	8.75	-	Glycoprotein	Stirpe *et al*. 1983
-	Seeds	*Asparagus officinalis*	32.5	≥9.5	-	Glycoprotein	Stirpe *et al*. 1983
-	Seeds	*Asparagus officinalis*	32.5	≥9.5	-	Glycoprotein	Stirpe *et al*. 1983
-	Seeds	*Asparagus officinalis*	32.5	≥9.5	-	Glycoprotein	Stirpe *et al*. 1983
-	Latex	*Hura crepitans*	28	≥9.5	-	Glycoprotein	Stirpe *et al*. 1983
Lamjapin	Kelp	*Laminaria japonica* A	36	8.4	0.69 nM	-	Liu *et al*., 2002

Table 4 (Continued)

RIPs	Tissue	Plant	M.W. (kDa)	pI	Cell-free translation inhibitory activity (IC_{50})	Glycosylation	References
Ricin	Seeds	*Ricinus communis*	64 A-chain 32 kDa B-chain 34 kDa	-	84 nM	Glycoprotein	Kumar *et al*., 2004; Barbieri *et al*., 2004
Abrin	Seeds	*Abrus precatrious*	64	-	-	-	Barbieri *et al*., 2004; Hegde *et al*., 1991
Volkensin	Roots	*Adenia volkensii*	62	8.2, 7.8	(LD_{50}) 84 nM	Glycoprotein	Stirpe *et al*., 1985
Modeccin	Roots	*Adenia digitata*	57 A-chain 25 kDa B-chain 32 kDa	-	45 nM	-	Barbieri *et al*., 2004; Gasperi-Campani *et al*., 1978
Cinnamomin	Seeds	*Cinnamomum camphora*	61 A-chain 31 kDa B-chain 34 kDa	-	30.5 nM	Glycoprotein	Barbieri *et al*., 2004; Xu and Liu, 2004; Hou *et al*., 2001
Bodinierin	Kernels	*Cinnamomum bodinieri*	A-chain 31 kDa B-chain 34 kDa	-	1.2 nM	Glycoprotein	Hou *et al*., 2001
Porrectin	Kernels	*Cinnamomum porrectum*	A-chain 30.5 kDa B-chain 33.5 kDa	-	-	Glycoprotein	Hou *et al*., 2001
Lanceolin	Caudices	*Adenia lancelolata*	61.2	5.7, 5.5, 5.4	-	Glycoprotein	Stirpe *et al*., 2007
Stenodactylin	Caudices	*Adenia stenodactyla*	63.1	5.0, 4.8	44	Glycoprotein	Stirpe *et al*., 2007
Viscumin	Mistletoe	Grown on *Acer platanoides*	60	-	-	-	Olsnes *et al*., 1982
Ebulin I	Leaves	*Sambucus ebulus* L.	A-chain 26 kDa B-chain 30 kDa	5.6	8.5 ng/ml	Glycoprotein	Girbes *et al*., 1993

Table 4 (Continued)

RIPs	Tissue	Plant	M.W. (kDa)	pI	Cell-free translation inhibitory activity (IC_{50})	Glycosylation	References
Nigrin b	Bark	*Sambucus nigra* L.	58 A-chain 26 kDa B-chain 32 kDa	-	0.03 nM	-	Girbes *et al*., 1993
Foetidissima	Roots	*Cucurbita foetidissima*	63	-	(LC_{50}) 25.9 nM	-	Zhang and Halaweish, 2003
Malanin	Seeds	*Malania oleifera*	61.9	5.5	-	Glycoprotein	Yuan *et al*., 2009
b-32	Kernels	*Zea mays*	34 (Inactive form)	6.5	28-60 pM	-	Hey *et al*., 1995
JIP 60	Leaves	*Hordeum vulgare* L. cv. Salome	66 (Inactive form)	-	-	-	Chaudhry *et al*., 1994; Dunaeva et al., 1999

2.2 Entry of plant RIPs into eukaryotic cells

Localization of RIPs is varied among different host cells. Most RIPs have N-terminal signal sequences used to target them for entering into the endomembrane system. RIPs can be transported both forward and in reverse through the endomembrane system. Synthesization of inactive proRIPs with short carboxyl terminal extensions is one of the strategies to protect a newly synthesized RIP back to the ribosome-containing cytosol. Once RIPs had been released from the plant cells, they would require binding of the RIP to cell surface, transport into the cell, entry into the cytosol of eukaryotic cells. The presence of the lectin B-chain of type 2 RIPs helps the binding of them and galactosyl moieties found on the surface of most eukaryotic cells. For example, trafficking of ricin into mammalian cells involves several steps: (i) binding of ricin to cell surface of mammalian cells through the B-chain with lectin activity (ii) uptake of ricin into cytoplasm by endocytosis and entry into early endosomes (iii) vesicular transport of ricin to ER through the trans-Golgi apparatus (iv) reduction of A- and B-chain of ricin (v) partial unfolding of ricin A-chain across the ER membrane by ER-associated degradation (ERAD) pathway (vi) refolding of ricin A-chain into active form with N-glycosidase activity (vii) interaction of ricin A-chain with 28s rRNA of mammalian ribosome followed by cleavage of *N*-glycosidic bond in the RNA and ribosome becomes inactive (Nielsen and Boston, 2001).

But type 1 and type 3 RIPs lack of B-chain leading to they enter into cells with difficulty therefore they are much less toxic than type 2 RIPs. Some type 1 RIPs such as gelonin and dianthin are glycosylated and bind to carbohydrate receptors on the cell membrane similar to ricin. Another type 1 RIPs such as α-sarcin can bind to membrane with receptor-independent mechanisms. It was a single-chain nonglycosylated protein with no hydrophobic zones for crossing of the membrane bilayer. For type 3 RIPs, they were less known that these proteins interact with mammalian cell membrane as the inactive form, the activated form or in both forms (Nielsen and Boston, 2001).

2.3 Mechanism of toxicity of plant RIPs

RIPs are a group of toxic proteins that can irreversibly inactivate ribosomes (He and Liu, 2003) leading to interruption of protein synthesis because they can alter conformation of the RNA and inhibit protein translation by their *N*-glycosidase activity (EC 3.2.2.22). The rRNA *N*-glycosidase can cleave the *N*-glycosidic bond between an adenine residue and ribose in 28s rRNA, releasing free adenine and leaving the rRNA depurinated at a single site, A_{4324} in the case of rat 28s rRNA (Endo and Tsurugi, 1987). This site is lied near the middle of the most highly conserved sequence (5′-AGUACGAGAGGA-3′), known as "Sarcin/Ricin Domain", present in large subunit rRNA that embeds in rat 28s rRNA, approximately 400 nucleotides from the 3′ end, or at A_{2660} in *E. coli* 23s rRNA (Hartley *et al.,* 1991). This domain is crucial for ribosome function. It forms part of a universally conserved stem-loop structure in rRNA that is part of the recognition/binding site for both the eukaryotic/prokaryotic elongation factor 1 (eEF-1/EF-Tu) and the eukaryotic/prokaryotic elongation factor 2 (eEF-2/EF-G) complexes (Moazed *et al.,* 1998). Ricin, a type 2 RIP from seed of *Ricinus communis* and abrin, type 1 RIP isolated from *Abrus precatorius* have the K_{cat} for non plant ribosomes greater than 10^{-3} min^{-1} (Nielsen and Boston, 2001).

RIPs possess a structural domain involved in the recognition of specific ribosomal proteins. In addition, conformational changes of rRNA which have been proposed to occur in the S/R loop during the elongation step may enhance or interrupt RIP interaction with the target sites (Marchant and Hartley, 1995; Park *et al*., 2002).

Trichosanthin (TCS), type 1 RIP from the root tuber of *Trichosanthes kirilowii* Maximowicz (Ke *et al*., 1997), was studied the catalytic mechanism of its RNA *N*-glycosidase. The *N*-glycosidase activity in TCS can be devided into two steps. The first step is substrate recognition. TCS have two subsites in the active pocket with four important active residues, Tyr70, Tyr111, Glu160 and Arg163. The first subsite is to initial substrate recognition and the second one is for catalysis. The second step is to hydrolysis of substrate. It has two different hypotheses for

protonation of adenine substrate. First, N7 of adenine may be protonated by Glu85 of TCS. However, this view was disagreed because Glu85 is not conserved and the distances from N7 to OE1 and OE2 of Glu85 in TCS-NADPH and TCS-ADE are quite far apart. Second, N3 of adenine is most likely the protonation site based on the hydrogen bond between N3 and Arg163. Recently, a stable water molecules was found to form hydrogen bonds with N3 of AMP and guanidinium of Arg163 in the [Glu85Ala or Glu85Gln]TCS-AMP complex and this AMP resides in a new position in the active pocket. It is hypothesized that the substrate first binds to the enzyme in a similar position to AMP in the [Glu85Ala or Glu85Gln]TCS-AMP complex where the stable water molecule acts as a proton donor and Arg163 stabilize the partial negative charge on the water molecule. Then, the adenine is oriented to the second subsite, as observed in NADP-TCS (Shaw *et al*., 2005).

2.4 Relationship between structure of plant RIPs and their rRNA *N*-glycosidase activity

Ricin was the first protein to have X-ray diffraction structure for plant RIPs. The 2.8-Å resolution structure showed a globular, glycosylated heterodimer with the monomer units joined by a single disulfide bond (Hartley *et al*., 1996). The family of plant RIPs shares only about eight invariant amino acids and most of these clusters in active site including Tyr78, Tyr80, Tyr123, Glu177, Arg180, Asn209 and Trp211 (Lord *et al*., 1994; Frankel *et al*., 1990). All RIPs except ricin A chain, residue 108 is a hydrophilic amino acid. This position is Lys in ricin A chain (Huang *et al*., 1995).

Ricin A chain is a 267-residues globular protein with a binding site clef and its B chain is a 262-residues dumbbell with galactose binding sites at both ends (Lord *et al*., 1994). The gene of ricin A chain was modified by site-specific mutagenesis in Arg180 and Glu177 because (i) Arg180 is a conserved amino acid residue, with a side chain protruding into a putative active-site clef, (ii) chemical modification studies with phenylglyoxal have suggested an important role of arginine in the enzyme reaction, and (iii) Arg180 and Glu177 coordinate an active-site water

which may be the ultimate nucleophile in the *N*-glycosidation reaction. Arg180 was changed to alanine, glutamine, methionine, lysine, or histidine. Separately, glutamic acid 177 was changed to alanine. The results showed that a positive charge at position 180 was found necessary for solubility of the protein and for enzyme activity and a negative charge at position 177 was critical for ricin A chain catalysis (Frankel *et al*.1990). After that some plant RIPs are determined the structure. The structure of TCS active site was studied by subjected to limited chymotrypsin digestion and three peptides fragment were generated. The RNA *N*-glycosidase and cytotoxic activities of all fragments were compared with intact TCS. The putative active site was supposed that it located at amino acid residue 110 to 174 based on the molecular model and the published crystal structure of TCS (Ke *et al*., 1997). Amino acid residue at position 120-123 was assumed that they may be important for biological activities of TCS and were improved by site-directed mutagenesis. The region of amino acid residues at position 120-123 lying at the entrance of the clef is one of the four hydrophilic areas of the nTCS molecule and has the highest homology of amino acid sequence in several type 1 RIPs (Wang *et al*., 2002; Nie *et al*., 1997). Lys-Ile-Arg-Glu (hydrophilic) was either deleted or changed to Ser-Ala-Gly-Gly (hydrophobic). Deletions and hydrophobic replacement of these residues caused completely lost and 4000-fold decrease in its ribosome-inactivating activity, respectively. This implied that position 120-123 of the native TCS molecule played a critical role in maintaining its biological activity (Nie *et al*., 1997).

Moreover, some RIPs were identified the protein sequence from their X-ray crystal structures. Luffaculin 1, a new type 1 RIP isolated from the seeds of *Luffa acutangula*, were determined the protein sequence using X-ray sequencing method. The results found that the crystal structure at 1.4-Å resolution showed residues Tyr70, Tyr110, Glu159, and Arg 162 defined the active site of luffaculin 1 as a RNA *N*-glycosidase. The amino acid sequence of luffaculin 1 showed high degree of sequence identities to other type 1 RIPs (Hou *et al*., 2007). Protein sequence of cucurmosin, a novel type 1 RIP from the sarcocarp of *Cucurbita moschata* (pumpkin), was confirmed using high resolution crystal structure analysis at 1.04-Å. Cucurmosin was contained two domains: a large N-terminal domain composed of 7 α-helices and 2 β-

strands. A high resolution structure displayed a glycosylation pattern of $GlcNAc_2Man_3Xyl$. Residue Asn225 was identified as a glycosylation site and other residues Tyr70, Tyr109, Glu158 and Arg161 were defined as the active site of cucurmosin with RNA *N*-glycosidase (Hou *et al*., 2008).

2.5 Specificity of plant RIPs and their substrates and cofactor requirements for rRNA *N*-glycosidase activity

Plant RIPs show a wide array of specificities toward their ribosomal substrates (Hartley *et al*., 1996). Ribosomes from different species have different sensitivities to RIPs. Those from prokaryotes and some from eukaryotes are completely or partially unaffected even though the rRNA sequence around A_{4324} is conserved. These findings would be explained if one or more specific contacts between RIPs and ribosomal proteins were necessary for the formation of a productive Michaelis complex. The sequence and organization of ribosomal proteins are more variable than the sequence of nucleotides at the target site of RIPs (Ippoliti *et al*., 1992). Some RIPs such as PAP are very active against both animal and plant ribosomes but those from cereals have low activity against plant ribosomes (Nielsen and Boston, 2001). Dianthin 29, a type 1 RIP isolated from leaves of *Dianthus barbatus*, was the first RIP shown to inactivate intact prokaryotic ribosomes in the same manner as eukaryotic ribosomes (Prestle *et al*., 1992). The depurination activity of RIPs depends on specific RIP-substrate interaction (Park *et al*., 2002). Three type 1 RIPs, ricin A chain (RTA), saporin-S6 (from *Saponaria officinalis*) and ME (from *Mirabilis expansa* R&P) were examined their substrate specificity to against the fungal ribosomes isolated from *Rhizoctonia solani* Kuhn, *Alternaria solani* Sorauer, *Trichoderma reesei* Simmons and *Candida albicans* Berkhout, and correlated the data with antifungal activity. Results showed that all tested RIPs exhibited depurination activity against fungal ribosomes in a substrate-specific manner. RTA showed overall the strongest activity toward fungal ribosomes (Park *et al*., 2002). Tritin-S and Tritin-L, a type 1 RIP purified from *Triticum aestivum* L. germ and leaves, differ in both their ribosome substrate specificities and cofactor requirement. Tritin-S showed highly ribosome-inactivating activity on wheat germ, tobacco leaf, and *E.coli*

ribosomes but active on rabbit reticulocyte and yeast ribosomes while Tritin-L is active on ribosomes from all of those sources (Andrea and Hartley, 1995).

One difference among RIPs contributing to their translation inhibitory activity is the requirement for cofactors. Bryodin, a type 1 RIP isolated from *Bryonia cretica* spp. Dioica and saponin inhibited protein synthesis by damaging ribosomes in the absence of any cofactor (Stirpe *et al*., 1986; Nielsen and Boston, 2001). Why some RIPs should show cofactor requirement is unknown.

2.6 Biological roles of plant RIPs

2.6.1 Antibacterial activity

Two novel type 1 RIPs named as ME1 and ME2 (from storage roots of *Mirabilis expansa*) showed antibacterial against 8 soil-borne bacterial species using a radial growth-inhibition assay. Five of all were plant pathogenic bacteria, *Pseudomonas syringae*, *Agrobacterium tumefaciens* C58, *A. rhizogenes* ATCC 15834, *Xanthomonas campestris* pv *versicatoria,* and *Erwinia carotovora* ATCC 15713. Others were nonpathogenic species, *Bacillus subtilis, Rhizobium leguminosarum,* and *Serratia marcescens* (Vivanco *et al*., 1999). *S. marcescens* can be human pathogenic under certain condition (von Graevenitz, 1980; Vivanco *et al*., 1999).

2.6.2 Antiviral and antifungal activity

It has been known since the 1920s that crude extracts of pokeweed inhibit plant virus infection when the extract and virus are mixed and rubbed onto the surface of a test plant. Ready *et al*. (1986) was the first team who proposed an explanation for the antiviral activity of RIPs. They found a largely PAP localized in a cell wall matrix of pokeweed and proposed that the local cellular damage caused by a viral infection would release RIP into the cytosol where it could inactivate ribosomes leading to prevention of virus replication. PAP and other RIPs were found to have antiviral activity against both plant and animal viruses, and to have some fungicidal

and insecticidal activities. All type 1 RIPs have antiviral against plant, fungal and animal viruses, whereas only a few type 2 RIPs were found to be active. The effect of RIPs on plant viruses led to transfection of plants with RIPs gene, to improve their resistance to viral infections (Park *et al*., 2004).

Several RIPs have been studied the antiviral and antifungal activity. ME1 and ME2 had antimicrobial activity against several fungi including *Pythium irregulare*, *Fusarium oxysporum solani*, *Alternaria solani*, *Trichoderma reesei*, and *Trichoderma harzianum* (Vivanco *et al*., 1999). Hypsin, a novel type 1 RIP from fruiting bodies of the mushroom *Hypsizigus marmoreus* had an inhibitory action against mycelia growth in various species including *Mycosphaerella arachidicola, Physalospora piricola, Fusarium oxysporum,* and *Botrytis cinerea* with an IC_{50} of 2.7, 2.5, 14.2, and 0.06 mM, respectively. In addition, hypsin also inhibited HIV-1 reverse transcriptase with IC_{50} of 8 μM (Lam and Ng, 2001). Hispin, a novel type 1 RIPs from the seeds of hairy melon *Benincasa hispida var chieh-gua* was found to display anti-fungal activity (Ng and Parkash, 2002). *Araucaria angustifolia* lectin (AaL) had significant antimicrobial activity, mainly against gram-positive bacteria, and anti-inflammatory effect in acute cellular inflammation (Santi-Gadelha *et al.*, 2006). Luffacylin isolated from *Luffa cylindrica* was the first ribosome-inactivating peptide with antifungal activity (Parkash *et al*., 2002). The antifungal activity of plant RIPs was summarized in Table 5.

Table 5 Summary of antifungal activity of plant RIPs

RIPs	Fungal species	IC_{50} (mM)	References
Hypsin	*Mycosphaerella arachidicola*	2.7	Lam and Ng, 2001
	Physalospora piricola	2.5	
	Fusarium oxysporum	14.2	
	Botrytis cinerea	0.06	

The antiviral activity against animal viruses has led to numerous studies on the effect of RIPs, especially PAP and trichosanthin, in HIV-infected cells. The replication of HIV in cells was inhibited by several RIPs and investigations were started with the hope that RIPs could be used in the therapy of AIDs. All RIPs with anti-HIV properties have ribosome-inactivating activity, but most of the RIPs with ribosome-inactivating activity and/or abortifacient activities do not possess anti-HIV activity (Wang and Ng, 2002; Zheng *et al*., 1998). The relationship between the anti-HIV-1 activity of TCS and its ribosome-inactivating activity was studied using site-directed mutagenesis to construct 3 TCS mutants with different ribosome-inactivating activities and tested the anti-HIV-1 activities of three mutants *in vitro*. Results showed that ribosome-inactivating activity of TCS had significant contribution to its anti-HIV-1 property, but the relationship may not be simple and direct, there should be other pathways involved in the effect of TCS and anti-HIV-1 (Wang *et al*., 2002). The Phe140, Tyr141, Tyr142, Asp176 and Lys177 were identified as key amino acid residues for the membrane translocation process (Zhao *et al*., 2010). The penetration behavior of TCS in viral particles may provide implications for design protein-based drug for AIDS therapy. Relationship between ribosome-inactivating activity and anti-HIV activity of the RIPs has been proposed by Stirpe (2004) and Stirpe and Battelli (2006).

Three explanations which may be possible: (i) RIPs act directly on the virus particles or viral nucleic acids, (ii) RIPs selectively gain entrance to the cytosol of infected cells and destroy the protein synthesis machinery so that virus cannot replicate and infect neighboring cells and (iii) RIPs act indirectly through an activation of the plant's defense system.

2.6.2 Antitumor and anticancer activity

The use of plant toxic RIPs as antitumor and anticancer agents have been investigated. Hypsin was determined antimitogenic and antiproliferative activities against mouse leukemia L1210, human hepatoma (HepG2), and human leukemia HL60 cells. Mouse leukemia L1210 cells were the most sensitive followed

by human hepatoma (HepG2) cells. Human leukemia HL60 cells were the least sensitive (Lam and Ng, 2001). Cinnamomin inhibited the growth of cultured carcinoma cells. The LC_{50} of cinnamomin to human hepatocarcinoma cell line 7721 and the melanoma M21 were 18.8 and 11.7 nmol, respectively (He and Liu, 2003). Cochinin B manifested strong antitumor activities on human cervical epithelial carcinoma (HeLa), human embryonic kidney (HEK293) and human small lung cancer (NCI-H187) cell lines with IC_{50} of 16.9, 114 and 574 nM, respectively (Chuethong *et al*., 2007). Riproximin was determined the antiproliferative activity against human breast cancer (MCF-7), cervix carcinoma (HeLa) and rat colon cancer (CC531-lacZ) cells with IC_{50} of 0.5, 1.1 and 0.6 pM, respectively (Voss *et al*., 2006). Marmorin was found to inhibit proliferation of hepatoma (HepG2) and breast cancer (MCF-7) cells with IC_{50} of 0.15 and 5 μM (Wong *et al*., 2008). The antitumor/anticancer of plant RIPs was summarized in Table 6.

Table 6 Summary of antitumor/anticancer activity of plant RIPs

RIPs	Cancer cell lines	IC_{50}	References
Cinnamomin	Human hepatocarcinoma cell line 7721	18.8 nmol	He and Liu, 2003
	Melanoma M21	11.7 nmol	
Cochinin B	Human cervical epithelial carcinoma (HeLa)	16.9 nM	Chuethong *et al*., 2007
	Human embryonic kidney (HEK293)	114 nM	
	Human small lung cancer cell line (NCI-H187)	574 nM	
Riproximin	Human breast cancer (MCF-7)	0.5 pM	Voss *et al*., 2006
	Cervix carcinoma (HeLa)	1.1 pM	
	Rat colon cancer (CC531-lacZ)	0.6 pM	
Mamorin	Human hepatoma (HepG2)	0.15 μM	Wong *et al*., 2008
	Breast cancer cell (MCF-7)	5 μM	

2.6.3 Insecticidal activity

Some RIPs have been found to have insecticidal properties against coleopteran and lepidopteran species (Vivanco *et al*., 1999). Two type 2 RIPs, ricin and saporin had very high toxicity to two Coleopteran species with average LD_{50} values of less than 10^{-2}% dry weight (Gatehouse *et al*., 1990). The protease-activated form of maize seed ribosome-inactivating protein (b-32) had significant toxicity to any caterpillar of *Trichoplusia ni*, *Spodoptera frugiperda*, *Ostrinia frugiperda* when fed in diets at 1 mg/ml of diet with mortality ranged 0-70% (Dowd *et al*., 1998). Cinnamomin, type 2 RIP from seeds of *Cinnamomum camphora* was tested the toxicity to bollworm (*Helicoverpa armigera*) and mosquito (*Culex pipens pallens*) during larval stage. The LC_{50} of cinnamomin to bollworm larvae was 1839 ppm and the LC_{50} to mosquito larvae was 168 ppm (Zhou *et al*., 2000). It also was tested the toxicity to domestic silkworm (*Bombyx mori*) larvae by oral feeding bioassay comparatively to ricin. The results showed that they exhibited a different toxicity to domestic silkworm larvae by oral feeding bioassay. The LC_{50} of ricin (489.4 ppm) to the silkworm larvae at the third instars was much lower than that of cinnamomin (165,996.4 ppm) about 33.9 times. In addition, the LC_{50} of both ricin and cinnamomin increased with the development of the domestic silkworm larvae (Wei *et al*., 2004). SNA-I, type 2 RIPs was investigated the insecticidal potency on two Hemipteran insect species using two different methods. (i) different concentrations of the purified SNA-I were supplemented to artificial diet. The result found that they can reduce survival and fecundity of pea aphids *Acyrthosiphon pisum*. (ii) feeding of tobacco aphids, *Myzus nicotianae*, on leaves from transfected plants constitutively expressing SNA-I resulted in a delayed development and reduced adult survival and also fertility parameters of the surviving aphids were reduced. The carbohydrate-binding activity of SNA-I is necessary for its insecticidal activity (Shahidi-Noghabi *et al*., 2008). Summary of insecticidal activity of plant RIPs are in Table 7.

Table 7 Summary of insecticidal activity of plant RIPs

RIPs	Insect species	IC_{50} (ppm)	References
Cinnamomin	*Helicoverpa armigera*	1,839	Zhou *et al*., 2000
	Culex pipens pallens	168	
	Bombyx mori	165,996.4	Wei *et al*., 2004
Ricin	*Bombyx mori*	489.4	Wei *et al*., 2004

2.6.4 Immunotoxins

Immunotoxins are a hybrid molecule created by coupling an antibody or antigen with a part or all of a toxin. The hybrid molecule combines the specificity of the antibody or antigen with the toxicity of the toxin. RIPs have been used to prepare immunotoxins either by chemical linkage or recombinant fusion proteins mostly with antibodies, but also growth factors, hormones and lectins (Stirpe and Battelli, 2006). Most researches on immunotoxin have focused on cancer therapy, but also for immunosuppression and the therapy of viral diseases. However, there are many difficulties in the use of immunotoxins in cancer therapy because they have several side effects such as formation of antibodies against the antibody and the RIPs with consequent allergic reactions, and even anaphylactic shock, capillary leak syndrome, hepatotoxicity, renal insufficiency, fatigue, and fever. Type 1 RIPs and A-chains of type 2 RIPs have been conjugated to antibodies to form immunotoxins specifically to the target cells of the antibody. Type 2 RIPs are not suitable because their B chains would bind unselectively to virtually any cell (Stirpe, 2004). Some type 1 RIPs were used to prepare the immunotoxin such as trichosanthin, moschatin. Trichosanthin-Hepama-1 was prepared by 2 iminothiolane modification and Hepama-1 conjugation. The hepatoma cytotoxicity of the immunotoxin was 500-fold higher than that of free TCS and was approximately 600-fold less cytotoxicity to HeLa cells (Wang *et al*., 1991). A novel immunotoxin, Moschatin-Ng76, was prepared using the anti-human melanoma McAbNg76 and it efficiently inhibited the growth of targeted melanoma cells M21 with IC_{50} of 0.04 nM, 1500 times lower than that of free Moschatin (Xia *et al*., 2003).

3. Ribosome-inactivating protein from *J. curcas*

Two types of RIP have been found in the seeds and leaves of *J. curcas*. The seeds of *J. curcas* contain toxic protein designated as "curcin" (Felke, 1914). General toxicities of curcin are nausea, severe abdominal pain, diarrhea and respiratory depression. It was classified into type 1 RIP (Barbieri, 1993). It has the molecular weight approximately of 28.2 kDa and has the pI of 8.4. It is a glycoprotein with total neutral sugar contents of 4.91% (Lin *et al*., 2010). Several biological properties of curcin were determined. It was found to have the cytotoxicity on gastric cancer cell line (SGC-7901), liver cancer cell line (human hepatoma) and mouse myeloma cell line (Sp2/0) (Lin *et al*., 2003). It showed acute toxicity in mice leading to abnormal change in their organs such as liver, kidney and lung.

The secondary structure of curcin was determined by Circular Dichroism (CD) spectrum. It contains 22.3% of α-helix, 43.5% of β-sheet and 34.2% of random coil (Lin et *al*., 2010). The three-dimension structure (3D) of curcin was depicted in Figure 3.

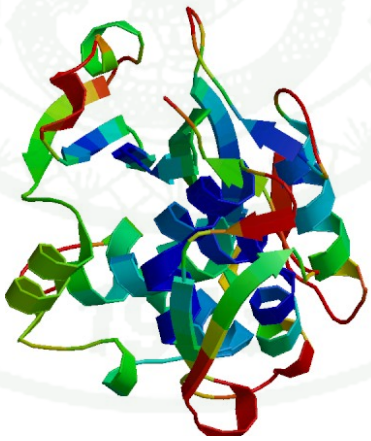

Figure 3 3D structure of curcin**.** It was performed with the SWISS MODEL program using submitted to Genbank sequence of curcin (accession number ABZ04128.1). Cucurmosin chain A (accession number 3bwhA) was used as template.

The full-length curcin cDNA was cloned using RT-PCR and 5′-RACE. The degenerate primers were designed based on the N-terminal partial sequence from purified curcin. The deduced amino acids sequence indicates that a preprotein with 293 amino acid residues was first translated and was processed to a mature protein with 251 amino acids. The comparison of the amino acid sequences of curcin with ricin A chain and trichosanthin were revealed (Figure 4). The percentages of identity between curcin and ricin A chain, and between curcin and trichosanthin were found to be 54% and 57% respectively. Curcin possesses conserved residues of similar amino acid around the proposed active site of ricin A chain and trichosanthin (Lin *et al*., 2003).

```
Ricin A chain      ----------------------GPGPKQYPIINFTTAG-----------------ATVQ
Trichosanthin      --------MIRFLVLSLLILTLFLTTPAVEGDVSFRLSG-----------------ATSS
curcin             MKGGKMNLSIMVAAWFCWSSIIFGWASAREIVCPFSSNQNYKAGSTPTLAITYDATTDKK
                                         .      *                      :  .

Ricin A chain      SYTNFIRAVRGRLTTGADVRHEIPVLPNRVGLPINQRFILVELSNHAELSVTLALDVTNA
Trichosanthin      SYGVFISNLRKALPN-ERKLYDIPLL--RSSLPGSQRYALVHLTNYADETISVAIDVTSV
Curcin             NYAQFIEDLREAFDF-SYLSHKIPVL--RATVAANQKFIVAKVINSGDIEVSVGLNVINA
                   .*  **  :*  :       :.**:*  *  :. .*:: :..: * .:  :::.::* ..

Ricin A chain      YVVGYRAGNSAYFFHPDNQEDAEAITHLFTDVQNRYTFAFGGNYDRLEQLAGNLRENIEL
Trichosanthin      YIMGYRAGDTSYFFN--EASATEAAKYVFKDAMRKVTLPYSGNYERLQTAAGKIRENIPL
Curcin             YLVAYKVGSNSYFFN-DSESLADAKKNLFTDTN-QQTLAFTGSYADFESRAKLHREEVDL
                   *::.*:.*..:***:  . . ::* . :*.*.  : *:.: *.*  ::  *   **:: *

Ricin A chain      GNGPLEEAISALYYYSTGGTQLPTLARSFIICIQMISEAARFQYIEGEM--RVP------
Trichosanthin      GLPALDSAITTLFYYNAN-----SAASALMVLIQSTSEAARYKFIEQQIGKRVDKTFLPS
Curcin             GVVALDNYVYTLEKSSQP----ADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPR
                   *  .*:. : :*   .        * .::  *:  .****:::** ::  ::

Ricin A chain      ------------------------------------------------------------
Trichosanthin      LAIISLENSWSALSKQIQIASTNNGQFETPVVLINAQNQRVTITNVDAGVVTSNIALLLN
Curcin             GDIISLENNWGDLSYQIQKS--VDDVFLKPVQLQRENYTNILVN--NVTQVKGLMGVLLN


Ricin A chain      ------------------------
Trichosanthin      RNDMAAMDDDVPMTQSFGCGSYAI
Curcin             AVNYKVSMEEIIFNDQKWLPWL—
```

Figure 4 Comparison of amino acid sequence of curcin and other RIPs. Sequence alignment was performed with the CLUSTALW2 program using submitted to GenBank sequence of curcin (accession number ABZ04128.1); ricin A chain (accession number ACZ56254.1) and trichosanthin (accession number AAB31048.1). Conserved sequences are shown in gray background.

The leaves of *J. curcas* contain a curcin-related RIP designated as "curcin 2". It is induced in leaves under stress conditions. It has the molecular weight approximately of 32 kDa from Western blot method under temperature stresses at 4°C and 50°C and fungal infections by *Pestalotia funereal*, *Curvularia lunata* (Walk) Boed, *Gibberelle zeae* (Schw.) Petch (Qin *et al*., 2005).

The open reading frame (ORF) encoding curcin 2 was cloned from total genomic and cDNA of leaves treated by drought, temperature stress and fungal infection by RT-PCR. The ORF has 927 bp that encodes a precursor protein of 309 amino acid residues. There are high similarities with curcin and the conserved domain of other RIPs (Qin *et al*., 2005).

4. Cloning and expression of plant RIPs gene

Plant RIPs have been cloned from many species through various strategies:

(1) cDNA expression library screened with antibodies (Legname *et al*., 1991).

(2) cDNA library screened with oligonucleotides constructed on known protein sequences (Chow *et al*., 1990).

(3) genomic DNA sequences obtained by direct PCR amplification or by screening with cDNA probes (Kataoka *et al*., 1992).

From genome analysis, protein sequence and the clones obtained from PCR of genomic DNA, it can be assumed that all RIPs are encoded by small multigene families (Benatti *et al*., 1989; Leah *et al*., 1991; Lin *et al*., 1991; Cho *et al*., 1999).

Most of all plant RIPs have been studied the molecular property. A genomic clone encoding α-TCS was isolated from leaves of *Trichosanthes kirilowii* Maxim. Results showed that α-TCS was synthesized as a preproproptein consisting of 289 amino acids, the first 23 residues of which comprise a putative secretory signal peptide. The last 19 residues comprise a carboxyl extension that had not been reported to be associated with the mature protein and that may be processed in the ER or Golgi

apparatus of cells producing α-TCS. The mature protein consisted of 247 amino acids residues (Chow *et al*., 1990). The sequencing of trichoanguin cDNA was reported. Total RNA was extracted from the mature seeds of *Trichosanthes anguina* and mRNA was reverse-transcribed to cDNA by RT-PCR method. The 3′- and 5′-end were amplified by Rapid Amplification cDNA Ends (RACE) method. Results showed the cDNA of trichoanguin consisted of 1039 nucleotides and an open reading frame (ORF) coding for a polypeptide of 294 amino acid residues (Chow *et al*., 1999). The expression of *curcin* 2 gene was induced under drought, temperature stress and fungal infection conditions. The ORF encoding *curcin* 2 was cloned from total genomic and cDNA of *J. curcas* leaves by PCR and RT-PCR. The ORF had 927 bp that encoded a precursor protein of 309 amino acid residues. There are high similarities with curcin and the conserved domain of RIPs (Qin *et al*., 2005). The RIP from Himalayan population of *Viscum album* (HmRIP) was cloned, characterized, and also correlated the properties with its amino acid sequence. Total RNA was isolated from young leaves and isolated poly A^+ RNA by affinity chromatography using oligo (dT) cellulose column. Denatured poly A+ RNA was used as template for single strand cDNA synthesis by RT-PCR. The nucleotide sequencing was done by chain termination method. ORF were translated and complete sequence was obtained by aligning the overlapping regions. cDNA cloning showed that HmRIP was 500 amino acids long and shortest among mistletoe RIPs (Mishra *et al*., 2005). The sequence encoding the mature curcin was obtained by RT-PCR. The fragment encoding the mature protein of curcin was inserted into *E. coli* strain M15 and recombinant protein was induced to express by the optimum inducer (0.5 mM IPTG). The recombinant protein was expressed in the form of inclusion body and was purified by Ni-NTA affinity chromatography. The target protein also can inhibit the growth of various tumor cells (Luo *et al*., 2007).

MATERIALS AND METHODS

Materials

1. Biological samples

1.1 Plant materials

Mature seeds of *J. curcas* KUBP 33 were from Suwanwajokkasikit Field Crops Research Station, Inseechandrastitya Institute for Crop Research and Development (IICRD), Kasetsart University, Thailand.

1.2 Human pathogenic bacterial strains

Human pathogenic microorganisms, 30 bacterial strains and 1 yeast strain were provided by Associate Professor Dr. Nuanchawee Wetprasit from Department of Biotechnology, Faculty of Science, Ramkhamhaeng University.

1. *Acinetobacter anitratus* DMST 4183
2. *Acinetobacter baumannii* ATCC 19066 (DMST 10437)
3. *Acinetobacter calcoaceticus* ATCC 23055 (DMST 10436)
4. *Acinetobacter lwoffii* ATCC 15309 (DMST 4229)
5. *Bacillus cereus* ATCC 11778 (DMST 5040)
6. *Burkholderia cepacia* ATCC 25416 (DMST 4205)
7. *Enterococcus faecalis* ATCC 29212 (DMST 4736)
8. *Escherichia coli* ATCC 25922 (DMST 4212)
9. *Pseudomonas aeruginasa* ATCC 27853 (DMST 4739)
10. *Pseudomonas fluorescens* DMST 6034
11. *Salmonella enteritidis* ATCC 17368
12. *Salmonella typhi* DMST 5784
13. *Shigella dysenteriae* DMST 15111
14. *Staphylococcus aureus* ATCC 25923 (DMST 8840)

15. *Staphylococcus aureus* DMST 20654 (MRSA)
16. *Staphylococcus epidermidis* ATCC 12228 (DMST 15505)
17. *Streptococcus agalactiae* DMST 17129
18. *Streptococcus pyogenes* DMST 17020
19. *Vibrio cholerae* nonO1, nonO139 DMST 2873
20. *Vibrio cholerae* O139 ATCC 51394
21. *Klebsiella pneumoniae* ATCC 27736
22. *Klebsiella oxytoca* DMST 16071
23. *Bacillus subtilis* ATCC 6633
24. *Escherichia coli* O157.H7 DMST 12743
25. *Listeria monocytogenes* DMST 17303
26. *Proteus mirabilis* DMST 8212
27. *Serratia marcescens* ATCC 8100
28. *Shigella flexneri* DMST 4423
29. *Shigella sonnei* (group D) DMST 2982
30. *Shigella boydii* DMST 7776
31. *Candida albican* ATCC 10231

1.3 Bacterial strain for molecular cloning

Escherichia coli strain DH5α was provided by Dr. Chotika Yokthongwattana

1.4 Rabbit erythrocytes

Rabbit erythrocytes were purchased from National Laboratory Animal Center, Mahidol University.

1.5 Cell cultures

A normal cell line, African green monkey kidney (Vero) cell were provided by Assistant Professor Dr. Teerakul Arpornsuwan from Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Thailand.

Two human carcinoma cell lines, breast cancer cell line (MCF-7, ATCC HTB-22) and human colon adenocarcinoma (SW620, ATCC CCL-228) were purchased from Sigma.

Breast adenocarcinoma cell line (HepG2) was given from Dr. Nattanan Panjaworayan.

1.6 *Aedes aegypti* Linn. and *Culex quinquefasciatus* Say larvae

The late third instars *Aedes aegypti* and *Culex quinquefasciatus* larvae were procured from Department of Medical Sciences, Ministry of Public Health.

1.7 *Spodoptera litura* and *Spodoptera exiguam* larvae

The second instars *Spodoptera litura* and *Spodoptera exigua* larvae were procured from Plant protection research and development office, Department of Agriculture, Ministry of Agriculture and Cooperatives.

2. Equipments

2.1 General equipments

Autopipette: Pipetteman (Gilson, France)
Balance (Satorious)
Centrifuge, refrigerated centrifuge: Model HARRIER 18/80
Centrifuge, refrigerated centrifuge: Model Sorvall® Biofuge Stratos
Centrifuge, refrigerated centrifuge: Model Sorvall® Legend Micro 17R
Centrifuge, microcentrifuge: Model Spectrafuge 16M (Labnet)
Carbondioxide (CO_2) Incubator: Model Lishen (Heal Force)
Hot plate (Fisher Scientific)
Incubator water bath (Grant Instruments)
Magnetic stirrer (Fisher Scientific)
NanoDrop® spectrophotometer: Model ND-1000 (Nanodrop, USA)
Microplate Reader: Model Sunrise (TECAN, AUSTRIA)
Microwave
pH meter: Model 215 (Denver Instrument)
Ultra low temperature freezer: Model Forma 900 series (Thermo Scientific)
UV Visible spectrophotometer (Beckman, USA)
Vortex mixer: Vortex-Genie 2™ (Scientific Industries)

2.2 Equipments for proteomics

MALDI-TOF mass spectrometer: Reflex IV (BRUKER, Germany)
Spectropolarimeter: Jasco J-175 (UK)

2.3 Equipments for molecular cloning

Agarose Gel Electrophoresis System (submerge): GelMate 200 (Toyobo)
Gel documentation: Model DNR Bio-Imaging system
High-pressure steam sterilizer: Model ES315 (TOMY)
Hot air oven: Model 838F (Fisher Scientific)
Incubator: Model IPR 150.XX2.C
Incubator shaker: Model IOC400.XX2.C (GALLENKAMP PLC)
Laminar flow (Biohazard Class II) (Microtech)
Thermal cycle: Model MultiGene Gradient Thermal Cycle (Labnet)

2.4 Miscellaneous

0.2 μm Millipore membrane filter (Schleicher & Schuell, Germany)
Liquid nitrogen (TIG)
Minerol oil (Sigma, USA)
Whatman 3MM paper

3. Chemicals and reagents

3.1 Chemicals and reagents for protein purification and characterization

Anion exchanger based on beaded cellulose (DEAE-Sephacel™) (GE Healthcare, Sweden)
Cation exchanger based on beaded cellulose (CM-Cellulose) (Sigma, USA)
Absolute ethanol (Merck, Germany)
Acetic acid glacial (BDH, UK)
Ammonium acetate (Merck, Germany)
β-mercaptoethanol (Sigma, USA)
Bromophenol blue (Merck, Germany)
Calcium chloride (Sigma, USA)

Chloroform (Merck, Germany)
Folin–Ciocalteu reagent (Fluka, USA)
Formaldehyde (BDH, Germany)
Glucose (Sigma, USA)
Glycerol (BDH, UK)
Hydrochloric acid (Merck, Germany)
Isoamyl alcohol (Merck, Germany)
Isopropanol (Merck, Germany)
2-mercaptoethanol (Sigma, USA)
N, N'-Methylene-bis-acrylamide (Pharmacia, USA)
Polyvinylpyrrolidone (Sigma, USA)
Potassium chloride (Sigma, USA)
Sodium acetate (Merck, Germany)
Sodium chloride (Merck, Germany)
Sodium dodecyl sulfate (SDS) (Biobasic, Canada)
Sodium hydroxide (BDH, UK)
Sulfuric acid (J.T. Baker, USA)
Xylene cyanol FF (Sigma, USA)

3.2 Chemicals and reagents for rRNA *N*-glycosidase activity assay

Absolute ethanol (Merck, Germany)
Acetic acid, Glacial (Mallinckrodt, USA)
Aniline (Univar, USA)
Chloroform (Merck, Germany)
DEPC (Biobasic, Canada)
Ethidium bromide (EtBr) (Sigma, USA)
Formamide (BDH, UK)
Magnesium chloride (Univar, USA)
Phenol (Sigma, USA)
Potassium chloride (Univar, USA)
Rabbit reticulocyte lysate (untreated) (Promega, USA)

RNA marker (Fermentus, Canada)

Sodium acetate (Biobasic, Canada)

Sodium dodecyl sulphate (Biobasic, Canada)

Tris (hydroxymethyl) aminomethane (Research Organics, USA)

Urea (Research Organics, USA)

Urea (for molecular biology) (Fluka, Germany)

3.3 Chemicals and reagents for antimicrobial activity assay

Amphotericin B (Sigma, USA)

Ampicilin (Biobasic, Canada)

Chloramphenicol (Sigma, USA)

Luria-Bertani (LB) Broth (Difco, USA)

Muller-Hinton Agar (Difco, USA)

Muller-Hinton Broth (Difco, USA)

Sodium chloride (Univar, USA)

3.4 Chemicals and reagents for anticancer activity assay

Dimethyl sulfoxide (Riedel de Haën, Germany)

Ellipticine (Sigma, USA)

Fetal bovine serum (Thermo scientific, USA)

Potassium cholide (Univar, USA)

Potassium dihydrogen phosphate (Biobasic, Canada)

MEM/EBSS+2.0 mM L-glutamine and Earle's balanced salts (Thermo scientific, USA)

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, USA)

Sodium chloride (Ajax Finechem, Australia)

Sodium phosphate (Biobasic, Canada)

3.5 Chemicals and reagents for molecular cloning

Agarose, low EEO (Research Organics, USA)

Ampicillin (Sigma, USA)

Cetyltrimethyl ammonium bromide (CTAB) (Biobasic, Canada)

Chloroform (Merck, Germany)

Diethyl pyrocarbohydrate (DEPC)

DNA markers : 100 bp ladder (Gibco,USA)

Ethanol, absolute (Merck, Germany)

Ethidium bromide (EtBr) (Sigma, USA)

Ethylene diamine tetraacetic acid, disodium salt dihydrate (EDTA) (Biobasic, Canada)

Formaldehyde (BDH, UK)

Formamide (BDH, UK)

Isopropyl-β-D-thiogalactoside (IPTG) (Sigma, USA)

Phenol (Sigma, USA)

Polyvinylpyrrolidone (PVP-40) (Sigma, USA)

Tris-(hydroxy methyl)-aminomethane (Sigma, USA)

X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) (Sigma, USA)

3.6 Kits for molecular cloning

Dream Taq™ Green PCR Master Mix (2X) (Fermentas, Germany)

GeneJET™ Plasmid Miniprep Kit (Fermentas, Germany)

HiYield™ Gel/PCR DNA fragment Extraction Kit (RBC BIOSCIENCE, Taiwan)

pGEM-T vector Systems (Promega, USA)

RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany)

Methods

1. Purification and characterization of the seed coat protein

1.1 Extraction of crude protein extract

1.1.1 Extraction of crude protein extract from seed coat of *J. curcas*

Dried ripe seed coat of *J. curcas* (Figure 5) were ground and extracted by continuous stirring for 48 h at 4°C with a 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl (1:3 w/v). The crude protein extract was dialyzed against a 50 mM Tris-HCl buffer (pH 7.5) overnight and centrifuged at 8,000 ×g for 30 min. The supernatant was stored at 4°C.

1.1.2 Extraction of crude protein extract from seed coat of *J. curcas* for larvicidal and insecticidal activity assay

Mature seed coat of *J. curcas* (100 g) were ground and extracted by maceration in 300 ml of distilled water for 24 h at 4°C. Extract was filtered and aqueous extract was kept at 4°C. The aqueous extract was defined as “JSCCP”

1.1.3 Extraction of crude protein extract from seed kernel of *J. curcas* and *Ricinus communis* for larvicidal activity assay

The seed kernels (100 g) of each plant were ground and extracted by maceration in 300 ml of distilled water for 24 h at 4°C. Extract was filtered and aqueous extract was kept at 4°C. The aqueous extract from seeds of *J. curcas* and *R. communis* were defined as “JSKCP” and “RSKCP”, respectively.

Figure 5 Dried ripe seed coat of *J. curcas* KUBP33.

1.2 Purification of the seed coat protein

1.2.1 Ammonium sulfate precipitation

Salt precipitation is a method used to purify protein by altering its solubility. The solubility of protein depends on the ionic strength of the solution and the salt concentration. At high ionic strength, the protein will be completely precipitated from the solution. Ammonium sulfate is a salt that is widely used because it has high solubility and is inexpensive. The ammonium sulfate concentration is increased stepwise and the precipitated protein is removed by centrifugation and dissolved in buffer or distill water for the next stage of purification procedures. This technique is used in this experiment to precipitate out the large amount of the total protein by ammonium sulfate at 80% saturation.

1.2.2 Ion-exchange chromatography

Ion-exchange chromatography is the most popular method for the purification of protein and other charged molecules. It separated molecules based on differences between net charges of the proteins. Cation-exchange chromatography, positively charged molecules are attracted to a negatively charged solid support and conversely anion-exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. The two most commonly used resins for ion-exchange chromatography of proteins are carboxymethyl cellulose (CM-Cellulose) and diethylaminoethyl cellulose (DEAE-Cellulose). These are granular

celluloses which have been chemically modified. CM-Cellulose is a resin which the $–CH_2OH$ groups of carbohydrate have been converted to $–CH_2OCH_2COOH$ groups. At neutral pH, these groups are ionized as $–CH_2OCH_2COO^-$ so that CM-Cellulose is a cation exchanger. DEAE-Cellulose contains an ionizable tertiary amine group instead. At neutral pH, it has a positively charged of $–OC_2H_5N(H^+)C_4H_{10}$ so that DEAE-Cellulose is an anion exchanger. Now, DEAE-Cellulose is also available in a bead form called DEAE-Sephacel™. It is the only spherical cellulose ion exchanger.

Ion-exchange involves 2 processes as binding of proteins to matrix and elution. Binding of all charged molecules can be optimized by using a low to medium conductivity mobile phase. The pH of the mobile phase buffer must be between the pI or pKa of the charged molecule and the pKa of the charged group of the solid support. The adsorption of the molecules to the solid support is driven by the ionic interaction between the oppositely charged ionic groups in the sample molecule and in the functional ligand on the support. The molecules with the weakest ionic interactions start to elute from the column first by increasing the salt concentration. Molecules that have stronger ionic interaction require a higher salt concentration and elute later.

After ammonium sulfate precipitation at 80% saturation, the pellet was dissolved and dialyzed against a 50 mM Tris-HCl buffer (pH 7.5). The crude protein was applied to a DEAE-Sephacel™ (1.5 × 20 cm) column which was equilibrated with the dialysis buffer. The unbound proteins were washed out with the same buffer at a flow rate of 40 ml/h. The adsorbed proteins were eluted with a step-wise of 0-2 M NaCl in a 50 mM Tris-HCl buffer. The fractions of 4 ml were collected and monitored at 280 nm. The unbound fractions were pooled and dialyzed against a 50 mM sodium acetate buffer (pH 5.0) before chromatography on a CM-Cellulose (fast flow, 1.5 × 20 cm) column at a flow rate 30 ml/h which was equilibrated and washed out the unbound proteins with the same buffer. A gradient of NaCl from 0-0.5 M in the same buffer were used to elute bound fractions. Fractions containing rRNA *N*-glycosidase activity were pooled, dialyzed against a 100 mM Tris-HCl (pH 7.5) and kept at -20°C to further analysis.

1.3 Hemagglutination and hemagglutination inhibition assay

1.3.1 Hemagglutination activity assay

To test the presence of lectin in Jc-SCRIP, hemagglutination activity assay was determined. When agglutination occurs, cross-linked red blood cells form a network that prevents the red blood cells from sedimenting to the bottom of the well whereas if there is no agglutination, the red blood cells are sedimented and formed a button on the bottom of the well.

Hemagglutination activity against rabbit erythrocytes of the seed coat protein was determined according to Ratanapo *et al.* (1998). Hemagglutination reaction was performed in a microwell plate using 2% (v/v) rabbit erythrocyte suspension in a 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. The activity was expressed in unit (U) of hemagglutination activity which was reciprocal of the highest dilution of lectin giving complete agglutination.

1.3.2 Hemagglutination inhibition activity assay

Sugar specificity of the purified protein was performed by inhibition of hemagglutination activity with various carbohydrates and glycoproteins.

The inhibition of agglutination was studied following the method of Ratanapo *et al*. (1998). Various carbohydrates and glycoproteins (see Appendix Table B1) were incubated with crude protein extract of *J. curcas* for 30 min at room temperature. The 2% (v/v) rabbit erythrocyte suspension was added and incubated for 45 min at room temperature. The hemagglutination activity was carried out as described earlier.

1.4 Assay for RNA *N*-glycosidase activity

The assay was conducted according to Lin *et al*. (2003) and Park *et al*. (2002). The test samples were mixed with 50 µl rabbit reticulocyte lysate (untreated) in 50 µl of reaction buffer (20 mM Tris-HCl, pH 7.6, 50 mM KCl and 5 mM $MgCl_2$) and incubated at 37°C for 10 min. Then, the reaction was stopped by adding 12.5 µl of 10% SDS. The reaction mixture was extracted with phenol/chloroform (see Appendix A) and the rRNA was recovered by ethanol precipitation (see Appendix A), and then treated with 1 M aniline/0.8 M acetic acid pH 4.5 in the darkness for 10 min at 60°C. The reaction products were analyzed by using 7 M urea/6% PAGE (see Appendix A). The gel was stained with ethidium bromide. The RNA bands were visualized on a UV transilluminator.

1.5 Molecular properties of the purified protein

1.5.1 Protein determination

Protein concentration was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Lowry method is used to determine the concentration of total protein in solution with concentration in range 0.01-1.0 mg/ml of protein. The method is based on both the Biuret reaction and the Folin-Ciocalteau reaction. The peptide bonds of proteins react with copper under alkaline conditions to produce Cu^+ which reacts with the Folin reagent. The Folin-Ciocalteau reaction is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions result in a strong blue color which depends partly on the tyrosine and tryptophan content.

The tested samples (100 µl) were mixed with alkaline copper sulphate reagent solution (3.0 ml) (see Appendix A) and incubated for 10 min at room temperature. Folin-Ciocalteau reagent solution (0.3 ml) was added and incubated for 30 min in the dark at room temperature. The absorbance was measured at 650 nm. Protein concentrations were calculated using standard curve of BSA (see Appendix Figure B1).

1.5.2 Determination of protein molecular weight

Sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE is useful for molecular weight analysis of proteins. SDS is a detergent that dissociates and unfolds oligomeric proteins into its subunits. The SDS binds to the polypeptides to form complexes with fairly constant charge to mass ratios. The electrophoretic migration rate through a gel is determined only by the size of the complexes. Molecular weights are determined by simultaneously running marker proteins of known molecular weights.

The crude protein extract and purified protein were dialyzed against distilled water overnight to eliminate salts and impurities. To concentrate the proteins, the samples were speed-vacuum dried and resuspended in appropriate volume of distilled water. The molecular mass of protein was analyzed by SDS-PAGE using 15% polyacrylamide as separation gel and 5% polyacrylamide as stacking gel (see Appendix A) and run at 30 mA for 35 min. Phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used as molecular markers. The estimated molecular weight of purified protein was calculated using standard curve of protein molecular weight markers (see Appendix Figure B2).

1.5.3 Mass spectrometry of native proteins

The native molecular mass of the purified protein was determined using a MALDI-TOF mass spectrometry. Matrix Assisted Laser Desorption/ Ionization Time-of-Flight (MALDI-TOF) is a mass analysis technique that was pioneered by Karas *et al*. (1985). Basically, the analyte is mixed with matrix solution to assist ionization and then dried on a target plate. A laser beam is fired at the sample crystals simultaneously with the application of a high voltage pulse. The time it takes for sample ions to drift through the flight tube to the detector is proportional to their molecular weight.

The purified protein from seed coat of *J. curcas* was dissolved in 0.5% TFA and 0.5 µl of this solution were mixed with 0.5 µl of matrix solution (10 mg/ml of sinapinic acid and 2,5-dihydroxybenzoic acid in acetonitrile /0.1%TFA with ratio 50:50 v/v). Finally, 1 µl of the mixture was deposited on multi-well stainless steel MALDI plate and air dried. Measurements were performed on a Bruker Reflex IV MALDI-TOF (Bruker, Germany) in the linear mode, at 20 kV of acceleration potential.

1.5.4 N-terminal amino acid sequence of the purified protein

Determining which amino acid forms the N-terminus of the peptide chain is useful for two reasons (1) to aid the ordering of individual peptide fragment sequences into a whole chain and (2) because the first round of Edman degradation is often contaminated by impurities and therefore does not give an accurate determination of the N-terminal amino acid. The Edman degradation is a very important reaction for protein sequencing because it allows the ordered amino acid composition of a protein to be discovered. The amino-terminal residue is labeled and cleaved from the peptide without disrupting the peptide bonds between other amino acid residues. Automated Edman sequencers are now in widespread use and are able to sequence peptides up to approximately 50 amino acids long. A reaction scheme for sequencing a protein by the Edman degradation follows: Phenylisothiocyanate is

reacted with an uncharged terminal amino group, under mildly alkaline conditions, to form a cyclical phenylthiocarbamoyl derivative. Then, under acidic conditions, this derivative of the terminal amino acid is cleaved as a thiazolinone derivative. The thiazolinone amino acid is then selectively extracted into an organic solvent and treated with acid to form the more stable phenylthiohydantoin (PTH)-amino acid derivative that can be identified by using chromatography or electrophoresis. This procedure can then be repeated again to identify the next amino acid. A major drawback to this technique is that the peptides being sequenced in this manner cannot have more than 50 to 60 residues (and in practice, less than 30). The peptide length is limited due to the cyclical derivitization not always going to completion. The derivitization problem can be resolved by cleaving large peptides into smaller peptides before proceeding with the reaction. It is able to accurately sequence up to 30 amino acids with modern machines capable of over 99% efficiency per amino acid. An advantage of the Edman degradation is that it only uses 10 - 100 pico-moles of peptide for the sequencing process.

N-terminal amino acid sequence analysis was carried out by the Bio-Synthesis Inc., Texas, U.S.A. Protein microsequencing was done by automated Edman degradation. The purified protein was loaded in to the cartridge of ABI492 Procise HT protein sequencer. It was rinsed with ethyl acetate and dried using a vacuum evaporator and sequenced.

1.5.5 Secondary structure of the purified protein

The secondary structure of protein is determined by Circular Dichroism (CD) technique. The electronic transitions of backbone peptide bonds in different conformations produce differential absorption spectra for left- and right-handed circularly polarized light in the far-UV range. The secondary structure of proteins does not conform to a single geometry. Deviations from ideal conformational angles lead to distortions such as bends and twists. Generally, secondary structure of proteins is classified into 4 groups: α-helix, β-sheet, turn and unordered. The α-helix structure can be divided into regular and distorted α-helix. The number of residues

per α-helix that are considered under distorted structure is varied from 2 to 6. The β-sheet structure is also separated into regular and distorted β-sheet. The number of residues per β-sheet that are considered under distorted structure is varied from 1 to 4. The observed protein CD spectrum is an average of CD signals from all conformations.

The purified protein (0.5 mg/ml) was prepared in a 50 mM phosphate buffer (pH 7.5) and was subjected into Jasco J-175 spectropolarimeter (UK). The CD spectra were scanned in the wavelength of 190-260 nm with 1 cm cell. The molar elipicity (θ) was calculated follow:

$$\theta\ (\text{deg.cm}^2/\text{dmol}) = \frac{S}{10 \times C \times n_{pept} \times L}$$

Where S is the raw protein signals (mDeg), C is protein concentration (M), n_{pept} is the number of peptide bond in protein or the number of amino acid residues, and L is the light pathlength (cm). The secondary structure content of the purified protein was calculated using three calculation algorithms, CDSSTR, CONTINLL, and SELCON3.

1.5.6 Carbohydrate content of the purified protein

1.5.6.1 Neutral sugar content of the purified protein

Neutral sugar content was determined by phenol-sulfuric acid method. It is an example of a colorimetric method that is widely used to determine the total concentration of carbohydrates present in foods. This method is simple, rapid, sensitive, and gives reproducible results. The reagent is inexpensive, stable and a given solution requires only one standard curve for each sugar. A clear aqueous solution of the carbohydrates to be analyzed is placed in a test-tube, then phenol and sulfuric acid are added. The solution turns a yellow-orange color as a result of the interaction between the carbohydrates and the phenol. The sulfuric acid causes all non-reducing sugars to be converted to reducing sugars, so that this method

determines the total sugars present. This method is non-stoichemetric and so it is necessary to prepare a calibration curve using a series of standards of known carbohydrate concentration.

The assay was conducted according to Dubois *et al*. (1956). The purified protein was mixed with 0.3 ml of phenol reagent. And then 2 ml of concentrated sulfuric acid was added and mixed immediately. The reactions were incubated for 30 min at room temperature. The absorbance was read at 484 nm. The content of neutral sugar was calibrated by comparing with standard curve of glucose (see Appendix Figure B3).

1.5.6.2 Determination of N-linked glycoprotein on SDS-PAGE

N-linked glycoprotein was determined using Endo Hf digestion. Endo Hf is a recombinant protein fusion of Endoglycosidase H and maltose binding protein. It cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins.

The purified protein (20 μg) was mixed with 2× glycoprotein denaturizing buffer and was boiled for 10 min. The 10× reaction buffer was added. The Endo Hf was added to the reaction mixture and was incubated at 37°C for 2 h. The reaction products were separated by SDS-PAGE.

1.5.7 Isoelectric point (pI) of the purified protein

The isoelectric point (pI) is the pH value at the molecule that has no electrical charge or the negative and positive charges are equal. The net charge on the molecule is affected by pH of surrounding environment and can become more positively or negatively charged due to the loss or gain of proton (H^+). Protein can be separated according to pI value on a polyacrylamide gel using an isoelectric focusing technique.

Isoelectric focusing technique was used to perform the first dimension. The desalted CM-Cellulose purified protein (100 µg) was mixed with 0.1% IEF buffer, mixed well and incubated in the dark for 30 min at room temperature. The reaction mixture was centrifuged at 12,000 ×g for 15 min and the supernatant was loaded onto IPG strip (11 cm, pH3-10 L) and was separated using Ettan IPGphor III isoelectric focusing system. The separation condition was run at 20°C as follows: 500 V for 1 h, 1000 V for 1 h, 8000 V gradient and finally 72000 volt × h (Vh). The strip was washed twice with DTT (10 mg/ml) and iodoacetamide (IAA) (5 mg/ml), mixed well and washed with distilled water for 5 min. DTT and IAA solution were added and shaked for 15 min. The second dimension was separated on 15% SDS-PAGE and protein band was observed by silver staining technique (see Appendix A).

1.5.8 Temperature stability of the purified protein

The purified protein (1 µg) was incubated at 37, 50, 60, 70, 80 and 100°C for 30 min. They were separately mixed with 50 µl rabbit reticulocyte lysate in 50 µl of reaction buffer (20 mM Tris-HCl, pH 7.6, 50 mM KCl and 5 mM $MgCl_2$) and incubated at 37°C for 10 min. Then, the reaction was stopped by adding 12.5 µl of 10% SDS. The reaction mixture was extracted with phenol/chloroform and the rRNA was recovered by ethanol precipitation, and then treated with 1 M aniline/0.8 M acetic acid pH 4.5 in the darkness for 10 min at 60°C. The reaction products were analyzed by using 7 M urea/6% PAGE. The gel was stained with ethidium bromide. The RNA bands were visualized on a UV transilluminator

1.6 Biological activities of the purified protein

1.6.1 Assay for antimicrobial activity

Minimum inhibitory activity of the purified protein against thirty-one strains of human pathogenic bacteria were determined by an agar dilution method (National Committee for Clinical Laboratory Standards, 1983). Mueller-Hinton agar

plate contained 18 ml agar plus a fixed volume 2 ml of serially diluted sample at each concentration was prepared. A loopful of bacterial culture from the slant was inoculated in Mueller-Hinton Broth and incubated at 37°C for 24 h with shaking. The culture was diluted to 10^8 CFU/ml (McFarland standard = 0.5) with sterile 0.85% NaCl and 10 µl was spreaded on to surface of the Mueller-Hinton agar plate. Incubation was performed at 37°C for 24 h and inhibition of the bacterial growth was determined by comparison with growth on the control plate prepared without sample. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of the sample to which no visible bacterial growth was observed. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as standard reference strains. The experiments were replicated three times. Three commercial drugs were used as reference inhibitors. Ampicillin is a very effective antibiotic drug against gram-positive bacteria. Chloramphenicol is an antibiotic drug effect to both gram-positive and gram-negative bacteria. Amphotericin B is an antifungal drug that is often used intravenously for systemic fungal infections. It is also commonly used in tissue culture to prevent fungi from contaminating.

1.6.2 Cytotoxicity of the purified protein to some human cancerous cell lines

1.6.2.1 Preparation of cell cultures

All cell lines and normal cell were cultured in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% activated fetal calf serum (FCS). They were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

1.6.2.2 Cytotoxicity to some human cancerous cell lines

Cytotoxicity to some human cancerous cell lines was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmmann, 1983) based on inhibition of the cellular conversion of a tetrazolium salt into a colored formazan product. African green monkey kidney

(Vero) cell, breast cancer (MCF-7), human colon adenocarcinoma (SW 620) and human hepatoma cell lines were prepared in 96-well tissue culture plates (2×10^4 cells/well). After a 24 h incubation at 37°C in a humidified 5% CO_2 atmosphere cell monolayer were confluent, the medium was removed from each well and replenished with 200 µl of the purified protein dilutions per well. For cell controls 200 µl of medium without the purified protein was added. After 24 h, at 37°C in a humidified 5% CO_2 atmosphere, medium was removed from each well and 100 µl MTT (0.35mg/ml) solutions were added to each well and the plates were incubated for 4 h at 37°C. The MTT solution was removed and 200 µl of DMSO was added to each well to dissolve formazan crystals. The absorbance was read by Microplate Reader at 570 nm. In this experiment, cytotoxicity to Vero cell, MCF-7 and SW620 were performed by Dr.Teerakul Arpornsuwan, Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University. Cell treated with a cytotoxic drug, ellipticine was used as a positive control, whereas untreated cells were used as a negative control. The percentage of cytotoxicity was calculated as following:

$\%\text{cytotoxicity} = 100 - [\{(A_{\text{treated cell}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}})\} \times 100]$; where control is only DMSO and blank is only untreated cells. The half inhibitory concentration (IC_{50}) was defined as the concentration that reduced the absorbance of treated cells to 50% when compared to cell controls.

1.6.3 Assay for HIV-1 reverse transcriptase inhibiting activity

HIV-1 RT inhibiting activity of the purified protein was examined by fluorimetric method as developed by Silprasit *et al*. (2008) using an EnzChek RT Assay Kit (Molecular Probe). In brief, 2-fold serial dilutions of purified protein were mixed with 2 µl of 350 bases polynucleotide template. Then 4 µl of the polymerizing buffer (containing 63 mM Tris-HCl, pH 8.1, 8 mM $MgCl_2$, 132 mM NaCl, and 13 mM DTT) was added and the reaction mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 5 µl of 200 mM EDTA and placed on ice for 15 min. 190 µl of the fluorescence dye (picogreen) was added to each well which was previously diluted in TE buffer at a ratio 1:2000. The fluorescence intensity was detected using excitation at 502 nm and emission at 523 nm.

1.6.4 Larvicidal activity assay against mosquitoes

The larvicidal activity of the purified seed coat protein was evaluated on *Aedes aegypti* and *Culex quinquefasciatus* larvae according to Chantraine *et al.* (1998). Ten healthy third late instars larvae were transferred into each plastic glass containing 10 ml of crude extract and purified protein at five various concentrations. Distilled water and two-fold serial dilutions of a 50 mM Tris-HCl buffer (pH 7.5) were used as control. During experiment, food was not given to the larvae. If the larvae cannot rise to surface when probe with needle, they were considered dead. The mortalities were recorded after 24, 48, and 72 h and were corrected using Abbott′s formula (1925).

1.6.5 Larvicidal activity assay against Spodoptera worm

Bioassay was conducted using leaf-dip method. It was evaluated on the second instars *Spodoptera litura* and *Spodoptera exigua* larvae. Fresh Chinese cabbage leaves were cut into discs (1 cm diameter) and were dipped into the tested solution for 30 s. The discs were removed from the tested solution and hung vertically for 5 min to allow excess solution to drip off. Each treated leaf disc was placed in a plastic cup. Insects were introduced on to the leaf disc. The mortalities were recorded after 12, 24, 48, 72, 96 and 120 h and were corrected using Abbott's formula (1925).

1.6.6 Statistical analysis

The Abbott's formula (1925) was used to correct mortality rates: corrected mortality (%) = $[(M_{obs}-M_{control})/(100-M_{control})]\times100$, where M_{obs} is mortality observed in the test and $M_{control}$ is mortality observed in the control. The lethal concentration (LC_{50}) was calculated by probit analysis and tested using the method of Finney (1971).

2. Molecular cloning of *RIP* gene from seed coat of *J. curcas*

2.1 Isolation of total RNA

The integrity and purity of total RNA used as starting material is an important element of high-quality cDNA synthesis. Fresh deionized (e.g. MilliQ-grade) water or water treated with DEPC (diethyl pyrocarbonate) was used directly. All glass wares were soaked with 0.5 M NaOH, followed by deionized water then baked at 160-180°C for 4-9 h. Single-use pipette tips and microcentrifuge tubes were used. Gloves were worn to avoid contamination and degradation of RNA.

An isolation to obtain a good quality and quantity of total and mRNA free from contaminants is important. The rRNA from plant seeds which are rich in oil, polysaacharides and other secondary metabolites may be difficult to isolate by commercial RNA isolation kit and to molecular analysis. Because those may limit RNA extraction yield and further enzymatic reaction so that CTAB extraction method would be used instead.

Total RNA was isolated from young seed coat of *J .curcas* according to the method of Provost *et al*. (2007) and Sangha *et al*. (2010) with some modifications.

Plant fresh tissue (100 mg) was ground to a powder in liquid nitrogen using a mortar and pestle. One hundred milligram of frozen powder was added to 1 ml of prewarmed (65°C) CTAB extraction buffer (see Appendix A) in 1.5 ml microcentrifuge tube. The reaction mixture was incubated at 65°C for 15 min with occasional gentle mixing. After incubation, the reaction mixture was centrifuged at 8,000 ×g for 5 min to pellet cell debris and the supernatant was transferred to clean microcentrifuge tube. The supernatant was extracted with an equal volume of chloroform: isoamyl alcohol (24:1 v/v). The sample was mixed well by inversion and then centrifuged at 8,000 ×g for 20 min at room temperature. The supernatant was transferred to new tube. After the addition of ¼ volumes of 10 M LiCl, the sample was mixed by inversion and incubated at -20°C for 1 h. After incubation, the sample

was centrifuged at 12,000 ×g for 30 min at 4°C and the pellet was collected. The pellet was resuspended in 500 µl of prewarm (60°C) SSTE buffer and then incubated at 60°C for 5 min. An equal volume of chloroform : isoamyl alcohol (24:1 v/v) was added and vortex for 15 s. The sample was spinned at 12,000 ×g for 20 min at room temperature and an aqueous phase was transferred to fresh eppendorf. Equal volume of 100% ethanol was added, mixed and the RNA was precipitated at -80°C for 1 h. The sample was centrifuged at 12,000 ×g for 20 min at 4°C. The supernatant was removed and the pellet was washed with 1 ml 70% ethanol and spin at 12,000 ×g for 20 min at 4°C. The supernatant was removed, spin briefly to collect residual ethanol and pipette off. The pellet was air-dried and then re-dissolved in 20 µl RNAse free H_2O.

2.2 Qualification and quantification of RNA

Two methods are widely used to measure the amount of nucleic acid.

2.2.1 Ethidium bromide (EtBr) determination

If the amount of nucleic acid is very small or if the sample contains significant quantities of impurities, the amount of nucleic acid can be estimated from the intensity of fluorescence emitted by EtBr after agarose gel electrophoresis.

Agarose gel electrophoresis was performed to identify, separate, and purify RNA fragment. Agarose was mixed with 0.5× Tris-borate EDTA (TBE) buffer (see Appendix A) to an appropriate concentration for separation the particular size of RNA fragment and heated in a microwave until complete solubilization. While the agarose solution was cooling, a clean and dry gel-casting tray was sealed the ends with tape and an appropriate comb was selected for forming the sample slot in the gel. A position of the comb was 0.5-1.0 mm above the plate. The warm agarose (55°C) was poured into the casting tray. After the gel set completely, the comb was removed carefully and the ends of the casting tray were unsealed. The gel is placed in an electrophoresis chamber containing TBE buffer.

The RNA sample was mixed with 6× loading buffer (see Appendix A) and the sample mixture was loaded slowly into the slots of the submerged gel. Standard RNA marker was loaded into the slots on both the right and left sides of the gel. The lid of the gel chamber was closed and the electrophoresis was carried out in 0.5× TBE running buffer. The RNA should migrate toward the positive anode at 100 volts and the bromophenol blue should migrate from the wells into the body of the gel within a few min. The gel was run until the bromophenol blue and xylene cyanol FF have migrated to an appropriate distance through the gel. After finishing, the gel was stained in 2.5 μg/ml ethidium bromide (EtBr) solution for 15 min and destained by submerging in an excessive amount of distilled water for 15 min. The nucleic acid bands were visualized under UV transilluminator.

The advantage of using EtBr in gel is that RNA bands can be stained and monitored with a UV lamp. Because the amount of fluorescence is proportional to the total mass of RNA, the quantity of RNA can be estimated by comparing the fluorescent intensity of the RNA sample with a series of exact concentration of standard RNA. It is used in the detection of nanogram levels of RNA.

2.2.2 Spectrophotometric determination of RNA concentration

If the sample is pure (i.e., without the contaminants such as proteins, phenol, or other nucleic acids), spectrophotometric measurement of the amount of (UV) irradiation absorbed by the bases is simple and accurate.

RNA concentration was determined by UV spectrophotometer at the absorbance 260 nm (A_{260}). An absorbance of 1.0 corresponded to 40 μg/ml of RNA (Sambrook and Russel, 2001). The RNA concentration of each sample was calculated in μg/ml by the following equations:

$$\text{RNA concentration} = A_{260} \times \text{dilution factor} \times 40\ \mu g/ml$$

The ratio of the A_{260} and A_{280} can be used as an indicator of nucleic acid purity. The ratio of pure nucleic acid should be 1.8-2.0. A low A_{260}/A_{280} ratio may indicate protein or phenol contamination. The measurements at A_{260} were quantitated for relatively pure nucleic acid preparation in microgram quantity. RNA was stored at -20°C. For long term storage, the total RNA was precipitated and kept in absolute ethanol at -80°C.

2.3 Cloning and sequencing of the partial cDNA fragments encoding Jc-SCRIP

2.3.1 Reverse transcription reaction

RT-PCR is a highly sensitive and specific method used in gene expression studies. RNA cannot serve as a template for PCR, so it is reverse transcribed into cDNA using oligo(dT) primers. The oligo(dT) primers anneal the 3′ poly(A)$^+$ tails of a mRNA molecule and facilitate the synthesis of the first-stranded cDNAs. Avian myeloblastosis virus (AMV) and Malony murine leukemia virus (MoMuLV) reverse transcriptases are commonly used for RT reaction. Complementary DNA of interest was amplified using first-stranded cDNA as a template. This powerful tool has utilized in numerous research applications, including library construction, the study of development gene expression and differential display.

First strand cDNA was reverse-transcribed from total RNA using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentus). The total RNA was reverse-transcribed by an oligo$(dT)_{18}$ primer and RevertAid^{TM} M-MuLV Reverse transcriptase. Twenty microlitres reaction in a 1.5 ml microcentrifuge tube contained 1 μg total RNA, 1 μL of RevertAid^{TM} M-MuLV Reverse transcriptase (200U), 1 μl oligo$(dT)_{18}$ primer, 4 μl of 5× Reverse Transcription buffer, 2 μl of 10 mM dNTP mix and 1 μl of RiboLock^{TM} RNase inhibitor (20U). The reverse transcription was performed at 42°C for 1 h. After incubation, the reaction mixture was heated at 70°C for 5 min to denature the RNA/cDNA hybrid and inactivated the RevertAid^{TM} M-

MuLV Reverse transcriptase then the reaction mixture was incubated on ice for 5 min. The first strand cDNA synthesis reaction was diluted to 100 µl with DEPC-treated water for further used as a template in PCR reaction or store at -20°C until use.

2.3.2 PCR amplification of Jc-SCRIP cDNA fragments

One of powerful methods for obtaining the specific gene is the replication of cDNA or genomic DNA using a technique called PCR. PCR is very sensitive, rapid and inexpensive for amplification and cloning. To perform PCR reaction, small quantity of the target DNA is added to the buffer reaction containing Taq polymerase from *Thermus aquaticus* and dNTP.

Primers typically have different sequences and are complementary to sequences that lie on the opposite strands of DNA template and flank the segment of DNA that is to be amplified. This technique is used genomic DNA as a template for PCR amplification. As a result, major PCR products including the target DNA sequence will be obtained.

The oligonucleotide primers as shown in Table 8 were used to amplify cDNA fragments encoding for Jc-SCRIP. The forward primers were designed based on the N-terminal amino acid sequence of Jc-SCRIP that determined by Edman degradation method and the peptide fragment using *De novo* sequencing method. The reverse degenerate primer was designed based on the peptide fragment using *De novo* sequencing method.

The partial cDNA sequence of Jc-SCRIP gene of *J. curcas* was amplified by polymerase chain reaction (PCR), using cDNA of *J. curcas*, as a template. The amplified reaction of 25 µl contained 1 µl of cDNA template, 12.5 µl of Dream Taq^{TM} Green PCR Master Mix (2×) (Fermentus) containing 0.05 units/µl *Taq* DNA Polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, 10.5 µl of nuclease-free water, and 2 µM of primers. The PCR was performed in a Perkin Elmer Thermocycle 480. PCR procedure was run as

following: preheated at 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. The final extension was carried out at 72°C for 10 min. The PCR product was separated on 1% agarose gel and then the DNA fragment was purified from agarose gel using HiYield™ Gel/PCR DNA fragment Extraction Kit (RBC Bioscience-Taiwan).

Table 8 Oligonucleotide primers were used for cloning of Jc-SCRIP cDNA

Primer	Method	Sequence
Forward	Edman degradation	F0: 5′GCTATTAATGGTGGTGGTGCT3′
	De novo sequencing	F1: 5′GCNGCNTTYYTNCANAAYGAYTAYAC3′
		F2: 5′GCNGCNTTYYTNCANGAYAAYTAYAC3′
		F3: 5′CCNGGNACNACNGTNGTNTA3′
		F4: 5′GGNGCNCANYTNCCNCARAA3′
Reverse	*De novo* sequencing	R1: 5′GTRTARTCRTTNGTNARRAANGCNGC3′
		R2: 5′GTRTARTTRTCNGTNARRAANGCNGC3′
		R3: 5′TANACNACNGTNGTNCCNGG3′
		R4: 5′TTYTGNGGNARNGTNGCNCC3′

Where N = A,C,G or T; Y = C or T; R = A or G

2.3.3 Purification of DNA fragment from agarose gel

Purification of DNA fragment from agarose gel was performed according to HiYield™ Gel/PCR DNA fragment Extraction Kit (RBC Bioscience) instruction. In brief, the desired DNA fragments on agarose gel were excised with razor blade and the agarose gel containing the band was transferred to a microcentrifuge tube. Five hundred microliters of DF buffer was added to the sample and mix by vortexing. The sample was incubated at 55°C for 10-15 min until the gel slice has been completely dissolved. During incubation, invert the tube every 2-3 min. The sample was loaded into DF Column which was in a 2-ml collection tube. The column was centrifuged at 10,000 ×g for 10 s at room temperature. After discarding

the flow-through, 600 µl of wash buffer were added to the column. The column was centrifuged at 10,000 ×g for 30 s. The DF Column was transferred to a clean 1.5-ml microcentrifuge tube. Thirty microlitres of nuclease-free water was added to the center of the column matrix and allow standing for 2 min until water is absorbed by matrix. The column was centrifuged at 10,000 ×g for 2 min to elute purified DNA. DNA yields were determined by UV spectrophotometry.

2.3.4 DNA ligation

The purified DNA fragment (section 2.3.3) was ligated into pGEM-T easy vector (Promega) which possessed a single 3′ deoxythymidine (T) overhanged at both ends. This vector allowed easy cloning of PCR product based on the fact that *Taq* polymerase used in PCR tend to add an additional nucleotide, usually a deoxyadenosine (A) to the 3′- end of each strand that it synthesized. Therefore, a double-stranded PCR product has a single adenosine nucleotide overhang. The molar ratio of the insert DNA to the vectors is usually 1:3 to 3:1. The amount of inserted DNA depends on the length of the inserted DNA fragment. The quantity of inserted DNA was estimated in ng by the following equation:

$$\text{x ng of insert DNA} = \frac{(\text{50 ng of pGEM-T vector})(\text{y bp of inserted DNA}) \times \text{insert:vector molar ratio}}{\text{3015 bp of pGEM-T easy vector}}$$

Where x was the amount of insert DNA, y (bp) was the length of inserted DNA to be ligated for 1:3 (vector : insert molar ratio).

The 5 µl of each ligation reaction composed of inserted DNA, 1 µl of 10× T4 DNA ligase buffer, 1 µl of pGEM-T easy vector (100 ng), and 1 µl of T4 DNA ligase (3 U/µl). Sterile water was added to the final volume of 5 µl. Each mixture was gently mixed by pipetting and then incubated at 16°C overnight.

2.3.5 Preparation of competent *Escherichia coli* cells

The basic recombinant DNA technique for competent cell preparation, transformation, and isolation of plasmid DNA were conducted using standard protocols of Sambrook and Russell (2001).

A single colony of *E. coli* DH5α was inoculated into 3 ml of LB broth and incubated at 37°C with shaking (220 cycles/min in a rotary shaker) for 12-16 h. One millilitre of microbial starter was inoculated into 100 ml of LB broth and the culture was incubated at 37°C with vigorous shaking for 3-5 h until the A_{600} of the cells was reached 0.5-0.7 (about 2-3 h). The bacterial cells were transferred to a sterile, ice cold 50 ml polypropylene tube and the tube was chilled on ice for 10 min. The cells were recovered by centrifugation at 2,700 ×g for 10 min at 4°C then the medium was removed from cell pellets as much as possible. Cell pellet was resuspended by gentle mixing in 2 ml of ice-cold 0.1 M $CaCl_2$ solution containing 15% (v/v) glycerol for each 50 ml original culture. Competent cells were preserved at -70°C not more than 4 months because there may be some deterioration in the efficiency of transformation during prolonged storage.

2.3.6 Bacterial transformation

The tube containing the ligation reaction (section 2.3.4) was centrifuged to collect contents at the bottom of the tube. *E. coli* DH5α was used as host cell. Competent cell vial was thawed at room temperature tap water or water bath for 10-20 seconds. Two hundred microlitres of the cell suspension was mixed with 3 µl of ligation mixture and was placed on ice for 30 min. The mixture was incubated at 42°C for 1-2 min and placed on ice for 2 min immediately. Eight hundred microlitres of LB broth were added to the mixture and then the transformed mixture was plated on LB ampicillin agar plates. The plates were incubated at 37°C for 12-16 h. The transformant colonies were counted and selected randomly to check for the presence of the inserted DNA by colony PCR analysis.

2.4 DNA Sequencing

2.4.1 Plasmid DNA preparation

The recombinant clones containing expected size product (from section 2.3.4) were purified using GeneJET™ Plasmid Miniprep Kit (Fermentus). Each clone, freshly cultured overnight in LB broth containing 100 µg/ml ampicillin, was transferred to a microcentrifuge tube and centrifuged at 6,800 ×g for 2 min at room temperature. The bacterial cell pellet was resuspended by vigorous vortex in 250 µl of resuspension solution until no cell clumps remained. After the pellet was completely resuspended, 250 µl of the lysis solution was added and mixed gently by inverting the tube 6-8 times until the solution becomes viscous and slightly clear. The neutralization solution (350 µl) was added and mixed immediately and thoroughly by inverting the tube 4-6 times and centrifuged for 5 min to pellet cell debris and chromosomal DNA. The supernatant was transferred into GeneJET™ spin column which was placed in a 2 ml collected tube and centrifuged at 12,000 ×g for 1 min. The flow-through was discarded and the column was placed back into the same collection tube. The column was washed with 500 µl of wash solution and centrifuged at 12,000 ×g for 1 min. The wash was repeated again. The flow-through was discarded and residual washed buffer was removed from the column by additional centrifugation at 12,000 ×g for 2 min and then the column was placed into a new 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 30 µl of nuclease-free water and incubated at room temperature for 2 min. After incubation, the tube was centrifuged at 12,000 ×g for 2 min and the plasmid DNA was collected.

2.4.2 Determination of inserted DNA size by PCR

To determine the size of the inserted cDNA in the plasmid pGEM-T, PCR was performed directly using purified plasmid as template. The reaction mixture of 25 µl contained 1 µl of purified plasmid, 12.5 µl of Dream Taq™ Green PCR Master Mix (2×) (Fermentus) containing 0.05 units/µl Taq DNA Polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM

dGTP, 0.4 mM dTTP, 10.5 µl of nuclease-free water, and 2 µM of primers. The PCR was performed using a Perkin Elmer Thermocycle 480. PCR procedure was as followed: preheated at 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. The final extension was carried out at 72°C for 10 min. The PCR product was separated on 1% agarose gel and stained in 0.5 µg/ml EtBr solution.

2.4.3 Sequencing of DNA

The sequencing of the cDNA clones were performed at the Macrogen Inc. (Korea) by 3730×l DNA analyzer. Homologous sequences were sought using the Basic Alignment Search Tool (BLAST) on National Center for Biotechnology Information (NCBI). Related sequences were cited from the GenBank database.

RESULTS AND DISCUSSION

Results

1. Purification of Jc-SCRIP

Crude extract of seed coat of *J. curcas* KUBP33 was initially found to agglutinate rabbit erythrocytes. The presence of lectin activity was suggested by the hemagglutination inhibition test using selected monosaccharides and glycoproteins in the preliminary study. It was found that the hemagglutination was inhibited by N-acetyl-D-galactosamine, 6-O-methyl-D-galactose and D-raffinose at concentration 0.03, 4.88×10^{-4} and 125 mg/ml, respectively (see Appendix Table B1). The crude extract was also found to have *N*-glycosidase activity. Based on the above preliminary results, purification of a protein from the seed coat of *J. curcas* with either lectin or *N*-glycosidase activity was attempted by ammonium sulfate precipitation at 80% saturation to precipitate out all the proteins, followed by anion-exchange chromatography on DEAE-Sephacel™ column as described in methods (section 1.2.1 and 1.2.2). It was found that the majority of proteins with hemagglutination activity and *N*-glycosidase activity were unbound to the column (Figure 6, peak I), while the others without lectin and *N*-glycosidase activity were bound to the column (Figure 6, peak II). The active unbound fractions were then pooled and subjected to a column of CM-Cellulose for cation-exchange chromatography as described in methods (section 1.2.2). Elution of the protein with hemagglutination activity was tried using a linear gradient of NaCl of 0-0.5 M (Figure 7). It was found that the hemagglutination and *N*-glycosidase activity determined after dialysis coincided only with the bound peak eluted at 0.26 M NaCl.

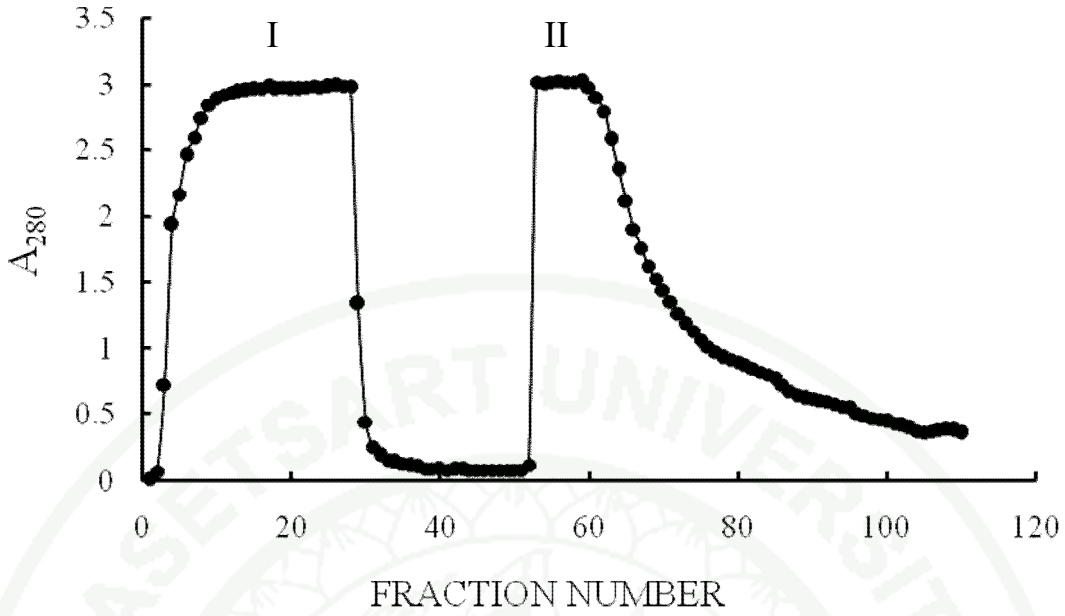

Figure 6 Ion-exchange chromatography on a DEAE-Sephacel™ column. Crude protein extract from seed coat of *J. curcas* (200 mg protein) was precipitated with ammonium sulfate at 80% saturation. After centrifugation, the pellets were dissolved with a 50 mM Tris-HCl (pH 7.5) and then dialyzed against the same buffer. The protein solution was applied on a DEAE-Sephacel™ column that had been pre-equilibrated with the same buffer. The column was washed out with a 50 mM Tris-HCl (pH 7.5) to elute unbound protein (peak I) and then 1.0 M NaCl in the same buffer was used to elute the bound protein (peak II) at a flow rate of 40 ml/h. Hemagglutination and *N*-glycosidase activity were coincided with the DEAE-Sephacel™ unbound fractions (peak I).

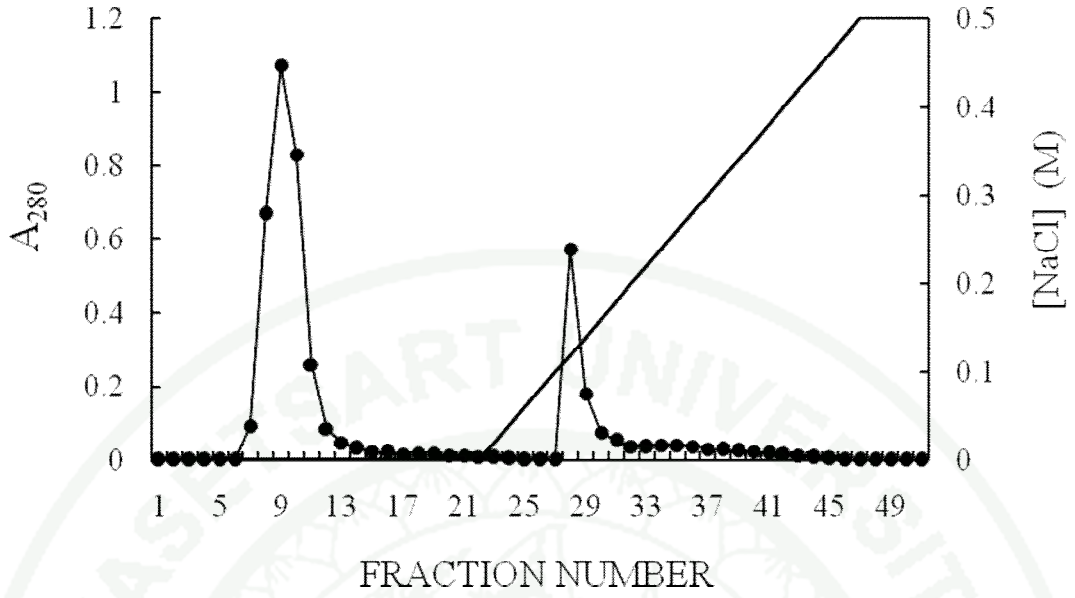

Figure 7 Ion-exchange chromatography on a CM-Cellulose (Fast flow) column. Pooled unabsorbed proteins from DEAE-Sephacel™ were dialyzed against a 50 mM sodium acetate buffer (pH 5.0) and then (30.48 mg protein) applied on a CM-Cellulose that had been pre-equilibrated with the same buffer. Peak II was eluted with a linear concentration gradient of 0-0.5 M NaCl in the same buffer at flow rate of 30 ml/h. Hemagglutination and *N*-glycosidase activity were coincided with peak II.

As shown in Table 9, the 80% ammonium sulfate precipitation was 2.2-folds in specific activity with 50.5% recovery of the hemagglutination activity. The anion-exchange chromatography of the protein on the DEAE-Sephacel™ column resulted in an increase of 21-folds in specific activity with 15.2% recovery of the hemagglutination activity. The specific activity of CM-Cellulose purified protein was 5.4-times of that of the DEAE-Sephacel™ purified protein and 113.5 times of that of the crude extract. By this step, only 1.12% recovery of the hemagglutination activity was found. A yield of 7.5 µg protein of the purified protein was obtained from 1 g of the seed coat protein. The purified protein was designated as **"Jc-SCRIP"**

Table 9 Purification of Jc-SCRIP from seed coat of *J. curcas*

Fractions	Total protein (mg)	Total HA (U)	Specific activity (U/mg protein)	Purification fold	Recovery (%)
Crude extract	200	4,000	20	1	100
80% ammonium sulfate	101	4,480	44	2.2	50.5
DEAE-Sephacel™	30.48	2,800	420	21	15.2
CM-Cellulose	2.25	5,120	2,270	113.5	1.12

2. Hemagglutination and hemagglutination inhibition activity of Jc-SCRIP

The hemagglutination activity of Jc-SCRIP was determined in comparison to two RIPs, ricin (crude extract of *Ricinus communis*) and curcin (type 1 RIP from seed kernel of *J.curcas*) as described in methods (section 1.3.1). As shown in Figure 8, Jc-SCRIP (Figure 8B) had hemagglutination activity 32 times lower than that of ricin (Figure 8C). No hemagglutination activity was found for curcin (Figure 8D).

The hemagglutination inhibition activity of crude protein extract from seed coat of *J. curcas* was studied with various carbohydrates and glycoproteins as described in methods (section 1.3.2) and Appendix Table B1. Agglutination was inhibited by 1.95 µM of fetuin, 0.49 µM of 6-O-Methyl-D-galactose, 0.03 mM N-

acetyl-D-galactosamine, and 125 mM D-(+)-raffinose pentahydrate. However, the purified protein, Jc-SCRIP was inhibited only by fetuin at 976.6 µM.

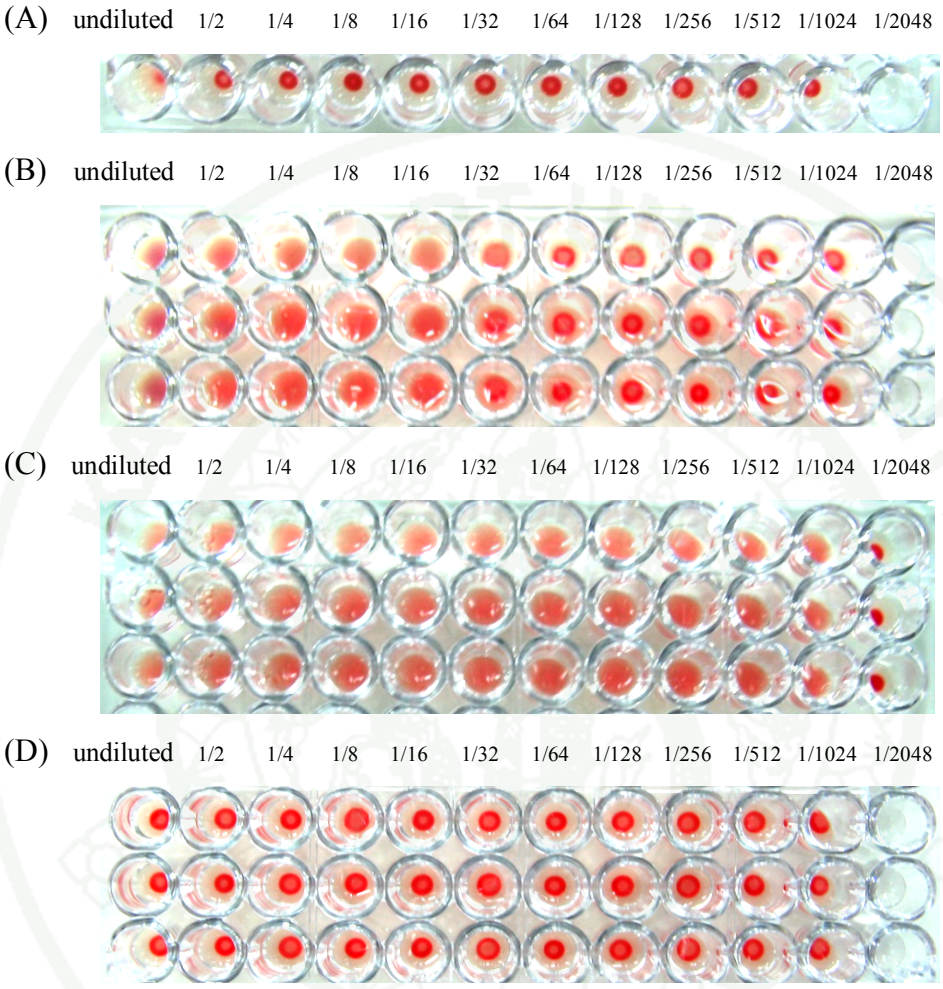

Figure 8 Hemagglutination activity of purified protein from seed coat of *J. curcas*. Hemagglutination activity assays were performed in microtitre plates. Each well contained 25 µl of a 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl, 25 µl of 2% (v/v) rabbit erythrocyte suspension and 25 µl of 2-fold serial dilutions of (B) 1 mg/ml of Jc-SCRIP, (C) 1 mg/ml of ricin (positive control) and (D) 1 mg/ml of curcin. (A) Negative control contained buffer only. Agglutination was examined after incubation for 45 min at room temperature (n=3). The agglutinated rabbit erythrocytes form a carpet that covers the whole well whereas if no agglutination occurs, red blood cells form a button at the bottom of the well.

3. *N*-glycosidase activity assay of Jc-SCRIP

To test that the purified protein, Jc-SCRIP was a ribosome-inactivating protein, it was determined by its RNA *N*-glycosidase activity using rabbit reticulocyte lysate as source of the 28S rRNA as described in methods (section 1.4). Results depicted in Figure 9, clearly showed that acidic aniline treatment of the Jc-SCRIP treated rRNA resulted in generation of a specific RNA fragment of approximately 560 nucleotides due to its *N*-glycosidase activity. On the contrary, without Jc-SCRIP treatment showed no released of such RNA fragment. Jc-SCRIP damaged the rabbit reticulocyte rRNA with strong *N*-glycosidase activity in the same manner as compare to the other two type I RIPs, ricin A chain from seeds of castor bean *Ricinus communis* and pokeweed antiviral protein (PAP) from the spring leaves of *Phytolacca americana*.

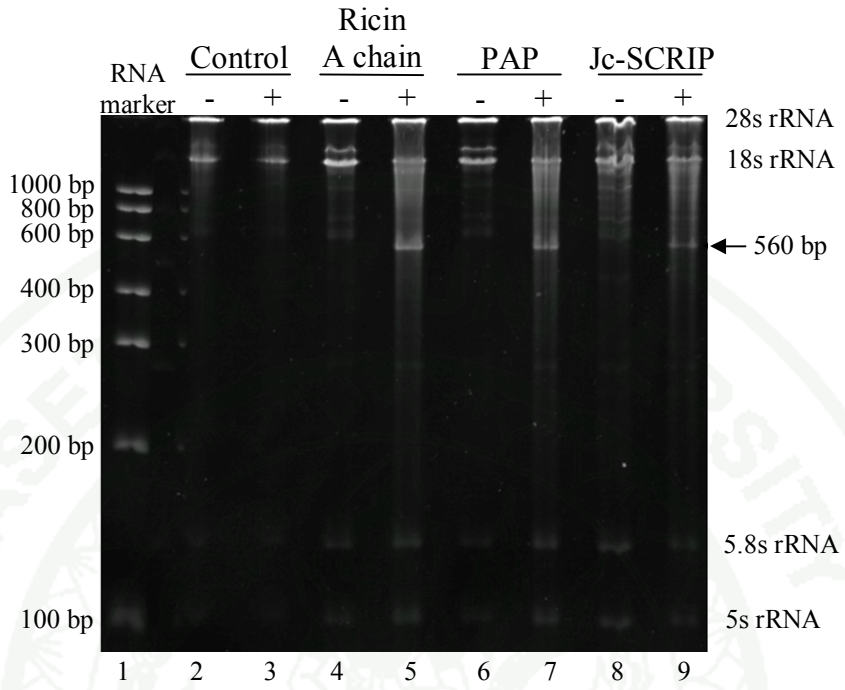

Figure 9 Characterization of the *N*-glycosidase activity of Jc-SCRIP in comparison to other type I RIPs.

Rabbit reticulocyte lysate (50 µl) was incubated with 1 µg of Jc-SCRIP at 37°C for 10 min and then aniline treatment. The resulted rRNA fragments were analyzed by 7M urea/6% PAGE. Ribosomes were incubated with 1 µg of ricin A chain and PAP, type I RIPs from seeds of *R. communis* and leaves of *P. Americana* respectively, as positive control. Lane 1, RNA marker; lane 2 and 3, negative control (without RIPs); lane 4, 6, 8 treatment with ricin A chain (1 µg), PAP (1 µg) and Jc-SCRIP (1 µg) and then with aniline. (+) and (-) indicate aniline treatment or absence of aniline treatment. The arrow indicated the approximately 560 nucleotides released from the ribosomes by aniline treatment of the modified rRNA.

4. Molecular properties of Jc-SCRIP

4.1 Subunit molecular weight of Jc-SCRIP

The crude protein extract (as described in methods, section 1.1.1), ammonium sulfate fraction (as described in methods, section 1.2.1), the partial purified protein from DEAE-Sephacel™ (as described in methods, section 1.2.2), and the purified protein, Jc-SCRIP from CM-Cellulose chromatography (as described in methods, section 1.2.2) chromatography were analyzed by SDS-PAGE under a reducing condition (Figure 10) as described in methods (section 1.5.2). Subunit molecular weights of the protein bands were calculated from the calibration curve (see Appendix Figure B1). The crude protein extract (Figure 10, lane 2) and ammonium sulfate fraction (Figure 10, lane 3) were found to contain many protein bands with molecular weights ranged from low to high molecular weights. After the DEAE-Sephacel™ chromatography, the unbound peak (Figure 10, lane 4) containing the hemagglutination activity was found to consist of one major band with molecular weight of approximately 41,800 Da and a few bands of higher molecular weights. These few bands were removed by cation-exchange chromatography using CM-Cellulose column. The bound peak containing the hemagglutination activity was found to contain a single band of purified protein, Jc-SCRIP (Figure 10, lane 5).

4.2 Molecular mass of Jc-SCRIP

The native molecular mass of Jc-SCRIP was analyzed by MALDI-TOF/MS as described in methods (section 1.5.3). The MALDI-TOF/MS spectrum of Jc-SCRIP was shown in Figure 11 and it was found that Jc-SCRIP possessed a major protein of molecular mass of 38,938.21 Da.

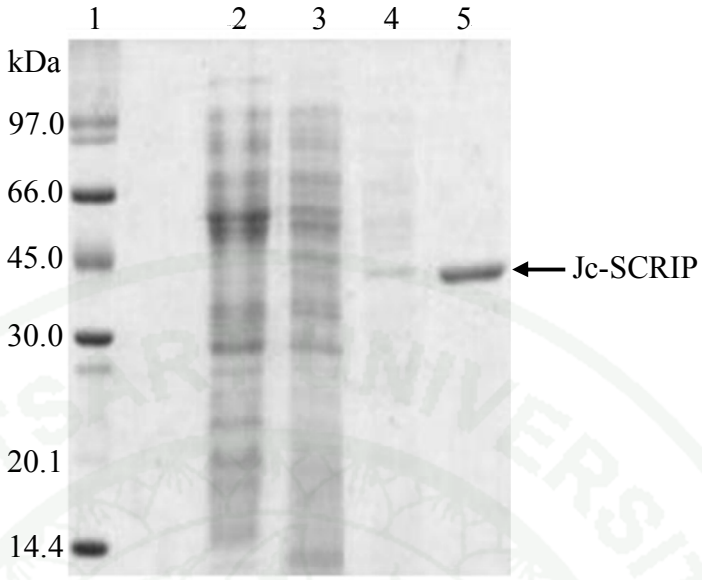

Figure 10 SDS-PAGE of each purification step.

From left to right; lane 1, Molecular mass marker containing α-lactalbumin (14,400 Da), trypsin inhibitor (20,100 Da), carbonic anhydrase (30,000 Da), ovalbumin (45,000 Da), albumin (66,000 Da), phosphorylase b (97,000 Da); lane 2, crude protein extract (30 µg); lane 3, 80% saturated ammonium sulfate-precipitated crude protein (15 µg); lane 4, unbound protein fraction obtained by DEAE-Sephacel™ (5 µg); lane 5, bound protein fraction obtained by CM-Cellulose (Fast flow) (5 µg).

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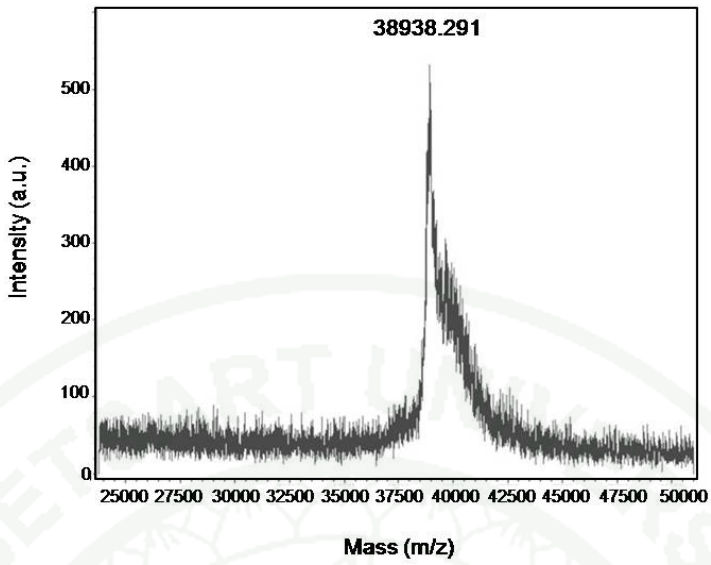


Figure 11 MALDI-TOF/MS spectrum of Jc-SCRIP.
The spectrum was recorded in the linear positive ion mode using sinapinic acid matrix. Jc-SCRIP purified CM-Cellulose column was subjected to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer equipped with a delayed extraction source and 337 mm pulsed nitrogen laser. One microliter of the sample was mixed with 1 µl of 50 nM sinapinic acid and applied to sample probe. The instrument was run in the linear mode using 20 kV acceleration.

4.3 N-terminal amino acid sequence of Jc-SCRIP

Analysis of N-terminal amino acid sequence of Jc-SCRIP was conducted with automated Edman degradation as described in methods (section 1.5.4). The results showed 7 amino acid residues AINGGVA at the N-terminal. It was analyzed in comparable to N-terminal of other the type 1 RIPs in the database (Table 10). It was found that the N-terminal sequence of Jc-SCRIP had no similarity with those of reported type 1 RIPs (Wang and Ng, 2000, 2001; Lam and Ng, 2001; Chuethong *et al*., 2007; Lin *et al*., 2010).

Table 10 Comparison of N-terminal sequences of Jc-SCRIP with the other type 1 RIPs

RIPs	Plants	N-terminal sequence	References
Jc-SCRIP	*Jatropha curcas*	AINGGVA	This study
Curcin	*Jatropha curcas*	A(G/Y)(S/K)(T/A)(P/D)TLTITYDA(T/A)ADKKNYAQFKDLREA(F/A)G	Lin *et al*., 2010
Ricin A	*Ricinus communis*	INFTTAGATVQSYINFIRAVRGRLTTGADVRHEIP	Lam and Ng, 2001
Trichosanthin	*Trichosanthes kirilowii*	VSFRLSGATSSSYGVFISNLRKALPNERKLY	Lam and Ng, 2001
PAP	*Phytolacca americana*	ITFDAGNATINKYATFMESLRNEAKDPSLKCYGIP	Lam and Ng, 2001
Bryodin I	*Bryonia dioica*	NVRFDLSGATSSSY	Chuethong *et al*., 2007
Cochinin B	*Momordica cochinchinensis*	DVSFDMSTASTESYKKFIAD	Chuethong *et al*., 2007
Flammulin	*Flammulina velutipes*	APSHFSHPGVLADRAQIDFIXGKVNEGAEPWXSAYN	Wang and Ng, 2000
Pleuturegin	*Pleurotus tuberregium*	ARTQPGNIAPVGDFTLYPNAPRQGHIVA	Wang and Ng, 2001

Note: PAP, pokeweed antiviral protein

4.4 Secondary structure of Jc-SCRIP

Secondary structure of Jc-SCRIP was determined by Circular Dichroism (CD) as described in methods (section 1.5.5). The far-UV CD spectrum of Jc-SCRIP showed a negative elipicity at 210 nm (Figure 12) which is characteristic of the β-sheet structure. Secondary structure of Jc-SCRIP was analyzed using three calculation algorithms including CDSSTR, CONTINLL, and SELCON3 as shown in Table 11. The result which was calculated from program CONTINLL was chosen to report because it had the lowest value of NRMSD (0.062). The NRMSD is a goodness-of-fit parameter. It should not be more than 0.1. If it is high (>0.1), the correspondence of the calculated secondary structure is unlikely to be corrected. The ratios of α-helix, β-

sheet, turn and unordered of Jc-SCRIP using CONTINLL program were about 7.3, 36.7, 23.5 and 32.5%, respectively.

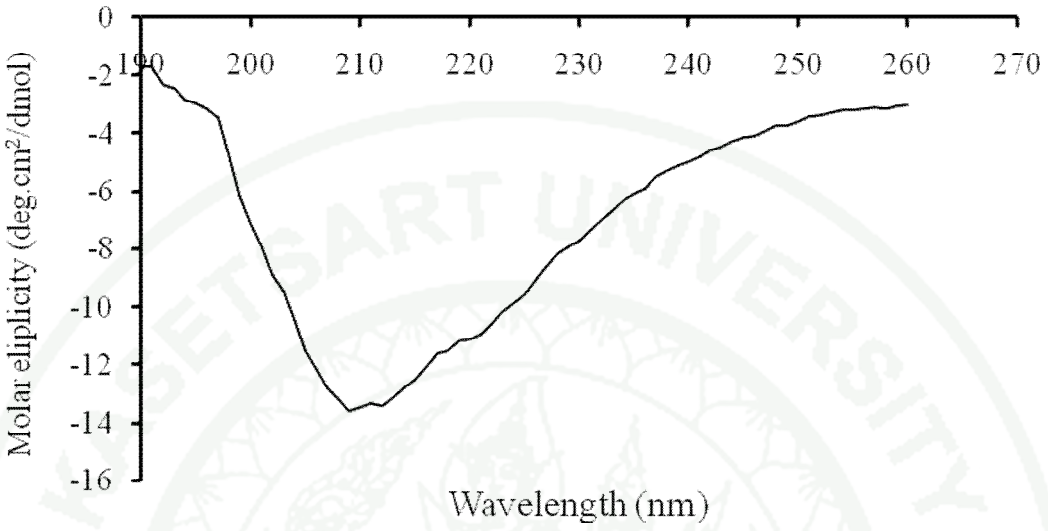


Figure 12 The far-UV CD spectrum of Jc-SCRIP.
The spectrum showed a negative peak close to 210 nm indicating that the β-sheet structures are predominant of protein.

Table 11 Estimation of secondary structure of Jc-SCRIP using three secondary structure calculation algorithms

Method	Secondary structure fractions (%)								
	α-helix (%)			β-sheet (%)			Turn	Unrd	NRMSD
	H(r)	H(d)	Total	S(r)	S(d)	Total			
CDSSTR	0.2	5.1	5.3	25.5	12.3	37.8	25.5	31.1	0.158
CONTINLL	2.3	5.0	7.3	23.8	12.9	36.7	23.5	32.5	0.062
SELCON3	1.1	4.8	5.9	24.3	13.0	37.3	25.6	33.5	0.261

Note: H(r), regular helix; H(d), distorted helix; S(r), regular sheet; S(d), distorted sheet; Unrd, unordered; NRMSD, Normalized Root Mean Square Deviation

4.5 Carbohydrate content of Jc-SCRIP

4.5.1 Neutral sugar content of Jc-SCRIP

Neutral sugar content of Jc-SCRIP was determined by phenol-sulfuric acid assay as described in methods (section 1.5.6.1) using a standard calibration curve of glucose (see Appendix Figure B3). Jc-SCRIP was found to be a glycoprotein having neutral sugar content of 4.80% (w/w) of the protein.

4.5.2 Determination of N-linked glycoprotein on SDS-PAGE

To determine whether Jc-SCRIP was N-glycosylated, it was treated with Endo Hf, an enzyme that cleaves high mannose complex type N-linked glycosylation as described in methods (section 1.5.6.2). As shown in Figure 13, the protein band of Endo Hf treated Jc-SCRIP (lane 2) did not shift to a lower appearance molecular weight upon SDS-PAGE. The results indicated that Jc-SCRIP was not N-linked glycoprotein.

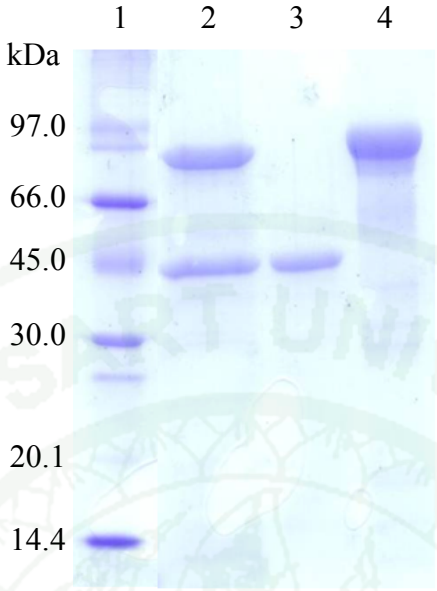


Figure 13 Determination of N-linked glycoprotein of Jc-SCRIP on SDS-PAGE. Jc-SCRIP (5 µg) was incubated with 2× glycoprotein denaturing buffer. After that the reaction mixture was inactivated by boiling at 100°C for 10 min and then cooled to room temperature. Concentrated reaction buffer (10×) and Endo Hf was added and incubated at 37°C for 2 h. The treated sample was analyzed on 15% SDS-PAGE. From left to right; lane 1, Molecular mass marker; lane 2, Jc-SCRIP+Endo Hf; lane 3, Jc-SCRIP and lane 4, Endo Hf only.

4.6 pI of Jc-SCRIP

pI of Jc-SCRIP was determined by isoelectric focusing technique as described in methods (section 1.5.7). The range of pI was separated to 3-10. Jc-SCRIP had pI of 4.8 (Figure 14). It was different from other type 1 RIPs which were basic protein (Table 4).

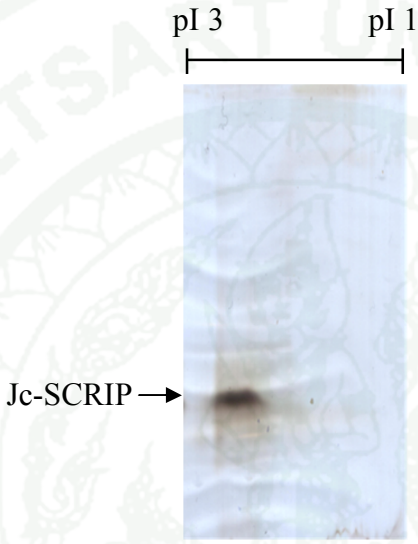


Figure 14 Isoelectric focusing (pI) of Jc-SCRIP.
Jc-SCRIP (100 µg) was mixed with 0.1% IEF buffer and incubated in the dark for 30 min at room temperature. The reaction mixture was centrifuged and the supernatant was loaded onto IPG strip. The first dimension was separated by Ettan IPGphor III isoelectric focusing system. The separation condition was run at 20°C. After that, the strip was washed with DTT and IAA solution. The second dimension was separated on 15% SDS-PAGE and protein bands were observed by silver staining technique.

4.7 Temperature stability of RNA *N*-glycosidase activity of Jc-SCRIP

Effect of temperature to the RNA *N*-glycosidase activity of Jc-SCRIP was determined by analyzing its RNA *N*-glycosidase activity by observing the intensity of the RNA fragment released after the Jc-SCRIP protein was incubated at various temperatures for 30 min as described in methods (section 1.5.8). The result showed that its *N*-glycosidase activity was maintained after heating at 37 to 100°C for 30 min (Figure 15).

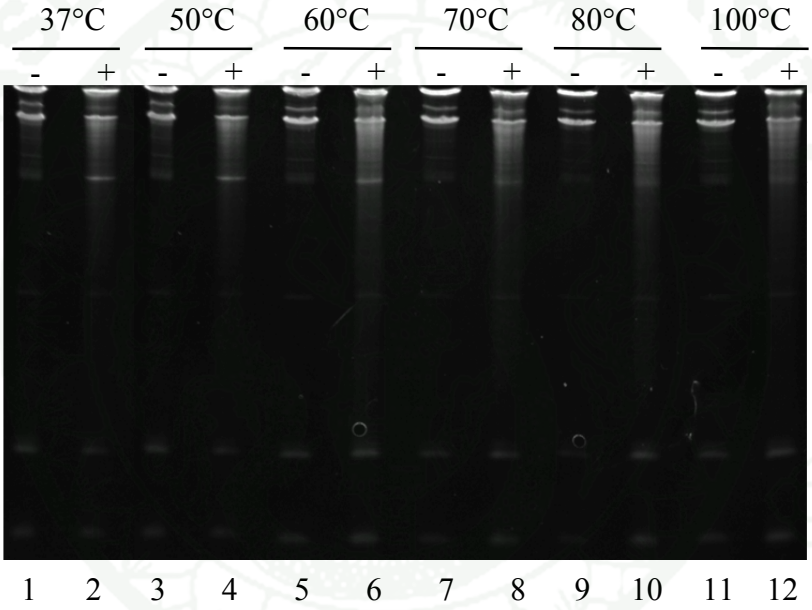


Figure 15 Effect of various temperatures on the *N*-glycosidase activity of Jc-SCRIP. Rabbit reticulocyte lysate (50 µl) was incubated with 1 µg of Jc-SCRIP by varying incubation temperature from 37 to 100°C for 30 min. The resulted rRNA fragments were analyzed by 7M urea/6% PAGE. Lane 1, 3, 5, 7, 9, and 11 treatment with Jc-SCRIP (1 µg) and were incubated at 37, 50, 60, 80 and 100°C; lane 2, 4, 6, 8, 10 and 12 treatment with Jc-SCRIP (1 µg) and were incubated at 37, 50, 60, 80 and 100°C and with aniline. (+) and (-) indicate aniline treatment or absence of aniline treatment. The arrow indicated the approximately 560 nucleotides released from the ribosomes by aniline treatment of the modified rRNA.

5. Biological activities of Jc-SCRIP

5.1 Antimicrobial activity of Jc-SCRIP

To explore the antimicrobial activity of Jc-SCRIP, minimum inhibitory activity of the purified protein against thirty-one strains of human pathogenic microorganisms were determined by an agar dilution method as described in methods (section 1.6.1). It showed inhibitory activity against both Gram-positive bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, and *Streptococcus pyogenes*) and Gram-negative bacteria (*Acinetobacter lwoffii*, *Burkholderia cepacia*, and *Shigella dysenteriae*), and yeast (*Candida albicans*) (Table 12). It showed the most potent inhibitory activity to a gram-positive bacterium, *S. epidermidis* ATCC 12228 at MIC value of 7.81 μg/ml. The inhibitory potency of Jc-SCRIP was compared to ampicillin, chloramphenicol, and amphotericin B. It was found that Jc-SCRIP had the lowest inhibitory activity as shown in Table 12. Jc-SCRIP showed lesser inhibitory activity than of all of tested microorganisms of antimicrobial drugs as shown in Appendix Table B2.

Table 12 Minimum Inhibitory Activity (MIC) of Jc-SCRIP and standard antibiotic against human pathogenic bacteria and fungi

Bacterial strains	Minimum Inhibitory Concentration (µg/ml)			
	Jc-SCRIP	Ampicillin	Chloramphenicol	Amphotericin B
Acinetobacter lwoffii ATCC 15309 (DMST 4229)	125	5	50	NT
Bacillus cereus ATCC 11778 (DMST 5040)	250	5	5	NT
Burkholderia cepacia ATCC 25416 (DMST 4205)	500	>2000	5	NT
Shigella dysenteriae DMST 15111	250	1	1000	NT
Staphylococcus aureus ATCC 25923 (DMST 8840)	500	0.25	5	NT
Staphylococcus aureus DMST 20654 (MRSA)	500	50	5	NT
Staphylococcus epidermidis ATCC 12228 (DMST 15505)	**7.81**	1	5	NT
Streptococcus agalactiae DMST 17129	62.5	0.25	5	NT
Streptococcus pyogenes DMST 17020	500	0.03125	5	NT
Bacillus subtilis ATCC 6633	250	1	5	NT
Candida albican ATCC 10231	125	NT	NT	0.061

Note: NT, Not tested; ATCC, American Type Culture Collection; DMST, Department of Medical Sciences Culture Collection; MRSA, Methicillin-resistant *Staphylococcus aureus*; MIC was not observed at concentration higher than 500 µg/ml

5.2 Cytotoxicity of Jc-SCRIP against cancerous cell lines

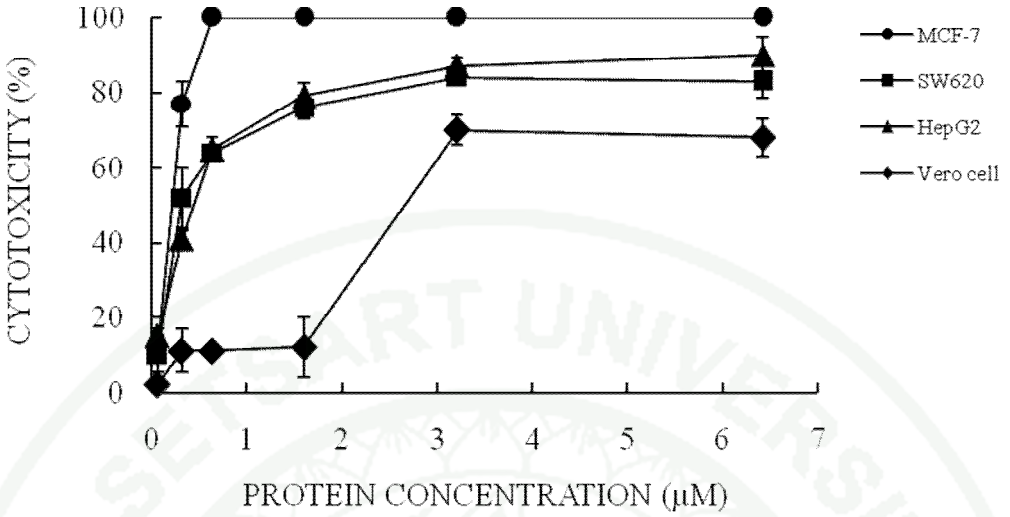
The effects of Jc-SCRIP from *J. curcas* seed coat on the cell viability of the three human cancerous cell lines, SW620, MCF-7 and HepG2 were examined as described in methods (section 1.6.2). A normal cell, Vero cell was used as control. Inhibitory concentrations of Jc-SCRIP at 50% inhibition or IC_{50} were shown in Table 13. Over the range of concentration tested, JcSCRIP exerted cytotoxicity in a dose response manner against all cancerous cells (Figure 16A). MCF-7 showed the most sensitive to Jc-SCRIP (Figure 16A) with IC_{50} at 0.15 μM, followed by SW620 at IC_{50} 0.25 μM, and HepG2 at IC_{50} 0.40 μM, respectively (Figure 16A).

Ellipticine, a commercial anticancer drug, was cytotoxic to SW620 and MCF-7 with IC_{50} at 0.024 and 0.008 μM, respectively (Figure 16B). Jc-SCRIP was cytotoxic to SW620 and MCF7 at lesser concentrations than ellipticine about 10 and 19 fold, respectively. However, Jc-SCRIP inhibited the proliferation of the normal cell lines, Vero cell with IC_{50} at 2.57 μM. (Figure 16B).

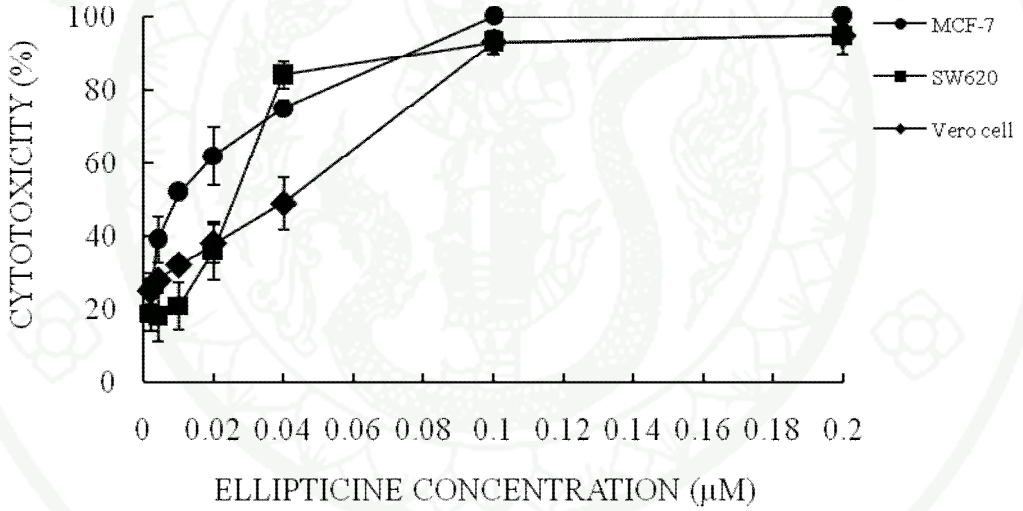
Comparison of the cytotoxicity of Jc-SCRIP and ellipticine against SW620, MCF-7 and Vero cell were shown in Figure 16C. At low concentration (2 μM), Jc-SCRIP showed the cytotoxicity against SW620 and MCF-7 about 2 and 3 times lesser than ellipticine, respectively. It had about 9 times lesser cytotoxic effect against Vero cell than ellipticine.

Figure 16 Cytotoxicity of the Jc-SCRIP against some human cancerous cell lines. Toxicities of Jc-SCRIP against SW620 (human colon adenocarcinoma), MCF-7 (breast cancer), HepG2 (human liver carcinoma), and normal cell (Vero cell) were tested by MTT assay based on inhibition of the cellular conversion of a tetrazolium salt into a colored formazan product. All cell lines were grown in 96-well plates at 37°C for 24 h. After that the medium was removed and replenished with 200 µl of the Jc-SCRIP dilutions per well. After 24 h at 37°C, medium was removed from each well and 100 µl MTT (0.35mg/ml) solutions were added to each well and the plates were incubated for 4 h at 37°C. The MTT solution was removed and 200 µl of DMSO was added to each well to dissolve formazan crystals. The absorbance was read by Microplate Reader at 570 nm. The percentage of cytotoxicity was calculated. Experiments were repeated 3 times (n=3). (A) Jc-SCRIP showed the inhibitory activity to SW620, MCF-7, HepG2 and normal cell. (B) Ellipticine was used as reference drug. It can inhibit the propagation both cancerous cell lines and normal cell. (C) Comparison of the cytotoxicity of Jc-SCRIP and ellipticine against SW620, MCF-7, and Vero cell at low concentration.

(A)



(B)



(C)

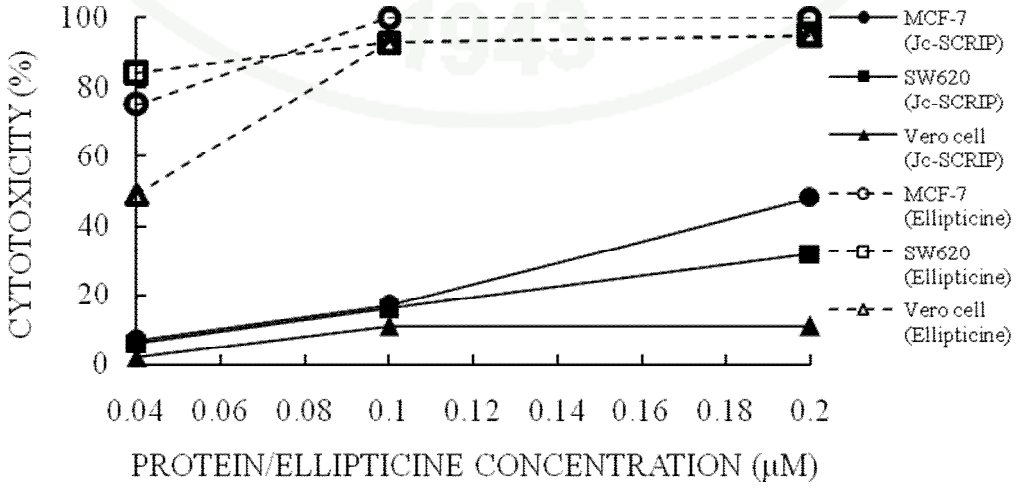


Table 13 *In vitro* cytotoxicity of Jc-SCRIP to human cancer cell lines

Cell lines	IC_{50} (μM)	
	Jc-SCRIP	Ellipticine
Vero cell (Normal cell)	2.57	0.04
SW620	0.25	0.024
MCF-7	0.15	0.008
HepG2	0.40	NT

Note: NT, Not tested

5.3 Assay for anti-HIV-1 reverse transcriptase activity of Jc-SCRIP

The anti-HIV-1 activity of Jc-SCRIP was determined by the inhibition of the enzyme HIV-1 reverse transcriptase which catalysed the immunodeficiency viral replication as described in methods (section 1.6.3). The inhibitory effect of the purified Jc-SCRIP against HIV-1 RT was determined by following the decrease in the enzyme activity by Jc-SCRIP. The result showed the inhibitory effect of Jc-SCRIP towards HIV-1 RT was a dose dependent manner with IC_{50} value of 16 μM (Figure 17).

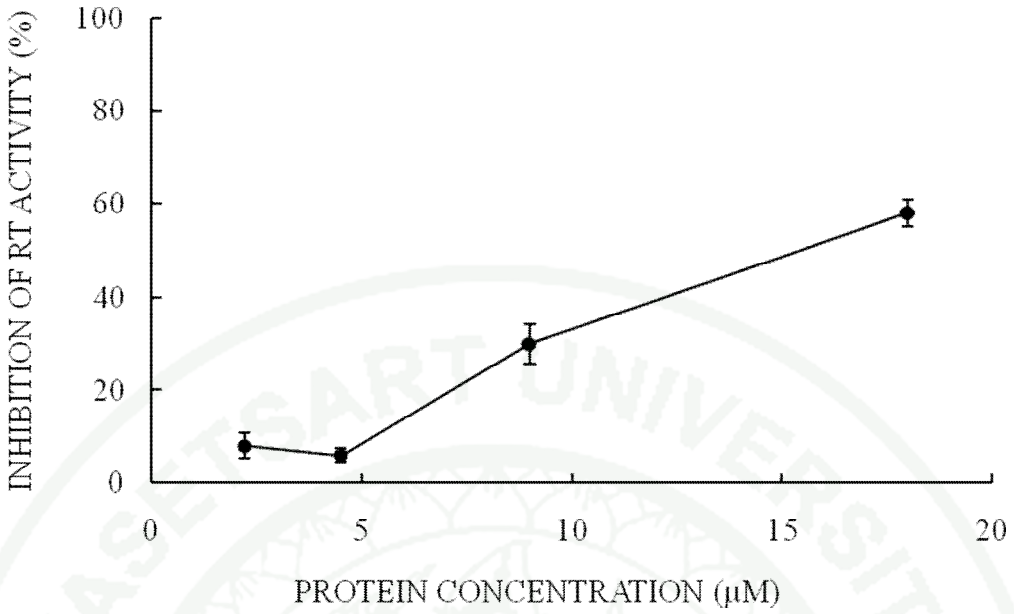


Figure 17 Anti-HIV-1 reverse transcriptase (RT) activity.
The 2-fold serial dilution of Jc-SCRIP was incubated with purified HIV-1 RT. A developed fluorometric method was used to detect the activity of RT. Jc-SCRIP showed the inhibitory activity on HIV-1 RT**.** Experiments were repeated 2 times (n=2).

5.4 Biopesticide activities of Jc-SCRIP

5.4.1 Larvicidal activity of Jc-SCRIP against mosquitoes

The *N*-glycosidase activity of the crude protein extract (JSCCP) and the purified protein (Jc-SCRIP) from seed coat of *J. curcas* were shown by the generation of a single band of the rRNA fragment product from the 28S rRNA substrate (Figure 18). The toxicity of the purified Jc-SCRIP on the late third instars larvae of two mosquitoes, *Aedes aegypti* and *Culex quinquefasciatus* as described in methods (section 1.6.4), were evaluated and compared to three crude protein extracts: *J. curcas* seed coat crude protein (JSCCP) (as described in methods, section 1.1.2), *J. curcas* seed kernel crude protein (JSKCP) (as described in methods, section 1.1.3), and seed kernel crude protein of castor bean, *R. communis* (RSKCP) (as described in methods, section 1.1.3). The difference in protein concentrations and exposure times required to induce the larval mortality was observed. Three crude protein extracts showed larvicidal activities against *Ae. aegypti* at a protein concentration of ≥ 0.375 mg/ml after 24 h of exposure (Table 14) and against *Cx. quinquefasciatus* at a protein concentration of ≥ 0.1875 mg/ml after 12 h of exposure (Table 15). Both larvae species remained viable in the control samples (absence of the extract or the purified protein). The Jc-SCRIP purified protein showed larvicidal activity against *Ae. aegypti* and *Cx. quinquefasciatus* at a protein concentration of ≥ 0.75 mg/ml after 24 h of exposure and ≥ 0.1875 mg/ml after 12 h of exposure, respectively. This protein induced 100% mortality in *Ae. aegypti* at a protein concentration of 3.0 mg/ml after 72 h of exposure (Table 14, Figure 19A) and 100% mortality in *Cx. quinquefasciatus* at a protein concentration of 1.5 mg/ml after 12 h of exposure (Table 15, Figure 20A). The larvicidal activity of all crude protein extracts against *Cx. quinquefasciatus* is more robust than against *Ae. Aegypti* and required lower protein concentrations and exposure times. The JSKCP and RSKCP crude protein extracts induced 100% mortality in *Cx. quinquefasciatus* at protein concentration of 1.5 mg/ml after 12 h of exposure, whereas the JSCCP crude protein extract induced 100% mortality in *Cx. quinquefasciatus* at protein concentration of 3.0 mg/ml after 12 h of exposure (Table 15, Figure 20B-20D). However, all crude protein extracts could not induce 100%

mortality in *Ae. aegypti* at any protein concentration or exposure time (Table 14, Figure 19B-19D).

The toxicity of Jc-SCRIP and each of the crude protein extracts to both mosquito larvae was dose dependent (Figure 19 and 20). The median lethal concentration (LC_{50}) of Jc-SCRIP, JSCCP, JSKCP and RSKCP to *Ae. aegypti* larvae after 24 h of exposure were 1.44, 3.89, 2.95 and 2.24 mg protein/ml, respectively (Table 16). The LC_{50} results indicated that the larvicidal activity towards *Ae. aegypti* of the Jc-SCRIP purified protein is approximately 2.7, 2.0 and 1.5 times more toxic than JSCCP, JSKCP and RSKCP, respectively (Table 16). *Cx. quinquefasciatus* larvae were more sensitive to all of the proteins tested, hence giving the lower LC_{50} values, than were the *Ae. aegypti* larvae. The LC_{50} of Jc-SCRIP, JSCCP, JSKCP and RSKCP for *Cx. quinquefasciatus* larvae after 24 h of exposure were 0.0303, 0.0575, 0.0468 and 0.0389 mg protein/ml, respectively (Table 16). The LC_{50} results indicated that the larvicidal activity of the purified protein, Jc-SCRIP, against *Cx. quinquefasciatus* was approximately 1.9, 1.5 and 1.3 times more toxic than JSCCP, JSKCP and RSKCP, respectively (Table 16). The LC_{50} values indicated that Jc-SCRIP is approximately 50 times more toxic towards *Cx. quinquefasciatus* than *Ae. aegypti*. RSKCP are the most toxic towards the larvae of both mosquito species followed by JSKCP and JSCCP, respectively.

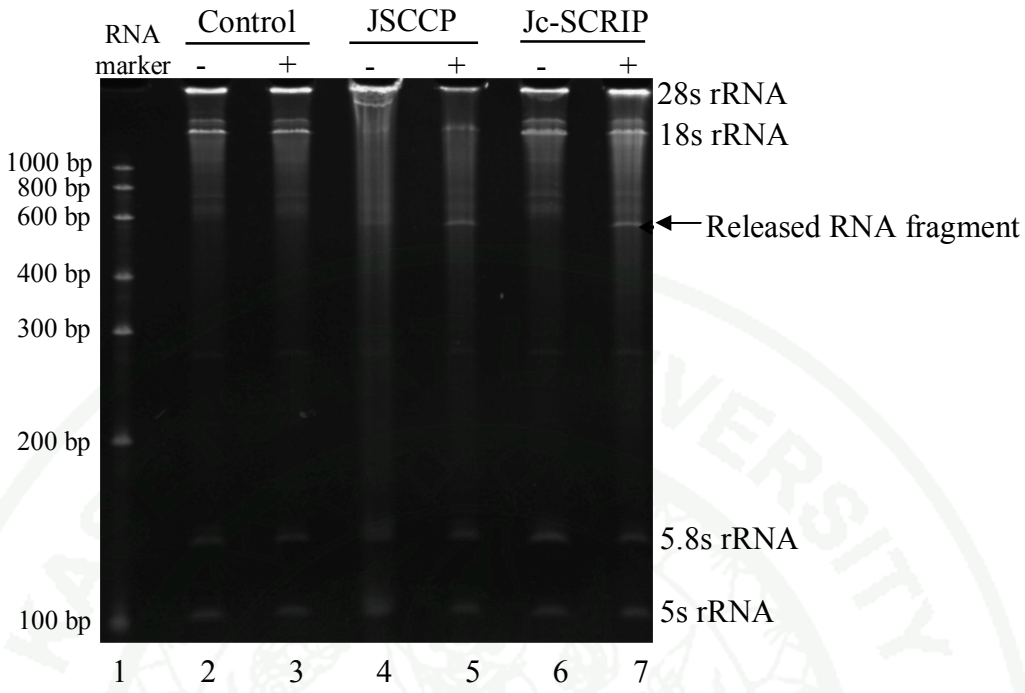


Figure 18 *N*-glycosidase activities of JSCCP and Jc-SCRIP.
A 7.5 M urea/6% PAGE analysis of rabbit reticulocyte lysate treated with crude protein extract (JSCCP) and purified protein from the seed coat of *J. curcas* (Jc-SCRIP). '+' and '-' indicate the presence and absence of aniline treatment, respectively. Lane 1: RNA marker; Lanes 2 and 3: negative control (no protein fraction) with and without aniline treatment, respectively; Lane 4: treated with JSCCP (1 µg) without aniline treatment; Lane 5: treated with JSCCP (1 µg) with aniline treatment; Lane 6: treated with Jc-SCRIP (1 µg) without aniline treatment; Lane 7: treated with Jc-SCRIP (1 µg) with aniline treatment. The arrow indicates the released fragment after aniline treatment, which was characteristic of the *N*-glycosidase activity of plant ribosome-inactivating proteins.

Table 14 The mean % mortalities induced by plant proteins on the third instars larvae of *Aedes aegypti* at various concentrations and exposure times

Proteins	Concentration (mg/ml)	Period of Exposure (h)			
		12	24	48	72
Jc-SCRIP	0.1875	0±0.0	0±0.0	0±0.0	0±0.0
	0.375	0±0.0	0±0.0	0±0.0	0±0.0
	0.75	0±0.0	8±8.4	30±10.0	34±13.4
	1.5	0±0.0	56±8.4	70±8.9	82±8.4
	3.0	0±0.0	82±8.4	94±4.5	0±0.0
JSCCP	0.1875	0±0.0	0±0.0	0±0.0	0±0.0
	0.375	0±0.0	6±5.5	8±4.5	8±4.5
	0.75	0±0.0	12±4.5	2±8.4	2±8.4
	1.5	0±0.0	8±4.5	26±5.5	8±8.4
	3.0	0±0.0	50±7.1	64±13.4	64±13.4
JSKCP	0.1875	0±0.0	0±0.0	0±0.0	0±0.0
	0.375	0±0.0	8±4.5	18±11.0	22±13.0
	0.75	0±0.0	12±4.5	24±5.5	32±8.4
	1.5	0±0.0	24±5.5	44±8.9	54±5.5
	3.0	0±0.0	58±8.4	64±8.9	70±7.1
RSKCP	0.1875	0±0.0	0±0.0	0±0.0	0±0.0
	0.375	0±0.0	12±8.4	20±0.0	20±0.0
	0.75	0±0.0	22±8.4	36±8.4	42±8.4
	1.5	0±0.0	28±8.4	52±8.4	62±11.0
	3.0	0±0.0	66±11.4	78±13.0	86±8.9

Note: 1. JSCCP, *Jatropha curcas* seed coat crude protein; JSKCP, *Jatropha curcas* seed kernel crude protein; RSKCP, *Ricinus communis* seed kernel crude protein

2. The experiments were performed in triplicate and values were expressed as mean ± SD

Figure 19 The larvicidal activity of the crude protein extract and purified toxin from the seed coat of *J. curcas* compared to the crude protein extract from the seed kernels of *J. curcas* and *R. communis* against the third instar larvae of *Aedes aegypti*. Experiments were repeated 5 times.

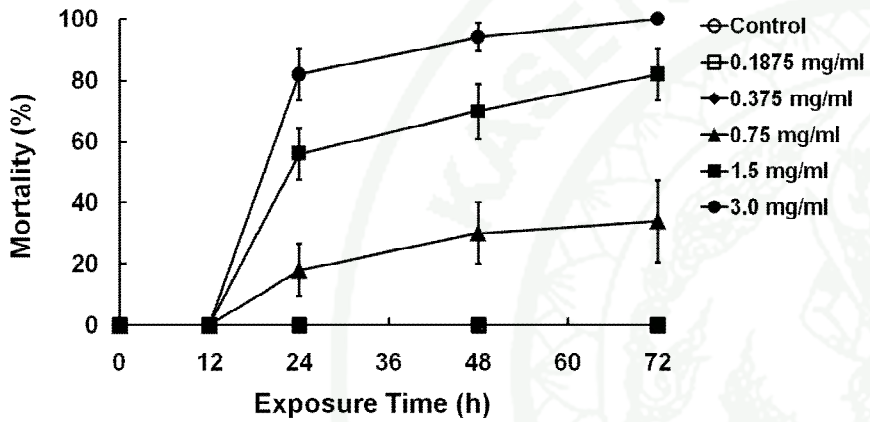
(A) Purified toxin from the seed coat of *J. curcas* (Jc-SCRIP)

(B) *J. curcas* seed coat crude protein (JSCCP)

(C) *J. curcas* seed kernel crude protein (JSKCP)

(D) *R. communis* seed kernel crude protein (RSKCP)

(A)



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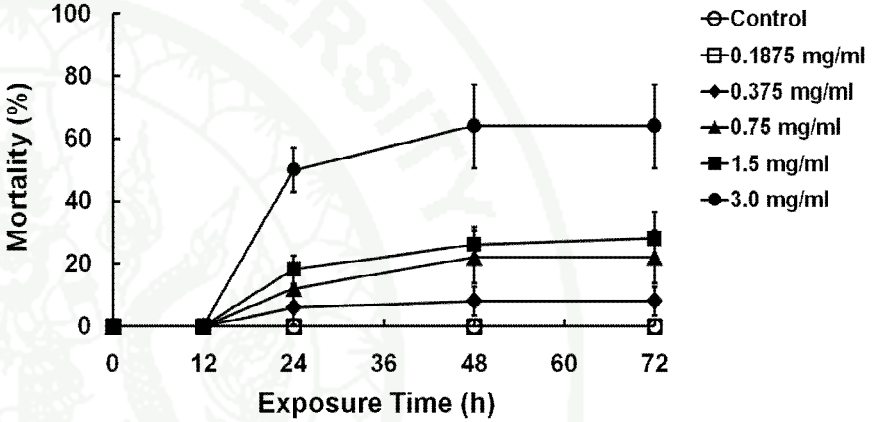

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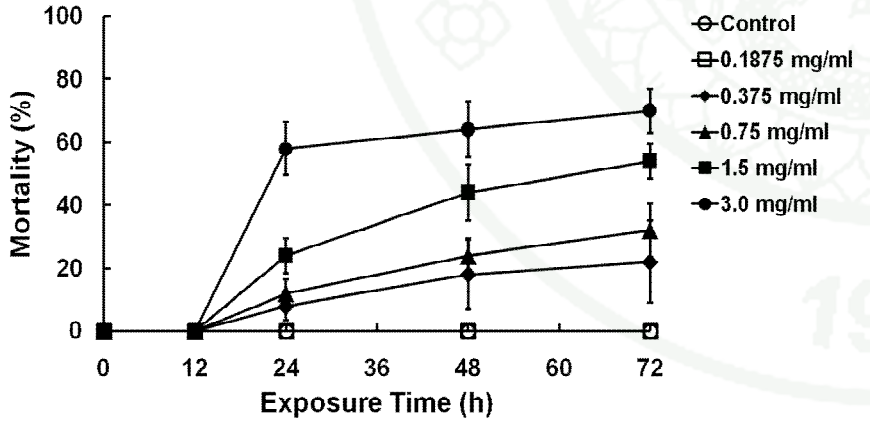

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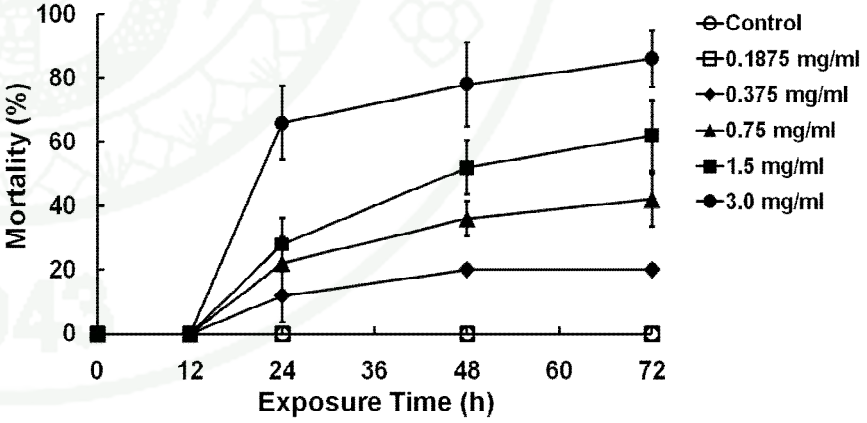


Table 15 The mean % mortalities induced by plant proteins on the third instars larvae of *Culex quinquefascitus* at various concentrations and exposure times

Proteins	Concentration (mg/ml)	Period of Exposure (h)			
		12	24	48	72
Jc-SCRIP	0.1875	64±8.9	76±5.5	84±5.5	90±0.0
	0.375	78±8.4	86±5.5	100±0.0	100±0.0
	0.75	86±8.9	90±5.5	100±0.0	100±0.0
	1.5	100±0.0	100±0.0	100±0.0	100±0.0
	3.0	100±0.0	100±0.0	100±0.0	100±0.0
JSCCP	0.1875	42±13.0	68±11.0	72±13.0	76±13.4
	0.375	66±11.4	82±13.0	94±5.5	94±5.5
	0.75	66±11.4	86±8.9	100±0.0	100±0.0
	1.5	94±5.5	100±0.0	100±0.0	100±0.0
	3.0	100±0.0	100±0.0	100±0.0	100±0.0
JSKCP	0.1875	58±8.4	76±5.5	80±7.1	84±5.5
	0.375	72±4.5	84±5.5	88±4.5	92±4.5
	0.75	84±5.5	92±4.5	100±0.0	100±0.0
	1.5	100±0.0	100±0.0	100±0.0	100±0.0
	3.0	100±0.0	100±0.0	100±0.0	100±0.0
RSKCP	0.1875	72±13.0	80±7.1	100±0.0	100±0.0
	0.375	76±16.7	86±11.4	100±0.0	100±0.0
	0.75	88±8.4	94±5.5	100±0.0	100±0.0
	1.5	100±0.0	100±0.0	100±0.0	100±0.0
	3.0	100±0.0	100±0.0	100±0.0	100±0.0

Note: 1. JSCCP, *Jatropha curcas* seed coat crude protein; JSKCP, *Jatropha curcas* seed kernel crude protein; RSKCP, *Ricinus communis* seed kernel crude protein

2. The experiments were performed in triplicate and values were expressed as mean ± SD

Figure 20 The larvicidal activity of the crude protein extract and purified toxin from seed coat of *J. curcas* compared to the crude protein extract from the seed kernels of *J. curcas* and *R. communis* against the third instar larvae of *Culex quinquefasciatus*. Experiments were repeated 5 times.

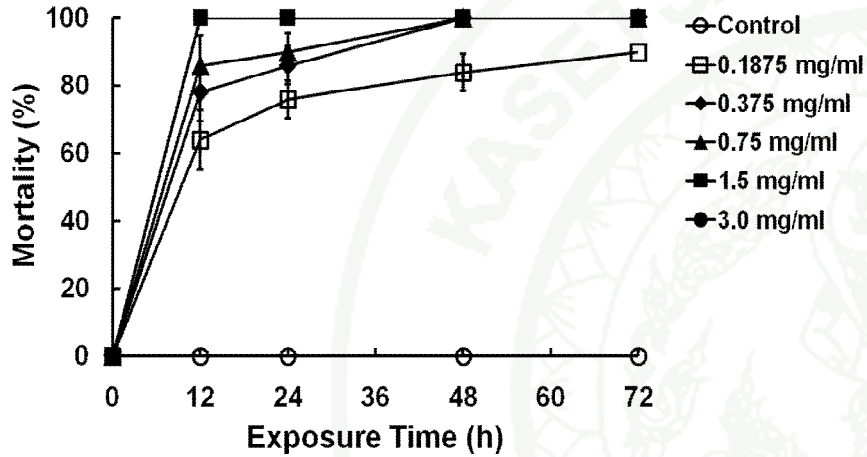
(A) Purified toxin from the seed coat of *J. curcas* (Jc-SCRIP)

(B) *J. curcas* seed coat crude protein (JSCCP)

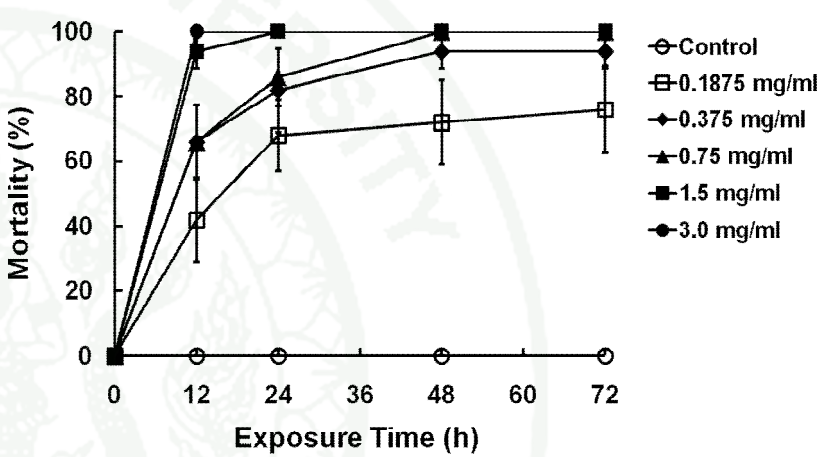
(C) *J. curcas* seed kernel crude protein (JSKCP)

(D) *R. communis* seed kernel crude protein (RSKCP)

(A)



(B)



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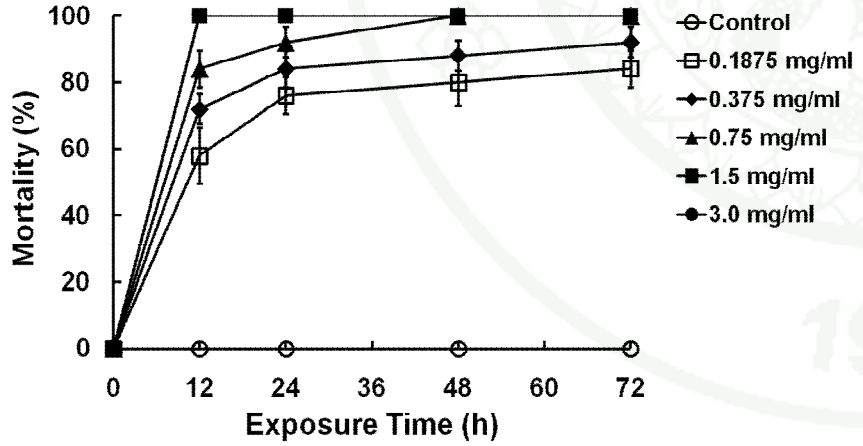


(D)

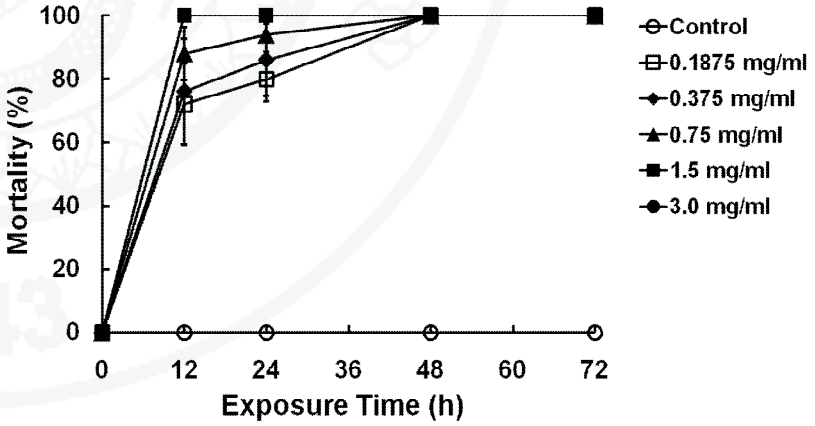


Table 16 The LC_{50} values of plant proteins for the third instars larvae of *Aedes aegypti* and *Culex quinquefasciatus* after 24 h of exposure

Proteins	Mosquito Species	LC_{50} (mg/ml)	Regression equations	R^2	95% confidence intervals (mg/ml)
Jc-SCRIP	*Aedes aegypti*	1.44	Y = 3.066X + 4.498	0.991	0.98 – 2.11
	Culex quinquefasciatus	0.0303	Y = 0.935X + 6.419	0.974	0.0095 – 0.097
JSCCP	*Aedes aegypti*	3.89	Y = 1.646X + 4.035	0.921	2.08 – 7.26
	Culex quinquefasciatus	0.0575	Y = 1.002X + 6.247	0.934	0.0180 – 0.184
JSKCP	*Aedes aegypti*	2.95	Y = 1.766X + 4.172	0.922	1.53 – 5.68
	Culex quinquefasciatus	0.0468	Y = 1.146X + 6.521	0.984	0.0170 – 0.129
RSKCP	*Aedes aegypti*	2.24	Y = 1.653X + 4.420	0.900	1.11 – 4.53
	Culex quinquefasciatus	0.0389	Y = 1.178X + 6.658	0.960	0.0144 – 0.100

Note: 1. JSCCP, *Jatropha curcas* seed coat crude protein; JSKCP, *Jatropha curcas* seed kernel crude protein; RSKCP, *Ricinus communis* seed kernel crude protein

2. LC_{50}, the lethal concentration at 50%; R, coefficient of regression equations
3. The experiments were performed in triplicate and values were expressed as mean ± SD

5.4.2 Larvicidal activity of Jc-SCRIP against Spodoptera worm

The toxicity of the crude protein extract (JSCCP) and the CM-Cellulose purified protein (Jc-SCRIP) from seed coat of *J. curcas* were tested against the second instars larvae of *Spodoptera litura* and *Spodoptera exigua* using leaf-disc method as described in methods (section 1.6.5). The damage of fresh cabbage leaf discs was observed after 6 h by feeding larvae of *S. litura* (Figure 21A) and *S. exigua* (Figure 21B). The damage of leaf discs was time dependent (Figure 22). The percentage of mortality against *S. litura* and *S. exigua* were represented in Table 17 and 18, respectively. No mortality of both larvae species was observed in the control (in the absence of the crude protein extract or purified protein). The crude protein extract had insecticidal activity against both larvae species at protein concentration of 0.50 mg/ml after 72 h of exposure and induced 100% mortality at protein concentration of 5.0 mg/ml after 120 h of exposure (Table 17 and 18, Figure 23B and 24B). The purified seed coat protein, Jc-SCRIP showed insecticidal activity against both insect species at a protein concentration of ≥ 0.05 mg/ml after 72 h of exposure and induced 100% mortality at a protein concentration of 0.25 mg/ml after 120 h of exposure for *S. litura* and that for *S. exigua* at a protein concentration of 0.50 mg/ml after 120 h of exposure (Table 17 and 18, Figure 23A and 24A).

The toxicity of the crude protein extract and purified protein towards both insect larvae was increased depending on both protein concentrations and exposure times (Figure 23 and 24). The median lethal concentration (LC_{50}) of crude protein extract against both insect species after 72 h of exposure were 0.511 and 0.786 mg protein/ml, respectively (Table 19), whereas the LC_{50} of purified protein against those were 0.0525 and 0.0692 mg protein/ml (Table 19). The LC_{50} results indicated that the insecticidal activity of the purified protein, Jc-SCRIP, against *S. litura* and *S. exigua* were approximately 9.7 and 11.3 times more toxic than crude protein extract, JSCCP (Table 19).

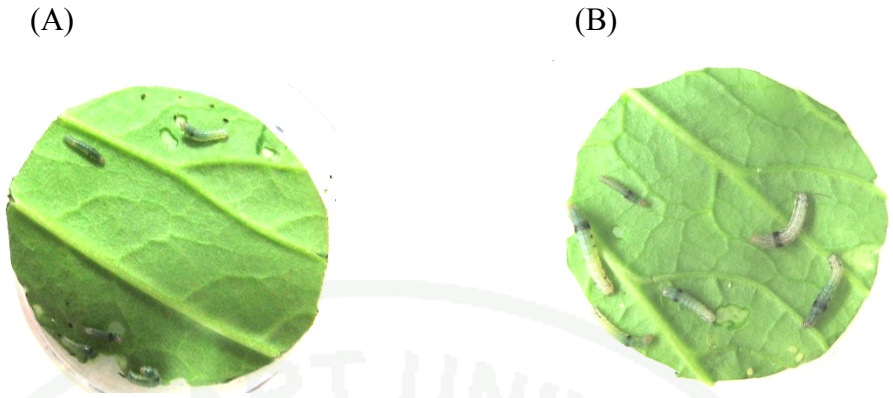


Figure 21 The damage of fresh Chinese cabbage leaf discs after 6 h by feeding larvae of (A) *Spodoptera litura* and (B) *Spodoptera exigua*.

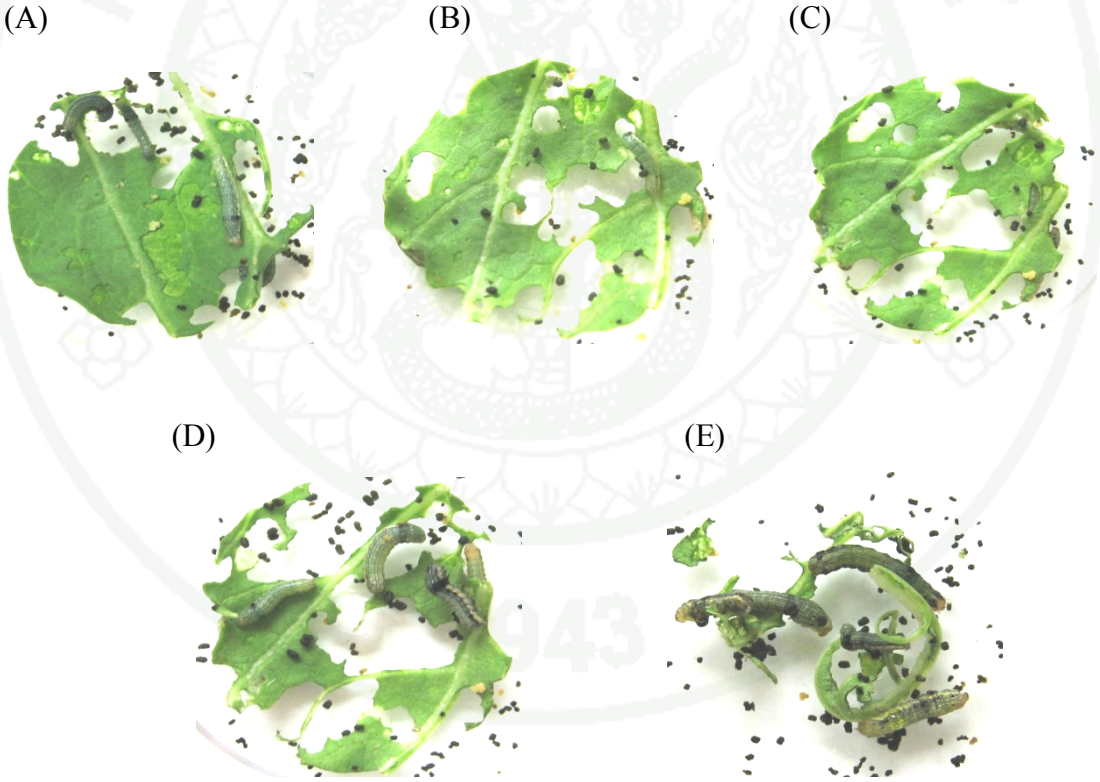


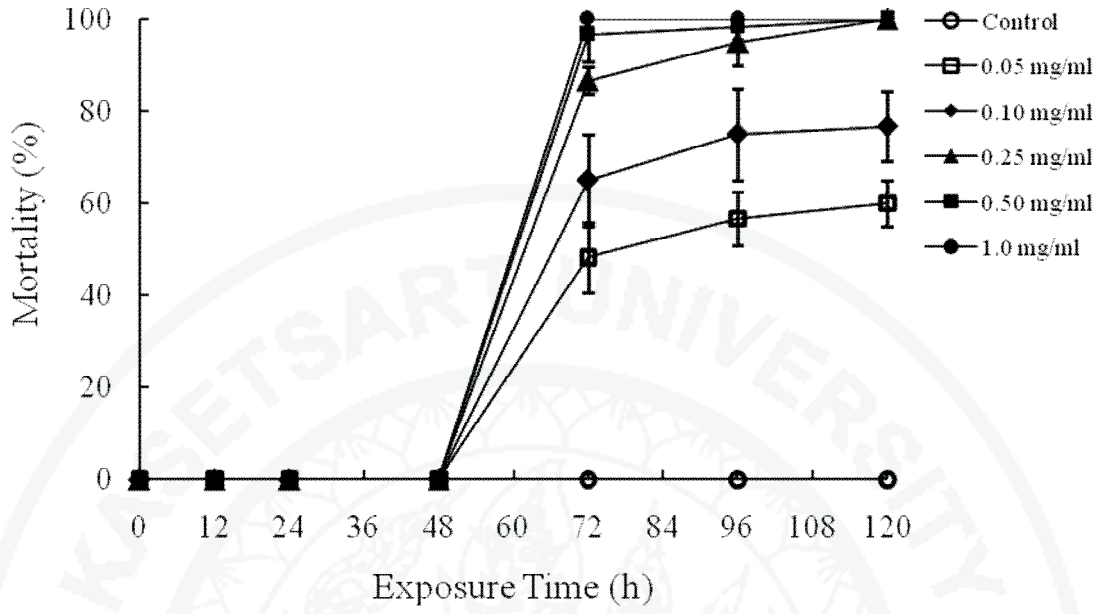
Figure 22 The damage of fresh Chinese cabbage leaf discs after (A) 24 h (B) 48 h (C) 72 h (D) 96 h and (E) 120 h by feeding larvae of *Spodoptera exigua.*

Table 17 The mean % mortalities induced by the crude protein extract (JSCCP) and purified protein (Jc-SCRIP) from seed coat of *Jatropha curcas* against the second instars larvae of *Spodoptera litura* at various concentrations and exposure times

Protein	Concentration (mg/ml)	Period of Exposure (h)					
		12	24	48	72	96	120
Jc-SCRIP	0.05	0±0.0	0±0.0	0±0.0	48.3±7.6	56.7±5.8	60.0±5.0
	0.10	0±0.0	0±0.0	0±0.0	65.0±10.0	75.0±10.0	76.7±7.6
	0.25	0±0.0	0±0.0	0±0.0	86.7±2.9	95.0±5.0	100.0±0.0
	0.50	0±0.0	0±0.0	0±0.0	96.7±5.8	98.3±2.9	100.0±0.0
	1.0	0±0.0	0±0.0	0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
JSCCP	0.5	0±0.0	0±0.0	0±0.0	45.0±8.7	56.7±7.6	70.0±0.0
	1.0	0±0.0	0±0.0	0±0.0	71.7±10.4	80.0±8.7	88.3±2.9
	2.0	0±0.0	0±0.0	0±0.0	81.7±5.8	95.0±0.0	96.7±2.9
	5.0	0±0.0	0±0.0	0±0.0	92.0±7.6	96.7±2.9	100.0±0.0
	10.0	0±0.0	0±0.0	0±0.0	100.0±0.0	100.0±0.0	100.0±0.0

Note: The experiments were performed in triplicate and values were expressed as mean ± SD

(A)



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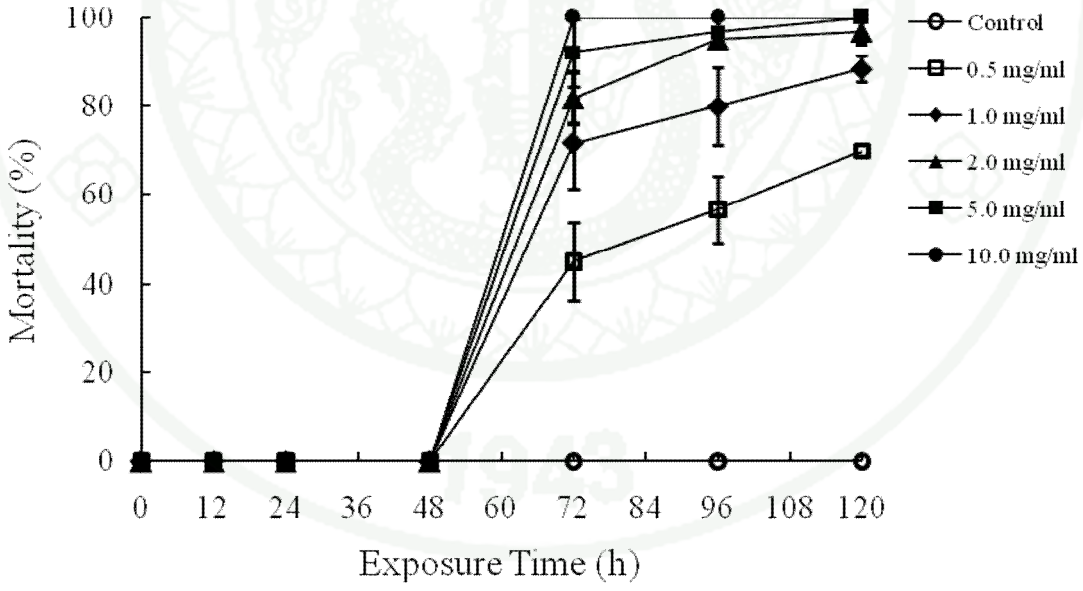

Figure 23 The insecticidal activity of the purified protein (Jc-SCRIP) (A) and crude protein extract (JSCCP) (B) from seed coat of *J. curcas* against the second instars larvae of *Spodoptera litura* at various exposure times and protein concentrations. The experiments were performed in triplicate and values were expressed as mean ± SD.

Table 18 The mean % mortalities induced by the crude protein extract from seed coat of *Jatropha curcas* against the second instars larvae of *Spodoptera exigua* at various concentrations and exposure times

Protein	Concentration (mg/ml)	Period of Exposure (h)					
		12	24	48	72	96	120
Jc-SCRIP	0.05	0±0.0	0±0.0	0±0.0	40.0±5.0	45.0±2.9	48.3±2.9
	0.10	0±0.0	0±0.0	0±0.0	61.7±7.6	73.3±7.6	75.0±5.0
	0.25	0±0.0	0±0.0	0±0.0	81.7±5.8	95.0±5.0	98.3±2.9
	0.50	0±0.0	0±0.0	0±0.0	93.3±7.6	98.3±2.9	100.0±0.0
JSCCP	1.0	0±0.0	0±0.0	0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
	0.5	0±0.0	0±0.0	0±0.0	38.3±7.6	46.7±5.8	61.7±5.8
	1.0	0±0.0	0±0.0	0±0.0	58.3±7.6	71.7±5.8	78.3±7.6
	2.0	0±0.0	0±0.0	0±0.0	70.0±5.0	85.0±0.0	91.7±7.6
	5.0	0±0.0	0±0.0	0±0.0	81.7±7.6	93.3±5.8	100.0±0.0
	10.0	0±0.0	0±0.0	0±0.0	100.0±0.0	100.0±0.0	100.0±0.0

Note: The experiments were performed in triplicate and values were expressed as mean ± SD

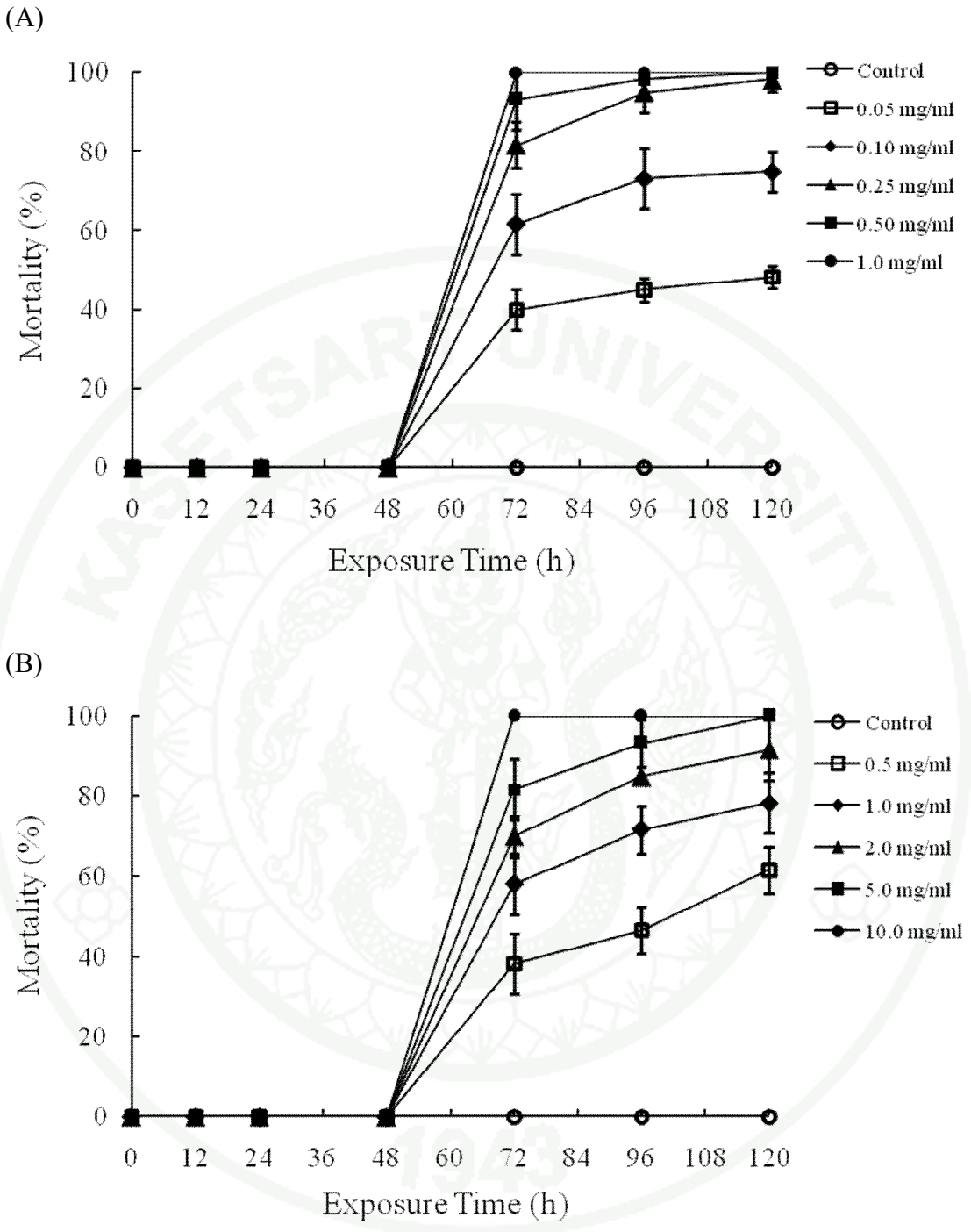

Figure 24 The insecticidal activity of the purified protein (Jc-SCRIP) (A) and crude protein extract (JSCCP) (B) from seed coat of *J. curcas* against the second instars larvae of *Spodoptera exigua* at various exposure times and protein concentrations. The experiments were performed in triplicate and values were expressed as mean ± SD.

Table 19 The LC_{50} values of the purified protein (Jc-SCRIP) and crude protein extract (JSCCP) from seed coat of *Jatropha curcas* against the second instars larvae of *Spodoptera litura* and *Spodoptera exigua* after 72 h of exposure

Proteins	Insect species	LC_{50} (mg/ml)	Regression equations	R^2	95% confidence intervals (mg/ml)
Jc-SCRIP	*Spodoptera litura*	0.0525	Y = 1.444X + 6.850	0.964	0.0321-0.0859
	Spodoptera exigua	0.0692	Y = 1.701X + 6.979	0.998	0.0454-0.1055
JSCCP	*Spodoptera litura*	0.511	Y = 1.489X + 5.434	0.967	0.316-0.825
	Spodoptera exigua	0.786	Y = 1.197X + 5.125	0.983	0.435-1.422

Note: The experiments were performed in triplicate and values were expressed as mean ± SD

6. Molecular cloning of Jc-SCRIP gene

Total RNA was extracted from 100 mg young seed coat (green color) of *J. curcas* using CTAB extraction method as described in methods (section 2.1) and was used as a template for RT-PCR. The isolated total RNA had an A_{260}/A_{280} ratio of 1.85 suggesting that the RNA had high quality without any degradation RNA and genomic DNA contaminated. Yield of the total RNA was approximately 7.5 µg per mg tissues. Two expected bands of 18s and 28s rRNA were observed as shown in Figure 25.

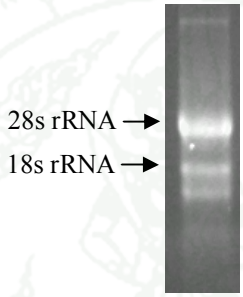

Figure 25 Agarose gel electrophoresis of total RNA from young seed coat of *J. curcas*.

First strand cDNA, resulting from reverse transcribed of total RNA, was amplified with 8 degenerate primers that were designed corresponding to the following peptide fragment from *De novo* sequencing method as shown in methods (section 2.3.2). The result of the amplification using all pair of primers did not generate DNA fragments (Figure 26A and 26B).

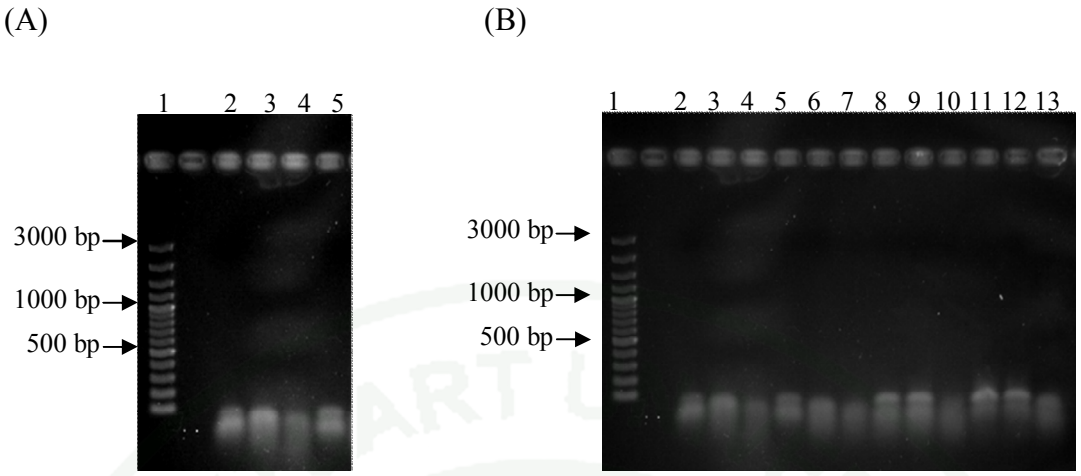

Figure 26 (A) Agarose gel electrophoresis of PCR fragments amplified by primers based on the peptide fragment using *De novo* sequencing method. Lane 1: 100 bp DNA marker; lane 2: F1+R1; lane 3: F2+R2; lane 4: F3+R3; lane 5: F4+R4.

(B) Agarose gel electrophoresis of PCR fragments amplified by primers based on the peptide fragment using *De novo* sequencing method. Lane 1: 100 bp DNA marker; lane 2: F1+R2; lane 3: F1+R3; lane 4: F1+R4; lane 5: F2+R1; lane 6: F2+R3; lane 7: F2+R4; lane 8: F3+R1; lane 9: F3+R2; lane 10: F3+R4; lane 11: F4+R1; lane 12: F4+R2; lane 13: F4+R3.

A new oligonucleotide primer was synthesized based on 7 N-terminal amino acid sequence of Jc-SCRIP using Edman degradation as shown in methods (section 2.3.2). Amplification of Jc-SCRIP cDNA using primer, F0 (5′GCTATTAATGGTGGTGGTGCT3′) and R1 (5′GTRTARTCRTTNGTNARRAA NGCNGC3′) generated a single band of approximately 500 bp (Figure 27, lane 8).

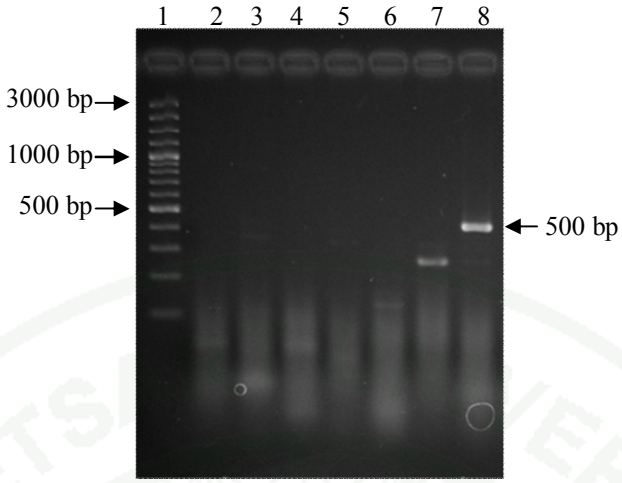

Figure 27 Agarose gel electrophoresis of PCR fragments amplified by primers designed based on N-terminal amino acid sequence of Jc-SCRIP. Lane 1: 100 bp DNA marker; lane 2: *ACTIN* gene; lane 3: *CURCIN* gene; lane 4: 18s rRNA gene of *J. curcas*; lane 5: F0+R4; lane 6: F0+R3; lane 7: F0+R2; lane 8: F0+R1.

DNA fragment of 500 bp was purified from agarose gel and ligated to pGEM-T easy vector. The ligation mixture was transformed into competent cells of *E. coli* DH5α and the transformant was screened on LB agar plates containing 100 μg of ampicillin and 20 mg/ml of X-gal. Approximately 3 white colonies and 5 blue colonies were obtained. Three white colonies were selected for plasmid isolation. All of these clones were found to have inserted fragment (Figure 28, lane 2). One clone containing inserted fragment was selected for sequencing.

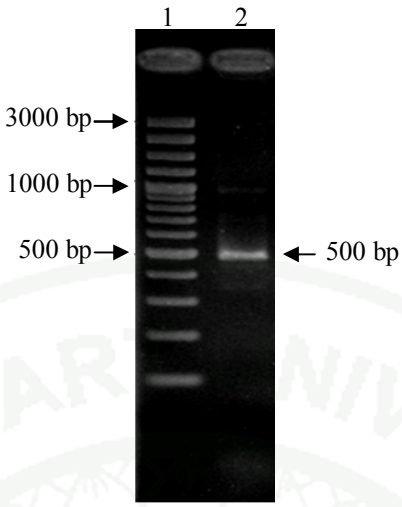

Figure 28 Determination of inserted DNA size by PCR. Lane 1: 100 bp DNA marker; lane 2: inserted DNA. PCR products were separated on 1.0% agarose gel in 0.5× TBE buffer at 100 volts for 1 hour, stained with EtBr and visualized under UV transilluminator.

Sequencing of the inserted cDNA was performed at the Macrogen Inc. (Korea) by 3730×l DNA analyzer. The data were recorded by the computer and the results were displayed either as a graph or as a text sequence. Homologous sequences were sought using the Basic Alignment Search Tool (BLAST). Related sequences were cited from the GenBank database. The nucleotide sequence of cloned cDNA shared 98% identity to aquaporin from *J. curcas* (Figure 29A), 89% identity to aquaporin of *Hevea brasiliensis*, and 87% identity to aquaporin of *Manihot esculenta*. The amino acid sequence of cloned cDNA shared 98% identity to aquaporin from *J. curcas* (Figure 29B), 95% identity to aquaporin of *Manihot esculenta*, and 93% identity to aquaporin of *R. communis*.

(A)

```
Cloned cDNA   65  TGGTGGTGTTGCTGCGGAAAGATCCCAAAGCTTTGATAGCTGCTGCTCTCAGTATGTATT  124
Aquaporin    838  TGGTGGGGTTGCTGCGGAAAGATCCCAAAGCTTTGATAGCTGCTGCTCTCAGTATGTATT  779
Cloned cDNA  125  GATGGTATGCAGCTGCTGCAATTGCTCCAACAAACGGTCCAACCCAGAAAATCCAATGAT  184
Aquaporin    778  GATGGTATGCAGCTGCTGCAATTGCTCCAACAAACGGTCCAACCCAGAAAATCCAATGAT  719
Cloned cDNA  185  CATCCCAGACTTTATCATTGTTGTAAATAACAACAACACCGAAGCTCCTCGCTGGGTTAA  244
Aquaporin    718  CATCCCAGACTTTATCATTGTTGTAAATAACAGCAGCACCGAAGCTCCTCGCTGGGTTAA  659
Cloned cDNA  245  TACCAGTACCAGTAATGGGTATGGTAGCCAAATGAACCATAAAAACTGCAAACCCAATTG  304
Aquaporin    658  TACCAGTACCAGTAATGGGTATGGTAGCCAAGTGAACCATAAAAACTGCAAACCCAATTG  599
Cloned cDNA  305  GAAGTGGAGCCAAAATAGGAACGTGTGAATCACGTGCACTTCTCTTGGGGTCAATTGCAG  364
Aquaporin    598  GAAGTGGAGCCAAAATAGGAACGTGTGAGTCACGTGCACTTCTCTTGGGGTCAGTTGCAG  539
Cloned cDNA  365  AGAAAACAGTGTAGACTAGAACAAAAGTGCCGATGATCTCAGCACCCAAAGCATTTCCTT  424
Aquaporin    538  AGAAAACAGTGTAGACTAGAACAAAAGTGCCGATGATCTCAGCACCCAAAGCAGTTCCTT  479
Cloned cDNA  425  TGCTATAGCCGGTATTTACAGAGTTAGCAACACCACCA  462
Aquaporin    478  TGCTATAGCCGGTATTTACAGAGTTAGCACCACCACCA  441
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(B)

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Cloned cDNA   85   GG ANSVN GYS GTALGAEIIGTFVLVYTVFSATDPKRSARDSHVP LAPLPIGFAVFM  264
Aquaporin    148   GGGANSVNTGYSKGTALGAEIIGTFVLVYTVFSATDPKRSARDSHVPILAPLPIGFAVFM  207
Cloned cDNA  265   VHLATIPITGTGINPARSFGAAVIYNNDKVWDDHWIFWVGPFVGAIAAAAYHQYILRAAA  444
Aquaporin    208   VHLATIPITGTGINPARSFGAAVIYNNDKVWDDHWIFWVGPFVGAIAAAAYHQYILRAAA  267
Cloned cDNA  445   IKALGSFRSN T  480
Aquaporin    268   IKALGSFRSNPT  279
```

Figure 29 Nucleotide and amino acid sequence of cloned cDNA from young seed coat (green color) of *J. curcas*.

Total RNA was extracted and amplified by PCR method. A single band of approximately 500 bp was generated. It was further purified and sequenced. Homologous sequences were sought using the Basic Alignment Search Tool (BLAST) and related sequences were cited from the GenBank database. Both nucleotide (A) and amino acid (B) sequence of cloned cDNA shared 98% and 98% identity with aquaporin from *J. curcas*, respectively.

A new condition for amplification of Jc-SCRIP cDNA was done. F0 (5′GCTATTAATGGTGGTGGTGCT3′) and poly $d(T)_{18}$ were used as forward and reverse primer, respectively. PCR condition was run as following: preheated at 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at various temperatures for 30 s, and extension at 72°C for 1.30 min. The final extension was carried out at 72°C for 10 min. Several annealing temperatures (T_m) were tested at 37, 40 and 45°C. Multiple bands of approximately 450, 800 and 1000 bp were generated (Figure 30).

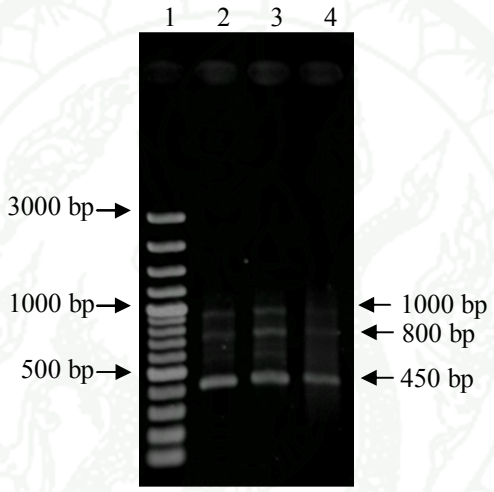

Figure 30 Agarose gel electrophoresis of PCR fragments from various annealing temperatures. Lane 1: 100 bp DNA marker; lane 2: T_m at 37°C; lane 3: T_m at 40°C; lane 4: T_m at 45°C.

DNA fragment of 1000 bp amplified using Tm at 40°C (Figure 30, lane 3) was chosen to purify because it showed the highest intensity. The purified DNA was ligated to pGEM-T easy vector and transformed into competent cells of *E. coli* DH5α. Inserted cDNA was selected to sequence at the Macrogen Inc. (Korea) by 3730×l DNA analyzer. The nucleotide and amino acid sequence of cloned cDNA had no similarity to any sequence from the Genbank database.

A new degenerate oligonucleotide primer was synthesized based on 7 N-terminal amino acid sequence of Jc-SCRIP and conserved sequences of several type 1 RIPs as shown in Table 20. PCR condition was run as following: preheated at 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1.30 min. The final extension was carried out at 72°C for 10 min.

Table 20 Degenerate primers were used for cloning of Jc-SCRIP cDNA

Primer	Sequence
Forward	F0: 5′GCTATTAATGGTGGTGTTGCT3′
	F5: 5′GCNATYAAYGGNGGNGTNGCN3′
Reverse	R5: 5′TTTGAATCTTGCTGCCTC3′
	R6: 5′RTANCKNGCNGCYTC3′

Where N = A,C,G, or T; Y = C or T; R = A or G; K = T or G

Amplification of Jc-SCRIP cDNA using primer F5+R6 generated a single band of approximately 400 bp (Figure 31, lane 4). It was purified for further cloning steps. Inserted cDNA was selected to sequence and the results showed that the nucleotide and amino acid sequence of cloned cDNA had no similarity to any sequence from the Genbank database.

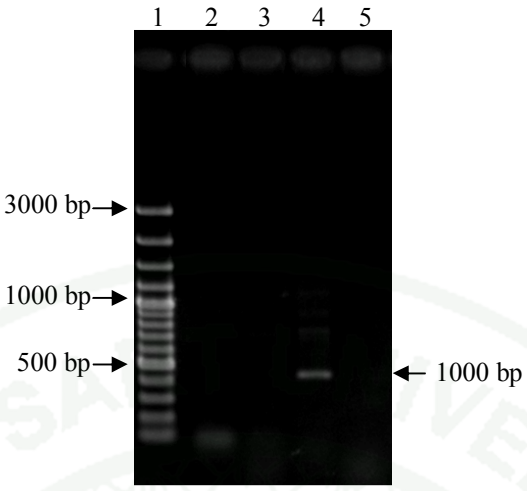

Figure 31 Agarose gel electrophoresis of PCR fragments amplified by degenerate primers designed based on N-terminal amino acid sequence of Jc-SCRIP and conserved sequences of several type 1 RIPs. Lane 1: 100 bp DNA marker; lane 2: F0+R5; lane 3: F5+R5; lane 4: F0+R6; lane 5: F5+R6

All pairs of oligonucleotide primers were used in cloning of Jc-SCRIP cDNA were summarized in Table 21.

Table 21 Summary of all pairs of oligonucleotide primers were used in cloning of Jc-SCRIP cDNA

Forward primer	Reverse primer	Size of DNA fragment (bp)
F0	R1	500
	R2	250
	R3	-
	R4	-
	R5	-
	R6	400
	$poly(dT)_{18}$	450, 850, 1000
F1	R1	-
	R2	-
	R3	-
	R4	-
F2	R1	-
	R2	-
	R3	-
	R4	-
F3	R1	-
	R2	-
	R3	-
	R4	-
F4	R1	-
	R2	-
	R3	-
	R4	-
F5	R5	-
	R6	-

Discussion

In this study, a new type 1 ribosome inactivating protein, designated Jc-SCRIP, has been discovered in the seed coat of *J. curcas*. In this thesis, four major parts of the results will be discussed. Firstly, the approach to purify Jc-SCRIP will be discussed. This will be followed by a discussion of its physiological activities and molecular properties. The last part will be deal with its application biological activities.

1. Purification and characterization of Jc-SCRIP

Due to the expectation that Jc-SCRIP may be present in small quantity in the seed coat of the Jatropha, an anion-exchange chromatography on DEAE-Sephacel™ has been used. Ion-exchange chromatography has been widely employed in the isolation of many ribosome-inactivating proteins from various sources including Flammin (Ng and Wang, 2004), Flammulin (Wang and Ng, 2000), Volvarin (Yao *et al*., 1998), Laffacylin (Parkash *et al*., 2002), Laffangulin (Wang and Ng, 2002) and Marmorin (Wong *et al*., 2008). Using the DEAE-Sephacel™ column at pH 7.5, Jc-SCRIP has not been retained in the column. The following chromatographic approach is then use cation-exchange chromatography on CM-Cellulose column at pH 5.5. This column step has worked satisfactorily since it gives only one bound peak (Figure 7) of 113.5 fold purification of Jc-SCRIP (Table 9). The low recovery of the hemagglutination activity (1.12%) at this purification step may be due to the hemagglutination activity in the crude extract is caused by lectins and other non-specific agents that can caused the observed hemagglutination.

2. Physiological activities of Jc-SCRIP

Jc-SCRIP has both lectin activity and RNA *N*-glycosidase activity. The lectin property is indicated by its induction of rabbit erythrocyte agglutination (Figure 8) and hemagglutination inhibition by a glycoprotein, fetuin at 976.6 μM. The lack of inhibition by specific monosugar of Jc-SCRIP may indicate that it may not a real

lectin. Lectins are known to be protein or glycoprotein of multiple binding sites for a specific carbohydrate (Rüdiger and Grabias, 2001; Ambrosi *et al*., 2005). Type 2 ribosome-inactivating proteins consist of a lectin subunit and *N*-glycosidase subunit (Peuman *et al*., 2001; Stirpe and Battelli, 2006). Jc-SCRIP has been shown to be a monomeric protein (Figure 10 and 11) with RNA *N*-glycosidase activity (Figure 15), therefore, it can be concluded that Jc-SCRIP is a type 1 ribosome-inactivating protein with lectin-like activity.

The RNA *N*-glycosidase activity of Jc-SCRIP may be the cleavage of glycosidic linkage between a specific adenine residue and pentose in 28S rRNA. In this experiment the cleavage has been shown by the release of a new rRNA fragment after acidic aniline treatment. Aniline treatment causes the phosphodiester bond to become sensitive to cleavage and release adenine. The size of the rRNA fragment released by Jc-SCRIP is about 560 nucleotides, which is larger than those obtained from the other type 1 RIPs (about 450–460 nucleotides) (Table 4, see Literature review). This may be due to different sensitivities of the various sources of 28S rRNA to the different RIPs (Ippoliti *et al*., 1992). Moreover, the release of an rRNA fragment of about 550 nucleotides from rabbit reticulocyte lysate by foetidissimin, a type 2 RIP from the roots of *Cucurbita foetidissima*, has been reported (Zhang *et al*., 2003). The RNA *N*-glycosidase activity of Jc-SCRIP is more or less strong as the crude extract of type 2 RIP, ricin (Figure 15). The result suggests that it should be very toxic against some eukaryotic cells if it can bind to the cell membrane or pass through it into the cytosol.

3. Molecular properties of Jc-SCRIP

SDS-PAGE analysis of Jc-SCRIP purified from CM-Cellulose column demonstrated an intense band of approximately 41,800 Da (Figure 10), indicating purification to homogeneity. Difference in molecular mass of Jc-SCRIP was observed by MALDI-TOF/MS, which showed a protonated molecular ion peak at m/z 38,938.291 (Figure 11). The results revealed that Jc-SCRIP was a monomeric protein. It is larger than curcin (Mw = 29,000 Da), the other type 1 RIP isolated from the seed

kernels of *J. curcas* (Kittikajhon *et al*., 2010). Its molecular size compares favourably with those of flammulin (Mw = 40,000 Da) from fruiting bodies of the mushroom, *Flammulina velutipes* (Wang and Ng, 2000); and pleuturegin (Mw = 38,000 Da) from sclerotia of the edible mushroom, *Pleurotus tuber-regium* (Wang and Ng, 2001).

Jc-SCRIP had β-sheet as a predominant secondary structure with minor α-helices, turn and unordered structure (Figure 12 and Table 11), similar to that of curcin (Lin *et al*., 2010). Some type 1 RIPs were mixed α+β structure. For example, saporin had the CD profile with the broad spectrum over 208-222 nm (Ghosh and Batra, 2006). Trichomislin had the ratios of α-helices and β-sheets of 13.6 and 18.0% while those of trichosanthin were 12.2 and 16.3%, respectively (Mi *et al*., 2005).

The sugar contents of Jc-SCRIP were about 4.80% (w/w) of protein. Type 1 RIPs have difference total neutral sugar content. For example, gelonin (Falasca *et al*., 1982), AAP-27 (Roy *et al*., 2006) and curcin (Lin *et al*., 2010) contain sugar content of 4.46, 4.0 and 4.91% (w/w) of protein, respectively whereas abelesculin from latex of *Hura crepitans* and Celosia AVPs from leaves of *Celosia cristata* have a high neutral sugar contents of 39.9 and 26-28% (w/w) of protein, respectively. Total neutral sugar content of other type 1 RIPs are summarized in Table 4 (see Literature review).

Jc-SCRIP was the heat-stable toxic protein because its *N*-glycosidase activity was not lost even with incubation of the protein at 37°C up to 100°C for 30 min. The high thermal resistance of Jc-SCRIP may be due to its glycosylation. Carbohydrate moieties have been suggested to play roles in stabilizing and folding of glycoproteins (Wang *et al*., 1996). Recently, in a study of various isoforms of ricin, the toxic type 2 RIP from the seeds of *R. communis* suggests that variations in the oligosaccharide structure present in ricin plays a significant role in the solubility, stability and thermal denaturation of the glycoproteins (Sehgal *et al*., 2011). Several unique molecular properties of Jc-SCRIP previously described including high molecular weight, low pI at 4.8, carbohydrate contents distinguish it from those known type 1 RIPs as shown in Table 4 (see Literature review).

4. Molecular cloning of Jc-SCRIP gene

RNA form several plant seeds which are rich in oil, polysaacharides and other secondary metabolites may be difficult to isolate by commercial RNA isolation kit (e.g. TRIZOL and QIAGEN) because those may limit RNA extraction yield and further enzymatic reactions (Sangha *et al*., 2010). Contamination of polysaccharide cause highly viscous of RNA preparation and may interfere several biological activity of several enzymes such as *Taq* polymerase (Fang *et al*., 1992), ligase and restriction endonuclease (Shioda *et al*. 1987). So that total RNA from young seed coat (green color) of *J. curcas* was extracted using the CTAB extraction method instead. CTAB, a cationic detergent, is used in the extraction buffer with a concentration of 2% (w/v) to aid lysis of cell membrane and for separation of polysaccharide from RNA (Doyle and Doyle, 1990). Polyvinylpyrrolidone 40 (PVP-40) and β-mercaptoethanol are used as reducing agents. PVP-40 can complex with secondary metabolites such as polyphenols, tannins and quinines. While β-mercaptoethanol protects RNA against oxidation of phenolic compounds (Jaakola *et al*., 2001). Total RNA was extracted from forest trees using CTAB extraction method. A high quality of total RNA was obtained by the visualization on agarose gel and spectrophotometry analysis indicated a high purity from protein contamination. Alternatively, a commercial protocol (QIAGEN plant extraction method) was used but the yield was very low (Provost *et al*., 2007).

Since limited or no sequence information about seed coat RIP of *J. curcas*, the degeneracy of the primers was designed based on the 7 N-terminal amino acid residues which were sequenced by Edman degradation method and the peptide fragment using *De novo* sequencing method. But attempts to clone cDNA encoding for Jc-SCRIP from seed coat of *J. curcas* was unsuccessful. Its nucleotide and amino acid sequence shared 98% identity to aquaporin from *J. curcas*. Jc-SCRIP may not be express in young seed coat. Expression patterns of Jc-SCRIP at various stages of growth should be followed. Primers may be not suitable so new primers should be tried.

5. Application biological activities of Jc-SCRIP

Plant RIPs have been reported to have various biological functions, including antifungal (Lam and Ng, 2001; Ng and Parkash, 2002; Parkash *et al*., 2002), antiviral (Stirpe *et al*., 1986; Zhang and Halaweish, 2003; Wong *et al*., 2008), anticancer (Lam and Ng, 2001; He and Liu, 2003; Chuethong *et al*., 2007; Wong *et al*., 2008), and insecticidal activities (Zhou *et al*., 2000; Carlini and Grossi-de-Sá, 2002; Wei *et al*., 2004; Shahidi-Noghabi *et al*., 2008). Their toxicities may be exploited for applications in both medicine and agriculture.

5.1 Antimicrobial activity of Jc-SCRIP

Jc-SCRIP has a broad spectrum of strong antimicrobial activity against various human-pathogenic bacteria and yeast. The mechanism of the antimicrobial action of Jc-SCRIP has not been elucidated in detail yet, but a possible mechanism can be suggested. A type 1 RIP, TRIP from the leaf of tobacco (*Nicotiana tabacum*), has shown inhibitory activity against *Trichoderma reesei* and *Pseudomonas solanacearum* due to its *N*-glycosidase activity which cleaves the microbial rRNA (Sharma *et al*., 2004). So it can be suggested that Jc-SCRIP also inhibits microbial growth by its *N*-glycosidase action.

5.2 Cytotoxicity of Jc-SCRIP against some human cancerous cell lines

Breast adenocarcinoma MCF-7, colon adenocarcinoma SW620, and hepatoma HepG2 cells exhibit differences in sensitivity to the potent cytotoxic activity of Jc-SCRIP. Breast adenocarcinoma MCF-7 cells are the most sensitive to Jc-SCRIP followed by SW620 cells and hepatoma HepG2 cells, respectively. This finding is in accordance with previous reports on the variation in cytotoxicity of other type 1 RIPs, in which the difference in cell type accounts for the different cytotoxic response against the RIPs. Curcin from the kernel of *J. curcas* strongly inhibits the growth of gastric cancer SGC-7901, mouse myeloma Sp2/0 and human hepatoma, respectively (Lin *et al*., 2003). Cochinin B from the seed of *Momordica*

cochinchinensis manifests strong anticancer activities on human cervical epithelial carcinoma (HeLa), human embryonic kidney HEK293 and human small cell lung cancer NCI-H187 cell lines, respectively (Chuethong *et al*., 2007). Marmorin from the mushroom *F. velutipes* possesses potent antiproliferative activity toward hepatoma HepG2 and breast cancer MCF-7 cells, respectively (Wong *et al*., 2008). It seems that the variation in cytotoxicity of RIPs to different cells may be dependent upon the mechanisms affecting their entry into the cells. A study with saporin and trichosanthin, two potent type 1 RIPs, has indicated that the alpha 2-macroglobulin receptors (alpha 2MR) expressed in cancer cell lines, including breast cancer T47D and BT-20, mediate the complex interaction between the RIPs and the cellular membrane (Cavallaro *et al*., 1995; Chan *et al*., 2000). Despite the lack of data on the expression of alpha 2-macroglobulin receptors on various cancer cells, it may be assumed that there is a difference in the expression of the Jc-SCRIP receptor on the different cells used in the present study, where MCF-7 shows the highest expression. In other words, the varying cytotoxicity of each RIP toward different kinds of cells may be explained by differences in the downstream apoptosis pathways or programmed cell death (Narayanan *et al*., 2005).

The cytoxic effect of ellipticine, the anticancer drug to the normal (Vero) cells has been previously reported with IC_{50} of 7.47 μM (Kumrit *et al*., 2010), 3.82 μM (Songsiang *et al*., 2011) and 2.7 μM (Isaka *et al*., 2011). Its mechanism of cytotoxicity is intercalation into DNA and inhibition of DNA topoisomerase II activity (Aimova *et al*., 2007). In this research, Jc-SCRIP (IC_{50} = 2.57 μM) has toxicity to normal cell about 60 times lesser than ellipticine (IC_{50} = 0.04 μM) (Figure 16C and Table 13). It can be noticed that Jc-SCRIP shows more far difference in IC_{50} values for cancerous cells and IC_{50} values for the normal cell than ellipticine. This may imply that application use of Jc-SCRIP as an anticancer agent should be more safety for the normal cell than the commercial anticancer drug.

Human breast cancer, colon cancer and liver cancer all are serious global health problems. In 2009, breast cancer was the most common cancer in Thai women. Colon cancer was the second most frequent in Thai men, and the third in Thai women. Liver cancer was third in frequency in Thai men and fifth in Thai women (National Cancer Institution, 2009). Based on the strongest anticancer activity of Jc-SCRIP to MCF-7 cell line, we suggest that Jc-SCRIP may be developed as an effective alternative agent to combat breast cancer.

5.3 Anti HIV-1 reverse transcriptase activity of Jc-SCRIP

Jc-SCRIP was also tested for the inhibitory activity of HIV-1 reverse transcriptase (Figure 17). Its inhibitory activity may be false positive due to the presence of 350 base pair-polyribonucleotide templates in the reaction mixture. Some RIPs can release adenine residue from non-ribosomal substrate. Saporin-L1 can release adenine residues from poly(A), mRNA, tRNA, and DNA (Barbieri *et al*., 1996; Park *et al*., 2004). But the N-glycosidase activity on a non-ribosomal substrate requires a high ratio of protein: substrate (Park *et al*., 2004). To confirm that Jc-SCRIP does not cleave the RNA-templates, the resulted RNA fragment should be determined after incubation Jc-SCRIP with the template.

5.4 Larvicidal activity of Jc-SCRIP against some mosquitoes

To develop the use of the seed proteins from *J. curcas* in mosquito bio-control, Jc-SCRIP has been evaluated for its potent larvicidal activity against the third instars larvae of two mosquitoes, *Ae. aegypti* and *Cx. quinquefasciatus*, and has been compared to the crude protein extracts of the plant seed coats (JSCCP) and seed kernels (JSKCP). Its larvicidal activity has also been compared to the crude protein extract of another plant, *R. communis* Linn. (RSKCP), which is the source of the highly toxic protein, ricin.

The parasporal crystal of *Bacillus thuringiensis* subsp. *israelensis*, or δ-endotoxin, is one of the biopesticides used to control the mosquito population (Panbangred *et al*., 1979; Temeyer, 1984). The larvicidal activity of δ-endotoxin involves ingestion of the crystal protein (solubilized in alkaline solution) of a host to induce lysis of the midgut epithelial cells, which leads to paralysis and death of the host within a short time (Khawaled *et al*., 1992; Cooper, 1994). The toxin is used to control highly concentrated mosquito populations of *Ae. aegypti* larvae (Liles and Dunn, 1959; Panbangred *et al*., 1979). The toxicity of this substance is heat sensitive (Panbangred *et al*., 1979). In addition to the bacterial toxin, a large number of plants have been examined for photochemical that may provide biological control of the medically important mosquitoes.

However, few studies are focused on plant protein toxins. Conversely, many studies have been performed on the application of plant toxic proteins in bio-pesticide development (Carlini and Grossi-de-Sá, 2002; Chowdhury *et al*., 2008; Kamaraj *et al*., 2011). Recently, cinnamomin, a new type 2 RIP found in the seeds of the camphor tree, *Cinnamomum camphora*, has been demonstrated to have toxic effects on the larvae of the mosquito, *Cx. pipines pallens* (Zhou *et al*., 2000). The extract of the castor bean, *R. communis* Linn*.,* has been reported to exhibit strong larvicidal effects on the diamondback moth, *Plutella xylostella*, due to the presence of the highly toxic type 2 RIP, ricin (Tounou *et al*., 2011). Ricin belongs to the group of type 2 RIPs, which are distinguished from type 1 RIPs by the presence of the B chain (Lord *et al*., 1994). The A chain of ricin possesses RNA *N*-glycosidase activity that results in the cleavage of a specific adenine base from ribosomal RNA, causing the inactivation of the ribosome and inhibition of protein synthesis. The lectin subunit, also known as the B chain, of ricin plays an important role of binding to the cell surface glycoconjugates of target cells and facilitates the internalization and translocation of the toxin to cytosol. The type 1 RIPs are much less cytotoxic due to the lack of the B chain. Although numerous plant RIPs and their biochemical properties were identified, the mechanism that leads to mosquito larvae toxicity is still unclear. The toxicity of those RIPs may be a result of their rRNA *N*-glycosidase activities, which inhibit protein synthesis in the larvae.

The toxicity of *J. curcas* is known to contain various toxic components, including phorbol ester, saponin, curcin, protease inhibitors and curcalonic acid (Kumar and Sharma, 2008; Acda, 2009), but no previous studies have reported the mosquito larvicidal activities of the *J. curcas* seed proteins. Many studies about the effect of methanol- and petroleum ether-extracted leaf lysate from *J. curcas* have been reported against several mosquito species, *Anopheles arabiensis*, *An. stephensi, Ae. aegypti, and Cx. quinquefasciatus*, (Rahuman *et al*., 2007; Sakthivadivel and Daniel, 2008; Kovendan *et al*., 2011; Zewdneh *et al*., 2011). Because both curcin (Lin *et al*., 2003; Kittikajhon *et al*., 2010) from the kernel and Jc-SCRIP from the seed coat of *J. curcas* are toxic type 1 RIPs with *N*-glycosidase activity. The highly active larvicidal activity of Jc-SCRIP as well as the crude protein extracts from the seed coat (JSCCP) and seed kernel (JSKCP) of *J. curcas* may reflect the presence of the rRNA *N*-glycosidase activities. However, the exact mechanism underlying the mortality of the mosquito larvae, the ecological effects and field trials should be examined for further understanding of the use of these proteins as biopesticides for mosquito control. To our knowledge, this is the first report describing the larvicidal activities of a type 1 RIP, Jc-SCRIP, from the seed coat of *J. curcas* compared to the crude protein extracts from the plant seed coat and seed kernel against the late third instars larvae of two medically important mosquitoes, *Ae. aegypti* and *Cx. quinquefasciatus*. In addition to being isolated from an abundant and inexpensive agricultural source, the strong larvicidal activity of JSCCP and Jc-SCRIP to the mosquito larvae of *Ae. aegypti* and *Cx. quinquefasciatus* suggests the possibility that further development of these seed proteins may provide new larvicides for use in mosquito control.

5.5 Insecticidal activity of Jc-SCRIP against Spodoptera worms

Due to several plant RIPs have been reported to involve defense mechanism with insecticidal activity, in this experiment Jc-SCRIP is explored for biopesticide activity against two agricultural important worms, *S. litura* and *S. exigua*. They are believed to have a high impact on destruction of several important economic plants such as corn, cotton, castor oil plant, cabbage and other green leafy vegetables. The second instars larva of *S. litura* is more susceptible to Jc-SCRIP than that of *S.*

exigua (Table 19). Two type 2 RIPs, ricin and saporin can extremely kill two Coleopteran species with average LD_{50} values of less than 0.01 % (w/dry weight) (Gatehouse *et al*., 1990). The protease-activated form of maize seed ribosome-inactivating protein (b-32) has significant toxicity to caterpillar of *Trichoplusia ni*, *Spodoptera frugiperda* and *Ostrinia frugiperda* when feed in diets at 1 mg/ml of diet with mortality ranged 0-70% (Dowd *et al*., 1998). Cinnamomin, a type 2 RIP from seeds of *Cinnamomum camphora* has been tested for the toxicity to bollworm (*Helicoverpa armigera*) during larval stage. The LC_{50} of cinnamomin to the bollworm larvae is 1839 ppm (Zhou *et al*., 2000). Its toxicity to domestic silkworm (*Bombyx mori*) larvae has been performed by oral feeding bioassay comparatively to ricin. The LC_{50} of ricin to the silkworm larvae at the third instars was much lower than that of cinnamomin. In addition, the LC_{50} of both ricin and cinnamomin increased with the development of the domestic silkworm larvae (Wei *et al*., 2004). SNA-I, a type 2 RIPs from the bark of elderberry, *Sambucus nigra* L. has been investigated for the insecticidal potency on two Hemipteran insect species using two different methods : (i) difference concentrations of the purified SNA-I have been supplemented to artificial diet. The result shows that they can reduce survival and fecundity of pea aphids, *Acyrthosiphon pisum*. (ii) feeding of tobacco aphids, *Myzus nicotianae*, on leaves from transfected plants constitutively expressing SNA-I results in a delayed development and reduced adult survival and also fertility parameters of the surviving aphids are reduced (Shahidi-Noghabi *et al*., 2008).

At the present, the exact mechanism of the entomotoxic action of RIPs is still not known. There are several studies to propose the toxicity of RIPs against some insect species. The carbohydrate-binding activity of lectin B-chain is expected to important for insecticidal activity of type 2 RIPs. The experiments with transgenic lines of a mutant SNA-I have been determined. Mutation of one carbohydrate-binding site strongly reduced the insecticidal activity of SNA-I, whereas mutation of both lectin sites completely abolished the SNA-I effect on tobacco aphids, *Myzus nicotianae* (Shahidi-Noghabi *et al*., 2008).

The previous study reveals that ricin with a single point mutation in one of the galactose binding sites of ricin B-chain results in a 20- to 40-fold lower cytotoxicity compared to the wild type ricin, whereas double mutations in the two carbohydrate-binding domains exhibits no detectable cytotoxicity (Newton *et al*., 1992; Shahidi-Noghabi *et al*., 2008). These results also confirm that carbohydrate-binding activity is crucial for cytotoxicity of ricin (Shahidi-Noghabi *et al*., 2008). The difference in toxicity of two type 2 RIPs, cinnamomin and ricin, is proposed to relate to the properties of their B-chains and is not related to their A-chains (Liu *et al*., 2002; Wei *et al*., 2004; Shahidi-Noghabi *et al*., 2008).

In addition, some RIPs have been determined for the stability in the gut of insects. Cinnamomin, the type 2 RIPs form seeds of *Cinnamomum camphora* has been incubated with gut extract of bollworm, *Helicoverpa armigera*. The gut extract displays strong ability to digest cinnamomin. Approximately 63% and 85% of cinnamomin are digested after incubation with gut extract for 30 min and 1 h, respectively. It has been digested absolutely after incubation for 32 h (Zhou *et al*., 2000). In order to consider whether the different toxicity between cinnamomin and ricin possibly results from the degradation of those RIPs in the midgut of larvae, they have been separately incubated with gut liquid of silkworm larvae at various incubation times. The results show that the gut liquid can digest approximately 28.8% of cinnamomin and 26.4% of ricin after incubation for 30 min, approximately 52.3% of cinnamomin and 48.5% of ricin after incubation for 1 h. They are completely digested after incubation for 8 h. These data reveals that the different toxicity between the two RIPs to silkworm larvae is not attributed to their hydrolysis rate by the midgut liquid.

For type 1 RIPs, most of them have the lower toxicity than type 2 RIPs because of lacking of a protein domain able to bind to, and translocate the toxin across cell membranes. Several type 1 RIPs, lychnin, momordin, gelonin, PAP-S and saporin S-6 have been studied for the toxicity against *Anticarsia gemmatalis* and *Spodoptera frugiperda*. After ingesting a total dose of 20 or 40 μg of the toxins, weight gain, survival rate, lesions in DNA and oxidative status of RIP-treated insects have been

followed. They show a 2- to 3-fold increase in DNA lesions as assessed by the comet assay. These results suggest that those single-chain RIPs are entomotoxic to Lepidoptera insects causing extensive DNA lesions (Bertholdo-Vargas *et al*., 2009). The entomotoxic action of the purified seed coat protein, Jc-SCRIP against the instars larva of both *Spodoptera* worms may be causing extensive DNA lesions, the same mechanism as those other type 1 RIPs or may be a result of their rRNA *N*-glycosidase activities, which inhibit protein synthesis in the larvae. However, the exact toxic mechanism of Jc-SCRIP against the larva should be explored both *in vivo* and *in vitro.*

The potent larvicidal activity of the crude extract and the type 1 RIP from the Jatropha seed coat, Jc-SCRIP suggests that they may be used as a low cost natural agents to control the agricultural pests of *Spodoptera* species. Further evaluation of biosafety to human and its toxic stability against the worms should be done before the application use.

CONCLUSION

1. A new type 1 ribosome-inactivating protein, designated as Jc-SCRIP was first purified from seed coat of *J. curcas* KUBP33 by ammonium sulfate precipitation and chromatography on DEAE-Sephacel™ and CM-Cellulose columns. Purification fold of Jc-SCRIP increased 113.5 times in final step with 1.12% yield of the total protein.

2. Jc-SCRIP exhibited lectin-like activity by agglutinate rabbit erythrocytes but it cannot be categorized as a lectin, which by definition must have multiple subunits and bind to specific carbohydrates.

3. It possessed strong *N*-glycosidase activity that released an RNA fragment of approximately 560 nucleotides from the rabbit reticulocyte rRNA after acidic aniline treatment.

4. SDS-PAGE analysis of subunit of Jc-SCRIP demonstrated a single band of approximately 41,800 Da indicating purification to homogeneity and it was a monomeric glycoprotein of a molecular mass of 38,938 Da, as determined by MALDI-TOF/MS.

5. Structural analysis of Jc-SCRIP indicated that its major structure was β-sheet with N-terminal amino acid sequence: AINGGVA.

6. The neutral sugar content of Jc-SCRIP was about 4.80% (w/w).

7. Jc-SCRIP had antimicrobial activity against 9 human-pathogenic bacteria and 1 fungus with the most potent inhibitory activity against *Staphylococcus epidermidis* ATCC 12228, with MIC value of 7.81 μg/ml.

8. Jc-SCRIP demonstrated *in vitro* cytotoxicity against human breast adenocarcinoma cell line (MCF-7), a colon adenocarcinoma (SW620), and a liver carcinoma cell line (HepG2), with IC_{50} values of 0.15, 0.25 and 0.40 μM, respectively.

9. Jc-SCRIP had larvicidal effects against the third instars larvae of mosquitoes, *Aedes aegypti* Linn. and *Culex quinquefasciatus* Say. It showed the toxicity to *Ae. aegypti* and *Cx. quinquefasciatus* with LC_{50} values of 1.44 and 0.0303 mg protein/ml, respectively. These results indicated that the larvae of *Cx. quinquefasciatus* were more susceptible to the toxin than that of *Ae. aegypti*.

10. Jc-SCRIP had insecticidal effects against the second instars larvae of Spodoptera worm, *Spodoptera litura* and *Spodoptera exigua*. It showed the toxicity to *S. litura* and *S. exigua* with LC_{50} values of 0.0525 and 0.0692 mg protein/ml, respectively.

11. Cloning of cDNA encoding for Jc-SCRIP from young seed coat of *J. curcas* was not successful yet. The nucleotide sequences obtained from the sequencing shared 98% identity to aquaporin from *J. curcas*.

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APPENDICES

Appendix A

Preparation of reagents

1. Reagents for purification and characterization of Jc-SCRIP

1.1 Preparation of potassium sodium tartrate - copper sulphate - sodium carbonate solution

- Solution A: Sodium carbonate	20	g
Distilled water	960	ml
3M sodium hydroxide	35	ml
- Solution B: Copper sulphate pentahydrate	1	g
Distilled water	100	ml
- Solution C: Potassium sodium tartrate	2	g
Distilled water	100	ml

All solutions were mixed well at ratio A:B:C = 100:1:1 (v/v)

1.2 Preparation of a 50 mM Tris-HCl buffer (pH 7.5)

- Tris (hydroxymethyl) aminomethane	6.06	g
- Distilled water	900	ml
- Adjust pH with concentrated HCl to pH 7.5		
- Distilled water to	1000	ml

1.3 Preparation of 30% acrylamide + 0.8% bis – acrylamide solution

- Acrylamide	75	g
- Bis – acrylamide	2	g
- Add distilled water to	250	ml

Filter through Whatman No.1 and kept at 4°C

1.4 Preparation of 4× sample buffer

- 0.5 M Tris – HCl buffer (pH 6.8)	5	ml
- SDS	2	g
- 2–mercaptoethanol	2	ml
- Glycerol	2	ml
- Bromophenol blue	0.03	g

1.5 Preparation of separating gel for 15% SDS-PAGE

- Distilled water	1.675	ml
- 30% acrylamide	2.0	ml
- 1.5 M Tris-HCl buffer (pH 8.8)	1.25	ml
- 10% SDS	0.050	ml
- 10% ammonium persulfate (fresh)	0.025	ml
- TEMED	0.005	ml

1.6 Preparation of stacking gel for 15% SDS-PAGE

- Distilled water	1.525	ml
- 30% acrylamide	0.325	ml
- 0.5 M Tris (pH 6.8)	0.625	ml
- 10% SDS	0.025	ml
- 10% ammonium persulfate (fresh)	0.0125	ml
- TEMED	0.003	ml

1.7 Preparation of 4× electrophoresis buffer for 15% SDS–PAGE

- Tris (hydroxymethyl) aminomethane	12.0	g
- Glycine	57.6	g
- 10% SDS solution	10	ml
- Distilled water to	1000	ml

1.8 Preparation of staining solution

- Coomassie Brilliant Blue	0.5	g
- Methanol	250	ml
- Glacial acetic acid	50	ml
- Distilled water to	200	ml

1.9 Preparation of destaining solution

- Methanol	250	ml
- Acetic acid	100	ml
- Distilled water	650	ml

1.10 Preparation of fixing solution for silver staining

- Methanol	500	ml
- Glacial acetic acid	120	ml
- Distilled water to	1000	ml

1.11 Preparation of washing solution for silver staining

- Ethanol	350	ml
- Distilled water to	1000	ml

1.12 Preparation of sensitizing solution for silver staining

- Sodium thiosulfate	0.2	g
- Distilled water to	1000	ml

1.13 Preparation of staining solution for silver staining

- Silver nitrate	2	g
- Distilled water to	1000	ml

1.14 Preparation of developing solution for silver staining

- Sodium carbonate	60	g
- 0.02% sodium thiosulfate solution	20	ml
- Distilled water to	1000	ml

1.15 Preparation of stopping solution for silver staining

- Sodium-EDTA	14.6	g
- Distilled water to	1000	ml

1.16 Preparation of 40% acrylamide for *N*-glycosidase activity assay

- Acrylamide	76	g
- Bis-acrylamide	4	g
- Distilled water to	100	ml

Filter through Whatman No.1 and kept at 4°C

1.17 Preparation of 6M urea-PAGE for *N*-glycosidase activity assay

- 10× TBE solution	0.75	ml
- Urea (molecular biology grade)	3.15	g
- 40% acrylamide/bis-acrylamide solution	1.50	ml
- DEPC-treated water	3.0	ml
- 10% ammonium persulfate solution (fresh)	100	µl
- TEMED	10	µl

1.18 Preparation of 10× TBE solution

- Tris (hydroxymethyl) aminomethane	108	g
- Boric acid	55	g
- 0.5 M sodium-EDTA solution	20	ml
- Distilled water to	1000	ml

2. Reagents for molecular cloning of Jc-SCRIP gene

2.1 Preparation of CTAB extraction buffer

- Hexadecyl trimethyl-ammonium bromide (CTAB)	10 g
- Polyvinylpyrrolidone 40K 29-32 (PVP-40)	10 g
- 1M Tris-HCl pH 8.0	50 ml
- 0.5M EDTA	25 ml
- NaCl	58.57 g
- Distilled water to	500 ml

CTAB extraction buffer was sterilized by autoclaving at 121°C for 20 min

2.1 Preparation of Chloroform : Isoamyl alcohol (24 : 1, v/v) solution

- Chloroform (Merck)	24.0	ml
- Isoamyl alcohol	1.0	ml

Both reagents were mixed together and stored in a dark bottle at 4°C

2.2 Preparation of Ethidium bromide (10 mg/ml) solution

- Ethidium bromide	100	mg
- Distilled water	10	ml

2.3 Preaparation of 6× gel-loading dye buffer

- Glycerol	500	µl
- 0.1 M sodium-EDTA solution	200	µl
- 1 % xylene Cyanol FF	60	µl
- 1 % Bromophenol blue	60	µl
- 1 M Tris HCl buffer (pH 7.5)	180	µl

2.4 Preparation of 20 mg/ml X-gal

5-bormo-4-chloro-3-indolyl-β-D-galactoside	100	mg

The chemical was dissolved in 2 ml of dimethyl-formamide. The solution was stored in a tube covered with aluminum foil and stored at -20°C.

3. Media for bacterial culture

3.1 Luria-Bertani medium (LB medium per liter)

- Tryptone (Difco)	10	g
- Yeast extract (Difco)	5.0	g
- NaCl	10	g

Adjust pH to 7.0 with NaOH. Then the solution was adjusted to the final volume of 1000 ml with distilled water and sterilized by autoclaving.

3.2 LB plates with ampicillin

Fifteen grams of agar was added to 1 lite of LB medium then the media was sterilized by autoclaving. The medium was allowed to cool to 50°C before adding ampicillin to a final concentration of 100 µg/ml. The medium (20-25 ml) was poured into 85 mm petri dishes. The agar was allowed to harden. Agar plates were stored at 4°C for up to 1 month or room temperature for up to 1 week.

3.3 LB plates with ampicillin/X-gal

Fifty microlitre of X-gal solution (50 mg/ml) were spreaded over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

Appendix B

Standard curves and Tables

1. Preaparation of standard curve of bovine serum albumin (BSA) to calculate protein concentration

To determine the concentration of protein from each purification step, the standard curve of BSA was prepared using Lowry′s method.

`

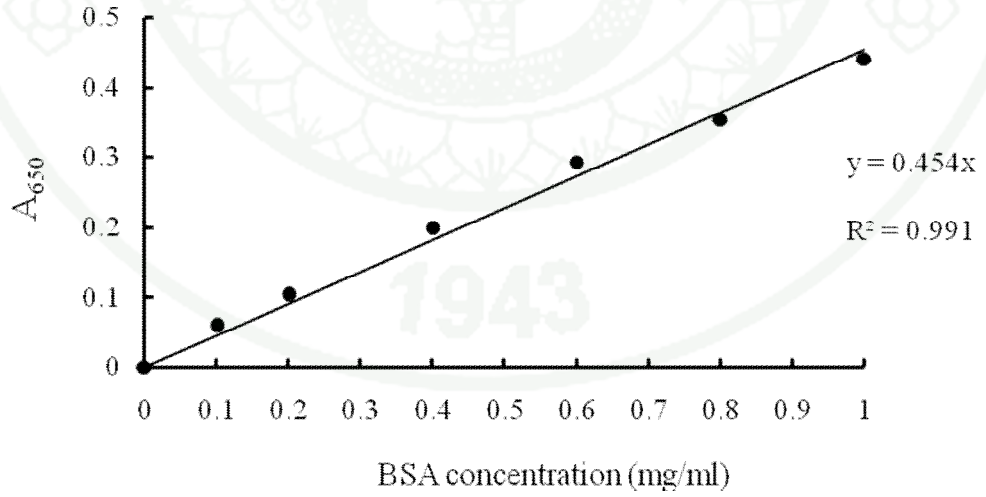
1. Stock BSA solution (1 mg protein/ml) was diluted to final concentration of 0.1, 0.2, 0.4, 0.6, 0.8 mg protein/ml into each tube.

2. Three millilitre of the mixed solution A:B:C (see Appendix A) were added to each tube, mixed well and incubated at room temperature for 10 min.

3. Three hundred microlitre of the Folin-Ciocalteau solution which was diluted with distlled water at ratio 1:1 was added to each tube, mixed well and incubated in the dark at room temperature for 30 min.

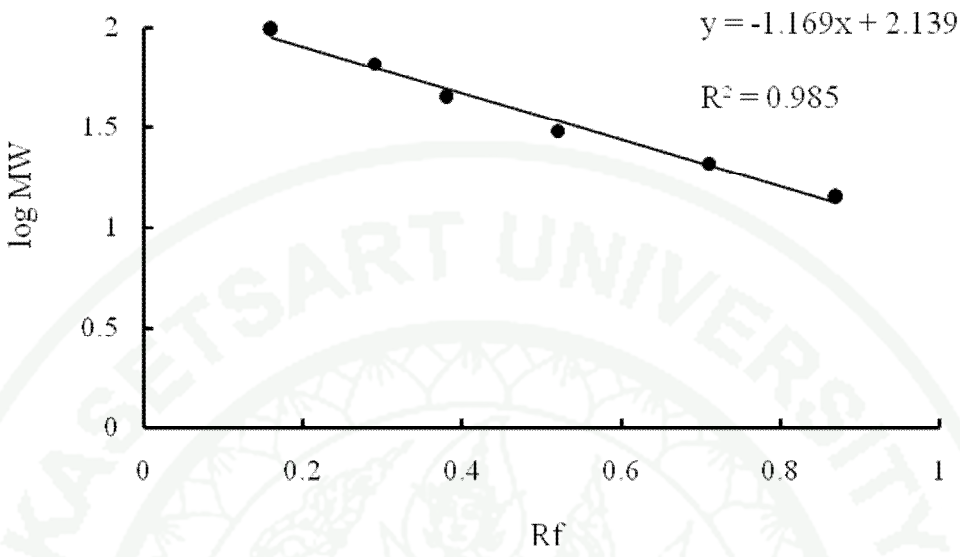
4. The absorbances were read at 650 nm and distilled was used as blank.

5. Prepare a standard curve by plotting the average absorbance at 650 against protein concentration (mg/ml) and calculated the concentration of protein from each purification step.



Appendix Figure B1 Standard curve of bovine serum albumin (BSA) using Lowry′s method

2. Standard curve of protein molecular weight markers



Appendix Figure B2 Standard curve of protein molecular weight markers

3. Preparation of standard curve of glucose to calculate neutal sugar content

To determine the content of neutral sugar of Jc-SCRIP, the standard curve of glucose was prepared using Dubois′s method. (All steps of this method should be prepared in the laboratory fume hood)

`

1. Stock glucose solution (1 mg/ml) was diluted using 2-fold serial dilutions with total volume of 0.5 ml into each tube.

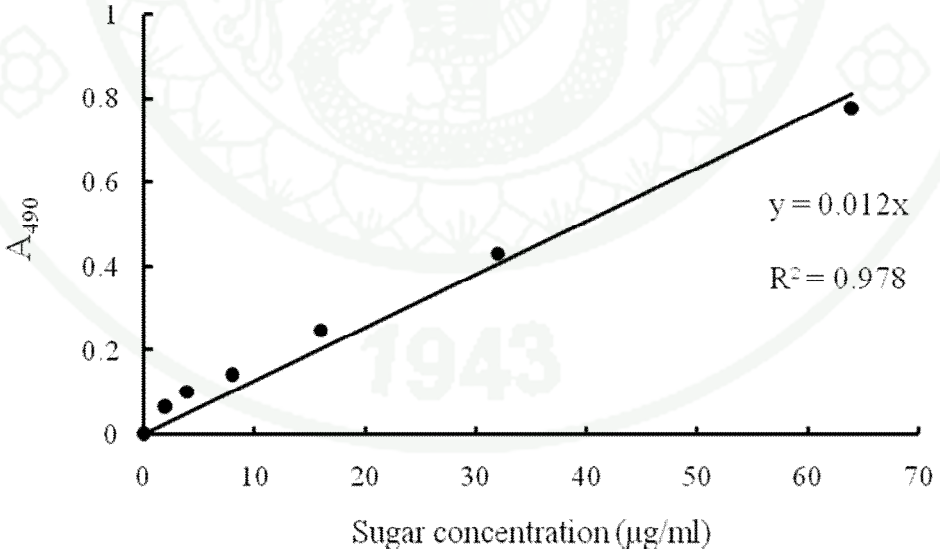
2. Three hundred microlitre of the 5% phenol reagent were added to each tube and mixed well.

3. Two milllitre of the contrated sulfuric acid were added to each tube and mixed immediately.

4. The reaction mixtures were incubated at room temperature for 30 min.

5. The absorbances were read at 490 nm and distilled was used as blank.

6. Prepare a standard curve by plotting the average absorbance at 490 against glucose concentration (µg/ml) and calculated the content of neutral sugar of Jc-SCRIP.



Appendix Figure B3 Standard curve of glucose using Dubois′s method

4. Extraction and precipitation of RNA by phenol/chloroform and ethanol method

1. Add an equal volume of TE-saturated phenol to the DNA/RNA sample contained in a 1.5 ml microcentrifuge tube and vortex for 15-30 s

2. Centrifuge the sample at 10000 ×g for 10 min at room temperature to separate the phases

3. Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous:phenol interface. At this stage the aqueous phase can be extracted a second time with an equal volume of 1:1 TE-saturated phenol:chloroform, centrifuged and removed to a clean tube as above but this additional extraction usually is not necessary if care is taken during the first phenol extraction.

4. Add an equal amount of 24:1 (v/v) chloroform: isoamyl alcohol

5. Centrifuge the sample at 10000 ×g for 10 min at room temperature to separate the phases

6. Remove about 90% of the upper, aqueous layer to a clean tube

7. Add a 0.1 volume of 3 M sodium acetate pH 5.5 to the aqueous phase and then 2.5 volume of cold 95% (or absolute) ethanol

8. Incubate at -20°C overnight or for shorter period at -80°C for 30 min

9. Centrifuge at 10000 ×g for 30 min at 4°C to recover the precipitated RNA and remove the ethanol with care.

10. Add 80% ethanol (corresponding to about two volume of the original sample) and centrifuge again for 20 min and decant and drain the tubes.

11. Dissolve in 20-50 µl of DEPC-treated water

12. Store the RNA at -20°C

Appendix Table B1 Hemagglutination inhibition of crude protein extract from seed coat of *J. curcas* by various carbohydrates and glycoproteins

Carbohydrates/Glycoproteins	Minimum concentration of carbohydrate (mM)
D-(+)-Glucose	-
Mannose	-
Fucose	-
Lactose	-
Galactose	-
Glycogen carrier	-
Fetuin from Fetal Calf Serum	1.95×10^{-3}
Mucin from BSM	-
6-O-Methyl-D-galactose	4.88×10^{-4}
α-acid glycoprotein	-
N-acetyl-D-glucosamine	-
N-acetyl-β-D-mannosamine	-
N-acetyl-neuraminic acid	-
N-acetyl-D-galactosamine	0.03
N-glycolyl-neuraminic acid	-
D-(+)-Raffinose pentahydrate	125
D-(+)-Melibiose	-
L-(+)-Rhamnose	-
L-(+)-Arabinose	-
D-(-)-Ribose	-
myo-Inositol (meso-Inositol)	-
D-(+)-Xylose	-

Appendix Table B2 Minimum Inhibitory Concentration (MIC) of standard antimicrobial drug against human pathogenic microorganisms

Microorganisms	Minimum Inhibitory Concentration (μg/ml)		
	Ampicillin	Chloramphenicol	Amphotericin B
1. *Acinetobacter anitratus* DMST 4183	10	100	ND
2. *Acinetobacter baumannii* ATCC 19066 (DMST 10437)	1000	100	ND
3. *Acinetobacter calcoaceticus* ATCC 23055 (DMST 10436)	NI	2000	ND
4. *Acinetobacter lwoffii* ATCC 15309 (DMST 4229)	5	50	ND
5. *Bacillus cereus* ATCC 11778 (DMST 5040)	5	5	ND
6. *Burkholderia cepacia* ATCC 25416 (DMST 4205)	NI	5	ND
7. *Enterococcus faecalis* ATCC 29212 (DMST 4736)	1	5	ND
8. *Escherichia coli* ATCC 25922 (DMST 4212)	5	5	ND
9. *Pseudomonas aeruginasa* ATCC 27853 (DMST 4739)	50	5	ND
10. *Pseudomonas fluorescens* DMST 6034	1000	1000	ND
11. *Salmonella enteritidis* ATCC 17368	1000	5	ND
12. *Salmonella typhi* DMST 5784	0.5	5	ND
13. *Shigella dysenteriae* DMST 15111	1	1000	ND
14. *Staphylococcus aureus* ATCC 25923 (DMST 8840)	0.25	5	ND
15. *Staphylococcus aureus* DMST 20654 (MRSA)	50	5	ND
16. *Staphylococcus epidermidis* ATCC 12228 (DMST 15505)	1	5	ND
17. *Streptococcus agalactiae* DMST 17129	0.25	5	ND

Appendix Table B2 (Continued)

Microorganisms	Minimum Inhibitory Concentration (µg/ml)		
	Ampicillin	Chloramphenicol	Amphotericin B
18. *Streptococcus pyogenes* DMST 17020	0.03125	5	ND
19. *Vibrio cholerae* nonO1, nonO139 DMST 2873	1	0.5	ND
20. *Vibrio cholerae* O139 ATCC 51394	5	5	ND
21. *Klebsiella pneumoniae* ATCC 27736	50	5	ND
22. *Klebsiella oxytoca* DMST 16071	NI	100	ND
23. *Bacillus subtilis* ATCC 6633	1	5	ND
24. *Escherichia coli* O157.H7 DMST 12743	1	10	ND
25. *Listeria monocytogenes* DMST 17303	1	5	ND
26. *Proteus mirabilis* DMST 8212	1	50	ND
27. *Serratia marcescens* ATCC 8100	1000	50	ND
28. *Shigella flexneri* DMST 4423	1000	5	ND
29. *Shigella sonnei* (group D) DMST 2982	1	5	ND
30. *Shigella boydii* DMST 7776	NI	1000	ND
31. *Candida albican* ATCC 10231	ND	ND	0.061

Note: Stock of Ampicillin and Chloramphenicol were 2000 µg/ml and they were diluted to 1000, 100, 50, 10, 5, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, µg/ml; NI, No inhibition at 2000 µg/ml; ND, Not determined

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PETTY PATENT : น้ ายากำจัดแมลงศัตรูพืชและยุงที่มีส่วนผสมของสารสกัดโปรตีนจากเปลือกหุ้มเมล็ดสบู่ดำ ยื่นคำร้องวันที่ 7 ตุลาคม 2554 (เลขที่คำร้อง 1103001131)