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Purification and Characterization of Trypsin Inhibitor from *Momordica cochinchinensis* Spreng. Seed and Its Effects to *Spodoptera litura*

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Abstract: This research was conducted to determine the effect of trypsin inhibitor on growth and development of *Spodoptera litura*. Trypsin Inhibitor from *Momordica cochinchinensis* (MMCTI) seed was extracted and purified using Ion Exchange Chromatography with CM-52 column. Trypsin Inhibitor Unit (TIU) was identified by using BApNA as substrate. The MMCTI showed 0.34 specific inhibitory units and mass numbers was 3165.03. In larval feeding studies, MMCTI were found to retard growth and development of *S. litura*. Larvae fed on diet containing MMCTI showed reduction in weight that was dose-dependent. Larval mortality ranged from 8 to 15% of the larval population. Reduction in pupal weight by 25% and pupal mortality by 27% were also observed with 12 TIU g^{-1} diet. Malformed adults (5-16%) were recorded as an effect of abnormal development. Fertility was affected as the egg-laying incidence reduced from 810 egg/female (control) to 298 egg/female (12 TIU g^{-1} diet). This result indicated that MMCTI is an efficient inhibitor of proteinases in the *S. litura* larvae. Being small peptide, it would be easy to express MMCTI in plants to confer protection against devastating pests such as *S. litura*.

Key words: Trypsin inhibitor, *Momordica cochinchinensis*, *Spodoptera litura*, mortality, pupal weight

INTRODUCTION

Spring bitter cucumber, *Momordica cochinchinensis* Spreng is an herb widely used in China and India (Perry, 1980). It belong to Cucurbitaceous or squash family. Pulp of seed contain 10 times β-carotene higher than carrot (Vuong and King, 2003), 12 times lycopene more than tomato and long chain fatty acid about 10% of the mass. Protein from the seeds show anti-cancer activity, reduce damage from free radicals (De Shan *et al*., 2001). Oil from the pulp has effective in the treatment of liver cancer (Vuong *et al*., 2002). In Thailand use spring bitter cucumber as authentic traditional medicines such as antimicrobial, resistance to cancer and diabetes. (Putiyanan, 2004) The seed contain cochinin B protein with inhibitory function of the ribosome and resist the growth of cancer cells *in vitro* (Chuethong *et al*., 2007). Trypsin is an important digestive enzyme in the stomach of a living thing (Wong *et al*., 2004). Also Lepidopteran pests such as *Helicoverpa armigera* and *Spodoptera litura* mainly use trypsin for protein digestion (Bown *et al*., 1997). Plant Trypsin Inhibitors (TIs) were found, most of it in tubers and seeds (De Leo *et al*., 2002). TIs are expressed during seed development and produced more in response to wound, insects and disease attracted. TIs interfere with the digestion of insects so that insects could not have normal growth (Ryan, 1990). The most recently study; the squash family inhibitors that consists of 28-32 amino acid residues, 6 of which are cysteine residues forming 3 disulfide bonds. In this study, the extraction and purification as well as trypsin inhibitory activity of TIs from *Momordica cochinchinensis* Spreng. was carried out.

MATERIALS AND METHODS

Preparation of crude extract: Ripe fruits of spring bitter cucumber were collected from plantation. Seeds were washed and dried at 50°C with hot air oven. Decoated seeds were crushed and homogenized in acetone and washed twice (1:5 w/v) to remove fat. The ground seed powder was collected by centrifugation and then dried under the air. The 50 g of defatted seed powder was extracted with 5 mM tris-HCl (pH 8.0) (1:10 w/v) for 2 h at room temperature with mechanical stirring (1:10 w/v). The supernatant was recovered by centrifugation at 7,000 rpm for 20 min at 4°C. The crude extract was heated at 100°C for 10 min to remove heat-labile protein as precipitate. The supernatant collected by centrifugation was adjusted pH to 3.5 with HCl, re-centrifuged and then the supernatant was subjected to purification steps.

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Purification of trypsin inhibitor: One hundred milligram protein of partial purification solution was applied to a Carboxymethyl cellulose (CM 52 1.5 cm×19 cm) pre-equilibrated with 0.01 M sodium acetate buffer (pH 3.5). After washing, bound proteins were eluted with a linear gradient of 0-1 M NaCl in the same buffer. The trypsin inhibitory activity was measured by change in absorbance at 405 nm using trypsin from hog pancreas (fluka) as an enzyme and N-alpha-benzoyl-L-arginine-p-nitroanilide (BApNA) as a substrate. Fractions with trypsin inhibitory activity were pooled.

Assay of trypsin inhibitory activity: Trypsin inhibitory activity was assay according to Roy and Rao (1971). The 0.2 mL of purified inhibitor and 0.5 mL of 2 mg mL^{-1} pure commercial enzyme were incubated at 30°C for 5 min in 1.3 mL of 0.1 M phosphate buffer pH 7.6, containing 1 mM HCl. The reaction was initiated by the addition 2 mL of 2% casein substrate. Incubation were carried out at 34°C for 20 min after which 6 mL of 5% trichloroacetic acid solution was added to stop the reaction and corresponding blank was run concurrently. Total volume of the mixture was 10 mL. The residual trypsin activity was measured by change in absorbance at 280 nm with a spectrophotometer (UV-1700, Shimadzu, Japan). One trypsin unit was defined as an increase of 0.01 absorbance unit at 280 nm in 20 min for 10 mL of reaction mixture and the trypsin inhibitory activity as the amount of trypsin units inhibited. This research was done during March 2010-June 2011. Mean, percentage and standard deviation for inhibition of *Momordica cochinchinensis* TI against insect growth and development were calculated using excel 2007.

RESULTS AND DISCUSSION

Ripe fruits of *Momordica cochinchinensis*: MMC (Fig. 1) have width of 11.5 cm length of 15.5 cm and fruit weight of 706±117.22 g. the ratio of seed: fruit pulp was 1:3. Seeds were washed and dried out at 40°C for 24 h, they have average 1.84±0.20×2.28±0.29 cm (Table 1).

Fifty gram of seed powder was extracted with 5 mM tris-HCl (pH 8.0) and gave 3,884 mg total proteins. After precipitated in acidic condition (pH 3.5) total proteins reduced to 2,422 mg with 6.378 g dried powder (Table 2). One hundred mg protein was applied to Carboxymethyl cellulose (CM 52) column (1.5×19 cm) determined protein concentration by change in absorbance at 280 nm. The elution profile obtained when the *Momordica cochinchinensis* seed extract was applied to a CM 52 as shown in Fig. 2. Most of proteins were unbound fractions. The bound fraction exhibited trypsin inhibitory activity. It shows that resin used has properties and the ability to bind to trypsin inhibitors because TIs would not wash out with buffer A. They were eluted when NaCl ion replaced the binding of TIs with the resin material. TIs were complete eluted with 0.3-0.5 M NaCl. This was consistent with reports of Wong *et al.* (2004). From 2,422 mg protein of partially purified of *Momordica cochinchinensis* seed extract, after ion exchange chromatography, the protein was remained 724.55 mg with 246.35 inhibitory units and 0.34 specific inhibitory units/mg protein (Table 2). Trypsin inhibitor fraction obtained by ion-exchange chromatography was further purified by reversed-phase HPLC (column:protein and peptide C18; 4.6×250 mm; Grace Vydac). The mass numbers measured by TOF mass spectrometry (REFLEX-III, Brucker, USA) was 3165.03. Previously, Huang *et al.* (1999) have found a potent trypsin inhibitor from *Momordica cochinchinensis* seeds with a molecular weight of 3479 Da which similar to our results. Giri *et al.* (2003) have identified potent inhibitors of *Helicoverpa armigera* gut proteinases from winged bean seeds, at least 14 TIs in the range of 28-6 kDa. Each of them has different binding potential towards gut proteinases although most of the gut proteinases activity is trypsin-like. They also developed a simple and versatile method for identifying and purifying proteinase inhibitors after two-dimensional separation using gel-X-ray film contact print technique. Even though, Plant trypsin inhibitors (TIs) were found, most of it in tubers and seeds (De Leo *et al*., 2002). Damle *et al.* (2005) showed higher accumulation of proteinase inhibitors in flowers than leaves and fruits of tomato (*Lycopersicon esculentum* Mill, cv. Dhanashree).

Effects of MMCTI on insect growth and development were investigated by incorporation of 4 different doses of inhibitors in artificial diet (3, 6, 9 and 12 TIU g^{-1} diet). Results were summarized in Table 3. Larvae fed on diet containing trypsin inhibitors showed reduction in weight that was dose-dependent, as compared with those fed on control diet. Larval weight was reduced by a maximum of 68% at the highest dose of inhibitor (12 TIU g^{-1} diet) after 10 days of feeding. Larval mortality ranged from 8 to 15% of the larval population. Reduction in pupal weight by

Table 1: Fruit size, fruit weight and seed size of *Momordica cochinchinensis* Spreng.

Fruit size (cm)			Fruit weight (g)			Seed size (cm)	
Diameter	Length	Fruit weight (g)	Seed and pericarp	Fruit pulp	Total	Diameter	Length
11.5	15.5	706±117.22	245	577	841	1.84±0.20	2.28±0.29

Fig. 1: Generality and composition of spring bitter cucumber fruit (*Momordica cochinchinensis* Spreng.)

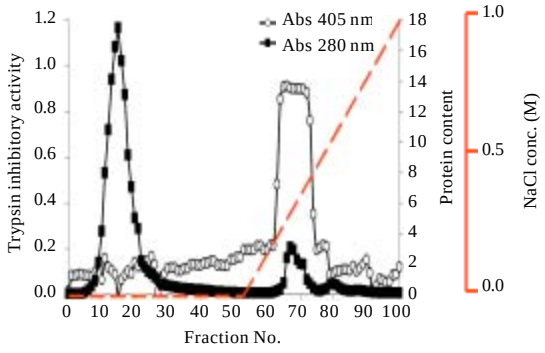

Fig. 2: Ion exchange chromatography of the partially purification of *Momordica cochinchinensis* seed extract on a carboxymethyl cellulose (CM 52) column. The extract was applied to a column equilibrated with 0.01 M sodium acetate buffer (pH 3.5). After washing, bound proteins were eluted with a linear gradient of 0-1 M NaCl in the same buffer. Fractions (No. 63-74) with trypsin inhibitory activity were pooled

25 and 27% pupal mortality were also observed with the highest dose. Larval period was delayed by up to 2 days. Malformed adults (5-16%) were recorded as an effect of abnormal development. Fertility was drastically affected as the egg-laying incidence reduced from 810 egg/female (control) to 298 egg/female (12 TIU g^{-1} diet).

Table 2: Step of purification of trypsin inhibitors extracted from spring bitter cucumber seed

Purification step	Volume (mL)	Amount of protein (mg)	Inhibitory activity (U)	Specific inhibitory activity (U mg^{-1})
Seed powder 50 g				
5 mM tris-HCl (pH 8.0)	400	3,884.00	-	-
Heat at 100°C, 10 min	383	2,056.71	-	-
pH 3.5 with HCl	350	2,422.00	-	-
Fractions No. 63-74	581.21	724.55	246.35	0.34

However, emergence of larvae from eggs was not affected. The female moth of *S. litura* typically lays about 800 eggs and this process requires a major input of proteins. Since the adults feed only on plant nectar, a poor protein source (Slansky and Scriber, 1985), it is apparent that the pool of reserve proteins is generated during actively feeding larval stage. Any disturbance in protein metabolism at larval stage is thus expected to reflect on the number and quality of eggs. Ingestion of diet containing trypsin inhibitors adversely affected the protein intake, at the larval stage, which caused developmental abnormalities and also reduced fertility and fecundity of the adult. Thus, these results are in agreement with Lee (2010). Lee (2010) studied that female caterpillar of *S. litura* selecting significantly more protein than males. Telang *et al.* (2001) showed that accumulation of proteins during larval stage of *Heliothis virescens* is critical to vitellogenesis.

Present results were supported by Franco with team (Franco *et al.*, 2003). They analyzed the inhibitory effects of black-eyed pea trypsin/chymotrypsin inhibitor (BTCI),

Table 3: Effect of MMC trypsin inhibitors on growth and development of *Spodoptera litura*

Parameters	Control	3 TIU g^{-1}	6 TIU g^{-1}	9 TIU g^{-1}	12 TIU g^{-1}
Larval weight (mg/larvae)					
DAT 6	9.30±2.05	9.00±0.52	7.5±1.17	7.8±0.54	6.4±0.49
DAT 8	59.10±1.15	58.50±3.52	51.5±4.28	51.6±1.15	50.3±3.15
DAT 10	139.30±18.15	132.90±12.05	130.4±21.45	113.4±23.18	95.4±13.15
Larval period (days)	17.00±0.75	18.00±0.95	18.0±1.04	18.0±1.95	18.0±0.50
Larval mortality (%)	0.00±0.00	8.00±0.45	11.0±1.25	11.0±0.85	15.0±0.92
Pupal weight (mg $pupa^{-1}$)	295.40±43.0	285.60±40.15	256.3±8.25	220.3±11.98	220.7±21.16
Pupal period (days)	16.00±0.55	17.00±1.87	18.0±1.70	18.0±0.80	18.0±0.82
Pupal mortality (%)	5.20±0.00	11.20±0.00	23.0±0.00	26.0±0.00	32.0±0.00
Malformed adults (%)	0.00±0.00	0.00±0.00	5.0±0.00	11.0±0.00	16.0±0.00
Fertility (eggs $female^{-1}$)	810.00±13.65	756.00±41.22	325.0±52.02	310.0±23.08	298.0±23.50
Fecundity (hatching; %)	85.51±0.00	70.19±0.00	110.7±0.00	82.4±0.00	93.5±0.00

TIU: Trypsin inhibitor unit, DAT: Days after treatment, MMC: *Momordica cochinchinensis*

towards trypsin and chymotrypsin from bovine pancreas and from midguts of *A. grandis* larvae and adult insects. BTCI, purified from *Vigna unguiculata* (L.) seeds, was highly active against different trypsin-like proteinases studied and moderately active against the digestive chymotrypsin of adult insects. Nevertheless, no inhibitory activity was observed against chymotrypsin from *A. grandis* larval guts. Telang *et al.* (2003) found that in larval feeding studies, proteinase inhibitors from the seed of bitter gourd (BGPIs) were found to retard growth and development of *H. armigera* and *Spodoptera litura*. The results showing that BGPIs mediated inhibition of insect gut proteinases directly affects fertility and fecundity. Afterward, Telang *et al.* (2009) isolated a DNA sequence encoding the mature peptide of a trypsin inhibitor MCTI-II, cloned and expressed as a recombinant protein using *Pichia pastoris*. The *H. armigera* larvae fed with recombinant MCTI-II-incorporated artificial diet suffered over 70% reduction in the average larval weight after 12 days of feeding. Moreover, ingestion of MCTI-II resulted in 23% mortality in the larval population. Trypsin inhibitor from *Albizia lebbeck* seeds caused mortality and suppressed larval growth of *Pieris brassicae* larvae. This may be due to direct inhibition of digestive enzyme and depletion of essential amino acids for larvae. It was also found to be effective against gut trypsin extracted from *Spodoptera littoralis* (Sharma *et al*., 2012).

There are several reports about proteinase inhibitor from Cucurbitaceous plant. Inanaga *et al.* (2001) generated novel type of Protein Inhibitors (Pis) with multi-inhibitory activities by replacement of phytocystatin domains in Sunflower Multicystatin (SMC) by the serine proteinase inhibitor BGIT from bitter gourd seeds. Then chimeric inhibitors (SMC-T3 and SMC-T23) and the recombinant SMC (r-SMC) were compared in relation to their effects on growth of larval *Spodoptera exigua*. When the second instar larvae were reared on a diet containing r-SMC, SMC-T3 or SMC-T23 for ten days, mean weights for r-SMC, SMC-T3 and SMC-T23 were 43, 32 and 43 mg, respectively, as compared with that (60 mg) for the absence of the inhibitor ($p<0.05$). This expressed that changing amino acids position might affect to inhibitory activity. Atiwetin *et al.* (2006) found that two squash family protease inhibitors were obtained from wax gourd. They were distinctly separated by reversed-phase chromatography, the amino acid of two inhibitors were identical. Both inhibitors were converted into each other, perhaps due to *cis-trans* isomerization of proline characteristic in the C-terminal region. Tamhane *et al.* (2007) have showed diverse forms of Pin-II family proteinase inhibitors from *Capsicum annuum* adversely affect the growth and development of *Helicoverpa armigera*.

CONCLUSION

In conclusion, we have characterized the inhibitory potential of trypsin inhibitor from *Momordica cochinchinensis* seed against *S. litura*. Being small peptide, it would be easy to express MMCTI in plants to confer protection against devastating pests. They will also bring a variety of applications because of their low molecular weight.

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