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Tannic Acid Enhancing Insecticidal Activity of Protoxin Produced in *Bacillus thuringiensis* subsp. *Kurstaki* KB100 Strain Against *Spodoptera exigua*

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Insecticidal activity was enhanced when *Bacillus thuringiensis* subsp. kurstaki KB100 strain containing insecticidal activity against *Spodoptera exigua* was mixed with tannic acid, a protease inhibitor. To investigate the cause of this result, inhibition rate of tannic acid against protease activity in midgut juice of *S. exigua* was measured. As a result, it was found that protease activity in midgut juice of *S. exigua* was about 83.1%, 77.6%, 68.0%, and 40.1% at concentrations of 10, 20, 40 and 80 mM, respectively. Such reduction of protease activity was measured with increasing concentration. Analysis of substrate reaction in several kinds of proteases was performed. Trypsin exhibited 91.4% and 89.4% of proteolytic activity in BApNA and BPVApNA substrates, respectively. Thus, when Trypsin was treated with tannic acid, proteolytic activity was 62.2% and 54.5% in BApNA and BPVApNA substrates, respectively. Trypsin's proteolytic activity was inhibited by 29.2% and 34.9% when mixed with tannic acid. However, proteolytic activity of chymotrypsin and elastase was not inhibited by tannic acid. Digestion of protoxin produced in *B. thuringiensis* KB100 strain by Trypsin was analyzed using SDS–PAGE. As a result, a band appeared at about 60 kDa–70 kDa and it seemed to be inhibited by tannic acid. Digestion patterns of protoxin were measured over time. As it was over–digested in a group of Trypsin treatment, a band of protoxin at 60 kDa completely disappeared. On the other hand, when it was treated with tannic acid, a digestion inhibitor, protoxin was maintained for up to 24 hours.

Key words: *Bacillus thuringiensis* KB100 strain, proteolytic enzymes, *Spodoptera exigua*, tannic acid

INTRODUCTION

Spodoptera exigua is classified as Order Lepidoptera, Family Noctuidae and Genus *Spodoptera* and it is the most common agricultural pest which is the most difficult one to control as a polyphagous pest (Ahn *et al*., 1989). Since *S. exigua* has a broad range of hosts, it gives a lot of damage to a variety of crops such as scallion, cabbage and watermelon (Park *et al*., 1991). As *S. exigua* is rapidly spread out after it passes the third stage and generations are mixed, it is the pest which is difficult one to control during packaging (Luo *et al*., 2000). Chemical insecticides such as organophosphate, carbamate and pyrethroid insecticides are being used to control this pest (Eveleens *et al*., 1973). However, *S. exigua* has a high resistance to most of commercially available insecticides (Noh, 2009). Moreover, larvae with increasing age exhibit significantly low sensitivity to chemical insecticides. On the other hand, the use of microbial insecticides is recently demanded for eco–friendly agriculture, but it is difficult to control this pest due to physiological and ecological characteristics of *S. exigua* and mixture of generation during packaging. Because *B. thuringiensis* agent being used as a microbial insecticide to control *S. exigua* in Korea exhibits low control threshold, effective studies on use methods and treatment time are needed (Jin *et al*., 2009).

B. thuringiensis which is a microbial insecticide produces insecticidal crystal proteins (ICPs) which are called δ–endotoxin during the formation of spores (Schnepf *et al*., 1998; Kumar 2003). These ICPs consist of Cry proteins which have activity against Lepidoptera (CryⅠ), Lepidoptera and Diptera (CryⅡ), Coleoptera (CryⅢ), Diptera (CryⅣ), and Lepidoptera and Coleoptera (CryⅤ) (De Maagd *et al*., 2003; Schnepf *et al*., 1998; van Frankenhuyzen, 2009). ICPs are produced by *B. thuringiensis* and their molecular weight is 130 kDa. However, it does not have insecticidal activity in steady state. It exhibits activity if it is hydrolyzed into proteins at 55–70 kDa by proteolytic enzymes in midgut juice once larvae eat it. Activated toxin acts on epithelial cells of midgut in larvae and punches the hole in the membrane. In final, it becomes toxin and kills insects (Soberon *et al*., 2009).

Enzymes digest insecticidal toxin proteins which kill lepidopteran pests and consist of three groups such as serine proteinases, cysteine proteinases and aspartic proteinases. It is known that proteolytic enzymes such as serine proteinases and cysteine proteinases are present in midgut juice of *S. exigua* (Jongsma, 1996). It has been known that proteases are the most important digestive enzymes in insecticidal activity of *B. thuringiensis* and

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serine proteases such as trypsin and chymotrypsin play a major role in the process of protein hydrolysis (Tojo and Aizawa, 1983; Zhu *et al*., 2007). It has been reported that trypsin and chymotrypsin are the most important digestive enzymes to activate protoxin in midgut of *B. thuringiensis* and play an important role to digest ICPs (Zhu *et al*., 2007; Opprert, 1999).

In previous studies, new *B. thuringiensis* containing strong activity against *S. exigua* was selected and tannic acid was selected as one of protease inhibitor which could prevent the over–digestion of protoxin of *B. thuringiensis* caused by strong digestive activity of midgut juice of pests (Jin *et al*., 2009). It was confirmed that addition of tannic acid into *B. thuringiensis* resulted in increase of activity (Jin *et al*., 2009). In addition, it has been reported that Tannis reduces the amount of strains eaten by larvae and enhances insecticidal activity of *B. thuringiensis* (Lord and Undeen, 1990; Navon *et al*., 1993). According to Salama *et al*. (1984), tannic acid increased the efficacy of δ–endotoxin by 2–4 folds and it increased pH in midgut of insects, resulting in increase of insecticidal activity of *B. thuringiensis* once tannic acid was added (Schnepf *et al*., 1998). However, mechanism of interaction between *B. thuringiensis* and tannic acid has not been clearly known yet. It has been reported that Tannins inhibited activity of proteases and thus inhibited growth of lepidopteran larvae (Chan *et al*., 1978; Klocke and Chan, 1982; Karowe, 1989; Morris *et al*., 1995).

In this study, we reviewed the mechanism of synergistic effects on interaction between *B. thuringiensis* showing activity against *S. exigua*. In addition, we sought the methods to effectively control *S. exigua* by examining the effects of treatment of tannic acid on inhibition of capacity of digestive enzymes which hydrolyzes protoxin of *B. thuringiensis* strain.

MATERIALS AND METHODS

Test insects

S. exigua used in this experiment was given by the insect physiology laboratory in Andong National University and artificial diet (Goh *et al*., 1990) was used for all insects being cultured over successive generations in the biological pest control laboratory in Chungnam National University. Culture conditions are as follows: Temperature of 25±1˚C, light conditions of 16L:8D. Adults were fed on 10% sugar water.

Test *B. thuringiensis* strain

Among *B. thuringiensis* strains isolated from domestic soils and being stored in the laboratory, we selected KB100 strain showing a synergistic effect when mixed with Tannic acid and we performed experiment. We sent it to Dr. M. Obha in Institute of Biological Control, Faculty of Agriculture, Kyusu University, Fukuoka, Japan to identify it. As a result of H serotype, it was identified as *B. thuringiensis* subsp. *Kurstaki*.

Preparation of midgut juice of *S. exigua*

Midgut juice was prepared to digest protoxin of *B. thuringiensis* as follows. After larvae of *S. exigua* at the fifth stage were placed at –4˚C for 10 seconds, midgut was dissected by using a sterile dissecting blade and put in a centrifugation tube on ice. It was centrifuged at 13,000 rpm for 15 minutes. Only light brown supernatant was transferred into a new eppendorf tube and stored at –20˚C.

Preparation of Parasporal crystal, Tannic acid, Trypsin and Chymotrypsin

For preparation of parasporal crystal, *B. thuringiensis* strain was inoculated into NA media and incubated at 27˚C for 5 days and then occurrence of autolysis was examined with a phase contrast microscope. Once it was confirmed, it was transferred into a centrifuge tube with PBS buffer and then centrifuged at 15,000 rpm at 4˚C for 10 minutes. After centrifugation, the supernatant was discarded and it was washed three times with washing buffer I (500 mM NaCl, 2% Triton X–100). It was washed twice with washing buffer Ⅱ (500 mM NaCl). After sterile water was added, washed parasporal inclusion was stored at –20˚C until it was used. Tannic acid (Sigma Co.) was diluted into four kinds of concentration (0.4, 4, 40 and 80 mM/l) using distilled water. 1 mg/ml Trypsin (Sigma Co.) and chymotrypsin (Sigma Co.) were used as proteases to examine the role of tannic acid as a protease inhibitor.

SDS–PAGE

Parasporal inclusion of *B. thuringiensis* was dissolved in 50 mM NaOH (pH12.5). To observe the inhibitory phenomenon over time, 7 µl parasporal inclusion of *B. thuringiensis* and 2 µl midgut juice of *S. exigua* were added and digested at 37 ˚C for 15 minutes. After 2 µl of 40 mM tannic acid was added, it was incubated at each time point (1 minute, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 5 hours and 24 hours) at 37˚C and used in the experiment. Parasporal inclusion was incubated at 37˚C for 30 minutes, after it was treated with 10 µl of trypsin (0.1 mg/ml) and 1 µl chymotrypsin (0.1 mg/ml). 10 µl parasporal inclusion, 1 µl Trypsin and 1 µl of 40 mM tannic acid were treated. For SDS–PAGE, a gel was made with 12% separating gel and 5% stacking gel according to the modified method of Laemmli (1970). After the electrophoresis was finished, a gel was stained with 0.5% Coomassie Brilliant Blue.

Measurement of protease activity of *S. exigua*

To measure protease activity, a method of Bradford (1976) was modified and used in this experiment. To measure activity of each digestive enzyme, substrates were used as follows: N–α–Benzoyl–DL–arginine 4–nitroanilide hydrochloride (BApNA) and N–Benzoyl–Phe–Val–Arg–p–nitroanilide hydrochlorid (BPVApNA) were used as substrates for trypsin. N–Benzoyl–L–tyrosine p–nitroanilide (BTpNA) and N–Succinyl–Ala–Ala–Pro–Phe p–nitroanilide (SAAPPpNA) and Ala–Ala–Val–Ala p–nitroanilide (AAVApNA) were used as sub-

strates for chymotrypsin. N–Succinyl–Ala–Ala–Ala–p–nitroanilide (SAAApNA) and N–Succinyl–Ala–Ala–Pro–Leu p–nitroanilide (SAAPLpNA) were used as substrates for elastase. To determine the inhibitory capacity of tannic acid against midgut juice of *S. exigua*, azocasein was used as a substrate at various concentrations (10, 20, 40 and 80 mM/l). After midgut juice of *S. exigua* and tannic acid were mixed in the ratio of 1:1, it was incubated at 37°C for 15 minutes. After 300 µl of each substrate was mixed with 100 µl of digested sample, it was incubated at 37°C for 15 minutes. The reaction was stopped by adding 200 µl of 10% TCA. After the sample was centrifuged at 15,000 rpm at 4°C for 30 minutes, supernatant was mixed with 1 M NaOH in the ratio of 1:1 to precipitate proteins. After that, protein concentration was measured by absorbance at 450 nm.

RESULTS AND DISCUSSION

Protoxin of *B. thuringiensis* KB100 strain of which insecticidal activity is reduced due to over–digestion in midgut juice of *S. exigua*

As eggs of *S. exigua* are laid and stuck as egg rafts in crops, it is difficult to biologically control them. Larvae live together once they hatch eggs. Once they pass the third stage, they begin spreading out and hiding to live. Thus, it is more difficult to control them. Due to the physiological and ecological characteristics of this pest in which adults continue to fly in from neighboring areas and lay eggs, it is one of most difficult pests to be controlled in the farm. *B. thuringiensis* is most commonly used to biologically control *S. exigua* occurring in organic crops and it shows very various insecticidal activities depending on its strains. Thus, in a previous study by Jin *et al*. (2009), the synergistic effect of *B. thuringiensis* mixed with tannic acid on insecticidal activity was examined. It was found that a group mixed with *B. thuringiensis* and tannic acid showed a higher extinction rate than a group treated with *B. thuringiensis* alone. Therefore, we reviewed mechanisms which may occur in the relationship between protoxin and digestive enzymes of midgut juice of *S. exigua* to increase the control effect. Five kinds such as tannic acid, PMSF, EDTA, TLCK and SBTI were selected as protease inhibitors containing the highest activity to inhibit digestive enzymes of midgut juice in *S. exigua*. As a result of measuring activity of five kinds of protease inhibitors against midgut juice of *S. exigua*, it was found tannic acid constantly inhibited activity of protease in midgut juice of *S. exigua* (Jin *et al*., 2009). To determine the most optimal activity reaction of tannic acid with protease present in midgut, it was measured at various concentrations. As shown in Table 1, protease activity of midgut of *S. exigua* was inhibited in about 83.1%, 77.6%, 68.0% and 40.1% with increasing concentration of tannic acid such as 10, 20, 40 and 80 mM, respectively. Through above results, we could expect that protease activity of midgut of *S. exigua* was effectively inhibited with increasing the concentration of Tannic acid. In addition, according to Ananthakrishnan *et al*. (1990), weight and survival rate of pupae were measured after giving foods of *H. armigera* treated with tannic acid at various concentrations. As a result, the weight of pupae feeding foods mixed with tannic acid got lighter and the survival rate got lower than those of pupae feeding regular foods. The reason was that foods were not digested because protease activity of midgut of larvae was inhibited by Tannic acid.

Proteases present in midgut of *S. exigua* consist of serine proteases such as Trypsin, chymotrypsin and elastase (Opprert, 1999). It was reported that the use of high substrate specificity was appropriate to determine the characteristics of digestive enzymes (Law *et al*., 1977). Thus, we measured activity of proteases after the reaction was performed by adding the specific substrate for proteases present in midgut of *S. exigua* in this experiment. If protease activity was measured by using azocasein which was a substrate for serine proteases as a control, the activity was 100%. As shown in Table 2, when protease activity was measured by using a substrate for Trypsin, activity was about 90% or higher. On the other hands, when it was measured by using a substrate for chymotrypsin, activity was about 55%, which was lower than that of trypsin by about 35%. Activity of elastase was about 44%, which was the lowest among three kinds of proteases. Through this result, we could expect that trypsin had the highest activity among proteases in midgut of *S. exigua*. According to results of a previous study showing the inhibition of protease activity in midgut juice of *S. exigua*, we performed the reaction treated with tannic acid. Tannic acid selected as an activity inhibitor was prepared at four kinds of concentration (10, 20, 40 and 80 mM/l). After it was mixed with midgut juice of *S. exigua*, we measured the degree of inhibition in activity of proteases (Table 3). As a result, when midgut juice was reacted with substrates for Trypsin such as BApNA and BPVApNA, activity was 91.4±1.8 and 89.4±0.7, respectively. However, when 40 mM tannic acid was added into above reaction, activity was significantly inhibited to 62.2±0.3 and 54.5±1.1, respectively. It suggested that 40 mM tannic acid inhibited activity of proteases in midgut juice of *S. exigua*. Among serine proteases included in midgut juice of *S. exigua* in which inhibitory effect of Tannic acid was shown, trypsin had the highest activity, but other three enzymes did not exhibit any significant difference (Fig. 1). As a result, it was found that the activity for a substrate in midgut juice of *S. exigua* was inhibited by about 30–40% when it was treated with 40 mM tannic

Table 1. Effect of tannic acid on protease activity of *S. exigua* midgut juice

Protease inhibitor	Working concentration of inhibitor (mM)	Protease activity (% control)
Tannic acid	10	83.1±2.1
	20	77.6±1.6
	40	68.0±0.4
	80	40.1±2.2

Table 2. Estimation of proteolytic activity in extracts from *S. exigua* midgut with the substrates of various proteolytic enzymes

Substrate	Midgut	Protease
	Protease activity (% control)	
Azocasein	100 ± 0	Serine
N–α–Benzoyl–DL–arginine 4–nitroanilidehydrochloride (BApNA)	91.4 ± 1.8	Trypsin
N–Benzoyl–Phe–Val–Arg–p–nitroanilidehydrochlorid (BPVApNA)	89.4 ± 0.7	
N–Benzoyl–L–tyrosine p–nitroanilide (BTpNA)	55.4 ± 0.6	
N–Succinyl–Ala–Ala–Pro–Phe p–nitroanilide (SAAPPpNA)	56.5 ± 3.9	Chymotrypsin
Ala–Ala–Val–Ala p–nitroanilide (AAVApNA)	52.7 ± 1.5	
N–Succinyl–Ala–Ala–Ala–p–nitroanilide (SAAApNA)	48.5 ± 1.6	Elastase
N–Succinyl–Ala–Ala–Pro–Leu p–nitroanilide (SAAPLpNA)	38.7 ± 4.9	

Table 3. Inhibitor analysis of proteolytic activity with various substrates of extracts from *S. exigua* midgut

Inhibitor	Concentration (mM)	Azocasein	BApNA	BPVApNA	BTpNA	SAAPPpNA	SAAApNA
		Serine	Trypsin		Chymotrypsin		Elastase
Tannic acid	10	100	86.9±1.2	87.1±.07	88.9±1.2	62.3±1.9	42.5±0.7
	20	100	83.5±0.7	81.2±1.3	86.2±2.5	51.2±2.3	44.2±2.7
	40	100	62.2±0.3	54.5±1.1	61±1.5	51.4±2.5	46.1±1.0
	80	100	61.4±2.1	63.3±0.5	57.3±2.1	46.8±±1.2	42.5±0.7

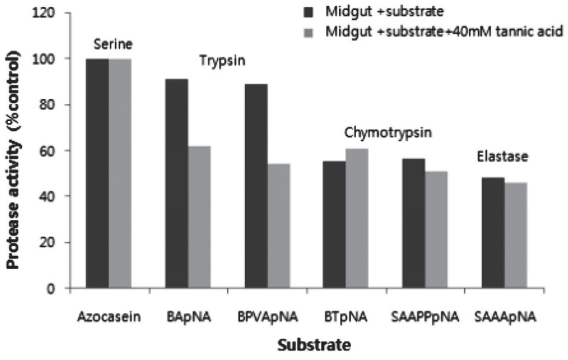

Fig. 1. Reductions of proteolytic activities of proteolytic enzymes of *S. exigua* to each substrate with 40 mM tannic acid.

acid. It was known that tannic acid effectively inhibited activity of trypsin among serine proteases.

Digestive roles of midgut juice, trypsin and chymotrypsin when tannic acid is mixed with protoxin produced by *B. thuringiensis* KB100 strain

In general, protoxin of *B. thuringiensis* showing insecticidal activity against lepidopteran pests consists of about 130 kDa and it is digested into active toxic protein at 60–70 kDa and a small protein by protease of midgut juice in insects (Li *et al*., 2011). Therefore, *B. thuringiensis* KB100 strain exhibited protoxin at 130 kDa and a partially digested protein at 70 kDa (Fig. 2A: lane 1). According to Li *et al*. (2011), it has been reported that protoxin of *B. thuringiensis* is activated by proteolytic enzymes such as trypsin and chymotrypsin. When protoxin of *B. thuringiensis* KB100 strain was activated by midgut protease and trypsin, it was broken down into proteins at 60–70 kDa over time. Activation of protoxin of *B. thuringiensis* KB100 strain by midgut juice, trypsin (0.1 mg/ml) and chymotrypsin (0.1 mg/ml) was compared (Fig. 2A). Protoxin broken down by midgut juice exhibited a pattern lower than 65 kDa (Fig. 2A: lane 2). The digestion ratio by Trypsin and chymotrypsin was smaller than that by midgut juice. When protoxin was digested by trypsin, it exhibited a protein band at

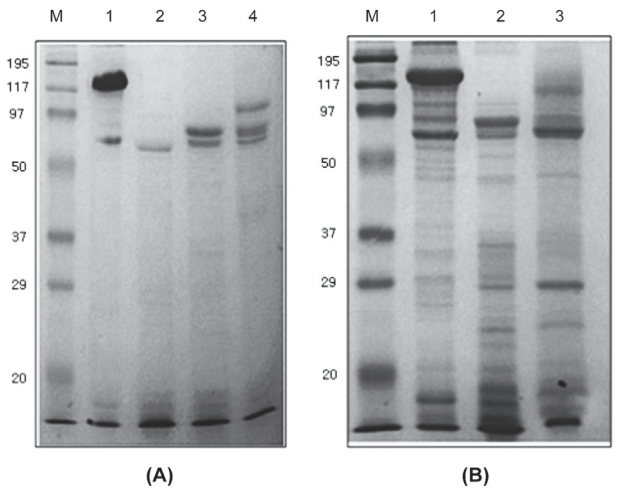

Fig. 2. (A) Digestion of protoxin by purified trypsin, chymotrypsin, and midgut juice from *S. exigua*. M: Standard Marker; lane 1: KB100 protoxin; lane 2: midgut juice; lane 3: trypsin; lane 4; chymotrypsin; (B) Effect of tannic acid on the protoxin activation in *S. exigua* midgut juice. M: Standard Marker; lane 1: KB100 protoxin; lane 2: trypsin; lane 3: trypsin+40 mM tannic acid.

60–70 kDa. When protoxin was digested by chymotrypsin, it exhibited several protein bands at 60–90 kDa (Fig. 2A: Lanes 3, 4). On the basis of previous results, we examined the digestion pattern of protoxin when 40 mM tannic acid was mixed with trypsin in order to determine whether tannic acid inhibited the digestion activity of trypsin when it was mixed with trypsin (Fig. 2B). It was found that activity of trypsin was inhibited when protoxin was decomposed by Trypsin together with 40 mM tannic acid rather than when it was digested by trypsin alone (Fig. 2B: lanes 2, 3). It could be expected that decomposition of protoxin was inhibited when 40 mM tannic acid was mixed with trypsin.

Tannic acid inhibiting the trypsin's role of over–digesting protoxin produced by *B. thuringiensis* KB100 strain

To compare the degree of decomposition of protoxin in *B. thuringiensis* KB100 strain by various kinds of digestive enzymes, SDS–PAGE was performed after it was treated in each condition. After *B. thuringiensis* KB100 protoxin was digested with midgut juice of *S. exigua* for 9 time points such as 1 minute, 5 minutes, 10 minutes, 15minutes, 30 minutes, 1 hour, 2 hours, 5 hours and 24 hours, protein patterns were examined. A band of protein at 60 kDa appeared on SDS–PAGE for up to 5 hours of treatment. The intensity of a band began to be reduced and a band almost disappeared at 24 hours (Fig. 3: lanes 1–9). Thus, it suggested that toxicity of *B. thuringiensis* showing high insecticidal activity against *S. exigua* may not appear in the presence of midgut proteases after pests eat it.

Thus, SDS–PAGE was performed to determine changes of protoxin of *B. thuringiensis* KB100 strain when it was digested by midgut juice together with tannic acid over time (Fig. 4). It was found that a band of protein with insecticidal activity at 60 kDa did not appear and activity was maintained in the sample combined with midgut juice + *B. thuringiensis* KB100 + 40 mM tannic acid showing the highest synergistic effect in a test of insecticidal activity against *S. exigua* (Fig. 4: lanes 1–9). It was similar to the previous results reporting that tannic acid inhibited the over–digestion of protoxin in *B. thuringiensis* KB100 strain by protease in midgut of *S. exigua* (Jin *et al*., 2009). In this experiment, a band of toxic protein at 60 kDa showing insecticidal activity against *S. exigua* was constantly maintained for up to 48 hours. In addition, a digestion pattern was examined over time after *B. thuringiensis* KB100 strain was digested by trypsin (Fig. 5). *B. thuringiensis* KB100 strain began to be digested over time after it was treated with trypsin. After 24 hours, a band of toxic protein at 60 kDa seemed lighter. On the other hand, changes of *B. thuringiensis* KB100 strain were observed over time when it was treated by trypsin together with tannic acid (Fig. 6). SDS–PAGE analysis exhibited that the digestion process of protoxin of *B. thuringiensis* KB100 strain by trypsin was inhibited by tannic acid. Therefore, it was found that decomposition rate of protoxin was substantially reduced with addition of tannic acid and its insecticidal activity lasted.

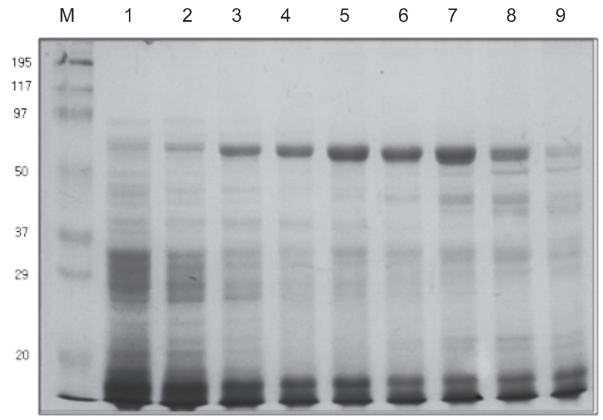

Fig. 3. Effects of incubation time of *S. exigua* midgut juice on KB100 toxin activation. M: Standard marker; lane 1: 1 min; lane 2: 5 min; lane 3: 10 min; lane 4: 15 min; lane 5: 30 min; lane 6: 1 h; lane 7: 2 h; lane 8: 5 h, lane 9: 24 h.

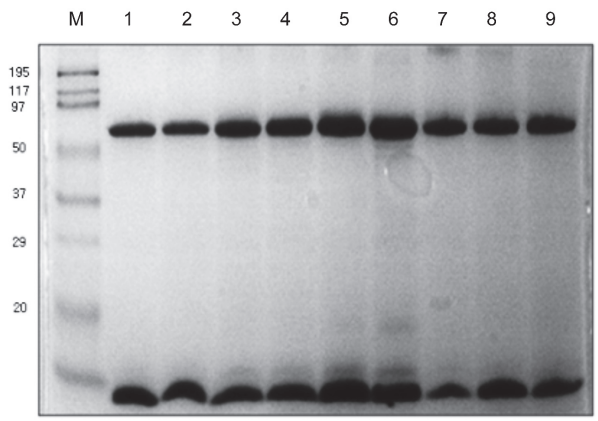

Fig. 4. Effects of incubation time of 40 mM tannic acid on KB100 toxin activation. M: Standard Marker; lane 1: 1min; lane 2: 5 min; lane 3: 10 min; lane 4: 15 min; lane 5: 30 min; lane 6: 1 h; lane 7: 2 h; lane 8: 5 h; lane 9: 24 h.

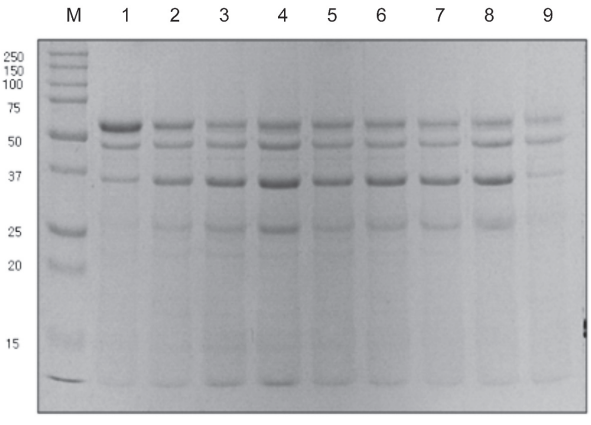

Fig. 5. Effects of incubation time of *S. exigua* midgut juice on typsin. M: Standard marker; lane 1: 1 min; lane 2: 5 min; lane 3: 10 min; lane 4: 15 min; lane 5: 30 min, lane 6: 1 h; lane 7: 2 h; lane 8: 5 h, lane 9: 24 h.

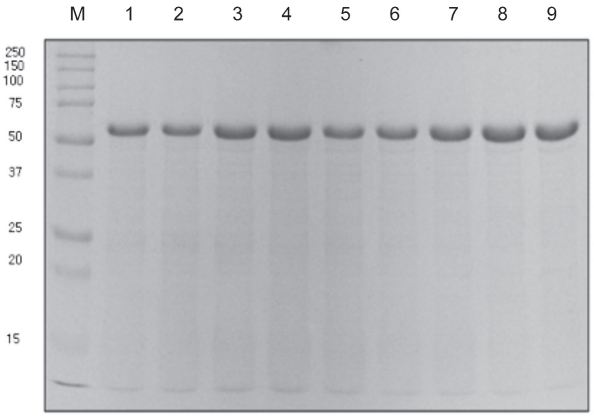

Fig. 6. Effects of incubation time of 40 mM tannic acid on trypsin. M: Standard marker; lane 1: 1 min; lane 2: 5 min; lane 3: 10 min; lane 4: 15 min, lane 5: 30 min; lane 6: 1 h; lane 7: 2 h; lane 8: 5 h; lane 9: 24 h.

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