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ORIGINAL ARTICLE

Inhibition of endogenous α-amylase and protease of *Aspergillus flavus* by trypsin inhibitor from cultivated and wild-type soybean

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Abstract The anti-*Aspergillus flavus* activity of trypsin inhibitor (TI) from cultivated and wild-type soybean (SBTI and WBTI) was investigated in order to confirm its ability to reduce the activity of endogenous α-amylase, protease enzymes and production of aflatoxin B_1 secreted by *A. flavus*. In the current study, it was demonstrated that purified SBTI/WBTI belonged to the family of Bowman-Birk TI, based on evidence from amino acid composition, the presence of two independent binding sites for trypsin and chymotrypsin, and a lysine residue as the active site for trypsin inhibition. Studying the inhibition of *A. flavus* showed that the effect of SBTI/SBTI on *A. flavus* α-amylase activity and aflatoxin B_1 production depended on TI concentration. However, no inhibitory effect was observed when sufficient exogenous α-amylase (EC 3.2.1.1, from *Bacillus subtilis*) was added. The resistance to *A. flavus* infection was partially due to the ability of SBTI/WBTI to inhibit α-amylase activity, thereby limiting the availability of hydrolyzed reducing sugar for fungal growth and further suppressing aflatoxin B_1 biosynthesis. In addition, the relationship between SBTI/WBTI levels and fungal protease expression revealed that *A. flavus* released a certain quantity of endogenous proteases into the culture medium, and the decreased activity of protease and production of aflatoxin B_1 suggested that the inhibition activity might also be mediated by SBTI/WBTI as *A. flavus* protease inhibitor activity.

Keywords *Aspergillus flavus* · Trypsin inhibitor · α-Amylase · Protease · Aflatoxin B_1

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Introduction

Aspergillus flavus is a common fungal species that is widely distributed in tropical and subtropical zones around the globe. It has gained significant agricultural importance because it produces potent aflatoxins, which are very active at very low levels and possess hepatotoxic, teratogenic and mutagenic properties, with a maximum allowable level permitted by the Food and Drug Administration of 20 ppb for foods and for most feeds and feed ingredients (Sandosskumar et al. 2007). The European Union has set an aflatoxin standard limit of 4 ppb for peanut (Mellon et al. 2007). Therefore, much effort has been devoted to the search for new antifungal materials from natural sources for food preservation to prevent or suppress these fungi and/or mycotoxin biosynthesis (Zhang et al. 2008; Reddy et al. 2009).

Protease inhibitors in the seeds of many plant species have been found to have activity against *A. flavus* infection, including trypsin inhibitor (TI) from peanut (Liang et al. 2004), lectin and α-amylase inhibitor from *Lablab purpureus* (Fakhoury and Woloshuk 2001), as well as the 22-kDa TI from corn (Huynh et al. 1992) and the 24-kDa cysteine protease inhibitor from pearl millet (Joshi et al. 1998). Huang et al. (1997) showed that kernels of Tex6 contain a 28-kDa protein that inhibited growth of *A. flavus* and a 100-kDa protein that inhibited aflatoxin biosynthesis without affecting fungal growth. The 14-kDa protein from corn purified by

Chen et al. (1998, 1999a) was identified as a TI that inhibited conidial germination and hyphal growth of *A. flavus*.

Alpha amylase secreted by *A. flavus* is an important α-1,4 linkage hydrolytic enzyme for breaking down starch or other carbon source to produce low molecular weight glucose, maltose, and maltotriose (reducing sugar; Nahas and Waldemrin 2002), which might induce aflatoxin B_1 biosynthesis (Woloshuk et al. 1997). Studies by Davis and Diener (1968) also showed that glucose, sucrose, and maltose produced during starch hydrolysis by endogenous *A. flavus* α-amylase induced high levels of aflatoxins. Woloshuk et al. (1997) also found that inducers of aflatoxin biosynthesis were generated by *A. flavus* α-amylase. Chen et al. (1998, 1999a) presented evidence that the 14-kDa TI from corn could inhibit α-amylase from *A. flavus*, showing that it belonged to a group of bifunctional trypsin and amylase inhibitors.

In addition, *A. flavus* produces different classes of polymer-degrading proteases to utilize a variety of natural substrates, the major classes of which appear to be serine proteases and metalloproteinases (Mellon and Cotty 1995). The 23-kDa protease (metalloproteinase) secreted by *A. flavus* was capable of utilizing a wide range of protein substrates, including both hydrophobic proteins (elastin, cottonseed storage protein, collagen, and zein) and soluble proteins (ovalbumin and bovine serum albumin; Mellon and Cotty 1996; Mellon et al. 2007). The 33-kDa protein present in *A. flavus*-infected maize embryo was identified as a fungal alkaline protease, which might play an important role in the infection of maize kernels and subsequent aflatoxin accumulation (Chen et al. 2009). However, there have been few reports on the control of the activity of endogenous protease by TI from cultivated [*Glycine max* (L.) Merr.] and wild-type (*Glycine soja* Sieb. & Zucc.) soybean resulting in inhibition of *A. flavus* growth and aflatoxin production.

Previous reports have demonstrated that purified TI from cultivated (SBTI) and wild-type (WBTI) soybean exerted strong inhibition on *A. flavus* germination and growth, with IC_{50} of 1.6 μM and 1.0 μM, respectively (Zhang et al. 2008, 2009a), which is stronger than some other antifungal peptides reported previously (Shivaraj and Pattabiraman 1998; Chen et al. 1999b; Wong and Ng 2003). In a different approach, chitosan-(TI extract)-glycerol blend films were prepared and used as potential bio-control packaging materials for peanut against *A. flavus* infection (Zhang et al. 2009b). The possible involvement of TI in plant defense against fungal pathogens had been implicated by their antifungal activities and ability to confer resistance against insects, e.g., upon expression of cowpea TI gene in tobacco (Hilder et al. 1987; Huynh et al. 1992). Thus, incorporation of anti-*A. flavus* genotypes into commercial hybrids could be further used to increase resistance to aflatoxin production in both field and laboratory studies.

However, no further results have been reported on the mechanism of action of soybean TI against *A. flavus* and its association with resistance to aflatoxin accumulation. Therefore, the objectives of this study were to further evaluate and identify the mechanism of *A. flavus* inhibition by SBTI/WBTI, in particular to determine whether SBTI/WBTI confer resistance to the activity of α-amylase and protease secreted by *A. flavus*, thereby inhibiting *A. flavus* growth and aflatoxin B_1 production.

Materials and methods

Materials and microorganisms

Cultivated soybean [*Glycine max* (L.) Merr.] from a local Chinese shop was used. Wild-type soybean (*Glycine soja* Sieb. & Zucc.) was collected manually from plants grown in Qingdao city (36°38′N, 120°45′E), Shandong province, China. Trypsin (EC 3.4.21.4, from porcine pancreas), chymotrypsin (EC 3.4.21.1, from bovine pancreas) and α-amylase (EC 3.2.1.1, from *B. subtilis*) were purchased from Amresco (http://www.amresco-inc.com). *Aspergillus flavus* strain 3.2890 was obtained from the General Microbiological Culture Collection Center of China (CGMCC, Beijing, China). Fungi were cultivated on Potato Dextrose agar medium (PDA) at 25°C for 7 days and the conidia harvested with 10.0 ml 0.1% Tween 80 solution sterilized by membrane filtration (0.45 mm). All other chemicals and reagents were of analytical grade.

Purification and characterization of SBTI and WBTI

TI from cultivated (SBTI) and wild-type soybean (WBTI) were isolated and purified using prepared chitosan resin-trypsin as filler on an affinity chromatography column based on our previous report (Zhang et al. 2008). TI inhibitory activity was analyzed using the improved BANA method (N-benzoy-arginine-2-naphthylamide, $C_{23}H_{25}O_2$·HCl; Sigma, St. Louis, MO) as reported by Carlton and Ines (1961) and Zhang et al. (2007).

Binary complexes of TI with either trypsin or chymotrypsin were prepared from mixtures of TI with excess enzyme after incubation at 4°C for 24 h. Ternary complex of TI with trypsin and chymotrypsin was obtained by adding an excess of isolated binary complex of TI and trypsin to chymotrypsin. This mixture was then applied to a Sephadex G-75 chromatography column (2 × 50 cm), equilibrated with 0.1 M citrate buffer (pH 7.2; $C_6H_8O_7$-Na_2HPO_4). SBTI/WBTI and the pooled fractions were then hydrolyzed in 6.0 M HCl solution at 110°C for 24 h and analyzed using a Hitachi L8800 amino acid analyzer (Hitachi, Tokyo, Japan).

The procedure for SBTI/WBTI lysine (Lys) residue modification with maleic anhydride followed the method previously reported by Bund and Singhal (2002) with slight modifications. SBTI/WBTI (2.0 mg/ml) was dissolved in 0.5 M Tris-HCl buffer, pH 8.6, in a reactor. A volume of maleic anhydride solution (0.02 M, 0–4.0 ml) was introduced into the container which was placed in an ice bath to maintain the temperature at 0–4°C. The pH of the reaction mixture was maintained at 8.6 by adding 0.2 M NaOH solution. After 30 min, the residual trypsin inhibition activity of SBTI/WBTI was assayed by the BANA method.

Effect of SBTI and WBTI on α-amylase activity and aflatoxin B_1 production

To test whether the anti-*A. flavus* activity of SBTI/WBTI was due to its activity as an α-amylase inhibitor, the following experiments were carried out (Zhang et al. 2009b). *Aspergillus flavus* conidia (10^2/μl), *A. flavus* conidia with SBTI/WBTI (1.8 μM), and *A. flavus* conidia with SBTI/WBTI plus different concentrations of exogenous α-amylase (5 μg/ml and 40 μg/ml, EC 3.2.1.1, from *B. subtilis*) were cultivated on Czapek culture medium. Mycelium diameter of *A. flavus* was measured after 2 days incubation at 28°C.

Another experiment examined the activity of endogenous α-amylase secreted by *A. flavus* in the presence of various concentrations of TI. SBTI/WBTI was added at a final concentration of 0.0–2.4 μM to conical flasks containing 10 ml modified Czapek liquid medium (where starch substituted sucrose as the substrate) and 100 μl (10^2/μl) freshly harvested *A. flavus* conidia. The conidia were incubated at 28°C for up to 6 days. Mycelial pellets were then separated from the liquid medium by centrifugal filtration (5,000 *g*, 15 min), and the activity of endogenous α-amylase in the medium was determined using the method of 3,5-dinitrosalicylic acid colorimetry (Li et al. 2002).

When starch was used as the sole substrate for *A. flavus* growth, the level of secreted α-amylase could be evaluated by the production of reducing sugar. SBTI/WBTI was added at a final concentration of 0.0–2.4 μM to Erlenmeyer flasks containing 50 ml basal medium (modified Czapek liquid medium where starch was substituted for sucrose as the substrate) with the following composition (g/l): $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.01 and starch, 20.0 and 400 μl (10^2/μl) freshly harvested *A. flavus* conidia. The inoculated flasks were agitated on a rotary shaker (120 rpm) at 28°C. Samples were taken every day. The concentrations of reducing sugars were determined according to the *p*-hydroxybenzoic acid hydrazide method with glucose as the standard (Lever 1972). Aflatoxin B_1 was measured by HPLC as previously described after 6 days incubation at the end of the experiments (Mahoney and Rodriguez 1996; Kim et al. 2008).

Effect of SBTI and WBTI on protease activity and aflatoxin B_1 production

Aspergillus flavus (15 μl, 10^2/μl) and *A. flavus* with SBTI/WBTI (15 μl, 1.8 μM) were grown on casein-agar medium (modified Czapek medium with casein substituted for sucrose as the substrate) having the following compositions (g/l): $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5; KCl, 0.5; FeSO4, 0.01; casein, 4.0; and agar, 20.0. *Aspergillus flavus* expressing high protease activity would exhibit a clear protease digestion zone around the margin of the colony on casein-agar medium. Furthermore, the influence of SBTI/WBTI on the activity of protease secreted by *A. flavus* was evaluated by incubating appropriate concentrations of SBTI/WBTI with the fungi. The protease was produced by *A. flavus* in modified Czapek liquid medium containing 10.0 g/l casein as the substrate. SBTI/WBTI was added at a final concentration of 0.0–2.4 μM to Erlenmeyer flasks (inoculated with 10^2/μl, 400 μl conidia suspension), which were incubated on a rotary shaker (120 rpm) at 28°C for 6 days. To obtain the secreted protease from fungal mycelial tissue, mycelial pellets were separated from the liquid medium by centrifugal filtration (5,000 *g*, 15 min), and the protease activity present in the filtrate was tested for the inhibition studies.

In addition, another experiment was conducted using the same conditions as described above, but the residual protein concentrations in culture filtrates were measured every 12 h by a Coomassie G-250 binding procedure with bovine serum albumin as the standard to evaluate the activity of the secreted protease (Bradford 1976). After 6 days, the concentration of aflatoxin B_1 produced was also assayed by HPLC (Mahoney and Rodriguez 1996; Kim et al. 2008).

Statistical analysis

Each property was measured at least three times in a randomized design, and all measurements were carried out at least twice. Statistical analysis was conducted using the SPSS package (SPSS, Chicago, IL). The Student's-Newman-Keuls (SNK) test was used to determine the statistical significance of differences ($P<0.05$).

Results and discussion

Purification and characterization of SBTI and WBTI

With the prepared chitosan resin-trypsin as matrix on the affinity chromatography column, SBTI and WBTI from

cultivated and wild soybean were purified with homogeneous MW of 8.2-kDa as estimated by SDS-PAGE—similar to that reported earlier for the Bowman-Birk TI (Zhang et al. 2006, 2008). In order to confirm that the purified SBTI/WBTI was a Bowman-Birk TI responsible for trypsin-inhibitory and chymotrypsin-inhibitory activities, the complexes formed by SBTI/WBTI with either trypsin or chymotrypsin or both of these enzymes were separated by gel chromatography on a Sephadex G-75 column (Fig. 1). When subjected to gel chromatography, trypsin/chymotrypsin eluted in a peak at 122/120 ml (chymotrypsin elution curve is not shown in Fig. 1, but was similar to the trypsin elution profile), and SBTI and WBTI were located in the same peak at 136 ml. The mixture of trypsin and SBTI/WBTI eluted with a protein peak at 105 ml that was judged to be the complex of trypsin and SBTI/WBTI. The second peak (122 ml) corresponded to non-reacted (excess) trypsin. When a mixture of chymotrypsin and excess isolated trypsin-SBTI/WBTI was subjected to gel chromatography, two peaks were observed with maximal protein values at 80 ml and 105 ml. The first peak was judged to represent a mixture of trypsin-SBTI/WBTI-chymotrypsin, and the second peak represented that portion of excess trypsin-SBTI/WBTI complex that had not combined with chymotrypsin.

Bowman-Birk TI (BBI) possesses two independent sites of inhibition, one at Lys 16–Ser 17 against trypsin and the other at Leu 43–Ser 44 against chymotrypsin. Therefore, it can form a 1:1 complex with either trypsin or chymotrypsin or a ternary complex with both enzymes (1:1:1; Zhang et al. 2006). Each of the complexes showed in Fig. 1 was analyzed for its amino acid composition. The results are given in Table 1. The amino acid compositions of SBTI/WBTI, trypsin/chymotrypsin-SBTI/WBTI, and trypsin-SBTI/WBTI-chymotrypsin agreed with the theoretical values of the BBI from other Leguminosae and their complexes (Wilcox 1970; Seidl and Liener 1971; Sessa and Nelsen 1991). These results suggest that SBTI/WBTI could also form a 1:1 molar complex with either trypsin or chymotrypsin and a 1:1:1 molar complex with both enzymes, confirming that the SBTI/WBTI belong to the BBI. In addition, chemical modification was used to assess the role of different amino acid residues in SBTI and WBTI. The Lys groups of SBTI/WBTI were modified with different concentrations of maleic anhydride (Fig. 2). The trypsin inhibitory activity of SBTI/WBTI decreased sharply with increasing amounts of maleic anhydride added. These results suggested the involvement of the Lys residues in the interaction between SBTI/WBTI and trypsin, in agreement with previous reports (Seidl and Liener 1971; Shivaraj and Pattabiraman 1998). Taken together, the amino acid composition, binding with trypsin and chymotrypsin, and the involvement of Lys residues in the active site for trypsin inhibition activity confirmed that SBTI and WBTI from cultivated and wild-type soybean can be classed as Bowman-Birk TI.

In our preliminary reports, we demonstrated that purified SBTI/WBTI exerted a strong inhibitory effect on *A. flavus* growth. Although TI from soybean has some disadvantages for food digestion as a native food grade inhibitor, it should be emphasized that all of these adverse effects were seen when TI was present in relatively high concentrations. At lower concentration it has been shown to be safe as a food additive (Friedman and Brandon 2001; Jiang et al. 2006). It

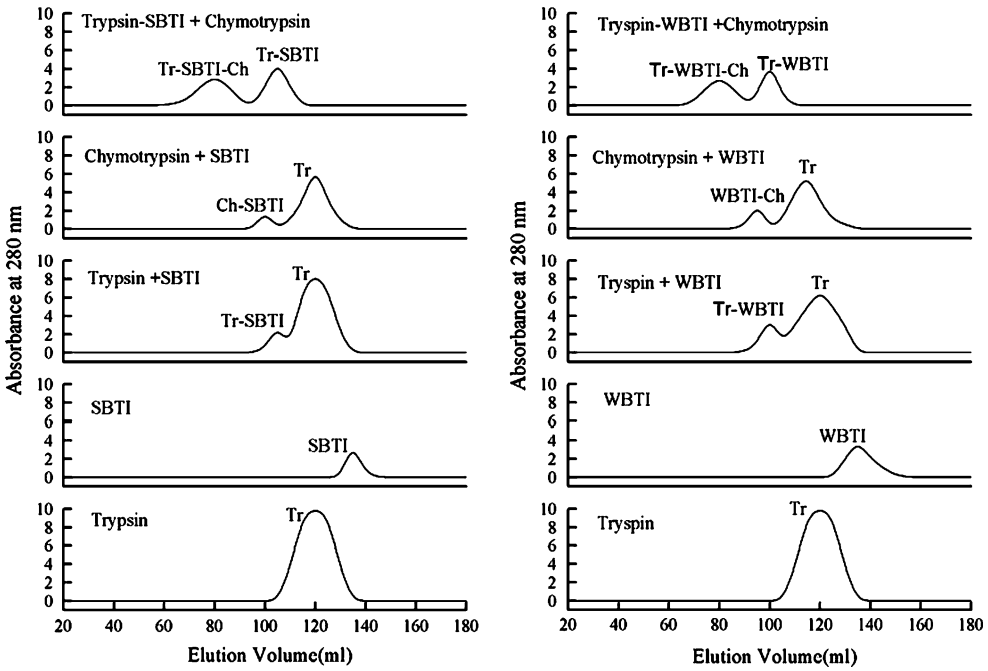

Fig. 1 Isolation by Sephadex G-75 filtration of complexes of cultivated and wild-type soybean trypsin inhibitor (SBTI/WBTI) with trypsin/chymotrypsin. Complexes: *Tr/Ch* Trypsin/chymotrypsin; *Tr-SBTI/WBTI* trypsin and SBTI/WBTI; *Ch-SBTI/WBTI* chymotrypsin and SBTI/WBTI; *Tr-SBTI/WBTI-Ch* trypsin, SBTI/WBTI and chymotrypsin

Table 1 Amino acid composition of complexes of cultivated (SBTI) and wild-type (WBTI) soybean trypsin inhibitor with trypsin or chymotrypsin. All values expressed as number of residues per mole of enzyme or complex

Amino acid	Bowman-Birk inhibitor[a]	SBTI[b]	WBTI[b]	Trypsin[c]	Chymotrypsin[d]	SBTI-Tr[b]	SBTI-Ch[b]	Tr-SBTI-Ch[b]	WBTI-Tr[b]	WBTI-Ch[b]	Tr-WBTI-Ch[b]
ASP	12	12.31	13.01	22	22	35.18	32.66	57.81	34.98	31.41	56.26
SER	8	8.05	7.32	33	27	43.72	36.73	66.32	44.22	36.14	67.46
GLU	7	7.76	6.85	14	15	20.36	21.34	35.05	21.17	22.53	36.14
GLY	0	0	0	25	23	27.12	23.55	47.23	27.92	24.37	48.63
ALA	4	4.64	4.33	14	22	18.40	26.87	41.34	17.91	26.12	40.33
VAL	1	1.39	1.72	17	23	17.22	23.41	40.93	18.27	22.67	41.98
MET	1	1.02	1.46	2	2	3.60	2.73	5.13	3.35	2.69	5.06
ILE	2	1.83	2.23	14	19	16.51	22.11	34.86	17.52	21.53	35.90
LEU	2	2.07	1.92	14	19	15.83	22.33	35.12	15.37	22.12	35.25
TYR	2	2.15	2.41	10	4	13.98	7.06	16.83	14.42	6.74	16.74
PHE	2	2.92	2.08	3	6	5.57	7.92	10.68	5.37	8.61	11.31
LYS	5	6.07	5.25	14	14	21.46	20.55	33.13	20.34	21.27	33.85
THR	2	1.95	2.25	10	22	11.55	26.16	34.95	12.57	25.92	34.87
HIS	1	1.30	1.12	3	2	3.93	2.82	6.72	3.75	2.94	6.25
ARG	2	2.01	2.10	2	3	4.22	6.37	7.38	4.18	6.51	7.11

[a] Theoretical values taken from Seidl and Liener (1971) and Sessa and Nelsen (1991)

[b] Values obtained by amino acid analysis as described in the text

[c] Theoretical values taken from Seidl and Liener (1971)

[d] Theoretical values taken from Seidl and Liener (1971) and Wilcox (1970)

was reported that the concentration of SBTI/WBTI required for 50% growth inhibition (IC_{50}) on *A. flavus* was about 1.6/1.0 μM (Zhang et al. 2009a), which is low compared with other soy foods. Reduced conidia germination and hyphal growth were readily observed in conidia suspensions of *A. flavus* treated with 0.6 μM SBTI/WBTI. Therefore, at such low concentrations, any residual adverse effects of SBTI/WBTI added to peanut or other agricultural crops in order to prevent or suppress *A. flavus* and/or mycotoxin contamination could be negligible.

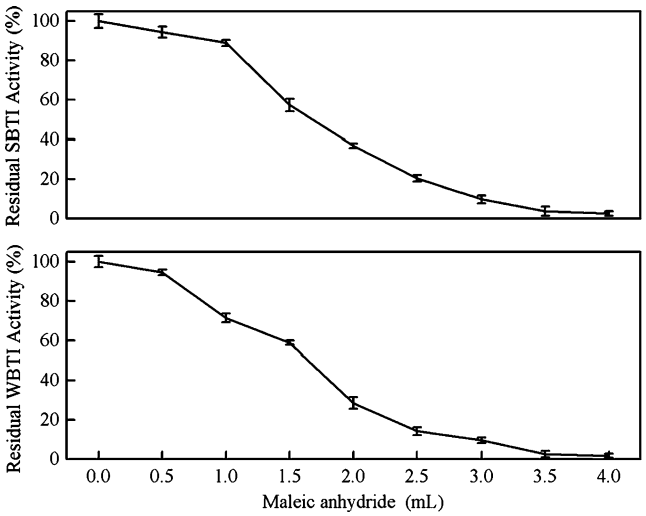

Fig. 2 Modification of Lys residues of SBTI/WBTI by maleic anhydride. Residual SBTI/WBTI activity was the residual trypsin inhibition activity determined by the BANA (N-benzoy-arginine-2-naphthylamide, $C_{23}H_{25}O_2{\cdot}HCl$) method

SBTI and WBTI inhibit α-amylase activity and aflatoxin B_1 production

The activity of α-amylase secreted by *A. flavus* after 6 days incubation was found to decrease rapidly with increasing TI concentrations (Fig. 3). The level of α-amylase activity in the presence of SBTI/WBTI at 1.8 μM was about one-fourth that of the non-treated control during the period studied, and *A. flavus* conidia could not germinate and grow normally at this concentration in the liquid medium. Furthermore, when *A. flavus* conidia were cultured with 1.8 μM SBTI/WBTI on Czapek solid culture medium, they were completely inhibited without germination at 28°C after 2 days (Table 2). In addition, *A. flavus* conidia also showed significant growth inhibition when they were treated with a lower concentration of exogenous α-amylase (5.0 μg/ml) plus 1.8 μM SBTI/WBTI (the mycelium diameter of *A. flavus* was about 3.1/2.6 mm). In contrast, the mycelium diameter of *A. flavus* with SBTI/WBTI (1.8 μM) plus higher exogenous α-amylase (40 μg/

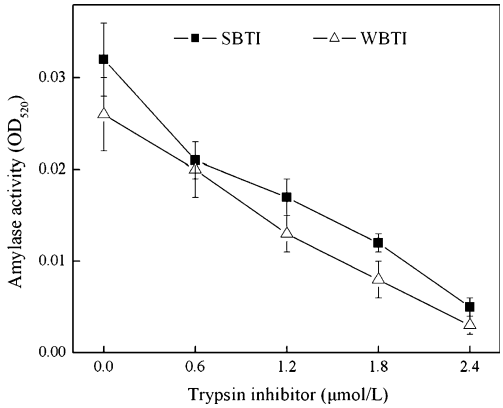

Fig. 3 Production of endogenous α-amylase secreted by *Aspergillus flavus* after 6 days in the presence of different SBTI/WBTI concentrations (modified Czapek medium with starch as the substrate)

ml) was about 15.6/15.2 mm, which was not significantly different from untreated *A. flavus* conidia growth (control, 15.3 mm). In other words, *A. flavus* conidia germination and growth was returned to normal when sufficient exogenous α-amylase added.

Alpha-amylase is an important hydrolyzing enzyme for breaking down starch to produce reducing sugar, the amount of which can be used to determine the activity levels of secreted α-amylase. It was previously demonstrated that constitutive levels of the 14-kDa TI in corn kernels were associated with resistance to *A. flavus* infection and aflatoxin production, and inhibited both conidia germination and hyphal growth (Chen et al. 1998). The results of Fakhoury and Woloshuk (2001) revealed that the resistance of certain corn genotypes to fungal infection might be related to the action of the corn TI in lowering the activity of α-amylase and consequently reducing the availability of simple sugars for fungal growth. Chen et al. (1999a) presented evidence showing that corn protein inhibited α-amylase of *A. flavus*, and suggested that it belonged to a group of bifunctional amylase and trypsin inhibitors. The above results and reports suggested that anti-*A. flavus* activity of SBTI/WBTI from soybean was probably due to inhibition of the activity of fungal α-amylase. Conidia germination and growth returned to normal when enough exogenous α-amylase was added, which combined with SBTI/WBTI to reduce the inhibition activity.

Adams and Deploey (1986) obtained high activity of α-amylase from *A. flavus* with starch as the substrate in the culture medium. Likewise Oso (1979) showed that α-amylase activity could be induced by using soluble starch as the sole substrate. Attia and Ali (1974) also demonstrated that starch, maltose, dextrin and glucose were effective inducers for *A. awamori* to secrete α-amylase. In the present study, *A. flavus* conidia with SBTI/WBTI (0–2.4 μM) were grown in modified Czapek liquid medium with starch as the substrate, the culture filtrates were harvested at various times and tested for the production of reducing sugars and α-amylase activity (Fig. 4). The production of reducing sugar essentially paralleled that of α-amylase activity. Production of reducing sugar (α-amylase activity) increased rapidly with prolonged incubation time for both the control and *A. flavus* with 0.6 μM SBTI/WBTI. However, when the concentration of SBTI/WBTI added was increased to 1.8 μM, little reducing sugar or α-amylase activity was detected in the filtrates collected after 6 days of growth. Low concentrations of SBTI/WBTI significantly inhibited the activity of α-amylase and the production of reducing sugar. The *A. flavus* growth-inhibiting activity of SBTI/WBTI is thus due to its α-amylase inhibitory activity by limiting the degradation of starch or other carbohydrates.

In addition, several attempts to identify inhibitory compounds for controlling aflatoxin biosynthesis have been reported (e.g., Reddy et al. 2009). It has been established that some types of carbohydrate available to *A. flavus* can greatly influence production of aflatoxin B_1.

Table 2 Diameter of *Aspergillus flavus* growth inhibition by TI with/without exogenous α-amylase in Czapek medium after 2 days. The concentration of *A. flavus* conidia, SBTI and WBTI were 10^2/μl, 1.8 μM and 1.8 μM, respectively. Values represent the means ± SD of measurements for five different samples. Experiments were repeated three times. Data with different letters were statistically different according to Student's-Newman-Keuls (SNK) test at $P<0.05$

Type	Diameter of *A. flavus* growth (mm)
A. flavus (control)	15.3±3.3 a
A. flavus with SBTI	–[c]
A. flavus with WBTI	–
A. flavus with SBTI plus exogenous α-amylase (5 μg/ml)	3.1±1.0 b
A. flavus with SBTI plus exogenous α-amylase (5 μg/ml)	2.6±1.4 b
A. flavus with SBTI plus exogenous α-amylase (40 μg/ml)	15.6±3.2 a
A. flavus with WBTI plus exogenous α-amylase (40 μg/ml)	15.2±3.5 a

[c] *A. flavus* conidia were inhibited completely (no germination or growth)

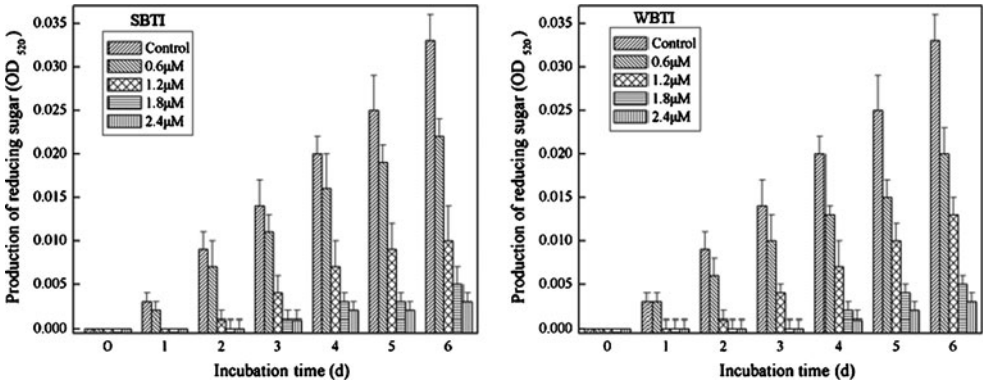

Fig. 4 Time course of production of reducing sugars in the presence of different SBTI/WBTI concentrations. SBTI (*left panel*)/WBTI (*right panel*) was added to the modified Czapek liquid medium with starch as the substrate at final concentrations of 0.0, 0.6, 1.2, 1.8 or 2.4 μM as indicated

Endogenous α-amylase can produce a burst of fermentable sugars (such as glucose, maltose and maltotriose), which form part of the aflatoxin B_1 inducers produced. Increases in α-amylase activity in maize kernel cultures paralleled the increase in aflatoxin-inducing activity, suggesting that the action of α-amylase on the maize starch played an important role in the induction. In theory, inhibition of the production of reducing sugars (or the activity of α-amylase) by SBTI/WBTI should also decrease aflatoxin B_1 biosynthesis. The data presented here obtained in the aflatoxin B_1 assay confirmed the above hypothesis. When *A. flavus* was cultivated with SBTI/WBTI at concentrations from 0.6 to 2.4 μM, a dramatic reduction in aflatoxin B_1 production was observed during the incubation (starch as substrate, Table 3). After 6 days of incubation, *A. flavus* treated with 2.4 μM SBTI/WBTI produced no aflatoxin B_1 but 70.4±4.6 ppb aflatoxin B_1 was produced when no SBTI/WBTI was present in the starch medium (control). These data show that an appropriate concentration of SBTI/WBTI can suppress aflatoxin B_1 biosynthesis effectively by limiting α-amylase activity that would otherwise hydrolyze carbohydrates to reducing sugar.

SBTI and WBTI inhibit protease activity and aflatoxin B_1 production

When used as a substrate, casein is subject to cleavage and hydrolysis by protease activities. Therefore, the level of protease secreted by *A. flavus* was determined by measuring hydrolysis of casein on a casein-culture medium (Fig. 5, inset). In this study, *A. flavus* (point A) exhibited a clear protein digestion zone around the margin of the colony on casein-agar medium, indicating significant protease activity. This result was also found with isolates of *A. flavus* obtained from cottonseed, corn, peanuts, insects and human sources, all of which displayed high protease activity (Mellon and Cotty 1996). However, no protease hydrolysis zone was seen in plate assays with *A. flavus* conidia

Table 3 Production of aflatoxin secreted by *A. flavus* with different concentrations of SBTI/WBTI in modified Czapek liquid medium with starch or casein as substrate. All inoculated media were agitated on a rotary shaker (120 rpm) at 28°C for 6 days. Production of aflatoxin secreted by *A. flavus* was then determined. Values are means ± SD. Data in columns with different letters are statistically different according to SNK test at $P<0.05$ and all treatments consisted of three replicates; experiments were repeated three times

Substrate	Compound	TI concentration (μM)	Production of aflatoxin B_1 (ppb)
Starch (20.0 g/l)	*A. flavus* (control)	0.0	70.4±4.6 a
	A. flavus with SBTI	0.6	55.6±5.1 b
		1.2	16.2±2.3 c
		1.8	1.7±0.3 d
		2.4	–[e]
	A. flavus with WBTI	0.6	54.1±3.3 b
		1.2	13.4±1.8 c
		1.8	1.5±0.5 d
		2.4	–
Casein (10.0 g/l)	*A. flavus* (Control)	0.0	64.2±4.8 a
	A. flavus with SBTI	0.6	42.3±2.4 b
		1.2	9.6±1.4 c
		1.8	1.1±0.2 d
		2.4	–
	A. flavus with WBTI	0.6	44.3±2.4 b
		1.2	8.3±1.7 c
		1.8	0.9±1.1 d
		2.4	–

[e] No aflatoxin B_1 detected

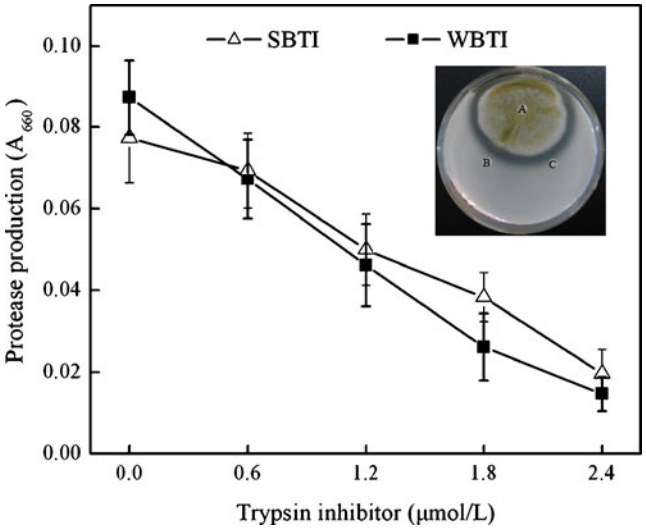

Fig. 5 Production of endogenous protease secreted by *A. flavus* in the presence of different SBTI/WBTI concentrations with casein as substrate. *Inset* Photograph of casein-digesting activity expressed by endogenous protease secreted by *A. flavus* on casein-agar medium ± SBTI/WBTI. *A* Control, *B* + SBTI, *C* + WBTI

cultivated with SBTI/WBTI (points B and C, *A. flavus* with SBTI and WBTI).

In order to evaluate whether protease expression patterns and aflatoxin B_1 contamination levels secreted by *A. flavus* were correlated with SBTI/WBTI treatment, a comparative study was carried out (Fig. 5). The results showed that the cultured *A. flavus* (control) had the maximum protease activity in liquid medium, whereas *A. flavus* in the presence of 2.4 μM SBTI/WBTI exhibited the lowest levels of proteolytic activity, which were reduced to about one-fifth the level of the control.

When *A. flavus* conidia were grown in the presence of SBTI/WBTI in liquid medium using casein as the substrate, the residual casein can provide an indirect reflection of the activity levels of the protease produced. Figure 6 shows time courses of residual protein in the presence of different SBTI/WBTI concentrations after incubation at 28°C for 3 days. The levels of residual protein were markedly dependent on the TI concentrations in the liquid medium. Increasing the SBTI/WBTI concentrations (0.6–2.4 μM) decreased the disruption or hydrolyzation of casein by inhibiting the activity of protease secreted by *A. flavus*. The serine protease secreted by *A. flavus* is disrupted or inhibited by some TIs (Huynh et al. 1992; Liang et al. 2004). Alkaline protease has been reported to be the dominant protease present in *A. flavus*-infected maize kernels. The activity of alkaline protease (33-kDa) from *A. flavus* was significantly inhibited by the maize 14-kDa TI at 200 μg/ml. Any reduction in this protease also significantly reduced the levels of aflatoxin accumulating in *A. flavus* cultures (Chen et al. 2009). Therefore, it can be concluded from the above results that the antifungal SBTI/WBTI might also exert their activity by inhibition of the serine protease and/or alkaline protease involved in fungal growth.

Furthermore, the production of aflatoxin B_1 by *A. flavus* in the casein liquid medium showed that it was completely inhibited at a concentration of 2.4 μM SBTI/WBTI after 6 days incubation (casein as substrate, Table 3). The results presented in Fig. 6 and Table 3 reveal the positive correlation between the aflatoxin B_1 level and protease activity of *A. flavus*. The filtered medium (control and 0.6 μM SBTI/WBTI added) was highly contaminated with aflatoxin B_1, which also displayed higher protease activity. The results presented in this paper support the view that endogenous protease activity is an important virulence factor involved in conidia germination and growth. The mechanism of inhibition of *A. flavus* conidia germination and aflatoxin B_1 production by SBTI/WBTI is also perhaps mediated by its activity as a fungal protease inhibitor.

Conclusions

This study, based on several lines of evidence (including amino acid composition, binding with trypsin/chymotryp-

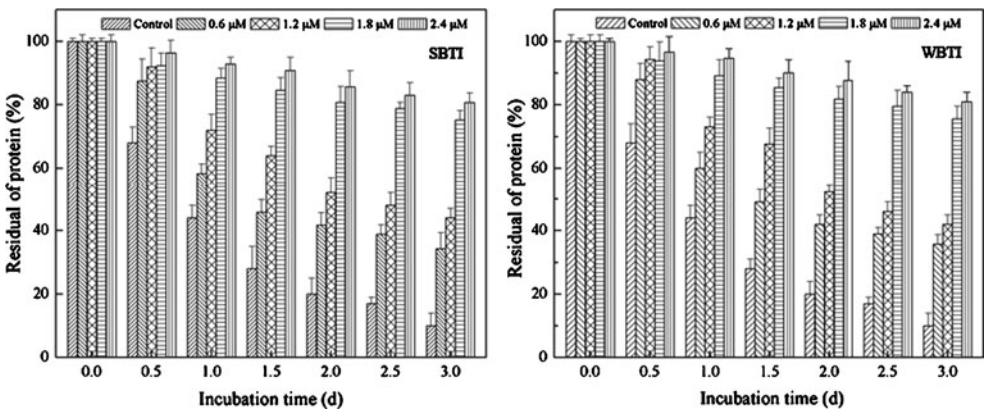

Fig. 6 Time course monitoring residual casein in the presence of different SBTI/WBTI concentrations. SBTI (*left panel*)/WBTI (*right panel*) were added final concentrations of 0.0, 0.6, 1.2, 1.8 or 2.4 μM, as indicated, to modified Czapek liquid medium containing 10.0 g/l casein

sin, and the involvement of the Lys residue at the active site for trypsin-inhibition), has shown that SBTI/WBTI from cultivated and wild-type soybean belong to the Bowman-Birk TI. A study of the mechanism of inhibition of *A. flavus* conidia and mycelia growth showed that the effect of SBTI/SBTI on *A. flavus* α-amylase and protease activity depended on the TI concentration, and that the proper concentrations of SBTI/WBTI would effectively limit the hydrolyzing activity of α-amylase and protease and further suppress the aflatoxin B_1 accumulation. Accordingly, our research indicates that SBTI and WBTI might be useful as potential biocontrol agents against *A. flavus* during food storage. Furthermore, isolation of TI genes from domesticated and wild-type soybeans could be used to develop innate anti-*A. flavus* growth plants.

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References

Adams PR, Deploey JJ (1986) Early starch catabolism by *Rhizomucor pusillus*. Mycopathologia 78:129–131

Attia RM, Ali SA (1974) Utilization of agricultural wastes by *Aspergillus awamori* for the production of glucoamilase. Rev Microbiol 5:81–84

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 72:248–254

Bund RK, Singhal RS (2002) An alkali stable cellulase by chemical modification using maleic anhydride. Carbohyd Polym 47:137–141

Carlton B, Ines M (1961) An improved test for the quantitative determination of trypsin, trypsin-like enzymes, and enzyme inhibitors. Anal Biochem 2:370–379

Chen ZY, Brown RL, Lax AR, Guo BZ, Cleveland TE, Russin JS (1998) Resistance to *Aspergillus flavus* in corn kernels is associated with a 14-kDa protein. Phytopathology 88:276–281

Chen ZY, Brown RL, Lax AR, Guo BZ, Cleveland TE (1999a) A corn trypsin inhibitor with antifungal activity inhibits *Aspergillus flavus* α-amylase. Phytopathology 89:902–907

Chen ZY, Brown RL, Lax AR, Cleveland TE (1999b) Inhibition of plant-pathogenic fungi by a corn trypsin inhibitor overexpressed in *Escherichia coli*. Appl Environ Microbiol 65:1320–1324

Chen ZY, Robert B, Jeffrey C, Kenneth D, Thomas C (2009) Characterization of an *Aspergillus flavus* alkaline protease and its role in the infection of maize kernels. Toxin Rev 28:187–197

Davis ND, Diener UL (1968) Growth and aflatoxin production by *Aspergillus parasiticus* from various carbon sources. Appl Microbiol 16:158–159

Fakhoury AM, Woloshuk CP (2001) Inhibition of growth of *Aspergillus flavus* and fungal α-amylases by a lectin-like protein from *lablab purpureus*. Am Phytopathological Soc 14:955–961

Friedman M, Brandon DL (2001) Nutritional and health benefits of soy proteins. J Agri Food Chem 49:1069–1086

Hilder VA, Gatehouse AMR, Sheerman SE, Barker RF, Boulter D (1987) A novel mechanism of insect resistance engineered into tobacco. Nature 330:160–163

Huang ZY, White DG, Payne GA (1997) Corn seed proteins inhibitory to *Aspergillus flavus* and aflatoxin biosynthesis. Phytopathology 87:622–627

Huynh QK, Borgmeyer JR, Zobel JF (1992) Isolation and characterization of a 22-kDa protein with antifungal properties from maize seeds. Biochem Biophys Res Commun 182:1–5

Jiang XJ, Zhang ZJ, Cai HN, Hara K, Su WJ, Cao MJ (2006) The effect of soybean trypsin inhibitor on the degradation of myofibrillar proteins by an endogenous serine proteinase of crucian carp. Food Biochem 94:498–503

Joshi BN, Sainani NN, Bastawade KB, Gupta VS, Ranjekar PK (1998) Cysteine protease inhibitor from pearl millet: a new class of antifungal protein. Biochem Biophys Res Commun 246:382–387

Kim JH, Yu JJ, Mahoney N, Chan KL, Molyneux RJ, Varga J, Bhatnagar D, Cleveland TE, Nierman WC, Campbell BC (2008) Elucidation of the functional genomics of antioxidant-based inhibition of aflatoxin biosynthesis. Int J Food Microbiol 122:49–60

Lever MA (1972) A new reaction for colorimetric determination of carbohydrates. Anal Biochem 47:273–279

Li W, Shao YZ, Chen WX (2002) Improved method for determining amylase activity. Plant Physiol Comm 41:655–666

Liang X, Guo B, Holbrook C, Robert L (2004) Resistance to *Aspergillus flavus* in peanut seeds is associated with constitutive trypsin inhibitor and inducible chitinase and β-1-3-glucanase. Proceedings of the American Peanut Research and Education Society. Clearwater, FL

Mahoney NE, Rodriguez SB (1996) Aflatoxin variability in pistachios. Appl Environ Microbiol 62:1197–1202

Mellon JE, Cotty PJ (1995) Expression of elastinolytic activity among isolates in *Aspergillus section flavi*. Mycopathologia 131:115–120

Mellon JE, Cotty P (1996) Purification and partial characterization of an elastinolytic proteinase from *Aspergillus flavus* culture filtrates. Appl Microbiol Biotechnol 46:138–142

Mellon JE, Cotty PJ, Dowd MK (2007) *Aspergillus flavus* hydrolases: their roles in pathogenesis and substrate utilization. World J Microbiol Biotechnol 77:497–504

Nahas E, Waldemrin MM (2002) Control of amylase production and growth characteristics of *Aspergillus ochraceus*. Rev Latinoam Microbiol 44:5–10

Oso BA (1979) Mycelial growth and amylase production by *Talaromyces emersonii*. Mycologia 71:521–529

Reddy KRN, Reddy CS, Muralidharan K (2009) Potential of botanicals and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. Food Control 20:173–178

Sandosskumar R, Karthikeyan M, Mathiyazhagan S, Mohankumai M, Chandrasekar G, Velazhahan R (2007) Inhibition of *Aspergillus flavus* growth and detoxification of aflatoxin B_1 by the medicinal plant zimmu (*Allium sativum* L. × *Allium cepa* L.). World J Microbiol Biotechnol 23:1007–1014

Seidl DS, Liener IE (1971) Identification of the trypsin-reactive site of the Bowman-Birk soybean inhibitor. Biochim Biophys Acta 251:83–93

Sessa D, Nelsen TC (1991) Chemical inactivation of soybean protease inhibitors. JAOCS 68:463–470

Shivaraj B, Pattabiraman TN (1998) Natural plant enzyme inhibitors. Biochem J 193:29–36

Wilcox PE (1970) Chymotrypsinogens-chymotrypsins. Meth Enzymol 19:64–108

Woloshuk CP, Cavaletto JR, Cleveland TE (1997) Inducers of aflatoxin biosynthesis from colonized maize kernels are generat-

ed by an amylase activity from *Aspergillus flavus*. Biochem Cell Biol 87:164–169

Wong JH, Ng TB (2003) Gymnin, a potent defensin-like antifungal peptide from the Yunnan bean (*Gymnocladus chinensis* Baill). Peptides 24:963–968

Zhang L, Wang DF, Zhang B, Sun LP (2006) Advances in the study of protease inhibitors from leguminous plants. Soybean Sci 3:314–319

Zhang B, Zhang L, Yu LN, Wang DF (2007) Preparation of immobilized trypsin chitosan resin and its absorption property for soybean trypsin inhibitor. Sci Technol Food Ind 7:65–68

Zhang L, Zhang B, Lin H, Wang DF (2008) Preparation of trypsin-immobilised chitosan beads and their application to the purification of soybean trypsin inhibitor. J Sci Food Agr 88:2332–2340

Zhang B, Wang DF, Fan Y, Zhang L, Luo Y (2009a) Affinity purification of trypsin inhibitor with anti-*Aspergillus flavus* activity from cultivated and wild Soybean. Mycopathologia 167:163–171

Zhang B, Wang DF, Li HY, Xu Y, Zhang L (2009b) Preparation and properties of chitosan-soybean trypsin inhibitor blend film with anti-*Aspergillus flavus* activity. Ind Crop Prod 29:541–548