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Research article

Purification and characterization of native and recombinant SaPIN2a, a plant sieve element-localized proteinase inhibitor

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Abstract

SaPIN2a encodes a proteinase inhibitor in nightshade (*Solanum americanum*), which is specifically localized to the enucleate sieve elements. It has been proposed to play an important role in phloem development by regulating proteolysis in sieve elements. In this study, we purified and characterized native SaPIN2a from nightshade stems and recombinant SaPIN2a expressed in *Escherichia coli*. Purified native SaPIN2a was found as a charge isomer family of homodimers, and was weakly glycosylated. Native SaPIN2a significantly inhibited serine proteinases such as trypsin, chymotrypsin, and subtilisin, with the most potent inhibitory activity on subtilisin. It did not inhibit cysteine proteinase papain and aspartic proteinase cathepsin D. Recombinant SaPIN2a had a strong inhibitory effect on chymotrypsin, but its inhibitory activities toward trypsin and especially toward subtilisin were greatly reduced. In addition, native SaPIN2a can effectively inhibit midgut trypsin-like activities from *Trichoplusia ni* and *Spodoptera litura* larvae, suggesting a potential for the production of insect-resistant transgenic plants.

Keywords: Insect; Phloem; Proteinase; Protease inhibitor; Proteolysis; Sieve element; *Solanum americanum*

1. Introduction

There are two conducting tissues in the vascular system of plants, xylem and phloem. Xylem is the water and minerals-conducting tissue, and phloem is responsible for the transport of various substances, especially photoassimilates and signaling molecules [1,2]. Unlike xylem, which consists primarily of dead cells, phloem is composed of living cells, including sieve elements (SEs), companion cells, and parenchyma cells [3]. During their differentiation, SEs undergo a unique selective autolysis, and the mature SEs eventually lose their nuclei and most of the cytoplasmic contents [4]. Although proteases are thought to be involved in the selective cytoplasmic degradation in SEs, no specific protease has been identified to be responsible for this proteolysis [4–6]. However, a large number of protease inhibitors have been identified in phloem [7–20], and some of them were suggested to regulate proteolytic activities during the differentiation of SEs [10,14,15,19].

We have previously shown that the nightshade (*Solanum americanum*) proteinase inhibitor II (PIN2) gene family contains two members, *SaPIN2a* and *SaPIN2b*, which are differentially expressed in plants [15,21]. SaPIN2a was highly expressed in phloem [15]. The localization of SaPIN2a protein to SEs [15] and the inhibitory activities of the heterogeneously expressed SaPIN2a toward endogenous trypsin- and chymotrypsin-like proteases in transgenic lettuce [22] suggest that SaPIN2a could have physiological role in the regulation of

Abbreviations: BAEE, N_α-benzoyl-L-arginine ethyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; PIN2, proteinase inhibitor II; SBTI, soybean trypsin inhibitor; SE, sieve element; TAME, N_α-*p*-tosyl-L-arginine methyl ester.

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proteolysis in SEs. Recently, SaPIN2a has also been shown to be involved in flower and seed development [23,24] and in resistance to insects [25]. In this study, the native SaPIN2a from nightshade stems and the recombinant SaPIN2a expressed in *Escherichia coli* were purified and characterized. The native SaPIN2a exists as three charge isomers (differing predominantly in net charge) of homodimers, and was weakly glycosylated. The purified native SaPIN2a significantly inhibited serine proteinases such as trypsin, chymotrypsin, and subtilisin, and also effectively inhibited midgut trypsin-like proteinases from larvae of *Spodoptera litura* and *Trichoplusia ni*. Recombinant SaPIN2a expressed in *E. coli* had a strong inhibitory effect on chymotrypsin, although its inhibitory activities toward trypsin and especially toward subtilisin were greatly reduced.

2. Results

2.1. Purification of native SaPIN2a from nightshade stems

SaPIN2a was purified from nightshade stems according to the purification procedure shown in Fig. 1A. Crude stem extracts were first partly purified and concentrated by ultrafiltration and ammonium sulphate precipitation. Salts and most of the impurity proteins were removed by gel filtration with Sephadex G-50 (Fig. 1B). The first trypsin-inhibitory peak separated on the Sephadex G-50 column (Fig. 1B) containing SaPIN2a, as revealed by the western blot analysis, was further purified by anion exchange chromatography on a column of DEAE-Sephadex A-50 (Fig. 1C), and followed by trypsin affinity chromatography (Fig. 1D). On both unreduced and reduced SDS-PAGE, the final purified SaPIN2a yielded a single polypeptide of approximately 18.5 kDa (Fig. 1E). A summary of a typical purification experiment is shown in Table 1.

2.2. N-terminal sequencing of the purified native SaPIN2a

The purified native SaPIN2a was transferred to polyvinylidenedifluoride (PVDF) membrane after SDS-PAGE, and the N-terminal amino acid sequence was determined by the Edman degradation method. The N-terminal analysis yielded 23 amino acid residues, KACTRECGHFSYGICPRSEGSPQ, which were found to be identical to the Lys^{28}–Gln^{51} segment of the precursor protein encoded by the SaPIN2a cDNA (GenBank accession no. AF174381).

2.3. Characterization of the purified native SaPIN2a

Based on the cDNA sequence [15], the calculated molecular mass of mature SaPIN2a is 13.3 kDa; however, SDS-PAGE analysis showed an apparent size of approximately 18.5 kDa (Fig. 1E). We suspected that native SaPIN2a was glycosylated. The glycosylation status of purified native SaPIN2a was examined by staining for carbohydrate with periodic acid–Schiff's reagent (Fig. 2A). The result revealed a faint band in SaPIN2a (Fig. 2A, line 2) not seen in negative control protein (soybean trypsin inhibitor, SBTI) (Fig. 2A, line 3). SaPIN2a showed a much weaker staining than the positive control protein (horseradish peroxidase, HRP) (Fig. 2A, line 1), suggesting that it has a low level of glycosylation.

The purified native SaPIN2a on non-denatured PAGE gel resolved into three discrete bands (Fig. 2C, lane 2). All of them have the trypsin-inhibitory activities (Fig. 2D, lanes 3 and 4). To examine the relationship between these three bands, the behavior of native SaPIN2a on non-denatured PAGE was studied according to the method of Hedrick and Smith [26]. When the log of mobilities of the three bands was plotted versus the percent gel concentration, three parallel lines were obtained (Fig. 2E), suggesting that they are charge isomers with the same molecular weight. The molecular weight of the native SaPIN2a was estimated to be ~37.0 kDa by native-PAGE, which is about twice of that determined by unreduced and reduced SDS-PAGE (Fig. 1E). Thus, the native SaPIN2a protein was assumed to exist as a charge isomer family of homodimers.

The thermal and pH stability of the purified SaPIN2a were determined by measuring the residual inhibitory activity following a 10-min preincubation at different temperatures and pHs. The purified SaPIN2a was stable up to 70 °C, and retained over 60% of its initial inhibitory activity even after being boiled for 10 min (Fig. 3A). As shown in Fig. 3B, SaPIN2a was stable in the pH range of 3–11. The high thermostability and pH stability of SaPIN2a are most probably conferred by the presence of a high number of disulfide bonds (8 within 121 residues) [15].

2.4. Inhibitory activities of the purified native SaPIN2a against various proteinases

To further characterize the purified native SaPIN2a, its inhibitory activity was tested with different types of proteinases. As shown in Table 2, native SaPIN2a significantly inhibited serine proteinases such as trypsin, chymotrypsin, and subtilisin with the most potent inhibitory activity on subtilisin; but did not inhibit cysteine proteinase papain and aspartic proteinase cathepsin D. The concentrations of SaPIN2a required to inhibit 50% (IC_{50}) of trypsin, chymotrypsin and subtilisin activities were determined using the data shown in Fig. 4. SaPIN2a appeared to be a potent inhibitor of chymotrypsin ($IC_{50} = 33.4$ nM), and subtilisin ($IC_{50} = 74.4$ nM); however, only a moderate inhibitor of trypsin ($IC_{50} = 137.1$ nM).

To further elucidate the inhibitory effects of SaPIN2a on trypsin, chymotrypsin, and subtilisin, the kinetic analyses of the inhibitory action were performed in the presence of two SaPIN2a concentrations. As shown in Fig. 5, the Eadie–Hofstee plot analysis revealed that SaPIN2a was a competitive inhibitor of trypsin with a K_i value of 6.4 nM, and a non-competitive inhibitor of chymotrypsin ($K_i = 22.8$ nM) and subtilisin ($K_i = 80.3$ nM).

To explore the potential of SaPIN2a for developing insect-resistant transgenic plants, inhibitory activities of SaPIN2a against midgut proteinases from larvae of *S. litura* and *T. ni*

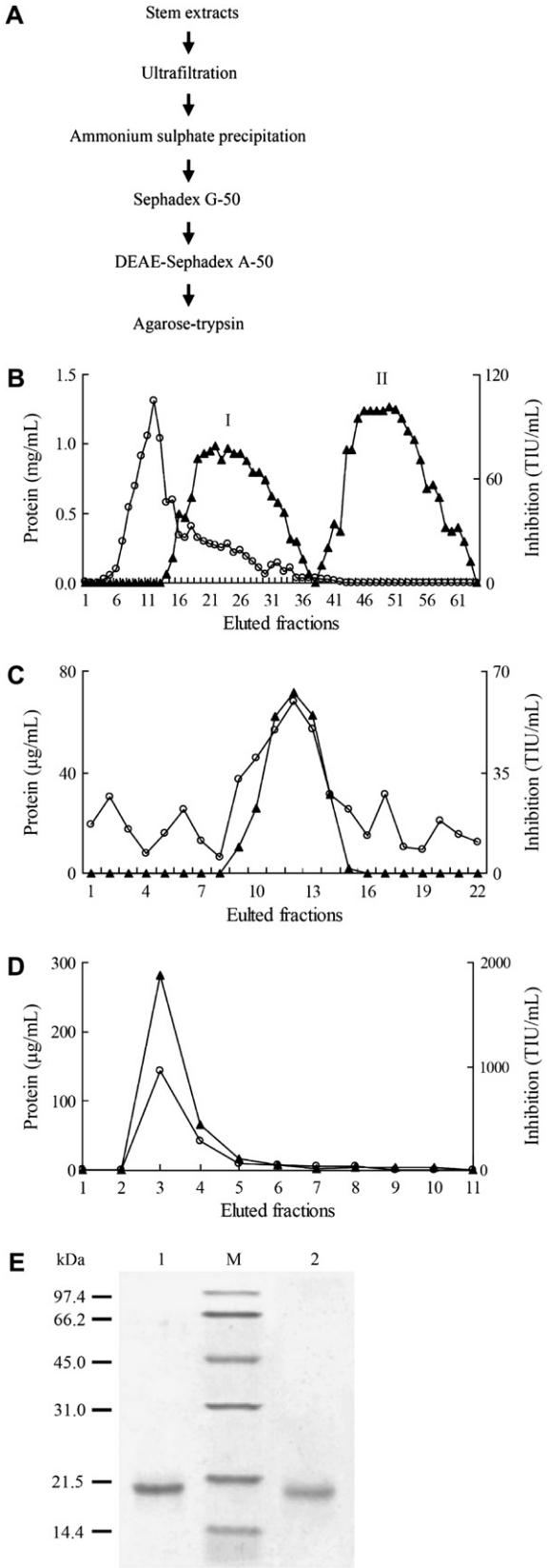

Fig. 1. Purification of SaPIN2a from *S. americanum* stems. (A) Scheme of the purification procedure. (B) Sephedax G-50 gel chromatography. (C) DEAE-Sephadex A-50 ion exchange chromatography. (D) Agarose–trypsin affinity chromatography. In (B)–(D), protein content (-○-) and trypsin inhibitory activity (-▲-) were determined as described under Section 4. (E) SDS-PAGE (15%) analysis of purified SaPIN2a. Samples (0.8 μg each) of reduced and unreduced SaPIN2a were run in lanes 1 and 2, respectively. Lane M is the SDS-PAGE low range molecular weight standard (3 μg, Bio-Rad Cat. no. 161-0304).

were analyzed. SaPIN2a showed very strong inhibitory activity against trypsin-like proteinases from both insects (Fig. 6), about 10 times that of the well-known soybean trypsin inhibitor (SBTI). Notably, both SaPIN2a and SBTI were approximately 3–4 times more effective against *S. litura* than *T. ni* midgut proteinases (Fig. 6). This suggests that SaPIN2a overexpression in transgenic plants might provide a greater resistance to *S. litura* than to *T. ni*, and the latter has been shown recently in transgenic lettuce [25]. In addition, no detectable inhibitory activity of SaPIN2a against chymotrypsin-like proteinases of insect midguts was found, although SaPIN2a was a potent inhibitor of bovine chymotrypsin (see above).

2.5. Production and characterization of recombinant SaPIN2a in *E. coli*

Since the yield of native SaPIN2a from nightshade stems was very low (ca. 0.5 mg/kg fresh tissue, see above), the recombinant SaPIN2a was expressed in *E. coli*, purified, and characterized. Initially, the full length cDNA encoding SaPIN2a precursor (called pre-SaPIN2a) was cloned into a glutathione *S*-transferase (GST) fusion expression vector and was expressed heterologously in *E. coli*. Most of the GST-pre-SaPIN2a fusion protein was found in the insoluble fraction of the bacterial extracts, which was excised from the SDS-PAGE gel and used to generate antisera in rabbits.

The soluble GST-pre-SaPIN2a fusion protein was purified by affinity chromatography using GST bind resin. The purified GST-pre-SaPIN2a, which did not exhibit protease inhibitory activity, was cleaved with thrombin to release the recombinant pre-SaPIN2a. However, like the His-tagged pre-SaPIN2a [27], the recombinant pre-SaPIN2a did not show protease inhibitory activity (data not shown), indicating the removal of the N-terminal signal peptide is critical for the inhibitory activity of SaPIN2a. Thus, an N-terminal truncation of the protein, where the N-terminal signal peptide (amino acids 1–27) was removed, was expressed as a GST fusion protein (GST-SaPIN2a) and purified as described above (Fig. 7A) and confirmed by the western blot analysis (Fig. 7C). Unlike the GST-pre-SaPIN2a, the GST-SaPIN2a fusion protein showed inhibitory activity against chymotrypsin (Fig. 7E, lane 1), but not trypsin (Fig. 7D, lane 1). After removal of GST by thrombin cleavage (Fig. 7B), the rSaPIN2a purified with the trypsin affinity column (Fig. 7A) exhibited inhibitory activities against both trypsin and chymotrypsin (Fig. 7D and E, lane 2).

Further quantitative analysis of the inhibitory activities of rSaPIN2a (Table 3) revealed that it had a strong inhibitory

Table 1
Purification summary for SaPIN2a from *S. americanum* stems

Step	Volume (mL)	Total protein (mg)	Total trypsin inhibitory activity[a] (TIU)	Special activity[b] (TIU/mg)	Purification[c] (fold)	Yield[d] (%)
Crude extract	655.0	1395.20	46,725	33.5	1.0	100.0
Ultrafiltration	58.0	405.50	16,437	40.5	1.2	35.2
Sephadex G-50	130.0	31.90	11,335	355.3	10.6	24.3
DEAE-Sephadex A-50	80.0	4.12	9551	2318.2	69.2	20.4
Agarose–trypsin	0.5	0.07	941	13,088.0	390.7	2.1

[a] Trypsin inhibitory activity = trypsin activity of uninhibited control − residual trypsin activity in the presence of inhibitor.
[b] Special activity = trypsin inhibitory activity/total protein.
[c] Purification = special activity of each step/special activity of the crude extract.
[d] Yield (%) = (total trypsin inhibitory activity of each step/total trypsin inhibitory activity of the crude extract) × 100.

effect on chymotrypsin, with an IC_{50} of 36.1 nM that was comparable to that of native SaPIN2a. The inhibitory activities of rSaPIN2a toward trypsin and especially toward subtilisin (Table 3) were greatly reduced as compared with those of the native inhibitor (Table 2). Thus, although about 0.5–0.8 mg of rSaPIN2a could be obtained from 1 L of *E. coli* cell culture, a much higher yield than that of recombinant potato PIN2 (50 μg/L) obtained by Beekwilder et al. [28], recombinant SaPIN2a could not substitute for native SaPIN2a in studies of inhibitory activity toward trypsin and subtilisin.

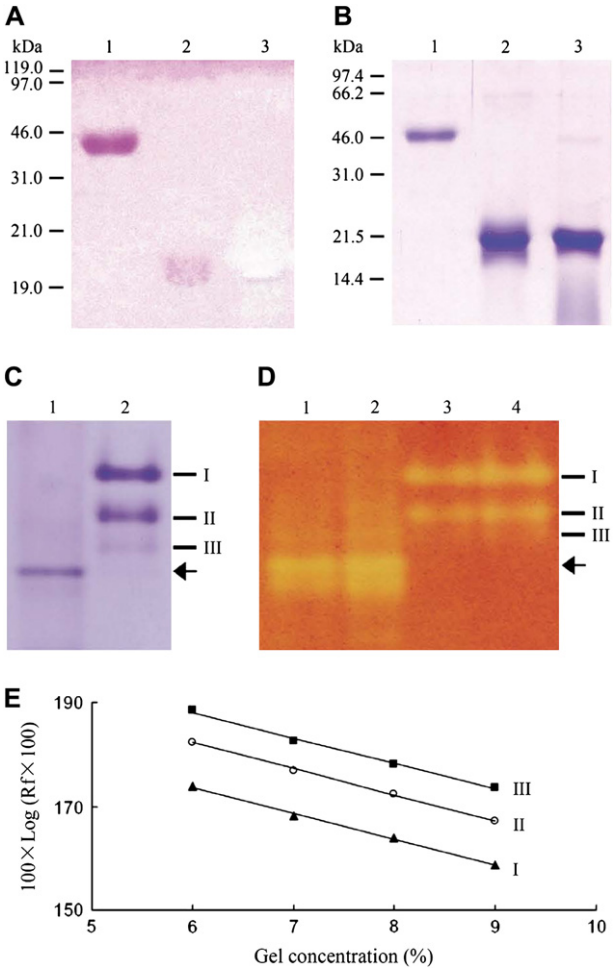

Fig. 2. Glycoprotein staining, non-denatured PAGE and inhibitory activity analysis of native SaPIN2a. (A) Glycoprotein staining. Lane 1, HRP (positive control, 5 μg); lane 2, purified SaPIN2a (20 μg); lane 3, SBTI (negative control, 20 μg). (B) Coomassie brilliant blue R-250 staining of samples shown in (A). Lane 1, HRP (1 μg); lane 2, purified SaPIN2a (4 μg); lane 3, SBTI (4 μg). (C) Non-denatured PAGE (12%) of SBTI (lane 1, 1 μg) and purified SaPIN2a (lane 2, 2 μg). (D) Gel (12% PAGE) staining for trypsin inhibitory activity of SBTI (lane 1, 1 μg; lane 2, 2 μg) and purified SaPIN2a (lane 3, 0.2 μg; lane 4, 0.4 μg). In (C) and (D), I–III indicate three isomers of SaPIN2a, arrows indicate SBTI. (E) Hedrick and Smith analysis of SaPIN2a isomers I–III shown in (C).

3. Discussion

We isolated and characterized a SE-localized proteinase inhibitor SaPIN2a, a member of the PIN2 family, from nightshade stems. The final yield of SaPIN2a was 72–120 μg, derived from 200 g of fresh nightshade stems. This low yield, as compared with that of tomato PIN2 (5 mg from 50 g of dry leaves) obtained by Plunkett et al. [29], appears to be due to the restricted accumulation of SaPIN2a in SEs [15]. N-terminal sequencing of the purified SaPIN2a confirms that SaPIN2a is synthesized as a preprotein whose

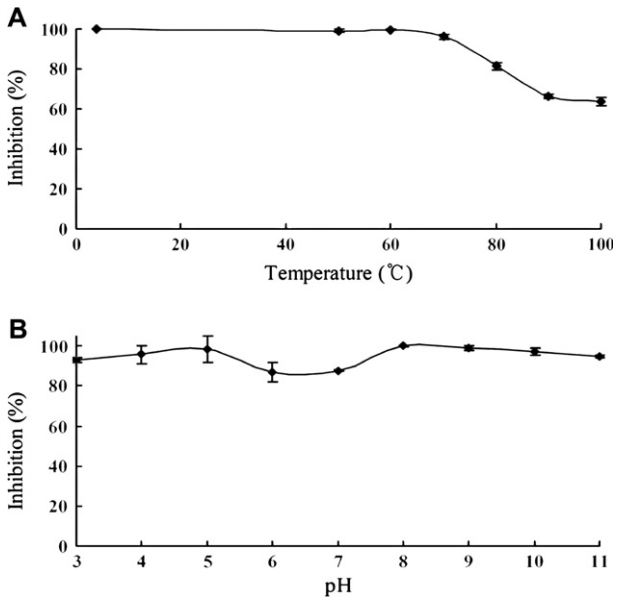

Fig. 3. Thermal and pH stability of native SaPIN2a. Purified SaPIN2a (1 μg) was preincubated at different temperatures (50, 60, 70, 80, 90 and 100 °C) for 10 min (A) or in the pH range 3–11 at 4 °C for 10 min (B), and the residual trypsin inhibitory activities were determined as described under Section 4. Each point represents the average of three experiments. The bars represent standard deviations.

Table 2
Inhibitory effect of native SaPIN2a on the activity of various proteinases

Proteinase (final concentration)	SaPIN2a[a] (nM)	*I*/*E*[b]	Inhibition[c] (%)	Substrate (final concentration)
Trypsin (7.5 nM)	404.0	53.9	73.0	TAME (1 mM)
Chymotrypsin (25.0 nM)	134.7	5.4	88.0	BTEE (0.5 mM)
Subtilisin A (167.0 nM)	224.3	1.3	95.3	Succinylcasein (14 nM)
Papain (25.0 nM)	404.0	16.2	0.0	BAEE (2 mM)
Cathepsin D (20.0 nM)	404.0	20.2	0.0	Bz-RGFFP-4-MeOβNA (0.02 mM)

[a] Proteinase and SaPIN2a were incubated for 3 min at 37 °C. Proteolysis was followed spectrophotometrically by the addition of substrate.
[b] *I*/*E* is the ratio of SaPIN2a concentration to proteinase concentration.
[c] Inhibition (%) = [1 − (velocity in the presence of inhibitor/velocity of uninhibited control)] × 100.

N-terminal signal peptide (27 amino acid residues) is removed during post-translational processing, as shown by the analysis of the *in vitro* transcription/translation product of the SaPIN2a cDNA [15].

The native SaPIN2a was found as a charge isomer family of homodimers, and was weakly glycosylated. Similar to SaPIN2a, the native PIN2s of both tomato and potato also exist as dimers, although they have a smaller molecular weight of about 23.0 kDa [29]. To our knowledge, SaPIN2a was the first member of the PIN2 family that was glycosylated, although some other plant protease inhibitors were found to be glycoproteins [30, 31]. SDS-PAGE analysis showed that the apparent size of native SaPIN2a was 18.5 kDa, which is larger than the calculated molecular mass (13.3 kDa) of mature SaPIN2a. The anomalous migration of native SaPIN2a on SDS-PAGE was probably not due to glycosylation, because the *E. coli*-expressed recombinant SaPIN2a on SDS-PAGE also gave a larger size than expected (Fig. 7A). It is possible that SaPIN2a has a particular conformational property that affects its migration on SDS-PAGE. Similar anomalous migration in SDS-PAGE has been observed for other native protein [32] and recombinant protein [33] of plants.

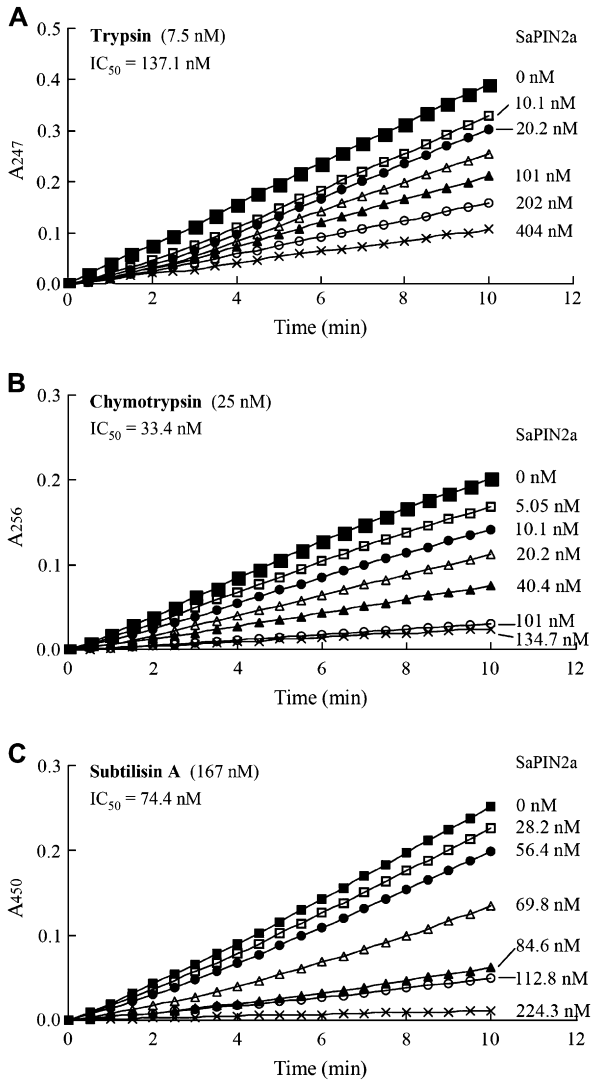

Fig. 4. Time course of inhibition of proteinases by native SaPIN2a. Proteinases and their concentrations, SaPIN2a concentrations, and corresponding IC_{50} values are listed in the figure. Substrates used in the assays were 1.0 mM TAME (A), 1.0 mM BTEE (B), and 14 nM succinylcasein (C), respectively.

The recombinant SaPIN2a (rSaPIN2a) expressed in *E. coli* was also purified and characterized in this study. rSaPIN2a showed a strong inhibitory effect on chymotrypsin, but its inhibitory activities toward trypsin and especially toward subtilisin were greatly reduced. The lower trypsin and subtilisin inhibitory activities of rSaPIN2a could be attributed to the N-terminal extension Gly−Ser− in rSaPIN2a and/or the lack of glycosylation or other post-translational modifications in *E. coli*. There is a high sequence identity between SaPIN2a and tomato PIN2 (71.7%) [15]. Both of them consist of two structurally similar inhibitory domains (domain I and domain II), which contain a trypsin reactive site loop and a chymotrypsin reactive site loop, respectively [34]. The three-dimensional structure of tomato PIN2 in complex with subtilisin revealed that each domain of tomato PIN2 interacts with a single subtilisin molecule and the reactive site loop in domain I lies at the N-terminus of the protein [35]. Therefore, the N-terminal extension Gly−Ser− in rSaPIN2a, which is located in the vicinity of the reactive site loop in domain I (the residues P2−P1−P1′ are Thr^4−Arg^5−Glu^6) [15], would be expected to interfere with the interaction of this domain with the target proteases. Consistently, the reduced inhibitory activities of rSaPIN2a toward trypsin and subtilisin, both of which bind to domain I, were observed in this study, whereas the inhibitory activity toward chymotrypsin that binds to domain II was not affected (Table 3). It is noteworthy that, as compared with that of the native SaPIN2a (Table 2), the inhibitory activity of rSaPIN2a toward subtilisin that binds to both domains of the PIN2s [35] was much more severely reduced than that toward trypsin (Table 3), which only binds to

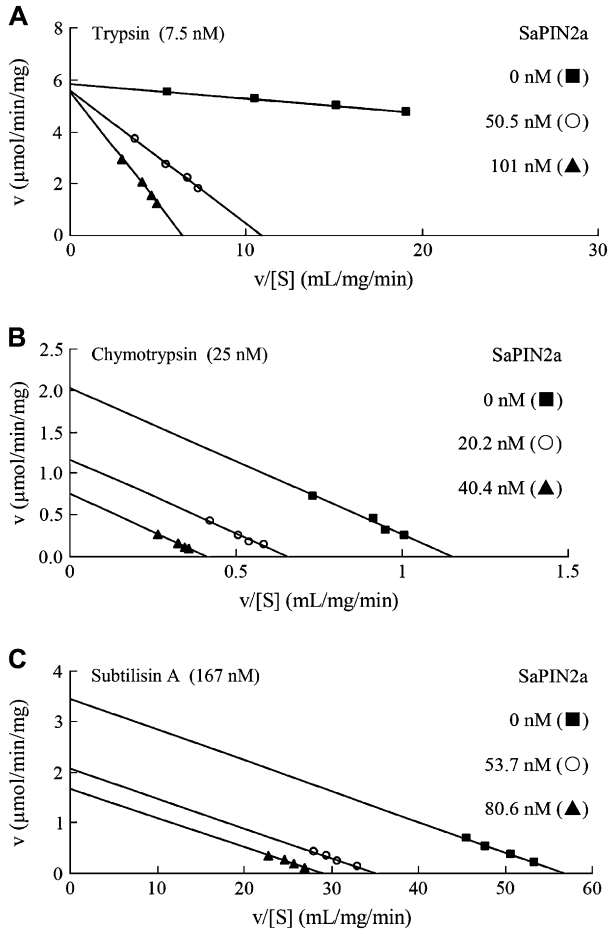

Fig. 5. Eadie–Hofstee plot analysis of the inhibitory effects of native SaPIN2a on serine proteinases. The lines in the plots represent the linear regression fits. Proteinases and their concentrations, and SaPIN2a concentrations are listed in the figure. Substrates used in the assays were 1.0 mM TAME (A), 1.0 mM BTEE (B), and 14 nM succinylcasein (C), respectively.

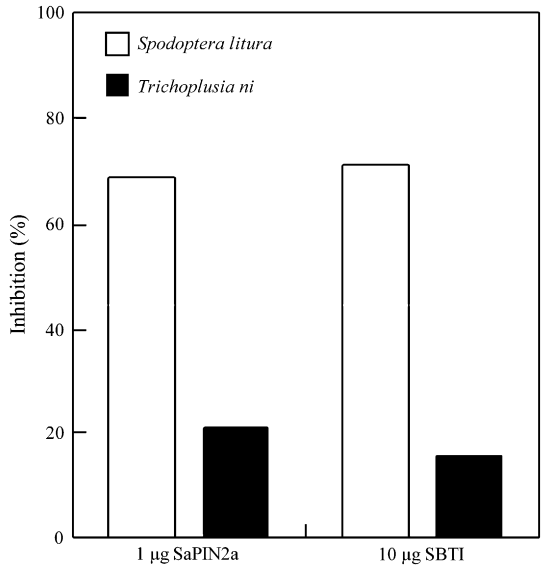

Fig. 6. Inhibitory activities of SaPIN2a against trypsin-like proteinases from insect midguts. Crude midgut proteins were extracted from midguts of 50 larvae of *S. litura* and 100 larvae of *T. ni*. One hundred micrograms of *S. litura* midgut protein and 300 μg of *T. ni* midgut protein were used in the assay, respectively. Midgut extracts and purified SaPIN2a or SBTI (soybean trypsin inhibitor) were incubated for 3 min at 37 °C. Proteolysis was followed spectrophotometrically by the addition of substrate. Inhibition (%) = [1 − (velocity in the presence of inhibitor/velocity of uninhibited control)] × 100.

domain I. This result suggests that, unlike tomato PIN2 [35], two domains of SaPIN2a do not independently bind subtilisin; or that domain II of SaPIN2a interacts with subtilisin through a contacting region that appears to be affected by the N-terminal extension in recombinant SaPIN2a and is different from that for chymotrypsin.

Purified SaPIN2a showed a strong inhibitory activity against insect midgut trypsin-like proteinases, indicating its potential application in the production of insect-resistant transgenic plants. Consistent with this result, increased resistance against insect pest *T. ni* was found on lettuce plants over-expressing SaPIN2a [25]. We also found recently that transgenic tobacco plants over-expressing SaPIN2a showed enhanced resistance to two polyphagous insect pests *Helicoverpa armigera* and *S. litura* (unpublished data). Since SaPIN2a occurs in phloem tissues, and serine proteases were found in phloem-feeding insects [36, 37], further studies are underway to determine the inhibitory activity of SaPIN2a against the gut proteases of phloem-feeding insects and to test the resistance of transgenic tobacco plants over-expressing SaPIN2a against aphids. To further elucidate the role of SaPIN2a in plants, the identification of the target endogenous protease(s) for SaPIN2a would be our future goal, which is inspired by the recent report of the inhibition of *Arabidopsis* metacaspase AtMC9 by an endogenous plant serine protease inhibitor AtSerpin1 [38].

4. Materials and methods

4.1. Plant materials and insects

American black nightshade (*S. americanum* Mill.) plants were grown in soil under natural conditions in a greenhouse. Larvae of *S. litura* Fabricius and *T. ni* Hübner were kindly provided by Dr. Guang-Hong Li (Sun Yat-sen University).

4.2. Extraction and purification of SaPIN2a

Stems (200 g) were homogenized and extracted in cooled buffer (100 mM Tris–HCl, pH 8.5, 10 mM EDTA, 2 mM $CaCl_2$, 1 mM β-mercaptoethanol, 4 mL/g tissue) [39, 40]. The extract was filtered through cheesecloth and centrifuged at 10,000 *g* for 20 min, and then was concentrated by ultrafiltration using Minitan™ Ultrafiltration System (5 kDa cutoff, Millipore, Bedford, MA, USA). To further concentrate the extract, the proteins were precipitated by adding solid ammonium sulphate to 80% saturation, the concentration required to precipitate all the proteinase inhibitory (PI) activity. The ammonium sulphate pellet was resuspended in 10 mL of 150 mM KCl, 10 mM Tris–HCl, pH 8.0, and centrifuged at 10,000 g for 1 h, and then loaded onto a Sephadex G-50 column (3.5 cm × 65 cm) equilibrated with the same buffer.

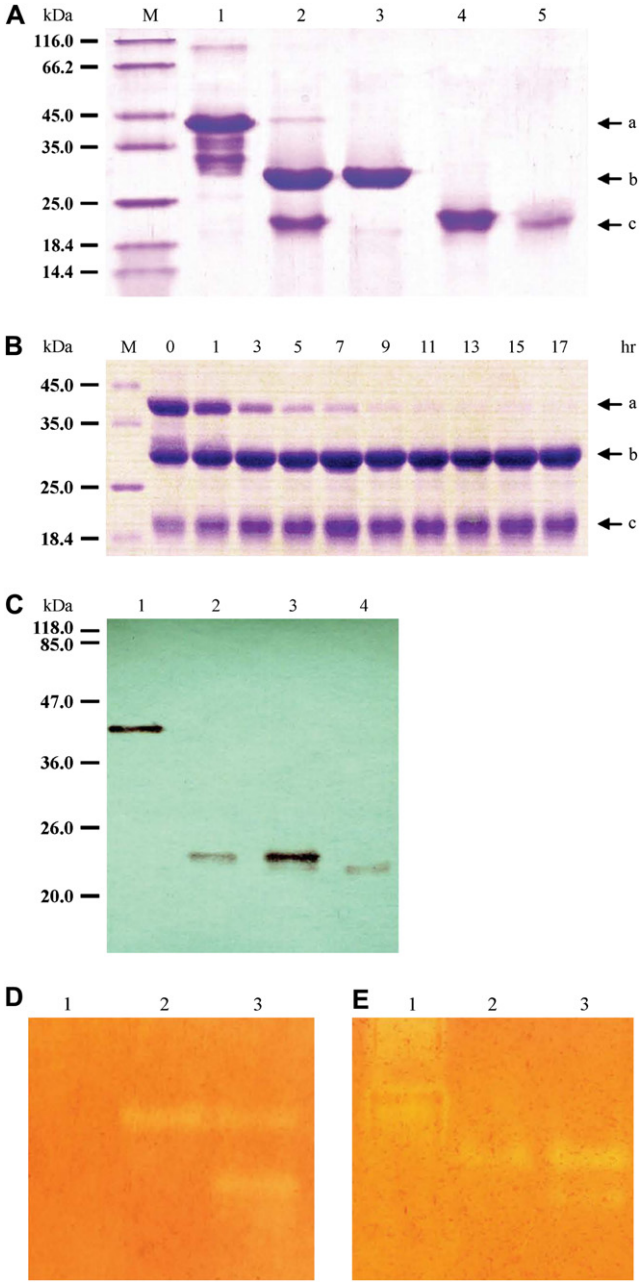

Fig. 7. Purification and activity assay of recombinant SaPIN2a (rSaPIN2a). (A) Purification of rSaPIN2a. Lane M, protein molecular weight marker (Fermentas, Cat. no. SM0431); lane 1, GST-SaPIN2a (13 μg) eluted from the GSTrap column; lane 2, cleavage of GST-SaPIN2a (13 μg) by thrombin for 7 h at 22 °C; lane 3, flow-through from the trypsin affinity column; lane 4, rSaPIN2a (4 μg) eluted from the trypsin affinity column; lane 5, purified native SaPIN2a (1 μg). Arrows indicate GST-SaPIN2a (a), GST-tag (b) and rSaPIN2a or native SaPIN2a (c), respectively. (B) SDS-PAGE of GST-SaPIN2a (13 μg) cleaved with thrombin (7.5 U/mg fusion protein) for different times at 22 °C. Lane M, protein molecular weight marker. Numbers on the top of the respective lane indicate the time of incubation in hours. Arrows indicate GST-SaPIN2a (a), GST-tag (b) and rSaPIN2a (c), respectively. (C) Western blot analysis of rSaPIN2a. Lane 1, purified GST-SaPIN2a (1.5 μg); lane 2, cleavage of GST-SaPIN2a (1.5 μg) by thrombin; lane 3, rSaPIN2a (0.25 μg); lane 4, native SaPIN2a (0.25 μg). (D) Trypsin inhibitory activity of the GST-SaPIN2a and rSaPIN2a. (E) Chymotrypsin inhibitory activity of the GST-SaPIN2a and rSaPIN2a. In (D) and (E): lane 1, GST-SaPIN2a (5 μg); lane 2, rSaPIN2a (0.2 μg); lane 3, native SaPIN2a (0.2 μg).

The fractions (8 mL) eluted from this column containing PI activity were pooled and applied to a DEAE-Sephadex A-50 column previously equilibrated with 100 mM KCl, 50 mM Tris–HCl, pH 8.0. The bound proteins were eluted with a liner gradient of 0.1–1 M NaCl in 50 mM Tris–HCl, pH 8.0. Fractions (3 mL) containing PI activity were collected and pooled, and then applied to an affinity column of agarose–trypsin (immobilized TPCK trypsin, Cat. no. 20230, Pierce, Rockford, IL, USA). The column was washed with 10 bed volume of 150 mM KCl/10 mM Tris–HCl, pH 8.0, prior to elution (0.5 mL fractions) of bound proteins with 10 mM HCl, 30 mM $CaCl_2$, 200 mM NaCl, pH 2.0 [7]. The eluate was neutralized immediately with 1 M Tris base (5 μL for each 0.5 mL of eluate), and dialyzed extensively against deionized distilled H_2O. Protein concentration was determined by the method of Bradford [41] using bovine serum albumin (BSA) as standard. Trypsin inhibitory activity was measured as described below.

4.3. N-terminal sequencing

Purified SaPIN2a was separated by SDS-PAGE (15%), and then was blotted to PVDF membrane (Immobilon-P^{SQ}, Cat. no. ISEQ10100, Millipore, Bedford, MA, USA). SaPIN2a on the PVDF membrane was sequenced at its N-terminus using the Edman degradation procedure by Wei-Qun Shen (Perking University).

4.4. Proteinases, chromogenic substrates, and soybean trypsin inhibitor

The following reagents were obtained commercially as indicated: papain (Cat. no. P4762), trypsin (Cat. no. T1426), N_α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE, Cat. no. B4500), *N*-benzoyl-L-tyrosine ethyl ester (BTEE, Cat. no. B6125), N_α-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME, Cat. no. T4626), 2,4,6-trinitrobenzenesulfonic acid solution (TNBS, Cat. no. P2297) from Sigma (St Louis, MO, USA); chymotrypsin (Cat. no. LS001432) from Worthington (Lakewood, NJ, USA); cathepsin D (Cat. no. 219398), Bz-RGFFP-4-MeOβNA-HCl (cathepsin D substrate I, Cat. no. 219399), subtilisin A (Cat. no. 572909), succinylcasein (Cat. no. 573464), and soybean trypsin inhibitor (SBTI, Cat. no. 650357) from Calbiochem (Darmstadt, Germany).

4.5. Proteinase inhibition assays and kinetic analyses

Trypsin and chymotrypsin inhibitory activity was assayed using either TAME (trypsin) or BTEE (chymotrypsin) as substrates previously described by Xu et al. [22]. Subtilisin inhibitory activity was assayed by the method of Hatakeyama et al. [42] and Bubnis and Ofner [43] using succinylcasein and TNBS. Papain inhibitory activity was determined according to the procedure of Rickauer et al. [44] using BAEE as a substrate. Cathepsin D inhibitory activity was assayed by the method of Yasuda et al. [45] using Bz-RGFFP-4-MeOβNA as a substrate. The 50% inhibitory concentrations (IC_{50}) for native and recombinant SaPIN2a were calculated by the

Table 3
Inhibitory effect of recombinant SaPIN2a (rSaPIN2a) on the activity of serine proteinases

Proteinase (final concentration)	rSaPIN2a[a] (nM)	*I*/*E*[b]	Inhibition[c] (%)	Substrate (final concentration)
Trypsin (7.5 nM)	1369	182.5	74.5	TAME (1.0 mM)
Chymotrypsin (25.0 nM)	224	9.0	91.4	BTEE (0.5 mM)
Subtilisin A (83.0 nM)	6202	75.0	30.0	Succinylcasein (14.0 nM)

[a] Proteinase and rSaPIN2a were incubated for 3 min at 37 °C. Proteolysis was followed spectrophotometrically by the addition of substrate.
[b] *I*/*E* is the ratio of rSaPIN2a concentration to proteinase concentration.
[c] Inhibition (%) = [1 − (velocity in the presence of inhibitor/velocity of uninhibited control)] × 100.

non-linear regression analysis, and K_i values were calculated from Eadie–Hofstee plots as described by Mitsudo et al. [46].

4.6. SDS-PAGE and western blot analysis

SDS-PAGE was conducted using the buffer system of Laemmli [47]. For the western blot analysis [47], proteins were separated on 15% (w/v) SDS-PAGE and blotted onto Hybond-C membrane (Amersham). The blot was probed with antibodies against recombinant SaPIN2a (see below) at 0.4 μg/mL. The amplified alkaline phosphatase goat anti-rabbit immuno-blot assay kit (Bio-Rad) was used to detect cross-reacting bands.

4.7. Non-denatured PAGE and inhibitory activity staining

Non-denatured PAGE was performed in a Laemmli [47] buffer system in the absence of SDS at 4 °C. Determination of protein molecular weights by the non-denatured PAGE was carried out by the method of Bryant et al. [48] and Hedrick and Smith [26] using the non-denatured protein molecular weight marker kit from Sigma (Cat. no. MW-ND-500). For trypsin and chymotrypsin inhibitory activity staining, gels were treated according to the procedure of Altpeter et al. [49].

4.8. Detection of SaPIN2a glycosylation

Glycoprotein detection was carried out with the GelCode glycoprotein staining kit (Cat. no. 24562, Pierce, Rockford, IL, USA) according to the manufacturer's procedure.

4.9. Determination of the thermal and pH stability of native SaPIN2a

The thermostability of native SaPIN2a was examined by incubating the purified SaPIN2a at different temperatures (50, 60, 70, 80, 90 and 100 °C) for 10 min. After being heated, the inhibitor solution was cooled in an ice bath and the residual inhibitory activity against trypsin was determined. The effect of pH on inhibitory activity was examined by incubating the purified SaPIN2a in the pH range 3–11 using the following buffers at final concentrations of 0.1 M at 4 °C for 10 min: glycine/HCl for pH 3; sodium acetate for pH 4–5; disodium phosphate for pH 6, Tris–HCl for pH 7–9; glycine/NaOH for pH 10–11. After that, the residual trypsin inhibitory activities were determined as described above.

4.10. Extraction and activity assay of proteinases from insect midguts

The fifth-instar larvae of *S. litura* and *T. ni* were immobilized on ice, and their midguts were dissected into the buffer (50 mM Tris–HCl, 100 mM NaCl, pH 8.0) [50,51]. The guts were homogenized in a cooled glass mortar, and the homogenate was centrifuged for 20 min at 12,000 *g* at 4 °C. The supernatant was aliquoted, snap frozen in liquid nitrogen, and stored at −80 °C until use. The inhibitory activities of native SaPIN2a and SBTI toward trypsin-like and chymotrypsin-like proteinases of insect midguts were determined as described above.

4.11. Expression and purification of recombinant SaPIN2a in E. coli

The full length cDNA encoding SaPIN2a precursor or the truncated cDNA encoding mature SaPIN2a was cloned into the *Eco*R I/*Xho* I (for SaPIN2a precursor, pre-SaPIN2a) or *Bam*H I/*Xho* I (for mature SaPIN2a) sites of the glutathione *S*-transferase (GST) expression vector pGEX-4T-1 (Amersham, Piscataway, NJ, USA), which resulted in two expression plasmids, pZY01 and pZY02, respectively. Maintenance of the correct reading frames of the pZY01 and pZY02 was confirmed by the DNA sequencing. Induction of recombinant protein expression by isopropylthio-beta-D-galactoside (IPTG) in *E. coli* BL21 (DE3), and preparation of cell extracts were carried out in principle as described by the manufacturer (Amersham). For the generation of antibodies against SaPIN2a, GST-pre-SaPIN2a fusion protein was excised from the SDS-PAGE gel and used for injection into rabbits. Antibodies were purified using Protein A-Sepharose CL-4B (Pharmacia) affinity column.

The soluble GST-pre-SaPIN2a and GST-SaPIN2a fusion proteins were purified by affinity chromatography on GST·Bind resin (Cat. no. 70541-3, Novagen, Darmstadt, Germany) according to the manufacturer's instruction. The fusion proteins were cleaved with thrombin (7.5 U of enzyme per mg of protein, Amersham Cat. no. 27-0846-01) to remove the GST-tag at 22 °C for 7 h. The recombinant pre-SaPIN2a and mature SaPIN2a were further purified by affinity chromatography with an agarose–trypsin column as described above.

5. Note added in proof

We recently found that overexpression of SaPIN2a in transgenic plants caused significant reduction in plant height of transgenic plants, and that chloroplast-like organelles, which are not present in enucleate sieve elements of wild-type plants, were found in the enucleate sieve elements of SaPIN2a-overexpressing transgenic plants [52].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.plaphy.2007.07.012.

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