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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.
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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*

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