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Short communication

Functional activity of sporamin from sweet potato (*Ipomoea batatas* Lam.): a tuber storage protein with trypsin inhibitory activity

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Abstract

Sporamin accounts for about 60% to 80% of total soluble protein in sweet potato tubers, and the predicted protein sequence of sporamin shares significant amino acid sequence identity with some Kunitz-type trypsin inhibitors. We constructed three recombinant plasmids with cDNAs that encode preprosporamin, prosporamin, and sporamin, and these three were expressed in *Escherichia coli* cells as fusion proteins. All three forms of sporamin expressed in *E. coli* were shown to have strong inhibitory activity to trypsin *in vitro*, suggesting that post-translational modifications are not essential for trypsin inhibitory activity. Northern blot analysis showed that sporamin transcripts could be systemically induced in leaf tissue of sweet potato by wounding. Therefore, sporamin may have a defense role as a protease inhibitor, in addition to its role as a storage protein.

Seed and tuberous roots of most plants contain a large amount of storage protein (ranging from 30% to 80% of total protein), such as glycinins of soybean [14], zein of maize [33], patatin of potato tuber [26], dioscorins of yam (*Dioscorea cayenensis*) [7], and sporamin of sweet potato (*Ipomoea batatas* Lam.) [21]. The major function of the storage proteins in plant tissues appears to be as a nutritional resource for seed germination or tuber regrowth. In certain cases, however, specific roles for some storage proteins are becoming evident; for example, it has been shown that zein functions as nitrogen sinks to regulate nutrient movement into the maize kernel [33], and patatin shows the enzymatic activity of lipolytic acylhydrolase [29].

Sporamin is the major storage protein in sweet potato tuberous roots, first described by Maeshima *et al.* [21]. It accounts for 60% to 80% of the total soluble protein in the sweet potato tuber. Expression of sporamin has been shown to be mainly associated with tubers, a very low amount in the stem and none in leaves [11]. Sporamins are encoded by a multigene family, which can be grouped into two subfamilies, namely sporamins A and B, based on nucleotide homology [12]. From studies of transgenic tobacco cells, transformed with a sporamin gene, it was revealed that post-translational processing of a sporamin precursor into two smaller – molecular – weight forms occurs [24]. The precursor, preprosporamin, gives rise to prosporamin by the removal of a signal peptide from the amino terminus. Prosporamin is 22–25 amino acid residues shorter than the precursor and is transported to the endoplasmic reticulum lumen. Mature sporamin is produced and stored as a vacuolar protein by removing a 16 amino acid long propeptide from the N-terminus of prosporamin [24].

Several reports have shown that a large amount of trypsin inhibitor is present in the tuber of sweet potato, and total soluble protein concentrations in tubers are shown to be positively correlated with trypsin inhibitor activity [4, 9, 18–20]. Recent studies have implied that the nucleotide sequences of sporamin cDNAs are homologous to the soybean trypsin inhibitor and win 3, a wound-responsive gene, of the poplar tree [5].

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence database under the accession number U17333 (spTi 1 cDNA).

Expression of the sporamin gene can also be induced in leaf-petiole cuttings by polygalacturonic acid, abscissic acid and sucrose [11, 25]. Together, this suggests that sporamin may have some role in the defense mechanism of the sweet potato tuber.

This information led us to attempt to identify the function activity of sporamin in sweet potato. In this study, a full-length sporamin cDNA (SPTI1) and two partial cDNA fragments deleted with 22 amino acid residues (SPTI1a) or deleted 37 amino acids (SPTI1b) from the N-terminus to precursor were subcloned in pGEX-2T expression vector respectively. Three forms of recombinant proteins corresponding to preprosporamin, prospormin and mature sporamin, were obtained from transformed *E. coli* cells by IPTG induction. They were assayed and showed strong trypsin inhibitory activity on SDS-polyacrylamide gel. The functional activity of sporamin was thus identified.

Isolation and sequence analysis of sweet potato sporamin genes

Standard procedures were used for DNA manipulation. To isolate cDNA clones encoding sporamin, a λgt11 cDNA library prepared from sweet potato (*Ipomoea batatas* Lam. cv. Tainong 57) tuberous root poly(A)$^+$ RNA was screened with radioactively labelled sp-B cDNA fragment, a putative antisense gene of sporamin (K.-W. Yeh, 1991, unpublished data). Four sporamin cDNAs, designated spTi-1 to -4 respectively, were isolated, and their sequence determined. Their nucleotide sequence show 92–94% identity to each other. The sequence analysis from GCG package showed that these four cDNAs all belonged to subfamily A group [12].

Sporamin sequence is related to Kunitz-type trypsin inhibitors

The deduced amino acid sequences of spTi-1 cDNA (sporamin A subfamily) and pIMO 336 cDNA (sporamin B subfamily) [13] were used for similarity search with the PILEUP program of the GCG package (version 7) of the Wisconsin Computer Group database [8]. Only the mature proteins, excluding the signal peptides, were used in the alignments. There was very low amino acid sequence identity (ca. 30%) with TIs of soybean, *Acacia confusa* [15] and *Erythrina variegata* [17] (Fig. 1). It was also observed that there was striking conservation of certain amino acids at various positions along the length of the polypeptides being compared, exemplified by Cys^{83}, Cys^{133}, Cys^{186}, and Cys^{195}. At the homologous location of the putative reactive site, the dipeptide composition for SPO A (spTi-1 cDNA) and SPO B (pIMO 336 cDNA) was observed to be Ala^{106}-Asp^{107}, which is different from the Kunitz-type TIs, which have a general reactive site defined by Arg^{64}-X^{65} or Lys^{64}-X^{65} [30]. However, the Conservation of four Cys residues may imply that a putative three dimensional common structure for trypsin inhibitors may result from cystein disulfide bonding. On the other hand, sporamin may possess the reactive site at a different location from those of the compared TIs, and this may therefore represent a distinct subset within the Kunitz-type family. Further investigation concerning the sporamin reactive site is indeed necessary.

Expression and purification of recombinant fusion proteins GST-SPTI from E. coli

In order to identify sporamin activity, we cloned the sporamin spTi-1 cDNA into the pGEX-2T expression vector to obtain recombinant proteins for assaying activity *in vitro*. In the cloning procedures, PCR was performed to generate correspondent DNA fragments. Employing sporamin cDNA spTi-1 as template, three nested sense primers, 5′-CATGAAAGCCCTCACACTG-3′, 5′-CCAATCCCATCCGCCTCCC-3′, and 5′-CCTCCTCTGAAACTCCAGT-3′ together with an antisense primer 5′-CATTACACATCGGTACCTTTG-3′ were used. The combination of the first sense and antisense primer generated a 0.66 kb full-length cDNA fragment SPTI1, which encodes sporamin precursor protein (preprosporamin). The other two combinations generated a 0.58 kb (SPTI1a) and 0.54 kb (SPTI1b) DNA fragments, which encode prosporamin (N-terminal 22 amino acids deleted to precursor) and mature sporamin (N-terminal 37 amino acids deleted to precursor) respectively. DNA fragments were subcloned into the pGEX-2T vector at *Sma*I site or *Eco*RI site behind the glutathione *S*-transferase (GST) gene (Fig. 2). Plasmid constructs with the various size DNA fragments were transformed into *E. coli* XL1-blue. Plasmid DNA was extracted and their sequences were determined to identify in-frame coding region fused to the GST gene. All three forms of recombinant proteins, preprosporamin, prosporamin and mature sporamin, were synthesized in the form of fusion protein joined with GST at the N-terminal region. Transformed bacteria cultures growing in LB broth were induced to express these chimeric genes by adding isopropy 1-β-

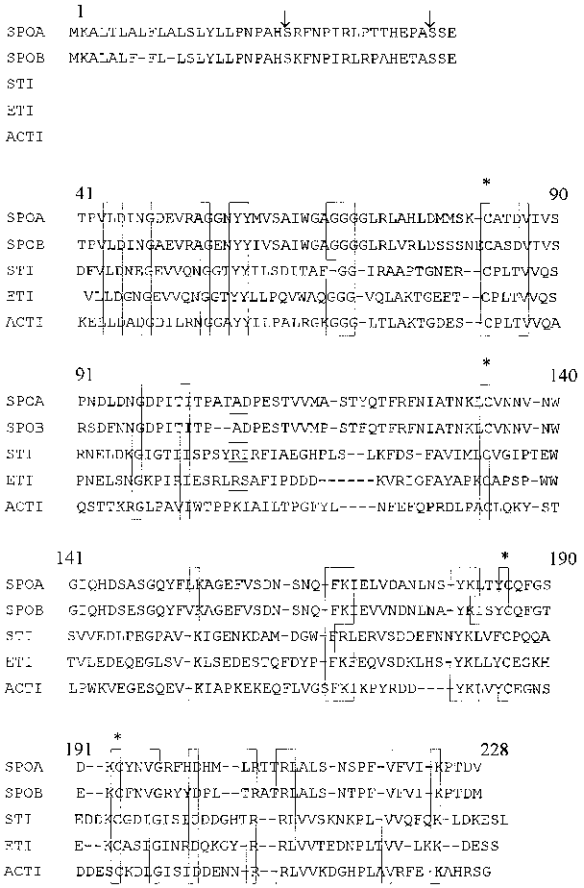

Figure 1. Alignment of the derived amino acid sequence of sopramin SPOA, sporamin SPOB, soybean Kunitz-type trypsin inhibitor (STI), *Acacia confusa* trypsin inhibitor (ACTI), *Erythrina variegata* trypsin inhibitor (ETI). The sequences showing homologous are boxed, and Cys^{83}, Cys^{133}, Cys^{186}, and Cys^{195} are indicated with *. Two sites of post-translational processing for signal peptide and propeptide [24] are indicated with ↓. The predicted reactive sites are underlined. SPOA is deduced from spTi-1 cDNA sequence, and SPOB deduced from pIMO 336 cDNA, which belongs to sporamin B gene family [11].

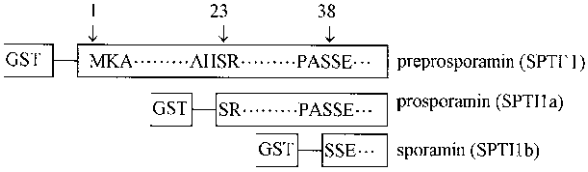

Figure 2. Plasmid constructs for expressing recombinant sporamin proteins in *E. coli*. Three cDNA portions coding for preprosporamin (SPTI1), Prosporamin (SPTI1a) and mature sporamin (SPTI1b) were generated by PCR, and ligated in the pGEX-2T vector. Proteins were fused with GST. The beginning site of three cDNA portions from N-terminus are denoted with ↓ and numbered.

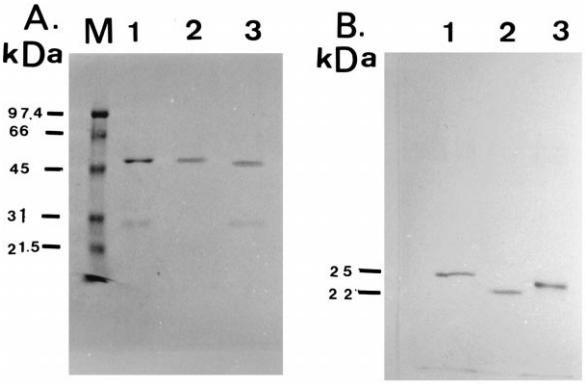

Figure 3. SDS-polyacrylamide gel (12.5%) analysis of recombinant sporamin proteins. A. Recombinant GST-sporamin proteins (2 μg each) purified through glutathione-sepharose 4B affinity chromatography. B. Free recombinant sporamin proteins (2 μg each) generated from treating fusion protein with thrombin. In panel A, lanes 1, 2 and 3 represent precursor, prosporamin and mature sporamin, respectively. All the size amount to ca. 50 kDa. In panel B: lane 1, precursor (ca. 25 kDa); lane 2, mature sporamin (ca. 22 kDa); lane 3, prosporamin (ca. 23 kDa). M = protein markers.

D-1-thiogalactopyranoside (IPTG, 1 mM) when a cell concentration of OD_{600} 0.4 to 0.6 was reached. After a 2 h incubation with IPTG, the cells were harvested and suspended in extraction buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM phenylmethanesulfonyl). Crude protein extracts were obtained from cells ruptured through sonication. Fusion proteins were column purified by affinity chromatography with a glutathione-sepharose 4B resin [32]. The yield of fusion protein was estimated to be ca. 20 mg in 1 liter of bacterial culture. The purified fusion protein was subsequently cleaved with thrombin at an enzyme-to-substrate molar ratio of 1:100 in 50 mM Tris-HCl buffer, pH 8.0, for 2 h at 25 °C. Cleaved proteins were further purified by affinity chromatography column packed with a trypsin-sepharose 4B gel (1.5 cm × 3 cm) [1]. Proteins from every purification step were resolved on a 12.5% SDS-polyacrylamide gel to check their purity and molecular size. All three fusion proteins had a molecular mass of ca. 50 kDa on SDS-PAGE analysis (Fig. 3A), while the molecular mass of the three free recombinant proteins after thrombin cleavage were ca. 25, 23 and 22 kDa, respectively (Fig. 3B). This is coincident with the putative molecular mass deduced from individual nucleotide sequences.

Assay for trypsin inhibitory activity of sporamin

Trypsin inhibitory activity was assayed on SDS-polyacrylamide gel using a modified version of the method of Chan and Delumex [6]. The three forms of recombinant protien samples and sporamin purified from tuber [21] were first dissolved in the sample buffer without 2-mercaptoethanol and without boiling. After electrophoresis, the gel was shaken gently in 25% 2-propanol solution for 30 min to remove SDS, and was further shaken in 20 mM Tris-HCl buffer, pH 8.0, for another 30 min to renature the proteins. The gel was then incubated in trypsin solution (40 μg bovine trypsin (Sigma) in 1 ml of 50 mM Tris-HCl pH 8.0, 50 mM $CaCl_2$) for 30 min before it was transferred to a dish containing freshly prepared substrate-dye solution, which consisted of 2.5 mg N-acetyl-DL-phenylalanine β-naphthyl ester (APNE, Sigma) in 1 ml of dimethylformamide (DMF) plus tetrazotized *O*-dianisidine dye solution (5 mg in 1 ml of 50 mM Tris-HCl pH 8.0, 50 ml $CaCl_2$). The gel was incubated for 1–2 h at room temperature, and destained with 2% acetic acid for 30 min. Though the three forms of recombinant proteins vary in size with a different N-terminal signal peptide, their inhibitory activities were not affected. Sporamin from tuber also exhibited the same TI activity (Fig. 4). This suggests that the extent of post-translational modifications had little effect on the activity. It appears that the cleavage of the N-terminal signal peptide and propeptide is a quite complex process for translocating storage protein without affecting the biochemical activity. Meanwhile, though there are reports that sporamins are modified by glycosylation post-translationally [22], this modification seems not to be a crucial step effecting the protease inhibitory activity.

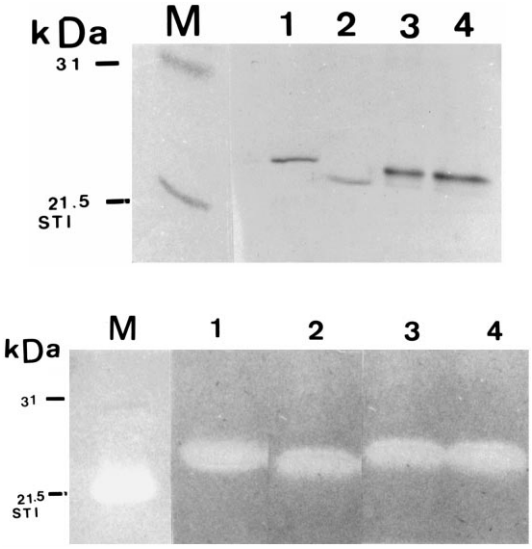

Figure 4. Inhibitory activity against trypsin. Three forms of recombinant sporamin proteins (1.5 μg each) and *in vivo* tuber sporamin were electrophoresed on 12.5% SDS-PAGE. One gel was for Coomassie blue staining (upper panel) and the other gel was assayed *in vitro* against bovine trypsin (lower panel). Lane 1, pre-prosporamin; lane 2, mature sporamin; lane 3, prosporamin; lane 4, sporamin purified from tuber. M, protein markers containing soybean Kunitz trypsin inhibitor protein (STI, 21.5 kDa, ca 1.2 μg) as a positive control.

Sporamin genes are expressed upon wound induction in leaves

Sweet potato plants were grown to a height of 30 cm about 12 leaves. The fifth leaf from the bottom of each plant was wounded using scissors and after 40 minutes, the wounded leaves together with the ones above and below them were collected respectively for RNA isolation to assay sporamin gene expression. Total RNA was extracted from freshly harvested sweet potato leaves according to a rapid extraction procedure described by Yeh *et al.* [34]. RNA was resolved, blotted onto NC membrane (Amersham) as described by the standard protocol [31]. The cDNA portion of spTi-1 was used as a probe to analyze the accumulation of sporamin transcripts in wounded and unwounded leaves. Northern blot analysis showned that a 0.9 kb sporamin gene transcripts were abundantly present in the leaves of wounded plants, but not in those of unwounded plants (Fig. 5). Sporamin transcripts accumulate not only in the wounded leaf (local) but also in distant, undamaged tissue. These results demonstrate that the expression of sporamin gene in sweet potato leaves is inducible both locally and systemically by wounding. It may be interesting to note that the levels of sporamin RNA detected are 2–3 times greater in unwounded leaves from a wounded plant than in either wounded leaves or tuber. The feature of systemic wounding-response pattern of gene expression was also found in potato and tomato proteinase inhibitor (PI) genes [10] and to some new Bowman-Birk trypsin inhibitors found in alfalfa and maize [23, 28]. These observation were though to be the most convincing evidence for a direct defensive role of protease inhibitors in plant protection [2, 3, 27]. The induction of sporamins in leaves by wounding implies that the activity of these protein inhibitors (sporamins) might play an important role in the defense of plants against insect infestations, as PI gene do. It

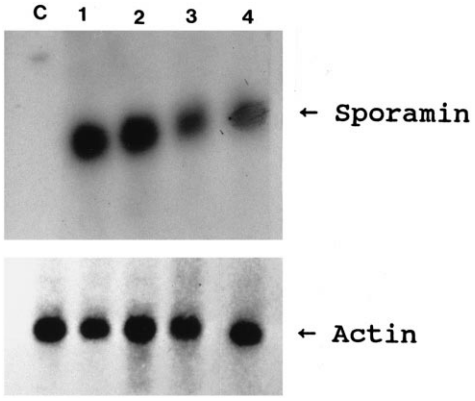

Figure 5. Sporamin transcripts are wound-induced in sweet potato leaves. About 10 μg of total RNA from the unwounded upper leaves, lower leaves and wounded middle leaves of a sweet potato plant were hybridized to sporamin spTi-1 cDNA open reading frame and sweet potato actin (Yeh, unpublished data) probes. The leaves were harvested 40 min after plants were mechanically wounded. RNA sample from leaves and tuber of unwounded plant were used concomitantly as controls. Lane 1, upper leaf RNA of a wounded plant; lane 2, lower leaf RNA of a wounded plant; lane 3, wounded middle leaf RNA; lane 4, tuber total RNA; C, leaf RNA of unwounded plant.

also strongly suggests that sporamin possesses a dual role in sweet potato tuber, one as a somatic storage protein and the other as a natural defense agent, i.e. as a trypsin inhibitor.

Thus far, several members of plant serine proteinase family, including soybean trypsin inhibitor (Kunitz-type), and cowpea (Bowman-Birk family) as well as inhibitors I (PI-I) and II (PI-II) from potato have been implicated in providing a defensive mechanism because of their ability to inhibit insect digestive enzymes [10, 28]. Transfer of these protein inhibitor gene into other plant species has been shown to be effective in functioning as endogenous insecticides against pest damage [13, 16]. Sporamins not only show a good inhibitory activity toward trypsin, but also they can be induced to express systemically in leaves by wounding stress. Moreover, recent studies on transgenic tobacco plants transformed with sporamin gene have already proven that sporamin is an effective anti-insect agent (under submission). Based on these observation, sporamin gene may be able to provide a viable alternative in designing transgenic crops to control insect pests.

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