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Site-directed mutagenesis evidence for a negatively charged trypsin inhibitory loop in sweet potato sporamin

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Abstract Sporamin, a sweet potato tuberous storage protein, has trypsin inhibitory activity. Sequence comparison with other plant trypsin inhibitors (TIs) of the Kunitz family reveals that, instead of the conserved Arg or Lys found in other Kunitz TIs, sporamin contains a negatively charged residue (Asp70 or Glu72) at the P1 reactive site. Using site-directed mutagenesis, six mutants were generated containing substitutions at the reactive site and at one of the disulfide bonds, and the recombinant proteins were assayed for TI activity. Mutants Asp70Val and Glu72Arg were found to have only 2–3% of the wild-type activity. These results provide the first evidence for a negatively charged trypsin inhibitory loop and a new mechanism of trypsin inhibition in the Kunitz family. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Sporamin; Sweet potato; Trypsin inhibitor; Site-directed mutagenesis; Trypsin inhibitory loop; Kunitz family

1. Introduction

Protease inhibitors that suppress the activity of specific proteases are widely distributed in all kinds of life forms [1–3]. The Kunitz-type soybean trypsin inhibitor (STI) family [4,5] is one of the best characterized protease inhibitors. X-ray crystallographic structural studies [6–10] have shown that, although members of the STI-Kunitz family exhibit a high degree of variation in amino acid sequence and protein structure, all contain a reactive site loop bearing the inhibitory residues. It is variations in these residues and in the conformation of the loop structure that largely confer inhibitory specificity for the cognate enzymes. The mechanism of inhibition is substrate-like blockade of the protease catalytic site, with or without a bond-cleaving reaction [1], and it is believed that an arginine or lysine residue must be present in the P1 subsite for trypsin inhibition [9,11].

In plants, many protease inhibitors have been isolated and characterized from legume seeds, cereals, and tubers [12,13]. In the tuber of the sweet potato (*Ipomoea batatas*), sporamins, the products of a multiple gene family which were originally purified by Maeshima et al. [14,15], are the most abundant storage proteins. An interesting aspect of sporamins revealed by their sequences is that they belong to the STI-Kunitz family and therefore may have dual functional roles as a storage protein and as a defense molecule against foreign stress. In recent years, we have demonstrated that various forms of sporamin possess strong trypsin inhibitory activity in vitro [16], and that sporamin can confer insect resistance on transgenic plants transformed with the sporamin gene [17].

The three-dimensional structure of STI-Kunitz protease inhibitors consists of a β-trefoil fold made up of three pseudo-symmetry-related trefoil units, each comprised of four β-strands [18]. The loop that connects the first and second trefoil units, termed the A4–B1 loop following the notation of McLachlan [19], contains the inhibitory residues. Sporamins share just under 30% amino acid sequence identity with other trypsin inhibitors (TIs) of the STI-Kunitz family. This weak, but significant, evolutionary relatedness appears to underlie the observed trypsin inhibitory activity of sporamins [16]. However, sequence comparison of sporamin and other Kunitz TIs (Fig. 1), including those from *Acacia confusa* (ACTI [20]), winged bean (WTI [21]), *Erythrina caffra* (ETI [22]), and soybean (STI [23]), indicates that sporamin may be quite distinct from other plant TIs in how it inhibits trypsin, since the sporamin A4–B1 loop is not only three amino acids longer than that of other plant TIs, but also does not contain any arginine or lysine residues, the signature amino acids acting as the amino-terminal residue of the scissile bond [9,11]. Moreover, the sporamin A4–B1 loop is uncharacteristically acidic, containing two negatively charged residues, Asp70 and Glu72 (Fig. 1). The major objective of the present study was, therefore, to determine whether the sporamin A4–B1 loop is indeed the reactive site loop responsible for inhibiting trypsin and, in particular, whether Asp70 and Glu72 are involved in this inhibition.

2. Materials and methods

2.1. Materials

CNBr-Sepharose and the expression vector, pGEX2T, were purchased from Pharmacia Biotech Co. (Sweden). Trypsin, *N*-acetyl-DL-phenylalanine β-naphthyl ester (APNE), and *N*-benzoyl-DL-arginine β-nitroanilide (BAPA) were obtained from Sigma.

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Abbreviations: ACTI, *Acacia confusa* trypsin inhibitor; APNE, *N*-acetyl-DL-phenylalanine β-naphthyl ester; BAPA, *N*-benzoyl-DL-arginine β-nitroanilide; ETI, *Erythrina caffra* trypsin inhibitor; GST, glutathione *S*-transferase; GST–SPOA, glutathione *S*-transferase–sporamin A fusion protein; STI, soybean trypsin inhibitor; TI, trypsin inhibitor; WTI, winged bean trypsin inhibitor

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2.2. Construction of the recombinant pUC19-sporamin plasmid for mutation

A 0.9 kb full-length cDNA encoding the sweet potato sporamin gene was previously cloned into pGEM-7Z, yielding the plasmid pGEM-TIA [16]. Two primers were designed to amplify the coding region of the sporamin gene by the PCR method. The 5′-end primer, containing a *Bam*HI site, was 5′-CT GGA TCC TCT GAA ACT-3′, while the 3′-end primer, containing an *Eco*RI site, was 5′-GAA TTC ATC CTC TGA AAC-3′. Since preliminary experiments indicated that the melting temperature of both primers was 58°C, the PCR conditions were set as 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. When the resulting PCR product was purified and analyzed by 1.5% agarose gel electrophoresis, only one DNA band of about 550 bp was observed. This is the mature form of sporamin DNA lacking the protein for two N-terminal signal peptides [16]. This fragment was then cloned into pUC19 at the *Bam*HI and *Eco*RI sites and the final construct, pUC-SPOA101, shown to contain the mature sporamin DNA fragment by dideoxy-sequencing, was used to transform *Escherichia coli* XL1-Blue.

2.3. Site-directed mutagenesis and expression of mutant sporamin

Based on the pUC-SPOA101 plasmid, six sporamin mutants were successfully produced. The following six oligonucleotides were used to create the indicated mutations (the bold type and underlining denote, respectively, the amino acid mutation and the corresponding codon): Ala69**Ser**: 5′-ATC ACG CCG GCG ACG AGT GAC CCG GAA TCC ACC GTG-3′; Asp70**Val**: 5′-ACG CCG GCG ACG GCC GTT CCG GAA TCC ACC GTG GTC-3′; Glu72**Arg**: 5′-GCG ACG GCC GAC CCG AGA TCC ACC GTG GTC ATG GCC-3′; Ser73-**Ile**: 5′-GCG ACG GCC GAC CCG GAA ATC ACC GTG GTC ATG GCG-3′; Cys45**Leu**: 5′-TTG GAC ATG ATG TCC AAA CTT GCC ACG GAC GTC ATC-3′; Arg15**Pro**: 5′-GGC GAC GAG GTC CCC GCC GGC GGG AAC TAC TAC-3′.

Oligonucleotide-directed mutagenesis was carried out according to the manual provided with the Transformer™ Site-Directed Mutagenesis kit (Clontech, PT1130-1). Mutations were verified by DNA sequencing of the entire fragment. The mutant pUC-SPOA DNAs were purified by minipreparation, and their *Eco*RI–*Bam*HI fragments ligated into pGEX2T at the *Eco*RI and *Bam*HI sites behind the glutathione *S*-transferase (GST) gene. The mutant pGEXT plasmids were then used to transform *E. coli* XL1-Blue to express GST–sporamin fusion proteins (GST–SPOA), as described previously [16].

2.4. Overexpression of GST–SPOA fusion proteins in E. coli

E. coli XL1-Blue cells harboring different pGEXT-sporamin mutant constructs were grown at 37°C in 50 ml of LB broth (1% NaCl, 1% Bacto-tryptone, 0.5% Bacto-yeast extract, pH 7.0) containing 100 μg/ml of ampicillin. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 1 mM to induce fusion protein expression and the cultures incubated for a further 4 h. A maximal harvest could be obtained under these conditions. Total soluble proteins were extracted in phosphate-buffered saline (10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.5) by repeated freeze-thawing, followed by centrifugation (10000×*g*, 10 min), the supernatant being the crude protein extract. 15 μg of crude protein extract was analyzed on 12.5% SDS–PAGE followed by Coomassie blue staining.

2.5. Purification of GST–SPOA fusion proteins

Mutant GST–SPOA fusion proteins were produced and purified to homogeneity as described previously for the wild-type protein [16]. In brief, crude protein extracts were loaded onto a glutathione-Sepharose-4B affinity column, then, after washing, the GST–SPOA fusion protein was eluted using 10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0.

2.6. Gel staining for TI activity of sporamin mutant proteins

The mutant sporamin protein was first resolved by SDS–PAGE (12.5%), then the gel was treated as described by Yeh et al. [16]. In brief, the gel was immersed in trypsin solution, then reacted with APNE, stained with *o*-dianisidine dye, and gently shaken in the dark at room temperature for 30 min. Sufficient 7% acetic acid was then added to cover the gel and stop the reaction.

2.7. Quantitative analysis of trypsin inhibitory activity of sporamin mutant proteins

Purified GST–SPOA wild-type or mutant protein was reacted at 37°C for 10 min with trypsin (1:1 molar ratio), then BAPA was added to a final concentration of 500 ppm, and the reaction continued for 30 min. The optical density of the reaction mixture was then measured at 410 nm. The percentage inhibition (*I*%) of trypsin by sporamin was calculated using the equation:

$$I\% = [(T - T^*)/T] \times 100\%$$

where T denotes the OD_{410} in the absence of GST–SPOA and T^* that in the presence of GST–SPOA. One inhibition unit was defined as the amount of inhibitor required to completely inhibit 1 μg of trypsin.

2.8. Molecular modeling

In order to select mutation sites, a standard homology modeling procedure was used to predict the three-dimensional structure of sporamin. The degree of sequence homology (~30% identity) between, and the conserved disulfide bridge cysteines found in, sporamin and other plant Kunitz TIs (Fig. 1) justified template-based structural modeling, which was performed using the tools provided by the Pro-Mod program in the Swiss-Model package (http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html) [24]. The model for sporamin, depicted in Fig. 2, was produced based on homology modeling principles and the use of multiple structural templates, including the X-ray structures of STI [8], ETI [6], and WTI [10].

3. Results and discussion

As shown in Fig. 1, six amino acids, Arg15, Cys45, Ala69, Asp70, Glu72, and Ser73, were chosen for mutation to address three questions: (1) whether the A4–B1 loop confers trypsin inhibitory activity, (2) whether the positively charged N-terminus residue (Arg15) plays a role in stabilizing the inhibitory loop, and (3) whether the first disulfide bond is re-

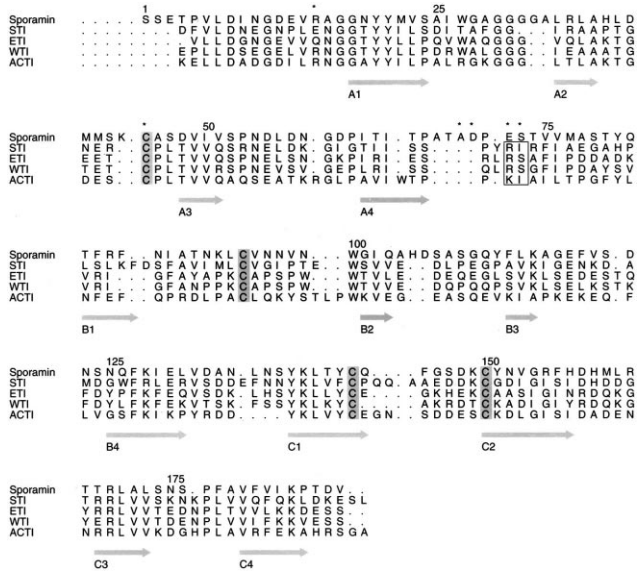

Fig. 1. Sequence comparison of sporamin and some related TIs from higher plants. These sequences were retrieved from the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) with the following accession numbers: sporamin (sporamin A) PIR S07465, STI SP P01070, ETI SP P81366, WTI SP P10821, ACTI SP P24924. The alignment was prepared using Alscript [30]. The four conserved cysteines are shown shaded and the P1–P1′ residues for canonical STI-Kunitz TIs in boxes (see text). The six residues that were mutated in this study are indicated by asterisks. The arrows below the sequences, labeled A1–C4, are the β-sheet positions as determined in the X-ray structure of STI [8].

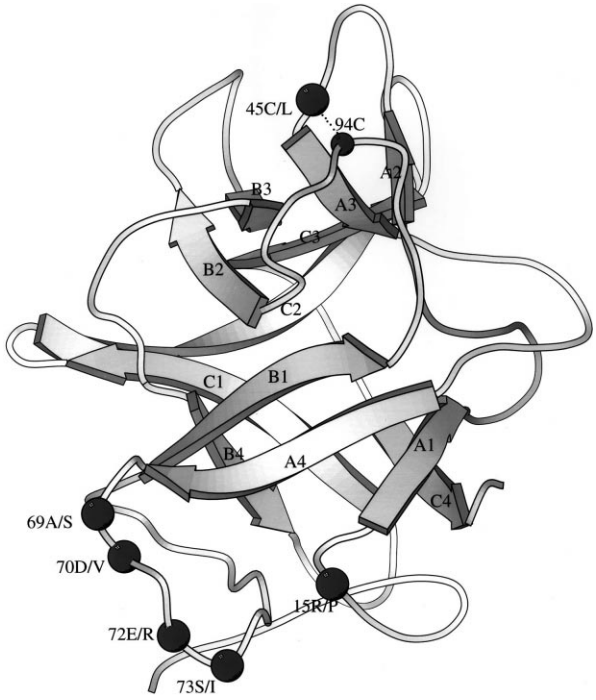

Fig. 2. Schematic representation of the Swiss-Model-predicted three-dimensional structure of sporamin. The six residues modified in this study are shown by a sphere and labeled with the wild-type and mutant amino acids separated by a slash. The first disulfide bridge, Cys45–Cys94, is indicated by a dashed line. This figure was prepared using Molscript [31].

quired for inhibitory activity. As described above, the GST-fused sporamin A mutants were individually expressed in *E. coli* XL1-Blue and assayed for trypsin inhibitory activity. The results of the SDS–PAGE trypsin inhibitory activity assay are shown in Fig. 3 for the four A4–B1 loop mutations (Ala69Ser, Asp70Val, Glu72Arg, and Ser73Ile) and in Fig. 4 for the Cys45Leu and Arg15Pro mutants. Fig. 5 provides a quantitative summary of the inhibitory activities of all the above-mentioned mutants. Of the six mutants examined, Ala69Ser and Arg15Pro had similar activities to the wild-type protein, whereas the other three A4–B1 loop mutants (Asp70Val, Glu72Arg, and Ser73Ile) and Cys45Leu exhibited greatly reduced trypsin inhibitory activity. These results support the conjecture derived from sequence analysis that the sporamin A4–B1 loop comprises the active site that interacts with trypsin and blocks its enzymatic function.

For defense against natural enemies, plants have evolved a diverse set of protease inhibitors, of which the members of two distinct families, the Kunitz family (~20 kDa) and the Bowman–Birk family (~8 kDa or ~16 kDa), are best characterized. In both families, the reactive P1 residue is always an arginine or lysine [9,21], so the finding that sporamin, which does not have an arginine residue or a lysine residue in the predicted reactive loop, can still inhibit trypsin [16] is therefore surprising. Nevertheless, the suggestion that the atypical A4–B1 loop of sporamin confers trypsin inhibitory activity was borne out by the present mutation experiments. Of the four A4–B1 loop amino acid mutants studied, three had essentially no trypsin inhibitory activity (Figs. 3 and 5); these included mutations at either Asp70 or Glu72, the two negatively charged residues near the middle of the loop (Fig. 1). Introduction of an arginine residue into the loop (in the Glu72Arg mutant) was, in fact, detrimental to the inhibitory function. The lack of effect of the Ala69Ser mutation may possibly reflect the fact that this was a relatively mild amino acid substitution.

It is of interest to note that a distorted A4–B1 loop resulting from the insertion of four amino acids is suggested to be the reason why winged bean albumin-1, a member of the STI-Kunitz family, does not exhibit trypsin inhibitory activity [9]. In contrast to the prevailing model of how STI-Kunitz proteins inhibit trypsin, sporamin has a longer than conventional A4–B1 loop, and the present results for its A4–B1 loop mutants therefore suggest that neither a canonical conformation of the A4–B1 loop nor a reactive arginine residue or a lysine residue are absolutely required for this family to inhibit trypsin. Although further biochemical and structural studies are needed to elucidate the details of this novel trypsin-inhibiting mechanism of sporamin, it should be noted that non-cleavage inhibition of proteases is seen in the larger Kunitz inhibitor family. For example, the wheat inhibitor of fungal protease K,

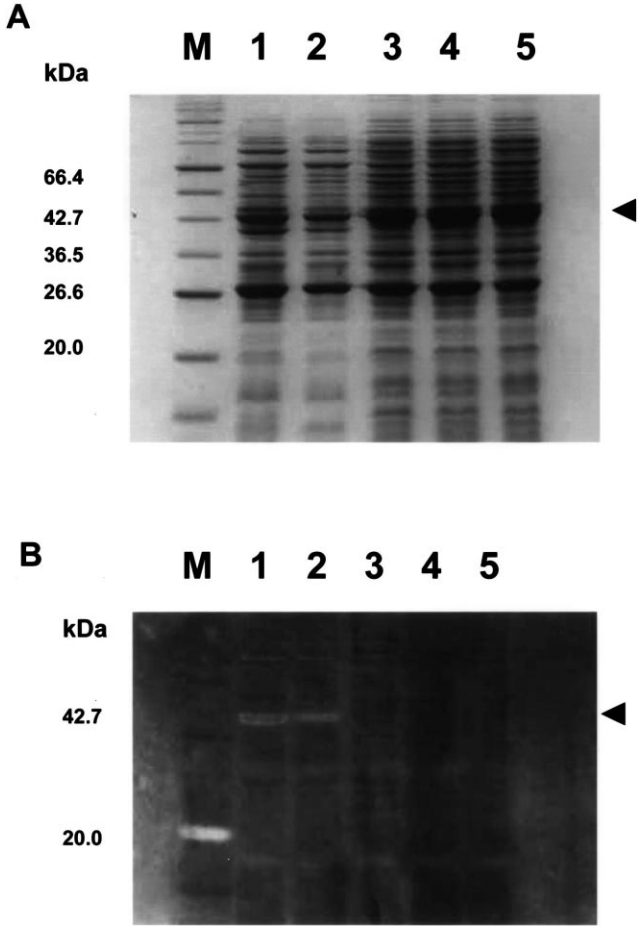

Fig. 3. Gel trypsin inhibitory activity assay of mutant sporamins. Samples of crude extracts of various mutant sporamins expressed in *E. coli* as GST fusion proteins (15 μg of protein) were analyzed on two 12.5% SDS–PAGE gels. One gel was stained with Coomassie blue (A) and the other tested by trypsin inhibitory staining (B). Lane 1, GST–SPOA wild-type; lane 2, GST–SPOA Ala69Ser; lane 3, GST–SPOA Asp70Val; lane 4, GST–SPOA Glu72Arg; and lane 5, GST–SPOA Ser73Ile. M, broad range protein markers (BioLab). ▶ indicates the recombinant GST–SPOA proteins.

PKI3, exerts its inhibitory activity by physically pushing away the active serine of the enzyme, thereby preventing the catalytic triad from forming an active conformation [7]. Another unique example is the case of barley β-amylase/subtilisin inhibitor, which blocks the access of substrate to the active site, but does not interact directly with the catalytic triad [26]. The Kunitz family is thought to have evolved from a storage protein [27], explaining the diverse functional specificity of this family. The present work on sporamin further suggests that, even for the TIs of this one family, the mechanism of inhibition could also be diverse.

The present mutational studies also attempted to explain how the trypsin inhibitory loop of sporamin is stabilized. In Bowman–Birk TIs, a disulfide bond bridging the ends of the inhibitory loop is pivotal in maintaining the loop in the inhibitory conformation [25]. Lacking a disulfide bridge and secondary structure constraints to stabilize the inhibitory loop, STI-Kunitz inhibitors compensate by employing an extensive hydrogen bond network that involves several N-terminal residues, including a conserved asparagine residue (Asn13 in STI) [8,27,28]. In sporamin, this asparagine residue is replaced by an alanine residue, as shown in the sequence alignment (Fig. 1). The homology-derived structural model (Fig. 2) of sporamin suggested that the preceding residue, Arg15, was close enough to the A4–B1 loop to allow the formation of salt bridges or hydrogen bonds with other residues (e.g. Glu72) in the loop. However, the data of the Arg15Pro mutant (Figs. 4 and 5) do not support the hypothesis that Arg15 plays a role analogous to that of the conserved asparagine residue found in other plant TIs. The factors responsible for the stabilization of the inhibitory loop of sporamin therefore remain to be determined.

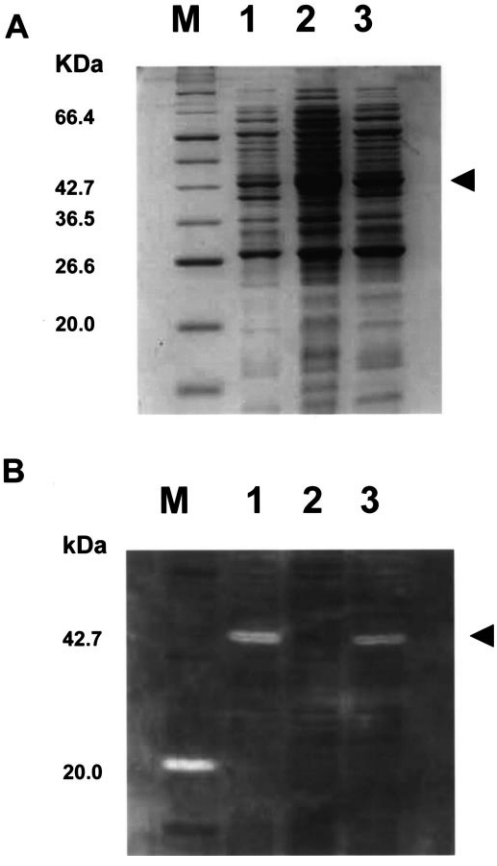

Fig. 4. Gel trypsin inhibitory activity assay of mutant sporamins. The experimental details are as in Fig. 3. Lane 1, GST–SPOA wild-type; lane 2, GST–SPOA Cys45Leu; and lane 3, GST–SPOA Arg15Pro. M, broad range protein markers (BioLab). ▶ indicates the recombinant GST–SPOA proteins.

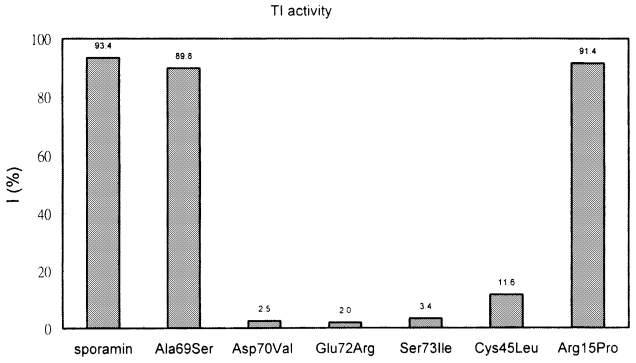

Fig. 5. Quantification of trypsin inhibitory activity of various mutant sporamins. The trypsin inhibitory activity of wild-type (WT) sporamin and the various mutants was quantified as the percentage inhibition (*I*%), as described in Section 2.

The two disulfide bonds, Cys45–Cys94 and Cys153–Cys160, are conserved in STI-Kunitz TIs (Fig. 1), but, despite this, their role in the inhibitory function is still controversial. Early experiments reported that at least one of the two disulfide bonds is required for substantial inhibitory activity, but a more recent redox experiment led to the conclusion that, in the case of ETI, whereas protein stability was affected when these disulfide bridges are disrupted, inhibitory function was not [29]. In the present study, the Cys45Leu sporamin mutant exhibited significantly reduced inhibitory activity (Figs. 4 and 5). Our data therefore favor the conclusion of the early experiments and suggest that, in the case of sporamin, the first disulfide bond is important for its inhibitory function. However, this conflicts with the results of the study by Hung et al. on ACTI [20], in which site-specific mutagenesis experiments showed that it is the second disulfide bond (Cys133–Cys141 in ACTI), and not the first, that is essential for the inhibitory activity. The reason for this disparity is not clear, but it should be noted that mature ACTI is a two-chain Kunitz inhibitor in which the constituent chains are connected by the second disulfide bond [20].

In summary, we have used site-directed mutagenesis to generate six sporamin mutants in order to probe the structure–function relationship for its trypsin inhibitory activity. The study yielded a number of results that appear to deviate from the current opinion on how the A4–B1 inhibitory loop of the STI-Kunitz family works and how it is stabilized. Most notably, our data demonstrated that the two acidic residues in the sporamin A4–B1 loop, Asp70 and Glu72, are critical for the inhibitory function, contradicting the long-held idea that a positively charged amino acid is required for inhibitory reaction in the STI-Kunitz family. Our findings thus suggest that trypsin inhibitory mechanisms are more diverse than previously thought, and should regenerate interest in further analysis of the Kunitz family, especially the less well studied members.

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