Supporting Information

Head-to-tail Cyclization after Interaction with Trypsin: A Scorpion Venom Peptide that Resembles Plant Cyclotides

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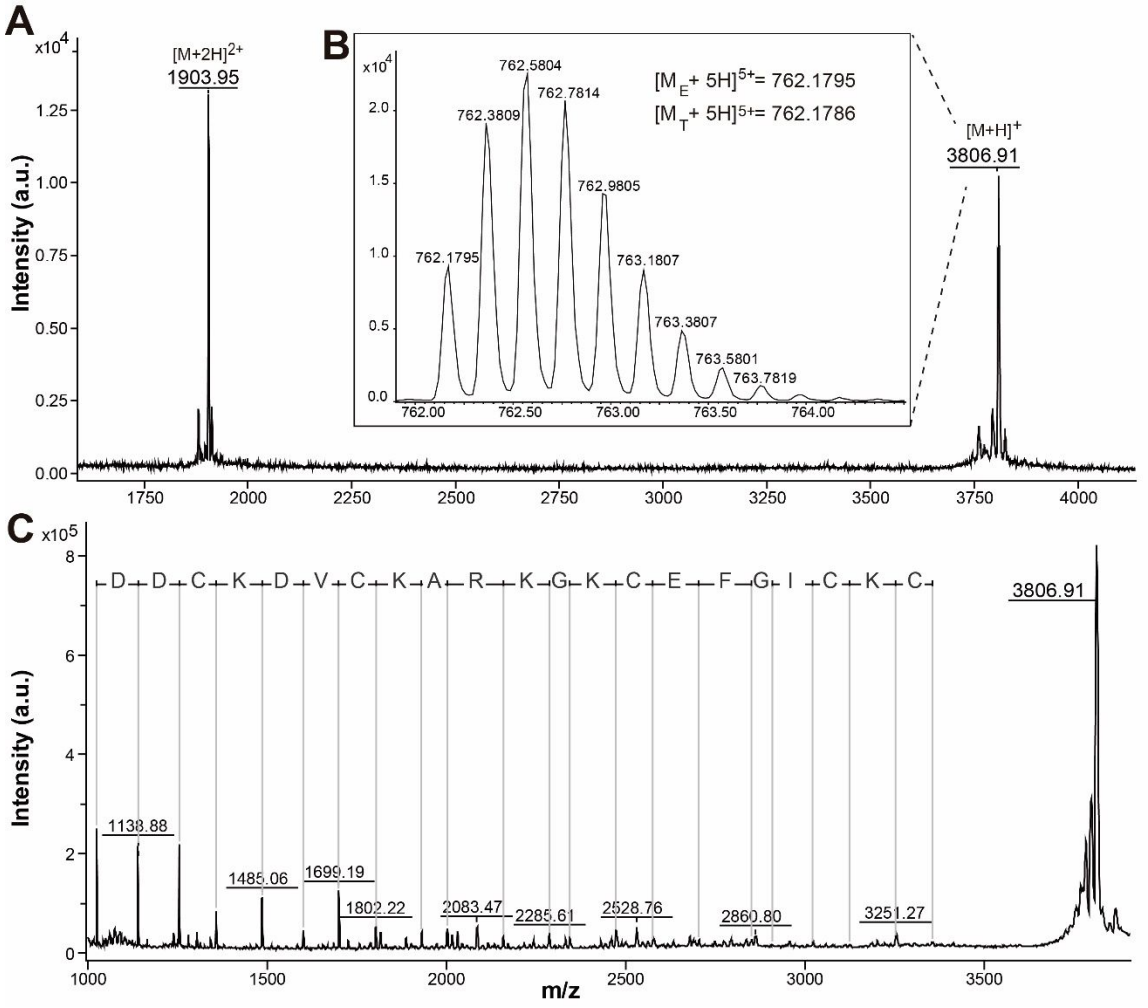

Figure S1. Molecular mass and partial amino acid sequencing of native ToPI1. (A) Mass spectrometry analysis of ToPI1 by MALDI-TOF/TOF MS Autoflex Speed (Bruker Daltonics), at positive reflector mode, using α-cyano-4-hydroxycinnamic acid as matrix, showing the +1 and +2 charged ions. **(B)** Mass spectrometry analysis of ToPI1 by micrOTOF-Q II (Bruker Daltonics), presenting the monoisotopic distribution of the +5 charged ion ($[M_E+5H]^{5+}$ = 762.1795), which is equivalent to $[M_E+H]^+$ = 3806.8975 Da. M_E and M_T correspond to experimental and theoretical molecular masses, respectively. M_T was calculated with the Isotopic Pattern tool, available from Bruker Daltonics analysis platform, considering the peptide with three disulfide bonds and a C-terminal amidation; this analysis was only performed after complete amino acid sequencing of ToPI1. As expected, the experimental molecular mass ($[M+H]^+$ = 3806.8975) matches with the theoretical molecular mass ($[M+H]^+$ = 3806.8930) of the mature toxin, with an accuracy of 1.2 ppm. **(C)** The amino acid sequence of ToPI1 native peptide was partially determined by MALDI - in source decay (ISD) method, using 1,5-diaminonaphthalene (DAN) matrix solution on an Autoflex Speed MALDI TOF/TOF MS (Bruker Daltonics). The spectra analysis revealed 21 amino acid residues related to c-series of ToPI1 structure, which are residues 9-29.

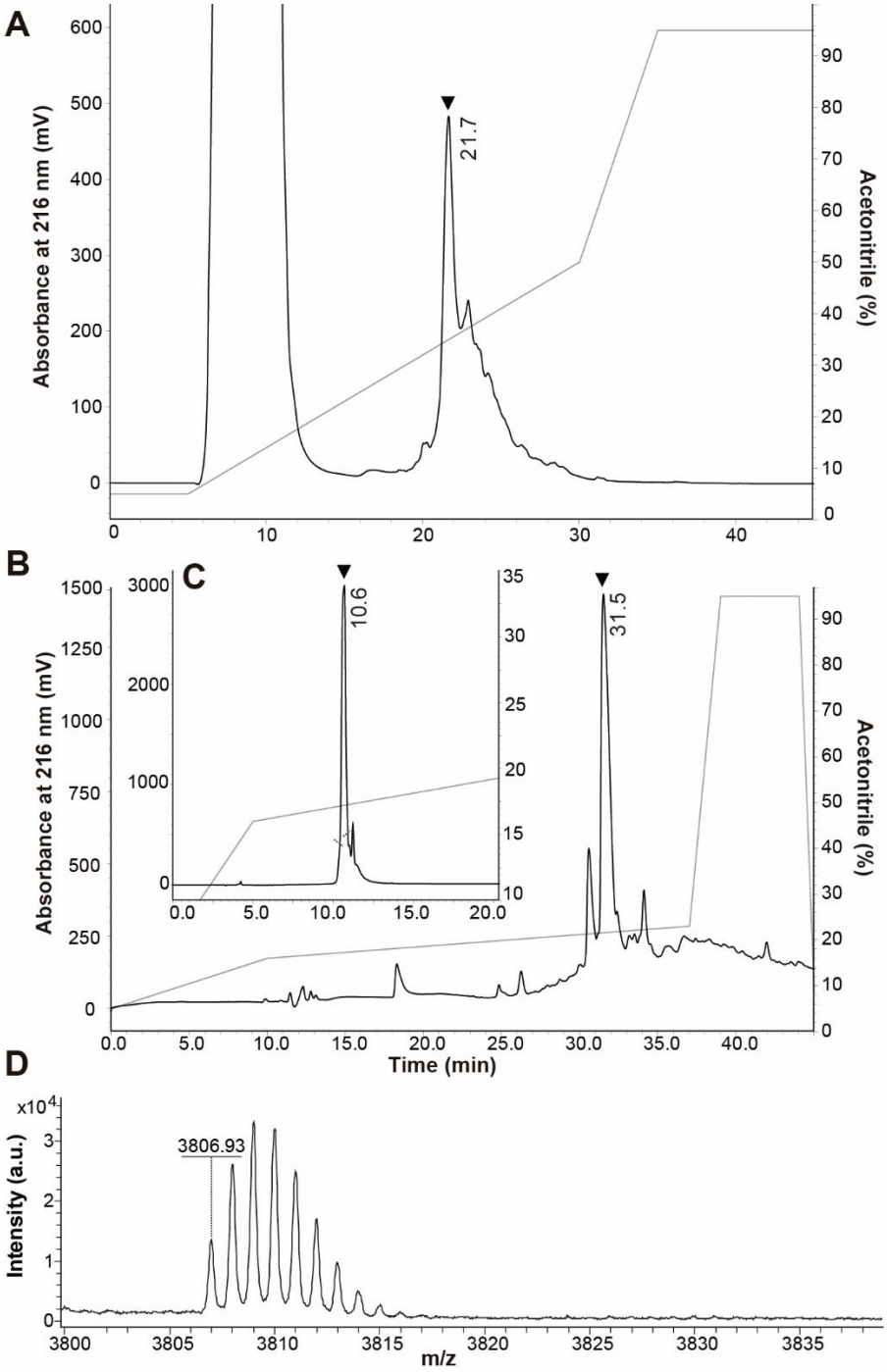

Figure S2. Purification and molecular mass analysis of the synthetic peptide ToPI1$_s$. (A) RP-HPLC fractionation of 25 mg of a synthetic and oxidized sample of ToPI1$_s$ in a preparative C_{18} column, using a gradient of acetonitrile (B solution) that is represented by the gray line, with a flow rate of 10.0 mL/min and absorbance monitored at 216 nm. The first fraction, at about 10 min, corresponds to salt elution, present in the oxidation buffer used to form the disulfide bonds. The component labeled with the arrow, with an elution time of 21.7 min (35.1% acetonitrile) corresponds to ToPI1$_s$. **(B)** Fractionation of about 5.0 mg of the sample eluted at 21.7 min in a semipreparative C_{18} column at 45°C, at 1.5 mL/min flow rate. The gray line represents the gradient of acetonitrile. The fraction eluted at 31.5 min (21.6% acetonitrile) corresponds to ToPI1$_s$. **(C)** Aliquots of about

1.0 mg of the sample eluted at 31.5 min were purified using an analytical C_{18} column maintained at 45°C, at 1.0 mL/min flow rate, using a gradient of acetonitrile that is represented by the gray line. The main fraction, eluted at 10.6 min (17.2% acetonitrile), corresponds to $ToPI1_s$. **(D)** Molecular mass analysis of purified $ToPI1_s$ by MALDI-TOF MS Autoflex Speed (Bruker Daltonics, Germany), at positive reflector mode, using α-cyano-4-hydroxycinnamic acid as matrix, showing the monoisotopic resolution of the monocharged ion. As expected, the experimental molecular mass ($[M+H]^+$ = 3806.93) matches with the theoretical molecular mass ($[M+H]^+$ = 3806.89), with a mass error of 0.04 Da. The molecular mass analysis of the main fraction was performed after each chromatographic step.

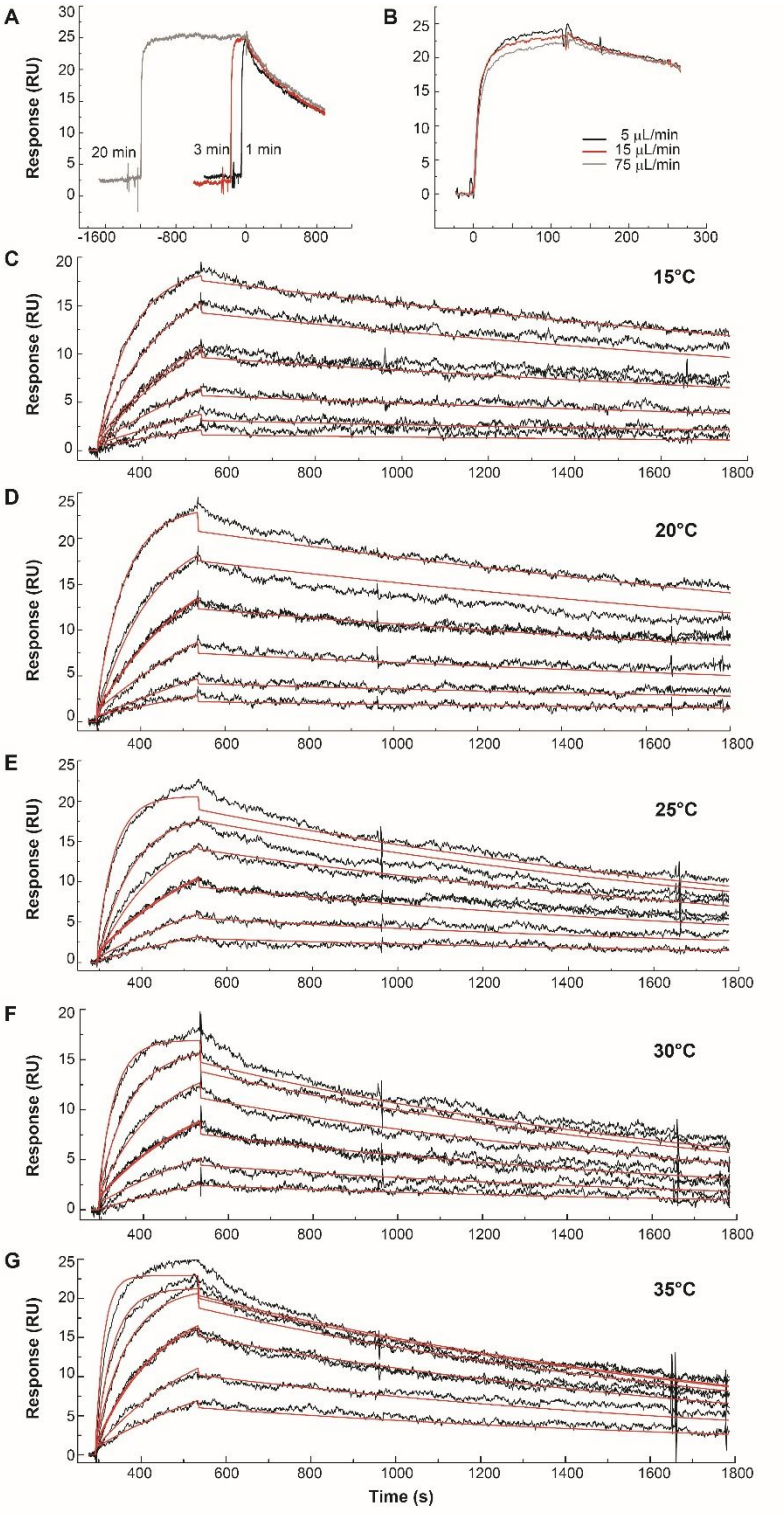

Figure S3. Kinetics of the interaction between ToPI1$_s$ and the surface immobilized trypsin evaluated by Surface Plasmon Resonance. Experiments were performed in a BIAcore 3000 biosensor (GE Healthcare, UK). **(A)** Linked reaction test and **(B)** mass transport for the interaction of ToPI1$_s$: trypsin complex, using a solution of 500 nM ToPI1$_s$ diluted in HPS-EP buffer (pH 7.4). In **(A)** the dissociation rate was monitored while varying the analyte injection period (1, 3 or 20 min)

during full ligand saturation. In **(B)** the mass transport effect in the complex interaction kinetics was evaluated at different flow rates (5, 15 or 75 µL/min). **(C-G)** Kinetics of ToPI1$_s$:trypsin complex interaction at 15, 20, 25, 30 and 35 °C. Black lines correspond to the differential binding responses obtained by injecting ToPI1$_s$ diluted in HPS-EP buffer (pH 7.4) in two-fold dilution series, from 25 to 0.78 nM, over a trypsin derivatized dextran CM5 sensor chip, at a 40 µL/min flow rate. Each concentration was randomly injected in triplicates. For better visualization, the response of only one injection was shown, with a concentration of each series being shown in duplicate in order to demonstrate data reproducibility. The concentration series were performed twice at each temperature, and only one representative sensorgram was shown. Data corresponding to the association phase of interaction were acquired for 4 min, and the dissociation phase was monitored for 25 min due to the slow dissociation kinetics. Red lines correspond to data fittings obtained by the adjustment to the simple bimolecular model (A+B ↔ AB), by using BIAevaluation 3.1 software.

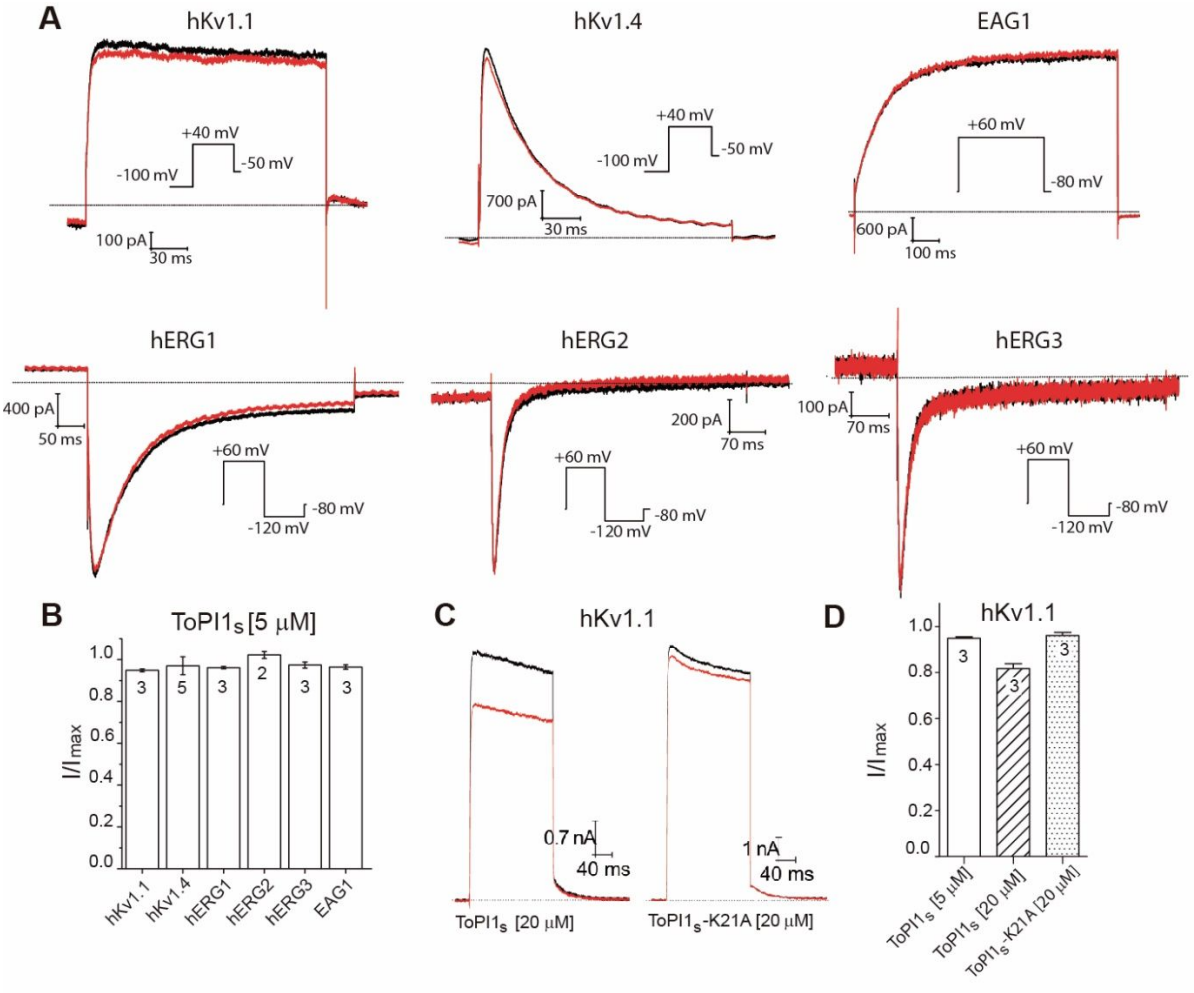

Figure S4. Effect of ToPI1$_s$ on the current of different subtypes of K$_v$ channels. (A) ToPI1$_s$, at 5 μM concentration, was tested against six different subtypes of potassium channels: hKv1.1, hKv1.4, EAG1, hERG1, hERG2 and hERG3. Potassium currents were recorded in the absence (control, in black) and in the presence (red) of 5 μM of ToPI1$_s$. The stimulus protocol is shown for each channel subtype. High potassium extracellular solution was used in the hERG currents determination. **(B)** There was no statistical difference between the different groups treated with 5 μM of ToPI1$_s$ by Kruskal-Wallis non-parametric test (*KW* = 5.463, p = 0.3621). **(C)** Comparison of ToPI1$_s$ and ToPI1$_s$-K21A activities, at 20 μM, on hK$_v$1.1 channel. The addition of 20 μM of ToPI1$_s$ caused a current reduction by 18.3% (I/I$_{max}$ = 0.8173 ± 0.022). In turn, the mutant analogue ToPI1$_s$-K21A reduced the current by only 3.9% (I/I$_{máx}$ = 0.9606 ± 0.014). **(D)** A comparison of the three treatments, however, showed that there was no significant statistical difference between groups (*KW* = 5.982, p = 0.0502). In **(B)** and **(D)**, data are presented as mean ± S.E.M., and the number of cells tested in each treatment is shown by the number in each column. Statistical analyses were performed with the GraphPad Prism version 6.0 software. All effects were reversible after washing the cell with external solution.

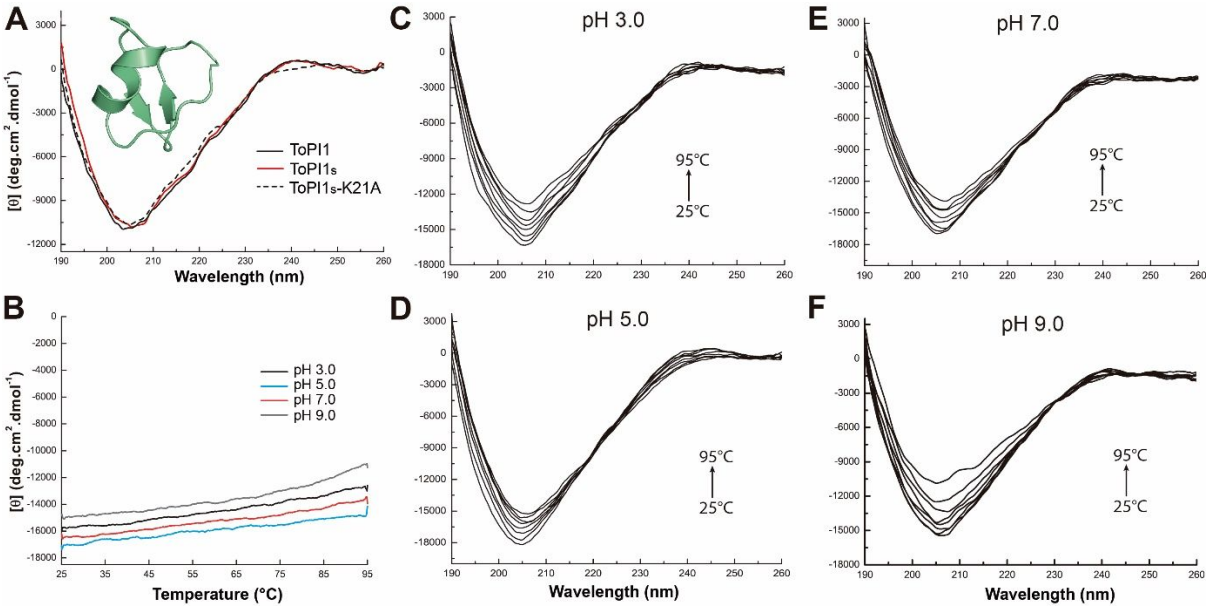

Figure S5. Circular dichroism spectra analyses of ToPI1, ToPI1$_s$ and ToPI1$_s$-K21A. (**A**) The CD spectra of the native ToPI1, the chemically synthesized ToPI1$_s$ and the mutant ToPI1$_s$-K21A were measured in the Far-UV range (190–260 nm) at a concentration of 0.04 mg/mL in water at 25°C. A cartoon representation of ToPI1$_s$ is also shown, based on the crystallographic analysis (PDB 6MRQ). (**B-F**) Effect of temperature and pH in ToPI1$_s$ secondary structure. The analyses were performed with 0.05 mg/mL of ToPI1$_s$, from 25 to 95°C, in sodium acetate buffer pH 3.0 (**C**) and pH 5.0 (**D**), and in Tris-HCl buffer pH 7.0 (**E**) and pH 9.0 (**F**). (**B**) Shows the temperature dependence of the molar ellipticity at 208 nm for ToPI1$_s$ at different pH values.

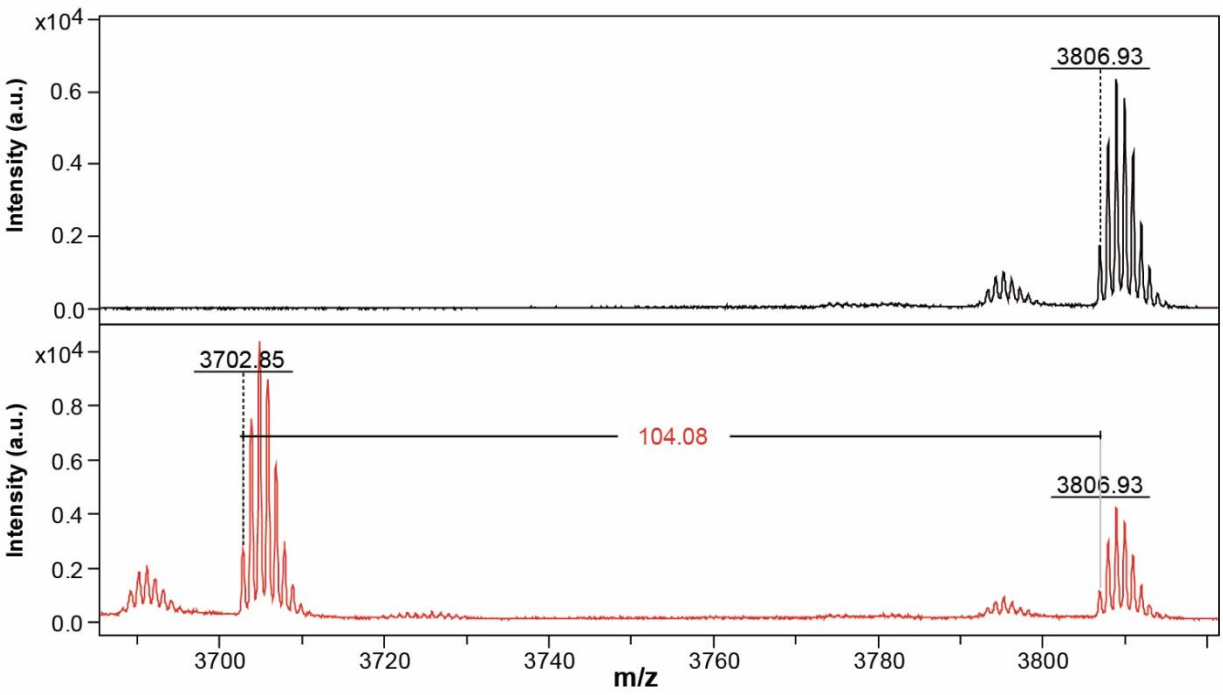

Figure S6. Molecular mass analyses of ToPI1$_s$ alone and ToPI1$_s$ after interacting with trypsin. At the top, the molecular mass of ToPI1$_s$ was analyzed in the presence of trypsin assay buffer (50 mM Tris-HCl, 20 mM $CaCl_2$, pH 8.2), resulting in the ion with 3806.93 Da, as expected for ToPI1$_s$. At the bottom, the molecular mass of ToPI1$_s$ was analyzed in the presence of trypsin (1:1, v/v), as performed for the chromogenic assay, after 1 h of incubation and without substrate addition. The molecular mass difference corresponds to the loss of the C-terminal residue Ser^{33}, which was amidated (-86.06 Da), and to the formation of a new peptide bond between the new C-terminal residue Lys^{32} and the N-terminal residue Ile^{1} (-18.02 Da). Thus, the ion with *m*/*z* 3702.85 corresponds to the cyclic ToPI1$_s$, formed after interacting with trypsin. The results were obtained by MALDI-TOF MS Autoflex Speed (Bruker Daltonics, Germany), at positive reflector mode, using α-cyano-4-hydroxycinnamic acid as matrix, showing the monoisotopic resolution of the monocharged ions.