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Purification and pH stability characterization of a chymotrypsin inhibitor from *Schizolobium parahyba* seeds

Rozeni C.L. Teles, Elizabeth M.T. de Souza, Leonardo de A. Calderon, Sonia M. de Freitas*

Universidade de Brasília, Depto de Biologia Celular, Laboratório de Biofísica, Campus Universitário Darcy Ribeiro, Asa norte. 70910-900, Brasília, DF, Brazil

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Abstract

Schizolobium parahyba chymotrypsin inhibitor (SPCI) was completely purified as a single polypeptide chain with two disulfide bonds, by TCA precipitation and ion exchange chromatography. This purification method is faster and more efficient than that previously reported: SPCI is stable from pH 2 to 12 at 25 °C, and is highly specific for chymotrypsin at pH 7–12. It weakly inhibits elastase and has no significant inhibitory effect against trypsin and α-amylase. SPCI is a thermostable protein and resists thermolysin digestion up to 70 °C.

Keywords: *Schizolobium parahyba*; Leguminosae; Protein purification; Protein stability; Chymotrypsin inhibitor

1. Introduction

Serine protease inhibitors are widely distributed in nature and can inhibit enzyme action in different organisms. These inhibitors vary in their specificity, and can either be synthetic or natural. Natural inhibitors have been classified into 20 different families (Laskowski and Qasim, 2000; De Leo et al., 2002), among which the most well studied are the Kunitz (Ascenzi et al., 2003) and Bowman–Birk types. These families mainly differ in molecular weight, disulfide bond content, three-dimensional structure, and stability to heat and denaturing agent. In biological systems, proteinases are inactivated either by proteolytic degradation or by interaction with inhibitors that act as pseudo-substrates displaying variable degrees of affinity to the enzyme catalytic sites (Laskowski and Kato, 1980; Bode and Huber, 2000). These inhibitors have been described as endogenous regulators of proteolytic activity (Ryan, 1991; Kato, 2002) and as storage proteins (Xavier-Filho, 1992). Interest in understanding the physiological roles of proteinase inhibitors has increased due to their importance in regulating diverse processes that involve proteinases, such as intracellular protein breakdown, transcription, cell cycle, cell invasion (Kataoka et al., 2002) and apoptosis (Thompson and Palmer, 1998; Fumagalli et al., 1996; Kato, 1999). Although serine proteinase inhibitors participate in reactions controlling proteinase activities involved in different physiological processes, their functions in the organism where they are found are not yet fully understood. However, plant protease inhibitors have been described as protective agents against pest attack (Sampaio et al., 1996; Shewry and Lucas, 1997; Walker et al., 1997, Franco et al., 2003; Shukle and Wu, 2003). Moreover, the serine proteinase–inhibitor complex has been studied as a model of protein–protein recognition (Richardson, 1977; Freitas et al., 1997; Bode and Huber, 2000; Ascenzi et al., 2003). A fundamental aim in biological sciences is to understand the mechanism by which a protein performs specific functions while preserving its three-dimensional

Abbreviations: BAPNA, Nα-benzoyl-DL-arginine-*p*-nitroanilide; CPY, carboxypeptidase Y; GPNA, glutaryl-L-phenylalanine-*p*-nitroanilide; MM, molecular mass marker; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPCI, *Schizolobium parahyba* chymotrypsin inhibitor; TCA, trichloroacetic acid.

* Corresponding author: Tel.: +55-61-3072192; fax: +55-61-349-8411.
E-mail address: nina@unb.br (S.M. de Freitas).

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structure. Studies of small model proteins such as serine proteinase inhibitors can lead to significant advances toward this aim (Ascenzi et al., 2003). The focus of the present study was therefore on SPCI, a chymotrypsin inhibitor, which may be further used as a structural model for the study of protein–protein interactions.

Schizolobium parahyba chymotrypsin inhibitor (SPCI) is a Kunitz type inhibitor isolated as a single polypeptide chain, with two disulfide bonds (Souza et al., 1995). It suppresses the proteolytic activity of chymotrypsin by forming a stable complex of 1:1 stoichiometry. SPCI has been studied structurally and is of particular interest as a model of a thermally stable protein. The secondary architecture of SPCI is mainly anti-parallel β-sheet and disordered structures (Teles et al., 1999), with principal forces maintaining native structure being hydrophobic and electrostatic interactions (Souza et al., 2000). Visualized by atomic force microscopy at high resolution in nanopure water, SPCI forms various oligomers, with a hexagonal form predominating (Leite et al., 2002).

The goal of this study is to investigate the physicochemical properties of SPCI to understand the features of its structural organization. SPCI has been previously partially purified to approximately 90% purity following the method described by Souza et al. (1995). Since the previous method did not produce SPCI of sufficient quality for structural and physicochemical studies, a new and improved method is presented here. Using highly purified protein, structural stability and physicochemical properties of SPCI were investigated as a function of pH. The enzyme–inhibitor association was studied by measuring high equilibrium constants at different pHs as well as the resistance towards proteolytic digestion; the results show that SPCI is a highly stable molecule.

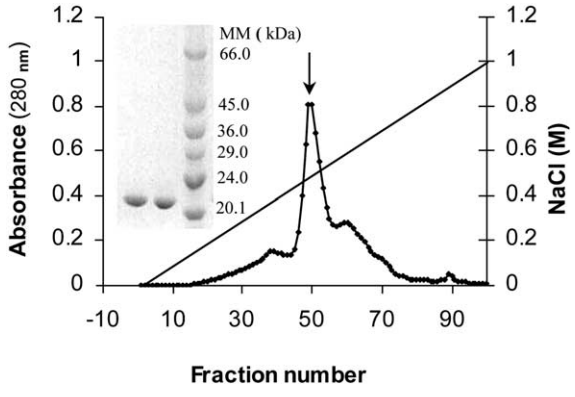

Fig. 1. Cation exchange chromatography of SPCI in SP-Sephadex. The proteins were eluted with a linear gradient from 0.2 to 1.0 M NaCl. The main peak, indicated by an arrow, displayed high inhibitory activity and corresponded to SPCI. Fig. 1 inset, 13% SDS–PAGE analysis of SPCI (10 μg) from main peak. Lanes 1 and 2: SPCI from SP-Sephadex pooled fractions. Lane 3: molecular mass markers. Protein bands were stained with Coomassie blue R-250.

2. Results and discussion

2.1. Purification of SPCI

SPCI was initially partially purified following the methodology described by Souza et al. (1995). According to the procedure, SPCI was obtained with ca. 10% of low molecular mass protein contaminant. Electrophoretic profiles of crude extract from different sources of *S. parahyba* seeds were quite different in terms of contaminants content (data not shown), which may be due to the different protein composition of crude extracts of *S. parahyba* seeds from different tropical areas. After testing several methods, it was found that protein precipitation from the crude extract with 1.0% aqueous TCA, followed by ion exchange chromatography afforded pure SPCI. The supernatants obtained from TCA precipitation showed high SPCI content, with small amounts of low molecular mass contaminants (data not shown). On SP-Sephadex, SPCI eluted as the main peak at 0.5 M NaCl (Fig. 1). The low molecular weight contaminant was not detectable after this ion exchange chromatography (Fig. 1, inset). This simple and efficient purification method is faster than that previously described (Souza et al., 1995). SPCI was judged as a homogeneous sample by 13% SDS–PAGE (Fig. 1, inset) with a corresponding molecular mass of approximately 20 kDa.

2.2. Effect of pH on stability of SPCI

SPCI inhibitory activity as a function of pH is shown in Fig. 2 A. SPCI displayed high stability at different pHs ranging from 2 to 12 at 25 °C, in agreement with those previously obtained for Kunitz and Bowman–Birk type inhibitors, such as the inhibitors isolated from *Concanavalis lineata* seeds (Terada et al., 1994) and the thermostable trypsin and chymotrypsin inhibitor from *Vigna unguiculata* (Freitas et al., 1999; Silva et al., 2001), respectively. Since inhibitory activity of SPCI on the proteolytic action of chymotrypsin remained constant at the several pHs tested, it was necessary to confirm if the residual activity could be due to a reversible process, when the initial condition at pH 7.6 was restored to test the specific chymotrypsin activity. Hence, the intrinsic tryptophan fluorescence emission intensity at 336 nm (the wavelength of native form) of SPCI was used to follow conformational changes as a function of pH at 25 °C. Fluorescence intensities were normalized by calculating the ratio of the equilibrium fluorescence intensity at each pH in relation to pH 7.0, with these being plotted as a function of pH (Fig. 2B). The plot showed clearly that there was no transition until at the extremes of pH, since the range of the emission intensity still remained up to 65% of that obtained at pH 7.0. No displacement of the emission band, with

exposition of the tryptophan residue, was observed in 2 h of incubation (Fig. 2C). The quenching in the emission intensity of approximately 35% (Fig. 2C) at extremes of acidic and alkaline conditions indicated that the range of tested pHs affects the SPCI conformation, but was not enough to induce complete denaturation, since a typical transition curve was not evident. These results were in agreement with data obtained from unfolding curves monitored by circular dichroism and differential scanning calorimetry (DSC) in which SPCI has shown high thermal stability under acidic, neutral and alkaline pH conditions (manuscript submitted).

2.3. Equilibrium dissociation constant (K_i) determination

Fig. 3 shows the inhibitory activity of SPCI against chymotrypsin as a function of pH. SPCI inhibition constants (K_i) for each pH value, as calculated from fitted inhibition curves, were 3.8×10^{-8} M (pH 2), 2.9×10^{-7} M (pH 4), 9.0×10^{-8} M (pH 7), and 5.9×10^{-8} M (pH 12). The K_is calculated from most pH assays were similar in order of magnitude, except those obtained at pH 4.0. In addition, these values are in agreement with those reported for Bowman–Birk and Kunitz type inhibitors (Birk, 1968; Terada et al., 1994). These results corroborate the fluorescence measurements indicating no change in inhibitory activity and conformational stability of SPCI as a function of pH. However, the K_i value at pH 4 suggests a weaker interaction of SPCI and chymotrypsin compared with other pH conditions. The isoelectric point of SPCI (pH 4.18) suggests that this high fluorescence intensity oscillation at pH 4.0 (Fig. 2B) may be due to protein precipitation.

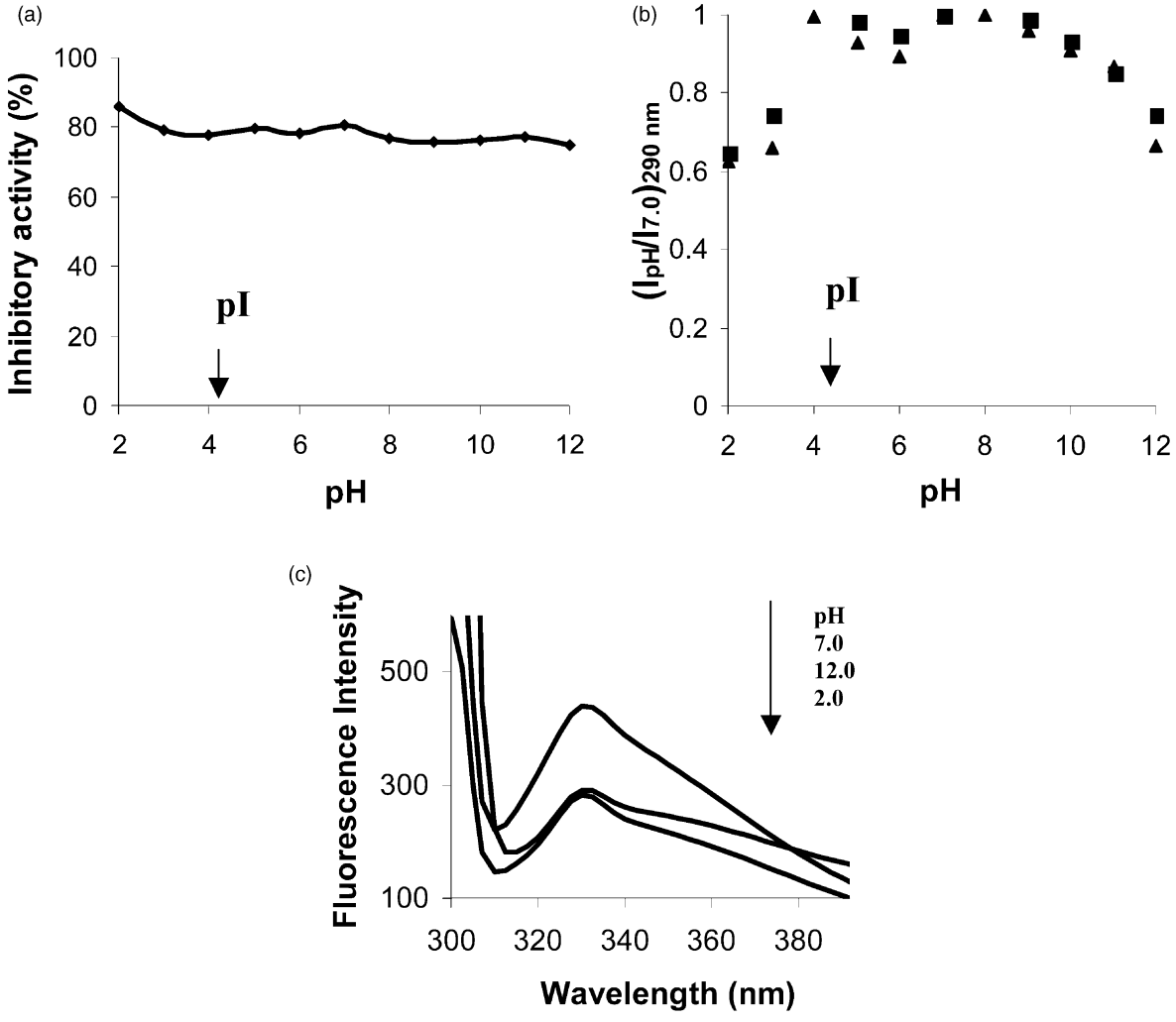

Fig. 2. Properties of purified SPCI. Panel A: Effect of pH on SPCI stability, analyzed by its inhibitory activity. Purified inhibitor was pre-incubated at different pH and residual chymotrypsin activity was determined as described in the Experimental. Panel B: Ratio of the buffer-corrected fluorescence emission intensity of SPCI at acidic and alkaline pHs to that of the native state at pH 7.0. Emission was monitored at 336 nm and excitation was at 290 nm. Fluorescence emission intensities were recorded before (■) and after (▲) incubation of SPCI for 2 h. Each point is an average of four individual sets of experiments. Panel C: Fluorescence emission spectra of SPCI 2 h after incubation at pH 2.0, 7.0 and 12.0. The λ_{ex} value was 290 nm. All spectra were recorded at 25 °C.

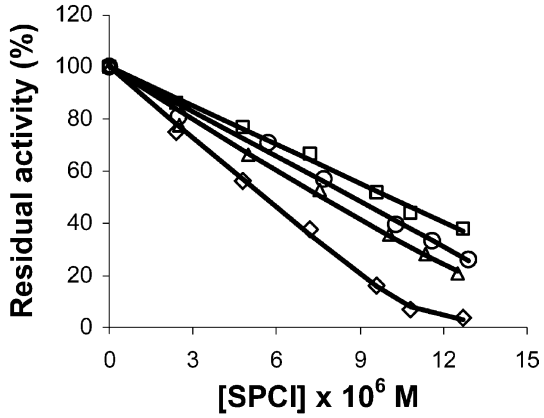

Fig. 3. Inhibitory activity of SPCI against chymotrypsin. Inhibition curves at pH 2 (□–□), pH 4 (△–△), pH 7 (○–○) and pH 12 (◇–◇), respectively. Residual activities of chymotrypsin were measured at 25 °C with GPNA (at pH 7.6).

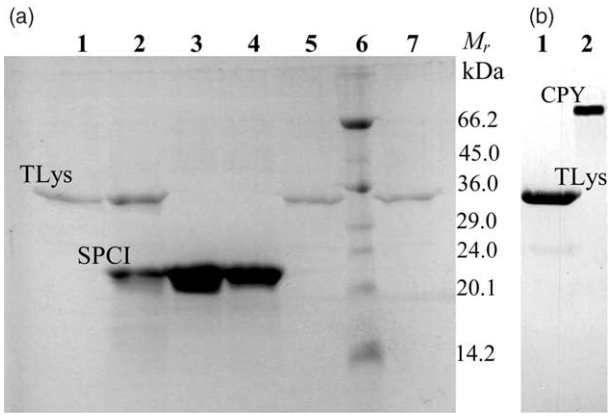

Fig. 4. Thermolysin (TLys) resistance of SPCI analyzed on 13% SDS–PAGE. Panel A: Lane 1: thermolysin (6 μg); lane 2: thermolysin (6 μg) and SPCI (8 μg); lane 3: SPCI (18 μg); lane 4: pre-incubated SPCI at 70 °C for 30 min. (18 μg); lane 5: incubation of CPY with thermolysin (6 μg, pre-incubated for 60 min at 70 °C), for 30 min; lane 6: molecular mass markers; lane 7: thermolysin (6 μg) incubated with carboxypeptidase Y (CPY) (6 μg) for 30 min at 70 °C. Panel B: Positive control for thermolysin activity. Lane 1: thermolysin (12 μg) incubated with carboxypeptidase Y (CPY) (12 μg) for 30 min at 70 °C. The single band of thermolysin (lane 1) indicates complete digestion of carboxypeptidase (lane 2).

2.4. Thermolysin resistance

Thermolysin is a thermostable enzyme (Inouye et al., 1998) widely used as a tool in structural events accompanying thermally induced transitions and structural stability relative to proteolysis resistance of proteins (Heinrikson, 1977; Johnson and Price, 1986; Signor et al., 1990; De-Laureto et al., 1997; Arnold and Ulbrich-Hofmann, 2001; Arnold et al., 2003). In particular, the wild and mutant thermolysins have been used to monitor the degradation of protease-resistance under extreme thermal conditions, as revealed by SDS–PAGE analysis (Almond and Dean, 1993; Van den Burg et al., 1998; Lindorff-Larsen and Winther, 2001). In the present study, the proteolytic digestions using thermolysin were performed and SPCI protease sensitivity was used to monitor its proteolysis resistance and thermal stability. Carboxipeptidase Y has been used as a comparative measurement of stability between native and pre-incubated SPCI at 70 °C. The pronounced thermal stability difference between carboxypeptidase Y and SPCI was indicated from their different resistance toward thermolysin digestion (Fig. 4). The electrophoresis profile of SPCI previously incubated at 70 °C (Fig. 4, panel A, lane 2) and carboxyipeptidase Y (Fig. 4, panel B, lane 1), in the presence of thermolysin, reveals that the SPCI is completely stable toward an approximately equal amount of thermolysin, whereas CPY was completely degraded. Furthermore, it was found that essentially all SPCI samples were recovered as a single band with a well defined mobility and no molecular mass smear, independent of the thermolysin presence (Fig. 4, panel A, lanes 2–4). This insensitive feature of SPCI to thermolysin proteolysis, even at 70 °C, suggests that the native and pre-incubated SPCI at 70 °C present no detectable structural differences. According to this criterion, SPCI was concluded by as a highly stable protein.

2.5. Specificity for serine proteinases

Protease assays revealed that SPCI has no significant inhibitory effect against trypsin and α-amylase, and no inhibitory effect against serine proteases from blood coagulation pathways (data not shown). However, SPCI was able to weakly inhibit elastase (18%) and presented high inhibitory activity against chymotrypsin (100%) (data not shown). These inhibitory assays indicated that SPCI is highly specific for chymotrypsin, since it did not inhibit proteases belonging to any other groups.

3. Experimental

3.1. Reagents

A-Chymotrypsin, carboxypeptidase Y (CPY), elastase, glutaryl-L-phenylalanine-*p*-nitroanilide, N_α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), elastin-congo-red, trichloroacetic acid (TCA) and SDS–PAGE molecular weight standard (MW ranges 10,000–100,000) were purchased from Sigma. Thermolysin was purchased from PIERCE, SP-Sephadex C25-120 from Pharmacia, and glycine from Life Technologies. All other chemicals were analytical grade.

3.2. Purification of SPCI

The crude protein extract was obtained from *S. parahyba* seeds (200 g) by continuous overnight stirring with

0.15 M NaCl (1 L) at room temp. The results precipitate, separated by centrifugation (13,000*g*, 40 min), was submitted to a second extraction with the same volume of 0.15 M NaCl solution. The supernatant was saturated with 60 and 70% (NH4)2SO4 (w/v) and the precipitate (crude extract) was dissolved in water, dialyzed and lyophilized. SPCI was purified following two steps consisting of TCA precipitation and ion exchange chromatography. Proteins from the crude extract were precipitated by adding 0.5–1.5% (v/v) TCA for 5 min in an Omnimixer homogenizer at medium speed. After centrifugation (13,000*g*, 30 min), the supernatant was dialyzed against water at 4 °C, lyophilized, and dissolved in 50 mM acetate buffer, pH 3.2. The soluble protein fraction was applied to a SP-Sephadex C 25–120 column (2 × 15 cm) equilibrated with 50 mM acetate buffer, pH 3.2. The column was washed with three volumes of buffer. The inhibitor was eluted with a linear salt gradient from 0.2 to 1.0 M NaCl in the same buffer. Fractions having inhibitory activities were pooled, dialyzed and lyophilized.

3.3. Electrophoresis

Molecular mass of SPCI was estimated by 13% SDS–PAGE (Laemmli, 1970) under reducing conditions, using molecular weight standards purchased from Sigma: bovine serum albumin (66,200), ovalbumin (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (STI) (20,100) and α-lactoalbumin (14,200). For protein detection, gels were stained with 0.01% Coomassie blue in MeOH–H_2O (4:6) and HOAc–H_2O (1:9).

3.4. Protein concentration

Protein concentrations were determined spectrophotometrically by means of the following parameters: MM = 20 kDa (SPCI), MM = 25 kDa (chymotrypsin—CT), and specific absorption coefficients $A^{1\%}_{280\ cm} = 6.18$ (SPCI) and $A^{1\%}_{280\ cm} = 20.4$ (CT).

3.5. SPCI inhibitory activity assay

The inhibitory activity of SPCI was assayed by evaluating the chymotrypsin and trypsin activity using the substrates *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA) and *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), respectively (Erlanger et al., 1961). Enzymes were incubated at room temp for 15 min with various amounts of SPCI in 50 mM Tris, pH 7.6, and prior to GPNA (0.8 mg/ml) and BAPNA (1 mM) addition. The reaction was stopped by addition of HOAc–H_2O (3:7, v/v) HOAc. The enzymatic hydrolysis of the substrate was evaluated by recording the absorbance at 410 nm in a Hitachi U-1100 spectrophotometer. Amylase inhibitory activities were measured according to Bernfeld (1995), in 0.1 M phosphate buffer (pH 5.5), containing 20 mM NaCl and 0.1 mM $CaCl_2$. Elastase inhibitory activities were measured using Elastin-congo-red substrate. The enzymatic hydrolysis of the substrate was evaluated by recording the absorbance at 570 nm. The relative activities of all enzymes, in the presence of inhibitor, were estimated considering the enzyme activity to be 100% without SPCI. All experiments were carried out in triplicate.

3.6. Effect of pH on stability of SPCI

The effect of pH on inhibitory activities of SPCI was investigated at different pHs ranging from 2 to 12 using the following buffers at final concentrations of 20 mM: KCl for pH 2; glycine–HCl for pH 3; sodium acetate for pH 4; disodium/sodium hydrogen phosphate for pH 5 and 6, Tris/HCl for pH 7–9; glycine–NaOH for pH 10, 11 and 12. After 10 min pre-incubation at each pH at 4 °C, residual chymotrypsin inhibitory activities were measured at pH 7.6 as described above. The experiments were carried out in triplicate.

3.7. Fluorescence measurements

Intrinsic protein fluorescence measurements were performed at 25 °C with a Jasco FP-77 Spectrofluorimeter. Spectra were measured from 300 to 400 nm after excitation at 290 nm with 5 nm bandwidth for both excitation and emission. The fluorescence measurements were performed in a 1 cm thermostatted cuvette in a Jasco electrical temperature controller. The sample of SPCI (concentration corresponding to $A_{280} < 0.05$) in different pH, were incubated for 2 h at 25 °C. Evaluations were made before and after the incubation.

3.8. Equilibrium dissociation constant (K_i) determination

Inhibition curves were obtained by plotting decreasing residual activities of chymotrypsin as a function of known amounts of the inhibitor. Dissociation constants of the enzyme–inhibitor complexes, K_i, for each pH inhibition assay were determined from fitted inhibition curves following the procedure described by Morrison (1982) and using a computational Grafit program (Knight, 1986).

3.9. Thermolysin resistance

For analyzing thermolysin resistance, 7 μg SPCI or 3 μg of the control substrate for carboxypeptidase Y (CPY) were incubated for 30 min in 0.5 ml of 20 mM $CaCl_2$, 1 mM $ZnCl_2$, 50 mM Tris pH 7.6 at 70 °C, in the

presence or absence of 5 μg thermolysin (Lindorff-Larsen and Winther, 2001). Assays were performed with pre-incubation of SPCI and control substrate for 5 min at 70 °C before thermolysin was added. In order to test thermolysin stability, this enzyme was pre-incubated for 30 min at 70 °C before addition of CPY. The enzymatic assays were stopped by addition of 100% TCA. The precipitated proteins were analyzed by 13% SDS–PAGE.

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