Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Purification, crystallization and preliminary crystallographic studies of SPCI–chymotrypsin complex at 2.8 Å resolution

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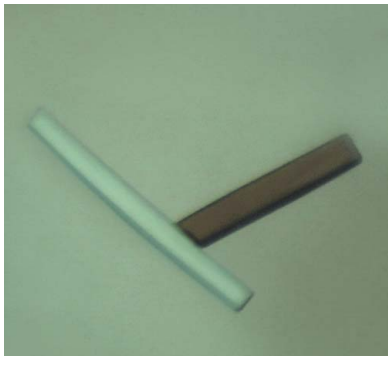
Received 6 August 2008
Accepted 20 August 2008



A binary complex of the *Schizolobium parahyba* chymotrypsin inhibitor (SPCI) with chymotrypsin was purified by size-exclusion chromatography and crystallized by the sitting-drop vapour-diffusion method with 100 m*M* MES–NaOH pH 5.5, 20%(*w*/*v*) PEG 6000, 200 m*M* LiCl as precipitant and 200 m*M* nondetergent sulfobetaine molecular weight 201 Da (NDSB-201) as an additive. SPCI is a small protein with 180 amino-acid residues isolated from *S. parahyba* seeds and is able to inhibit chymotrypsin at a 1:1 molar ratio by forming a stable complex. X-ray data were collected to 2.8 Å resolution from a single crystal of the SPCI–chymotrypsin binary complex under cryogenic conditions. The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters $a = 45.28$, $b = 64.57$, $c = 169.23$ Å, and the $R_{\rm merge}$ is 0.122 for 11 254 unique reflections. A molecular-replacement solution was found using the preliminary crystal structure of SPCI and the structure of chymotrypsin (PDB code 4cha) independently as search models.

1. Introduction

Proteinases are crucial for a wide variety of biological processes (Neurath, 1997) that are essential to life and their activity is of great importance both *in vivo* and for therapeutic intervention (Phillips & Fletterick, 1992). Naturally occurring canonical serine proteinase inhibitors (PIs) are proteins that inactivate the functions of serine proteinases by providing a reactive site, which in turn serves as a substrate analogue for the cognate enzyme (Laskowski & Kato, 1980; Bode & Huber, 1992). Proteinase inhibitors are found in microorganisms, plants and animals and play an important role in the regulation of metabolic pathways in which proteinases are one of the major components (Joanitti *et al.*, 2006). Natural inhibitors have been classified into at least 18 different families (Laskowski & Qasim, 2000); among the most extensively studied are the Bowman–Birk and Kunitz serine proteinase inhibitors.

Kunitz inhibitors are proteins with one or two polypeptide chains, a molecular weight of about 6–20 kDa and one reactive site. These inhibitors are cross-linked by two or three disulfide bonds that confer a compact and stable tertiary structure. They have been the subject of several studies, especially in the Graminaceae, Leguminosae and Solanaceae (Richardson, 1977), and are mainly found in the seeds of the Caesalpinoideae and Mimosoideae (Norioka *et al.*, 1988).

A soybean Kunitz trypsin inhibitor (STI) has been reported to have the potential to suppress ovarian cancer cell invasion and peritoneal disseminated metastasis *in vivo* (Kobayashi *et al.*, 2004). STI inhibits cell invasiveness through suppression of the urokinase-type plasminogen activator signalling cascade and its specific receptor observed in HRA human ovarian cancer cells. In addition, STI has an adverse effect on insect development and might serve as a transgenic resistance factor (Shukle & Wu, 2003). Bioassays with this inhibitor revealed that it caused a significant delay in the development of *Sitotroga cerealella* (one of the major storage pests of cereals) larvae when incorporated into the diet. These results indicated that a plant protease inhibitor could provide antibiotic resistance toward *S. cerealella* larvae.

Schizolobium parahyba chymotrypsin inhibitor (SPCI) is a Kunitz chymotrypsin inhibitor with a single polypeptide chain of 180 amino-acid residues isolated from *S. parahyba* seeds (Teles *et al.*, 2004). This inhibitor suppresses the proteolytic activity of chymotrypsin through the formation of a stable complex with a 1:1 stoichiometry and has been characterized as a highly stable protein over a broad pH and temperature range (Teles *et al.*, 2005).

The secondary structure of SPCI is formed by β-strands and its native structure is stabilized by hydrophobic forces and electrostatic interactions (Souza *et al.*, 1995, 2000; Teles *et al.*, 1999, 2004). Disulfide-bond reduction caused conformational changes in SPCI, as evidenced by the maximum fluorescence emission shifting from 336 to 328 nm with increased intensity without alteration of the inhibitory activity. Indeed, 1 m*M* dithiothreitol (DTT) did not affect the time course of thermoinactivation of SPCI (Souza *et al.*, 1995, 2000) at 363 K after 1 h incubation and did not interfere in the secondary-structure content as evidenced by far-UV circular-dichroism spectroscopy (unpublished data). These findings suggest that disulfide bonding plays no crucial role in maintaining either the inhibitory function or the conformational stability of SPCI. The molecular arrangements of SPCI at pH 7.0, as visualized by atomic force microscopy at high resolution in nanopure water, indicated an organization in various oligomeric states, with a predominance of hexagonal forms (Leite *et al.*, 2002). In order to elucidate the three-dimensional structure of SPCI, this inhibitor was crystallized (Teles *et al.*, 2007) and its structure determination is in progress.

The three-dimensional structure of an inhibitor–proteinase complex reveals the residues and intermolecular contacts that contribute to the specificity profile and tight association. For this reason, the structural studies of inhibitor complexes with chymotrypsin are helpful not only to comprehend models of protein–protein recognition but also to understand conformational changes during enzyme–inhibitor interactions, aiming towards biotechnological applications. In this work, we present the purification of the SPCI–chymotrypsin complex, its crystallization, data collection and phasing at 2.8 Å resolution.

2. Materials and methods

2.1. Purification of SPCI and SPCI–chymotrypsin complex

SPCI was initially purified from a crude extract of triturated *S. parahyba* seeds following trichloroacetic acid (TCA) precipitation and ion-exchange chromatography (Teles *et al.*, 2004). Proteins from the crude extract were precipitated with 1.2%(*v*/*v*) TCA for 5 min in an Omnimixer homogenizer at medium speed. After centrifugation at 13 000*g* for 30 min, the supernatant was dialyzed against water at 277 K and lyophilized. The sample (100 mg) obtained from TCA precipitation was dissolved in 5–10 ml 50 m*M* acetate buffer pH 3.2. The soluble protein fraction was applied onto a SP-Sephadex C 25-120 column (2 × 15 cm) equilibrated with 50 m*M* acetate buffer pH 3.2. The inhibitor was eluted with a linear salt gradient from 0 to 1.0 *M* NaCl in the same buffer. Eluted samples were monitored at 280 nm and purity was confirmed by 13% SDS–PAGE (Laemmli, 1970).

The binary complex (SPCI–chymotrypsin) was obtained by mixing SPCI with α-chymotrypsin (bovine pancreatic α-chymotrypsin type II, thrice crystallized; Sigma-C4129, St Louis, Missouri, USA) in a 1:1 molar ratio at a concentration of 500 µ*M* in 50 m*M* Tris–HCl, 0.2 *M* KCl pH 7.5 for 30 min at room temperature. The concentrations of the proteins were determined from absorbance measurements using a Jasco V-530 spectrophotometer (Jasco, Tokyo, Japan) and using the following values: α-chymotrypsin (MW = 25 kDa), $A^{1\%}_{280,\,1\ \mathrm{cm}}$ = 20.4; SPCI (MW = 20 460 Da), $A^{1\%}_{280,\,1\ \mathrm{cm}}$ = 6.18 (Souza *et al.*, 1995). The resulting binary complex was purified from the mixture by size-exclusion chromatography using a Sephadex G-75 (3 × 100 cm) with a flow rate of 25 ml h^{-1} previously equilibrated with the same buffer as indicated above. The purified complex was dialyzed against water and lyophilized for storage at 253 K.

2.2. Crystallization and data collection and processing

The SPCI–chymotrypsin binary complex was crystallized using the sitting-drop vapour-diffusion method (McPherson, 1990) at 291 K and an automatic system for crystallization using Matrix Maker and Honeybee robots to set up 96-well crystallization plates. Initial crystallization trials were performed using different precipitant types from commercial screens: the sparse-matrix screen from the Joint Center for Structural Genomics (JCSG; Page *et al.*, 2003), the PACT Suite for a systematic analysis of the effect of pH, anions and cations (Newman *et al.*, 2005), precipitant synergy (Majeed *et al.*, 2003), Wizard Screens I and II (Hol *et al.*, 2001), SaltRx (Gilliland *et al.*,

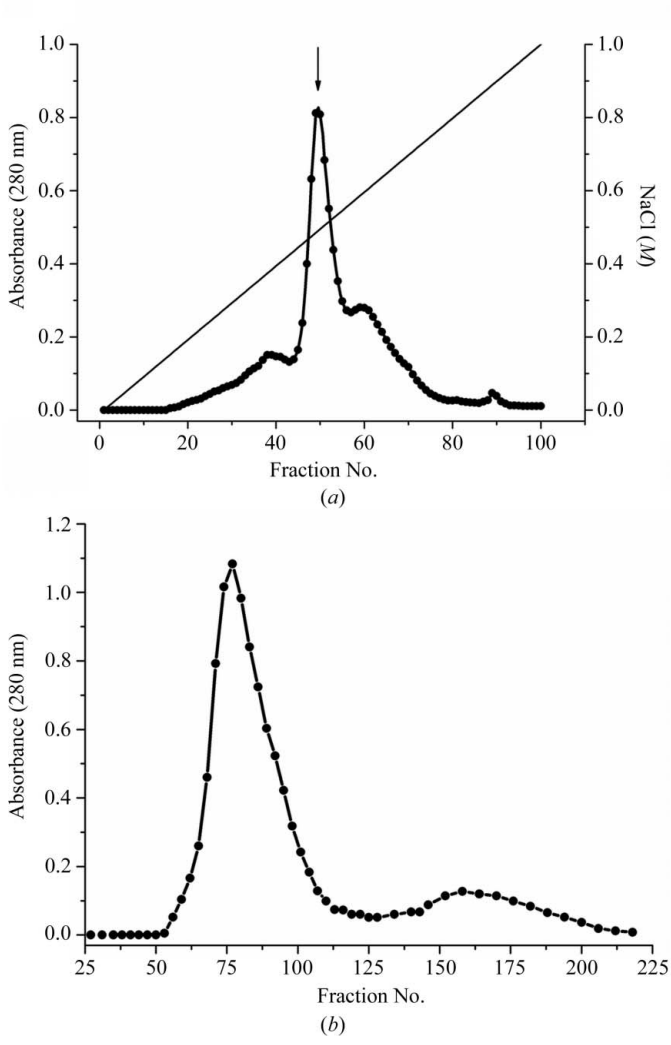


Figure 1
(*a*) Cation-exchange chromatography of SPCI on SP-Sephadex eluted with a linear gradient (0–1.0 *M*) of NaCl. The arrow indicates the eluted SPCI. (*b*) Size-exclusion chromatography of the SPCI–chymotrypsin binary complex on Sephadex G-75 with 50 m*M* Tris–HCl pH 7.6. First peak, SPCI–chymotrypsin complex; second peak, free SPCI and chymotrypsin.

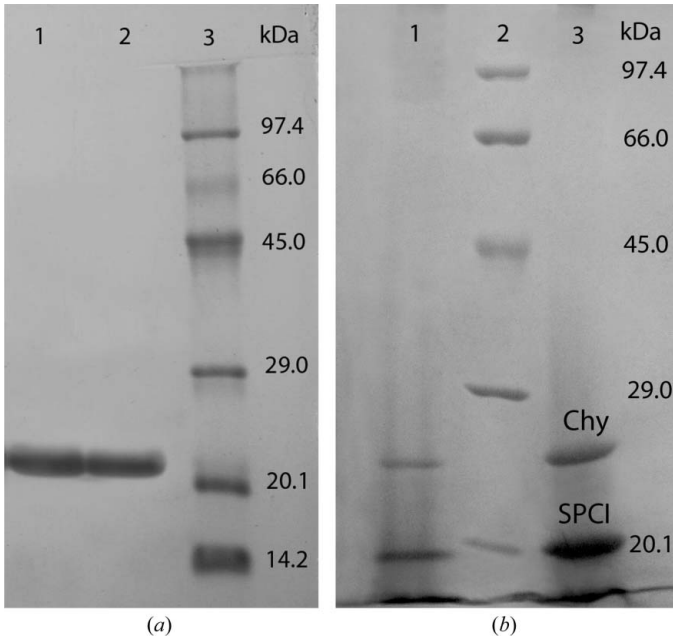
1994; Kanaujia *et al.*, 2007), and Crystal Screen and Crystal Screen 2 from Hampton Research (Jancarik & Kim, 1991). The reservoirs contained 80 µl solution and the drops were prepared by adding 0.2 µl reservoir solution to 0.2 µl protein solution (lyophilized protein dissolved in Milli-Q water) at concentrations of 10 and 20 mg ml^{-1}.

After screening, optimization of the crystallization conditions was performed by varying the pH of the buffers and the precipitant concentration and using the Additive Screen 2 HR-430 kit (Hampton Research). The drops were prepared by adding 0.9 µl reservoir solution (300 ml) to 0.9 µl protein solution (14 mg ml^{-1}) and 0.2 µl additive. The best crystals were grown in drops using a solution consisting of 100 m*M* MES–NaOH pH 5.5, 20%(*w*/*v*) PEG 6000, 200 m*M* LiCl as precipitant and 200 m*M* nondetergent sulfobetaine molecular weight 201 Da (NDSB-201) as an additive. Crystals were soaked in reservoir solution containing 20%(*v*/*v*) glycerol as a cryoprotectant for less than 30 s and mounted in a loop.

Diffraction data were collected on the W01B-MX2 beamline of the Brazilian Synchrotron Light Laboratory (LNLS; Campinas, Brazil), which is equipped with a two-crystal monochromator and a MAR CCD detector with a square X-ray-sensitive surface of 225 × 225 mm combined with a MAR DTB goniostat. A wavelength of 1.459 Å was used together with oscillations of 1°. Crystals were cryocooled in a stream of nitrogen gas at 100 K in order to minimize radiation damage. A total of 360 frames were recorded. Data processing was performed with the *HKL*-2000 software package (Otwinowski & Minor, 1997).

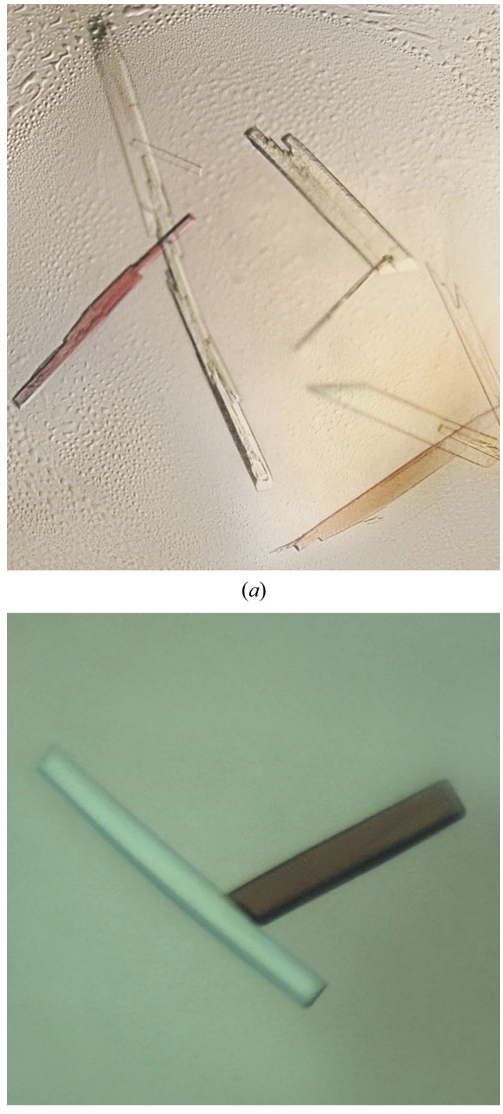
2.3. Molecular replacement

The Matthews coefficient (Matthews, 1968) was calculated using 45 kDa as the molecular weight of the binary complex in order to indicate the number of molecules per asymmetric unit. A calculated Matthews coefficient of 2.74 Å^3 Da^{-1} is compatible with the presence of one heterodimer of the SPCI–chymotrypsin complex in the asymmetric unit with a solvent content of 55%. A molecular-replacement (MR) solution was found using the partially refined crystallographic structure of SPCI (Teles *et al.*, 2007) and the structure of chymotrypsin (Tsukada & Blow, 1985; PDB code 4cha) independently as a search models. This procedure was performed using the *MOLREP* program (Vagin & Teplyakov, 1997) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). The first MR was carried out using the structure of chymotrypsin (Tsukada & Blow, 1985; PDB code 4cha) as the search model. The solution was used in the second MR as a fixed model and the structure of SPCI was used as the search model. In both searches, the chymotrypsin and SPCI molecules contained all the protein atoms; all other atoms, such as waters and other ligands, were removed.



(*a*) (*b*)

Figure 2
Coomassie-stained (13%) SDS–PAGE of SPCI and the SPCI–chymotrypsin complex. (*a*) Lanes 1 and 2, SPCI (20.46 kDa). Lane 3, molecular-weight markers from Amersham Pharmacia Biotech UK (part No. 17-0446-01): rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and bovine milk α-lactalbumin (14.2 kDa). (*b*) Lanes 1 and 3, SPCI (20.46 kDa) and chymotrypsin (25 kDa) from the binary complex under denaturing conditions; lane 2, molecular-weight markers.



(*a*)

(*b*)

Figure 3
Crystals of the SPCI–chymotrypsin complex obtained by sitting-drop vapour diffusion at 291 K with an average length of 400 µm in the longest dimension. (*a*) Crystals obtained using 100 m*M* MES–NaOH pH 5.5, 20%(*w*/*v*) PEG 6000, 200 m*M* LiCl as precipitant. (*b*) Optimized crystals diffracting to 2.8 Å obtained in the same precipitant solution with the additive 200 m*M* NDSB-201 from the Additive Screen 2 HR-430 kit (Hampton Research).

Table 1
SPCI–chymotrypsin complex data-collection and processing statistics.

Values in parentheses are for the last resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 45.28, b = 64.57, c = 169.23
Mosaicity (°)	0.94
Temperature (K)	291
Wavelength (Å)	1.459
Frame oscillation (°)	1
Crystal-to-detector distance (mm)	170.00
No. of frames	360
Resolution limits (Å)	16.00–2.80 (2.90–2.80)
$\langle I/\sigma(I)\rangle$ after merging	23.4 (1.3)
Completeness (%)	88.3 (51.8)
Redundancy (%)	12.0 (6.0)
$R_{\rm merge}$†	0.12 (0.59)
No. of reflections	135446
No. of unique reflections	11254 (638)

† $\sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

3. Results and discussion

SPCI was purified from a crude extract by a precipitation step with 1.2%(*v*/*v*) TCA followed by ion-exchange chromatography (Fig. 1*a*) as described by Teles *et al.* (2004) and corresponds to a band of approximately 21 kDa on SDS–PAGE (Fig. 2*a*). The SPCI–chymotrypsin complex was purified by size-exclusion molecular chromatography (Fig. 1*b*). Both proteins present in the complex are shown in the SDS gel, which contains two bands of the expected molecular weights for chymotrypsin (25 kDa) and SPCI (21 kDa) (Fig. 2*b*).

The crystallization conditions for this binary complex were obtained using the vapour-diffusion method with a robotic system. After two weeks, small crystals of SPCI–chymotrypsin complex were observed in several screening conditions, but the best crystals grew in (i) 100 m*M* sodium acetate–HCl pH 5.0, 20%(*w*/*v*) PEG 6000, 10 m*M* $ZnCl_2$, (ii) 100 m*M* MES–NaOH pH 6.0, 20%(*w*/*v*) PEG 6000, 200 m*M* LiCl, (iii) 100 m*M* bis-tris–HCl pH 5.5, 17%(*w*/*v*) PEG 10 000, 100 m*M* ammonium acetate and (iv) 200 m*M* potassium formate pH 7.3, 20%(*w*/*v*) PEG 3350. Optimization of these initial conditions was performed using the same precipitants, varying the pH and PEG concentration, combined with the Additive Screen 2 HR-430 kit (Hampton Research) and the protein solution at concentration of 14 mg ml^{-1}. Improved crystals grew using 100 m*M* MES–NaOH pH 5.5, 20%(*w*/*v*) PEG 6000, 200 m*M* LiCl as precipitant (Fig. 3*a*) and 200 m*M* NDSB-201 as an additive (Fig. 3*b*). The best and largest monocrystals (Fig. 3*b*) suitable for X-ray analysis were used for data collection.

After data collection and processing, the crystals were found to belong to space group $P2_12_12_1$, with unit-cell parameters a = 45.28, b = 64.57, c = 169.23 Å. Data were collected in the resolution range 16.00–2.80 Å with an overall $R_{\rm merge}$ of 0.12, a completeness of 88.3% (11 254 unique reflections recorded) and an $\langle I/\sigma(I)\rangle$ of 23.4. Details of the data-collection and processing statistics are presented in Table 1. First using the structure of the enzyme as a search model in the molecular-replacement protocol and then fixing it and using the inhibitor molecule in a new search resulted in rotation-function and translation-function peaks that clearly corresponded to one binary complex in the asymmetric unit, as corroborated by the Matthews coefficient of 2.74 Å^3 Da^{-1}. The rotation function over σ, translation function over σ, score and R factor for the first (correct solution) and second highest peaks in the chymotrypsin search of the MR procedure are 12.0 and 4.0, 16.3 and 4.7, 0.58 and 0.32, and 0.49 and 0.62, respectively. The same data for the SPCI search yielded values of 8.0 and 4.7, 44.2 and 18.4, 0.64 and 0.48, and 0.45 and 0.54, respectively. These data clearly represent the correct solution of the binary complex.

At present, refinement is being performed and new crystallization attempts are being carried out in order to improve crystal quality and resolution.

This work was supported in part by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Associação Brasileira de Tecnologia de Luz Síncrotron (ABTLuS).

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