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PURIFICATION AND PARTIAL CHARACTERIZATION OF A *SCHIZOLOBIUM PARAHYBA* CHYMOTRYPSIN INHIBITOR

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Key Word Index—*Schizolobium parahyba*; Leguminosae; proteinase inhibitor; chymotrypsin, Kunitz inhibitor; proteinase

Abstract—*Schizolobium parahyba* seed chymotrypsin inhibitor (SPC) is a protein with M_r of 20000 and four half-cystine residues and no free thiol group. SPC is stable at temperatures up to 75° at pH 7 but gradually loses activity when kept at 95° for 1 hr and total inactivation occurs after 5 hr. Amino acid analysis shows a high content of glycine, aspartate, glutamate and alanine residues. A pI of 4.52 predicted from the amino acid content agrees with experimental results. A stable binary complex with M_r of 45000, **Ki** = 5.85×10^{-8} M and molar ratio of 1:1 is formed between SPC and chymotrypsin. The determined single *N*-terminal sequence of SPC shows homology with Kunitz type soybean trypsin inhibitors.

INTRODUCTION

Serine protease inhibitors have been isolated from various Leguminosae seeds [1]. The trees of the genus *Schizolobium* belong to the subfamily Caesalpinoideae and are distributed throughout tropical to subtropical regions. The partial characterization of a strong trypsin inhibitor from seeds of *Schizolobium parahyba* (Vell.) Toledo was previously reported [2]. *Schizolobium parahyba* trypsin inhibitor is a Bowman–Birk type inhibitor with M_r of 12000 and high cystine content. During the isolation of this trypsin inhibitor, traces of a higher M_r trypsin inhibitor were detected but not further characterized; a larger amount of an alpha-chymotrypsin inhibitor was also detected in the seeds. This report deals with the partial characterization of the novel inhibitor, with M_r of 20000 and four half-cystine residues per molecule. *Schizolobium parahyba* chymotrypsin inhibitor (SPC) may be classified as a high M_r, low cystine legume inhibitor, distinct from *Schizolobium parahyba* trypsin inhibitor.

RESULTS AND DISCUSSION

Schizolobium parahyba seeds contain not only trypsin inhibitors but also a specific chymotrypsin inhibitor. The prior partial removal of the trypsin inhibitors by ammonium sulphate fractionation (0.6 saturation) was a prerequisite step in the purification of the chymotrypsin inhibitor. A gel filtration profile on a Sephadex G-100 column of the proteins separated between 0.6 and 0.7 ammonium sulphate saturation is shown in Fig. 1a, with at least two different groups of serine protease inhibitors present. Pooled fractions from several Sephadex G-100 chromatographies, containing the major inhibitory activity against chymotrypsin, were rechromatographed on Sephadex G-100 and yielded a large and symmetrical protein peak with a constant chymotrypsin inhibitory activity. Chromatographed on a Superose 12 column of an FPLC system, this material is eluted as a single peak with antichymotryptic activity. No trypsin inhibitory activity was detected in this column (Fig. 1b). The purity of the isolated inhibitor preparations was assessed by SDS–PAGE, and electrofocusing indicated a monophoretic band (data not shown). The reverse phase HPLC system demonstrated the presence of a major peak and a minor contaminant (Fig. 1c). The chymotrypsin inhibitory activity was present only in the fractions eluted with 42% (v/v) acetonitrile. *Schizolobium parahyba* alpha-chymotrypsin inhibitor was named SPC.

The M_r of SPC is 20000 as determined by SDS–PAGE gradient and by Sephadex G-75 calibrated column chromatography. SPC appears as a protein of M_r of 20700 and a Stokes radius of 1.98 nm. A Stokes radius of 2.71 nm and M_r 45000 characterize the SPC–chymotrypsin complex.

The inhibitory activity of SPC against bovine alpha-chymotrypsin activity was measured by increasing levels of the inhibitor with *N*-glutaryl-DL-phenylalanine-*p*′-nitroanilide (GPNA) as substrate. The resulting titration

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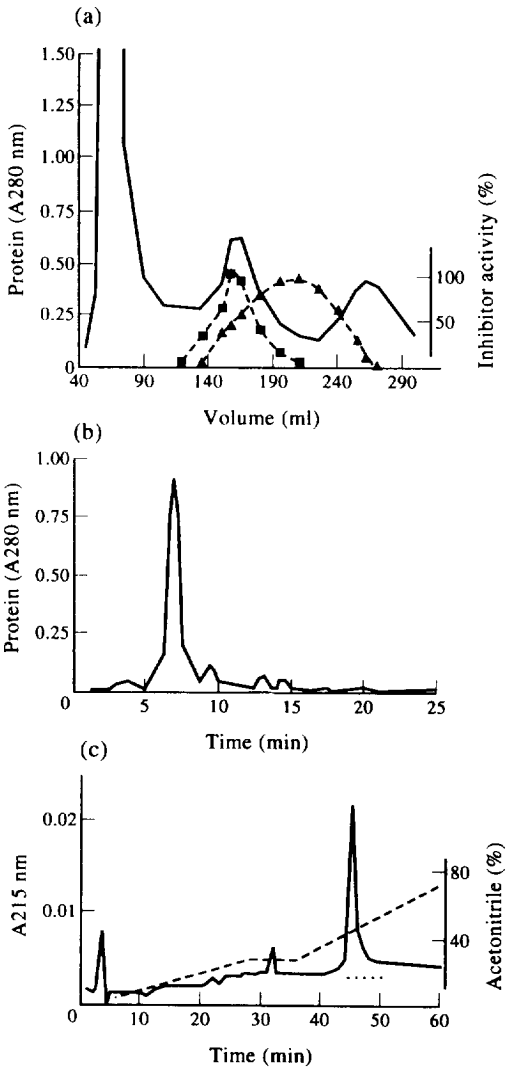

Fig. 1. (a) Chromatography of ammonium sulphate 0.6–0.7 fraction of *S. parahyba* chymotrypsin inhibitor (SPC) on Sephadex G-100. Sample (700 mg lyophilized material) dissolved in 4 ml equilibrium buffer. Flow rate: 25 ml hr^{-1}. Fractions: 3 ml $tube^{-1}$. (——) Absorbance, (■) inhibitory activity against chymotrypsin, (▲) inhibitory activity against trypsin. (b) Chromatography on Superose 12. Column equilibrated with 0.05 M sodium phosphate, pH 8. Sample 2.0 mg in 200 µl of the pool of active fractions on chymotrypsin from Sephadex G-75 column; 1 ml min^{-1} flow-rate. (c) Reverse phase on C4 Vydac column equilibrated with 0.1% TFA. Sample 200 µg protein of the pool from Superose chromatography. SPC was eluted with an acetonitrile gradient, with 1.0 ml min^{-1} flow-rate. (– – – –) Indicates chymotrypsin inhibitory activity.

curve was linear up to 85% inhibition and extrapolation of this data indicates that 1 mol of SPC reacts with 1 mol of chymotrypsin. The apparent dissociation constant calculated by the Green and Work method [3] was 5.8 $\times 10^{-8}$ M. Similar results are obtained using casein as substrate (**Ki** = 1.9×10^{-8} M). Higher SPC concentrations react with bovine trypsin and the dissociation constant (in the order of 10^{-5} M) is consistent with a typical substrate interaction.

Gel filtration experiments also reveal the 1:1 relationship between SPC and chymotrypsin in the binary complex. Figure 2 illustrates the elution profile of the preincubated mixture of SPC and chymotrypsin, in a Sephadex G-75 column, at pH 8. The first eluted peak exhibits neither inhibitory nor enzymatic activity and corresponds to a M_r of 45 000, a value that corresponds to the sum of the masses of the two proteins. The second peak, which corresponds to a M_r of 24 000, is detected in the elution volume of the two proteins in their free forms, and shows only chymotrypsin activity. Since alpha-chymotrypsin is in excess in the applied mixture, it can be concluded that this trailing peak contains mainly the free enzyme.

The SPC–chymotrypsin complex was isolated, reincubated with trypsin and applied to the same Sephadex G-75 column. The elution profile is very similar to that shown in Fig. 2 with a leading peak corresponding to a M_r of 45 000 and devoid of any inhibitory activity against the two involved enzymes but with the trailing peak showing only trypsin activity. These results suggest that under the experimental conditions a ternary complex is not formed and that the SPC–chymotrypsin complex is stable and no hydrolysis products are detectable.

The simultaneous incubation of SPC with chymotrypsin and trypsin also gave only the SPC–chymotrypsin complex. Since the affinity of SPC for chymotrypsin is higher than that for trypsin and since there is no evidence of proteolytic activity on the complex, it is possible that the SPC–chymotrypsin complex is relatively protected against the hydrolytic action of trypsin.

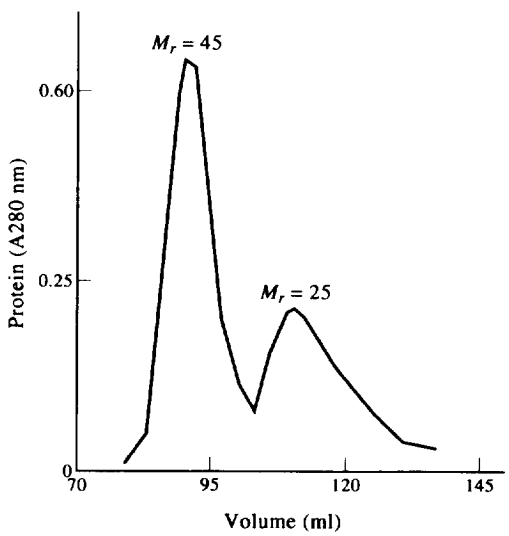

Fig. 2. Gel filtration of the chymotrypsin–SPC complex on Sephadex G-75. SPC inhibitor (15 mg) and chymotrypsin (17 mg) were incubated in 2 ml 0.05 M Tris-HCl, 0.2 M KCl, pH 7.6 buffer for 30 min at room temperature and applied to a calibrated Sephadex G-75 column (3.0 × 38 cm). The column was equilibrated and eluted with the same buffer; fraction volume was 2.5 ml $tube^{-1}$, flow rate was 25 ml hr^{-1}.

The inhibitory activity against chymotrypsin and the conformation of SPC as revealed by circular dichroism studies (in preparation) are not significantly affected by the addition of the reducing agent DTT in a concentration 200 times greater than that of SPC.

Results of thermostability do not show loss of inhibitory activity when SPC is heated up to 75° for 1 hr at pH 7. Above 75° the inhibitory activity decreases and at 95° it is completely abolished in 5 hr. Therefore, SPC is a relatively heat stable protein although its molecule contains only two disulphide bonds. The thermostability of low M_r serinoprotease inhibitors is generally associated with high disulphide content. The unusual thermostability of SPC has also been observed for other proteins with low content of disulphide bonds, such as the chymotrypsin inhibitor isolated from winged bean that also has two S–S bridges [4].

Amino acid composition analysis is presented in Table 1. The value of pI = 4.52 was obtained by theoretical determination [7] on the basis of its amino acid composition, and it agrees with the pI = 4.46 obtained from electrofocusing on polyacrylamide gel. SPC is rich in dicarboxylic amino acids and hydroxyl amino acids which together account for *ca* 36% of the total amino acid composition. SPC aromatic amino acid and cystine content is low and 12 alanine residues occur in the molecule. This amino acid composition resembles that of the Kunitz type inhibitors from different *Erythrina* species studied by Joubert and Sharon [8]. These authors associated the *Erythrina* high alanine content (about 11–12 residues mol^{-1}) with potent alpha-chymotrypsin inhibitory activities. Since no sulphydryl groups were detected in the intact SPC, they are probably cross-linked by two intramolecular disulpide bonds.

Table 1. Amino acid composition of SPC inhibitor*

Residues	Mol/mol† Experimental	Integer
Cys‡	3.8	4
Asp	14.2	14
Thr	4.5	5
Ser	14.6	15
Glu	24.1	24
Pro	5.6	6
Gly	28.9	29
Ala	11.8	12
Val	9.1	9
Ile	9.4	9
Leu	7.7	8
Tyr	2.4	3
Phe	5.3	5
Lys	12.5	12
His	3.3	3
Arg	7.9	8
Trp§	1.2	1
Met	0.6	1
Total		169

*Calculations are based on a M_r of 20000.

†Values were obtained from 24 hr hydrolysates.

‡Values obtained as SH content by Ellman method in the presence of 6 M guanidine-HCl [5].

§Determined by the spectrophotometric method of Goodwin and Morton [6].

Figure 3 shows the sequence of residues from the *N*-terminus of SPC inhibitor and the comparison with those from soybean trypsin inhibitors. Although serine protease inhibitors show high structural diversity in the *N*-terminal region, similarities between the *N*-terminal sequence of SPC and the *Glycine max* Kunitz inhibitor were identified through homology analysis. The exact family classification of SPC inhibitor will be possible only after determination of its complete primary structure.

EXPERIMENTAL

Materials. Mature seeds of *Schizolobium parahyba* (Vell.) Toledo were collected in the outskirts of Brasília, DF, Brazil. Alpha-chymotrypsin (crystallized × 3 from bovine pancreas, type II), trypsin (crystallized × 2 from bovine pancreas, type III), *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA), equine skeletal muscle myoglobin, bovine serum albumin, ovalbumin and blue dextran were purchased from Sigma. Ampholine was from LKB-Produkter. *N*-glutaryl-L-phenylalanine-*p*′-nitroanilide (GPNA) was from Serva. Sephadex G-100 and G-75 were obtained from Pharmacia.

Purification. Ground defatted seeds (200 g) were extracted with 0.15 M NaCl (1 l) by continuous overnight stirring, at room temp. The solid residue sepd by centrifugation was submitted to a second extraction with the same vol. of NaCl soln. The supernatants were combined, brought to 0.6 satn with solid $(NH_4)_2SO_4$ and the ppt was discarded. The resulting supernatant was brought to 0.7 $(NH_4)_2SO_4$ saturation. The sediment was dissolved in H_2O, dialysed and lyophilized. The material was subjected to a Sephadex G-100 column (2.5 × 60 cm) equilibrated with 0.05 M NaPi, 0.2 M NaCl, pH 7.6. The second peak collected, containing the frs active against chymotrypsin, from several runs, were combined, dialysed, lyophilized and chromatographed on the same column. The *A* of the eluted fr. was measured at 280 nm. The major fr., with inhibitory activity against chymotrypsin was applied to a Superose 12 column, equilibrated with 0.05 M NaPi, pH 8, and eluted with a 1 ml min^{-1} flow-rate on a FPLC system.

Electrophoresis. Slab gel electrophoresis (SDS–PAGE) was performed using 10–20% polyacrylamide continuous gradient [13]. Protein bands were stained by Coomassie Brilliant Blue.

Determinations of pI. Disc gel isoelectrofocusing was carried out in 8% polyacrylamide gel containing ampholytes over the range of pH 3.5–10 and 3.0–6.0, according to manufacturer's instruction. The amino acid composition was utilized to calculate the theoretical pI of SPC,

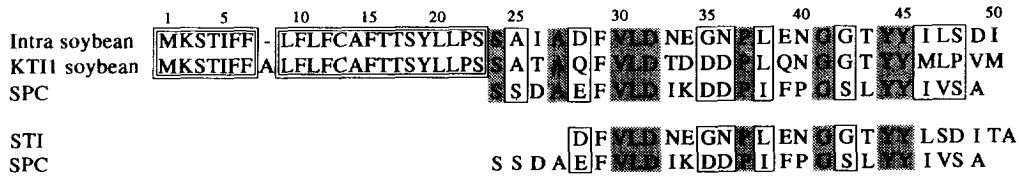

Fig. 3. Alignment of the *N*-terminal amino acid sequences of SPC and *Glycine max* Kunitz-family inhibitors [9]. Intra soybean—Kunitz-type trypsin inhibitor A and C precursor [10], KTI1 soybean—Kunitz-type trypsin KTI1 precursor [11], STI—Kunitz-type trypsin inhibitor active form [12]. Shadowed areas indicate identical residues; boxes indicate conserved residues; boxes with double line show identical residues in precursors.

according to ref. [7]. The application of the polynominal equation of the fourth degree was resolved with the help of a computer program developed by M.M. Ventura (personal communication).

M_r *and Stokes radius.* The estimation of the M_rs of the free and complexed inhibitor were carried out on a column (3.0 × 38 cm) of Sephadex G-75 (fine grade) packed and developed with 0.05 M Tris-HCl, 0.2 M KCl, pH 7.5. The column was calibrated using as standard proteins (5.0 mg ml^{-1}) BSA, ovalbumin, myoglobin and chymotrypsin. Stokes radii were obtained from the linear relationship between (− log KAV)1/2 and Stokes radius of the proteins used as markers. KAV is the distribution coefficient [14].

Inhibitor assay. SPC inhibitory activity on alpha-chymotrypsin was determined by measuring the inhibition of the enzyme hydrolysis of GPNA [15] and by the Kunitz caseinolytic method [16]. The inhibitory activity on trypsin was determined by measuring the inhibition of trypsin catalysed hydrolysis of BAPNA. The dissociation constant (**Ki**) values of the enzyme–inhibitor complexes were determined by the procedure of ref. [3]. The concn of the native inhibitor was determined by *A* at 280 nm; the extinction coefficient at 280 nm was found by experimental determination. The trypsin active site was titrated with *p*-nitrophenyl-*p*-guanidinobenzoate [17].

HPLC. Reverse phase HPLC of the purified inhibitor was performed on a Vydac Protein C4 column (0.2 × 12 cm). The adsorbed samples were eluted with a linear gradient from 0.1% TFA to 80% acetonitrile–0.1% TFA. Flow rate: 1 ml min^{-1} at room temp.

Isolation of inhibitor–serine protease complexes. The study of the interaction of the inhibitor with either chymotrypsin or trypsin and the isolation of the complexes were carried out on a Sephadex G-75 column. The inhibitor (5–15 mg) and enzymes (5–20 mg) were incubated together at pH 7.5 (0.05 M Tris-HCl, 0.2 KCl) in a total vol. of 2 ml and after 30 min at room temp. the mixt. was applied to the column. The formation of the complexes was carried out in different mixts: (a) inhibitor and chymotrypsin; (b) inhibitor and chymotrypsin and trypsin simultaneously; (c) inhibitor–chymotrypsin complex previously isolated and trypsin.

Amino acid analysis and sulphydryl contents. Protein samples were hydrolysed under vacuum in 6 M HCl for 24 hr at 110° and the amino acid composition was analysed by the method of ref. [18]. Liberated amino acids reacted with 2% ninhydrin. Tryptophan content was determined from the absorption spectrum of the inhibitor in 0.1 M NaOH, as described in ref. [6]. Sulphydryl groups were assayed as described in ref. [5], using 5-5′-dinitrobenzoic acid in 6 M guanidine chloride. The number of disulphide bonds was determined by comparing the number of free thiol groups in the unfolded protein prior to and after reduction with dithiothreitol (molar ratio DTT: protein equal to 204). Excess of DTT was removed by gel filtration on a Sephadex G-25 column using acidic conditions.

Effect of temp. on the stability of the inhibitor. The inhibitor in aq. soln was incubated in a sealed tube at various temps for 1 hr and then cooled in ice, and aliquots were assayed for inhibitory activity. The same procedure was performed at 95° for 5 hr.

N-*terminal sequence determination.* Manual Edman degradation was performed by the method of ref. [19]. Automatic Edman degradation [20] was carried out with a model 477A/120A liquid-phase Applied Biosystems (USA). Phenylthiohydantoin (PTH) derivatives of amino-acids were identified on a PTC-222 C18 column.

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