

Isolation and Characterization of a Trypsin Inhibitor from *Vigna sinensis* Seeds

J. XAVIER—FILHO* and FRANCISCA D. N. DE SOUZA

Department of Biochemistry and Molecular Biology, Federal University of Ceará,
Fortaleza, Ceará*

Abstract. The major trypsin inhibitor of *Vigna sinensis* cv. seridó was isolated and shown to be devoid of chymotrypsin inhibiting activity. It has a molecular weight of 9800 as determined by gel electrophoresis and a pI of 5.0. The activity of the inhibitor was decreased by treatment with trinitrobenzenesulfonic acid, suggesting that it is a LYS-X type trypsin inhibitor. Self-association of the molecule was demonstrated both in 1% sodium dodecylsulfate and in acidic (pH 2.4) conditions.

The physiological significance of proteinase inhibitors in plant tissues is subject to controversy, several roles being ascribed to them. Of these, the control of proteinases of the plant, protection against attack by insects or microorganisms, and their role as reserve proteins have recently been much substantiated (RYAN 1973).

Vigna sinensis (L) Savi seeds are known to contain trypsin and chymotrypsin inhibitors (BORCHERS *et al.* 1947, VENTURA and XAVIER-FILHO 1966) as seems to be the case with all leguminous seeds so far studied. A chymotrypsin-trypsin inhibitor from *V. sinensis* cv. seridó seeds was isolated in this laboratory (VENTURA and XAVIER-FILHO 1966) and some of its properties were further studied by VENTURA *et al.* (1971). GENNIS and CANTOR (1976a) isolated two double-headed proteinase inhibitors from a North American variety of *V. sinensis* and showed them to be different from the inhibitor isolated by us.

Trypsin inhibitors in germinating *V. sinensis* seeds were shown to be mobilized differently from the bulk of reserve proteins and at least for 25 to 50% of the activity this mobilization seems to be under the control of the axis (XAVIER-FILHO 1973). We have also shown that both trypsin and chymotrypsin inhibiting activities are preferentially mobilized in the axis and proximal regions of the cotyledon during germination (unpublished results).

XAVIER-FILHO (1973), ROYER *et al.* (1974) and GENNIS and CANTOR (1976a) have shown that at least part of the trypsin inhibitor pool of *Vigna* interacts with endogenous proteases of the seed.

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* Address: Caixa Postal 1065, 60000 Fortaleza, Ceará, Brazil.

The multiplicity of inhibitors of mammalian proteinases found in leguminous seeds further complicates the study of their role during germination. We can only obtain meaningful results if we isolate these substances in a pure form and develop means to localize and follow them through germination. In this study we report the isolation and purification of one of the two major trypsin inhibitors in *V. sinensis* cv. seridó seeds, a necessary step in our continued effort to understand the physiological significance of these substances.

Material and Methods

Dry seeds of *Vigna sinensis* (L.) Savi cv. seridó were ground to a fine powder (60 mesh) in a Wiley mill. A "crude inhibitor" preparation was obtained essentially as described in a previous paper (VENTURA and XAVIER-FILHO 1966). Trypsin and chymotrypsin inhibitors were obtained through gradient elution chromatography in DEAE-Cellulose of the "crude inhibitor" preparation, essentially as described in the above cited paper. The fractions containing the main trypsin inhibiting activity peak and partially free of chymotrypsin inhibiting activity were pooled and saved for further purification. This material, "pooled trypsin inhibitor", was submitted twice to gradient (NaCl 0—1.5 M) elution chromatography in a glass column ($\varnothing$ 2.0 cm) packed with DEAE-Cellulose to a height of 28.0 cm and equilibrated with 0.005 M phosphate buffer, pH 7.0. An essentially pure trypsin inhibitor was obtained.

Measurements of trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) inhibitory activity were carried out by the Kunitz caseinolytic assay (KUNITZ 1947) using the Folin phenol reagent to detect the trichloroacetic acid soluble products. One unit of trypsin or chymotrypsin inhibiting activity (TIU or CIU) is taken as the amount of inhibitor that reduces by 50% the activity of a preparation of enzyme that produces an absorbance difference of 0.500 at 750 nm in the above assay (XAVIER-FILHO 1974). The activity of the inhibitor was also taken as the residual trypsin activity expressed as the percentage of a trypsin standard without inhibitor.

Polyacrylamide gel electrophoresis at pH 8.1 was performed according to CLARKE (1964). Protein loads from 50 μ g to 500 μ g were used per gel column. Voltages from 140 to 160 V and currents of 4—6 mA per gel rod were used. The protein bands were stained with 1% Amido Black in acetic acid and destained by electrophoresis in 7% acetic acid. 8 M urea-polyacrylamide gel electrophoresis at pH 8.1 was performed as described by XAVIER-FILHO (1969). The same loads of protein, electrophoresis conditions and detection of protein bands were used as described above.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the β -mercaptoethanol reduced inhibitor was performed according to the method of WEBER and OSBORN (1964). The inhibitor (800 μ g ml⁻¹) was reduced by 1% β -mercaptoethanol in 0.01 M phosphate buffer, pH 7.0 containing 1% SDS,

Abbreviations used: Diethylaminoethyl cellulose (DEAE-Cellulose), trypsin inhibitor (TI), chymotrypsin inhibitor (CI), unit of trypsin inhibiting activity (TIU), unit of chymotrypsin inhibiting activity (CIU), trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS), trinitrobenzenesulfonic acid (TNBS), 1, 2 cyclohexanedione (CHD), polyacrylamide gel electrophoresis (PAGE), β -mercaptoethanol (β -ME), N-acetyl-DL-phenylalanine- β -naphthyl ester (APNE).

at 40 °C during 2 h. Protein loads of up to 100 µg per gel column were used. The protein bands were stained with Coomassie Brilliant Blue in methanol : acetic acid (1 : 9) and destained in 5% methanol, 7.5% acetic acid. The negative staining method described by XAVIER-FILHO and MOREIRA (1978) was used to visualize the trypsin inhibitory activity in polyacrylamide gel electrophoresis developed in SDS. For this type of experiment loads of 0.5 to 10 µg were used.

The absorption spectrum of the purified inhibitor (0.942 mg ml⁻¹) in phosphate buffer, $\mu = 0.1$, pH 7.6, was measured in the near UV region in a Beckman DU spectrophotometer. At the same time the absorptivity at 280 nm was calculated by taking absorbance readings of sequential dilutions of the above inhibitor solution.

A Sephadex G-75 column (2.5 × 48.0 cm) was equilibrated with 0.1 M formic acid, pH 2.4 and a 2 ml aliquot of a solution of the "pooled trypsin inhibitor", containing 25 mg ml⁻¹ was applied to the column. The flow rate was adjusted to 25 ml h⁻¹ and fractions of 4.2 ml were collected. The absorbance of each fraction was read at 280 nm. The same column was equilibrated with 0.01 M TRIS-HCl, pH 8.0 buffer and again a 2 ml aliquot of a solution of the "pooled trypsin inhibitor" containing 25 mg ml⁻¹, in TRIS buffer was applied to the column. The same conditions used in the first experiment were maintained. The molecular weights of the inhibitor were calculated according to the equation derived by DETERMAN and MICHEL (1966).

TABLE 1

Specific activities and purification factors of preparations from different steps of the purification of the trypsin inhibitor

	Specific activity		Purification factor	
	TIU mg ⁻¹	CIU mg ⁻¹	TI	CI
Seed flour (aqueous extract)	2.5	0.7	1	1
"Crude inhibitor"	447.7	160.5	179	229
"Pooled trypsin inhibitor"	1617.0	108.0	647	154
2nd DEAE-Cellulose rechromatography	1676.0	0.0	670	—

Amino groups in the purified inhibitor were modified according to HABEER (1966). 1 ml of a 4% solution of sodium bicarbonate, pH 8.0 and 1 ml of 0.1% trinitrobenzene sulfonic acid in water were added to 1 ml aliquots of a 0.5 mg ml⁻¹ solution of the inhibitor. The absorbance of the solutions at 345 nm was immediately read. After standing for 2 h, at 40 °C in a controlled bath the reaction was stopped by the addition of 1 ml of a 10% solution of SDS followed by 0.5 ml of 1 N HCl.

The absorbance was again read at 345 nm. Convenient aliquots were taken for the determination of the trypsin inhibiting activity.

Arginine residues in the inhibitor were modified by 1,2-cyclohexanedione (LIU *et al.* 1968). To 4.5 ml of a solution of the inhibitor containing 0.64 mg ml⁻¹ in 0.1 M triethanolamine, pH 10, was added 1.5 ml of an aqueous solution of CHD (22.2 mg ml⁻¹). The solution was left at room temperature,

in total darkness, for 15 h. After dialysis against water for 24 h the trypsin inhibitory activity was measured.

Gel slab isoelectric focussing was performed according to AWDEH *et al.* (1968). Acrylamide (7.5%), containing bis-acrylamide (1%), LKB Ampholine ampholytes (pH 3–10, 1%) and ammonium persulfate (0.058%) was polymerized between two glass plates (12 × 9 cm) spaced by rubber bands (0.5 mm thick). As soon as the gel was set one of the plates was removed and the inhibitor-containing solutions were applied with a micropipet at the cathode side. Focussing was performed over graphite electrodes in a humid box at a maximum potential difference of 100 V. After 4 h and when the current was stable (0.5 mA) the slab was removed from the glass plate and dipped in 15% TCA or sequentially treated with trypsin (0.1 mg ml⁻¹ in phosphate buffer 0.1 M, pH 7.4) and 15 ml of a mixture made of 1.5 ml of N-acetyl-DL-phenylalanine- β -naphthyl ester (2.4 mg ml⁻¹ in dimethylformamide) and 13.5 ml of tetrazotized *o*-dianisidine (0.55 mg ml⁻¹ in phosphate buffer, 0.1 M, pH 7.4) (URIEL and BERGES 1968). Before either staining procedure was performed a 1 cm strip of gel was cut in 0.5 cm portions and eluted overnight with 2 ml of water for pH measurements.

Results

Inhibitor Purification

The major trypsin inhibitor found in mature seeds of *V. sinensis* cv. seridó was isolated by traditional methods employing water extraction, TCA precipitation and ion-exchange chromatography through DEAE-cellulose. The final product was purified 670-fold and was completely devoid of antichymotryptic activity. It was shown to be homogenous by polyacrylamide gel electrophoresis in 8 M urea at pH 8.1, and polyacrylamide electrophoresis in SDS and β -mercaptoethanol.

Action of the Inhibitor on Trypsin and Chymotrypsin

The inhibitor was totally inactive towards chymotrypsin. Its activity against trypsin was such that we could calculate a minimum molecular weight of 11 040 assuming an interaction ratio of 1 mole inhibitor per mole trypsin (Fig. 1).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of the purified inhibitor at pH 8.1 showed several bands when stained with Amido Black even when the experiments were done with small quantities of inhibitor. When 8 M urea was used only one strong band was seen using the same sample size. Only one band was also seen at M_r^* of 9800 in PAGE-SDS when the inhibitor was reduced by 1% β -ME for 2 h at 40 °C. The native inhibitor showed the same molecular weight in PAGE-SDS as the reduced inhibitor. In these experiments, when the negative staining procedure was used we could see, besides a very strong trypsin inhibitor band at 9800, two very faint bands at 19 000 and 26 000, corresponding to less than 2% of the total sample.

* Molecular weight relative to the standards.

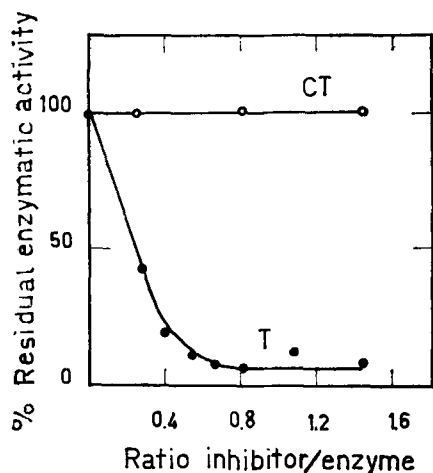


Fig. 1. Action of the purified inhibitor on the proteolytic activity of trypsin (—●—) and chymotrypsin (—○—).

Slab Isoelectric Focussing

Polyacrylamide gel slab isoelectric focussing of aqueous extracts of *V. sinensis* cv. seridó seed powder coupled with the trypsin negative staining described in Methods showed the presence of at least 10 trypsin inhibiting species with pI's ranging from 4.5 to 7.0. The two most important bands correspond to the double-headed chymotrypsin-trypsin inhibitor already isolated and characterized (VENTURA and XAVIER-FILHO 1966) and to the trypsin inhibitor reported in this paper. The purified trypsin inhibitor has an isoelectric point of 5.0 and was obtained more than 95% pure by this method determined either by the negative staining or 15% TCA visualization techniques. A slightly more acidic (pI about 4.9) contaminant was consistently seen in all experiments.

Sephadex G-75 Gel Filtration

Gel filtration of the "pooled inhibitor" at both acidic and basic pH is shown in Fig. 2. The molecular weight calculated for the inhibitor in acidic conditions was 11 220 and is similar to the monomer weight as calculated by PAGE-SDS in β -ME and by the trypsin equivalent. The pattern of the gel filtration experiment at pH 8 shows that the inhibitor has now an apparent molecular weight of 19 910, clearly indicative of pH-dependent association.

UV Absorption Spectrum

The purified inhibitor shows a characteristic absorption spectrum (Fig. 3) with no maximum in the 280 nm region. The calculated molar absorptivity was 3308 mol cm⁻².

Reactive Groups

A total of seven amino groups were determined by TNBS coupling. Of these, two were very reactive inasmuch as they reacted at 25 °C as soon as the reaction was started. Trypsin inhibitory activities of TNBS treated inhibitor were reduced to 24% of the controls after 2 h at 40 °C.

Fig. 2.

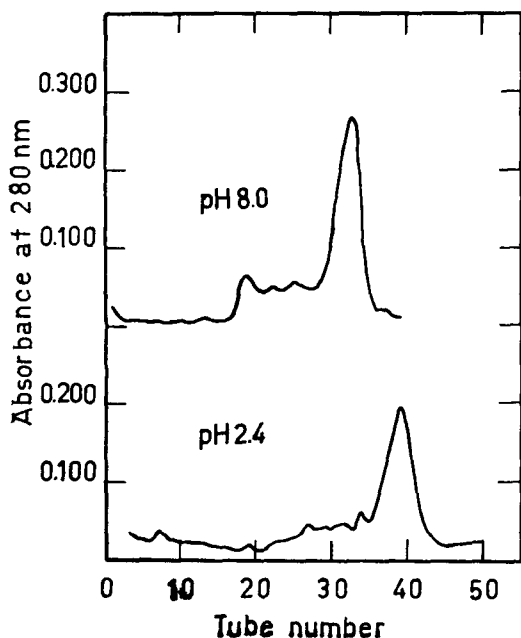


Fig. 3.

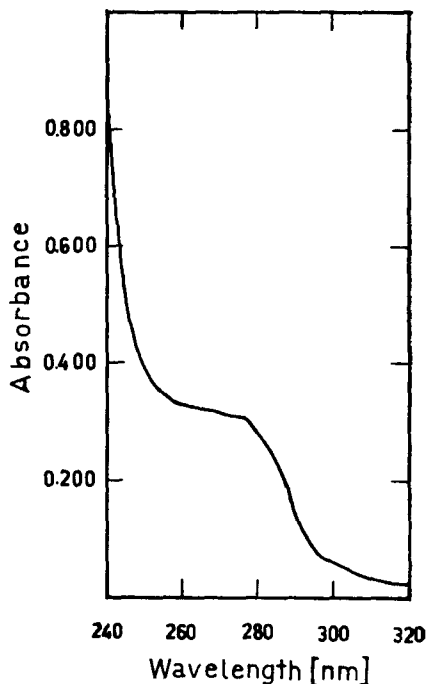


Fig. 2. Sephadex G-75 gel filtration of the "pooled inhibitor" in both pH 2.4 and pH 8.0. The calculated molecular weight of the main peak was 11 220 and 19 910, respectively.

Fig. 3. Ultraviolet absorption spectrum of the inhibitor at pH 7.6 and 0.942 mg ml⁻¹.

The activity of the inhibitor was not modified by the action of CHD. Controls with soybean trypsin inhibitor, an arginine type inhibitor (LASKOWSKI, JR. and SEALOCK 1971) showed considerable reduction (about 40%) of the activity.

Discussion

Aqueous extracts of mature *Vigna sinensis* cv. seridó seeds show a great number of trypsin inhibitors when examined by polyacrylamide gel slab isoelectric focussing. At least 10 clearly visible species are seen with pI's between 4.5 and 7.0 when viewed by the APNE negative staining technique. In these extracts, the two most conspicuous TI inhibitors are the double-headed chymotrypsin-trypsin inhibitor of pI 4.7 previously isolated in this laboratory (VENTURA and XAVIER-FILHO 1966) and the trypsin inhibiting species reported in this study, which has a pI of 5.0.

Using the above isolation procedure we obtained a product which is apparently more than 95% pure as determined by isoelectric focussing. The inhibitor is totally inactive towards chymotrypsin and strongly inhibits trypsin with an apparent 1 : 1 stoichiometry. It is of interest to note that a double-headed trypsin inhibitor was isolated from *Vigna sinensis* cv. Redbow by GENNIS and CANTOR (1976a, b). The Redbow inhibitor is probably

different from the one reported in this communication although they share some properties.

The inhibitor, like the majority of the trypsin inhibitors isolated from other leguminous seeds, is a self-associating protein of molecular weight around 10 000. The monomer predominates in acidic pH whereas in high pH a high-molecular weight form, apparently a dimer species, becomes the main component as seen by gel filtration. The self-association of the inhibitor can also be seen in PAGE at pH 8.1 and even in PAGE-SDS where three distinct bands are seen at 9800 (monomer), 19 000 (dimer), and 26 000 (trimer) positions. Only after prolonged contact with β -ME do the dimer and trimer bands disappear. A similar self-association was already seen in PAGE-SDS for the trypsin-chymotrypsin inhibitor but in this case only one band at 24 700 was consistently seen (XAVIER-FILHO and MOREIRA 1978). GENNIS and CANTOR (1976a) also reported a similar self-association in PAGE-SDS for their two double-headed trypsin and chymotrypsin inhibitors of Redbow *Vigna sinensis*.

The UV spectrum of the inhibitor is one characteristic of a protein containing small proportions of aromatic amino acids. It is interesting to compare the spectrum of this inhibitor with the spectra for the inhibitors isolated by GENNIS and CANTOR (1976b). These authors have correlated the spectra found for their inhibitors with a small proportion of aromatic amino acids and a high content of anomalous UV-absorbing cystine residues. We suggest that the inhibitor studied here also has a low aromatic amino acid content but a somewhat lower percentage of cystine than the ones isolated by the above authors.

Of the seven free amino groups found by titration with TNBS, two were more reactive, suggesting that they are probably at the surface of the molecule. The trypsin inhibiting activity is strongly decreased by reaction with TNBS, indicating that the inhibitor is a LYS-X type inhibitor, that is, it has a lysine residue at its active center (LASKOWSKI JR. and SEALOCK 1971). Reaction with cyclohexanedione, a typical arginine modifying reagent, did not affect the activity of the inhibitor, further substantiating the LYS-X linkage at the active center of the molecule.

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BOOK REVIEW

Moore, P. D., Webb, J. A.: *An Illustrated Guide to Pollen Analysis*. — Hodder and Stoughton, London—Sydney—Auckland—Toronto 1978, 133 pp., 48 plates, £ 4.95.

The book summarizes various aspects of palynology. Its main concern is research of dispersed pollen grain and spores, which can be recovered from the sediments of Quaternary age (peats, lake sediments etc.). The authors discuss the types of sediments suitable for the preservation of palynomorphs and give a useful outline of field and laboratory techniques. A substantial part of the book is devoted to morphology of spores and pollen grains and to the structure of the wall. The pollen and spore key is modified to enable the identification of dispersed palynomorphs even to less experienced workers. The chapter is conveniently supplemented by plentiful illustrations, including SE micrographs.

Selected sets of microscope slides are available on request. The authors draw attention to potentialities and on the other hand limitations of pollen analysis, especially in constructing and interpreting the pollen diagrams. The volume can be recommended for its simple, lucid style, practical approach and condensed information to botanists, paleobotanists as well as undergraduates. It shows pollen analysis as a challenging field for original research of students, utilizable in dating of sediments, reconstruction of vegetational changes and in tracing the effect of man on his environment.

MILADA VAVRDOVÁ (Praha)