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PURIFICATION AND CHARACTERIZATION OF PROTEINASE INHIBITOR FROM *ARTOCARPUS INTEGRIFOLIA* SEEDS

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Key Word Index—*Artocarpus integrifolia*; Moraceae; proteinase inhibitor; purification; properties; chemical modification.

Abstract—A trypsin-chymotrypsin inhibitor has been isolated and purified from seeds of the jackfruit plant, *Artocarpus integrifolia*. Its purity has been checked by PAGE, isoelectric focusing, immunochemical tests and sedimentation in the ultracentrifuge. The inhibitor has an M_r of 44 200 and a pI of 4.5. It consists of two disulphide-linked polypeptide chains having M_rs of 24 700 and 18 600. Amino acid analyses have shown that it contains three half-cystine residues and is rich in aspartic acid, glutamic acid and leucine. It contains 3% neutral sugar and 1.8% glucosamine. It inhibits trypsin and chymotrypsin at independent reactive sites by forming enzyme-inhibitor complexes at a molar ratio close to unity. Chemical modification of amino groups with trinitrobenzene sulphonic acid has reduced its inhibitory activity against trypsin with no loss of chymotrypsin inhibitory activity.

INTRODUCTION

Plants are rich sources of proteinase inhibitors. A number of inhibitors have been extensively studied in Leguminosae, Gramineae and Solanaceae [1]. Most of them are devoid of a carbohydrate moiety and contain a single polypeptide chain.

The jackfruit plant, *Artocarpus integrifolia* Linn. is cultivated in the tropics for its large, edible fruits. Its seeds are nutritious and may be widely used as a food source. The presence of antitryptic and antichymotryptic activities in the crude extract of jackfruit seeds has been reported earlier [2]. In the present paper, we describe the purification and characterization of a serine proteinase inhibitor obtained from the same source. It is a glycoprotein composed of two disulphide-linked polypeptide chains and is specific for inhibition of trypsin and chymotrypsin.

RESULTS AND DISCUSSION

Purification of inhibitor

The trypsin-chymotrypsin inhibitor was purified from the seeds of *A. integrifolia* by ammonium sulphate precipitation followed by ion-exchange chromatography on DEAE-cellulose and on CM-Sephadex. Its rechromatography on CM-Sephadex was necessary to obtain a peak with constant inhibitory activity across the peak. The purification procedure is summarized in Table 1. The overall yield of the inhibitor was 300 mg per kg of decorticated seeds.

Electrophoresis and isoelectric focusing

The purified inhibitor gave a single band on PAGE at pH 8.3 and two major bands on SDS-PAGE in the presence of 2-mercaptoethanol (Fig. 1). Isoelectric focusing of the inhibitor yielded a single peak with an isoelectric point at pH 4.5.

Sedimentation velocity

The inhibitor sedimented as a single symmetrical peak at different protein concentrations (0.5–1.5%) in 0.036 M Na-Pi–NaCl buffer, pH 7.45. Its sedimentation coefficient ($s_{20,w}$) was dependent of protein concentration and the value of $s_{20,w}$ was determined to be 3.42*S* at zero protein concentration.

M_r

The M_r of the inhibitor was estimated to be 43 000 by gel filtration [3] through Sephadex G-100. Its diffusion coefficient calculated from the boundary spreading in the ultracentrifuge [4] was 6.86×10^{-7} cm^2/sec and its limiting viscosity number was 4.62 ml/g in water. From the sedimentation-diffusion data using the Svedberg equation and diffusion-viscosity data the M_rs of the inhibitor were found to be 45 100 and 45 500 respectively. SDS-PAGE without 2-mercaptoethanol yielded a single band with the M_r of 44 000. In the presence of reducing agent, however, it yielded two bands of M_rs of 24 700 and 18 600, indicating that the inhibitor was composed of two disulphide-linked polypeptide chains. Its amino acid composition gave an M_r of 44 100. From the above results the average M_r of the inhibitor was calculated as 44 200. The molecular radius (Stokes radius) of the inhibitor determined by gel filtration [5] was found to be 28 Å.

Immunodiffusion and immunoelectrophoresis

The purity of the inhibitor was shown by immunodiffusion and immunoelectrophoresis where it gave a single precipitin line of identity (Fig. 2). Its antiserum did not cross-react with well-characterized trypsin inhibitors of

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Table 1. Summary of the purification of trypsin-chymotrypsin inhibitor of jackfruit seed

Step	Protein (mg)	Specific activity (units/mg)	Recovery of activity (%)
Homogenate	7324	0.07	100
$(NH_4)_2SO_4$ precipitation	3213	0.10	60
Chromatography on DEAE-cellulose	500	0.22	21
Chromatography on CM-Sephadex	128	0.49	12
Rechromatography on CM-Sephadex	100	0.50	10

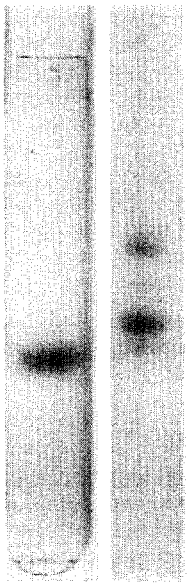

Fig. 1. PAGE of jackfruit seed inhibitor on (left) 10% gel at pH 8.3 and (right) 7.5% gel containing 0.1% SDS at pH 7.0.

Table 2. Amino acid composition of trypsin-chymotrypsin inhibitor of jackfruit seed

Amino acid	Residues/mol
Lys	19
His	13
Arg	9
Asp	41
Thr	11
Ser	32
Glu	40
Pro	29
Gly	31
Ala	15
1/2 Cys	6
Val	20
Met	4
Ile	21
Leu	36
Tyr	21
Phe	20
Trp	5
Total	373

soybean and limabean, suggesting that the antigenic determinants of the latter two proteinase inhibitors differed from that of jackfruit seed inhibitor.

Amino acid composition

The amino acid composition of the inhibitor is shown in Table 2. No free sulphydryl group were detected with Ellman's reagent [6] even in the presence of 8 M urea, suggesting that all of the half-cystine residues are present in the inhibitor as disulphide bonds. It contained 3% neutral sugar and 1.8% glucosamine.

Inhibitor properties

Figures 3 and 4 show that the inhibition of trypsin and chymotrypsin respectively at alkaline pH by increasing amount of the inhibitor. The inhibition was linear up to 80% with trypsin and 85% with chymotrypsin and extrapolation of the data gave molar ratios of enzyme-inhibitor complexes close to unity. The complex was stable for at least 24 hr. The dissociation constants of complexes of the inhibitor with trypsin and chymotrypsin were calculated [7] to be 3.63×10^{-9} M and 1.35×10^{-9} M respectively.

Effect of 2,4,6-trinitrobenzene sulphonic acid (TNBS) on inhibitory activity

The time course for modification of the inhibitor with TNBS and the change in its inhibitory activity are shown in Fig. 5. The modification of free amino groups reduced its trypsin inhibitory activity sharply, suggesting that its reactive site [8] for trypsin inhibition is lysine. Since the inhibition of chymotrypsin was unaffected by the modification of lysine residues, it is apparent that the inhibitor has independent inhibition sites for these two enzymes.

Modification of arginine residues of the inhibitor with 1,2-cyclohexanedione did not affect its inhibitory activity against trypsin or chymotrypsin.

The results of this study show that the jackfruit seed inhibitor differs from the well-characterized inhibitors of legume seeds such as soybean, lima bean, garden bean, chick pea [1] and winged bean [9] in its high M_r and the presence of two polypeptide chains and a carbohydrate

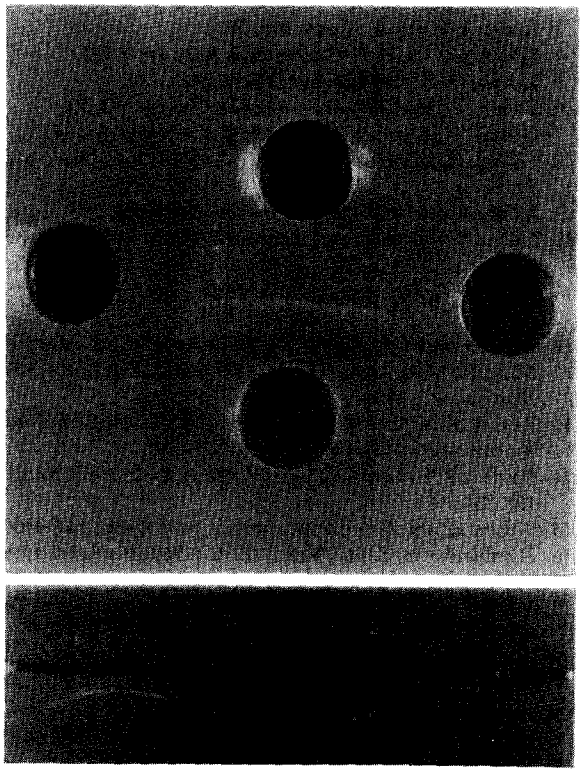

Fig. 2. Immunodiffusion of jackfruit seed inhibitor (top). The lower well contained the antiserum to jackfruit seed inhibitor and the upper, left and right wells contained inhibitors of jackfruit, soybean and limabean respectively. Immunoelectrophoresis of jackfruit seed inhibitor (bottom). The central slot contained the antiserum to jackfruit seed inhibitor and upper and lower slots contained inhibitors of jackfruit and soybean respectively.

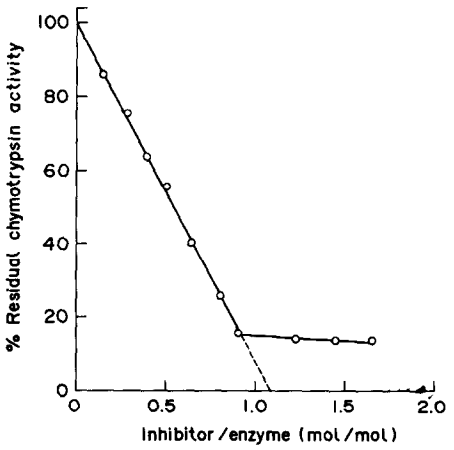

Fig. 3. Inhibition of jackfruit seed inhibitor by bovine trypsin.

moiety. In this respect it is similar to some extent to the inhibitors [10–12] of the Mimosoideae subfamily which contain two disulphide-linked polypeptide chains, but they have lower M_r and lack a carbohydrate moiety.

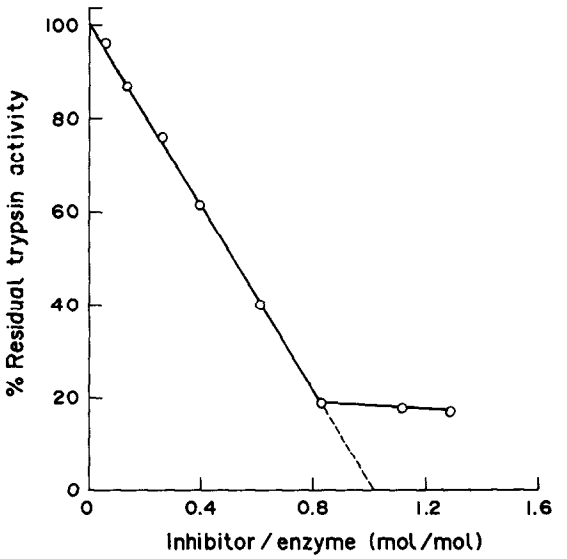

Fig. 4. Inhibition of jackfruit seed inhibitor by bovine α-chymotrypsin.

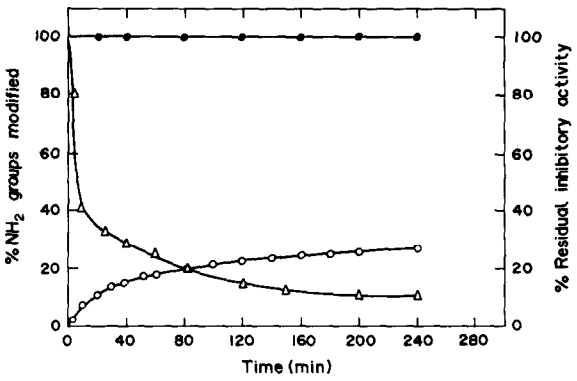

Fig. 5. Effect of modification of amino groups (○—○) with TNBS on inhibitory activities of jackfruit seed inhibitor against trypsin (Δ—Δ) and chymotrypsin (●—●).

EXPERIMENTAL

Purification of trypsin-chymotrypsin inhibitor. Matured seeds of *A. integrifolia*, collected locally, were homogenized with 0.05 M Na-Pi–0.1 M NaCl buffer, pH 7.5, and the homogenate, clarified by centrifugation, was treated with $(NH_4)_2SO_4$ to 85% saturation. The ppt. was dissolved in a minimum vol. of H_2O and the soln was applied to a column (3 × 50 cm) of DEAE-cellulose equilibrated with 0.05 M Na-Pi buffer, pH 7.5. Elution was done with a linear gradient of 0 to 0.5 M NaCl in the same buffer. Fractions containing inhibitor were dialysed against 0.1 M NaOAc buffer, pH 5.0, and the dialysed soln. was applied to a column (3 × 34 cm) of CM-Sephadex C-50 equilibrated with the same buffer. The inhibitor was eluted with the equilibrium buffer. The eluate was dialysed against water and lyophilized.

Activity and concentration measurements. Trypsin and chymotrypsin activities were determined as described in ref. [13]. The inhibitor activity was calculated as the difference between enzyme activity in the absence and presence of inhibitor. One unit

of inhibitory activity is expressed as a mg enzyme inhibited by the inhibitor. Sp. activity is expressed as units of inhibitory activity/mg inhibitor. Protein conc. of the purified inhibitor was determined spectrophotometrically at 280 nm using $E_{1\%}^{1cm} = 13.8$.

PAGE. Disc electrophoresis in 10% polyacrylamide gel was performed at pH 8.3 [14]. SDS-PAGE was carried out as described in ref. [15].

Immunodiffusion and immunoelectrophoresis. These were done as described in ref. [16] using rabbit antiserum to the purified inhibitor.

Isoelectric focusing. This was performed in an LKB 8100 electrofocusing column using 1% ampholine in the pH range 3–6 [17].

Ultracentrifugation. For the determination of sedimentation coefficient and diffusion coefficient of the inhibitor, ultracentrifugal analyses were carried out with a Spinco Model E analytical ultracentrifuge at 56000 rpm and 8000 rpm respectively, as described in ref. [13]. Its partial specific vol. was taken as 0.732 ml/g calculated from the amino acid composition [18].

Amino acid analyses. These were performed on a Beckman Multichrom amino acid analyzer. Samples were hydrolysed with 6 M HCl at 110° for 24, 48, and 72 hr under vacuum. Half-cystine was determined as cysteic acid [19]. Tryptophan was determined after hydrolysis with *p*-toluenesulphonic acid [20].

The neutral sugar was determined by the phenol–H_2SO_4 method [21] and amino sugar according to ref. [22].

Modification of lysine and arginine. Lysine residues were modified with TNBS at pH 9.5 [23, 24] and arginine residues with 1,2-cyclohexanedione at pH 9.0 [25].

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