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PSOPHOCARPIN B_1, A STORAGE PROTEIN OF *PSOPHOCARPUS TETRAGONOLOBUS*, HAS CHYMOTRYPSIN INHIBITORY ACTIVITY

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Key Word Index—*Psophocarpus tetragonolobus*: Leguminosae; Winged bean; Psophocarpin B_1; Chymotrypsin inhibitory activity; Western blotting.

Abstract—Psophocarpin B_1, a major storage protein of winged bean (*Psophocarpus tetragonolobus*) seeds, inhibits the activity of bovine pancreatic chymotrypsin. This inhibitory activity is abolished by heat treatment above 70°. In germinating seeds total chymotrypsin inhibitory activity (CIA) declines steadily as germination proceeds. By means of the Western blotting technique, it has been demonstrated that both psophocarpin B_1 and CIA disappear concurrently during germination.

INTRODUCTION

Psophocarpin B_1 is one of the major seed storage proteins of *Psophocarpus tetragonolobus* (winged bean), an outstanding crop of humid tropical regions. In a recent communication, we reported on its purification, physicochemical characterization, tissue distribution and levels in germinating seeds [1]. It is one of the major components of a highly heterogeneous group of proteins of similar size, collectively known as psophocarpin B fraction [2]. Although all the components have not been individually characterized, some lectins and protease inhibitors have been isolated from this fraction [3–5]. This led us to investigate whether psophocarpin B_1, which has been previously shown to possess the characteristics of a vicilin-type seed storage protein [1, 2], has any additional biological properties, such as hemagglutinating or protease inhibitory activities. The chymotrypsin inhibitory activity of the purified protein and some additional information of its metabolic fate during germination are presented.

RESULTS

Metabolic fate of psophocarpin B_1 *in germinating seeds*

The metabolic fate of psophocarpin B_1 in germinating seeds was investigated by means of the Western blotting technique. The results clearly indicate that there was a gradual decrease in psophocarpin B_1 in the cotyledons of germinating seeds and no lower M_r fragment was detectable (Fig. 1).

In a separate experiment, the protein profiles of the cotyledons during germination were studied by SDS–PAGE, followed by Coomassie Blue staining. The appearance of any additional small polypeptides (M_r < 19 000) as a result of degradation of the larger ones, was not detectable (Fig. 2).

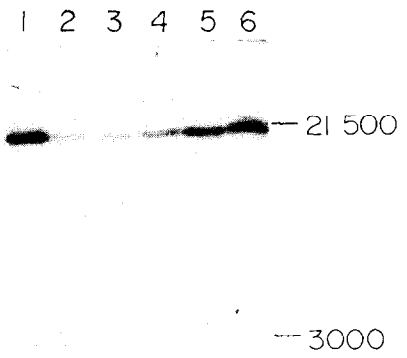

Fig. 1. Western blot analysis of the proteins of germinating winged bean seeds. Seeds were germinated under controlled contitions and extracts were prepared in 10 volumes of Tris–saline containing EDTA, PMSF and NaN_3; and suitable aliquots were resolved by SDS-PAGE (18% acrylamide) and analysed by Western blot as described in the text. Soybean trypsin inhibitor (21 500) and α and β chains of insulin (3000) were used as standard M_r markers. Their positions are shown on the right hand margin. Cotyledons from different days of germination were analysed: lane 1, purified psophocarpin B_1 (150 ng); 2, 21 days of germination (1.1 μg protein); 3, 15 days (1.5 μg); 4, 10 days (1.4 μg); 5, 5 days (1.9 μg); 6, 0 day (2.1 μg).

Amino acid composition of psophocarpin B_1

The amino acid composition of psophocarpin B_1 was found to be very similar to that of the chymotrypsin inhibitor, previously characterized by Kortt [5] (Table 1).

Protease inhibitory and hemagglutinating activities of psophocarpin B_1

Purified psophocarpin B_1 was found to inhibit bovine pancreatic chymotrypsin. The inhibition was increased as increasing concentrations of the protein were pre-incubated with the protease (Fig. 3). More than 50% inhibition was observed at a molar ratio of about 1

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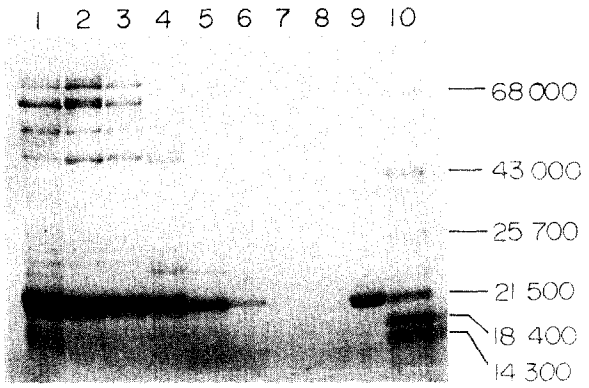

Fig. 2. Protein profile of germinating winged bean seeds by SDS-PAGE. Seed germination, preparation of extract and SDS-PAGE (12.5% acrylamide) were carried out as described in Fig. 1. Proteins were stained with Coomassie Blue. Lanes: 1, 0 day of germination; 2, 1 day; 3, 3 days; 4, 5 days; 5, 10 days; 6, 15 days; 7, 20 days; 8, 25 days; 9, purified psophocarpin B_1 and 10, various standard M_r marker proteins. The numbers on the right hand margin represent the M_r of these standard.

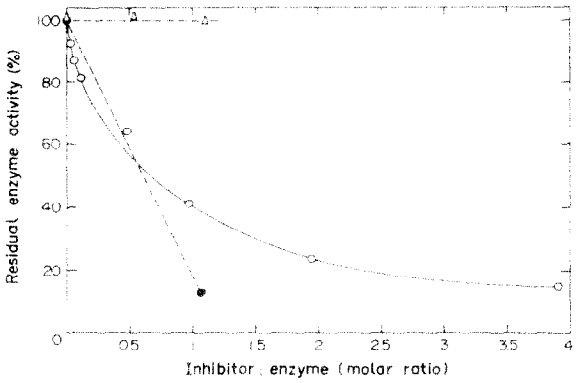

Fig. 3. Protease inhibitory activity of psophocarpin B_1. Proteases, trypsin (10 μg) and chymotrypsin (12.8 μg), were incubated separately with different amounts of psophocarpin B_1 (0–40 μg) in a total volume of 100 μl for 10 min and 50 μl aliquots were assayed for the residual enzyme activity as described in the text. ○—○, preincubation of chymotrypsin with psophocarpin B_1; and △—△, preincubation of trypsin with psophocarpin B_1; and ●—●, preincubation of trypsin with soybean trypsin inhibitor. For the calculation of molar ratio, 25000, 24000, 21500 and 20000 were used as the M_r of chymotrypsin, trypsin, soybean trypsin inhibitor and psophocarpin B_1, respectively.

Table 1. Amino acid composition of psophocarpin B_1

Amino acid	Residue per mole*	
	Psophocarpin B_1	Chymotrypsin inhibitor (Kortt)†
Lys	13	11
His	6	6
Arg	8	7
Asp	20	19
Thr	8‡	7
Ser	15‡	14
Glu	21	20
Pro	12	14
Gly	15	14
Ala	11	10
Cys	3§	4
Val	13	16
Met	0	0
Ile	10	9
Leu	19	19
Tyr	6	5
Phe	5	5
Try	2‖	3

* Calculations are based on a M_r of 20000 for psophocarpin B_1.

† Values taken from Kortt [5].

‡ Values obtained by extrapolation to zero time of hydrolysis.

§ Determined by the DNTB method after sodium borohydride reduction [20].

‖ Determined after hydrolysis with 4M methane sulphonic acid [21].

(psophocarpin B_1: chymotrypsin). However, complete inactivation of chymotrypsin could not be achieved even when a large excess of the purified protein was used (a molar ratio of about 4).

Psophocarpin B_1 had no effect on the activity of trypsin whereas more than 80% inhibition of its activity was observed in the control experiment with soybean trypsin inhibitor (Fig. 3). The hemagglutination assays revealed that it did not agglutinate normal rabbit erythrocytes even at a concentration as high as 1 mg/ml, although in the controls, Con A agglutinated the erythrocytes at a concentration of about 0.5 μg/ml.

Heat inactivation of psophocarpin B_1 and the loss of chymotrypsin inhibitory activity (CIA)

Heat treatment of purified psophocarpin B_1 at 70° for 5 min neither denatured the protein nor affected CIA. But at higher temperatures, the protein was denatured with concomitant loss of CIA (Fig. 4). At 80° for 5 min, about 90% of the protein as well as CIA was lost from the solution while at 90° there was total denaturation of the protein. No CIA was detectable in the denatured protein.

CIA during germination

Total CIA in the cotyledons of germinating seeds showed a steady decline with the progress of germination (Fig. 5). There was also a similar decrease in the total protein content of the cotyledons.

DISCUSSION

The results of the Western blot experiment confirm our earlier finding that psophocarpin B_1 undergoes a time-dependent depletion in the cotyledons during germination [1]. The earlier studies which were carried out employing a radio-immunoassay (RIA) technique, did not distinguish between an intact protein and its proteolytic

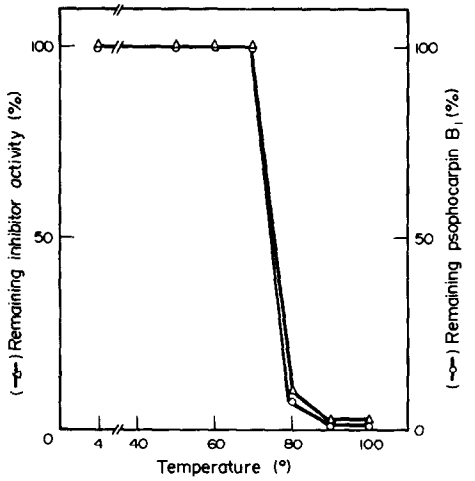

Fig. 4. The effect of temperature on the stability of psophocarpin B_1 and its chymotrypsin inhibitor activity. 100 μl aliquots of psophocarpin B_1 (4 mg/ml) in 100 mM Tris–HCl, pH 8.0, containing 10 mM $CaCl_2$ were heated separately at appropriate temperatures for 5 min and immediately cooled in an ice-bath, centrifuged at 15000 g for 15 min. The supernatant solutions were assayed for residual protein and chymotrypsin inhibitor activity, as described in the text. △—△, remaining inhibitor activity; ○—○, remaining phosphocarpin B_1.

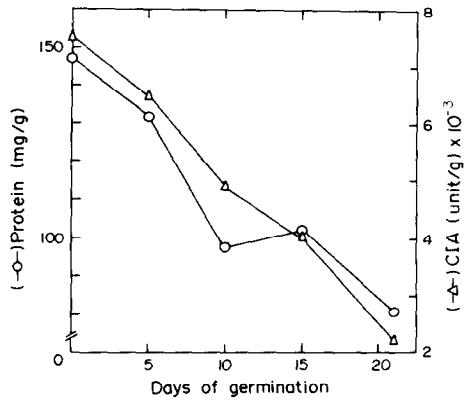

Fig. 5. Chymotrypsin inhibitor activity in germinating winged bean seeds. Seeds were germinated under controlled conditionons and extracts were prepared in 0.1 M Tris–HCl, pH 8.0. Total protein and CIA were measured as described in the test. The protein (○—○) and CIA (△—△) values are expressed as mg or units per g of fresh seed weight, respectively.

fragments if the latter happened to cross-react with the antisera. Our inability to detect any such fragments by the Western blot technique (Fig. 1), indicates the absence of any such immuno-reactive fragments in the cotyledons and the values obtained by RIA would represent only the intact psophocarpin B_1. Additional evidence that psophocarpin B_1 has a storage role, is provided by the results of SDS-PAGE analysis of the proteins in the germinating seeds. Moreover, these studies do not indicate the presence of any significant peptide pool during the degradation of the storage protein(s) since the appearance of a newly generated small peptide is not obvious (Fig. 2). This also suggests that once the proteolytic process starts, it is quite rapid and extensive, generating small peptides and amino acids to meet the nutritional demands of the growing seedling.

Some storage proteins have been found to possess additional properties and therefore, the present finding of CIA for psophocarpin B_1, is not unique. It has been shown that the G_2 storage globulin from *Phaseolus vulgaris* possesses hemagglutination property [6, 7], while urease in the seeds of *Canavalia ensiformis* appears to have a secondary storage role [8]. Chymotrypsin inhibitor I of potato also appears to serve as a nitrogen source during the growth of young plants [9] and may, therefore, be considered as a secondary storage protein.

The present finding that psophocarpin B_1 has chymotrypsin inhibitory activity (Fig. 3), raises the question as to whether or not this protein is the same as the one previously purified by Kortt, by a chymotrypsin-Sepharose affinity chromatography [5]. The close similarity in the physico-chemical properties of psophocarpin B_1 as reported earlier by us [1] and those of the chymotrypsin inhibitor by Kortt [5], lends support to the idea that these two proteins, prepared by two quite different methods are the same. The striking resemblance of the amino acid composition of psophocarpin B_1 to that of the chymotrypsin inhibitor (Table 1), further substantiates the idea that these two preparations may represent the same protein. Moreover, complete inhibition of chymotrypsin even in the presence of a large excess of the inhibitor could not be demonstrated either in the present studies or in the earlier work by Kortt. This inability to bring about a complete inhibition of the enzyme may be accounted for by the low affinity of the inhibitor for the enzyme; this observation is not at all surprising since chymotrypsin is not of plant origin. However, it is possible to speculate on the significance of the occurrence of a chymotrypsin-like protease in winged bean seeds for which psophocarpin B_1 is the naturally occurring inhibitor. It is quite tempting to suggest that the existence of such a protease and its specific inhibitor in the same system could have vital regulatory functions as has been demonstrated in the case of human elastase and α_1-antitrypsin [10]. With this objective in mind, studies are underway to identify a protease in winged bean, which is specifically inhibited by psophocarpin B_1.

EXPERIMENTAL

Materials. Bovine pancreatic chymotrypsin (Type VII) and trypsin (Type XI), *N*-benzoyl-L-tyrosine ethyl ester (BTEE), *p*-tosyl-L-arginine methyl ester (TAME), SDS, BSA, phenyl methyl sulphonyl fluoride (PMSF), soluble protein A from *S. aureus*, soybean trypsin inhibitor, Antifoam C emulsion and sodium deoxycholate were purchased from Sigma. Nitrocellulose (BA 85) paper was obtained from Schleicher and Schuell, Gmbh. NP-40 was purchased from BRL, USA and $Na^{125}I$ (carrier-free) was supplied by Bhabha Atomic Research Centre, India. Other chemicals were analytical grade reagents. Purified psophocarpin B_1 and monospecific antisera against it were prepared as described earlier [1].

Plant material. Dry seeds of winged bean were obtained from the farmhouses of Manipur (India), where it is a common long-standing crop.

Germination of seeds and preparation of tissue extract. Dry winged bean seeds were sterilized with a soln of 0.01 % $HgCl_2$ and

washed thoroughly with sterile H_2O and allowed to germinate on moist filter paper at 30° under sterile conditions. The cracking of the seed coat was counted as day zero and germination was continued on moist, sterilized sand.

Tissues were homogenized in 2–10 vols (g fr. wt) of 100 mM Tris–HCl, pH 8.0, and centrifuged at 15000 *g* for 15 min at 4°. The clear supernatant solns represented the tissue extracts. For the extracts used in SDS-PAGE, the buffer also contained 5 mM EDTA, 5 mM PMSF and 0.02% NaN_3.

Western blot analysis. This was performed essentially as described in ref. [11] with minor modifications in the washing procedures. Proteins resolved by SDS-PAGE by the Laemmli procedure [12] under reducing conditions, were electrophoretically transferred to nitrocellulose paper. The latter was treated with 3% BSA in Tris–saline (10 mM Tris–HCl, pH 7.5, containing 0.9% NaCl) and a few drops of Antifoam C for 2 hr at 37° and then incubated with 0.5% (v/v) anti-serum in the same buffer at 25° for 16 hr. It was successively washed with (i) 10 mM Tris–HCl, pH 7.5, containing 0.9% NaCl, (ii) 0.2% NP-40, 0.1% SDS, 0.25% sodium deoxycholate and 5% gelatin in Tris–saline, (iii) Tris–saline. It was then incubated with 20 ml [^{125}I]-protein A (1.5×10^6 cpm/ml), iodinated by the chloramine T method ref. [13], in 10 mM Tris–HCl, pH 7.5, 0.2% NP-40 and 5% BSA for 45 min at 25° with gentle shaking. The nitrocellulose paper was thoroughly washed as described above, dried and autoradiographed. The sensitivity of detection was 50 ng of psophocarpin B_1.

Hemagglutination assay. The hemagglutinating activity of the purified psophocarpin B_1 was carried out by the method of ref. [14] as described earlier [15], using Con A as the positive control.

Trypsin inhibitor assay. Trypsin inhibitor was assayed using TAME as the substrate as described in Worthington manual [16].

Chymotrypsin inhibitor assay. This was done according to ref. [17] as modified by ref [5] using BTEE as the substrate and one inhibitor unit was defined as the amount of protein which can inhibit the chymotrypsin activity by 50% under the assay conditions. Protein was assayed by the procedure of ref. [18] using BSA as the standard.

Amino acid analysis. This was carried out according to the method of ref. [19]. Samples were hydrolysed at 110° in 6 M HCl for 24, 48 and 72 hr in evacuated, sealed tubes. The total half-cystine residue was determined according to ref. [20] For the determination of the tryptophan content, the sample was hydrolysed in 4 M methane sulphonic acid [21]. Analyses were performed on a JEOL (JLC-5AH) amino acid analyser.

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