Abundant Class III Acidic Chitinase Homologue in Tamarind (*Tamarindus indica*) Seed Serves as the Major Storage Protein

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The phyla Leguminosae contains protease inhibitors, lectins, chitinases, and glycohydrolases as major defense proteins in their seeds. Electrophoretic analysis of the seed proteins of tamarind (*Tamarindus indica* L.), an agri-waste material, indicated the unusual presence of two major proteins comparable to overexpression of recombinant proteins. These proteins were identified by amino-terminal analysis to be (1) Kunitz-type trypsin inhibitor and (2) class III endochitinase (34000 Da). These two proteins were purified to apparent homogeneity by a single-step chitin bead affinity chromatography and characterized. The Kunitz inhibitor was specific toward inhibiting trypsin with a stoichiometry of 1:1. The 33000 ± 1000 Da protein, accounting for >50% of the total seed protein, is an acidic glycoprotein exhibiting a very low endotype hydrolytic activity toward chitin derivatives. SDS-PAGE followed by densitometry of tamarind seed germination indicates the disappearance of the chitinase with the concomitant appearance of a cysteine endopeptidase. On the basis of its abundance, accumulation without any pathogenesis-related stimulus, temporal regulation, amino acid composition, and very low enzyme activity, this 34000 Da protein designated "tamarinin" physiologically serves as the major storage protein.

KEYWORDS: Tamarinin; germination; Kunitz-type trypsin inhibitor; kernel protein; chitin bead affinity chromatography; densitometry

INTRODUCTION

Legume seeds are an important component of the human diet. Their high contents of protein, vitamins, and minerals, together with their relatively low cost, render them the major source of protein in the human diet (*1*). In developing countries extensive research has been diverted toward the proteins of traditional legumes, namely, peas, beans, and lentils (*2*). In comparison, the proteins of tree legume seeds as valuable sources of protein have not been studied.

The tamarind tree (*Tamarindus indica* L.) of the family Caesalpinaceae is found in both tropical and subtropical regions of the world. It is grown extensively in India for its sour fruit pulp. The fruit pulp is used extensively in the local confectionary industry and is a common article of trade in India (*3*). This results in the seed (34% by weight of the fruit) as a byproduct. The major industrial use of the seed is as tamarind kernel powder (TKP), a sizing material used in the textile and jute industry (*3*). Jellose, the major polysaccharide in the seed, accounts for 70% of the carbohydrate, which is used as a substitute for fruit pectin in the food industry. Marangoni et al. (*1*) evaluated the seed proteins as a potential source of food proteins. The sulfur-containing amino acids of the TKP are the limiting amino acids,

resulting in a low chemical score (*4*). Despite its many uses, there have been relatively few investigations on the biochemical and structural characterization of the proteins of TKP. Electrophoretic investigations on the crude extract of TKP indicated the presence of two major proteins, accounting for >60–70% of the total protein. These two proteins were identified by their amino-terminal sequence to be a class III endochitinase and a Kunitz-type trypsin inhibitor. It is well documented that seeds accumulate constitutively or after induction a wide array of defense proteins that confer resistance against phytophagous predators and pathogenesis. However, tamarind seed in the absence of any such external stimuli accumulates a chitinase and a trypsin inhibitor, both of which are classified as plant defense pathogenesis-related proteins (PRPs). In this paper we report the purification and characterization of the endochitinase and Kunitz-type trypsin inhibitors. We further demonstrate that the 34000 Da protein is an acidic class III endochitinase the physiological role of which is not as a PRP but serves as the major storage protein designated "tamarinin". We also show that a cysteine protease induced on germination can degrade tamarinin to mobilize nitrogen to the developing seedling.

MATERIALS AND METHODS

Tamarind seeds (*T. indica*) were obtained locally. Chitin beads were purchased from New England Biolabs, Inc., Beverly, MA. Bovine serum

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albumin, chitosan, glycolchitosan, N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA), N-α-benzoyl-L-tyrosine-p-nitroanilide (BTPNA), N-acetyl-DL-phenylalanine β-naphthyl ester (APNE), and amino acid standards were purchased from Sigma Chemical Co., St. Louis, MO. HPLC grade solvents were from Spectrochem Pvt. Ltd., Mumbai, India. Protein molecular weight markers for SDS-PAGE were from Genei, Bangalore, India. All other chemicals were of the highest purity available commercially.

Enzyme Extraction and Purification. Tamarind seeds were steeped in water containing 0.02% sodium azide followed by the removal of testa manually. The kernels were air-dried and powdered. This served as the tamarind kernel powder. TKP (10% w/v) was extracted for 16 h at 4 °C in 0.1 M NaOH, pH 10.0, containing 1 M NaCl. The extract was centrifuged at 8000 rpm for 30 min at 4 °C. The clear supernatant was dialyzed against 20 mM Tris-HCl buffer, pH 7.0, at 4 °C (4 × 1 L). The retentate was passed through a 10000 Da cutoff centrifugal device. The retentate was loaded onto a chitin bead affinity column (9 × 2.5 cm) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.0, containing 0.5 M NaCl. The column was washed with the same buffer. The trypsin inhibitor was recovered as the unbound protein fraction in the buffer wash. The fractions showing trypsin inhibitory activity were pooled and dialyzed against the same buffer, minus NaCl. The bound chitinase was eluted using 20 mM NaOH containing 0.5 M NaCl. The chitinase fractions were pooled, dialyzed against 20 mM Tris-HCl, pH 7.0, and stored at 4 °C until used.

Chitinase Assay. Commercial chitosan (C-3646, Sigma Chemical Co.) was used as the substrate. The enzyme reaction was initiated by adding enzyme (10–100 μg) in 0.1–0.5 mL of 0.5% chitosan in 1% acetic acid and incubated at 37 °C for 60 min. The reaction was stopped by adding 2 N NaOH (0.4 mL), which renders the undigested chitosan insoluble above pH 7.0. The precipitate formed was removed by centrifugation (10000 rpm for 10 min). The increase in reducing sugar ends, released by chitinase was analyzed according to the method of Imato and Yagishita (*5*). To the control, enzyme was added after stopping the reaction. One unit of chitinase activity is defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute at 37 °C.

Trypsin Inhibitory Assay. The amidase activity of trypsin and its inhibition were assayed using the chromogenic substrate BAPNA at pH 8.2 in 50 mM Tris-HCl containing 20 mM CaCl₂ according to the method of Kakade et al. (*6*), The amidase activity of chymotrypsin and its inhibition were assayed using the chromogenic substrate BTPNA in 80 mM Tris-HCl buffer, pH 7.8, containing 20% DMSO and 20 mM CaCl₂. One unit of trypsin/chymotrypsin enzyme activity is defined as the increase in the absorbance of 0.01 at 410 nm under the assay conditions. One inhibitory unit is defined as the amount of inhibitor that reduces the enzyme activity by 1 unit.

Protein Estimation. Protein concentration was determined according to the method of Bradford (*7*) and using bicinchoninic acid (BCA) (*8*). Bovine serum albumin was used as the standard.

Polyacrylamide Gel Electrophoresis (PAGE). Native-PAGE (10% T, 2.7% C) was performed as described by Flurkey (*9*). Duplicate samples were run for simultaneous protein and enzyme staining. The gel was stained for protein with 0.1% Coomassie Brilliant Blue R-250. APNE staining was performed for trypsin inhibitor activity. Glycoprotein staining was carried out following the periodic acid Schiff (PAS) glycoprotein staining method (*10*). SDS-PAGE (12.5% T, 2.7% C) was performed according to the method of Laemmli (*11*).

Glycolchitin-Embedded Polyacrylamide Gel Electrophoresis (Glycolchitin-PAGE). Glycolchitin-PAGE was performed by adding glycolchitin (0.05% w/v final concentration) to the polyacrylamide gel (10% T, 2.7% C). Following electrophoresis at pH 8.8, the gel was washed two times with distilled water and then incubated at 37 °C in 0.1 M sodium acetate buffer, pH 4.5, for 3 h. The gel was then washed with distilled water and stained with Congo Red (0.1%) for 10 min. The excess dye was removed by washing the gel with 0.1 M KCl solution. The contrast was enhanced for the development of a dark blue color by the addition of 5% acetic acid. Chitinase activity was observed as a clear area against a dark blue background (*12*).

Molecular Weight Determination. The apparent molecular weight of the native enzyme was determined by HPLC on a Biosep-SEC-S-2000 (300 × 7.8 mm) column, using a Waters Associate HPLC system equipped with a 1525 binary pump and a Waters 2996 photodiode array (PDA) detector. The eluent used was 0.1 M sodium phosphate buffer, pH 7.0, at a flow rate of 0.5 mL/min. The proteins were detected at 230 and 280 nm. The column was calibrated using aprotinin (6500 Da), cytochrome *c* (12400 Da), carbonic anhydrase (29000 Da), and bovine serum albumin (66000 Da). The molecular weight was also determined by SDS-PAGE (12.5% T, 2.7% C).

Matrix-Assisted Laser Desorption Ionization−Time of Flight−Mass Spectroscopy (MALDI-TOF-MS). The exact molecular mass of purified chitinase was obtained from MALDI-TOF-MS on a Bruker Daltonics Ultraflex MALDI TOF/TOF system, in the refractive positive ion mode. The sample was prepared in water/TFA (100:0.1 v/v). Data were collected between 10000 to 120000 Da.

Amino Acid Composition and Amino-Terminal Sequence Analysis. The purified protein was transferred from SDS-PAGE to polyvinylidine difluoride (PVDF) membrane in 10 mM CAPS/10% methanol buffer, pH 11.0, by electroblotting at 0.8 mA/cm² of constant current for 1.5 h. The membranes were stained with Coomassie Brilliant Blue R-250 and destained according to the method of Matsudaira (*13*). The bands corresponding to chitinase and trypsin inhibitors were excised. The excised bands were washed with methanol and loaded directly to the automated gas phase sequenator (Applied Biosystems 491-A Procise 4.0) for amino-terminal sequencing by automated Edman degradation.

The excised protein band was hydrolyzed in vacuum at 110 °C in constant boiling HCl for 24 h, using the Pico-Tag workstation. Amino acid analysis was performed by precolumn derivatization using phenyl isothiocyanate. The phenyl thiocarbamoyl amino acids were analyzed by RP-HPLC (*14*).

Multiple Sequence Alignment. To reveal subtle conserved family characters of chitinases and trypsin inhibitor families with the newly identified chitinase and trypsin inhibitors, multiple alignment was carried out. Seven closely related chitinases and trypsin inhibitor sequences were selected from the Protein Data Bank (SWIS PROT) released on March 2, 2007, and reported in the literature for comparative studies in Kumar et al. (*15*). The program MULTALIN (*16*) was used to align all of the chitinase and trypsin inhibitor sequences. In this method, all possible pairs of sequences were aligned initially using the Lipman and Pearson (*17*) FASTP algorithm. The similarity scores were then computed to determine the hierarchical order of clustering sequences. The alignments of sequences that have the highest score were initially accepted, and then the aligned pair was treated as a single sequence. Each further step then combines either two sequences or clusters or a sequence and a cluster. The similarity measures were re-evaluated after each combination. Aligned sequences were then analyzed using the PHYLIP program (*18*). The sequences were analyzed by the maximum parsimony method using the PROTOPARS program. The unrooted phylogenic tree was drawn.

Determination of p*I*. The isoelectric point (p*I*) was determined using an ampholine precast PAGE gel (245 × 110 × 1 mm, 5% T and 3% C), pH range 3.5−9.5. Orthophosphoric acid (1 M) and NaOH (1 M) were used as anode and cathode buffers, respectively. A Multiphore II electrophoresis unit and an EPS 3501 power supply were used. The gel was focused at 1500 V, 25 mA, and 15 W for 1.5 h. The gel was fixed in 10% TCA followed by staining with 0.1% Coomassie Brilliant Blue R-250.

Carbohydrate Estimation. The neutral sugar content was determined according to the phenol−sulfuric acid method (*19*). Glucose was used as standard (0–60 μg).

Effect of pH and Temperature. The effect of pH on the enzyme activity of purified chitinase was investigated in the pH range of 2.5−7.0. Chitosan (0.5%) solutions at various pH values were prepared by adjusting the pH with either 0.1 N HCl or 0.1 N NaOH. Chitosan precipitates above pH 7.0. Therefore, pH ranges above 7.0 could not be used. The effect of temperature on the chitinase activity was evaluated by incubating the substrate at temperatures ranging from 25 to 90 °C.

Thermal Stability. The purified chitinase was incubated at 45, 75, and 90 °C in a water bath preset to the appropriate temperature. Aliquots were removed at different time intervals (10–120 min) and assayed for the residual chitinase activity as described above.
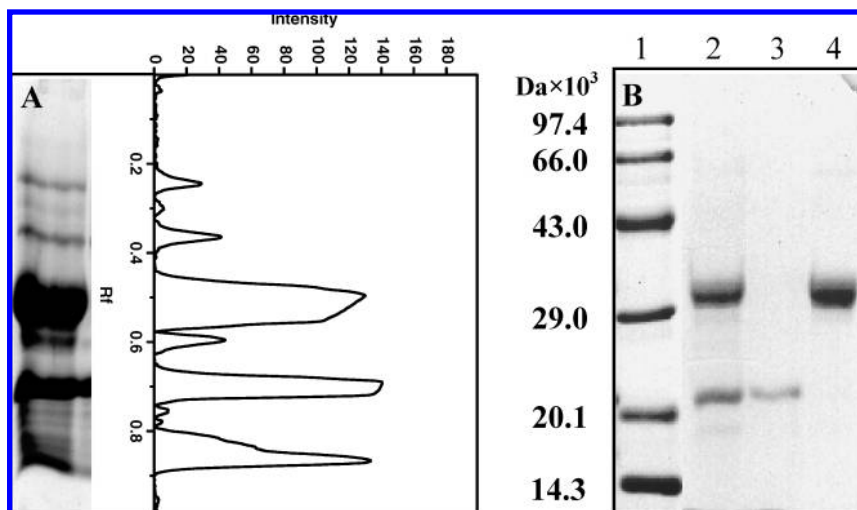
Tamarinin Is the Major Storage Protein of *T. indica* Seeds

J. Agric. Food Chem., Vol. 56, No. 6, 2008   **2177**

Figure 1. (**A**) Densitometric analysis of proteins resolved by SDS-PAGE (12.5% T, 2.7% C) of an alkaline extract of TKP and (**B**) SDS-PAGE analysis of the TKP [lane 1, molecular weight markers; lane 2, crude extract (10 μg of protein); lane 3, purified TKTI; lane 4, purified chitinase].

Seed Germination. Thirty healthy tamarind seeds were soaked in water for 48 h and then dispensed into the soil (2–3 in. deep). Germination of seed was monitored. Cotyledons were collected at different intervals of growth period. Proteins were extracted as mentioned above and analyzed by SDS-PAGE. Proteins extracted from the flowers, tender leaves, and mature leaves and samples were analyzed by SDS-PAGE.

Densitometric Analysis. After SDS-PAGE, the gels were scanned, and the protein bands were quantified using densitometry (Gel-Doc Multimager, Bio-Rad Laboratories) and expressed as OD values. Quantity One quantitation software (Bio-Rad Laboratories) was employed for the image analysis. Height and area ratios of selected chitinase bands were calculated from densitometric scans and compared. This method permitted the quantitation of chitinase at different days of germination.

RESULTS AND DISCUSSION

Identification of the Major Proteins of TKP. The abundance of proteins and polypeptides with varied biological activities in legume plants has made them the subject of several investigations. However, tree legumes have not gained much attention as alternative sources of protein. Solubilization of TKP proteins was higher in the alkaline extracts when compared to the acid extracts. Preliminary SDS-PAGE results showed that the number of peptides in the alkaline extracts was higher than those obtained from the acidic extracts (results not shown). The SDS-PAGE profile of the alkaline extract shows the presence of a range of proteins between 6500 and 66000 Da (**Figure 1A**). Densitometric analysis of the gel indicated the constitutive expression of two major polypeptides having molecular masses of ∼21000 and 34000 Da, accounting for 18 and 51% of the total protein, respectively (**Figure 1A**). These proteins were electroblotted and subjected to amino-terminal sequencing by Edman degradation. The amino-terminal sequences are listed in **Table 1**. When these were used in a search with the NCBI-BLAST program, the most abundant 34000 Da protein exhibited considerable sequence similarity to the class III endochitinases of legumes. The amino-terminal sequence of the second most abundant ∼21000 Da protein showed maximum homology to legume Kunitz-type trypsin inhibitors. The minor proteins were identified as enzymes of the carbohydrate metabolic pathway (**Table 1**). Both the Kunitz-type trypsin inhibitor and endochitinase are reportedly plant defense related molecules. Marangoni et al. (*1*) showed that SDS-PAGE of an alkaline extract of TKP resolved into three bands, of which one represented the principal

Table 1. Identification of the Proteins in the Alkaline Extract of Tamarind Kernel Powder

R_f value	protein abundance (%)	amino-terminal sequence	identified protein
0.244	5.43	HLRPSQG...	β-1,4-endoglucanase methyl transferases
0.363	6.80	GKPYGSAQQ...	glycoside hydrolases
0.495	48.10	WDDAAYAGVIS...	chitinases
0.694	16.72	DTVHDTDGKPV...	trypsin inhibitors
0.866	9.60	EERHEQG...	α-mannosidases, glucose-6-phosphate isomerases

protein fraction. However, no further effort was made to identify this protein or estimate the average molecular mass. They reported that electrophoresis in the absence of dissociating agents did not result in the further fractionation of these proteins. This led them to conclude that the TKP proteins were different from other legume seed proteins as reported earlier. Our results are in concurrence with the observations of Marangoni et al. (*1*). The reported size exclusion profile of an alkaline extract of TKP revealed one major fraction with at least five related minor fractions (*1*). It is reported that 70% of the nitrogen is solubilized by dilute alkali. The SDS-PAGE profile of the crude extracts therefore represents almost, if not, all of the TKP proteins.

Purification of the Endochitinases and Trypsin Inhibitor (TKTI). These results further provided the impetus to characterize these two proteins and ascertain their physiological role. Chitinases (EC 3.2.1.14) catalyze the hydrolytic cleavage of β-1,4 glycosidic bonds that occur in the biopolymer of *N*-acetyl glucose amine, mainly chitin. The chitinolytic activity of the crude extract of TKP assayed using chitosan (deacetylated chitin) was 1.5 units/mg of protein. This measured chitinase activity is unique to TKP despite the fact that chitin is not found in TKP, and yet it is the most abundant protein. It is reported that jellose accounts for 70% of the total polysaccharide (*3*). The high content of jellose in the extract hampered further purification of chitinase. Jellose was partially removed by allowing the crude extract to gel at 4 °C overnight. The gel was removed by centrifugation at 10000 rpm at 4 °C for 30 min. The clear supernatant concentrated by vacuum evaporation was applied to a chitin bead affinity column (2.5 × 8.5 cm) equilibrated in Tris-HCl buffer, pH 7.0, containing 0.5 M NaCl.
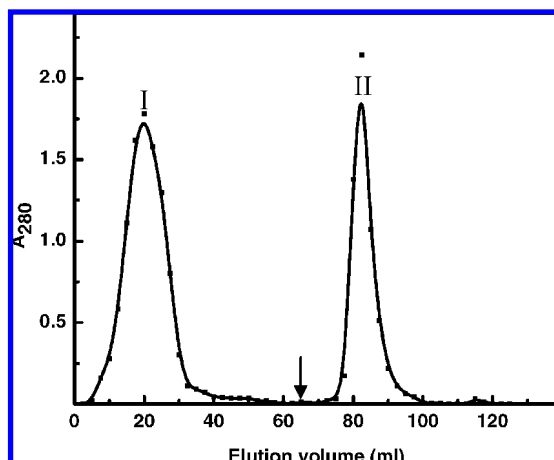
Figure 2. Chitin bead affinity chromatography profile of the alkaline extract of TKP. Peak I is the unbound fraction showing trypsin inhibitory activity, and peak II is the bound fraction showing chitinase activity. Arrow indicates start of elution with 20 mM NaOH containing 0.5 M NaCl.

A large amount of protein eluted as an unbound fraction in the buffer wash (**Figure 2**). The unadsorbed protein fractions showed trypsin inhibitory activity and no chitinase activity. These fractions were pooled and labeled as fraction I (TKTI, **Figure 2**). The bound protein was desorbed from the column using 20 mM NaOH containing 0.5 M NaCl. A single symmetrical protein peak that eluted with NaOH exhibited very low chitinase activity. These fractions were pooled as shown, the pH was adjusted to 7.0 (**Figure 2**), and the sample was stored at 4 °C. The specific activity of the purified chitinase was 2.46 units/mg with 64.6% yield and 1.6-fold purification (**Table 1**). The chitin binding affinity of the TKP chitinase was very strong, as revealed by the highly alkaline solution of 20 mM NaOH required to elute the bound chitinase. Such high-affinity binding has been reported for a class III chitinase of pumpkin leaves (*20*). Using a single affinity step, two plant defense proteins, TKTI and chitinase, were purified to homogeneity. The purified TKTI had a specific activity of 3000 units/mg of protein. Plants defend themselves against microbial and insect pests by accumulation of pathogenesis-related proteins, which include protease inhibitors and hydrolytic enzymes such as endochitinases capable of degrading cell wall polysaccharides (*21*). Therefore, the presence of these two defense proteins in the tamarind seed is not unexpected. However, the high abundance of these proteins is intriguing.

Homogeneity of the Purified Chitinase and TKTI. The homogeneity of the purified chitinase and TKTI was assessed by native-PAGE, amino-terminal sequencing, and RP-HPLC. The purified chitinase was electrophoresed in Tris—glycine buffer, pH 8.3, and located by enzyme staining with Congo Red. The purified enzyme revealed a single activity band by specific enzyme staining (**Figure 3A**, lane 1) and Coomassie Blue (**Figure 3A**, lane 2) indicating the presence of a single protein species. The RP-HPLC profile of the purified chitinase shows a single homogeneous peak confirming the presence of a single species (**Figure 4A**). The release of a single amino-terminal amino acid also indicated the purified chitinase to be homogeneous.

The purified TKTI was subjected to electrophoresis on a 10% PAGE at pH 8.3 and inhibitor located by specific inhibitor staining. The purified inhibitor resolved into three distinct inhibitor activities by APNE staining (**Figure 3B**, lane 1) and Coomassie staining (**Figure 3B**, lane 2) indicating the presence of three isoforms. The p*I* values of the three isoinhibitors were 5.97, 5.29, and 5.0, respectively. SDS-PAGE of the purified
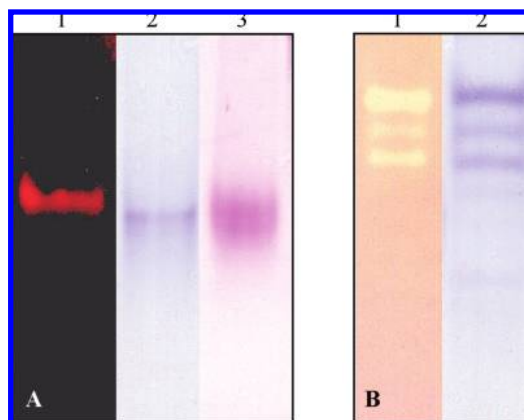

Figure 3. Native-PAGE (10% T, 2.7% C) of (**A**) purified chitinase (lane 1, chitinase activity; lane 2, protein; lane 3, glycoprotein staining) and (**B**) purified TKTI (lane 1, APNE zymography; lane 2, protein staining).

inhibitor indicates a single species of mass 21000 ± 1200 Da (**Figure 1B**). A comparison of the amino-terminal sequences of the isoinhibitors determined by Edman degradation indicates that the inhibitor (TKTI-I) with the highest R_f (relative mobility) differs at residue 4 from the TKTI-II and III (**Table 4**). The differences in the amino-terminal sequence, relative mobility in native-PAGE, and p*I* and the similar molecular masses reckon that the trypsin inhibitors exists as three isoforms, of which TKTI-I is the most abundant. The purified TKTI was evaluated for trypsin, chymotrypsin, and elastase inhibition. TKTI was found to inhibit only bovine trypsin and had no effect against chymotrypsin or elastase. The stoichiometry of trypsin inhibition was 1:1, indicating that it is a single-headed inhibitor. Serine protease inhibitors related to the Kunitz family of trypsin inhibitors have been purified and characterized from a variety of sources including tamarind seeds (*22*). Fractionation by Sephacryl 200-SH showed three inhibitor fractions, of which only one fraction was characterized. Araujo et al. (*22*) showed that the trypsin inhibitor (TTI) of mass 21420 Da possesses bioinsecticidal activity against different insect orders such as Coleoptera, Lepidoptera, and Diptera.

Molecular Weight of Purified Chitinase. Size exclusion chromatography of the purified enzyme was carried out by HPLC using a Biosep-SEC-2000 and FPLC using a calibrated Superdex-75 column. The native molecular mass of the purified enzyme was estimated to be 33000 ± 1000 Da. The exact molecular weight as determined by MALDI-TOF showed a monomeric mass of 33886.6 (**Figure 4B**). SDS-PAGE of the purified chitinase followed by protein staining (**Figure 1**, lane 4) showed a single polypeptide of mass ~33000 ± 1000 Da. The apparent subunit size was the same irrespective of the enzyme being reduced with β-mercaptoethanol or not. This value is in close agreement with the general molecular weight reported for chitinase from seeds (*23–25*). The molecular weight of most plant chitinases is ~30000 (*26*), whereas those of corn are much smaller, ranging from 10000 to 11500 Da (*27*). The legume chitinases from chickpea, bean leaves, and mung bean (*28*) have molecular masses over 30000 Da.

Amino-Terminal Sequence and Amino Acid Composition of Chitinase. The purified chitinase after SDS-PAGE was electroblotted onto PVDF and subjected to 28 cycles of automated Edman degradation (**Table 4**). The obtained sequence fell among the class III chitinases. The tamarind seed chitinase, like the pumpkin leaf class III Cchitinase (*20*), lacks the putative chitin binding domain (*29*) of other class III chitinases, yet binds strongly to the chitin bead affinity column (**Figure 2**). As
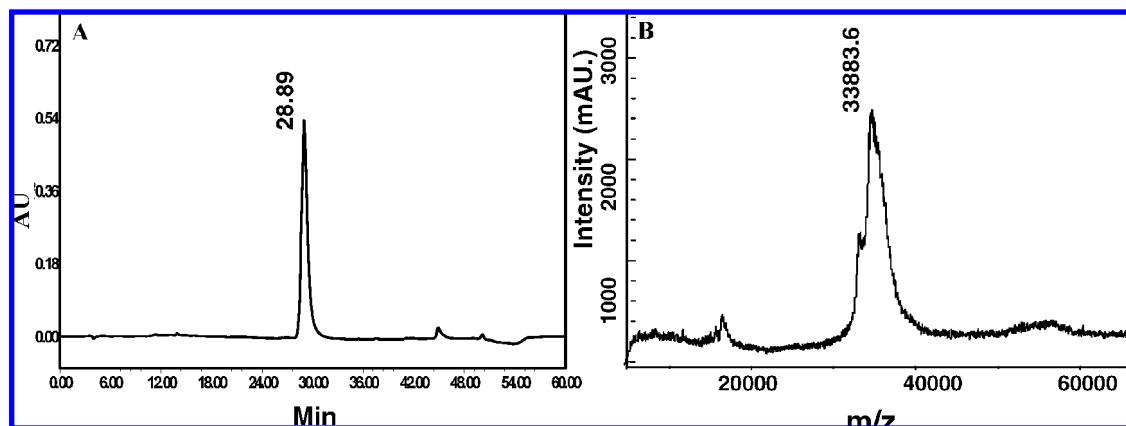
Figure 4. (**A**) RP-HPLC profile of the purified chitinase. The protein was analyzed on a Waters Symmetry Shield C18 column (150 × 4.6 mm, 5 μm) using a gradient of 0.1% TFA in water and 70% acetonitrile in 0.05% TFA at a flow rate of 0.7 mL/min and detected at 280 nm. (**B**) MALDI-TOF MS profile of chitin bead affinity chromatography purified chitinases.

Table 2. Summary of the Purification of Tamarind Kernel Chitinase[a]

purification step	total activity (μmol/min)	total protein (mg)	specific activity (μmol/min/mg)	yield (%)	fold purification
crude extract	49.5	33	1.5	100	
chitin bead affinity chromatography	32	13	2.46	64.6	1.64

[a] These are the results of a typical purification starting from 2 g of TKP. These values are reproduced in three separate purifications.

Table 3. Summary of the Purification of Tamarind Kernel Trypsin Inhibitor[a]

purification step	total trypsin inhibitory units (TIU)	total protein (mg)	specific activity (TIU/mg)	yield (%)	fold purification
crude extract	39000	33	1180	100	
chitin bead affinity chromatography	8400	2.8	3000	21.5	2.54

[a] These are the results of a typical purification starting from 2 g of TKP. These values are reproduced in three separate purifications.

Table 4. Amino-Terminal Sequence of Purified Proteins of Tamarind Kernel Powder

protein	amino-terminal sequence
tamarind acidic chitinase class III	WDDAAYAGVISVYWGQNGSDNSEGSLY...
tamarind kernel trypsin inhibitor I	DTVADTDGVPMKNNAGTYYIKPWASGGG...
tamarind kernel trypsin inhibitor II	DTVHDTDGXPVL...
tamarind kernel trypsin inhibitor III	DTVHDTDGXPVLNNA...

Table 5. Relative Amino Acid Composition of Purified Chitinase

amino acid	mol %	amino acid	mol %
Asx	10.72	Tyr	3.63
Glx	11.53	Val	5.97
Ser	8.16	Met	0.44
Gly	6.74	Cys	0.15
His	1.85	Ile	6.99
Arg	6.22	Leu	10.81
Thr	5.04	Pro	3.91
Ala	7.52	Lys	5.95
Pro	4.37		

suggested by Kim et al. (*20*), it is plausible that tamarind chitinase binds to another structural chitin binding domain dispersed in the enzyme. The amino acid composition of purified chitinase is listed in **Table 5**. The levels of Ser, Gly, Arg, Ala, Val, Ile, and Leu were high. The amino acid composition of the purified chitinase is very similar to that reported for the

total protein TKP. This observation is not unexpected, taking into consideration that the chitinase accounts for >50% of the total protein (**Figure 1A**).

Effect of Temperature and pH on Chitinase Activity. The maximum hydrolytic activity of the purified chitinase using partially acetylated chitin as the substrate was found at pH 5.0. The chitinase was relatively active at acidic pH (**Figure 5A**). Greater than half the maximal activity was measurable at pH 3.5. Most of the bacterial chitinases reported are active over a wide pH range of 4–7.5 (*30*). The chitinases purified from sorghum flour were found to be more reactive at an acidic pH of 5.0 (*31*). The optimal pH of the purified TKP chitinase was similar to those of the chitinase of mung bean (*28*), sweet potato leaves (*26*), cabbage (*32*), corn (*27*), and rye (*33*). The hydrolytic activity could not be evaluated beyond pH 7.0 owing to the insolubility of the substrate. The p*I* of the purified chitinase was 4.4. The only other chitinase of a leguminous plant with a known p*I* is that of mung bean, which is 6.33 (*28*).

The hydrolytic activity of the purified chitinase was maximal at 45 °C, but was active over the range of 25–80 °C. The purified TKP chitinase was thermostable, retaining its activity at 70 °C for 90 min. Thereafter, a decrease in activity was observed (**Figure 5C**). A mung bean chitinase optimally active between 40 and 50 °C was rapidly inactivated above 50 °C (*28*). The optimum temperatures of the chitinases purified from corn and cabbage were 45–55 °C (*27*) and 40–50 °C (*32*), respectively

Chitinase Is an Acidic Glycoprotein. Neutral carbohydrate analysis shows a total carbohydrate content of 6–7%. The glycoprotein nature of the chitinase was revealed by the positive PAS stain (**Figure 3A**, lane 3). The acidic p*I* and carbohydrate
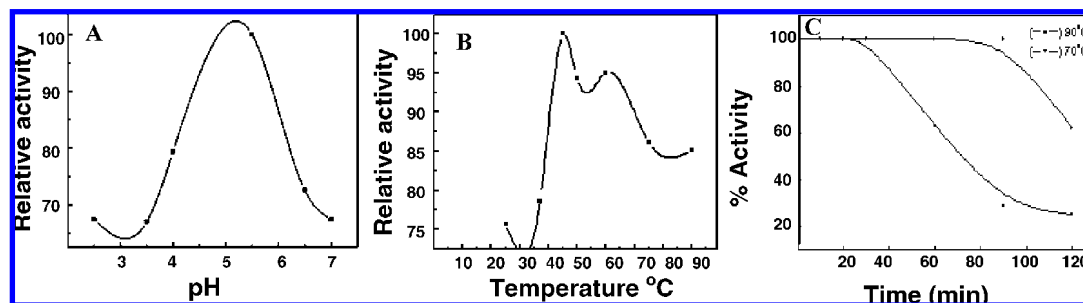
Figure 5. (**A**) Effect of pH on chitinase activity, (**B**) effect of temperature on chitinase activity, and (**C**) thermal stability profile of chitinase.
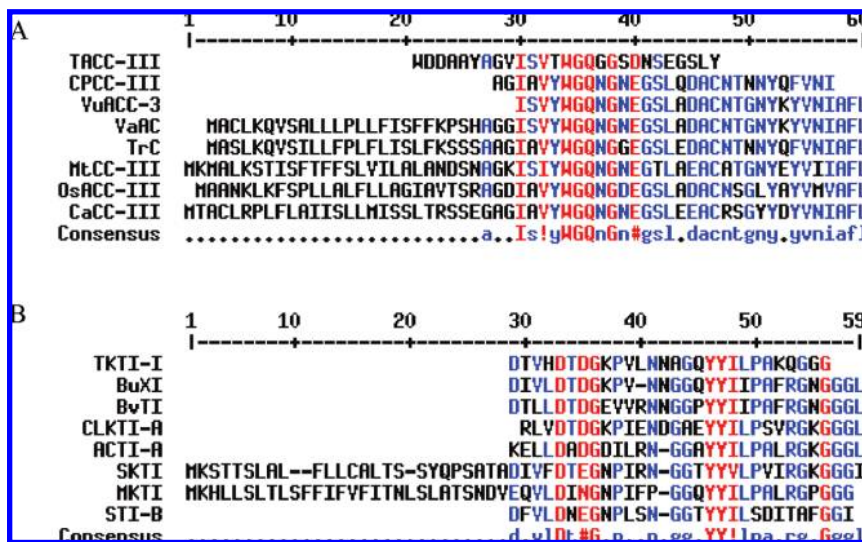

Figure 6. (**A**) Sequence alignment of tamarind acidic chitinase class III (TACC-III) with other chitinases from PDB: chickpea chitinase class III (CPCC-III), *Vigna unguiculata* acidic chitinase 3 (VuACC-3), *Vigna angularis* acidic chitinase (VaAC), *Trifolium repens* chitinase (TrC), *Medicago truncatula* chitinase class III (MtCC-III), *Oryza sativa* acidic chitinase class III (OsACC-III), and *Coffea arabica* chitinase class III (CaCC-III). (**B**) Sequence alignment of tamarind kernel trypsin inhibitor (TKTI) with other trypsin inhibitors from PDB: Bauun factor Xa inhibitor (BuXI), Bauva trypsin inhibitor (BvTI), *Copeifera langsdorffii* Kunitz trypsin inhibitor A chain (CLKTI-A), *Acacia confusa* trypsin inhibitor A chain (ACTI-A), soy Kunitz trypsin inhibitor (SKTI), *Medicago truncatula* Kunitz trypsin inhibitor (MKTI), and soy trypsin inhibitor B (STI-B).

content render it an acidic glycoprotein. The glycoprotein nature of the protein could well be the contributing factor for high thermal stability. The thermostability of TKP chitinase is higher than that of chitinase reported for mung bean (*28*).

Absence of Antifungal Activity. According to Schlumbaum et al. (*34*), plant chitinases are potent inhibitors of fungal growth. Therefore, the antifungal activity toward *Fusarium oxysporum*, *Curvularia lunata*, *Drechslera turcica*, *Sclerotium sclerotiarum*, and *Verticillium* species was evaluated. The tamarind seed acidic class III chitinase was devoid of any antifungal activity. This was unexpected as class III chitinases of mung bean, chickpea, and other legumes have been reported to inhibit the growth of *Fusarium solani*, *Trichoderma viride*, and *Sclerotium rolfsii* (*28*). A class III chitinase-like protein isolated from *Dolichos lablab* displayed antifungal activity in three different fungal species including *Rhizoctonia solani*, *F. oxysporum*, and *Coprinus comatus* (*35*). The absence of antifungal activity may be attributed to the negligibly low chitinase activity in the crude extracts (**Table 2**).

Physiological Role of Chitinase. Class III chitinases belong to the class of pathogenesis-related proteins, which act to destroy the cell wall of the invading fungi (*36–38*), have molecular masses of 30000–40000 Da, and are either basic or acidic (*39*). The purified chitinase is acidic by virtue of its pI with a mass of ~33883.6 Da and a very low chitinase activity and does not exhibit any antifungal activity and therefore differs from other plant chitinases. The alkaline extract of the pathogen-free

tamarind kernel shows that this protein is the most abundant protein (**Figure 1A**). The purified protein therefore does not fall under the group of PRPs. An abundant 31000 Da catalytically inactive homologue of class III acidic chitinase is reported to serve as the transient vegetative storage protein in banana pulp, which supplies amino acids for the synthesis of ripening-associated proteins (*40*). Therefore, it seemed plausible that the tamarind endochitinase with low enzyme activity serves as a storage protein in this tissue. Storage proteins are a heterogeneous group of proteins with no biochemical assays for their function, which display the following traits: (1) they are localized in protein bodies; (2) they account for 5% or more of the total protein; (3) they are stored for nutritional needs of a later developmental stage; and (4) they lack any other metabolic or structural role (*41*). The properties of the chitinase can be related to the above traits, and it is therefore designated "tamarinin". It is the most abundant protein in the alkali and acid extract of TKP (**Figure 1A**), accounting for >50% of the total soluble protein. The protein accumulates and is present only in the seed extracts. SDS-PAGE of the tamarind leaf and flower extracts reveals the absence of tamarinin (results not shown). A chitinase-related protein (CRP) accounts for 20–30% of the total soluble protein in banana pulp (*42*). Further evidence supporting a storage protein role for tamarinin emerges from the preferential degradation during germination. The SDS-PAGE profile indicated that the tamarinin content progressively decreased upon germination and eventually disappeared in the
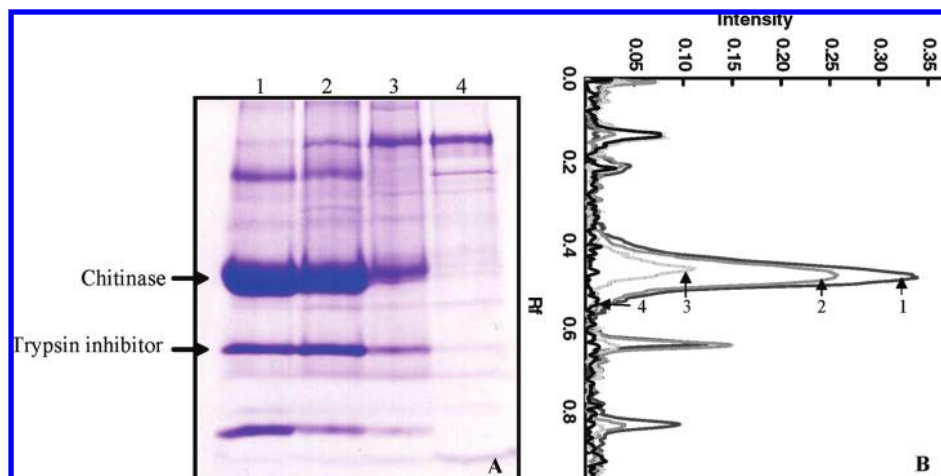
Figure 7. (**A**) SDS-PAGE (12.5% T, 2.7% C) analysis of the germination profile of tamarind seeds (lane 1, TKP crude extract; lanes 2, 3, and 4, 2−3, 6−8, and 13−15 days after germination, respectively); (**B**) densitometric analysis of the SDS-PAGE gels.

Table 6. Protein Profiling of the Germination of Tamarind Seeds

days after germination	2–3	6–8	13–15
protein concentration (mg/mL)	1.27	1.22	0.51
trypsin inhibition (TIU/mg)	1.53×10^3	1.48×10^3	0.69×10^3
chitinase activity (units/mg)	2.41	1.99	1.12
protease activity (units/mg)	0	0	0.7

cotyledon prior to senescence (**Figure 7**, lane 4). Densitometric analysis also showed that the protein with an R_f 0.45 corresponding to tamarinin was <1% of the initial level in the cotyledons. Tamarinin is preferentially degraded, wheres new proteins are synthesized (**Figure 7**, lanes 3 and 4). Germination of seeds requires the action of proteolytic enzymes, which hydrolyze the seed storage proteins to provide amino acids to the developing plant (*43*). Consistent with this observation is the appearance of a papain-like cysteine protease (**Table 6**) with a specific activity of 0.7 unit/mg with purified tamarinin as the substrate. The appearance of the cysteine protease occurs after 30–40% of the tamarinin is degraded. The activity of the protease increased 2-fold in the presence of 0.6 mM DTT. Aspartic proteases are often dominant in the resting seeds, but germination appears to increase the level of cysteine proteases (*44, 45*). Papain-like cysteine endopeptidases represent the major storage protein degrading enzymes in legumes (*46*). A papain-like cysteine endopeptidase from *Vicia faba* (*47*) and *Phaseolus vulgaris* (*48*) reaches maximum activity after a major portion of the stored protein is degraded. It has been suggested that seed aspartic proteases may initiate the hydrolysis of seed storage proteins before the massive de novo synthesis of cysteine proteases (*49*). Similar endopeptidases have been found in the cotyledons of developing, mature, and germinating soybean (*50*).

The amino acid composition of tamarinin indicates that 37% of the residues have N-containing side chains. This is approximately the same proportion of N-containing amino acids as in the vegetative storage protein of banana (*42*) and soybean. These data suggest that tamarinin serves as a nitrogen sink source. It is well established that storage proteins typically lack any metabolic or structural activity (*41, 51*). Tamarinin exhibits a very low or negligible endochitinase activity as measured using soluble chitosan (**Table 2**). However, tamarinin has no effect on chitobiose, chitotriose, or any of the chromogenic substrates commonly used to assay chitinase. The abundance of tamarinin in the tamarind seed, the amino acid composition, preferential degradation during germination, and the absence of a catalytic role in plant defense unambiguously demonstrate that it is a

"seed storage protein". This is the first report that the major seed storage protein of a legume is a constitutive acidic endochitinase-related protein. The low catalytic activity of tamarinin may have a role in the protection of the embryo and young seedling and in addition may serve as a protectant of stored reserve.

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