JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification, Characterization, and Solvent-Induced Thermal Stabilization of Ficin from *Ficus carica*

KAMSAGARA BASAVARAJAPPA DEVARAJ, PARIGI RAMESH KUMAR, AND VISHWESHWARAIAH PRAKASH*

Department of Protein Chemistry and Technology, Central Food Technological Research Institute (A Constituent Laboratory of the Council of Scientific and Industrial Research), Mysore 570020, India

Ficin (EC 3.4.22.3), a cysteine proteinase isolated from the latex of a *Ficus* tree, is known to occur in multiple forms. Although crude ficin is of considerable commercial importance, ficin as such has not been fully characterized. A major ficin from the commercial crude proteinase mixture preparation of *Ficus carica* was purified and characterized. The purified enzyme was homogeneous in both sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and gel-filtration chromatography and is a single polypeptide chain protein with a molecular mass of 23 100 ± 300 Da as determined by matrix-assisted laser desorption ionization−time of flight (MALDI−TOF). The enzyme was active in the pH range of 6.5−8.5, and maximum activity was observed at pH 7.0. The N-terminal core sequence of ficin has homology with N-terminal sequences of plant cysteine proteinases. The enzyme contains three disulfide bonds and a single free cysteine residue at the active site. The effect of co-solvents, such as sorbitol, trehalose, sucrose, and xylitol, on the thermal stability of ficin was determined by activity measurements, fluorescence, and thermal denaturation studies. The apparent thermal denaturation temperature (T_m) of ficin was significantly increased from the control value of 72 ± 1 °C in the presence of all co-solvents. However, the maximum stabilization effect was observed in terms of thermal stabilization by the co-solvent trehalose.

KEYWORDS: ***Ficus*; fig; cysteine protease; ficin; co-solvents; stabilization; sorbitol; trehalose; thermal denaturation temperature**

INTRODUCTION

Proteolytic enzymes play fundamental roles in many physiological processes, from generalized protein degradation and protein maturation to more specific regulated functions. Thus, characterization of specific proteolytic enzymes helps in better understanding their roles in physiological processes as well as in commercial applications. Proteinases being the most widely used enzymes in the food, pharmaceutical, and detergent industries constitute about 60% of the total worldwide sale of enzymes (*1*). The number of industrially used proteases of plant origin is small. Papain, bromelain, and ficin are the most commercially important endopeptidases from the plant sources. Among these plant proteases, only papain and bromelain have been extensively used in medicine and many industrial applications as compared to ficin (*2*). This is probably because little information is available on physical, chemical, and enzymatic properties of ficin.

Ficin (EC 3.4.22.3), a proteolytic enzyme present in the latex of fig trees and recognized as a sulfhydryl enzyme, contains a cysteine residue at the active site essential for its activity (*3*). Until now, most of the work has been carried out on the enzyme prepared from the latex of *Ficus glabrata* and a very few studies have been carried out on the ficin isolated from the latex of *Ficus carica* species. The ficin isolated from both of these species is known to occur naturally in multiple forms, distinguishable by ion-exchange chromatography (*3*). Englund et al. have studied a major ficin among the several active components of the latex of *F. glabrata* and showed that ficin is a single polypeptide chain protein (*4*). Sgarbieri et al. reported the separation of several proteolytic components by CM-cellulose chromatography from the lattices of both *F. glabrata* and *F. carica* (*5*). The available information indicates that ficin apparently shares many common properties with papain with regard to substrate specificity, esterase activity, transpeptidase reactions, and activation by reducing agents (*3*). The amino acid sequence determined for neighboring residues of active-site cysteine was found to resemble closely the corresponding one in papain (*6*). In this study, we have purified and characterized ficin from the commercial crude proteinase mixture preparation from the latex of *F. carica* for its enzymatic and chemical properties, which would help for its understanding structure and function of ficins in general from different *Ficus* species and also other plant cysteine proteases.

* To whom correspondence should be addressed: Central Food Technological Research Institute, Mysore 570 020, India. Telephone: +91-821-2517760. Fax: +91-821-2516308. E-mail: prakash@cftri.com.

10.1021/jf802205a CCC: $40.75
Published on Web 11/08/2008

Protein stability is one of the most important issues that determine the commercial viability of enzymes. The problem of stability is of great concern when these proteins/enzymes are used in biotechnological process, such as therapeutics, diagnostics, bioreactors, biosensors, and fine chemicals. Hence, the stability with respect to structure and biological activity needs to be considered during production, isolation, purification, storage, and ultimate application of the protein product. Upon extraction of proteins from their biological sources, they are subjected to different environments, which may bring the conformational changes directly related to protein stability. The introduction of co-solvents, such as sugars and polyhydric alcohols, into the solvent medium has been found to stabilize biological macromolecules in solutions (*7*−*9*). The co-solvents interact with protein in a diverse way, depending upon the physicochemical properties of the proteins. The co-solvents are known to prevent the loss of enzymatic activities, inhibit irreversible aggregation, and increase the thermal transition temperature of macromolecules (*8*−*10*). The detailed and through studies of Timasheff and co-workers have proven that the ability of co-solvents to stabilize the structure and function of proteins is related to the preferential hydration of macromolecules (*9*−*11*). Preferential hydration is a thermodynamic phenomenon that reflects the inability of co-solvent molecules to interact with proteins; thus, it leads to an exclusion of these solvent molecules from the protein surface (*10, 12*). Among the several ways of enzyme thermostability enhancement, the use of additives is of much importance, owing to their structure thermal stabilizing effect.

MATERIALS AND METHODS

Chemicals. Ficin (*F. carica*) Lot 031K76652, benzoyl-D,L-arginine *p*-nitroanilide hydrochloride (BAPNA), *p*-chloromercuribenzoate (PCMB), iodoacetamide, sodium tetrathionate, polyvinyledine difluoride membrane (PVDF), bovine serum albumin (BSA), acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), 2-mercaptoethanol, casein, cysteine hydrochloride, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), sucrose, D-sorbitol, trehalose, and D-xylitol were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. All other chemicals used were of analytical/HPLC grade obtained from E-Merck, Mumbai, India.

Purification of Ficin. Ficin was purified using a SP-Sepharose cation-exchanger column on fast protein liquid chromatography (FPLC). The crude preparation of ficin obtained commercially from Sigma was dissolved in sodium acetate buffer at pH 5.0 (0.05 M) and centrifuged. The clear supernatant ficin solution was directly applied to a Hiload 16/10 SP-Sepharose high-performance column (Amersham Biosciences AB, Uppsala, Sweden), pre-equilibrated with the same buffer at 20 °C. The column was washed with 2 column volumes (about 40 mL) of buffer to remove unbound fractions. The bound proteins were eluted with a linear gradient of 0−0.6 M NaCl in the same buffer at the flow rate of 1.0 mL/min. The total gradient elution volume was 100 mL, which constitutes about 6 column volumes. The elution profile of proteins as shown in **Figure 1** separated into five fractions. The major ficin fraction II was collected separately and pooled. The pool was dialyzed extensively against 0.05 M sodium phosphate buffer at pH 7.0, and this fraction was used in all further studies.

Ficin Assay. Activity of ficin was measured using casein and benzoyl-D,L-arginine *p*-nitroanilide hydrochloride (BAPNA) as substrates. The assay with casein as the substrate was based on the method described by Kunitz (*13*). The casein concentration of 1% (w/v) was prepared in sodium phosphate buffer at pH 7.0 containing 5 mM cysteine hydrochloride. To 1 mL of substrate, 1 mL of purified enzyme containing 0−10 μg of ficin was added, and the reaction mixture was incubated at 55 °C for 20 min. The reaction was stopped by the addition of 2 mL of 10% trichloroacetic acid (TCA), incubated for 10 min at room temperature, and centrifuged. The absorbance of soluble peptides in the supernatant was measured at 280 nm. In the case of the blank, substrate was added after the enzyme was first inactivated by TCA. One unit of activity is defined as the amount of enzyme that increases the absorbance by 0.001 min^{-1} under given assay conditions.

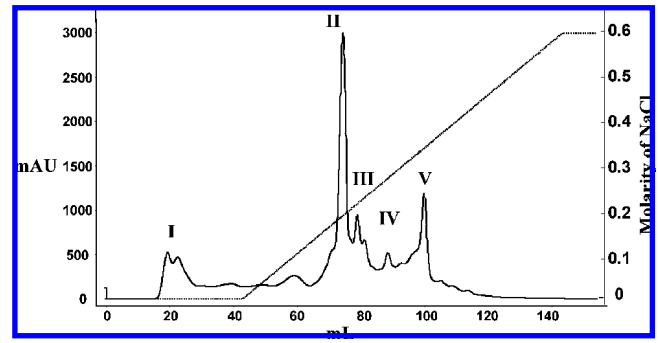

Figure 1. Chromatographic separation of crude preparation of ficin on FPLC using a Hiload 16/10 SP-Sepharose high-performance column (Amersham Biosciences AB, Uppsala, Sweden). The column was equilibrated with 0.05 M sodium acetate buffer at pH 5.0. The bound proteins were eluted using a linear gradient of 0−0.6 M NaCl in the same buffer, and the eluted fractions were read at 280 nm.

Enzyme activity of ficin was also quantified by measuring its ability to cleave an amide bond in small-molecular-weight synthetic substrate BAPNA (*4*). The extent of hydrolysis was determined by measuring the product (*p*-nitroaniline) formed at 410 nm (ε = 8800 for *p*-nitroaniline). Activity of ficin is expressed as the amount of *p*-nitroaniline formed by 1 mol of protein per min. In the case of thermal inactivation studies, all of the enzyme activity was measured in both the presence and absence of the third component in 0.05 M sodium phosphate buffer containing 5 mM cysteine hydrochloride.

Protein Determination. The protein concentration was quantified by the dye binding method of Bradford (*14*). BSA was used as the standard protein.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electophoresis (SDS−PAGE). SDS−PAGE was carried out using a discontinuous buffer system according to the method of Laemmli (*15*), using 12% gel (acrylamide concentration of 12% with a bisacrylamide cross-linker concentration of 2.7%). Electrophoresis was performed after denaturing the ficin with SDS and β-mercaptoethanol, and the gel was stained with Coomassie brilliant blue R-250.

Gel-Filtration Chromatography. The homogeneity and apparent molecular mass of the purified ficin was determined on FPLC using a Superdex-75 HR 10/30 column (Amersham Biosciences AB, Uppsala, Sweden) calibrated with ovalbumin (43 kDa), carbonic anhydrase (29 kDa), myoglobin (17.6 kDa), ribonuclease (13.7 kDa), and aprotinin (6.5 kDa). The column was equilibrated with 0.02 M sodium phosphate buffer at pH 7.0, and proteins were eluted at a flow rate of 30 mL/h.

Mass Spectrometry. The molecular mass of the purified ficin was determined by matrix-assisted laser desorption ionization−time of flight (MALDI−TOF). MALDI−MS analysis were performed on a Bruker Daltonics Ultraflex MALDI−TOF/TOF system (Bruker-Daltonics, Bremen, Germany) in the reflective positive-ion mode. The purified enzyme was dialyzed extensively against water. The samples were prepared by mixing equal volumes of protein and matrix. Matrix was saturated with α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie GmbH), prepared separately in CH_3CN/H_2O/TFA (80:20:0.1). The samples were then dried at 25 °C under atmospheric pressure and loaded onto a probe slide for measurements. The instrument was calibrated with the masses ranging between 10 000 and 100 000 Da.

Effect of pH on Enzyme Activity. The effect of pH on the enzyme activity of ficin was evaluated from pH 4.0 to 9.5 using the following buffers: 50 mM citrate buffer (pH 4.0−6.0), 50 mM sodium phosphate buffer (pH 6.5−7.5), and 50 mM Tris-HCl buffer (pH 8.0−9.5). Activity of the ficin was determined at 55 °C using BAPNA as the substrate.

Effect of Group-Specific Reagents. The purified ficin was pre-incubated with 5 mM solutions of PCMB, iodoacetamide, and 10 mM

sodium tetrathionate in buffer at pH 7.0 and 37 °C for 30 min, and the residual activities were measured. The enzyme activity without these reagents was taken as the control (100%), and the relative activities were calculated.

N-Terminal Sequence. The purified ficin was transferred following SDS−PAGE to a polyvinylidene difluoride (PVDF) membrane in 10 mM CAPS−10% methanol buffer by electroblotting at 0.8 A/cm^2 at constant current for 1.5 h and stained with Coomassie brilliant blue R-250. The band corresponding to the ficin was excised and loaded directly to the gas-phase sequencer (Applied Biosystems 447A, Rotkreu, Switzerland) for automated Edman degradation. β-Lactoglobulin (Applied Biosystems) was used as the standard to calibrate the instrument.

Amino Acid Analysis. Amino acid analysis of purified ficin was performed according to the method of Bidlingmeyer et al. (*16*) using the Waters Associates Pico-Tag amino acid analysis system (Waters Corporation, Milford, MA). Tryptophan content of ficin was estimated by the spectroscopic titration method (*17*) as described by Spande and Witkop using *N*-bromosuccinimide (NBS). Free cysteine and disulfide of ficin were determined using the sulfhydryl-specific reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The assay was performed according to the method described by Ellman (*18*).

Extinction Coefficient. The extinction coefficient of ficin was determined by the Kjeldahl nitrogen estimation method (*19*). The absorbance of different concentration solutions of purified ficin was recorded at 280 nm. These solutions were used for nitrogen estimation, and a conversion factor of 6.25 was used to calculate the protein content of the samples. The specific extinction coefficient was calculated using Beer−Lambert's law: $E^{1\%}_{280\ \mathrm{nm}} = Acl$, where A is absorption at 280 nm, c is the concentration of protein in mg/mL, and l is the path length of the cuvette in centimeters (1 cm).

Effect of Co-solvents on Thermal Stability. Thermal stability of purified ficin was determined by incubating the enzyme solution at 70 °C in the presence of different concentrations of co-solvents for 10 min in sodium phosphate buffer at pH 7.0 using a water bath with shaker. The enzyme was immediately cooled in ice after incubation, and the remaining activity was measured at 55 °C using BAPNA as the substrate. The enzyme activity in the absence of the co-solvent served as the control, and the percentage residual activity was calculated on the basis of its original activity. The percentage residual activity was plotted against different concentrations of co-solvents. Thermal inactivation of ficin was also performed at 70 °C at different time intervals in the presence of different co-solvents [30% (w/v) concentration].

Thermal Denaturation Studies. The effect of pH and different concentrations of co-solvents on the thermal denaturation profile of ficin was performed using a Cary Varian 100-bio UV−vis spectrophotometer (Varian, Victoria, Australia). The spectrophotometer was equipped with an electronically controlled thermal cuvette holder, which can accommodate six thermal quartz cuvettes. The thermal unfolding of ficin was monitored by recording the absorbance at 287 nm as a function of the temperature in the range of 30−90 °C at a scan rate of 1 °C per min. The concentration of protein solution throughout the experiment was 1.05×10^{-5} M. The apparent T_m of ficin was obtained after normalizing the absorbance of the native and denatured states. From the thermal denaturation profile, the fraction of ficin unfolded was calculated using standard equation (*20*)

$$F_u = \frac{Y_F - Y}{Y_F - Y_U}$$

where Y_F is the absorbance of the protein solution in the native state, Y_U is the absorbance of the protein solution in the unfolded state, and Y is the absorbance of the protein solution at different temperatures. The apparent thermal denaturation temperature (T_m) is defined as the temperature at which the value of F_u is 0.5.

Fluorescence Spectroscopy. Fluorescence measurements were recorded on a Shimadzu RF-5000 spectrofluorometer (Shimadzu, Kyoto, Japan) using a 10 mm path-length quartz cell at 25 °C. For both, excitation and emission slit widths were set at 5 nm. The protein was excited at 280 nm, and emission was recorded between 300 and 400 nm. Appropriate blanks were used for baseline correction of the fluorescence intensity.

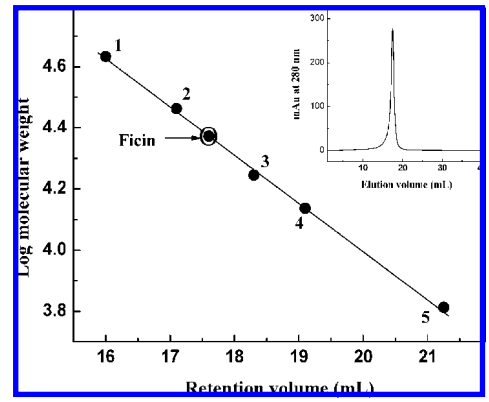

Figure 2. Assessment of the homogeneity and molecular mass by gel-filtration chromatography using a Superdex 75 column on FPLC (Amersham Biosciences AB, Uppsala, Sweden). The column was calibrated with (1) ovalbumin, (2) carbonic anhydrase, (3) myoglobin, (4) ribonuclease, and (5) aprotinin. The column was equilibrated using 0.02 M sodium phosphate buffer at pH 7.0, and the proteins were eluted at the flow rate of 30 mL/h. (Inset) Elution profile of purified ficin from the column.

RESULTS AND DISCUSSION

Purification of Ficin. The lattices of *F. glabrata* and *F. carica* are known to contain multiple forms of proteolytic components (*3*). These multiple forms of ficin can be distinguished by ion-exchange chromatography. Hence, the commercial crude preparation of ficin from the *F. carica* was subjected to cation-exchange chromatography using a SP-Sepharose column on FPLC. The column was equilibrated with sodium acetate buffer at pH 5.0 (0.05 M). **Figure 1** shows the chromatographic separation of proteolytic components of the crude preparation of ficin. These components have been designated as ficin I, II, III, IV, and V in order of their elution from the column. A previous report by Sgarbieri et al. has shown the separation of nine proteolytic components when salt-fractionated *F. glabrata* latex was subjected to CM-cellulose chromatography (*5*). Similar to the results in this study, the presence of six active fractions of ficin from the latex of *F. carica* was also reported by Richter et al., when the crude ficin was subjected to a CM-cellulose column on FPLC (*21*).

The major ficin fraction II (which constituted about ∼60% of total activity) was collected separately and subjected to further studies. Hence, throughout this paper, ficin simply refers to fraction II. The purified ficin showed the activity of 6824 ± 26 units per mg of protein against casein. The activity of ficin was also studied using synthetic substrates BAPNA and showed the activity that 1 mol of enzyme hydrolyses 0.65 mol of BAPNA per min. These results indicated a relatively low level of activity with BAPNA when compared to casein hydrolysis. The exhibition of the higher activity of ficin from *F. glabrata* with casein and a relatively low level of activity with BAPNA was also reported by Englund et al. (*4*).

Criteria of Purity and Molecular Mass. The homogeneity of the ficin fraction II was evaluated by SDS−PAGE and gel-filtration chromatography on FPLC using a Superdex 75 column. In both cases, the purified enzyme was found to be homogeneous. The purified ficin eluted as a single peak on gel-filtration chromatography, and the molecular mass was determined to be 23 400 ± 500 Da (**Figure 2**). As shown in the **Figure 3a**, the purified ficin migrated as a single band on SDS−PAGE. These results put together indicated that the purified ficin is homoge-

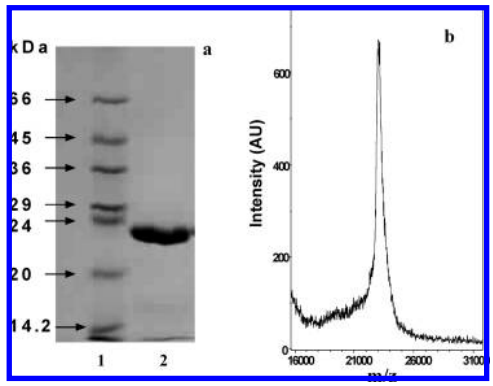

Figure 3. (a) SDS−PAGE pattern of the purified ficin using 12% gel. Lane 1, molecular-mass markers; lane 2, purified ficin. (b) MALDI−TOF spectrum of the purified ficin.

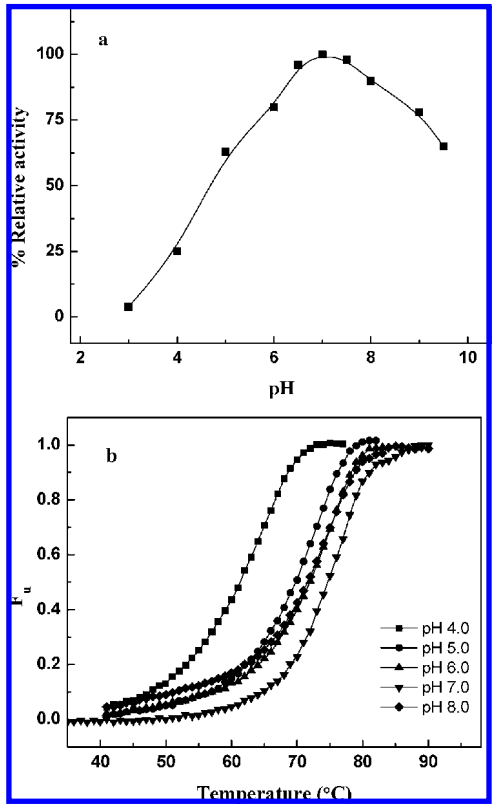

Figure 4. (a) Effect of pH on the activity of the purified ficin. Experimental conditions and buffer used are as specified in the Materials and Methods. (b) Thermal denaturation profile of ficin as a function of pH.

Table 1. Comparison of the N-Terminal Sequence of Ficin to N-Terminal Sequences of Other Plant Cysteine Proteases

proteases	N-terminal sequences
ficin (*Ficus carica*)	L P E S V D W A R F G A V N
papain (*Carica papaya*)	I P E Y V D W R S K G A V T
chymopapain (*Carica papaya*)	Y P Q S I D W R A K G A V T
bromelain (*Ananus comosus*)	A V P Q S I D W R D Y G A V
papaya proteinase Ω (*Carica papaya*)	L P E N V D W R K K G A V T
actinidin (*Actinidia chinensis*)	L P S Y V W R S A G A V V D

neous and the enzyme is a single polypeptide chain protein. The molecular mass of ficin is also determined by MALDI−TOF, and it was found to be 23 100 ± 300 Da (**Figure 3b**). These results are comparable to earlier reports. The estimated molecular mass of purified ficin falls in the range of 20 000−35 000

Table 2. Amino Acid Composition of Purified Ficin

amino acids	gram percentage	number of residues (value to the nearest integer)
Asp	8.0 ± 0.15	15
Glu	12.2 ± 0.30	22
Ser	6.0 ± 0.05	16
Gly	7.4 ± 0.10	29
His	1.2 ± 0.10	2
Arg	8.8 ± 0.20	13
Thr	4.0 ± 0.20	9
Ala	6.8 ± 0.15	21
Pro	2.5 ± 0.10	6
Tyr	7.1 ± 0.10	10
Val	7.7 ± 0.10	16
Met	0.6 ± 0.05	1
Cys	3.1 ± 0.05	7
Ile	4.4 ± 0.10	9
Leu	8.3 ± 0.20	17
Phe	3.8 ± 0.10	6
Trp[a]	4.8 ± 0.10	6
Lys	3.3 ± 0.15	5

[a] Percent tryptophan is determined by a spectrophotometric method using NBS.

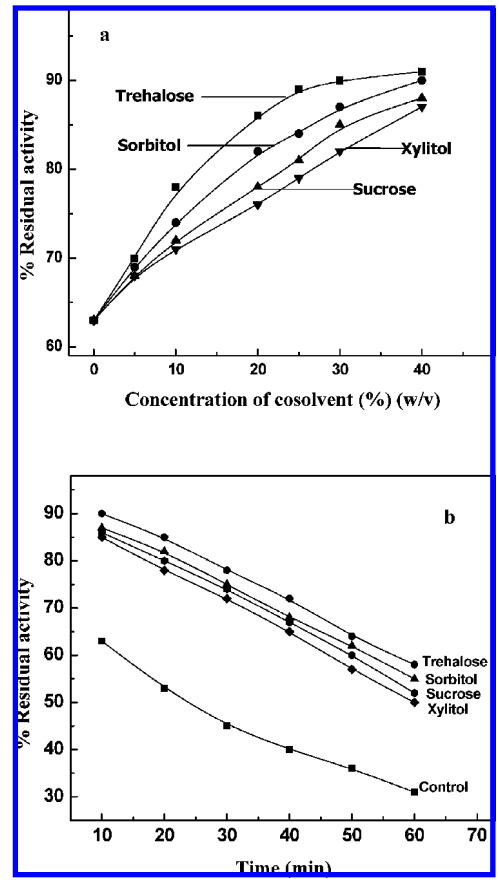

Figure 5. Effect of co-solvents on thermal inactivation of ficin. (a) Residual activity of ficin heated at 70 °C for 10 min in the presence of different concentrations of co-solvents at pH 7.0. (b) Time course thermal inactivation of ficin at 70 °C in the presence of co-solvents [at 30% (w/v) concentration].

Da, which is also the reported range of values for many other cysteine proteases (*22*). The molecular mass of ficin fractions isolated from *F. glabrata* is in the range between 25 000 and 26 000 Da (*4, 23, 24*).

pH Optima and Stability. The purified ficin has a pH activity profile as shown in the **Figure 4a**. The maximum activity was observed between pH 6.5 and 8.5, and its optimum was shown at pH 7.0. The results show that the enzyme is more active in the

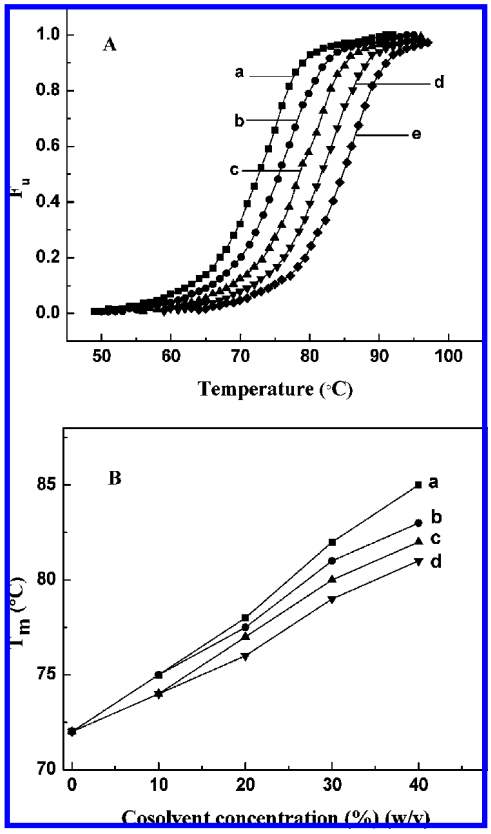

Figure 6. (A) Effect of trehalose on the thermal denaturation profile of ficin as monitored by changes in the absorbance at 287 nm as function of the temperature: (a) control, (b) 10%, (c) 20%, (d) 30%, and (e) 40% trehalose. (B) Apparent thermal denaturation temperature (T_m) of ficin as a function of different concentrations of co-solvents: (a) trehalose, (b) sorbitol, (c) sucrose, and (d) xylitol.

neutral range as compared to the acidic range. The lowest activity was observed in the acidic range, indicating the liable nature of ficin in acidic conditions. The thermal denaturation profile of ficin as a function of pH is shown in the **Figure 4b**. The apparent thermal denaturation temperature (T_m) decreased as the pH decreases or increases from the optimum pH 7.0. The results again confirmed that the enzyme is unstable at acidic pH and the enzyme is most stable at pH 7.0. Similar results have been reported earlier by Kramer and Whitaker (*25*) that the different ficin fractions from *F. carica* are more active in the neutral pH range, and the acid-liable nature of ficin from *F. glabrata* was also observed by Englund et al. (*4*).

Effect of Specific Reagents on Ficin. The activity of the purified ficin was determined in the presence of cysteine-group-specific inhibitors, such as PCMB, iodoacetamide, and sodium tetrathionate. The ficin activity was completely inhibited in the presence of these inhibitors, which clearly confirms the participation of the cysteine residue at the active site of the enzyme.

N-Terminal Sequence. The first 14 residues of the N-terminal sequence determined for the purified ficin showed the following sequence from the amino-terminus end, NH_2-L P E S V D W A R F G A V N. The first amino-terminal residue of ficin is Leu, and the previous investigations have also shown the presence of Leu at the amino-terminus end of ficin (*21, 24*). Although there was some variation, the core of this sequence was common to proteases of 18 species that we found using the blast network service search machine (http://www.ch.embnet.org/index.html), in the protein databases (www.expasy.org) hosted by Swiss Institute of Bioinformatics (SIB). The comparison of the N-terminal sequence of ficin to similar sequences of other plant cysteine proteases (*26*) is shown in **Table 1**. The core sequence has homology in all plant proteases of the cysteine protease family, indicating the conservation of residues at the amino-terminus end.

Table 3. Apparent Thermal Denaturation Temperature (T_m) of Ficin in Different Co-solvents

co-solvent concentration (%) (w/v)	apparent $T_m \pm 1$ (°C)			
	trehalose	sorbitol	sucrose	xylitol
control[a]	72	72	72	72
10	75	75	74	74
20	78	77	77	76
30	82	81	80	79
40	85	83	82	81

[a] Ficin in 0.05 M sodium phosphate buffer at pH 7.0.

Amino Acid Analysis. Amino acid composition of purified ficin is shown in the **Table 2**. With few exceptions, the amino acid composition of purified ficin is similar to the ficin isolated from *F. glabrata* (*27*). The free cysteine and disulfide content of the ficin was determined by the Ellman method using DTNB. The purified ficin contains three disulfide bonds and a single free cysteine residue, upon which the activity depends. The cysteine content and disulfide bridges of the purified ficin are similar to the ficin from *F. glabrata* (*4*) and other plant cysteine proteases (*22*). The above-determined analytical observations reveal many similarities with respect to enzymatic activity, molecular mass, pH optima, thermal stability, amino acid composition, and the role of disulfide bonds among the ficin fractions of *F. carica* and *F. glabrata*.

Extinction Coefficient. The extinction coefficient value ($E^{1\%}_{280\,nm}$) for the purified ficin was determined using a nitrogen estimation method, and it was found to be 20.9 ± 0.2. This value is consistent with the amino acid composition of ficin. The extinction coefficient value range of 15−25 has been reported for many plant cysteine proteases (*22*), and our value is found to occur in this range. The $E^{1\%}_{280\,nm}$ value of 20.2 ± 0.7 for different fractions of ficin was previously reported by Kramer and Whitaker (*25*). Englund et al. have also shown the $E^{1\%}_{280\,nm}$ value of 21.0 for the major ficin isolated from *F. glabrata* (*4*). These results further indicate the similarity of ficin fractions from *F. carica* and *F. glabrata*.

Effect of the Co-solvent on Thermal Stability. The effect of different co-solvents on the thermal inactivation of ficin was examined by enzyme activity measurements. Ficin was heated in the presence of different concentrations of co-solvents at 70 °C for 10 min, and the remaining activity was measured. **Figure 5a** shows the percent residual activity of ficin in the presence of different concentrations of co-solvents. Ficin looses 37% of its activity at 70 °C after 10 min of incubation, and in the presence of co-solvents, the activity was protected up to 91%. The protection of enzyme activity by co-solvents increased with an increasing concentration of each co-solvent. The maximum protection was shown by trehalose and followed by sorbitol, sucrose, and xylitol. Enhanced thermal stability of enzymes in the presence of co-solvents in a concentration-dependent manner are reported (emperically) in some of the earlier studies (*11, 12*). Ficin was also incubated at 70 °C at different time intervals in the absence and presence of co-solvents (at 30% concentration) to determine the stabilizing capacities of co-solvents. Ficin looses nearly 50% of its activity after 20 min of incubation at 70 °C. **Figure 5b** shows the increased thermal stability of ficin in the presence of co-solvents. In the presence of trehalose, the enzyme retained 85% of its activity after 20 min and 53% even

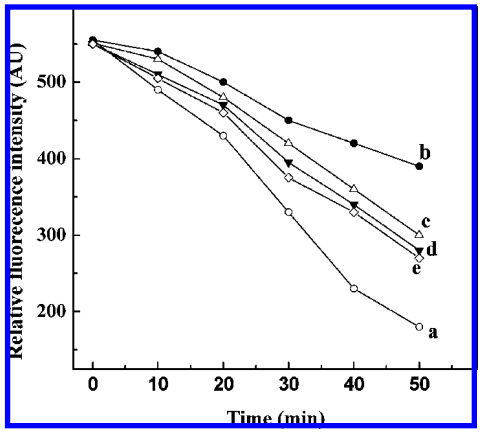

Figure 7. Time course of thermal denaturation of ficin at 70 °C, in the presence of co-solvents (at 30% concentration). The fluorescence intensity at 347 nm was monitored after incubation of ficin for different time intervals: (a) control is without co-solvents in 0.05 M sodium phosphate buffer at pH 7.0 only and in the presence of (b) trehalose, (c) sorbitol, (d) sucrose, and (e) xylitol.

after 60 min of incubation. In the case of sorbitol, sucrose, and xylitol, the enzyme retained the activity of 82, 80, and 78%, respectively, after 20 min of incubation. The results presented here are most likely that of the co-solvents, which stabilize the various proteins/enzymes (*7*−*10*). The presence of co-solvents has been shown to prevent the thermal inactivation of many enzymes (*11, 12*). Recently, Sathish et al. (*8*) have shown the stabilizing effect of co-solvents on papain, which is also a cysteine protease.

Thermal Denaturation Studies. The thermal denaturation profile of ficin was studied in the presence of co-solvents by monitoring the absorbance at 287 nm as function of the temperature in the range of 30−95 °C. These results further demonstrated the stabilization effect of co-solvents. The apparent denaturation temperature (T_m) of ficin was found to be 73 °C. **Figure 6A** shows the denatutration profile of ficin in the presence of different concentrations of trehalose. The apparent T_m shifted from the control value of 73 to 85 °C in the presence of 40% trehalose. All co-solvents used were able to increase the apparent T_m of the ficin (**Figure 6B**). The presence of different co-solvents has been shown to elevate the thermal denaturation temperature of various proteins (*8, 12*). The apparent T_m of ficin in the presence of 10, 20, 30, and 40% sorbitol was 75, 77, 81, and 83 °C, respectively. The presence of other co-solvents also showed the same effect, and the results are summarized in **Table 3**. The increments in the thermal denaturation temperature of proteins/enzymes in the presence of different co-solvents have been extensively studied (*8, 11, 28, 29*).

Fluorescence Studies. The effect of the temperature on the conformation of ficin in the presence and absence of co-solvents was also tested by fluorescence measurements. Fluorescence emission signals are very sensitive to the structural changes of a macromolecule. Ficin was exposed at 70 °C for different time intervals in the presence and absence of co-solvents, and their fluorescence emission spectra were recorded. **Figure 7** shows a significant reduction in the intensity of intrinsic fluorescence at 347 nm in the case of the control. The reduction in intrinsic fluorescence intensity reflects the unfolding of the enzyme tertiary structure, which is dependent upon the time of exposure (*30*). The presence of all co-solvents protected this intensity reduction at different time intervals. The co-solvents, such as sorbitol, trehalose, sucrose, and xylitol, significantly enhance the thermal stability of ficin, and the maximum stabilization effect was observed with trehalose. The results presented in this study perhaps will help in understanding the properties of ficin from different *Ficus* species and plant cysteine proteases.

ABBREVATIONS USED

SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate; BAPNA, benzoyl-D,L-arginine *p*-nitroanilide hydrochloride; SP-Sepharose, sulfopropyl-Sepharose; TCA, trichloroacetic acid; HCl, hydrochloric acid; NaCl, sodium chloride; BSA, bovine serum albumin.

LITERATURE CITED

(1) Rao, M. B.; Tanksale, A. M.; Ghatge, M. G.; Deshpande, V. V. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 597–635.
(2) Uhlig, H. Description of enzymes: Proteases. In *Industrial Enzymes and Their Applications*; Linsmaier-Bednar, E. M., Ed.; John Wiley and Sons, Inc.: New York, 1998; pp 146−179.
(3) Liener, I. E.; Friedenson, B. Ficin. *Methods Enzymol.* **1970**, *19*, 261–273.
(4) Englund, P. T.; King, T. P.; Craig, L. C.; Walti, A. Studies on ficin I. Its isolation and characterization. *Biochemistry* **1968**, *7*, 163–175.
(5) Sgarbieri, V. C.; Gupte, S. M.; Kramer, D. E.; Whitaker, J. R. *Ficus* enzymes I. Separation of the proteolytic enzymes of *Ficus carica* and *Ficus glabrata* lattices. *J. Biol. Chem.* **1964**, *239*, 2170–2177.
(6) Liener, I. E.; Friedenson, B. The active site sequence of multiple forms of ficin. *Arch. Biochem. Biophys.* **1972**, *149*, 169–174.
(7) Arakawa, T.; Timasheff, S. N. Stabilization of protein structure by sugars. *Biochemistry* **1982**, *21*, 6536–6544.
(8) Sathish, H. A.; Kumar, P. R.; Prakash, V. Mechanism of solvent induced thermal stabilization of papain. *Int. J. Biol. Macromol.* **2007**, *41*, 383–390.
(9) Timasheff, S. N.; Lee, J. C.; Pittz, E. P.; Tweedy, N. J. The interaction of tubulin and other proteins with structure-stabilizing solvents. *Colloid Interface Sci.* **1976**, *55*, 658–663.
(10) Timasheff, S. N.; Arakawa, T. Stabilization of protein structure by solvents. In *Protein Structure: A Practical Approach*, 2nd ed.; Creighton, T. E., Ed.; IRL Press: Oxford, U.K., 1997; pp 349−364.
(11) Gekko, K.; Timasheff, S. N. Mechanism of protein stabilization by glycerol: Preferential hydration in glycerol−water mixture. *Biochemistry* **1981**, *20*, 4667–4676.
(12) Rajendran, S.; Radha, C.; Prakash, V. Mechanism of solvent-induced thermal stabilization of α-amylase from *Bacillus amyloliquefaciens*. *Int. J. Pept. Protein Res.* **1995**, *45*, 122–128.
(13) Kunitz, M. Crystalline soybean trypsin inhibitor: General properties. *J. Gen. Physiol.* **1947**, *30*, 291–310.
(14) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(15) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(16) Bidlingmeyer, B. A.; Cohen, S. A.; Travin, T. L. Rapid analysis of the amino acids using pre-column derivartization. *J. Chromatogr.* **1984**, *336*, 93–104.
(17) Spande, T. F.; Witkop, B. Determination of the tryptophan content of proteins with *N*-bromosuccinimide. *Methods Enzymol.* **1967**, *11*, 498–506.
(18) Habeeb, A. F. S. A. Reaction of protein sulfhydryl groups with Ellman's reagent. *Methods Enzymol.* **1972**, *25*, 457–464.
(19) Jaenicke, L. A rapid micromethod for the determination of nitrogen and phosphate in the biological material. *Anal. Biochem.* **1974**, *61*, 623–627.

(20) Pace, C. N.; Scholtz, J. M. Measuring the conformational stability of a protein. In *Protein Structure: A Practical Approach*, 2nd ed.; Creighton, T. E., Ed.; IRL Press: Oxford, U.K., 1997; pp 299−321.
(21) Richter, G.; Schwarz, H. P.; Dorner, F.; Turecek, P. L. Activation and inactivation of human factor X by proteases derived from *Ficus carica*. *Br. J. Haematol.* **2002**, *119*, 1042–1051.
(22) Turk, B.; Turk, V.; Turk, D. Structural and functional aspects of papain like cysteine proteases and their protein inhibitors. *Biol. Chem.* **1997**, *378*, 141–150.
(23) Williams, D. C.; Whitaker, J. R. Multiple molecular forms of *Ficus glabrata* ficin: Their separation and relative physical, chemical, and enzymatic properties. *Plant Physiol.* **1969**, *44*, 1574–1583.
(24) Jones, I. K.; Glazer, A. N. Comparative studies on four sulfhydryl endopeptidases (Ficins) of *Ficus glabrata* latex. *J. Biol. Chem.* **1970**, *245*, 2765–2772.
(25) Kramer, D. E.; Whitaker, J. R. *Ficus* enzymes II. Properties of the proteolytic enzymes from the latex of *Ficus carica* variety *kodata*. *J. Biol. Chem.* **1964**, *239*, 2178–2183.
(26) Lee, K. L.; Albee, K. L.; Bernasconi, J.; Edmundus, Y. Complete amino acid sequence of ananain and a comparison with stem bromelain and other plant cysteine proteases. *Biochem. J.* **1997**, *337*, 199–202.
(27) Kortt, A. A.; Hamilton, S.; Webb, E. C.; Zerner, B. Ficins (EC 3.4.22.3). Purification and characterization of the enzymatic components of the latex of *Ficus glabrata*. *Biochemistry* **1974**, *13*, 2023–2028.
(28) Park, H.; Kidman, G.; Northrop, D. B. Effect of trehalose on pressure-induced inactivation of yeast alcohol dehydrogenase. *Protein Pept. Lett.* **2005**, *12*, 597–599.
(29) Zancan, P.; Sola-Penna, M. Trehalose and glycerol stabilize and renature yeast inorganic pyrophosphatase inactivated by very high temperatures. *Arch. Biochem. Biophys.* **2005**, *444*, 52–60.
(30) Eftink, M. R. The use of fluorescence methods to monitor unfolding transitions in proteins. *Biophys. J.* **1994**, *66*, 482–501.

Received for review July 18, 2008. Revised manuscript received September 11, 2008. Accepted September 26, 2008. A Senior Research Fellowship to Mr. K. B. Devaraj from the Council of Scientific and Industrial Research, New Delhi, India, is gratefully acknowledged.

JF802205A