MATERIALS AND METHODS
MATERIALS and METHODS

MATERIALS

Molecular biology grade guanidine hydrochloride (GdnHCl), Sephadex G - 50 superfine, blue dextran, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, soybean trypsin inhibitor, cytochrome C, CAPS, TPCK treated α - trypsin (Lot - 121K7692), TLCK treated α - chymotrypsin (Lot - 109H7485), sinapic acid, phytic acid from rice, allyl isothiocyanate, dithiothreitol (DTT), iodoacetamide, acrylamide, bis-acrylamide, sodium dodecyl sulfate, tris base, TNS, TNBS, EDC, BHT, DTNB, succinic anhydride, and urea were from Sigma Chemical Company (St. Louis, MO, USA). Bio - Gel P-30 was from BIO-RAD laboratories (Richmond, CA). CM-Sephadex C - 50 was from Pharmacia Fine Chemicals, Upssala, Sweden. Ampholine PAGplate and isoelectric focusing standards of broad pl (3.5 - 9.3) were from Amersham Biosciences, UK. 8 - anilino - 1 - naphthalene sulfonic acid (ANS) was from Aldrich Chemical Co., Milwaukee, USA. cis - Parinaric acid and PRODAN was from Molecular probes. Standard low molecular weight electrophoresis marker kit was from Genei, Bangalore. Solvents used were of HPLC grade. Milli-Q grade water was used for preparing reagents. All other chemicals were of analytical grade.

Mustard seeds (Brassica juncea) ‘Argani’ variety, was procured from National Seed Corporation (NSC) Ltd, Mysore, India, were finely ground, defatted with hexane, dried and stored at 27°C. All experiments were carried out in 0.02 M phosphate buffer pH 7.0 (buffer A).
Isolation and Purification

Purification of napin

Napin was purified as reported earlier with minor modifications (Aruna and Appu Rao, 1988). Purification was carried out using 0.02 M phosphate buffer pH 7.0 (buffer A) unless indicated. The protein from defatted *B. juncea* meal (5 g) were extracted with buffer A containing 1.0 M NaCl for 60 min (1:10) at 10°C. The suspension was centrifuged for 20 min to recover supernatant (2980 x g, 10°C), which was subjected to ammonium sulfate precipitation. The ammonium sulfate pellet (15 – 40%) was dissolved in buffer A containing 0.1 M NaCl and dialyzed against the same buffer. The dialyzed protein was loaded onto a Sephadex G - 50 (1.5 x 200 cm, 350 mL) pre-equilibrated with buffer A containing 0.1 M NaCl. The peak corresponding to 2S protein (170 - 185 mL) was pooled dialyzed against buffer A and fractionated on a CM-Sephadex column (2.1 x 7 cm, 25 mL). Bound protein was eluted with a linear gradient of 0 M – 0.5 M NaCl in buffer A at a flow rate of 20.0 mL/h. Protein concentration was determined by measuring the absorbance at 280 nm using a value of $E^{1\%}_{1\text{cm}} = 4.8$ (Aruna and Appu Rao, 1988).

Determination of homogeneity and molecular weight of napin

Electrophoresis

Homogeneity of the preparation was ascertained by non-reducing and reducing SDS–PAGE on a 15% vertical slab gel in presence of 0.1% SDS
according to the method of Laemmli (Laemmli, 1970). β- Mercaptoethanol was added to reduce the protein.

**Native gel**

Native-PAGE (10%) was run in 0.2 M glycine-HCl buffer, pH 4.0 and stained with 1% amido black in 7% acetic acid. The polarities were reversed during the run. Methylene green was used as the tracking dye.

**Gel permeation chromatography using HPLC**

Gel filtration was performed on Waters HPLC system (Waters, Milford MA), equipped with a 1525 binary pump and Waters 2996 photodiode array detector. Gel filtration was carried out using TSK-Super SW2000 (4.6 mm x 300 mm, 4.0μ) column. The column was pre equilibrated with buffer A, and 20 μg of protein was injected. The elution of the sample was carried out isocratically using the same buffer. The flow rate was maintained at 0.2 mL/min at 25°C and detected at 230 and 280 nm by PDA detector. The analysis of the data was done by Waters Millennium software provided by the manufacturer. The column was calibrated with standard proteins (Cytochrome C-12,500 Da, Insulin- 5800 Da, Soybean trypsin inhibitor-20,000 Da, Carbonic anhydrase-29,000 Da, BSA-66,000 Da).

**RP-HPLC**

Homogeneity was also ascertained by reversed phase HPLC using symmetry shield™ RP<sub>18</sub> (5.0 μ, 4.6 mm X 250 mm) Waters column, with solvent A (0.1% TFA in water) and solvent B (100% acetonitrile containing
0.1% TFA). The column was washed with solvent A for 10 min. Protein sample containing 20 μg / 20 μl was injected at a flow rate of 1 mL/min. The bound protein was eluted by a shallow gradient of acetonitrile from 0-50% over time span of 70.0 min. The eluted protein was monitored at 230 and 280 nm. Gel filtration chromatography was carried out using the above column, in presence of 0.1 and 1 mM DTT. The column was equilibrated with buffer A containing DTT. All other conditions were as given above.

**Mass spectrometry**

The molecular weight of the napin protein was analyzed by MALDI-TOF after RP-HPLC. The peak fraction was collected, concentrated under vacuum (speed vac Sawant, Mumbai) and analyzed. Matrix Assisted Laser Desorption Ionization Mass Spectroscopy (MALDI MS) analyses were performed on a Bruker Daltonics Ultraflex MALDI TOF/TOF system (Bruker-Daltonics, Bremen, Germany) in the reflective positive ion mode, equipped with a nitrogen laser of 337 nm. The samples were prepared by mixing equal volumes of samples (prepared in H₂O/TFA 100:0.1 and dialyzed against H₂O) and saturated matrix prepared separately in CH₃CN/H₂O/TFA (80:20:0.1) α-cyano-4-hydroxycinnamic acid (Sigma Aldrich Chemie GmbH). The amount of protein loaded on the probe slide was ~ 10 pmol. The samples were then dried at 25°C under atmospheric pressure. Data was collected between 0-60,000 Da.
Western blotting

PVDF membrane preparation: The membrane was cut to the required size and soaked for 2 min in methanol. Membranes are transferred into buffer containing 10% methanol and equilibrated for 10 min. Napin was reduced and loaded onto 15% SDS - PAGE. Gels were washed 4 - 5 times to remove glycine after the completion of electrophoresis. The gel was equilibrated in CAPS buffer for 10-15 min. Electrophoretic transfer (Mini Trans-Blot ®; Bio-Rad) was [in 10 mM 3-[cyclohexylamine]-1-propane sulfonic acid (CAPS) (pH 11.0) with 10% (v/v) methanol] carried out in a semi-dry blotting apparatus for 4 h using a current of 0.8 mA / cm² of the filter paper, at 100 V and 25°C. The membranes were stained with Ponceau stain, subunit bands excised and subjected to N- terminal sequencing by automated Edman degradation on an Applied Biosystems Procise® 4.0 sequencer.

N- terminal sequencing

The cut membrane was placed directly onto the fibre disc. The coupling reaction was carried out with Edman reagent phenyl isothiocyanate in presence of gaseous trimethylamine. Excess reagents were removed and cleavage was carried out with gaseous TFA. Free ATZ - amino acids (Anilino thiazolinone) were converted to PTH - amino acids with 25% TFA. These are separated on RP - HPLC and recovery was calculated using CR4A system. The results were recorded.
Trypsin inhibitor assay

Trypsin inhibition assay was carried out according to Smith et al., (Smith et al., 1980) using synthetic substrate BAPNA. To 1 mL of trypsin solution (4 mg/100 mL in 0.001N HCl), different amounts of napin were added and the volume is made upto 2 mL with water. This was incubated at 37°C for 5 min. Freshly prepared BAPNA solution (40 mg/mL) in tris-HCl buffer of pH 8.0 containing 0.05 M CaCl₂ was added and the resulting mixture was incubated for 10 min at 37°C. The reaction was terminated by the addition of 1 mL of 30% acetic acid and the absorbance was measured at 410 nm against the reagent blank. Trypsin was added after the addition of all other regents for blank. A trypsin standard was run in absence of inhibitor. Inhibition was tested for napin, subunits and peptide.

Tryptophan estimation in napin

The tryptophan content in napin was determined according to Spande and Witkop. Protein solution (1.2 mg/mL) at pH 4.0 was titrated with 10 mM NBS in water. Titration was continued until minimum optical density at 280 nm was observed. A molar extinction coefficient of $\varepsilon_{280}$ for tryptophan – 5500 M⁻¹cm⁻¹ was used for the calculation (Spande and Witkop, 1967)

Cysteine estimation

Cysteines were estimated according to the method of Riddles and Blackley. Napin was dialyzed against 0.1 M phosphate buffer pH 7.2 (1 mM EDTA) with 5 - 6 changes for 24h at 8°C. Stock solution of Ellman’s reagent in
buffer was added to reference and sample cuvette. Increase in the absorbance was monitored at 412 nm. Molar extinction coefficient of $\varepsilon_{412} = 14150 \text{ M}^{-1} \text{ cm}^{-1}$ was used for the calculation of total number of cysteines (Riddles et al., 1983).

**Tyrosine ionization**

Tyrosine ionization was carried out according to the method of Donovan (1973). Ionization of phenolic group increases with increasing the pH. Napin solution (1% w/v) in glycine - HCl varying pH (8 – 11.5) was prepared by dialyzing against respective pHs. Absorbance was read at 280 nm after centrifugation. The number of hydroxyl group ionization was calculated using molar extinction coefficient of $\varepsilon_{295} = 2480 \text{ M}^{-1}\text{cm}^{-1}$. A titration curve was constructed by plotting the number of phenolic group ionized as a function of pH (N-acetyl tyrosine ethyl ester).

**Amino acid composition**

The peak fraction of gel filtration purified napin and concentrated peptide fraction from HPLC were analyzed directly for total amino acids. The composition was determined according to the method of Bidlingmeyer et al., (1984) using Waters Pico-tag amino acid analysis system. Napin (5 mg/mL) was hydrolyzed for 24 h at 110°C with dry 6N HCl containing 1 % (v/v) phenol, in test tubes that had been sealed under vacuum. The total cysteine content in the protein was also determined by performic acid oxidation (Hirs, 1967). Performic acid was prepared by mixing formic acid, methanol and hydrogen
peroxide in the ratio of 9.6: 0.2: 1. The resultant solution was mixed well allowed to stand for 2h at room temperature, cooled and stored at 4°C in dark.

Protein was dissolved in formic acid mixture and cooled on ice for 30 min. Performic acid oxidation was allowed to precede for overnight (12h) at -5°C. The solution was diluted with water and freeze-dried. The freeze-dried protein was digested with 6N HCl for 24 h at 110°C. The standard amino acids as a mixture (Pierce H) containing 25 nmol of each amino acids were placed samples were dried under vacuum along with hydrolyzed protein. The samples were redried after treating (10 - 20 µL) with ethanol: water: triethanolamine (2 : 2 : 1). Amino acids were derivatised with phenyl isothiocyanate. The phenyl thiocarbonyl amino acids were analyzed by RP - HPLC using specific PICO-TAG™ amino acid analysis column (15 x 3.9 cm) with binary gradient. Temperature was maintained at 37 ± 1°C with column heater. The solvents used were 0.14 M sodium acetate containing 0.5 mL trimethylamine and titrated with glacial acetic acid to get pH 6.4 and 6% acetonitrile (Solvent A) and 60% acetonitrile in water (Solvent B). A gradient was run from 0-46% in 10 min at a flow rate of 1 mL/min. The peaks were detected at 254 nm.

**Stability: Contribution of electrostatic & hydrophobic interactions**

**Isoelectric focusing**

Isoelectric focusing was carried out according to the method described elsewhere (Jyothi et al., 2005). Isoelectric focusing of the napin and separated subunits was performed at 10°C on an Amersham Biosciences multiphor II
apparatus. The polyacrylamide gel, Ampholine PAGplate with a pH range 3.5 – 9.5 was used. The electrode wicks for the anode and cathode were soaked for 4 – 5 min in 1.0 M phosphoric acid and 1.0 M sodium hydroxide, respectively, and the excess electrolyte was removed. Broad pl kit (3.5 – 9.3) was used as standard. Protein (3 mg/mL) and separated subunits (2 mg/mL) along with markers were loaded directly onto the gel with sample application pieces. The gel was electrofocused for 90 min at constant 15W with a maximum of 1500V and 50 mA. The electrofocused gel was stained with coomassie brilliant blue R – 250.

**Conformational studies using proteolytic enzymes**

Conformational studies were carried out using TPCK treated α- trypsin. Napin was digested with trypsin using an enzyme to substrate ratio of 2 : 100. The digestion was performed in 0.05 M ammonium bicarbonate buffer (pH 8.1) at 37°C. Samples were drawn at regular intervals of 15 min and the degree of hydrolysis estimated by TNBS method (Nissen, 1979).

The hydrolytic behavior of trypsin on napin was also monitored by SDS – PAGE and RP - HPLC.

**Peptide purification**

Trypsin digested napin was loaded onto CM - Sephadex column (0.8 x 6 cm, 12 mL) which was pre-equilibrated with buffer A. Bound peptides were eluted with 0 - 0.5 M NaCl in buffer A gradient. Also for N - terminal sequencing of peptide purification was carried out using RP-HPLC, symmetry shield™ RP18 (5.0 μ, 4.6 mm x 250 mm) Waters column. Solvent 0.1% TFA in
water and 100% acetonitrile containing 0.1% TFA were used for gradient elution. The column was washed with Solvent 0.1% TFA in water for 10 min. Trypsin digested napin (5 mg/mL) was injected at a flow rate of 1 mL/min. The bound protein was eluted by a shallow gradient of acetonitrile from 0 - 50% over time span of 70.0 min. The eluted peptide was monitored at 230 nm and peak fraction was collected and concentrated under vacuum.

The amino terminal sequence of the purified peptide was carried out on Applied Biosystems Procise® 4.0 instrument. Molecular weight of the peptide was analyzed by MALDI - TOF. The purified peptide was spectroscopically characterized using CD, fluorescence and absorption.

**Separation of subunits**

The large and small subunits were separated according to reported procedures (Menendez-Arias et al., 1987) with a little modification. Napin was denatured with 8.0 M urea for 30 min and reduced by 0.1 M dithiothreitol at 27°C for 2 h. Free cysteines were blocked using 0.2 M iodoacetamide in dark for an hour. Subunits were separated on G - 50 Sephadex gel filtration column (140 x 0.5 cm, 110 mL). Purity of the two separated peaks (Elution volume: 40 - 48 mL, 60 - 66 mL) was ascertained by 17% SDS-PAGE and gel filtration on HPLC. Further structural characterization of separated subunits was carried out by absorption, fluorescence and CD spectroscopy. Purity of the separated subunits was ascertained by 17% SDS-PAGE and gel filtration HPLC.
Hydropathy plot

Hydropathy plot was constructed according to Kyte and Doolittle (1982). The plot was constructed using the available amino acid sequence (Brassica juncea CAA46785). Scores assigned for the most hydrophobic and for most hydrophilic amino acid were +4.5 and -4.5, respectively.

Surface hydrophobicity measurements

Napin surface hydrophobicity was measured using ANS, TNS, CPA and PRODAN. Hydrophobicity was calculated according to Cardamone and Puri (1992). The binding constants were determined using Scatchard plot. A stock solution of ANS ($\varepsilon_{350} 4.95 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (Cardamone and Puri, 1992) and TNS ($\varepsilon_{366} 4.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (McClure and Edelman, 1967) was prepared in buffer A whereas CPA ($\varepsilon_{303} 7.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), and PRODAN ($\varepsilon_{360} 1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) were prepared in ethanol and methanol (analytical grade), respectively. For CPA, 10 µg of butylated hydroxyanisole (BHA) was added per mL of ethanol, to prevent oxidation. The stock solution was transferred into a brown bottle and stored at 4°C (Alizadeh - Pasdar and Li-Chan, 2000). All reagents were prepared fresh.

Excitation wavelengths were 375, 366, 325 and 365 nm for ANS, TNS, CPA and PRODAN, respectively. The emission spectra were collected from 400 - 500 nm using a 1 cm path length cell. The excitation and emission slit widths were 5 and 5 nm, respectively. Appropriate blanks in the corresponding solvents were subtracted to obtain the fluorescence enhancement caused by the fluorescent probe. Binding constants for the
above ligands with napin were also determined in presence of NaCl and Na$_2$SO$_4$. The association constant was calculated using Scatchard plot (Scatchard, 1949)

The ligand concentration after additions given by,

$$[L]_T = \frac{SV_t}{V_i + V_t}$$

Where,

$S = $ the stock concentration of ligand

$V_t = $ the net volume of ligand added

$V_i = $ the initial volume of protein

After each addition, the sample was mixed, allowed to equilibrate at 27°C and the fluorescence emission intensity was recorded. Values of $\Delta F$ were also corrected for the sample dilution arising from successive ligand additions.

$$\frac{[L]_B}{[L]_F} = K_n p - K [L]_B$$

(1)

$p = $ Protein concentration
n = No of ligand binding sites

K = Association constant

[L]_B = ligand bound per mole of protein

[L]_F = Concentration of free ligand

\[ Q = \frac{\Delta F_{\text{max}}}{[L]} \]

\[ [L]_F = [L]_T - [L]_B \]

**Fluorescence quenching measurements**

To determine the solvent exposure of tryptophan residues, fluorescence-quenching measurements were made with the progressive addition of acrylamide and potassium iodide in absence or presence of 0.5 M NaCl and 0.2 M Na₂SO₄. Protein solutions were centrifuged at 10,000 rpm for 30 min before the scans. Titration was carried out with excitation and emission slit widths of 5 and 10 nm respectively. Napin concentration was fixed at 0.1 mg/mL.

Sodium thiosulfate was added (0.1mM) to KI solution to prevent I⁻³ formation. Since the absorption of the KI at the excitation wavelength is not detectable, no correction was given for the inner filter effect. The absorption of acrylamide at 280 nm was corrected using the Lehrer and Leavis equation.

\[ F_{\text{corr}} = F_{\text{obs}} \times 10^{A/2} \]
From the recorded titration spectra, the accessibility of tryptophan was calculated using Stern-Volmer equation (Eftink, 1991) namely,

\[(F_0/F) - 1 = K_{SV} [Q] \quad (2)\]

\(F_0\) and \(F\) are the fluorescence intensities in the absence and in the presence of acrylamide quencher. The \(K_{SV}\) is the Stern-Volmer constant. The slope of \(F_0/F\) vs. \([Q]\) gives \(K_{SV}\). The experiment was also carried out for the separated subunits.

**Circular dichroism measurements**

CD measurements were performed with Jasco-810 automatic recording spectropolarimeter calibrated with d (+)-10-camphor sulfonic acid. Dry nitrogen was purged continuously into the instrument before and during the experiment. The light path length of the cell used was 1 mm in the far-UV region (260 - 190 nm) and 10 mm in the near-UV region (320 - 240 nm). All measurements were made at 27°C and the mean residue ellipticity was calculated using a value of 110. Average of three scans at a speed of 10 nm/min, with a bandwidth of 1 nm and response time of 1s, were recorded. Protein concentration of 0.4 mg/mL and 2.5 mg/mL was used for far and near UV measurements, respectively.

The effect of sodium chloride (0 – 1 M) or sodium sulfate (0 – 0.2 M) on the secondary and tertiary structure of napin were carried out after incubation of the protein in salt for a period of 30 min. Changes in the secondary structure of napin, upon interaction with different concentrations of monohydric alcohols (methanol, ethanol, propanol and butanol), were
followed in the range 2 – 18 M (alcohol concentration), at 222 nm. The studies were also made in presence of 3 – 12 M trifluoroethanol (TFE).

**Gel filtration chromatography**

Gel filtration was carried out on a Bio-gel P-30 column (0.6 x 35 cm) with a flow rate of 4.0 mL/ h. The column was calibrated with marker proteins whose Stokes radius were known ($R_s$). Blue dextran (1 mg/mL) was used for determining the void volume (void volume, $V_o = 12$ mL). The protein sample (4.0 mg/mL) was centrifuged at 10°C, 10000 rpm for 20 min before loading onto the column pre-equilibrated with buffer A. The experiment was repeated in presence of 0.5 M NaCl and 0.2 M Na$_2$SO$_4$. The column was equilibrated with required salt concentration in buffer A for the above.

**Thermal stability**

Thermal stability of napin, in presence or absence of 0.5 M NaCl, was followed by CD. The change in secondary structure, as a function of temperature, was monitored at 222 nm. Protein was heated in the temperature range 27 – 75°C, at 1°C/min using a peltier attachment (PMH 356WI). Protein concentration used for the study was 0.4 mg/mL in buffer A.

**DNA - binding studies**

**Gel retardation assay**

The studies on binding of napin to bacterial DNA were studied by DNA gel retardation assay. The napin (5 µg/mL) was incubated with bacterial DNA (100 µg/mL) in 10 mM Tris, 1 mM EDTA buffer, pH 8.0 for 30 min and the
mixture was loaded on to a 0.8% agarose gel. The electrophoresis was run in TBE buffer at constant voltage of 100V for 60 min. The DNA in the gel was visualized by staining with ethidium bromide (Hsu, et al., 2005).

**Amphipathic plot:** Helical wheel diagram was generated using BioEdit Sequence Alignment Editor software (ver.7.0.5.3).

Methylene blue assay was done as per the method of Greiner-Stoeffele et al., (1996). Protein stock solution of 10 mg/mL was used for the assay. RNA solution was prepared by dissolving 100 mg yeast RNA in 10 mL Mops buffer (0.1 M Mops – HCl, pH 7.5, 2 mM EDTA). Methylene blue buffer was prepared by dissolving 1mg Methylene blue buffer in 100 mL Mops buffer and the absorbance at 688 nm was adjusted to 0.5 ± 2% using Mops buffer. Due to light sensitivity of the dye, the Methylene blue buffer was stored in dark. DNA solution (100 µL) was mixed with varying concentration of napin and methylene blue buffer to a final volume of 1 mL in cuvette of 1 cm light path. The sample was preincubation at 25°C for 10 min in the spectrophotometer in the dark. RNA methylene blue complex causes an absorbance decrease at 688 nm. The decrease in the absorbance was monitored for 1 min (Greiner-Stoeffele et al., 1996). The IC₅₀ and Kᵢ were calculated.

The Kᵢ is calculated according to the method of Sluyterman and Wijdenes (1973). In the Line Weaver - Burk plot the lines of various inhibitor concentration intersect in the first quadrant. This is due to the association between substrate and inhibitor.

**Thermodynamic and structural stability of napin**
Proteolytic digestion

Purified napin protein (4.0 mg/mL) was extensively dialyzed against 0.05 M, pH 8.15 ammonium hydrogen carbonate buffer, for 12 h at 8°C. Dialyzed protein was centrifuged at 12000 rpm for 30 min. Prior to digestion protein and α-trypsin stock (10 mg/mL) were kept at 37°C for four minutes to attain the temperature. The protein was digested with α-TPCK treated trypsin and α-TLCK treated chymotrypsin at 1:100 enzymes to protein. Aliquots were drawn for every fifteen minutes and the extent of hydrolysis was measured by trinitrobenzene sulfonic acid (TNBS) method (Nissen, 1979). The same procedure has been repeated for α-chymotrypsin digestion. The proteolytic digestion was also carried out at 27 and 60°C and the digest was loaded onto 15% non reducing SDS-PAGE.

Thermal unfolding of napin

Circular dichroism studies

Circular dichroism spectra in the far UV (260-200 nm) and near UV (240-320 nm) region were recorded on a Jasco-810 spectropolarimeter (Jasco, Tokyo, Japan) calibrated with d-10 camphor sulfonic acid. Protein was heated in the temperature range 27 – 75°C using a peltier attachment (PMH 356WI). Dry nitrogen was purged before and during the measurements. The light path length of the cell used was 1mm in the far-UV region and 10 mm for near UV region. All the samples filtered through 0.22 μ membrane (millipore) filter before taking the spectra. During the course of measurement concentration of the protein was kept constant. An average of three scans at a
speed of 10 nm/min with a bandwidth of 1 nm and a response time of 1 s were recorded. The mean residue ellipticity, $\theta_{MRW}$ was calculated using a value of 110 for napin through out the CD experiment. Buffer base line was subtracted in each scan and the change in structure as a function of temperature was measured at $\theta_{222 \text{ nm}}$. The concentration protein used for the study was 0.4 mg/mL in buffer A. DTT (1 mM) was used for following thermal denaturation under reducing conditions. For all the CD measurements structural analysis were made according to Yang et al (1986)

**Fluorescence measurements**

Fluorescence emission spectra were recorded on a shimadzu RF-5000 automatic recording spectrofluorimeter in the temperature range of 15 - 70°C. The temperature of the cell was maintained by circulating water (Huber, Germany). The excitation and emission slit widths were set at 5 and 10 nm respectively. The excitation wavelength was fixed at 280 nm, and emission spectra were recorded from 300 - 400 nm. Samples were incubated at the test temperature for fifteen minutes before recording the fluorescence intensity. Unfolding was monitored at the emission maxima 343.0 nm. All the unfolding and refolding measurements were made at 27°C with 0.16 mg/mL protein in buffer A.

**DSC studies**

Change in the heat capacity as a function of temperature was measured on the ultrasensitive differential scanning calorimeter, VP-DSC (Microcal Inc.). The scans were performed as a function of protein concentration (10 - 106
μM), pH (3.0 - 10.0) and rates (10 - 60 K/h). All DSC measurements were carried out with a protein concentration of 50 μM (0.8 mg/mL) in buffer A. The sample and dialysate were filtered through 0.22μ filter and degassed prior to loading the DSC cell. Glycine–HCl buffer (pH 3.0) 0.05 M, sodium acetate buffer (pH 3.5 - 5.5) 0.05 M, phosphate buffer (pH 6.0 - 7.0) 0.02 M, Hepes (pH 8.0 - 9.0) 0.05 M and glycine-NaOH (pH 10.0) at 0.05 M concentration were used for the pH dependent calorimetric scans of samples.

Napin was dialyzed against buffer A containing 1 mM DTT (5 changes over 24 h) before DSC, to understand the effect of reduction on thermal denaturation. At desired pH, buffer and the protein samples were dialyzed extensively with four intermittent changes (1:500, 8°C). The 0.5 mL of protein and dialysate buffer was introduced in to sample cell and reference cell. The calorimeter was up scanned at a constant rate of 60 K/h. Using origin software supplied along with the instrument, the data were accumulated and analyzed. For the analysis of data the calorimeter is interfaced with microcomputer. The analysis of normalized data using progress base line, connection of pre and post transition base line of the DSC thermogram yielded the van’t Hoff enthalpy (ΔHV), midpoint of transition (TM). The ratio of number of moles in the cell yields the calorimetric enthalpy (ΔHC). The ratio of ΔHV and ΔHC yields the cooperativity of transition (Privalov and Khechinashivli, 1974).

Thermal unfolding of napin in presence and in absence of 1 mM DTT was carried out on Carry Bio100. The TM was calculated using the software supplied along with the instrument.
Gel filtration chromatography for following the state of association

Gel filtration was carried out on a water-jacketed Sephadex-G50 superfine column (0.48 x 135 cm, 98 mL) at a flow rate of 8.0 mL/h in the temperature range 10 - 70°C ± 2°C. The column was calibrated with known molecular weight proteins (Sigma Chemical Co., St. Louis, USA). 0.8 mL fractions were collected and the absorbance measured at 280 nm. Blue dextran (1 mg/mL) was used to determine the void volume (V₀ = 30.1 mL). Ovalbumin (43,000), carbonic anhydrase (29,000), trypsinogen (24,000) and RNase (14,500) were run independently at 27°C. Protein samples (8.0 mg / mL) were centrifuged for 20 min at 10,000 rpm and 27°C before loading onto the column pre-equilibrated at the test temperature in buffer.

Cross-linking of napin by carbodiimide

Cross-linking of napin was carried out according to Enami et al., (1998). Protein (130 μM) was heated at 45°C for 15 min and the protein was cross linked by incubating in the presence of 1mM EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide) for 12 h. The reaction was stopped by adding 100 mM sodium acetate. The cross-linked protein was extensively dialyzed against 0.02 M phosphate buffer with four changes. The pH of the solution was adjusted to 9.5 and dialyzed against 0.02 M phosphate buffer pH 7.0. The cross linking of the napin was confirmed by non - reducing SDS - PAGE. The 15% gel was stained by coomassie brilliant blue R - 250.

Isothermal unfolding of napin

Isothermal equilibrium unfolding induced by guanidinium hydrochloride
Preparation of GdnHCl

Stock solutions of GdnHCl (8.2 M) were prepared fresh, in buffer A containing 0.002% sodium azide. The pH of stock solution was adjusted by adding 0.01 M NaOH. Concentration of GdnHCl was determined by measuring refractive index (RI) of solution (Nozaki, 1972).

The equilibrium unfolding was monitored with the help of fluorescence spectrometer, circular dichroism and gel filtration chromatography as a function of guanidinium hydrochloride. Protein samples were equilibrated for 12h at 27°C. (Where there is no further increase in the fluorescence intensity). During the course of incubation, there was no precipitation of napin as confirmed by recording the absorption spectra from 300 - 600 nm. The temperature of the CD and fluorescence cell was maintained at 27°C. The stock solutions of GdnHCl and protein were prepared using buffer A, containing 0.002% sodium azide. The concentration of the protein was kept constant during the measurement. All fluorescence-unfolding measurements were made with 0.12 mg / mL protein.

Excitation and emission slit widths were set at 5 and 10 nm respectively. The excitation wavelength was fixed at 280 nm, and emission spectra were recorded from 300 - 400 nm. Unfolding was monitored at the emission wavelength 343.0 nm. Each data point is an average of five individual experiments. GdnHCl induced isothermal denaturation curves were collected in the temperature range 15 - 55°C. A circulating Huber water bath was used to maintain the temperature of the sample within ± 0.5 °C of the set temperature.
Similarly far-UV and near UV CD spectra were recorded for napin in GdnHCl (0 - 7 M). The data points are an average of three scans at a speed of 10 nm/min. Corresponding base lines were subtracted and the change in structure was measured at $[\theta]_{222}\text{nm}$. The protein concentration was 0.4 mg/mL and 2.5 mg/mL for far and near UV, respectively.

Reversibility of napin unfolding

The reversibility of unfolding was checked by refolding the unfolded protein by dialyzing against buffer A for 24h with five changes and diluting with the same buffer. Refolding was confirmed by measuring secondary, tertiary structure, fluorescence emission spectra, gel filtration, and hydrophobicity.

The hydrophobic surface exposure of native, denatured and refolded protein (25 $\mu$M) were measured by incubation in dark with the hydrophobic probe ANS (Engelhard and Evans, 1995). A concentrated stock of ANS was added to get a final concentration of 25 $\mu$M. The excitation wavelength was 375 nm and emission spectra collected from 400 - 550 nm using 1cm path length cell. The excitation and emission slit widths were 5 and 5 nm. Appropriate blank spectra of ANS were used for correcting the protein bound ANS spectra obtained.

Unfolding of napin by gel filtration chromatography

Gel filtration was carried out on a Bio-gel P-30 column (0.6×35 cm) with a flow rate of 4.0 mL/h. The column was calibrated with known molecular weight proteins for which stokes radius is known (Rs). Blue dextran (1 mg/mL) was used for determining the void volume (void volume $V_o = 12$ mL). Alcohol
dehydrogenase (1,500,000 Da, $R_S = 44.3 \text{ Å}$), bovine serum albumin (66,000 Da, $R_S = 33.5 \text{ Å}$), cytochrome C (12,500 Da, $R_S = 17 \text{ Å}$) and soybean trypsin inhibitor (20,000 Da, $R_S = 20 \text{ Å}$) were run independently (Uversky, 1993). The protein samples (4.0 mg/mL) were incubated for 12h in various concentrations of denaturant (0-7.0 M) at 27°C and centrifuged for 20 min at 10000 rpm. The incubated protein was loaded onto the column which was pre equilibrated with the desired concentration of the denaturant in buffer A. The void volume ($V_v$) correction was given for each increase in the denaturant concentration. The elution volume was calculated (Uversky and Ptitsyn, 1994).

Elution volume$= (V_e - V_v) / (V_t - V_v)$

Where, $V_e$ = Elution volume of the protein $V_v$=Void volume $V_t$=Total bed volume

**Dynamic light scattering measurements**

The dynamic light scattering measurements were done using Dyna Pro-MS800 dynamic light scattering equipment (Proterion, Protein solutions, Wyatt Technology, Santa Barbara, CA). The protein concentration used was 1.0 mg/mL in buffer A.

**Computational analysis of the sequence for the internal repeats**

Internal repeat regions in the sequence of napin were identified using the software ‘RADAR’, (Rapid Automatic Detection and Alignment of Repeats; [http://www.ebi.ac.uk/Radar/index.html](http://www.ebi.ac.uk/Radar/index.html), European Bioinformatics Institute). The software uses an automatic algorithm, for segmenting the interested
sequence into repeats. It identifies short composition biased as well as gapped approximate repeats and complex repeat architectures involving many different types of repeats in sequence (Heger and Holm, 2000). This program identifies both sequence similarities and duplications in the sequence.

Sequence alignment

Brassica family napin sequences were aligned using CLUSTAL W version 1.83 from European Bioinformatics Institute. Sequences of napins used in the alignment were taken from GenBank as well as protein databank databases and include Brassica juncea (CAA46785), Brassica oleracea (CAA46783), Brassica campestris (CAA46782), Brassica nigra (CAA46784), Brassica carinata (CAA52813), Brassica rapa (CAA46171) and Brassica napus (1pnb).

Homology modeling

A model of napin was constructed based on the NMR structure of Brassica napus protein (PDB code: 1PNB). The Swiss-model software was used for this purpose was obtained from the website (http://swissmodel.expasy.org/SWISSMODEL.html).

Intrinsic ligands

Preparation of phenolic acid free napin
1. Activated charcoal: Purified napin was treated with 1% activated charcoal and was incubated at 27°C for 15 min, the resulting mixture was centrifuged.

2. Napin was treated with 1% PVP and fractionated on Sephadex G-50 column, pre-equilibrated with buffer A.

3. Protein bound sinapic acid (15 mg) was removed by increasing the pH to 10, followed by gel filtration on LH-20 (partly hydroxy propylated Sephadex G-25) column (140 x 0.5 cm, 110 mL). Protein was centrifuged at 10000 rpm for an hour, before loading onto the column. The column was pre - equilibrated with 0.02 M Glycine – NaOH buffer (pH 9.5) and protein was eluted from the column with same buffer. Protein fractions corresponding to napin were pooled and used for interaction studies.

**Fluorescence measurements**

Interaction of sinapic acid, phytic acid and allyl isothiocyanate with napin has been monitored following the quenching of relative fluorescence intensity of napin. Fluorescence measurements were carried out using Shimadzu RF 5000 spectrofluorimeter attached with thermostated circulating water bath. The solution in the cuvette was stirred using a Hellma cuv-o-stir®. Excitation and emission slit widths were set at 5 and 10 nm, respectively. Measurements were made using a 10 mm pathlength cuvette with the sample in buffer A. Fluorescence quenching of napin was followed at 27 ± 1°C. All samples were centrifuged at 10000 rpm for an hour to remove any aggregates. Inner filter effect was corrected by following the quenching of N-acetyl tryptophanamide, equivalent in absorption of napin at 280 nm. The
binding constant was calculated using established procedures (Appu Rao and Cann, 1981).

The binding constant $K$ is given by

$$K = \beta / (1-\beta) \cdot 1/C_f,$$  \hspace{1cm} (3)

where $\beta = Q/Q_{\text{max}}$ and $C_f = C_T - n\beta T$, in which $Q$ is the corrected percentage quenching; $Q_{\text{max}}$, the maximum quenching, $C_f$, the molar equilibrium concentration of unbound sinapic acid; $C_T$, the molar constituent concentration of sinapic acid; $T$, the molar constituent concentration of napin; and $n$ is the binding stoichiometry (Lee et al., 1975).

The value of $K$ (binding constant) is given by the slope of a plot of $\beta/(1-\beta)$ against $C_f$. $Q_{\text{max}}$ has been determined by extrapolation of a double reciprocal plot of $1/Q$ vs. $1/C$, to $1/C = 0$. In both cases, the data are fitted to a straight line by the method of least squares.

**Sinapic acid- binding**

**Determination “$\varepsilon$” for Sinapic acid**

Molar extinction coefficient was determined by measuring the absorbance of sinapic acid (0 - 150 $\mu$M) in ethanol. The absorbance spectrum of sinapic acid is having maxima at 321, 238 and 204 nm.

Stock solution of sinapic acid was prepared in ethanol (10 mM) using $\varepsilon_{321\text{ nm}} = 2.2 \times 10^4 \text{ ML}^{-1}$, and added in increments of 5 $\mu$L to napin (7$\mu$M) in
buffer A. The excitation and emission wavelengths were set at 295 nm and 340 nm respectively. The effect of temperature in the range 17 - 47°C, on the binding constant was determined by fluorescence quenching studies. Effect of ionic strength on the binding constant of sinapic acid with napin was determined by increasing the concentration of sodium chloride (0 - 200 mM) in buffer A. The effect of salt on the Stokes radius of napin was studied by gel filtration. Gel filtration measurements were carried out using TSK super SW 2000 (300 x 4.6 mm) column. The absorbance was detected at 280 nm. Standard proteins from a molecular weight marker kit for gel filtration (Sigma) with known stokes radius were used for calibrating the column. Blue dextran (1 mg/mL), was used for determining the void volume.

**Determination of intrinsically bound sinapic acid by HPLC**

Intrinsically bound sinapic acid to napin was quantitated by HPLC method. Purified napin (40 mg) was extracted with 80% methanol for 3h at 70°C, centrifuged at 10000 rpm for an hour and concentrated under nitrogen. Dried napin was redissolved in 2 mL mixture of ammonium di hydrogen phosphate (75% v/v) and methanol (25% v/v). An aliquot of 20 µL was directly injected to symmetry shield™ RP<sub>18</sub> (5.0 µ, 4.6 mm x 250 mm) Waters column equipped with a C<sub>18</sub> guard column. The solvent system used was, solvent A: 0.02 M ammonium di hydrogen phosphate buffer (pH 2.15)/methanol (75:25), solvent B: methanol. The gradient was 5% solvent B (0 - 15 min), 5 - 35% solvent B (15 – 20 min), 35 – 100% B (35 – 45 min), 100 – 5% B (45 – 50 min), 5% B (50 – 52 min) with an 18 min post run period at 5% solvent B. Elution profile was monitored at 325 nm with a flow rate of 1 mL/min at 27°C
(Vuorela et al., 2004). A standard curve was constructed by injecting standard sinapic acid (0 – 0.1 mg). The amount of intrinsically bound ligand was calculated from the curve.

**Phytic acid – napin interaction**

Stock solution of phytic acid was prepared in buffer A. Protein solution of 1 mg/mL was taken and the desired pH was adjusted (1 – 7) and known amount of phytic acid solution was added. After 30 min of incubation at room temperature, napin was centrifuged and protein in the supernatant was measured at 280 nm. Napin at respective pH were taken as controls. The precipitation was also followed as a function of phytic acid concentration.

Inorganic phosphate was estimated according to the method of Chifflet et al (1988). Supernatant solution of protein-phytic acid complex, after precipitation with 3% TCA was used for the determination of inorganic phosphorus. To 50 µl napin (10 mg/mL), 50 µl of 12% SDS was added and mixed. The color developing was started after the addition of 100 µl mixture of 6% ascorbic acid in 1N HCl and 1% ammonium molybdate solution. The samples were left at 27°C for 10 min and terminated by the addition of 150 µl stopping solution (2% Sodium citrate, 2% Sodium metaarsenite, 2% acetic acid). This mixture was incubated at room temperate for 20 min at 27°C and absorbance was read at 850 nm. All the reagents were prepared freshly. NaH₂PO₄ (0 - 15 µM) was used to construct standard curve.

**Determination of binding constant of phytic acid by HPLC**
Quantitative estimation of phytic acid binding to napin was carried out on RP-HPLC. The amount of free phytic acid in the napin-phytic acid complex was determined by this technique. Protein-phytic acid complex (2 mg/mL) was precipitated with 3% TCA and centrifuged after 30 min at 10000 rpm. The supernatant was filtered through 0.22 µ membranes (Millipore) filter. An aliquot of 20 µL was injected directly to the symmetry shield™ RP 18 (5.0 µ, 4.6 mm x 250 mm) Waters column. Bound phytic acid was isocratically eluted using 0.005 M sodium acetate for 15 min at a flow rate of 1 mL/min. The eluted phytic acid was monitored at 254 nm (Tangendjaja et al., 1980). A standard curve of phytic acid was constructed (0 – 0.2 mg). The amount of free phytic acid calculated from the standard curve. Binding constant was calculated from Scatchard plot (Scatchard, 1949).

**Circular dichroism measurements**

Circular dichroism spectra in the far UV (260 - 200 nm) and near UV (240 - 320 nm) region were recorded. All the samples filtered through 0.22 µ membranes (Millipore) before taking the spectra. An average of three scans at a speed of 20 nm/min, with a bandwidth of 1 nm and response time of 1s, was recorded. The mean residue ellipticity, \([\theta]_{M_{R W}}\) was calculated using a value of 110 for napin.

The spectra were recorded for napin and napin-phytic acid (0.07 and 0.3 mM phytic acid) and napin-AIT complex (0.1 and 0.3 mM AIT). Secondary structural analysis was performed according to Yang et al (1986).

**Modification of amino acids**

81
Lysine modification by Succinylation

Modification of lysine was carried out according to the method of Hass et al (1964). To a 2 mL of 10 mg protein 100 mg of solid succinic anhydride was added. The solution was stirred continuously at pH 8.0 for 2 hr. The pH was adjusted with 1N NaOH. Resultant solution was dialyzed extensively against buffer A (24 hr at 4°C). Extent of modification was measured by estimating “available lysine”.

Modification of Tryptophan residue

The tryptophan residue on napin was modified using N – bromosuccinamide (NBS) according to the method of Spande and Witkop (1967).

AIT- binding

Stock solution of AIT was prepared in ethanol (10 mM) concentration was determined by using $\epsilon_{240 \text{ nm}} = 770 \text{ M}^{-1}\text{cm}^{-1}$ (Schwimmer, 1961). Intrinsically bound AIT to napin was determined by RP – HPLC according to Matthäus and Fiebig (1996). AIT was extracted from pure napin with 80% ethanol for 3h at 60°C. Resulting mixture was centrifuged and injected on to RP C$_{18}$ column. Mobile phase used was water and 100% acetonitrile, for a total run time of 60 min. The column was pre equilibrated for 8 min with 20% acetonitrile. Gradient was started from 40% acetonitrile to 60% acetonitrile in 27 min. The column was equilibrated at 20% acetonitrile for 25 min. The gradient elution of AIT was monitored at 240 nm.
Binding of AIT - napin complex was studied by measuring the available lysine content. AIT concentration was varied from 0 to 0.6 mM at napin concentration 100 μM. After 2 h incubation at 27°C the available lysine content was measured. The experiment was also carried out in the pH range 4 – 10 and concentration 10 – 200 μM (napin).

Change in the electrophoretic mobility of napin and napin – AIT (1 mg/mL) complex was carried out on 10% native – PAGE. Glycine – HCl buffer (0.2 M) at pH 4.0 was used as running buffer. Methylene green was the tracking dye. Gels were stained in 0.5% amido black in 7% acetic acid and destained using 7% acetic acid. Electrophoretic mobility was calculated using the software (Software Syngene tools from Synoptics technologies, UK).

**Available lysine**

Available lysine was estimated according to Sashidhar et al (1994). To 1 mL of Napin (1 mg/mL) solution, 4% NaHCO₃ (1 mL, pH 8.5) and 1 mL of freshly prepared 0.01% TNBS were added. The mixture was incubated at 40 ± 2°C for 2h and terminated by adding 1 mL of 10% SDS followed by 0.5 mL of 1N HCl. The absorbance of the solution was monitored at 335 nm against appropriate blanks. A standard lysine (0 – 25 μg) curve was constructed.

Effect of AIT concentration was studied by measuring the available lysine content. AIT concentration was varied from 0 – 0.6 mM, and napin concentration was kept constant (100 μM). After 2h incubation available lysine was measured. Also, interaction of AIT (0.3 mM) with napin (100 μM) was
studied as a function of temperature in the range 17 – 47°C. After two hours of incubation available lysine content was measured.

Effect of tyrosine ionization on napin and napin – AIT complex was studied in the pH range 7.0 –11.9. The pH of the napin solution was adjusted by the addition of 0.1 M NaOH. The sample was centrifuged and absorbance read at 280 nm.