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Carnein, a Serine Protease from Noxious Plant Weed *Ipomoea carnea* (Morning Glory)

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A new serine protease from the latex of *Ipomoea carnea* spp. *fistulosa* (Morning glory), belonging to the Convolvulaceae family, was purified to homogeneity by ammonium sulfate fractionation followed by cation exchange chromatography. The enzyme, named carnein, has a molecular mass of 80.24 kDa (matrix-assisted laser desorption/ionization time-of-flight) and an isoelectric point of pH 5.6. The pH and temperature optima for proteolytic activity were 6.5 and 65 °C, respectively. The extinction coefficient ($\epsilon_{280}^{1\%}$) of the enzyme was estimated as 37.12, and the protein molecule consists of 35 tryptophan, 76 tyrosine, and seven cysteine residues. The effect of several inhibitors such as iodoacetic acid, diisopropylfluorophosphate, phenyl-methanesulfonyl fluoride, chymostatin, soybean trypsin inhibitor, $HgCl_2$, 3*S*-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-ethylbutyl}carbamoyl)oxirane-2-carboxylic acid, *N*-ethyl maleimide, ethylene glycol-bis(α-amino ethyl ether)tetraacetic acid, ethylenediamminetetraacetic acid, and *o*-phenonthroline indicates that carnein belongs to the family of serine proteases. The enzyme is not prone to autolysis even at very low concentrations. The N-terminal sequence of carnein (T-T-H-S-P-E-F-L-G-L-A-E-S-S-G-L-X-P-N-S) exhibited considerable similarity to those of other plant serine proteases; the highest similarity was with alnus AG12, one of the subtilase family endopepetidases.

KEYWORDS: ***Convolvulaceae*; *Ipomoea carnea*; latex; serine proteases; purification; characterization**

INTRODUCTION

Proteolysis is fundamental for the normal functioning of multicellular organisms, and proteolytic enzymes are intricately involved in many aspects such as development, physiology, defense and stress responses, and adaptation to the changing environment. It regulates protein processing and intracellular protein levels and removes abnormal or damaged proteins from the cell, working as a cellular housekeeper. In addition, proteolysis appears to play key roles in the regulation of biological processes in plants, such as the recognition of pathogens and pests and the induction of effective defense response. Plant proteases are thus involved in all aspects of the plant life cycle ranging from mobilization of storage proteins during seed germination to the initiations of cell death and senescence programs (*1*). Moreover, plant proteases show broad substrate specificities and activities over a wide range of pH and temperature values; therefore, they find extensive applications in pharmaceutical, medicinal, food, and biotechnology industries (*2*).

Serine proteases are one of the largest groups of proteolytic enzymes involved in numerous regulatory processes. They catalyze the hydrolysis of specific peptide bonds in their substrates, and this activity depends on a set of amino acids in the active site of the enzyme, one of which is always a serine. They show a wide range of activities, including exopeptidase, endopeptidase, oligopeptidase, and ω-peptidase activities (*3*). The MEROPS database (*4*), an information resource for peptidases and their inhibitors, lists over 40 serine protease families (set of homologous peptidases). These families are grouped in six clans; groups of families having similar tertiary structures thus are likely to share a common ancestor (*5*). Structures are known for several of the clans; these appear to be totally unrelated, suggesting at least four evolutionary origins of serine peptidases and possibly many more. Despite their different evolutionary origins, there are similarities in the reaction mechanisms of several peptidases (*5*). Three of them, chymotrypsin, subtilisin, and carboxypeptidase C clans, share a catalytic triad of serine (S), aspartate (D), and histidine (H) in different orders (e.g., HDS in chymotrypsin, DHS in subtilin, and SDH in the carboxypeptidase clan) (*6*).

Serine proteases are involved in numerous regulatory processes; therefore, they are the best-characterized groups of proteolytic enzymes in mammals (e.g., the trypsin chymotrypsin family) and microorganisms (e.g., the subtilisin family). Despite being the largest class of proteases in plant, the functions and regulatory roles of plant serine proteases are poorly understood, probably due to a lack of identification of their physiological

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substrates (*7*). However, once thought to be rare in plants, in recent years, several of the serine proteases have been isolated and duly purified from various plant species in which they occur in distinct parts, ranging from the seeds to the latex and the fruits (*7*). Among other families, special mention is given to the subtilisin family, the second largest encompassing serine proteases.

The subtilisin-like proteases, named subtilases for short, may in turn be subdivided into two different subfamilies: the kexins and the pyrolysins; most mammalian subtilases belong to the former, whereas most plant subtilases purified to date are members of the latter (*8*). The first subtilase to be purified from plants was cucumisin, which is found in the sarcocarp of developing *Cucumis melo* fruits (*2*). Siezen and Leunissen (*8*) classified serine proteases from *Lilium longiflorum*, *Arabidopsis thaliana*, and *Alnus glutinosa*, the P69 protease from *Lycopersicon esculentum*, and the antifreeze protein af70 from *Picea abies* as subtilases. A number of subtilases have been isolated from various cucurbitaceous plants; yet, cucumisin, an enzyme purified from melon (*C. melo* L.), is best characterized to date. There is evidence suggesting the occurrence of large gene families encoding subtilases in rice and *Arabidopsis* (*9*). Despite the involvement of plant serine proteases, namely, subtilases, in many physiological processes, functions of these enzymes in plants are not clear due to a lack of information on their processing sites and substrates. Therefore, the search for valuable serine proteases and their physiological roles in plants is still an open field of research.

Ipomoea carnea spp. *fistulosa*, Convolvulaceae family (morning glory), is a toxic plant (weed) found in abundance in India, Brazil, the United States, and other countries. *I. carnea*, an aggressive weed in wetlands, is toxic to cattle and difficult to eradicate (*10*). The toxic principles of the plant were identified as two nortropane alkaloids, calystegines B2 and C1, and an indolizidine alkaloid, swainsonine (SW) (*11*). The first two are powerful glycosidase inhibitors affecting α-glucosidase and β-galactosidases, leading to human genetic lysosomal storage defects, Gaucher's and Fabry's disease, respectively (*12*), while the latter (SW) is a potent inhibitor of lysosomal mannosidase. The water and 80% ethanol extracts of *I. carnea* spp. *fistulosa* exhibited HIV type 1 reverse transcriptase inhibitory activity; therefore, it may be useful in the treatment of AIDS (*13*). Although many organic compounds are identified and isolated from plant sources, less attention is paid toward the protein component. In view of the potential applications of the latex, efforts were focused to identify and purify the protein constituents, if any. While screening different parts of the plant for biological activities, the latex exhibited a considerable amount of proteolytic activity. In this paper, the identification, purification, and biochemical characterization of a serine protease from latex of *I. carnea* spp. *fistulosa* are reported.

MATERIALS AND METHODS

Safety. The latex of *I. carnea* (morning glory) is toxic, so latex was collected wearing gloves. Acrylamide is a potent neurotoxin and carcinogen, and it was handled with safety gloves. The handling of phenol and trichloroacetic acid (TCA) was done carefully because of their highly corrosive nature to skin. All other experiments were carried out with the utmost precaution and care.

Latex was collected by superficial incisions of young stems of *I. carnea* plants found abundantly on the Banaras Hindu University campus (Varanasi, India). SP-Sepharose was purchased from Amersham Pharmacia. BSA, ribonuclease A, hen egg white lysozyme, azocasein, azoalbumin, hemoglobin, 5,5μ-dithiobis(2-nitrobenzoic acid) (DTNB), diisopropylfluorophosphate (DFP), iodoacetic acid (IAA), chymostatin, $HgCl_2$, 3*S*-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-ethylbutyl}-carbamoyl)oxirane-2-carboxylic acid (E-64), guanidine hydrochloride (GuHCl), glycerol, urea, *o*-phenanthroline, ethylene diammine tetraacetic acid (EDTA), ethylene glycol-bis(α-amino ethyl ether)tetraacetic acid (EGTA), soybean trypsin inhibitor (SBTI), *N*-ethyl maleimide (NEM), β-mercaptoethanol, phenyl-methanesulfonyl fluoride (PMSF), Coomassie brilliant blue R-250, all synthetic substrates, Freund's complete and incomplete adjuvants, agarose, and Tween-20 were obtained from Sigma Chemical Co. (United States). Coomassie brilliant blue G-250 was purchased from Eastman Kodak. Trifluoroacetic acid (TFA) was obtained from Applied Biosystems. Acetonitrile was of high-performance liquid chromatography (HPLC) grade. Ampholine carrier ampholytes were from LKB. All other chemicals were of the highest purity available commercially.

Protease Purification. All steps for purification of the enzyme were carried out at 4 °C.

Step 1: Gum Removal. Latex (5 mL) was collected by superficial incisions made on young stems of *I. carnea* plants into 45 mL of 0.01 M sodium acetate buffer, pH 4.5, and stored at −20 °C for 24 h. The latex was thawed to room temperature and centrifuged at 28000*g* for 20 min to remove any insoluble material and gum. The clear supernatant thus obtained was referred to as crude latex and used further.

Step 2: Ammonium Sulfate Precipitation. Crude latex obtained in the first step was subjected to 80% (w/v) ammonium sulfate precipitation at 4 °C with continuous stirring. Ammonium sulfate was added slowly to the supernatant over a period of 30 min, allowing the salt to slowly dissolve. After 24 h, the resultant precipitate was recovered by centrifugation (28000*g* for 20 min at 25 °C) and dissolved in 80 mL of 0.01 M acetate buffer, pH 4.0, followed by overnight dialysis against the same buffer. The protease activity and protein concentration were measured, and the specific activity was calculated.

Step3: Cation Exchange Chromatography. The dialyzate from the previous step was subjected to cation exchange chromatography on a SP-Sepharose fast flow column pre-equilibrated with 0.01 M sodium acetate buffer, pH 4.0. The column was washed thoroughly with the same buffer until no protein was detected in the eluate. The bound proteins from the column were eluted with a linear gradient of NaCl from 0 to 0.5 M in 0.01 M acetate buffer, pH 4.0. Fractions of 3 mL in volume were collected at a flow rate of 3 mL/min. All of the fractions were assayed for protein content, extent of homogeneity and proteolytic activity by absorbance at 280 nm, and sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE) and proteolytic activity measurement, respectively. Typically, the elution resolved the retained proteins into two peaks. The first was homogeneous with no activity, whereas the latter two peaks had an activity of which the descending shoulder of the second peak exhibited a considerable amount of proteolytic activity as well as a greater homogeneity.

Protein Concentration. The protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm as well as by the Bradford method (*14*) using bovine serum albumin (BSA) as a standard.

Assay for Enzyme Activity. The hydrolyzing activity of the protease was monitored using denatured substrate casein, hemoglobin, and azoalbumin (*15*). For assay, the enzyme was added to 0.05 M Tris-HCl buffer, pH 8.0, and incubated at 37 °C for thermal equilibration. After 15 min, 0.5 mL of 1% (w/v) substrate was added and the reaction was allowed to proceed for 1 h at 37 °C. The reaction was terminated by an addition of 0.5 mL of 10% TCA and allowed to stand for 10 min. The resultant precipitate was removed by centrifugation for 10 min, and the absorbance of TCA soluble peptides in the supernatant was measured by absorbance at 280 nm. A control assay was done without any enzyme in the reaction mixture and was used as reference. When azoalbumin was used as a substrate, the resulting supernatant obtained after TCA precipitation was mixed with an equal volume of 0.5 M NaOH. The color developed was measured by absorbance at 440 nm after 5 min. One unit of enzyme activity is defined as the amount of enzyme that, under the assay conditions described, gives rise to an increase of one unit of absorbance at 280 or 440 nm per minute of digestion. The specific activity is the number of units of activity per milligram of protein.

Electrophoresis. Homogeneity of the enzyme preparation and estimation of molecular mass (M_r) of the purified carnein were

performed on 12% SDS-PAGE under nonreducing and reducing conditions (*16*). The proteins were stained with Coomassie brilliant blue R-250. A See Blue prestained ladder from Invitrogen was used as a molecular weight marker.

Zymography. A zymogram was performed to visualize the proteolytic activity (in run). For zymography, 1.2% casein was included in 12% polyacrylamide gel. The gel was run at 200 V for 1 h and soaked in 2.5% Triton X-100 for displacement of the SDS. Gels were then incubated in reaction buffer (50 mM Tris buffer, pH 8.0, 1 mM $CaCl_2$) for 15 h at 37 °C and later stained with Coomassie brilliant blue R-250. The gels were destained overnight with 40% methanol and 10% acetic acid until clear bands appeared against a blue background. The unstained region of gel reflected the proteolytic activity of the proteases.

Mass Spectrometry. The purity degree as well as the molecular weight of purified endopeptidase was determined by mass spectrometry (matrix-assisted laser desorption/ionization time-of-flight, MALDI-TOF). A mass spectrum was acquired on a Bruker Daltonics Flex Analysis system. A protein of known molecular mass (BSA) was used as the standard for mass calibration.

Assay for Proteolytic Activity toward Synthetic Substrates. The amidolytic activities of the enzyme were studied using NR-benzoyl-larginine *p*-nitroanilide (BAPA), L-alanine alanine *p*-nitroanilide, succinyl phenylalanine *p*-nitroanilide, L-glutamyl *p*-nitroanilide, L-alanine *p*-nitroanilide, *N*-succinyl alanine alanine alanine *p*-nitroanilide, and L-leucine *p*-nitroanilide by the method of Arnon (*15*) with some modifications. The synthetic substrates (5−20 mM) were prepared by dissolving the required amount of synthetic substrate in a minimum volume of dimethyl sulfoxide (DMSO) and diluted to final volume with 0.05 M Tris−HCl buffer, pH 8.0, at 37 °C. The reaction mixture contained 10 μg of active enzyme in 0.5 mL of buffer and 0.5 mL of the synthetic substrates. Before assay, the enzyme was thermally equilibrated as described previously. After 30 min of incubation at 37 °C, the reaction was terminated by addition of 0.2 mL of 30% acetic acid. The liberated *p*-nitroaniline was monitored spectrophotometrically by absorbance measurement at 410 nm against a blank sample containing no enzyme (*17*).

pH Optima. The reaction mixture consisted of 10 μg of carnein and buffer of desired pH; the final volume was made up to 0.5 mL. It was thermally equilibrated for 30 min at room temperature followed by addition of 0.5 mL of substrate (1% hemoglobin or 1% casein) dissolved in the buffer of a similar pH. The assay was carried at 37 °C as described above. Enzyme activity measurements could not be done with casein due to their insolubility below pH 4.0. Therefore, denatured hemoglobin was used as the substrate for assays at low pH (*18*). A control assay was done at each pH without enzyme in the reaction mixture and used as reference blanks. The buffers used were as follows: KCl-HCl (pH 0.5−1.5), glycine-HCl (pH 2.0−3.5), sodium-acetate (pH 4.0−5.5), sodium-phosphate (pH 6.0−7.5), tris-HCl (pH 8.0−10.0), and glycine-NaOH (pH 10.5−13.0). A buffer of 0.01 M was used throughout the experiment.

Temperature Optima. The effect of temperature on the proteolytic activity of carnein was studied using 1% casein in 0.05 M Tris-HCl buffer, pH 8.0. The enzyme was equilibrated in the 0.05 M Tris-HCl buffer, pH 8.0, at different temperatures (25−95 °C) for 15 min. An aliquot was used for assay at 37 °C as described previously.

Effect of Inhibitors on Proteolytic Activity. The effect of different inhibitors on the hydrolysis of casein by the enzyme was monitored. The inhibitors used were IAA, mercuric chloride ($HgCl_2$), E-64, DFP, PMSF, chymostatin, SBTI, EGTA, EDTA, and *o*-phenanthroline. In each case, 20 μg of the activated enzyme was incubated in the presence of increasing concentrations of the inhibitor in 0.05 M Tris-HCl buffer, pH 8.0, for 30 min at 37 °C and assayed as described previously. A control assay was done with enzyme solution without inhibitors, and the resulting activity was considered as 100%.

Effect of Substrate Concentration on Reaction Velocity. The effect of increasing substrate concentration on the reaction velocity of the enzyme-catalyzed reaction was studied using casein dissolved in 0.05 M tris-HCl, pH 8.0, buffer. In each assay, 20 μg of carnein was added and the substrate was taken in a concentration range of 0−0.5 mM. The reaction was allowed to proceed for 30 min. A Lineweaver−Burk plot was plotted, and the value of the Michaelis−Menten constant (K_m) was calculated (*19*). The value of the catalytic constant (K_{cat}) was estimated by dividing the V_{max} value by the amount of enzyme and assuming that 80 mg of protein (M_r 80000) represents 1 μmol of enzyme.

Isoelectric Focusing. The isoelectric point (pI) of purified carnein was determined by isoelectric focusing on polyacrylamide disc gels as described (*20*). Electrophoresis was carried out with ampholine carrier ampholytes at a pH range of 5−8.

Extinction Coefficient. The extinction coefficient of carnein was determined by a spectrophotometric method (*21*). Several solutions of the enzyme were prepared by serial dilutions, and the concentration of the enzyme in each sample was determined by the Bradford method. The absorbance of each sample at 280 nm was measured, and the extinction coefficient of the enzyme was calculated using Beer−Lambert's law. In the spectrophotometric method, the extinction coefficient was determined using a formula given by Aitken and Learmoth (*21*).

Tyrosine and Tryptophan Content. The tyrosine and tryptophan contents of the enzyme were measured spectrophotometrically using the method of Goodwin and Morton (*22*). The absorbance spectra of the enzyme in 0.1 M NaOH were recorded between 300 and 220 nm using a Beckman DU 640 B spectrophotometer, and the absorbance values at 280 and 294.4 nm were deduced from the spectra. The standard formula given by Goodwin and Morton was used to estimate the tryptophan and tyrosine contents. To validate these measurements, tyrosine and tryptophan contents of papain, ribonuclease, BSA, and lysozyme were determined in similar conditions.

Free and Total Sulfhydryl Contents. The exposed and total cysteine residues of carnein were estimated by Ellman's (*23*) method, where the release of thionitrobenzoate (TNB) due to reduction of the thiol with DTNB was determined by an increase in the absorbance at 412 nm (*24*). The molar extinction coefficient of TNB anion at 412 nm is 14150 M^{-1} cm^{-1} (*25*). For exposed sulfhydryl group estimation, the purified enzyme was activated with 0.01 M β-mercaptoethanol in 0.05 M Tris-HCl buffer, pH 8.0, for 15 min and then dialyzed against 0.1 M acetic acid at 4 °C for 24 h with frequent changes. For the estimation of the total sulfhydryl content, the enzyme was reduced in the presence of 6 M GuHCl for 15 min at 37 °C and dialyzed against 0.1 M acetic acid. To validate these estimations, exposed and total sulfhydryl contents of papain, ribonuclease, BSA, and lysozyme were determined in similar conditions.

Carbohydrate Content. The carbohydrate content of carnein was determined using the phenol-sulfuric acid method (*26*). Different amounts of protein and galactose (standard) were taken in microtiter plates (96 wells), and the absorbance was measured at 492 nm.

Autodigestion. Proteases are prone to autolysis, and the extent of autolysis is dependent on the concentration of the enzyme, type of activator, and the other external conditions such as pH, temperature, and time of incubation. Therefore, the extent of autodigestion of carnein, under neutral conditions, was studied as a function of enzyme concentration from 0.02 to 1 mg/mL. At every concentration, the enzyme sample was incubated at 37 °C in 0.05 M Tris-HCl buffer, pH 8.0. At different time intervals, an aliquot of the enzyme was withdrawn from the incubation mixture and assayed for the proteolytic activity using casein as the substrate as mentioned before. A control sample without enzyme was incubated under similar conditions. The autodigestion experiment was performed after each 24 h for 6 successive days. The activity of the enzyme in the first 30 min of incubation was used as 100% for estimating the residual activity.

Stability. The ability of the purified carnein to retain its activity under conditions of extreme pH, strong denaturants, temperatures, detergents, and organic solvents was studied by exposing the enzyme to the respective conditions. For determining the stability at different pH values, the enzyme was incubated for 24 h in buffers of a pH range of 0.5−12.0. Similarly, enzyme was incubated at different temperatures and varying concentrations of denaturants, detergents, and organic solvents for 24 h and assayed for proteolytic activity using casein. In the case of temperature, the enzyme was incubated at different temperatures for 15 min and assayed at 37 °C as already described.

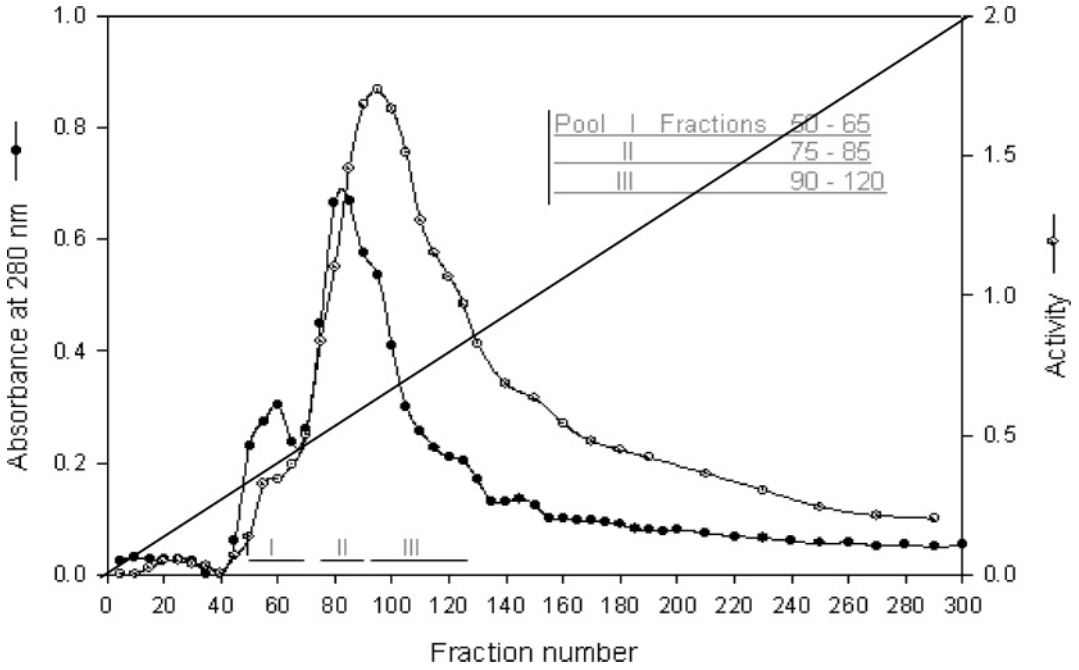

Figure 1. Elution profile of carnein on cation exchanger: A SP-Sepharose fast flow column was pre-equilibrated with 10 mM sodium acetate buffer, pH 4.0. The bound proteins were eluted with a linear salt gradient of 0.0–0.5 M NaCl at the same pH. Fractions of 3 mL were collected at a flow rate of 3 mL/min. All fractions were assayed for activity (○) and for protein content (●). Upon activity and SDS-PAGE analysis, three pools were collected as follows: pool I (fractions 50–65), pool II (fractions 75–85), and pool III (fractions 90–120).

Substrate Specificity. The carnein activity toward BSA was compared to that of a few selective serine proteases. Equal amounts (5 μg) of activated samples of protease carnein, proteinase K, milin (*27*), cryptolepain (*28*), and a serine protease from *Wrightia tinctoria* were incubated with 50 μg of BSA at 37 °C for 15 min. The resulting fragments were separated on a tricine-SDS-PAGE (*29*).

Antigenic Properties. Antibodies to the purified enzyme were raised in a male albino rabbit (1 kg body weight) as described (*20*), and the presence of antibodies was confirmed by immunoassays. The pure enzyme in 0.05 M Tris-HCl, pH 8.0, buffers was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites. After 1 week, 500 μg of enzyme was emulsified with an equal volume of Freund's incomplete adjuvant and administered as a booster dose. Furthermore, two similar doses were administered at intervals of 7 and 15 days. After the seventh day of the last dose, the rabbit was bled through the marginal ear vein. Blood was allowed to clot initially for 1 h at room temperature and later for 12 h at 4 °C followed by supernatant collection by centrifugation. Preimmune serum was obtained from the rabbit before the first injection of antigen. All sera were stored at −20 °C. The presence of antibodies was confirmed by immunodiffusion studies. Ouchterlony's double immunodiffusion was performed as described by Ouchterlony and Nilsson (*30*). A 1% agarose amount in phosphate-buffered saline containing 0.02% sodium azide was solidified in Petri dishes, and appropriate holes were punched into it. Various antigens (40 μg) of trypsin, milin, carnein, and proteinase K in peripheral wells and 100 μL of antiserum were loaded in the central well and left at room temperature for 24 h. Similarly, control assays were performed with preimmune serum.

Amino-Terminal Sequence Analysis. One milligram of the purified enzyme was dialyzed against distilled water and subjected to reverse phase HPLC on a C18 column. The column was eluted with a linear gradient of 0−60% water−acetonitrile solvent system containing 0.1% trifluroethanol (TFE) at a flow rate of 1 mL/min over a period of 1 h using a LKB Bromma HPLC system. Fractions of 1 mL were collected, and the elution was monitored by absorbance at 280 nm using a variable wavelength detector. The homogeneity of the fractions was determined on SDS-PAGE. The homogeneous fractions were pooled and freeze-dried on a Virtis lyophilizer, and the N-terminal sequence was determined by Edman's automated degradation using an Applied Biosystems 477A peptide sequencer.

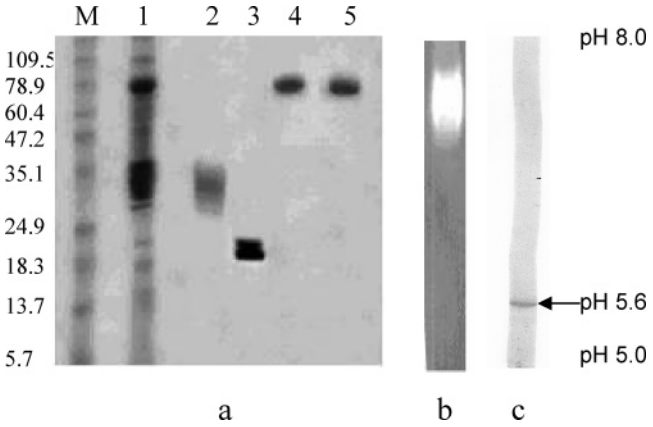

Figure 2. Electrophoretic analysis of purified proteases. (**a**) SDS-PAGE of different pools (mentioned in **Figure 1**) includes 10 μg of prestained molecular weight marker (lane M), 50 μg of crude latex (lane 1), 20 μg of pool I (lane 2), 20 μg of pool II (lane 3), 20 μg of pool III (lane 4) under nonreducing conditions, and 20 μg of pool III (lane 5) under reducing conditions. (**b**) Zymogram showing in-gel proteolytic activity of protease carnein. Twenty micrograms of carnein was loaded on SDS-PAGE with casein for the zymogram study. The unstained region of gel reflects hydrolysis of casein by carnein (**c**). Isoelectric focusing of carnein: 50 μg of carnein was loaded, and electrophoresis was performed using 5% polyacrylamide gels with ampholine carrier ampholytes (pH 5–8) for 3 h at a constant current of 2 mA/rod.

RESULTS

Purification. The crude latex was subjected to saturated ammonium sulfate precipitation (85%), dissolved in acetate buffer, pH 4.0, dialyzed, and loaded on SP-Sepharose fast flow column. The unbound fraction showed no proteolytic activity. The bound protein resolved into two peaks (**Figure 1**), and SDS-PAGE of the fractions showed three different pure proteins. The first protein (fractions 50−65, $M_r \sim 35$ kDa) lacks proteolytic activity, the second protein (fractions 75−85, $M_r \sim$

Table 1. Purification of Carnein from the Latex of *I. carnea*[a]

steps	volume (mL)	protein concn (mg/mL)	total protein (mg)	total activity (units)	specific activity (unit/mg)	purification-fold	yield %
1. crude latex	467	0.40	187	2500	0.134	1	100.0
2. ammonium sulfate precipitation 80% (w/v)	80	1.40	112	1750	0.156	1.2	70.0
3. SP-Sepharose fractions (90–120)	90	0.26	23	1375	0.598	4.5	55.0

[a] Definition of 1 unit: 1 unit of enzyme activity is defined as mentioned in the Materials and Methods.

Table 2. Physicochemical Properties of Carnein in Comparison with Other Plant Serine Proteases[a]

enzyme	plant	mol mass (kDa)	pH optimum	temp optimum (°C)	isolectric point (pI)
carnein	*I. carnea*	80.2	6.5	65	5.6
IBSP82[b]	*I. batatas*	82.0	7.9	40	NR
Ara 12[c]	*A. thaliana*	76.1	5.0	80	NR
MCA protease[d]	*N. tobacum*	68.0	5–9	NR	5.8 ± 0.1
cucumisin[e]	*C. melo* L.	54.0	7.1	70	NR
serine protease[f]	*C. cochinchinensis*	76.0	11	60	NR
KLSP[g]	*P. vulgaris*	72.0	9.9	60	4.6
taraxilisin[h]	*T. officinale*	67.0	8.0	40	4.5
artocarpin[i]	*A. heterophyllus*	79.5	8.0	60	6.3
serine protease[j]	*P. hindsii*	82.0	6.5–10.5	30	NR
serine protease[k]	*T. aestivum*	110.0	8–10	60	NR
RSIP[l]	*Z. mays* L.	59.0	6.0–6.5	NR	4.55
hordolisin[m]	*H. vulgare* L.	74.0	6.0	60	6.9

[a] NR, in the table represents data not reported. [b] Ref *31.* [c] Ref *32.* [d] Ref *33.* [e] Ref *34.* [f] Ref *35.* [g] Ref *36.* [h] Ref *37.* [i] Ref *38.* [j] Ref *39.* [k] Ref *40.* [l] Ref *41.* [m] Ref *42.*

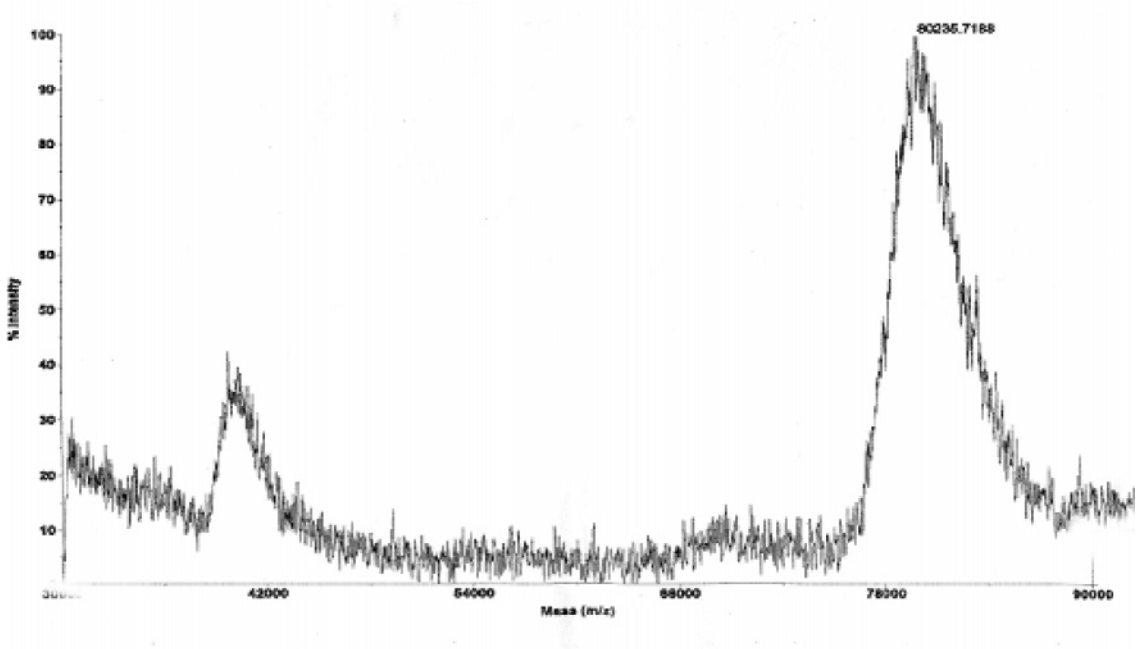

Figure 3. Mass spectrometry of protease carnein: MALDI-TOF. Carnein (10 pmol) was used for MALDI-TOF analysis. BSA was used as a standard for calibration of the instrument, and data were collected in linear mode.

20 kDa) has less activity, and the third protein (fractions 90−120, $M_r \sim 80$ kDa) has good homogeneity as well as caseinolytic activity (**Figure 2a**). The highly pure and active protein (fractions 90−120) was used for further characterization. Fractions 90−120 were pooled, concentrated, dialyzed against 50 mM Tris-HCl, pH 8.0, buffer, and stored at 4 °C. The protease was named carnein as per the rule of protease nomenclature.

Casein zymography (in gel) showed a clear region against a stained background, indicating the proteolytic activity of carnein (**Figure 2b**). The details of the purification analysis are summarized in **Table 1**.

Physical Properties. Carnein showed a single band in SDS-PAGE with an estimated molecular mass (M_r) of 80 kDa (**Figure 2a**); a value of 80.236 kDa was obtained by MALDI-TOF (**Figure 3**). The extinction coefficient measured by spectrophotome-

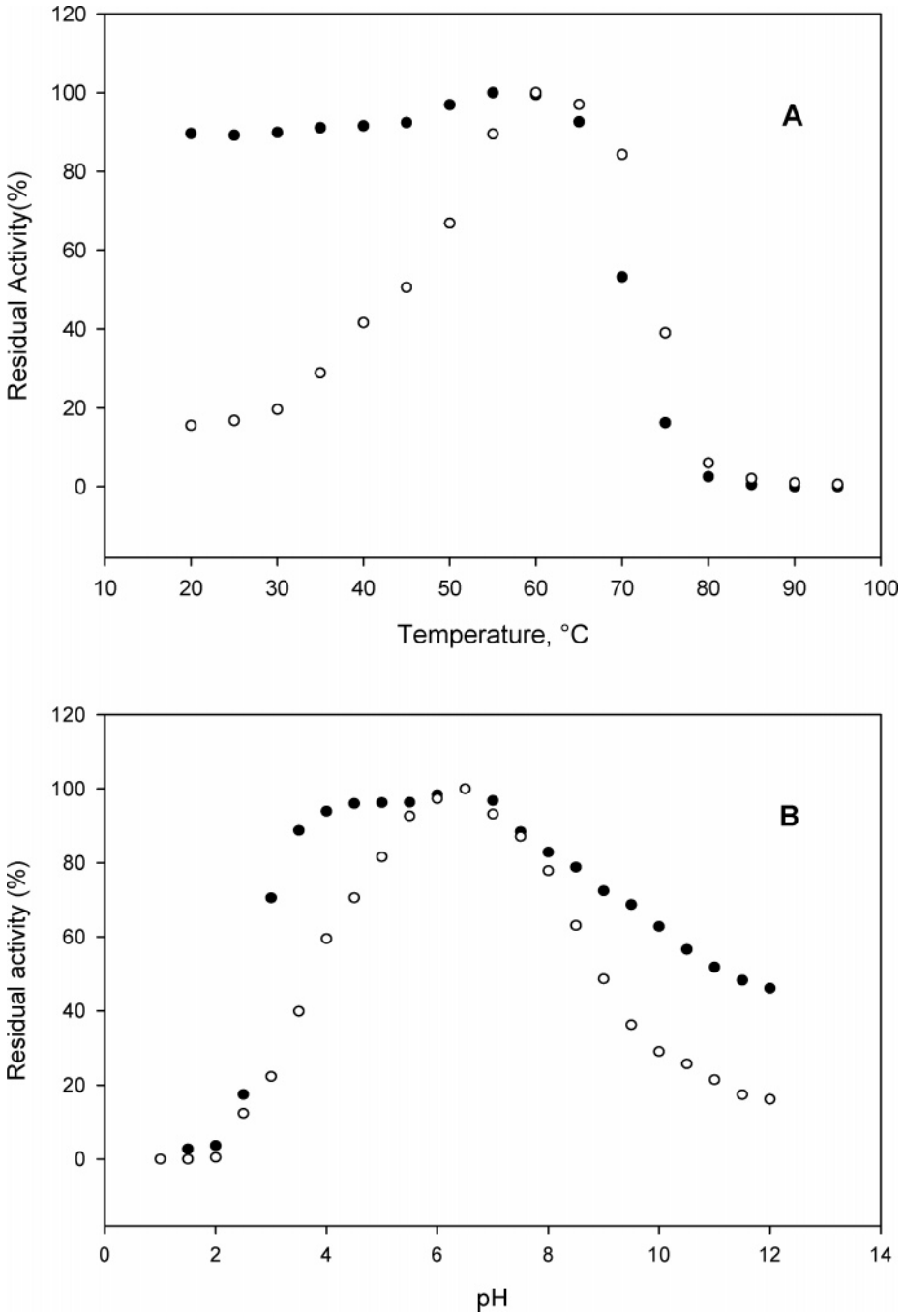

Figure 4. Effects of temperature (**A**) and pH (**B**) on stability (●) and optimum (○) of carnein. To see the effect of temperature on activity, 10 μg of carnein was incubated at a required temperature for 15 min, 0.5 mL of substrate was added to it, and the activity was measured at the same temperature. For stability experiments, 10 μg of enzyme was incubated at the required temperature for 15 min, and activity was measured at 37 °C and pH 8.0. For pH optima, 10 μg of activated enzyme in 0.5 mL of buffer of required pH was used and the activity was determined using substrates prepared in corresponding buffers, as described in the Materials and Methods. The pH stability was determined by overnight incubation of 10 μg of enzyme at room temperature. The maximum activity obtained was taken as 100%, and the residual activity was calculated based on it.

tric methods was 37.12; it was used for all of the practical applications. There was no detectable carbohydrate moiety in carnein. A single band was observed on isoelectric focusing with a pI of 5.6 (**Figure 2c**). Even at very low concentrations (0.02 mg/mL), carnein did not undergo autolysis (data not shown). The comparison of physiological and biochemical properties of carnein with other serine proteases is given in **Table 2**.

pH and Temperature Optima. Carnein retained its proteolytic activity over a broad range of pH and temperature. The enzyme effectively retains its activity toward casein at temperatures in the range of 35−75 °C, and the optimum activity is observed at 60 °C (**Figure 4A**). Similarly, casein hydrolysis can be accomplished at pH 3.0−10.0 with a maximal activity at pH 6.5 (**Figure 4B**).

Effects of Inhibitors. The enzyme was completely inhibited after incubation with 1 mM PMSF for 30 min, and it retained only 0.54% activity with PMSF, 4.01% with DFP, and 0.5% with chymostatin. Cysteine-specific (IAA, $HgCl_2$, E-64, and NEM) and metallopeptidase-specific (EGTA, EDTA, and *o*-phenonthroline) inhibitors did not hinder the proteolytic activity.

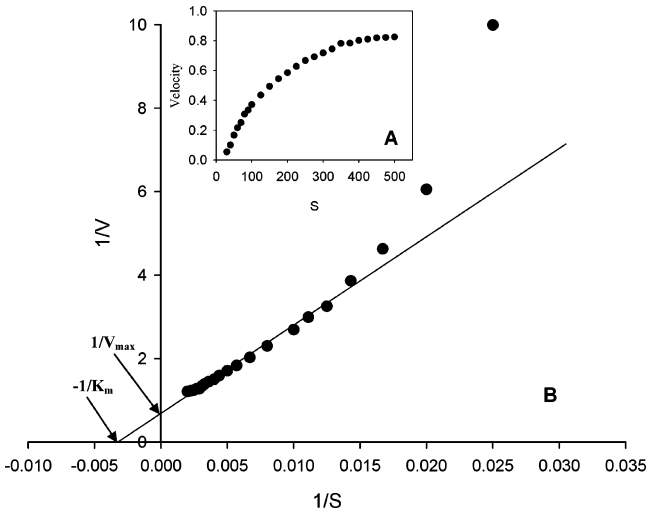

Figure 5. Effect of substrate concentration on reaction velocity of carnein. (**A**) The activity of carnein undergoes saturation at the higher substrate concentration in accordance with the Michaelis–Menten equation. A different concentration of substrates (0.0–0.5 mM) was taken with an equal amount of enzyme (20 μg), and the activity was measured. (**B**) A Lineweaver–Burk plot, 1/(*S*) vs 1/(*V*), has been used for the determination of K_m. The K_m was calculated to be 0.31 mM using casein as a substrate.

Table 3. Effect of Various Compounds on the Activity of Carnein

inhibitor type	name of inhibitor	[I][a] (mM)	residual activity (%)
serine protease	PMSF	1	0.54
	SBTI	2.5	1.70
	DFP	1	4.01
	chymostatin	1	0.5
cysteine protease	IAA	1	98.9
	sodium tetrathionate	1	90.7
	E-64	1	96.7
	$HgCl_2$	1	99.5
	NEM	1	97.2
	PCMB	1	95.0
metallo-protease	EDTA	5	98.3
	EGTA	5	97.4
	o-phenanthroline	5	98.0

[a] Minimum amount of inhibitor for maximum inhibition.

Table 4. Stability of Carnein under Various Conditions

conditions	concentration[a]	% residual activity
urea	5.5 M	86
GuHCl	1.5 M	82
GuSCN	0.5 M	81
DMSO	45%	86
acetonitrile	40%	92
TFE	35%	91
methanol	35%	90
ethanol	30%	90
isopropanol	25%	91
butanol	20%	92
SDS	0.02%	0
Triton X-100	3.5%	97
exalin (a laboratory cleaner)	1.0%	91

[a] Minimum concentration for activity more than 80%.

These results suggest that carnein is likely to be a serine protease. The minimum amount of effective inhibitor required for maximum inhibition is reported in **Table 3**.

Effects of Substrate Concentration on Reaction Velocity. The effects of increasing substrate concentration on reaction velocity follow the typical Michaelis−Menten equation with casein as substrates (**Figure 5A**). Generally, the nature of kinetics with respect to the substrates is typically hyperbolic, and at higher concentrations of the substrate, the enzyme activity attains saturation. The Michaelis−Menten enzyme kinetic model is not appropriate for proteases where the substrate is a native protein with multiple cleavage sites per mole of protein. This may be the reason for the observed deviation at lower protein concentrations. However, such deviation is not seen with

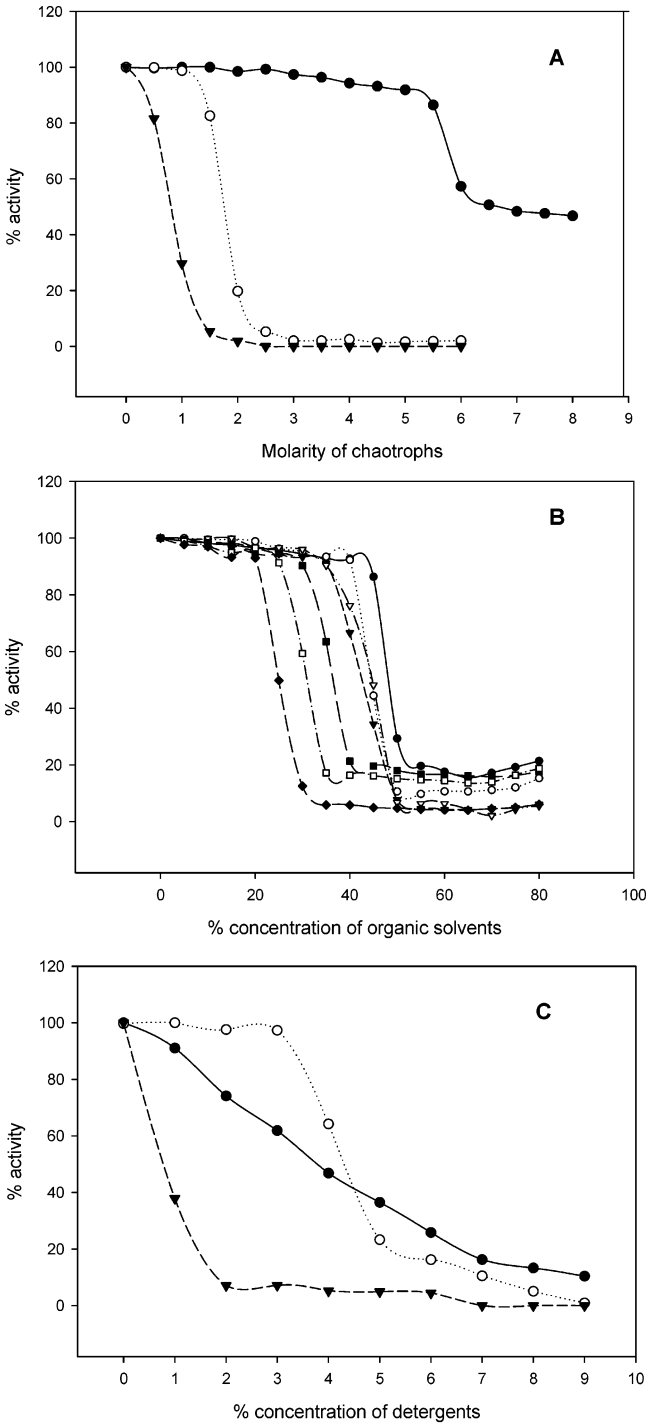

Figure 6. Stability of carnein against chaotrops (**A**): GuHCl (○), urea (●), and GuSCN (▼); organic solvents (**B**): DMSO (●), acetonitrile (○), TFE (▼), methanol (gray triangle), ethanol (■), isopropanol (□), and butanol (◆); detergents (**C**): Triton X-100 (○), SDS (▼), and exalin (●). Ten micrograms of carnein was incubated for 24 h at a given concentration of the additive, and the proteolytic activity was measured.

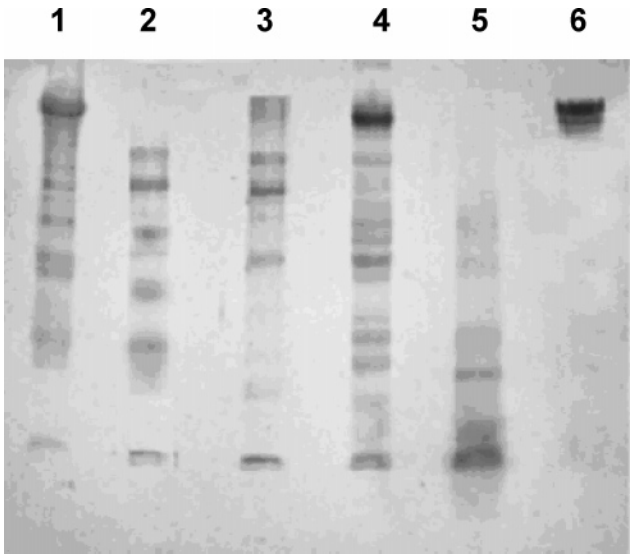

Figure 7. Comparative substrate specificity studies using digestion of BSA. Equal amounts of BSA (50 μg) were digested with various serine proteases. In each case, an equal amount of enzyme (5 μg) was used, and the substrate digestion was allowed to proceed for 15 min. The resulting fragments were electrophoresed on a Tricine SDS-PAGE as discussed in the Materials and Methods. Lanes 1–5 show BSA digestion by a serine protease purified from *W. tinctoria*, carnein, cryptolepain, milin, and proteinase K, respectively; lane 6, BSA alone.

proteases studied in our laboratory. Therefore, this kind of deviation may be protease-dependent. The apparent K_m value obtained from the Lineweaver−Burk plot was 0.30 ± 0.05 mM with casein as the substrate (**Figure 5B**). The values of V_{max} and K_{cat} were determined to be 9.9 ± 0.1 μM min^{-1} and 0.794 min^{-1}.

Substrate Specificity. The enzyme carnein hydrolyzed denatured natural substrates such as casein and hemoglobin with high specific activities. The enzyme shows reasonable amidolytic activity on synthetic substrate, *N*-Ala-Ala-*p*-nitroanilide, L-alanine-*p*-nitroanilide, and L-leucine-*p*-nitroanilide. Proteolysis of BSA by serine protease purified from *W. tinctoria*, carnein, cryptolepain, milin, and proteinase K gave fragments of different lengths on tricine-SDS-PAGE **(Figure 7**), indicating the distinct substrate specificity of carnein.

Specific Amino Acid Residues. The tryptophan and tyrosine contents of the protein were 35 (measured value, 35.2) and 76 (measured value, 75.6), respectively. The total sulfhydryl content of the protein was found to be 7 (measured value, 7.18) with three (measured value, 3.07) exposed cysteines and the remaining four forming two disulfide bonds. Under similar experimental conditions, ribonuclease, papain, proteinase K, and lysozyme gave the reported values.

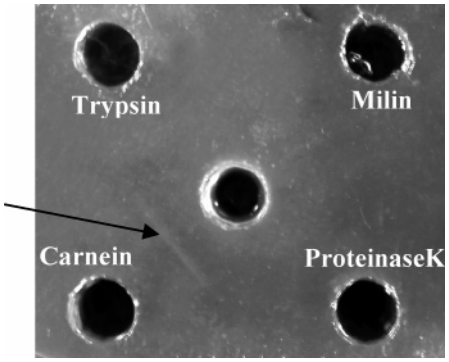

Figure 8. Ouchterlony's double immunodiffusion was carried out in (2%) agarose in phosphate-buffered saline containing 0.02% sodium azide. Anti-carnein serum (100 μL) was added in the central well, and 40 μg of carnein was added in one well; 40 μg of trypsin, milin, and proteinase K was added in the remaining three peripheral wells. The appearance of precipitin bands was observed after 24 h of incubation. This observation identifies unique antigenic determinants of carnein.

Stability. The enzyme is thermostable as it retains almost complete activity up to 70 °C (**Figure 4A**). Like other serine proteases, even after prolonged exposure, the enzyme retained its activity over the pH range 3.0−10.0 as shown in **Figure 4B**. Furthermore, the enzyme's stability has been monitored against chaotrophs [such as guanidine isothiocynate (GuSCN), GuHCl, and urea], organic solvents (DMSO, acetonitrile, TFE, methanol, ethanol, isopropanol, and butanol), and detergents (Triton X-100, SDS, and exalin). The stability of carnein against chaotrophs, organic solvents, and detergents is shown in **Figure 6A−C**, respectively. Concentrations of chaotrophs, organic solvent, and detergent at which the enzyme retains more than 80% activity are tabulated in **Table 4**.

Polyclonal Antibodies and Immunoassays. The presence of polyclonal antibodies in anti-rabbit serum was checked by immunodiffusion. Precipitin lines start appearing after about 10−12 h of incubation at room temperature and are distinctly visible by about 24−30 h toward antigen carnein. A control experiment was performed with preimmune serum in the central well surrounded by carnein antigen in the peripheral wells, and no precipitin line was observed (data not shown). The precipitin line is formed as a result of precipitation of antigen−antibody complexes near the equivalence zone. Antisera to carnein did not cross-react with trypsin, milin (*29*), and proteinase K (**Figure 8**), suggesting that the antigenic determinants of carnein are different from that of trypsin, milin, and proteinase K.

Table 5. Comparison of N-Terminal Sequences of Carnein and Other Plant Endopeptidases[a]

enzyme	botanical name	family	amino terminal sequence (first 20 residues)	identity (%)
carnein	*I. carnea*	Convolvulaceae	**TTHSP**E**FLGLAESSGL**X**PNS**	**100**
IBSP82	*I. batata*	Convolvulaceae	**T**PRT**P**Q**FLGLAES**BF**LVPN**D	**60**
alnus ag12	*A. glutinosa*	Betulaceae	**TTH**T**PRFL**SLNPTG**GL**W**P**A**S**	**55**
protease B	*E. supina*	Euphorbiaceae	**TT**RT**PNFLGL**VDD**SGL**WLDG	**50**
P69A	*L. esculentum*	Solanaceae	**TTH**TSS**FLGL**QQNN**G**VMKD**S**	**45**
P69B	*L. esculentum*	Solanaceae	**TTRSPTFLGL**EGRESRSFFP	**40**
ARA12	*A. thalliana*	Cucurbitaceae	**TT**RT**PLFLGLDE**HTADLFPE	**40**
cucumisin	*C. melo*	Cucurbitaceae	**TTRS**WD**FLG**FPLTVPRRSQV	**30**
kiwano protease	*C. metuliferous*	Cucurbitaceae	**TTRS**WD**FLG**FPQNVPRVSQV	**30**
lily LIM 9	*L. longiflorum*	Liliaceae	**TTH**T**PDYLG**IQTGVWPELGG	**30**
cucumisin-like protease	*B. criapra*	Cucurbitaceae	**TTRS**WD**FL**NFPQNIQRVNQV	**25**

[a] N-terminal sequence references of other proteases were taken from ref *2*. The amino acids are abbreviated with one letter symbols, and the identical amino acid residues are represented in bold.

Amino-Terminal Sequence. The N-terminal sequence of carnein was generated by the Edmann degradation method. The first 20 amino residues, T-T-H-S-P-E-F-L-G-L-A-E-S-S-G-L-X-P-N-S showed high % identity with that of subtilisin-like serine proteases (**Table 5**). Thus, it appears that carnein may belong to family of subtilisin-like endopeptidases.

DISCUSSION

Three proteins have been identified in the latex of *I. carnea*; two out of three are serine proteases, indicating the occurrence of a multiplicity of serine proteases in plant lattices. A simple purification procedure has been developed to simultaneously purify three proteins. The purification procedure was very efficient in terms of time, labor, total enzyme units, specific activity, yield, and fold purification (**Table 1**). The protein having a high protease activity (80 kDa) has been emphasized for further studies. Such an economic purification procedure combined with the easy availability of the latex makes large-scale preparation of the enzyme possible, allowing a broad study of its various aspects and hence probable applications. Endopeptidases such as papain and stem bromelain have been extensively used in medicine, brewing wine, and food industries (*2*). In fact, 60% of the enzymes used in industry are proteolytic; hence, the study of proteolytic enzymes such as carnein is essential.

A single band in SDS-PAGE, tube gel isoelectric focusing, a symmetrical peak in gel filtration (data not shown), and a single precipitin line in Ouchterlony's double immunodiffusion confirm the purity of the enzyme.

The enzyme is inhibited by serine specific inhibitors (chymostatin, DFP, SBTI, and PMSF) and not by cysteine- and metallopeptidase-specific inhibitors, suggesting carnein to be a serine protease. The estimated molecular mass of carnein (80.236 kDa) was in the range of molecular mass (60−85 kDa) reported for the majority of plant serine proteases (**Table 2**).

Carnein, like some other serine proteases, showed amidolytic activity in addition to proteolytic activity. Like proteinase K, it hydrolyzes synthetic substrates such as L-Ala-Ala-*p*-nitroanilide, L-alanine-*p*-nitroanilide, and L-leucine-*p*-nitroanilide. It does not hydrolyze BAPA, an ideal substrate for papain, ficin, and other plant endopeptidases. This indicates that the enzyme acts mostly on the nonpolar R group amino acids, whereas its activity over polar and aromatic amino acid is not detectable.

Most of the conserved N-terminal residues observed in plant serine proteases are found to be conserved in carnein. The N-terminal residues of carnein when compared to those of known plant serine proteases, especially subtilases, showed a high degree of identity. Therefore, carnein is likely to be a member of the subtilisin family. Further sequence analysis, catalytic site studies, and structural determinations are essential to categorize carnein.

Carnein is a fairly stable protease and resembles subtilases in its stability toward pH, strong denaturants, temperature, and organic solvents. It is stable up to a temperature of 70 °C and over a broad pH range of 3.0−10. Carnein retains more than 80% activity in 5.5 M urea, 1.5 M GuHCl, 45% DMSO, 40% acetonitrile, 35% TFE, and 30% ethanol. Such a high stability against pH, temperature, and various organic solvents enables one to explore carnein in industrial and biotechnological applications. Carnein did not undergo autolysis even at very low concentrations (0.02 mg/mL), demonstrating the resistance of the enzyme to autodigestion, and thus its value in various industrial applications.

Polyclonal antibodies specific to carnein have been raised, and the immunoassays confirm that anticarnein serum does not cross-react with other related serine proteases. The polyclonal antibodies of carnein may facilitate in purification and detection as well as serve as a probe in biophysical investigation.

By virtue of its remarkable physiochemical properties, carnein may have potential applications in several industries. On the other hand, carnein is an excellent model system to study the structure−function of plant serine proteases as well as protein folding problem.

ABBREVIATIONS USED

BAPA, NR-benzoylarginine *p*-nitroanilide; BSA, bovine serum albumin; DFP, diisopropylfluorophosphate; DMSO, dimethyl sulfoxide; DTNB, 5,5μ-dithiobis (2-nitrobenzoic acid); E-64, 3*S*-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-ethylbutyl}carbamoyl)oxirane-2-carboxylic acid; EDTA, ethylenediamminetetraacetic acid; EGTA, ethylene glycol-bis(α-amino ethyl ether)tetraacetic acid; GuHCl, guanidine hydrochloride; GuSCN, guanidine isothiocynate; IAA, iodoacetic acid; NEM, *N*-ethyl maleimide; PAGE, polyacrylamide gel electrophoresis; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TFE, trifluroethanol.

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