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Studies on Low Molecular Mass Phytocystatins Purified from *Phaseolus mungo* (Urd)

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Abstract—In the present study two phytocystatins (thiol protease inhibitors) have been isolated and purified to homogeneity from *Phaseolus mungo* by a simple two-step procedure using ammonium sulfate fractionation and gel filtration on Sephacryl-100 HR. The latter procedure yielded two peaks of the inhibitors (PMC I and PMC II). The pH optimum of both phytocystatins was pH 7.0; the temperature optima for PMC I and PMC II were 65 and 70°C, respectively. The molecular masses of the purified phytocystatins were 19 and 17 kD, respectively, as determined by SDS-PAGE and mass spectrometry. Antibodies raised against the purified cystatins gave a single precipitin line in Ouchterlony double immunodiffusion. Kinetics of inhibition showed that PMC I and PMC II strongly inhibit papain and ficin but not trypsin and chymotrypsin. Binding stoichiometry of PMC I and PMC II with both papain and ficin was 1 : 2. The effect of urea on PMC I and PMC II was analyzed by fluorescence and circular dichroism spectroscopy. The CD results suggest an unfolding of PMC I and PMC II accompanying a decrease in the amount of extended (hydrated) coil structure and an increase in sheet-like structure. FTIR results show that PMC I is structurally similar to PMC II. Hydrophobic interactions are observed over a long time scale (5-150 min). Furthermore, fluorescence spectroscopy results were found to be in accordance with CD results, by showing quenching of fluorescence intensity of PMC I and PMC II, although to different extents, due to perturbations of the environment of aromatic residues in the protein. Both cystatins showed strong inhibitory activity against *Escherichia coli* and *Staphylococcus aureus*.

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The cystatin superfamily includes three families: type I cystatins (stefins), type II cystatins, and type III cystatins (kininogens) [1]. Type I cystatins are single chain proteins with molecular mass of 11 kD that contain no disulfide bonds or carbohydrate. Type II cystatins with molecular mass of 13 kD contain single chain proteins with two disulfide bonds near the carboxyl terminus [1, 2]. Members of the third cystatin family, the kininogens, are large molecules with three type II-like domains and bound carbohydrate. During the last two decades, a fourth group belonging to the cystatin superfamily has emerged, that is the plant cystatins specifically named phytocystatins [3]. Homology searches show that some plant cystatins resemble family II cystatins of animal origin, while others resemble family I cystatins of the mammalian system [4].

Phytocystatins have been identified and studied in many plant sources such as rice [5, 6], maize [7], soybean [8], cowpea [9], potato [10], Chinese cabbage [11], and carrot [12]. Thiol protease inhibitors present in the plant system perform a variety of functions, and they are regulated by phytocystatins. They are important in a variety of ways, including their role in storage proteins [13], as regulators of endogenous proteolytic activity [14], and as participants in the mechanism of programmed plant cell death [15]. Apoptosis has been implicated in several plant processes such as xylogenesis, some forms of senescence, and in response to attack by pathogens [16]. Furthermore, proteinase inhibitors are expressed in abiotic stress [17] and in plant defense processes against insect attack [18]. Phytocystatins present in cereal seeds like rice and maize have been used to prevent certain types of cancer [19].

Rice contains three species of cysteine proteinase inhibitors, Oz α, Oz β, and Oz λ, which play a role in maturation of storage proteins like glutenin, rice seed

Abbreviations: PMC I and II) *Phaseolus mungo* cystatins I and II; FTIR) Fourier transform infrared spectroscopy; CD) circular dichroism.

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ripening, and proteolysis of storage proteins during germination [20]. Furthermore, purified sugarcane cystatin is found to inhibit the growth of the filamentous fungus *Trichoderma reesi* [21], which is a potential biotechnological application of phytocystatins.

Most studies give details of cystatins purified from mammalian species, and very few studies elucidate cystatins purified from plant sources. Moreover, gaps exist in the characterization of cystatins from a widely used legume (urd). Therefore, the aim of this study was to obtain in-depth knowledge about this cystatin. This study reports the purification and some characteristics of a phytocystatin purified from this new source, *Phaseolus mungo*. The purified phytocystatin has been characterized on the basis of molecular mass, kinetic behavior towards various proteases, and secondary structure analysis by CD, FTIR, and fluorescence spectroscopy.

MATERIALS AND METHODS

All chemicals used in the study were of analytical grade.

Purification of PMC I and PMC II. PMC I and PMC II were purified by a modification of the method of Wu and Haard [22]. One hundred grams of urd seeds were soaked in 25 mM sodium phosphate buffer (pH 7.0) containing 0.15 M sodium chloride overnight at 4°C. The seeds were homogenized and subjected to centrifugation in a Sigma refrigerated centrifuge (Japan) at 8000 rpm for 20 min at 4°C. The supernatant was collected and supplemented with ammonium sulfate to 40% saturation. The suspension was kept for 4 h at 4°C and then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant thus obtained was made 70% saturated with ammonium sulfate. After 4 h, the pellet was recovered by centrifugation and dissolved in 10 ml of 25 mM sodium phosphate buffer (pH 7.0). This pellet was extensively dialyzed against several changes of the same buffer to remove ammonium sulfate. The dialyzed sample was then loaded on a Sephacryl S-100 gel filtration column. Five milliliter fractions were collected and assayed for cystatin inhibitory activity and protein concentration. Results of the characterization of PMC I and PMC II are presented; however, wherever the properties of PMC I and PMC II are similar, only the PMC I data are shown.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) of PMC I and PMC II was performed at pH 7.0 in 7.5% gel [23]. SDS-PAGE in the presence and absence of β-mercaptoethanol was done using 12.5% polyacrylamide gel for both PMC I and PMC II. The proteins were silver stained [24].

Assay of protease inhibitory activity. Cystatin inhibitory activity was determined by a modification of the method of Kunitz [25]. Commercial papain and ficin were incubated with various amounts of PMC I and PMC II in 1 ml of 25 mM sodium phosphate buffer, pH 7.0, containing 50 mM cysteine and 20 mM EDTA at 37°C for 30 min. One milliliter of 2% casein solution was added, and the mixture was incubated further at 37°C for 30 min. The reaction was terminated by adding 1 ml of 10% trichloroacetic acid. Acid insoluble material was removed by centrifugation at 3000 rpm for 15 min. The supernatant was analyzed for acid soluble peptides with Folin phenol reagent by the method of Lowry et al. [26]. Inhibitory activities of PMC I and PMC II on trypsin, chymotrypsin, and bromelain were also measured using casein as the substrate.

Mass spectrometry of PMC I and PMC II: matrix-assisted laser-desorption time-of-flight (MALDI-TOF) mass analysis. To further check the purity, cystatins I and II were again loaded on the Sephacryl S-100 gel filtration column and eluted with 25 mM sodium phosphate buffer (pH 7.0). Samples were then freeze-dried, desalted, and prepared for analysis on a Voyager Bioworkstation (Perspective Biosystems).

The samples were dissolved in 1% trifluoroacetic acid (TFA) and the matrix—sinapinic acid (saturated solution in acetonitrile–0.1% TFA, 1 : 1 v/v; Sigma, USA)—was added. This preparation was then vortexed and 1.2 ml (1 mg/ml) of each cystatin (I and II) was applied on the sample plate. The spectrophotometer equipped with delayed extraction system accessory, was operated in linear mode. Sample ions were evaporated using a N_2 laser at 330 nm wavelength and accelerated at a potential of 20 kV with a delay of 134 nsec. Around 150 shots of 3 nsec pulse width laser light were required to ionize the sample. Finally, the signal was digitized at a rate of 480 MHz and averaged data were presented to the data system for correction.

Fourier transform infrared spectroscopy. Infrared spectroscopy was used to examine the secondary structure components present in PMC I and PMC II. The spectra were truncated between 1740 and 1520 cm^{-1} and baseline corrected. The equipment used was a Nicolet (Esp) 560 spectrophotometer (USA) equipped with transmission Omnic Esp 5.1 software and a DTGS detector; data were analyzed and quantified using Grams 32 software. PMC solutions (0.15 mg/ml) were prepared in 25 mM sodium phosphate buffer (pH 7.0) and original spectra of native PMC I and PMC II were taken with a resolution of 4 cm^{-1} and 128 scans.

Immunoassay. Immunodiffusion was performed by the method described by Ouchterlony and Nilsson [27]. The reaction was allowed to proceed for 24 h at room temperature.

Carbohydrate estimation. Neutral sugars of PMC I and PMC II were determined by the phenol sulfuric acid method of Dubois et al. [28] using glucose as a standard.

Temperature and pH stability of PMC I and PMC II. The thermal stability of PMC I and PMC I was investigated by measuring their inhibitory activity in 25 mM

sodium phosphate buffer (pH 7.0). For this investigation, the cystatins were incubated at 4, 25, 50, 75, and 100°C for 30 min.

The pH stability of PMC I and PMC II was investigated in the range pH 3-10 at 37°C for 24 h. PMC I and PMC II were incubated with 25 mM sodium acetate buffer (pH 3-6), 25 mM sodium phosphate buffer (pH 7-8), and 25 mM Tris-HCl buffer was used for adjusting pH 9-10; pH 6-8 was adjusted with 1 N HCl or NaOH. Tris-HCl buffer was used for adjusting pH 9-10. After incubation, the mixtures were neutralized and remaining inhibitory activity was analyzed using casein as the substrate.

Circular dichroism (CD) spectroscopy. Solutions (0.1 mM) of purified PMC I and PMC II were prepared in 3 ml of 25 mM sodium phosphate buffer, pH 7.0, and allowed to equilibrate for 24 h. Urea was added to a final concentration of 6 mM, and the resulting solution was stirred and warmed to 37°C on a magnetic stirring plate/heater. Samples were prepared by placing a 40 µl aliquot of PMC I and PMC II solutions between two quartz windows with a 0.02 cm path length. CD spectra (180-280 nm, 0.5 nm steps) were collected using a dry nitrogen-purged Jasco-715 spectropolarimeter. Spectra were collected from PMC aliquots before urea was added, just after urea was added, and every 25 min, until no additional changes in the spectra were observed. Since CD spectroscopy is particularly sensitive to α-helical content but less reliable in predicting changes in the β-sheet or extended coil structure, popular self-consistent basis methods [29-31] were successful at modeling the changes observed. The difference spectra were generated by subtracting the spectrum of intact PMC I and PMC II from spectra of urea-treated PMC at each time point using Grams 32 software. The changes in the peak frequency and intensity were then compared to the standard CD spectra of primary α-helix, β-sheet, and coil structure [32] to qualitatively interpret structural changes. The time course of the structural changes was determined by plotting the change in the ellipticity versus time at 200 and 225 nm, where the largest structural changes occurred. Time constants were determined by fitting with Prism software.

Fluorescence spectroscopy. For fluorescence measurements, samples were prepared as described above and fluorescence emission spectra were taken before and after urea addition after each time interval up to 330 min in the wavelength range of 300 to 400 nm. The equipment used for this investigation was a Shimadzu RF-1501 spectrofluorophotometer (Japan), and spectra were recorded using a xenon arc lamp as the light source.

Antibacterial activity of PMC I and PMC II. The antibacterial activity of PMC I and PMC II was checked by determination of inhibition zone diameter [35]. Different bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*) were allowed to grow overnight in nutrient broth at 37°C, and then 0.3 ml of the fresh culture was overlaid with soft agar on nutrient agar plates to aid the formation of bacterial lawns. Whatman filter discs were placed in 25 µg/ml, 50 µg/ml, and 1 mg/ml of the inhibitor to be absorbed on discs. After 2 h, the discs were placed on the bacterial lawn and the inhibition zone diameter was determined.

RESULTS AND DISCUSSION

Purification of the inhibitor. The two step procedure described in the present study for the purification of PMC I and PMC II from *Phaseolus mungo* is simpler and more convenient than that reported earlier for other cystatins [34, 35]. The protein retained on the gel filtration column was obtained as two peaks, I and II (Fig. 1). Both peaks showed approximately 90% inhibitory activity. The papain inhibiting fractions with the highest inhibitory activity in both peaks were pooled and checked for their homogeneity. When subjected to native PAGE, they yielded single bands, as shown in Fig. 2A (lanes *a* and *b*). The subunit structure of PMC I and PMC II was assessed by SDS-PAGE under reducing (in the presence of β-mercaptoethanol) as well as non-reducing conditions, which also yielded single bands with apparent molecular masses of 19 and 17 kD for PMC I and PMC II, respectively (Figs. 2B and 2C). A total of 2.9 and 3.7 mg of PMC I and PMC II with 1340-fold enrichment and yields of 18 and 16% were purified, respectively (Table 1). The yield and purification fold were higher than that reported by Wu and Haard [22] for phytocystatin isolated from methyl jasmonate treated tomato plants. It has also been reported that most of the phytocystatins are 6-12 kD in size and contain no disulfide bonds [36]. The carbohydrate contents in PMC I and PMC II were found to be 1 and 1.4%, respectively. The purified inhibitor is placed in

Table 1. Purification of phytocystatins from *P. mungo*

Step	Volume, ml	Total protein, mg	Specific activity*, units/mg
Homogenate	100	1200	0.33
Ammonium sulfate fractionation (40-70%)	10	110	15
Dialysis	12	108	19
Gel filtration			
PMC I	5	5.0	440
PMC II	5	5.5	445

* One unit of inhibitor enzyme activity is defined as the amount of inhibitor bringing a 0.001 change in absorbance/min per ml.

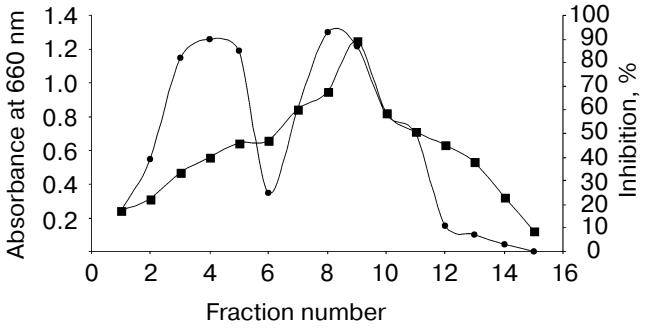

Fig. 1. Gel filtration of PMC I and PMC II on Sephacryl S-100 HR (1.2 × 80 cm): protein profile (■); elution profile (•).

the family of type IV cystatins (phytocystatins) and is found to possess properties of both type I and type II cystatins of the mammalian system.

MALDI-TOF analysis. Molecular masses of the cystatins as analyzed by mass spectrometry were found to be 19,124 and 17,510 daltons for cystatins I and II, respectively (Fig. 3), which is very similar to that determined by SDS-PAGE (under reducing condition). It has been reported that the molecular masses of type I and II animal cystatins are in the range of most of the phytocystatins [9, 37]. A cysteine proteinase inhibitor isolated from the fruit bodies of *Clitocype nebularis* has been reported with a molecular mass of 17 kD [38].

Fourier transform infrared spectroscopy. In the IR spectra of proteins, the secondary structure is most clearly reflected by the amide I and, in particular, amide II bands [39-41]. The amide I band absorbs at 1657 cm^{-1} (mainly C=O stretch) and the amide II band absorbs at 1542 cm^{-1} (C–N stretching coupled with N–H bending modes) [42, 43]. It has also been reported that, for a native protein, the amide I component for the α-helical structure is located at 1656 ± 2 cm^{-1}, and the band components for β-sheet structure are located between 1622 and 1642 cm^{-1} (lower wave number β-sheet bands) and between 1690 and 1698 cm^{-1} (higher wave number β-sheet bands) [39-41, 44]. Figure 4 shows the original spectra of native PMC I and PMC II. Quantitative analysis of the protein secondary structure for native PMC I and PMC II summarized in Table 2 shows the secondary structures of both the cystatins are similar, which is also evident by the peak intensities of the two proteins. The difference was that the peak intensity for PMC I was recorded at 1646 cm^{-1}, while for PMC II it was recorded at 1652 cm^{-1}.

Immunoassay. For immunodiffusion, antigen (cystatin) was loaded in the central well and antiserum was loaded in other wells in varying concentrations (Fig. 5, a and b). A single precipitin line was observed for both cystatins, which shows that PMC I and PMC II are immunologically pure. Furthermore, cross-reactivity was also

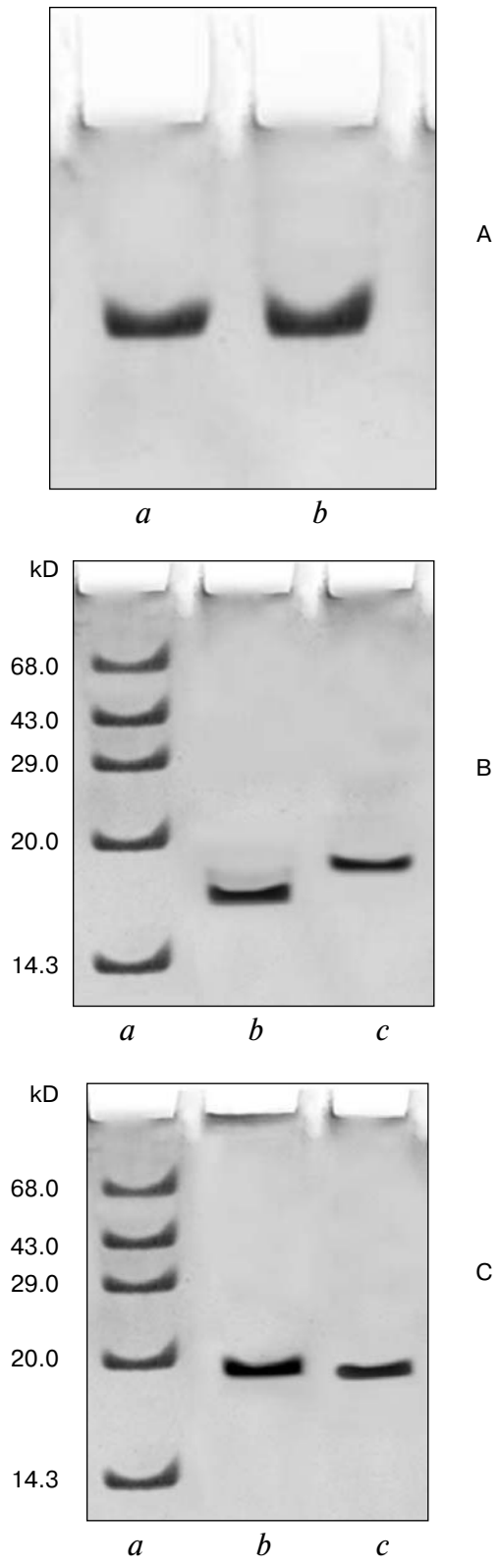

Fig. 2. A) Native PAGE of PMC I (*a*) and PMC II (*b*). B, C) SDS-PAGE of PMC I and PMC II in the presence (B) and in the absence of β-mercaptoethanol (C). In panels (B) and (C), lane *a* shows marker proteins, lane *b* is PMC II, and lane *c* is PMC I.

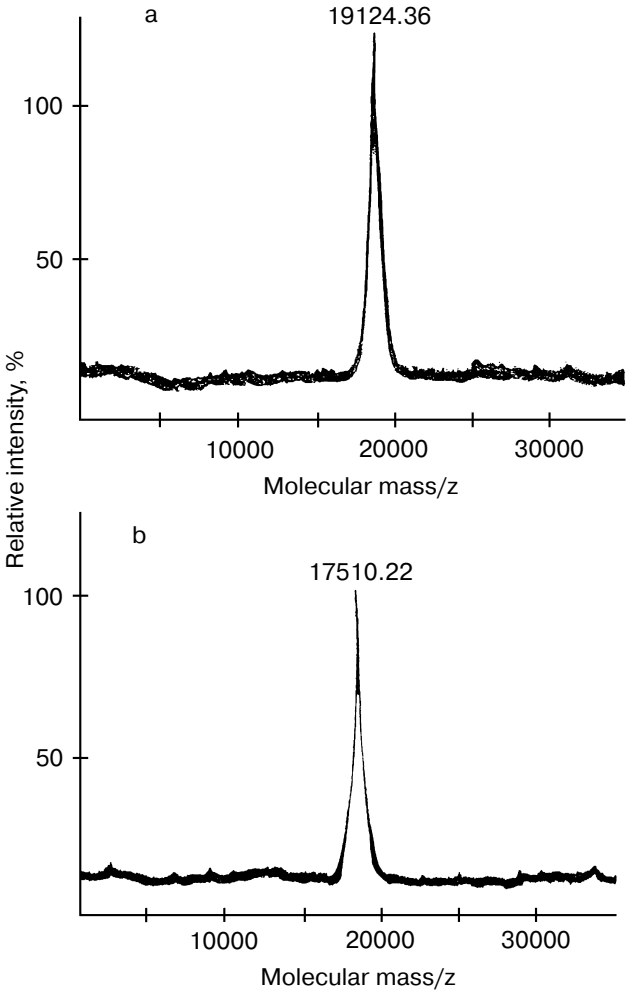

Fig. 3. Molecular masses of cystatins determined by mass spectrometry (MALDI-TOF): a) PMC I; b) PMC II.

checked in PMC I and PMC II (Fig. 5, c and d). With both, a single precipitin line was obtained, which suggests that they are immunologically pure and identical.

Cystatin autolysis. There was no autolysis of PMC I and PMC II as well as no change in the inhibitory activity even after 24 h of incubation.

Temperature and pH stability. PMC I and PMC II retained about 79% inhibitory activity at pH 4.0 and 83% inhibitory activity at pH 8.0 after prolonged exposure (24 h), and their activity was significantly decreased below pH 4.0 and above pH 8.0. The inhibitors retained 100% activity after 30 min of incubation at up to 75°C at pH 7.0, while there was 19% decline in the activity at 90°C. These properties are similar to phytocystatin isolated from tomato plants [9]. Furthermore, it has also been reported that most of the purified phytocystatins are stable to extremes of pH and temperature [34]. These results show that PMC I and PMC II are extremely stable protein inhibitors, as are other phytocystatins like that of *Clitocype* [38].

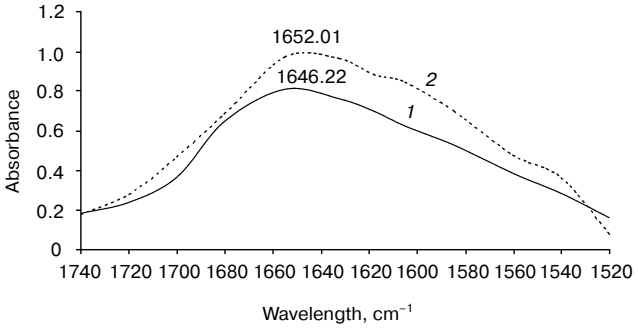

Fig. 4. FTIR spectra of native PMC I (*1*) and PMC II (*2*).

Circular dichroism (CD) spectroscopy. Far-UV CD spectra in the presence and absence of urea are shown in Fig. 6. Figure 6a shows far-UV CD spectra at different time points (30-330 min) during the unfolding of PMC I, while curves in Fig. 6b show the change in the ellipticity at 200 and 220 nm as a function of time. Analysis of model CD spectra [32] was used in interpreting the spectral changes. Positive and negative changes near 200 nm are representative of β-sheet and extended coil conformations, respectively. Results in Fig. 6 suggest that, as PMC unfolds, a decrease in the negative feature near 200 nm is consistent with the loss of extended coil and an increase in sheet-like structure (k = 0.0061 ± 0.001 min^{-1}, $t_{1/2}$ = 83.5 min, R^2 = 0.835). The observed increase in the negative feature near 220 nm also supports an increase in sheet-like conformation, as PMC I and PMC II unfold (k = 0.0048 ± 0.001 min^{-1}, $t_{1/2}$ = 102 min, R^2 = 0.851). Thus the changes observed in the CD spectra of unfolding PMC I and PMC II are consistent with a loss of hydrated, extended coil structure and an increase in sheet-like hydrophobic interactions.

Fluorescence spectroscopy. Fluorescence spectroscopy was also used to investigate spectral changes during the interaction of PMC I and PMC II with urea.

Table 2. Secondary structure determination of native PMC I and PMC II by Fourier transform infrared spectroscopy

Wave number, cm^{-1}	Structure	Content, %	
		PMC I	PMC II
1692-1680	β-anti	7 ± 1	7.8 ± 1
1673-1666	β-turn	8 ± 1	6.7 ± 1
1658-1650	α-helix	58 ± 2	56 ± 3
1640-1615	β-sheet	27 ± 1	29 ± 2

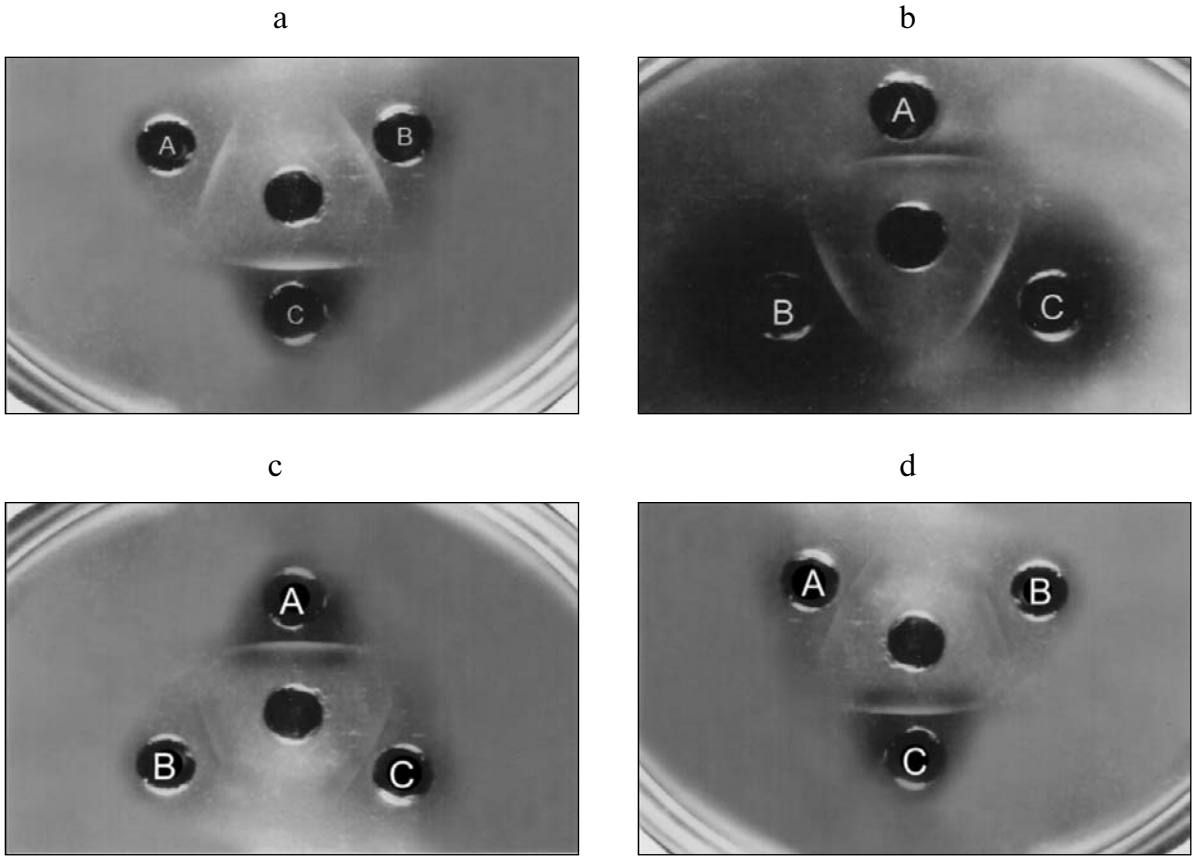

Fig. 5. Ouchterlony double immunodiffusion of PMC I and PMC II. a) The central well contained PMC I (50 µl) while wells A, B, and C contained antisera against PMC I in different concentrations (50 µg, 100 µg, and 1 mg). b) The central well contained PMC II (50 µl) while wells A, B, and C contained antisera against PMC II (50 µg, 100 µg, and 1 mg). c) Cross reactivity between PMC I and PMC II. The central well contained antiserum against PMC I (50 µl) while wells A, B, and C contained PMC II (50 µg, 100 µg, and 1 mg). d) Cross reactivity between PMC I and PMC II. The central well contained antiserum against PMC II (50 µl) while wells A, B, and C contained PMC I (50 µg, 100 µg, and 1 mg).

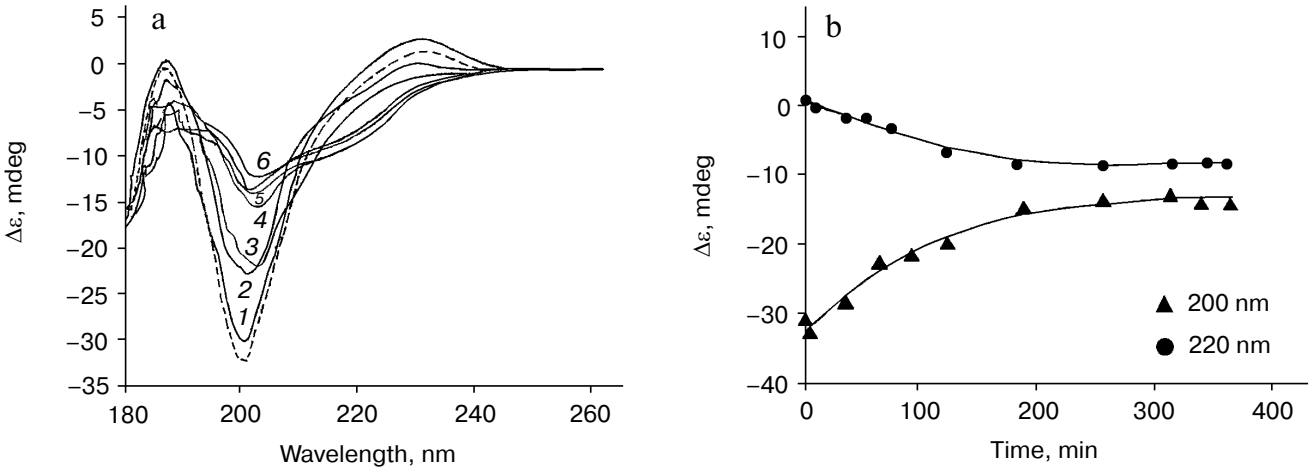

Fig. 6. a) CD spectra at different time points during the unfolding of PMC I. The dashed line represents the spectrum before the addition of urea. The solid lines (*1-6*) represent spectra collected in 30, 60, 120, 240, 300, and 330 min after the addition of urea, respectively. b) Change in the ellipticity at 200 and 220 nm as a function of time.

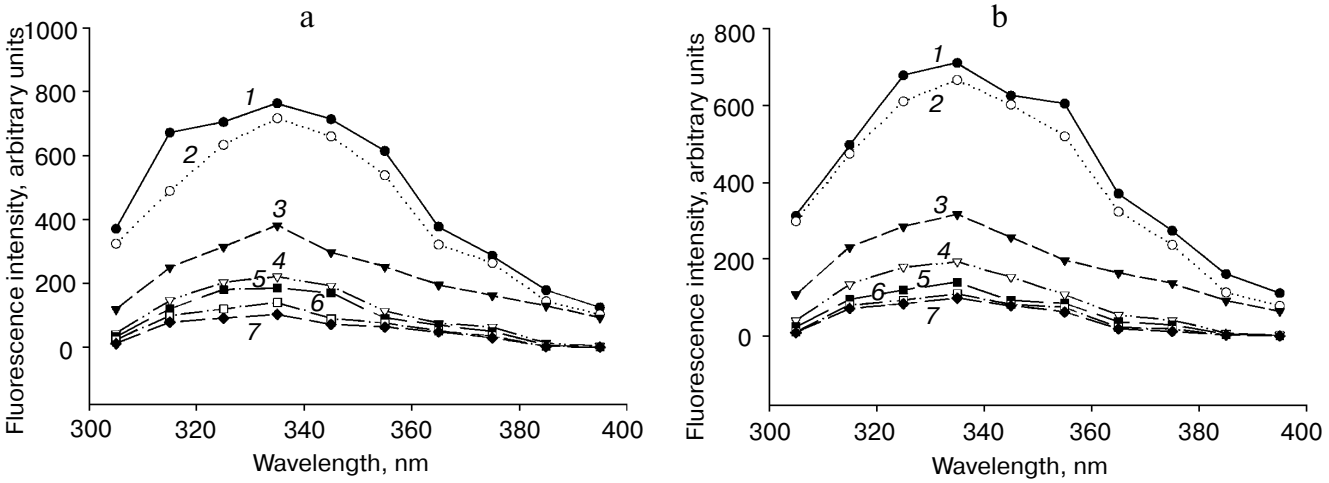

Fig. 7. Fluorescence spectra of PMC I (a) and PMC II (b) taken before (*1*) and 30, 60, 120, 240, 300, and 330 min (*2-7*, respectively) after addition of urea.

The results obtained are in accordance with the CD results and implicate quenching of fluorescence emission, which is minimal upon 25 min of incubation of PMC I and PMC II with urea, but increases successively with increasing time (Fig. 7). These spectroscopic changes resulting in decrease in fluorescence emission may be due to perturbations in the environment of aromatic residues in the protein. Quenching of fluorescence increased to 8.2% in PMC II as compared to PMC I.

Antibacterial activity of PMC I and PMC II. The antibacterial effect was quantified based upon the inhibition zone diameter. The results illustrated in Table 3 show that PMC I and PMC II have strong antibacterial activity against *E. coli* and *S. aureus*, which is a striking observation, while they were found to be ineffective against *B. subtilis*. Antifungal activity of thiol protease inhibitors like sugarcane cystatin has also been reported in earlier studies [22].

These results suggest that the discovery of PMC I and PMC II should broaden the spectrum of specific cysteine proteinase inhibitors available for potential use in human medicine and in agricultural crop protection. Furthermore, detailed conformational analysis of phytocystatins must await the results of X-ray crystallographic studies of phytocystatin–urea complex.

Table 3. Antibacterial effects of PMC I and PMC II

Inhibitor concentration, μg/ml	Inhibition zone diameter, mm*		
	B. subtilis	*S. aureus*	*E. coli*
25	—	13-14	11-12
50	—	13-14	11-12
100	—	≥17	15-16
1000	—	≥17	15-16

* Symbol "–" means no effect.

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