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PHENOLIC HYDROXYL IONIZATION IN CALOTROPINS FROM *CALOTROPIS GIGANTEA* LATEX

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Key Word Index—*Calotropis gigantea*; Asclepiadaceae; madar plant; cysteine proteases; phenolic hydroxyl ionization; chemical modification.

Abstract—The ionization of the phenolic hydroxyl groups in calotropins DI and DII isolated from the latex of *Calotropis gigantea* has been studied by spectrophotometric titration at 295 nm in the pH range 6–13.2. Of the 12 tyrosine residues of calotropin DI and 13 tyrosine residues of calotropin DII, only four residues were ionized reversibly in the pH range 8.9–10.7 with the apparent pK of 9.7. The remaining tyrosine residues were ionized irreversibly in the pH range 11.2–13.2 with the apparent pK of 11.5. Both calotropins showed time-dependent ionization of phenolic groups at 295 nm in the pH range 11.5–12.0. Chemical modification with tetranitromethane suggested the presence of three tyrosine residues on the surface of each calotropin molecule.

INTRODUCTION

Calotropins DI and DII isolated from the latex of madar plants, *Calotropis gigantea*, are classified as plant cysteine proteases like well-studied papain, ficin and stem bromelain [1, 2]. Earlier communications [3, 4] from this laboratory describe the crystallization and physicochemical, enzymatic and immunological studies of both calotropins. These two enzymes are closely similar but marked differences in detail have been found, including differences in auto-digestion behaviour, amino acid composition, peptide mapping and carboxyterminal amino acid sequence.

We have continued the comparative approach to the problem of the structure–activity relationship in calotropins and present in this paper the results of studies on the spectrophotometric titration of phenolic hydroxyl groups in two enzymes. In order to correlate the spectrophotometric data with the chemical reactivity, the reaction of tetranitromethane with calotropins has also been described.

RESULTS AND DISCUSSION

Ultraviolet absorption spectra

The UV absorption spectra of calotropins DI and DII at three different pH values are shown in Fig. 1. The patterns of the spectral shift at alkaline pHs are closely similar to those reported for papain [5] and stem bromelain [6]. It was noted that in both calotropins the change in absorption at 295 nm, characteristic of tyrosine ionization [7], was increased with increasing pHs and maximum change was observed in the region of pH 13.

Titration curves

Figure 2 shows the plots of molar A at 295 nm vs. pH of solutions of calotropins DI and DII. In both cases the ionization of the phenolic hydroxyl groups begins at pH 8 and is complete at pH 12.5–13. The change in molar A at 295 nm is from 18 570 at pH 7 to a maximum of 47 260 at pH 13 for calotropin DI and from 18 010 to 47 620 for calotropin DII over the same pH range. Amino acid analyses [3] have indicated the presence of 12 tyrosine residues in calotropin DI, and 13 tyrosine residues in calotropin DII. Thus the changes in molar A at 295 nm per tyrosine residue are 2390 for calotropin DI and 2280 for calotropin DII. These values are in good agreement with reported values of other proteins [8, 9].

It was observed that in the pH range 7–11.5 the change in A at 295 nm was instantaneous after adjustment of neutral solution of calotropins to the desired pH. At pH values higher than 11.5, a time-dependent ionization of phenolic groups in both calotropins was observed and the change in A with time gradually increased as the pH increased (Fig. 3). In these instances an ionization equilibrium seems to be attained within 1 hr. It appears from the wavelength dependence of this increase in A that the phenomenon is probably due to slow denaturation of the proteins, but not due to aggregation or precipitation [5, 6].

The reverse titration curves for both calotropins are shown in Fig. 2. The data were obtained by reverse titration of calotropin solutions after they had been exposed to pH 13 for 1 hr to obtain complete ionization equilibrium. As shown in this figure, exposure to pH 13 leads to irreversible changes in the ionization behaviours of both calotropins. The reverse titration could not be continued below pH 10 because of the appearance of a precipitate below this pH. In the pH range 6–10.5 the ionization was found to be reversible in both calotropins (not shown in the figure).

As found for a number of proteins [6–9] the ionization

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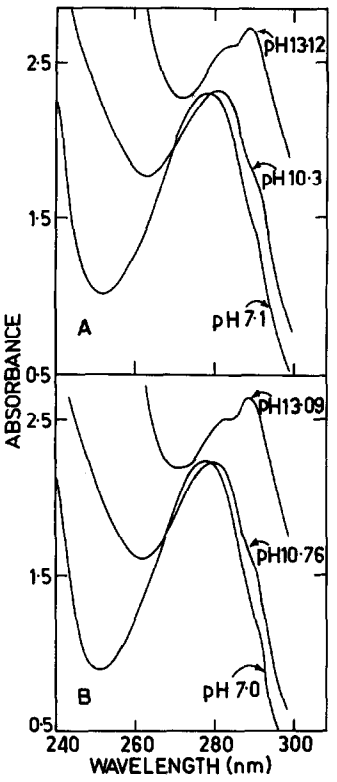

Fig. 1. UV absorption spectra of (A) calotropin DI and (B) calotropin DII as a function of pH. Spectra were taken after achieving complete ionization equilibrium. Protein concentrations were 4.80×10^{-6} M for calotropin DI and 4.76×10^{-6} M for calotropin DII.

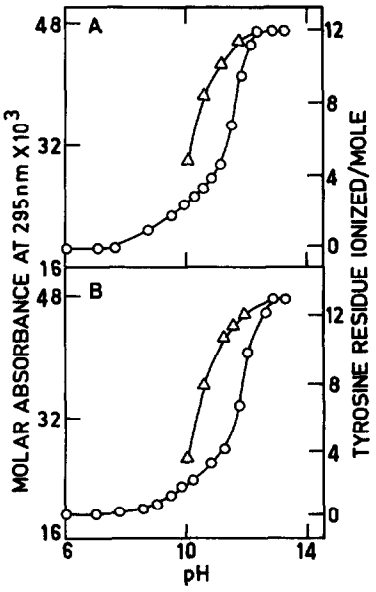

Fig. 2. Spectrophotometric titration curves of (A) calotropin DI and (B) calotropin DII at 295 nm and ionic strength of 0.2. Protein concentrations were 4.74×10^{-6} M for calotropin DI and 4.83×10^{-6} M for calotropin DII. O——O, Forward titration; △——△, reverse titration, reversed after exposure to pH 13 for 1 hr.

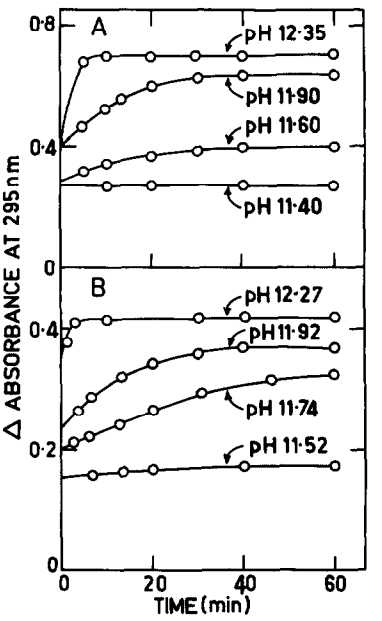

Fig. 3. Time dependence of the UV absorption of (A) calotropin DI and (B) calotropin DII at 295 nm. Protein concentrations were 13.86×10^{-6} M for calotropin DI and 8.26×10^{-6} M for calotropin DII.

behaviours of tyrosine residues of both calotropins are complex. At least two types of tyrosine residues can be easily distinguished in their forward titration. In the pH range 8.9–10.7 where no time-dependence of increase in absorption was observed, four tyrosine residues of each calotropin were ionized reversibly with the apparent pK of 9.7. The remaining tyrosine residues of both calotropins ionized irreversibly in the pH range 11.2–13.2 with the apparent pK of 11.5.

Reaction with tetranitromethane

Tetranitromethane is known to react selectively with tyrosine residues in proteins to form 3-nitrotyrosyl residues [10]. Nitration of calotropins at pH 7.5 with 5-fold molar excess of tetranitromethane per mole of protein for about 60 min resulted in the modification of three tyrosine residues with 30% loss of activity for calotropin DI and 21% for calotropin DII. The results obtained are shown in Fig. 4. The nitrated derivatives were found to be homogeneous as judged by ion-exchange chromatography on SP-Sephadex and by SDS–polyacrylamide gel electrophoresis at pH 7. Amino acid analyses of calotropins before [3] and after nitration revealed that tetranitromethane nitrated only three tyrosine residues without affecting other amino acid residues present in calotropins. It was observed that increase in pH, tetranitromethane concentration or reaction time did not modify additional tyrosine residue, but led to the aggregation of protein molecules, particularly at higher pH.

Results presented in this study suggest that three tyrosine residues of each calotropin are present on the surface of the molecule and that an additional residue becomes accessible to the solvent as the pH rises up to 10.8 (Fig. 2). The remaining tyrosine residues in both calo-

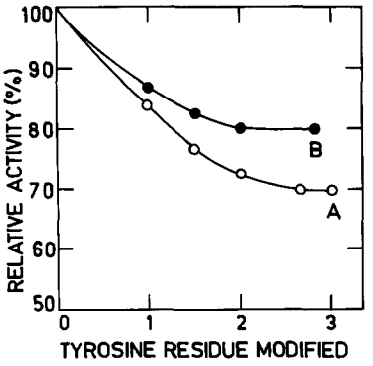

Fig. 4. Relationship of proteolytic activity of calotropin DI (A) and calotropin DII (B) to the number of tyrosine residues modified by tetranitromethane.

tropins appear to be buried, and their ionization is probably associated with an irreversible disorganization of protein molecules as indicated by reverse titration.

EXPERIMENTAL

Enzyme purification and assay. Calotropins DI and DII were isolated and purified from the latex of *C. gigantea* as described previously [3]. Both calotropins were reversibly inactivated with Na tetrathionate to avoid autodigestion at alkaline pH. Protein concn was determined spectrophotometrically at 280 nm using $E^{1\,cm}_{1\%} = 19.5$ for each calotropin and M_rs of 23 800 for calotropin DI and 24 200 for calotropin DII [3].

Spectrophotometric titration. The titration of phenolic hydroxyl groups of tetrathionate—inactivated calotropins was carried out in a Beckman recording spectrophotometer with 10-mm quartz cells at 295 nm at 25° ± 0.5° in the pH range 6–13.2, according to ref. [11]. Compositions of buffers used were 0.02 M Tris–HCl plus 0.2 M KCl for pH 6.0–9.0; 0.02 M glycine–KOH plus 0.2 M KCl for pH 9.47–10.73 and mixtures of 0.2 M KOH and 0.2 M KCl with varying mutual ratios for pH 10.55–13.2. In all solns KCl was added to maintain the ionic strength of 0.2. Enzyme concn was in the range $4.74–4.83 \times 10^{-6}$ M. Optical readings were taken until the maximum difference at 295 nm was obtained, usually within 1 hr. Reverse titrations were performed at 295 nm and 0.2 ionic strength using 0.2 M HCl with the reaction mixture exposed to pH 13 for 1 hr.

Measurements of pH were made in a Radiometer pH-meter at the same temp. at which the spectra were recorded. The meter was calibrated with standard buffers of pH 6.48 and 9.18 at 25°. A current of pure N_2 satd with H_2O was blown over the solns to avoid absorption of atmospheric CO_2. The apparent pK values of tyrosine groups were calculated from the titration data as described [6].

Reaction of tyrosine residues with tetranitromethane. The tyrosine residues of tetrathionate-inactivated calotropins DI and DII were nitrated with tetranitromethane in 0.1 M NaPi buffers, pH 7–8.5 at room temp. according to ref. [10]. The degree of nitration was estimated from the A at 428 nm at pH 9 using $E_{428} = 4200\ M^{-1} \cdot cm^{-1}$ for nitrophenoxide ion [10] and by amino acid analysis [3]. The purity of the nitrated calotropins was checked by chromatography on SP-Sephadex C-50, under conditions described in ref. [3], and by SDS–polyacrylamide gel electrophoresis [12].

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