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CHEMICAL MODIFICATION AND AMINO TERMINAL SEQUENCE OF CALOTROPIN DI FROM *CALOTROPIS GIGANTEA*

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Key Word Index—*Calotropis gigantea*; Asclepiadaceae, madar plant, cysteine protease, chemical modification, *N*-terminal sequence

Abstract—The effect of chemical modification of histidine, lysine, arginine, tryptophan and methionine residues on the enzymatic activity of calotropin DI has been studied. 1,3-Dibromoacetone inhibited the enzyme completely, indicating that a single histidine residue and a cysteine residue are involved in its catalytic activity. Its second histidine residue was modified with diethyl pyrocarbonate without loss of activity. Modification of seven of its 13 lysine residues with 2,4,6-trinitrobenzene sulphonic acid led to 90% loss of its activity, but no single lysine residue appears to be essential for its activity. Four of the 12 arginine residues by 1,2-cyclohexanedione can be modified with little loss of activity. Modification of a single tryptophan residue and two methionine residues did not inhibit enzymatic activity. The blocked amino-terminal amino acid residue of calotropin DI has been identified as pyroglutamic acid. Its amino-terminal amino acid sequence to residue 14 has been determined and compared with that of papain. They show an extensive homology in their amino-terminal amino acid sequences.

INTRODUCTION

Calotropin DI which is classified as a plant cysteine protease like the well-studied papain, ficin and stem bromelain [1, 2] has been isolated in crystalline form from the latex of the madar plant, *Calotropis gigantea* [3]. There are substantial similarities between calotropin DI and papain in their physicochemical and enzymatic properties [3] and overall three-dimensional structure [4, 5]. However, distinct differences have been noted in their immunological behaviour, amino acid composition, carboxyterminal amino acid sequence and specificity towards synthetic substrates [6]. Calotropin DI does not hydrolyse synthetic ester and amide substrates commonly used for the hydrolysis by papain and ficin.

The necessity of a cysteine residue for the enzymatic activity of calotropin DI and the reactivity of its tyrosine residues with tetranitromethane have been reported earlier [3, 7]. In the present communication we report the results of modification studies on lysine, histidine, arginine, tryptophan and methionine residues aimed at establishing whether these residues are critical for the enzymatic activity of calotropin DI. The determination of its amino-terminal amino acid sequence and a comparison with the corresponding sequence of papain are also described.

RESULTS AND DISCUSSION

Lysine modification

2,4,6-Trinitrobenzene sulphonic acid (TNBS) has been used as a modifying agent to arylate the amino groups in proteins [8]. Since the *N*-terminal amino acid of calotropin DI is blocked, its lysine residues have been modified by this reagent. Figure 1 shows the effect of modification of lysine residues by TNBS on the enzymatic activity of calotropin DI. Enzymatic activity decreases gradually with the increasing number of lysine residues modified. It appears that no single lysine residue is essential for the activity of calotropin. There are 13 lysine residues in calotropin, seven of which were modified by TNBS with the loss of 90% of its enzymatic activity. In contrast, papain lost only 5% of its activity on modification of seven of its 10 lysine residues and probably the *N*-terminal amino group. The result is consistent with that reported by others [9] who showed that seven lysine residues of papain were modified with *O*-methylisourea with retention of full activity.

Histidine modification

1,3-Dibromoacetone (DBA) is a bifunctional alkylating reagent known to react with cysteine and histidine residues in cysteine proteases [10, 11]. At pH 5.6 it completely inhibited calotropin DI. The M_r of the inhibited enzyme was found to be indistinguishable from that of the native enzyme by gel filtration, indicating the absence of cross-linking between enzyme molecules. Amino acid analysis indicated the loss of 0.8 mol histidine residue and 0.5 mol half-cysteine residue per mol of calotropin when compared with the native enzyme. The loss of a half-cystine residue was less clear because of the instability of this residue during acid hydrolysis. It is suggested that, as with papain [10], ficin and stem bromelain [11], both cysteine and histidine residues are involved in the catalytic activity of calotropin DI.

Diethyl pyrocarbonate (DEPC) at 1 mM modified a single histidine residue of tetrathionate-inactivated calo-

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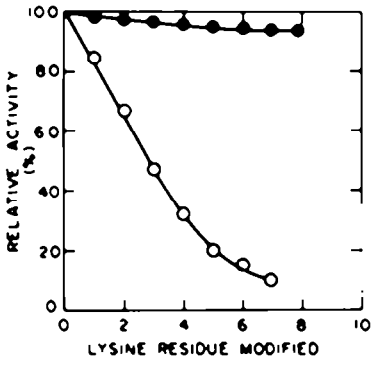

Fig. 1. Relationship of proteolytic activity of calotropin DI (O——O) and papain (●——●) to the number of lysine residues modified by TNBS. Enzymes were incubated with excess TNBS in 0.1 M Na borate buffer, pH 9.5 and at room temperature. The number of amino groups modified was determined spectrophotometrically and assays were performed on azoalbumin as a substrate.

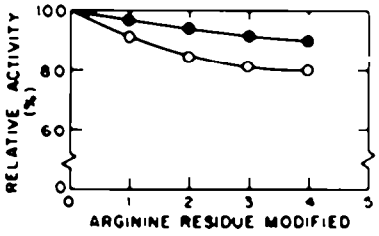

Fig. 2. Relationship of proteolytic activity of calotropin DI (O——O) and papain (●——●) to the number of arginine residues modified by CHD. Enzymes were incubated with excess CHD in 0.2 M Na borate buffer, pH 9 and at 37°. The number of arginine groups modified was estimated from amino acid analysis, and assays were performed on azoalbumin as a substrate.

tropin DI at pH 7.5 in 2 min. An increase in the DEPC concentration or reaction time did not modify additional histidine residues, but led to the precipitation of protein molecules after 30 min. The UV absorption spectra of native and DEPC-treated calotropin DI were different. The difference had a maximum near 242 nm, which indicated a reaction with a histidine residue [12]. The absence of any detectable difference between the spectra in the region of 280 nm, suggested that DEPC did not react with the tyrosine residue [13]. The modified calotropin possessed full enzymatic activity when its blocked cysteine residue was regenerated by reaction with 2-mercaptoethanol. There are two histidine residues in calotropin, one of which reacted with DEPC and had no obvious catalytic function. The second histidine residue which was catalytically important, was probably not accessible to DEPC. It was observed that under identical conditions active calotropin lost essentially all of its activity when treated with DEPC. The loss of activity was due to the reaction of its active cysteine residue with DEPC as judged by titration with Ellman's reagent [14].

Arginine modification

1,2-Cyclohexanedione (CHD) is known as a specific reagent for modification of arginine residues of proteins [15]. Calotropin DI has 12 arginine residues, four of which were modified with CHD in 2 hr with 20% loss of its enzymatic activity (Fig. 2). No further modification of the arginine residues was observed on prolonged treatment with CHD. A similar result was obtained with papain in which four of 12 arginine residues were modified with CHD in 60 min with a 10% loss of activity.

Tryptophan modification

2-Hydroxy 5-nitrobenzoyl bromide (HNBB) was used as a modifier [16]. Of the three tryptophan residues of calotropin DI, only one residue was modified with HNBB without affecting its enzymatic activity significantly.

Methionine modification

Calotropin DI has two methionine residues which were modified with 0.1 M H_2O_2 at pH 8.8. The amino acid analysis of oxidized enzyme showed a quantitative conversion of its two methionine residues to methionine sulphone [17]. Modification of methionine residues resulted in 10% loss of activity, indicating their non-involvement in the catalytic activity of calotropin DI.

Amino-terminal amino acid sequence

Calotropin DI has a blocked *N*-terminal amino acid residue, as reported earlier [6]. Mild alkaline treatment of *S*-amidomethyl calotropin DI yielded a glutamic acid residue as a major amino acid together with trace amounts of other amino acids such as alanine, leucine and isoleucine, suggesting that pyroglutamic acid was the *N*-terminus of the enzyme. This was confirmed by treatment of the enzyme with pyroglutamate aminopeptidase which removed the blocked *N*-terminal amino acid residue of calotropin DI and exposed arginine as its new terminal amino acid.

After removal of pyroglutamic acid, *S*-amidomethyl calotropin DI was subjected to Edman degradation and the first 13 amino acid residues were identified. Figure 3 shows the *N*-terminal amino acid sequence of calotropin DI in comparison with that of papain whose complete amino acid sequence [18] is known. It appears that homology between them is notably high, with nine of the 14 residues the same. When compared with the *N*-terminal sequences of other plant cysteine proteases such as stem bromelain [19], actinidin [20] and asclepains [21], several amino acid residues have been observed to be highly conserved. On the other hand, there was no homology among calotropins, papain and stem bromelain in their *C*-terminal amino acid sequences [6].

EXPERIMENTAL

Materials. Papain, pyroglutamate aminopeptidase, catalase and azoalbumin were purchased from Sigma. Protein modification reagents were obtained from Aldrich. Sequencing reagents were from Pierce.

Preparation and assay of calotropin DI. Calotropin DI was isolated and purified from the latex of *C. gigantea* as described earlier [3]. Its proteolytic activity was determined at pH 7.5 at 37° using azoalbumin as a substrate [22]. In modification studies

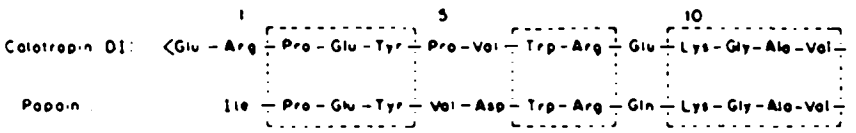

Fig. 3. Amino-terminal amino acid sequences of calotropin DI and papain. Numbers refer to the position of the amino acid in papain taken from Mitchel *et al.* [18]. ∠Glu indicates pyroglutamic acid. Identical amino acid residues are boxed.

Na tetrathionate-inactivated calotropin DI was used by conversion of its active sulphydryl group to a sulphenyl thiosulphate $(SSSO_3)^-$. Prior to enzyme assay its activity was regenerated by treatment with a thiol reagent, 2-mercaptoethanol. The conc. of calotropin DI was determined spectrophotometrically using $E_{1cm}^{1\%}$ = 19.5 at 280 nm and a M_r of 23800 [3].

Lysine modification with TNBS. The lysine residues of tetrathionate-inactivated calotropin DI and papain were modified with TNBS according to ref. [23]. The number of lysine residues modified was calculated from the change in A at 367 nm by using E_{367} = 1.1×10^4 M cm for the trinitrophenyl amino group.

Histidine modification with DBA. The histidine residue of active calotropin DI was modified with 3-fold molar excess of DBA per mol of protein according to ref. [10]. The number of modified groups was determined by amino acid analysis.

Histidine modification with DEPC. The modification of histidine residue of both active and tetrathionate-inactivated calotropin DI was done with 1 mM DEPC in 0.2 M KPi buffer, pH 7.5 at room temp. according to ref. [12]. The number of histidine residues that reacted with DEPC was calculated from the change in A at 242 nm by using E_{242} = 3200 M cm.

Arginine modification with CHD. The arginine residues of tetrathionate-inactivated calotropin DI and papain were modified with a 20-fold molar excess of CHD per mol of protein in 0.2 M Na borate buffer, pH 9.0 at 37° according to ref. [15]. The number of modified groups was determined by amino acid analysis.

Tryptophan modification with HNBB. This was done with HNBB according to ref. [16] on tetrathionate-inactivated calotropin DI in 0.05 M NaPi buffer, pH 7 at room temp. for 24 hr. Excess reagent was removed by dialysis against the same buffer in the cold. The dialysed soln. was diluted with an equal vol. of 2 M NaOH soln. and A measured at 410 nm. The extent of tryptophan modification was determined by using E_{410} = 1800 M cm.

Methionine modification with H_2O_2. The methionine residues of tetrathionate-inactivated calotropin were oxidized with 0.1 M H_2O_2 at room temp. in 0.1 M Na borate buffer, pH 8.8 according to ref. [24]. After 1 hr, residual H_2O_2 was destroyed by the addition of 1 μg of catalase. The number of modified groups was determined by amino acid analysis.

Preparation of S-amidomethyl calotropin. The active calotropin DI was inactivated by blocking its active SH group with iodoacetamide at pH 8.5 [25] to obtain the *S*-amidomethyl derivative.

Amino acid analyses. Amino acid analyses of native and modified calotropins were performed on a Beckman-Multichrom amino acid analyser after hydrolysis under red. pres. in constant boiling HCl for 22 hr at 110° [26].

Amino-terminal amino acid analysis. The blocked *N*-terminal amino acid of calotropin DI was determined by mild alkaline treatment of its *S*-amidomethyl derivative with 1 M NaOH according to ref. [27] followed by dansylation and thin-layer chromatography [28].

Its blocked *N*-terminal amino acid residue, pyroglutamic acid, was removed by treatment with pyroglutamate aminopeptidase by the method of ref. [29].

Sequence analyses. The *N*-terminal amino acid sequence of pyroglutamate aminopeptidase-treated *S*-amidomethyl calotropin DI was determined manually by the phenyl isothiocyanate method [30]. The PTH-amino acids were identified by TLC [31]. Ninhydrin–collidine spray was used to develop the chromatograms [32]. PTH arginine was identified by spot reaction on a filter paper with modified Sakaguchi reagent [33]. In some cases the PTH-derivatives were characterized by identification of amino acids liberated on hydrolysis with 5.7 M HCl containing 0.1% $SnCl_2$ at 150° for 4 hr in evacuated sealed tubes [34].

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