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STUDIES ON PROTEINASES FROM *CALOTROPIS GIGANTEA* LATEX

II. PHYSICO-CHEMICAL PROPERTIES OF CALOTROPAIN-FI AND FII

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Summary

The molecular weights of purified calotropain-FI and FII were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and by gel filtration on Sephadex G-100.

Activation of calotropain-FI and FII by different sulfhydryl activators was studied. The results obtained from inhibition studies by various enzyme-modifying reagents suggest the possible role of cysteine and histidine residues in the active site of both the enzymes.

The free and total sulfhydryl contents of both the enzymes were determined by the use of 5-5'-dithio-bis-2-nitrobenzoic acid. Total amino acid compositions of both the enzymes were also determined.

A comparative study of the esterase, amidase, milk-clotting and caseinolytic activities of calotropain-FI and FII are also presented.

Introduction

We previously reported [1] the purification of two proteinases containing carbohydrate (calotropain-FI and FII) from *Calotropis gigantea* latex. In addition to the differences in chromatographic, electrophoretic behaviour, as well as carbohydrate content, N-terminal amino acid and specific activities of the

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Abbreviations: Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester-HCl; Bz-Arg-*p*Na, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide-HCl.

two enzymes, the present paper demonstrates other physico-chemical differences between these two enzymes.

Materials and Methods

All the chemicals used were of analytically pure grade. Water distilled in an all-glass unit was used throughout.

Sephadex G-100 (fine) was obtained from Pharmacia Fine Chemicals. Crystalline bovine serum albumin, ovalbumin, cytochrome *c*, 5,5′-dithio-bis-2-nitrobenzoic acid, α-*N*-benzoyl-L-arginine ethyl ester-HCl (Bz-Arg-OEt) and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide-HCl (Bz-Arg-*p*Na) were from Sigma. Crystalline pepsin and trypsin were from Nutritional Biochemicals Corporation. Papain (electrophoretically homogeneous) was obtained from V.P. Chest Institute (New Delhi).

Molecular weights determinations. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis for the determination of molecular weights of calotropain-FI and FII was carried out by the method of Weber et al. [2]. Crystalline bovine serum albumin, ovalbumin, pepsin, papain and cytochrome *c* were used as standards. Electrophoresis was carried out for 3.5 h using 20 μg protein samples and standards on 10% acrylamide gel (0.6 × 7.0 cm gel) with a current of 8 mA/tube.

Molecular weight determination of both enzymes by gel filtration on a Sephadex G-100 column (2.4 × 24 cm, 100 ml gel) was carried out according to the method of Andrews [3,4]. The column was equilibrated and eluted with 0.01 M sodium phosphate buffer (pH 7.0), containing 0.145 M NaCl at 25°C (flow rate, 30 ml/h, 2-ml fractions). Crystalline bovine serum albumin, ovalbumin, pepsin, trypsin and cytochrome *c* were used as standards. Protein content was estimated according to Lowry et al. [5].

Effect of various reducing agents. The effect of various reducing agents (effective concentration of 0.005 M) in the presence of 0.002 M EDTA on the caseinolytic activity [1] of the two enzymes (20 μg/ml) was studied (Table II).

Effect of various inhibitors and photooxidation. Calotropain-FI and FII (*p*-chloromercuribenzoate inhibited) were separately activated at 30°C for 15 min as described earlier [1] and passed through a Sephadex G-25 column (1.7 × 10 cm, equilibrated with 0.2 M sodium phosphate buffer pH 7.0), to remove the activating agent.

20 μg of the above enzymes (0.4 ml) were mixed with 0.4 ml of inhibitor solution prepared in distilled water (effective inhibitor concentration of $2.5 \cdot 10^{-3}$ M), kept at 37°C for 15 min and subsequently assayed for caseinolytic activity, with and without activating agent [1] (Table III).

For photooxidation of calotropain-FI and FII (*p*-chloromercuribenzoate inhibited) in the presence of methylene blue or rose bengal, 1 ml of 100 μg/ml enzyme in 0.05 M sodium phosphate buffer (pH 7.0) was mixed with 1 ml of 0.02% dye solution (prepared in the same buffer). Photooxidation was carried out for 15 min at 12—15°C. The enzyme (*p*-chloromercuribenzoate inhibited) with dye solution in the dark, or with buffer in the dark or in light, served as controls. After dialysis with 0.1 M sodium phosphate buffer (pH 7.0) in the dark at 4°C for 6 h, enzyme activity was determined [1] (Table III).

Determination of the sulfhydryl content. The free sulfhydryl content of calotropain-FI and FII was determined by Ellman's 5,5′-dithio-bis-2-nitrobenzoic acid titration method [7]. Acetone-precipitated purified enzyme was dissolved in 0.1 M sodium phosphate buffer (pH 8.0) containing the activating agent [1]. After 15 min at 37°C, the activating agent was removed by passage through a Sephadex G-25 column (1.7 × 10 cm, equilibrated previously with 0.1 M sodium phosphate buffer, pH 8.0). The fraction having maximum protein content was collected under N_2 and immediately used for the sulfhydryl titration. Readings were recorded at 412 nm on a Beckman DU-2 spectrophotometer using cuvettes of 1 cm path length. This titration was also carried out in the presence of 8 M urea (AnalaR, free from thio-urea) and in 1% SDS (Table IV).

Determination of the total sulfhydryl content of calotropain-FI and FII was carried out by the method of Cavallini et al. [8] for enzymes with and without heat denaturation (97°C, 10 min) (Table IV).

Amino acid analysis. Acetone-precipitated calotropain-FI and FII (purified) were dried over P_2O_5 in vacuum. 5-mg samples of the enzymes were hydrolysed at 105°C in the presence of 6 N HCl for 24, 48 and 72 h and were analysed on a Beckman 'Unichrome' automatic amino acid analyser (Table V).

Esterase and amidase activity. The esterase activity (Bz-Arg-OEt as substrate, pH 6.2, 35°C) and amidase activity (Bz-Arg-*p*Na as substrate, in 0.05 M sodium phosphate buffer, pH 6.0, 37°C) of calotropain-FI and FII were determined as described for papain [9] (Table VI).

Milk-clotting and caseinolytic activity. The milk-clotting activity of calotropain-FI and FII was determined according to the method of Hata et al. [10] with slight modification.

1 ml 0.1 M sodium phosphate buffer (pH 6.0), containing up to 25 μg calotropain-FI and FII, 0.01 M cysteine and 0.004 M EDTA, was incubated at 32.5°C for 10 min and the reaction was initiated by adding 1 ml 1% casein in 0.1 M sodium phosphate buffer (pH 6.0) at 32.5°C. The readings were recorded at 610 nm on a Beckman DU-2 Spectrophotometer at 30-s intervals using a 1 ml cuvette (1 cm path length). Activity was expressed as the reciprocal of time ($1/T$) required for increase by 0.5 absorbance units at 610 nm. Although the increase in absorbance at 610 nm is not proportional to the time (T), $1/T$ is proportional up to 12.5 μg/ml enzyme.

The caseinolytic activities of both the enzymes were determined as described earlier [1] except the assay was carried out at pH 6.0 in 0.1 M sodium phosphate buffer at 32.5°C (Table VI).

Results and Discussion

Since the molecular weights obtained by SDS-polyacrylamide gel electrophoresis and gel filtration for calotropain-FI (25 700 and 27 230, respectively) and calotropain-FII (24 550 and 23 300, respectively) (Table I) were in reasonably good agreement, both the enzymes are likely to be a single polypeptide chain. The molecular weights obtained for calotropain-FI and FII are of the same magnitude as of other latex proteinases [11—16].

Among the reducing agents studied on the activation of both the enzymes

TABLE I

COMPARISON OF THE MOLECULAR WEIGHT OF CALOTROPAIN-FI AND FII

Method	Calotropain-FI	Calotropain-FII
SDS-polyacrylamide gel electrophoresis	25 700	24 550
Gel filtration	27 230	23 300

TABLE II

EFFECT OF VARIOUS REDUCING AGENTS ON CALOTROPAIN-FI AND FII ACTIVITY

Percent activity on the basis of cysteine/activation as 100%. EDTA was present with each reducing agent.

Reducing agent	% activity	
	Calotropain-FI	Calotropain-FII
Glutathione	93.33	93.87
Mercaptoethanol	93.33	89.79
Thioglycollicacid	91.11	89.79
Sodium cyanide	73.33	58.16

TABLE III

EFFECT OF VARIOUS INHIBITORS AND PHOTOOXIDATION ON CALOTROPAIN-FI AND FII ACTIVITY

Effective concentrations of inhibitors were $1 \cdot 10^{-3}$ M.

Inhibitor	Calotropain-FI		Calotropain-FII	
	Inhibition (%)	Reactivation (%)	Inhibition (%)	Reactivation (%)
Iodoacetic acid	100.0	0.0	100.0	0.0
Iodoacetamide	100.0	0.0	100.0	0.0
N-Ethylmaleimide	100.0	7.2	100.0	8.8
Trinitrobenzenesulphonic acid	61.5	100.0	53.0	77.2
N-Bromosuccinimide	100.0	7.7	100.0	0.0
Dithio-bis-nitrobenzoic acid	88.5	119.0	100.0	70.2
p-Chloromercuribenzoate	100.0	98.9	100.0	98.2
$HgCl_2$	100.0	90.5	100.0	89.5
$ZnSO_4$	100.0	90.5	100.0	100.0
$CuSO_4$	100.0	42.9	100.0	35.1
$AgNO_3$	100.0	66.7	100.0	87.7
$FeSO_4$	100.0	0.0	100.0	0.0
$Cd(CH_3COO)_2$	100.0	90.5	100.0	98.3
$AlCl_3$	3.9	95.3	41.2	98.3
Photo oxidation	% loss of activity in 15 min			
	Calotropain-FI		Calotropain-FII	
Methylene blue *	10.8		31.7	
Rose bengal *	21.6		31.0	

* Effective concentrations of the dyes were 0.01%.

(*p*-chloromercuribenzoate inhibited), maximum activation was observed with cysteine, whereas the activation by NaCN was minimal (Table II). Increase in concentration of any reducting agents or EDTA did not affect the percent activation significantly. Various plant sulfhydryl proteinases [9,17—21] are all reported to be maximally activated by reducing agents in the presence of a metal-binding agent such as EDTA.

The inhibition of calotropain-FI and FII due to iodoacetamide, *N*-ethylmaleimide, trinitrobenzene sulphonic acid, *N*-bromosuccinamide, dithio-bis-nitrobenzoic acid and *p*-chloromercuribenzoate may be due to the presence of an active site cysteine -SH in the enzyme molecule. Metal ions like Hg^{2+}, Cu^{2+}, Ag^{+} and Fe^{2+} all inhibited calotropain-FI and FII for the same reason, and the enzymes could be reactivated (Table III). Both the enzymes were blocked with *p*-chloromercuribenzoate (at pH 7.0) before photooxidation to protect the free -SH from the reaction. However, photooxidation in the presence of methylene blue and rose bengal result in considerable loss of enzyme activity (Table III). This loss in activity may be attributed to the presence of another active site, possibly histidine. The inhibition by Zn^{2+} and Cd^{2+}, besides other heavy metals, is in accord with this possibility. The presence of active sites cysteine and histidine were reported for other plant -SH proteinases [9,12,17—19,21,22,28].

Both the enzymes were found to have one free -SH/mol of enzyme (Table IV). Since there is no increase in the titer value in the presence of urea or SDS (Table IV), it is likely that no masked free -SH is present in these enzymes. Calotropain-FI and FII after reducing with $NaBH_4$ were estimated to have a total -SH content of seven and five residues/mol enzyme, respectively (Table IV). No increase in titer value was observed after 60 min reduction, but heat-

TABLE IV

SULFHYDRYL CONTENT OF CALOTROPAIN-FI AND FII

Molecular weights of 26 500 and 24 000 for calotropain-FI and FII, respectively, were used for calculation.

	Calotropain-FI		Calotropain-FII	
	Calculated number of residues	Assumed number of residues	Calculated number of residues	Assumed number of residues
Free -SH content *				
In buffer	0.73		0.54	
In presence of 8 M urea	0.90	1.0	0.54	1.0
In presence of 1% SDS	0.65		0.46	
Total -SH content **				
$NaBH_4$ reduction without heat denaturing the enzyme				
30 min reduction	2.95		1.79	
60 min reduction	6.44		3.65	
90 min reduction	6.45	7.0	4.4	5.0
$NaBH_4$ reduction after heat denaturing the enzyme				
30 min reduction	5.91		4.33	
60 min reduction	6.44		4.40	5.0
90 min reduction	6.45	7.0	4.50	

* Molar extinction coefficient of 13 600 $M^{-1} \cdot cm^{-1}$ [7] was used for 2-nitro-5-thiobenzoate anion.

** Molar extinction coefficient of 12 000 $M^{-1} \cdot cm^{-1}$ [8] was used for 2-nitro-5-thiobenzoate anion.

TABLE V

AMINO ACID COMPOSITION OF PAPAIN, FICIN, BROMELAIN, CALOTROPAIN-FI AND FII

Amino acid	Papain [26]	Ficin [27]	Bromelain [28]	Calotropain-FI	Calotropain-FII
Lysine	10	5	23	13	15
Histidine	2	1	2	2	2
Arginine	12	10	12	5	6
Aspartic acid	19	17	29	16	18
Glutamic acid	20	25	23	28	25
Threonine	8	8	14	2	4
Serine *	13	14	28	17	18
Proline	10	11	14	18	17
Glycine	28	28	35	42	40
Alanine	14	20	35	13	19
Valine	18	18	22	14	16
Isoleucine	12	7	21	14	15
Leucine	11	15	10	5	7
Tyrosine *	19	15	21	7	7
Phenylalanine	4	5	9	4	3
Tryptophan ***	5	6	8	4	3
Half-cystine **	7	8	10	7	5
Methionine	0	5	5	1	2

* Value corrected for destruction during acid hydrolysis by extrapolating to zero time.
** Estimated independently by 5,5'-dithio-bis-2-nitrobenzoic acid method [8].
*** Estimated independently by *p*-dimethylaminobenzaldehyde method [29].

denatured enzymes are more susceptible to $NaBH_4$ reduction than the native form. The presence of three disulphide bridges and one free -SH in calotropain-FI and two disulphide bridges and one free -SH in calotropain-FII can be deduced from the above data. Papain has one free -SH and three disulphide bridges [9]. Ficin is reported to have one free and one buried -SH/mol enzyme [30]. Bromelain has one free -SH/mol enzyme [21].

Amino acid analyses (Table V) show a close similarity of both enzymes except in the case of threonine, glutamic acid, alanine, half-cystine and methionine. In the present work the ammonia contents of the samples were not estimated. Both calotropain-FI and FII are rich in glycine.

Calotropain-FI and FII widely differ in their esterase and amides activities (Table VI). Calotropain-FII shows higher amidase, esterase and caseinolytic

TABLE VI

COMPARISON OF ESTERASE, AMIDASE, MILK-CLOTTING AND CASEINOLYTIC ACTIVITIES OF CALOTROPAIN-FI AND FII

Enzyme	Esterase activity *	Amidase activity *	Milk-clotting activity **	Caseinolytic activity *	Milk-clotting activity / caseinolytic activity	Milk-clotting activity / amidase activity
Calotropain-FI	0.384	2.33	134	5.540	24.18	57.50
Calotropain-FII	1.52	5.34	102	7.015	14.54	19.10

* Activity units/min per mg protein.
** Activity units/mg enzyme protein.

activities than calotropain-FI, whereas calotropain-FI shows higher milk-clotting activity than calotropain-FII. The ratio of the milk-clotting to caseinolytic activity for calotropain-FI was found to be approximately twice that of calotropain-FII, whereas the ratio of the milk-clotting to amidase activity of calotropain-FI is approximately three times higher than that of calotropain-FII resembles a papain-type enzyme. Other plant proteinases also differ digestion activity of chymopapain was found to be exactly twice that of papain [23]. In this respect, calotropain-FI is a chymopapain-type enzyme and calotropain-FII resembles papain-type enzyme. Other plant proteinases also differ in their ratio of milk-clotting to caseinolytic or amidase activity units [24,25].

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