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PARTHENAIN, A PROTEASE FROM *PARTHENIUM ARGENTATUM*

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Key Word Index—*Parthenium argentatum*; Compositae; protease; isolation; active site; serine.

Abstract—A homogeneous protease, parthenain, was isolated from *Parthenium argentatum*. The enzyme has serine at the active site and a dependence on free histidine residues. It is a glycoprotein of M_r about 63 000 and is composed of a single unit of pI 4.6.

The enzyme has a preference for neutrally charged amino acid residues in oxidized insulin B chain. It displays anomalous partial activation in the presence of a large molar excess of *p*-chloromercuribenzoate.

INTRODUCTION

We have purified, to homogeneity, a number of serine-centred proteases from latices of various members of the Euphorbiaceae which were designated euphorbains [1–6] and hevains [7, 8]. As an extension of that work, we have investigated the protease of guayule (*Parthenium argentatum* A Gray), which is a latex carrying shrub native to semi-arid regions of Mexico and the U. S. A. Because the latex of this species occurs in small isolated sacs, we have worked with extracts of the whole plant.

The protease, which was given the trivial name parthenain, was obtained as a homogeneous glycoprotein of M_r 63 000 which has an essential serine residue. In these respects it is comparable with the enzymes previously isolated in this laboratory from members of the Euphorbiaceae [1–6]. Other properties of parthenain are reported here, along with its amino-acid composition.

RESULTS AND DISCUSSION

Parthenain, which was homogeneous in HPLC on a TSK column, gave a single band on PAGE (Fig. 1a). It had a M_r of 64 000 on SDS gel electrophoresis (Fig. 1b) thus confirming the homogeneity, and showing that the enzyme is composed of a single unit. The M_r was also estimated by the method of Andrews [9] using a Bio-Gel P-150 column calibrated with ribonuclease, ovalbumin, bovine serum albumin and phosphorylase. Parthenain was then found to have a M_r of 61 000. Isoelectric focusing showed that the protease has a pI of 4.6 (Fig. 2). Using *Z*-glycine-*p*-nitrophenyl ester as substrate, maximal activity occurred between pH 7 and 8. The amino acid composition of parthenain is shown in Table 1. As glucosamine is present, the enzyme is a glycoprotein. This observation was confirmed by attachment of parthenain to an agarose-concanavalin A affinity column [10]. It is notable that a number of proteases which are glycoproteins have been isolated from members of the families Euphorbiaceae [1–8] and Moroceae [11]. The hydrolytic assay for tryptophan [12] consistently gave a charred product, so that amino acid residue was estimated spectrophotometrically [13]. Comparison of the per cent compositions given in Table 1, with data published

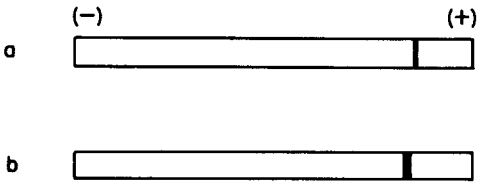

Fig. 1. (a) Pathenain stained with Amido Black after cationic gel electrophoresis [24]; marker dye at (–) end; R_f 0.14. (b) SDS-gel electrophoresis [25] of parthenain, stained with Coomassie Brilliant Blue.

Table 1. Amino acid composition of parthenain

Amino acid	Residues/mol	% composition
Cys	15	2.5
Asx	62	11.4
Thr	40	6.3
Ser	49	6.8
Glx	42	8.7
Pro	29	4.5
Gly	65	5.9
Ala	53	6.0
Val	42	6.6
Met	3	0.6
Ile	28	4.9
Leu	46	8.3
Tyr	15	3.9
Phe	27	6.2
His	7	1.5
Lys	28	5.6
Arg	16	4.1
Trp	3	0.9
GlcN	13	3.4

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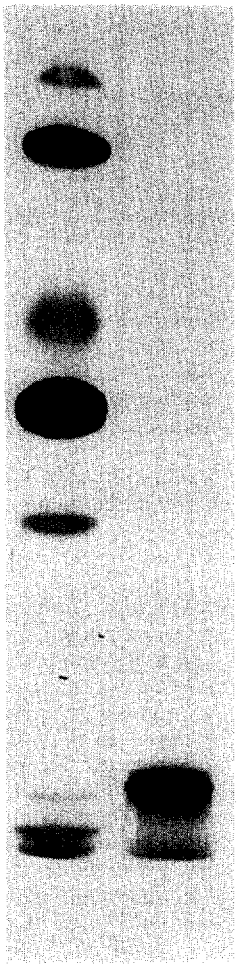

Fig. 2. Isoelectric focusing [26] of partnenain (right) and of standards comprising (in order, from top to bottom) amyloglucosidase (pI 3.50), soybean trypsin inhibitor (pI 4.55), bovine carbonic anhydrase (pI 5.85), horse myoglobin (pI 6.85, 7.3), lentil lectin (pI 8.5) and trypsinogen (pI 9.3). Staining was with Coomassie Brilliant Blue.

Table 2. Effect of protease inhibitors on parthenain in 100 mM Tris–HCl, pH 7.0, 22°

Inhibitor [ref.]	% inhibition at	x-fold molar excess
PhMeSuF [14]	70	10^5
	41	5×10^4
Diethylpyrocarbonate [15]	90	10^5
	83	5×10^4
	73	10^4
Dibromoacetophenone [16]	60	10^6
	45	10^5
Elastatinal [17]	0	10^6
Leupeptin [18]	0	10^6
Ovomucoid trypsin inhibitor	0	10^6
Bovine pancreas trypsin inhib.	0	10^6
Lima bean trypsin inhibitor	0	10^6
Hirudin	0	10^6
Potato inhibitor I	60	10^5
	55	5×10^4
	45	10^4
Chymostatin [19]	90	10^5
	75	10^4
Pepstatin [20]	20	10^5
Iodoacetic acid	0	10^6
Hg^{2+}	70	10^5
EDTA*	0	10^6
1,10 Phenanthroline*	0	10^6
Mg^{2+}, Mn^{2+}, Ca^{2+}	0	10^6
Zn^{2+}, Ca^{2+}	40	10^7

*Reagent mixed with enzyme 16 hr before assay.

elsewhere for other serine-centred proteases derived from plants [1–8] shows a general familiar resemblance.

The results of Table 2 show that parthenain is a member of the family of serine-centred proteases (inhibition by PhMeSuF) in which histidine plays an essential role (inhibition by diethylpyrocarbonate and by dibromoacetophenone). Many of the common trypsin inhibitors (ovomucoid, bovine pancreas, lima bean, hirudin) were without effect on the activity of parthenain, as were the bacterially derived elastatinal and leupeptin. However chymostatin and potato I inhibitor each were effective, suggesting that parthenain resembles chymotrypsin rather than trypsin.

To obtain the results reported in Table 2, large molar excesses of reagents were required. Other plant proteases isolated in this laboratory (e.g. refs [5, 8, 11]) have similarly been significantly inhibited only with 10^3 to 10^6 fold molar excesses of the reagents employed in Table 2. This suggests that the active sites of these enzymes are not readily accessible to the inhibitors, which also explains the incomplete reaction of, for example, the potato I inhibitor (compare ref. [11]).

Parthenain was only slightly inhibited by pepstatin (Table 2). Thus there can be only a minor role for acid residues in the reactivity of this enzyme. The metal complexing agents EDTA and 1,10 phenanthroline were without effect on parthenain which is, then, probably not a metallo-protein. The enzyme is unaffected by the presence of Mg^{2+}, Mn^{2+} or Ca^{2+}, and partly inhibited by Zn^{2+} and Cd^{2+}.

Parthenain activity is unaffected by iodoacetic acid (Table 2), suggesting that free cysteinyl groups have no role in catalysis by that enzyme. However, *p*-chloromercuribenzoate in 10^6 molar excess caused a 35% activation of parthenain; this rose to 46% when the concentration of reagent was doubled and was 15% when the inhibitor concentration was halved. A comparable example of anomalous activation of two proteases, euphorbains d_1 and d_2, by this reagent has recently been observed and ascribed to conformational changes in the enzyme [6]. Parthenain, however, is effectively inhibited by Hg^{2+}, a 10^5 molar excess causing a 70% loss of activity. The contradictory nature of these data may be the result of the employment of large molar excesses of the reagents: only a 7% enhancement of parthenain activity was observed with a 10^5-fold molar excess of *p*ClHgBzO. Both reagents may be acting at sites other than

Table 3. Michaelis–Menten parameters for reactions of parthenain with various *N*-substituted amino acid esters, at pH 7.0 in Tris–HCl (100 mM) at 22°

Substrate	K_m (M)	V_{max} (M/sec)	V_{max}/K_m (sec^{-1})
Z-Ala-*p*-NPE*	5.0×10^{-5}	1.43×10^{-6}	2.83×10^{-2}
Z-Leu-*p*-NPE	2.5×10^{-5}	5.0×10^{-8}	2.0×10^{-3}
Z-Asn-*p*-NPE	2.85×10^{-4}	3.3×10^{-7}	1.15×10^{-3}
Z-Gly-*p*-NPE	2.17×10^{-4}	2.5×10^{-7}	1.15×10^{-3}
N-Benzoyl-gly-*p*-NPE	2.56×10^{-4}	9.52×10^{-8}	3.71×10^{-4}
N-Benzoyl-ala-methyl ester	3.3×10^{-3}	2.17×10^{-7}	0.66×10^{-4}

*NPE-nitrophenyl ester.

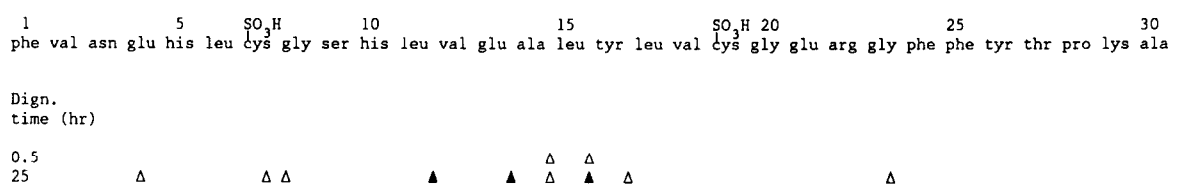

Fig. 3. Scission points in oxidized insulin B chain. Digestion with parthenain was in 100 mM ammonium acetate at 21°. Δ indicates a single and ▲ more than two bond breaks at the positions indicated.

sulphydryl centres and so modifying the conformation of the protease in different ways, a hypothesis supported by the lack of inhibition with iodoacetic acid (Table 2).

Parthenain displayed no reactivity to 10^{-4} M *p*-nitrophenyl esters of acetic, propionic, *n*-butyric, valeric and caprylic acids and so is not a simple esterase. It is, also, unreactive to l-alanine-*p*-nitroanilide, suggesting that it has no amidase activity.

In an examination of other synthetic substrates hydrolysed in the presence of parthenain, *p*-nitrophenyl esters of *Z*-isoleucine, -lysine, -proline, -tryptophan and -valine were unaffected, and their analogues which are susceptible to reaction are listed, with values of K_m and V_{max} in Table 3. The data were collected at the optimal pH for these substrates (7–8). The *Z*-ala-*p*-nitrophenyl ester is a notably favoured substrate among those tested, having not only the lowest K_m, but also the highest V_{max}. Blocking at the amine group by *Z* is preferred to that by *N*-benzoyl, and comparison of the esters tested shows that parthenain is highly sensitive to the nature of the substitution on the amino acid residue.

Oxidized insulin B chain is a convenient peptide substrate for testing proteases as it contains most of the common amino acids in its thirty residue chain. For this reason it has been widely used in comparative work with proteolytic enzymes (e. g. [21]). When oxidized insulin B chain was digested in the presence of parthenain, the initial scissions occurred between ala and leu at positions 14 and 15, and leu-tyr at positions 15 and 16, showing that the enzyme is an endopeptidase with preference for neutral residues. Prolonged digestion of the insulin substrate produced the hydrolysis shown in Fig. 3 where it is evident that, like the proteases of other plants examined in this laboratory, parthenain is not highly specific.

EXPERIMENTAL

Reagents. Unless noted all reagents used were of analytical grade. The Bio-Gels P-30 and P-150, the reagents used in the disc-gel electrophoresis (cationic, SDS and isoelectric fousing) as well as the HPLC Bio-Sil TSK 250 (300 × 7.5 mm) gel filtration column, were obtained from Bio-Rad Chemicals, Richmond, CA. Pharmacia (Canada) Ltd supplied the DEAE-Sepharose CL-6B. Elastatinal and chymostatin were from the Protein Research Foundation, Osaka, Japan. Bovine pancreatic and ovomucoid trypsin inhibitors, leupeptin and pepstatin were purchased from the Sigma Chemical Co., St. Louis, MO as were *p*-chloromercuribenzoate (*p*Cl-HgBzO), diethylpyro-carbonate, phenyl methyl sulphonyl fluoride (PhMeSuF) and oxidized insulin B chain. The same company supplied the *N*-carbobenzoxy (*Z*) *p*-nitrophenyl esters of alanine, asparagine, glycine, isoleucine, leucine, lysine, proline, tryptophan and valine as well as *N*-benzoyl alanine methyl ester, L-alanine-*p*-nitroanilide and the *p*-nitrophenyl esters of acetic, propionic, *n*-butyric, valeric and caprylic acids. *N*-Benzoyl glycine *p*-nitrophenyl ester was a gift from Dr A. C. Storer of this Division. Eastman Chemical Co., Rochester, NY, supplied the *p*-bromophenacyl bromide and the Pierce Chemical Co., Rockford, IL the methane sulphonic acid. Potato inhibitor I and lima bean trypsin inhibitor were isolated as described [22, 23].

Assays. Activity measurements with *Z*-glycine-*p*-nitrophenyl ester were made as described [1–6]. Protein was estimated either as A_{280} or by the Lowry procedure [24]. PAGE. Cationic gels were prepared following ref. [25]; SDS-gels after ref. [26].

Isoelectric focusing. Bio-lyte ampholytes were used in the procedure of ref. [27]. Protein staining was with 0.05% Coomassie Brilliant Blue–0.5% $CuSO_4$ in 10% HOAc/27%EtOH.

M_r determinations. The method of ref. [9] was used with a Bio-Gel P-150 column.

HPLC gel filtration. A Bio-Sil TSK 250 column (Bio-Rad Chemicals) and guard-column was employed with a Beckman model 110A pump and Varian model 2050 UV detector. 200 mM K-Pi pH 7.0, at a flow-rate of 0.5 ml/m was the solvent system.

Amino acid analyses. Hydrolysates were prepared in 6 M HCl or, for tryptophan determination, in 4 M methane sulphonic acid [12] under vacuum at 110° for 22 hr. Cysteine content was measured after oxidation and hydrolysis [28], and amino sugars

after hydrolysis in 4 M HCl for 6 hr under vacuum at 110°. The tryptophan content was also estimated spectrophotometrically [13]. Amino acid and amino sugar determinations were made on an automatic analyser.

Kinetic measurements. Michaelis–Menten parameters were estimated in 100 mM Tris–HCl, pH 7.0, at 22° using concentrations of between 10^{-4} and 10^{-5} M substrate. It was under those conditions that all tests on synthetic substrates were made.

Inhibition studies. Enzyme in 100 mM Tris–HCl, pH 7.0, at 22° was mixed, with increasing concentrations of inhibitor solns, for five minutes. Residual activity was monitored with *Z*-glycine *p*-nitrophenyl ester as substrate.

Parthenain digestions of insulin B chain. Digestions were made in 100 mM ammonium acetate at 21° for varying times using a ratio of enzyme to substrate of 1:100. Samples were injected directly on to a Synchropak RP-4 (25 × 10 mm) (Synchron Inc., Linden, IN) column on a Varian Vista 5500 liquid chromatography apparatus equipped with a Varian UV 200 detector. A gradient of H_2O–MeCN (0.1 % in trifluoracetic acid) was used at a flow rate of 3 ml/m. Peptide peaks were collected, hydrolysed as described above and subjected to amino acid analysis. Scission points in the oxidized insulin B chain could then be ascertained.

Purification of parthenain. Parthenium argentatum was grown from seeds in a sand–soil mixture (1:1) and an 18 hr daylight cycle, at 23°. Twenty mature plants were cut from their roots, chopped, macerated in 2 l 100 mM Tris–HCl, pH 7.0, at 4° using a Waring blender, then stirred at 4° for 3 days. The supernatant was decanted and the residue extracted twice more in the same manner. The supernatants were pooled, cond with polyethylene glycol, pptd with 1 M $(NH_4)_2$ SO_4, the pellet (17000 *g* for 30 min) discarded, and the resulting soln dialysed against 100 mM Tris–HCl, pH 7. 91 ml were obtained containing 1342 A_{410}/min (measured with *Z*-glycine *p*-nitrophenyl ester) and 10 mg protein/ml estimated by the Folin–Lowry procedure.

Gel-filtration on Bio-Gel P-30 (2 × 50 cm) in 100 mM Tris–HCl, pH 7.0, 4° gave a single esterolytically active peak with total recovery of activity.

After dialysis in 10 mM Tris–HCl, pH 7.0, the active protein was applied to a column (1.5 × 3.0 cm) of DEAE Sepharose CL-6B in that buffer, at 4°. The column was washed with the same buffer (100 ml) then subjected to a linear gradient composed of 200 ml each 10 mM and 2000 mM Tris–HCl, PH 7.0. The major active peak was recovered with 45% of the applied activity. The minor second peak of Fig. 4 suggests the presence of other proteases, but these were not investigated.

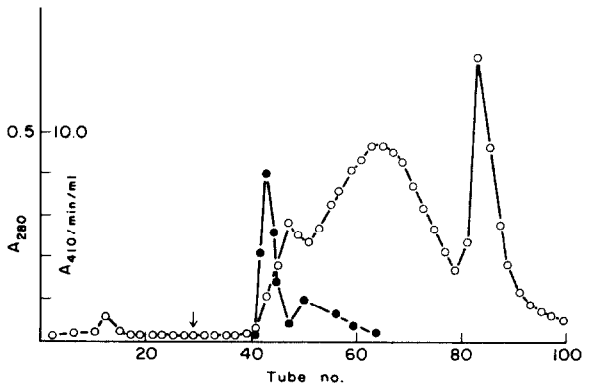

Fig. 4. Elution profile of parthenain from a 25 × 2 cm column of DEAE-Sepharose CL-6B at pH 4. After washing with 100 ml, 10 mM Tris–HCl pH 7.0, elution was with a linear gradient (↓) from 10 to 2000 mM Tris–HCl pH 7.0, 100 ml of each. A_{280} - ○; Enzyme activity - ●.

The enzyme from the ion-exchange column was concentrated on a Diaflo membrane (UM-10) and applied to the TSK gel filtration column in an isocratic HPLC apparatus using a Varian 2050 detector, the solvent being 200 mM potassium phosphate, pH 7.0, at 21° pumped at 0.5 ml/min. Activity was recovered in 88% yield, in a single symmetrical peak which was unchanged on re-cycling. The specific activity of the final product was 36.8 A_{410}/min/mg, a 25-fold increase over the extract applied to the P-30 column. The yield was 40% of the original activity.

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