Biochimica et Biophysica Acta, 612 (1980) 119—125

BBA 68922

MULTIPLE FORMS OF THE ASCLEPAINS

CYSTEINYL PROTEASES FROM MILKWEED *

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(Received July 31st, 1979)

Key words: Asclepain; Cysteinyl protease; (Milkweed)

Summary

Two groups of asclepains (EC 3.4.22.7) isolated from the latex of *Asclepias syriaca* L. (milkweed) were each separated into five homogeneous enzymes. The members of each group are of similar amino acid composition, and leucine is the common N-terminal residue. Michaelis values are reported for each of the component cysteinyl proteases of milkweek latex, and are compared with those of analogous enzymes from other plant sources. The asclepains all catalysed the hydrolysis of insulin B chain to yield similar two-dimensional maps. The peptides produced from one such digestion were characterized and scission points were defined and compared with those for papain.

Introduction

Latex of the milkweed (*Asclepias syriaca* L.) has been known for many years to contain proteases [1,2]. These have recently been separated into two groups of sulphydryl enzymes, asclepains A and B (EC 3.4.22.7), each composed of several members [3]. A representative of each of the two forms of asclepain was purified and examined. They were shown to be of different amino acid composition, but with a common N-terminal leucine residue. The reactivities of the A and B asclepains to synthetic and natural substrates differed, as did their pH profiles [3].

In this report further data from studies with the two types of asclepains are presented, following isolation of each of the components as a homogeneous protein. Amino acid analyses of the asclepains are reported, showing that the members of each group are closely related in primary structure, as has been found for other plant proteases [4]. Michaelis data have also been collected for

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the several enzymes from milkweed which are discussed here and are compared with results obtained from other sulphydryl enzymes of the papain family.

The peptides produced by digestion of oxidized insulin B chain with asclepain have been determined, and are shown to be somewhat different from those reported for the proteases of papaya latex [5].

The work reported here is a continuation of studies (Refs. 3,4,5 and references therein) of the comparative biochemistry of plant sulphydryl proteases [21]. From such investigations data elucidating the roles of the multiple forms of such enzymes from papaya [5,11,17], ficus [15,16,18], ananas [4], as well as asclepias [1,2,3] families may be obtained.

Experimental

Unless otherwise specified, the reagents and techniques used in this work were as previously described [3].

Isolation and purification of the asclepains. The methods used have been described in detail elsewhere [3], except that the Sepharose CL-CM ion-exchange column was eluted with a 0.1—0.5 M sodium acetate linear gradient followed by another of 0.5—0.7 M, both of pH 7. Five fractions of the A and B groups of enzymes were purified, homogeneity being established by recycling on the ion-exchange column, and by disc gel electrophoresis with protein and activity staining [3]. Isolation of a single N-terminal dansyl derivative for each fraction [6] further confirmed the homogeneity of the isolated enzymes.

Amino acid analyses. The procedures previously described were used, namely hydrolysis in both constant boiling HCl and in methanesulphonic acid, and persulphuric acid oxidation followed by hydrolysis [3]. Amino acid analyses were performed on a Durrum analyser.

Determination of Michaelis constants. The method employed, with carbobenzoxylysine *p*-nitrophenyl ester (Cyclo Chemicals), was essentially that of Bender et al. [7]. Measurements were made with several different preparations of the asclepains, and the data obtained were treated as described by Eadie [8].

Digestion of oxidized insulin B chain. The peptide was obtained from Schwartz-Mann. Digestions proceeded in 0.1 M NH_4HCO_3 for 5 h at 21°C with an enzyme to substrate ratio of 2%. After extensive freeze-drying, peptide maps were prepared on thin-layer sheets by electrophoresis and chromatography under conditions previously described [5].

The peptides from the digests were separated on a column of BioRad AG 50WX2 (1.5 × 200 cm) maintained at 40°C, using a linear gradient from 0.2 M pyridine/acetic acid (pH 3.1) to 2 M pyridine/acetic acid (pH 4.95) (1 l of each). This was followed by elution with 400 ml of 3 M pyridine/acetic acid (pH 6.53). Peptides were located by alkaline hydrolysis followed by ninhydrin treatment, and the relevant fractions were further purified by thin-layer chromatography on Macherey-Nagel Polygram Cell 400 sheets with the solvent system previously employed [5]. These peptides were then hydrolysed in constant boiling HCl at 110°C for 22 h under vacuum. The component amino acids were identified by two-dimensional thin-layer mapping: electrophoresis followed by chromatography [5]. From the known sequence of the insulin B chain it was then possible to specify the peptides produced in the digestion with asclepain.

Results

As has been reported, application of a linear salt gradient to the CM-Sepharose column made a clear separation of both the A and B groups of asclepains possible [3]. The separated fractions were recycled on the ion-exchanger column. Disc gel electrophoresis [9] demonstrated the homogeneity of the enzymes separated: sodium dodecyl sulphate (SDS) gel electrophoresis [10] confirmed that each of the members of the A and the B series were of molecular weights 23 000 and 21 000, respectively [3].

All members of both series had N-terminal leucine residues, which was determined by the method of Gray [6] and compared with results reported for the A_3 and B_5 asclepains.

The amino acid compositions of the enzymes here described are presented in Table I where it is apparent that members of the A and of the B groups are closely related, differing from each other in only a few residues. The asclepain B family differs significantly from the A group in acid, basic and neutral residues. Differences in the contents of aromatic residues are not great. While the A series has two residues of methionine per molecule, only one was found in the B group.

Table II gives collected Michaelis data from the members of both asclepain groups of cysteinyl enzymes. Data reported from other laboratories for papain [11], ficin [12] and actinidin [13] are included for comparison, as well as those for other members of the papaya family of enzymes which have recently been separated [5].

Maps of the representative digestions of the insulin B chain by the two types

TABLE I

AMINO ACID COMPOSITIONS OF ASCLEPAINS EXPRESSED AS RESIDUES PER MOLECULE OF M_r 23 000 FOR THE A AND 21 000 FOR THE B SERIES

Amino acid	A					B				
	1	2	3	4	5	1	2	3	4	5
Asp	16	16	16	16	16	13	13	13	13	13
Thr	5	6	6	7	7	4	4	4	4	4
Ser	13	14	14	14	14	11	11	11	11	11
Glu	22	23	22	22	22	18	18	18	18	18
Pro	6	6	6	6	6	5	5	5	5	5
Gly	25	25	26	25	25	20	19	19	19	20
Ala	14	14	18	16	18	9	9	9	9	10
Val	14	14	14	14	14	15	15	15	16	16
Met	2	2	2	2	2	1	1	1	1	1
Ile	12	12	14	12	13	9	8	9	9	10
Leu	6	7	7	7	6	7	7	8	7	8
Tyr	14	14	14	12	14	15	14	15	14	15
Phe	7	7	6	6	6	7	7	8	9	8
His	2	2	2	2	2	1	1	1	1	1
Lys	13	16	16	16	16	9	9	9	9	9
Arg	9	8	8	8	8	12	13	13	14	14
$Cys_{1/2}$	5	5	5	5	5	7	7	7	7	7
Trp	7	7	6	6	6	7	5	6	6	7

TABLE II

MICHAELIS VALUES FOR HYDROLYSIS OF *N*-CARBOBENZOXYLYSINE *p*-NITROPHENYL ESTER IN 0.1 M PHOSPHATE BUFFER, pH 6.3 AT 21°C

		K_m (μM)	K_{cat} (s^{-1})
Asclepain	A_1	11.4 ± 0.2	54.7 ± 3.5
	A_2	10.5 ± 0.1	46.2 ± 0.5
	A_3	14.2 ± 0.2	42.9 ± 1.2
	A_4	12.2 ± 0.2	59.6 ± 2.6
	A_5	9.7 ± 0.3	22.3 ± 0.5
	B_1	24.8 ± 0.3	34.6 ± 2.1
	B_2	38.9 ± 0.8	49.2 ± 0.9
	B_3	12.6 ± 0.1	25.8 ± 0.5
	B_4	27.0 ± 0.1	33.8 ± 0.6
	B_5	27.2 ± 0.4	47.6 ± 0.3
Papain [a]		1.71 ± 0.25	44.8 ± 1.8
Chymopapain [b]		31.0 ± 2.0	18.2 ± 0.6
Papaya peptidase A		22.7 ± 2.1	5.2 ± 1.5
Papaya peptidase B		37.5 ± 0.4	1.4 ± 0.2
Ficin [c]		2.7 ± 0.2	32.4 ± 0.5
Bromelain [d]		57.0	7.4
Actinidin [e]		22.0 ± 2.0	29.0 ± 2.0

[a] Ref. 11; pH 6.2.
[b] This laboratory, pH 6.5.
[c] Ref. 12; pH 6.4.
[d] Ref. 19; pH 4.7, unspecified buffer.
[e] Ref. 13, pH 6.0.

of asclepain are shown in Fig. 1. Results strictly comparable with these were obtained from the other members of each group of enzymes, differences being commonly found in only a few peptides in the maps. The two types of proteases (A and B) act proteolytically in similar fashions, and the components

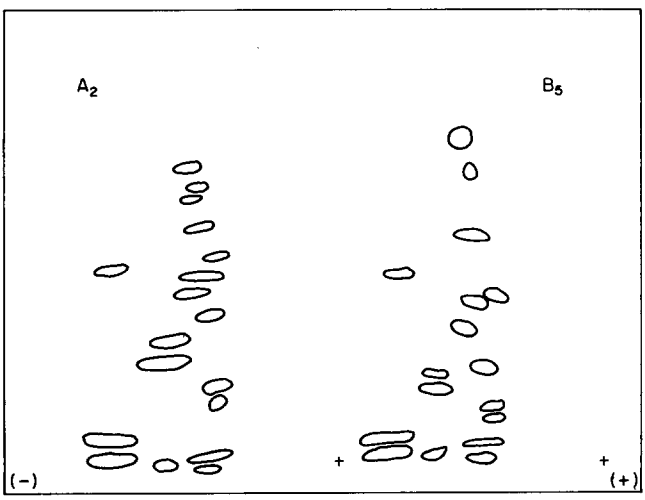

Fig. 1. Peptide maps, prepared as described elsewhere [5] from digests of oxidized insulin B chain by asclepain fractions A_2 and B_5: horizontal dimension, electrophoresis; vertical, chromatography.

TABLE III

PEPTIDES PRODUCED, IN YIELDS OF 10% OR GREATER' FROM DIGESTION OF INSULIN B CHAIN WITH ASCLEPAIN A_3

Peptide	%
Phe-Val-Asn	14
Ser-His-Leu-Val-Glu-Ala-Leu	22
Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-CysA-Gly-Glu	15
Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala	10
Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala	10
Gly-Phe-Phe-Tyr-Thr	13

of the A and B forms resemble each other even more closely in their reactivity to insulin B chain.

Maps have been prepared from digests of the same peptide with papain, chymopapain and papaya peptidase (A and B), which are all derived from papaya latex and behave in a similar fashion to each other in their digestion of that substrate [5,14]. Comparison shows that the asclepains differ in their mode of proteolysis from that group of cysteinyl proteases.

In Table III the peptides produced are listed, in yield of ten percent or more, from digestion of insulin B chain with asclepain A_3. In Fig. 2 are shown the points of hydrolysis of the peptide bonds of the substrate peptide. Data from a similar study with papain are included for comparison [14]. The differences remarked with the two-dimensional maps are clearly confirmed.

Discussion

It is apparent from the data presented here and elsewhere that the latex of *Asclepias syriaca* L. contains two distinct types of cysteinyl proteinases, categorized A and B, which are related in their modes of action but significantly differ in amino acid compositions. The relationship between the two types of asclepains is comparable with that found for the four sulphydryl enzymes of papaya latex [5,14]: differing compositions but essentially the same selectivities for substrates. The occurrence of plant cysteinyl proteases in

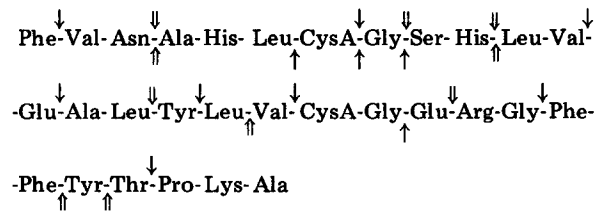

Fig. 2. Comparison of the digestions by asclepain A_3 and by papain of the oxidized B chain of insulin. Weak (<10%) and strong (10—25%) cleavage points for asclepain and papain are indicated by single and double arrows respectively. Asclepain cleavage points are indicate above, papain cleavage points below the peptide chain.

a number of closely related forms, as reported here for both types of asclepain, is not unusual; it has been reported for ficin [15,16], bromelain [4] and chymopapain [17]. There is no evidence from this or other work [18] that the multiplicity of enzymic forms observed is caused by self-hydrolysis. That the two families of asclepains differ somewhat in reactivities is seen in Table II. While there is some overlap of values, in general K_m for the A series (average 11.6 μM) is lower than that for the B series (average 26.1 μM). It is notable that some members of each group (for example A_5, B_3 show distinct differences from the others.

Comparison with Michaelis values for other cysteinyl proteases using the same synthetic substrate under comparable buffer conditions shows that the asclepains resemble chymopapain and actinidin, but have values of K_m which are significantly larger than those for papain or ficin, and of k_{cat} which are notably higher than those for the less active proteases of papaya latex (papaya peptidases A and B) and of bromelain [18].

The peptide maps of Fig. 1, which are typical of those found for the asclepains, show that the two groups of enzymes react in very similar ways with a protein substrate. Consequently only the digestion of insulin B chain by asclepain A_3 was examined in detail. The results are presented in Fig. 2 with the scission points reported when papain was used for the proteolysis [14].

These data show that asclepain is apparently even less specific than papain. While there are a number of peptide bonds hydrolysed which are common to both enzymes, there are also notable differences: sites adjacent to aromatic residues are less readily, those adjacent to basic residues more readily hydrolysed in the presence of asclepain than in the presence of papain. A similar observation was made in studies with synthetic substrates [3]. Because of the change of preference described above (if, as is reasonable, asclepain is mechanistically analogous to papain) the former enzyme must require a set of subsites [20] different from those of the latter protease. The data of Fig. 2, however, do not permit definition of these.

Acknowledgements

The amino acid analyses were performed by Dr. M. Yaguchi.

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