

Institute of Pharmacy, Free University Berlin, Berlin, Germany

Proteolytic activity in latex of the genus *Euphorbia* - a chemotaxonomic marker?

A. DOMSALLA, C. GÖRICK, M. F. MELZIG

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Prof. Dr. Matthias F. Melzig, Free University Berlin, Institute of Pharmacy, Königin-Luise Str. 2+4, D-14195 Berlin, Germany

matthias.melzig@fu-berlin.de

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Eighteen species of the genus *Euphorbia* are known to have proteolytic enzymes in their latices, 9 of them are characterized by the type of endopeptidases (Cysteine-, Serine-, Metallo- or Aspartatic-endopeptidase) which are responsible for the activity, and all nine are serine endopeptidases. In our study we examined the latices of 64 different species of the genus *Euphorbia* concerning proteolytic activity and serine protease activity, five of them are mentioned in the literature to be proteolytic active and four are known to contain at least one serine endopeptidase. All tested samples were able to degrade labelled casein, the activity of six latices were completely inhibited by specific serine protease inhibitors, 15 samples were not influenced, and in 43 latices a remaining activity was measured, indicating that other types of endopeptidases seem to be involved.

1. Introduction

More than 110 latices of different plants are known to contain at least one proteolytic enzyme. The enzyme subclass of endopeptidases (EC 3.4) is in turn divided into sub-subclasses: enzymes belonging to subclass EC3.4.21 (serine proteases) possess a Ser residue in the active site; those belonging to EC 3.4.22 (cysteine proteases) have a Cys residue instead; those belonging to EC 3.4.23 (aspartatic proteases) depend on an Asp residue for their catalytic activity; and those belonging to EC 3.4.24 (metalloproteases) containing metal ions (normally Zn^{2+}) in their catalytic mechanism (Antao and Malcata 2005). Latex proteases are known in different plant families such as Anacardiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Caricaceae, Convolvulaceae, Euphorbiaceae and Moraceae. Most of them belong to the cysteine or serine endopeptidases family and one (*Ficus racemosa* L., Moraceae) is known to belong to aspartatic family (Domsalla and Melzig 2008). Nearly half of the commercially available enzymes are also proteases, frequently used in food processing, tenderization of meat, brewing/cheese elaboration, bread manufacturing, leather and textile industries. Besides, some proteases have also been used as model systems for studies on their structure–function relationship, and in protein folding problem (Patel and Jagannadham 2003; Dubey and Jagannadham 2003; Kundu et al. 2000). Proteolytic enzymes from plant latex have also received special attention in pharmaceutical industry and biotechnology due to their property of being active over wide range of temperature and pH.

In the literature only 18 species of the genus *Euphorbia* are known to contain proteolytic active enzymes in the latex, 9 of them are characterized as serine proteases. The others are not characterized concerning this property (Domsalla and Melzig 2008).

In our investigation we want to proof if proteolytic activity in the latex of species of the genus *Euphorbia* might be a chemo-

taxonomic marker. Therefore we tested the latex of 64 different species of the genus *Euphorbia* for proteolytic activity and we also investigated if the proteolytic activity is inhibitable by specific serine proteases inhibitors because all of the known specified species of the genus *Euphorbia* contain serine protease activity in their latices (Domsalla and Melzig 2008).

2. Investigations and results

2.1. Proteolytic activity

The latices of 64 Euphorbiaceae of the genus *Euphorbia* were analyzed for proteolytic activity. To classify the activity the change in fluorescence was compared to trypsin as known serine endopeptidase; with a volume of 100 µl of each trypsin (1 µg/ml–10 mg/ml) concentration; the latices were divided into two groups: group I expresses an activity in the range of minimal detectable 1 µg/ml and maximum detected activity at 2.5 mg/ml trypsin concentration, and group II has a strong proteolytic activity higher than an activity compared to 2.5 mg/ml trypsin. To intimate which plant belongs to the defined group, we used a score, explained in the Fig. The following examined plants are known in the literature to contain proteolytic enzymes in their latices *E. amygdaloides* L., *E. cyparissias* L., *E. esula* L., *E. coerulescens* Haw., *E. lathyris* L., *E. milii* Des Moul. var. *mili*, *E. royleana* Boiss., *E. tirucalli* L., and *E. trigona* Haw. These findings could be confirmed by our investigation.

The proteolytic activity of the other 55 *Euphorbia* latices was not published in the past. In our study all tested latices were able to digest the substrate used. Nine samples have a strong proteolytic activity; the others are all of moderate activity. Interestingly, four of the samples *E. balsamifera*, *E. trigona*, *E. pilansii* and *E. teixeira* show no proteolytic activity after the procedure of freeze drying but using the supernatant after centrifugation of the fresh latex the samples showed proteolytic activity (Table).

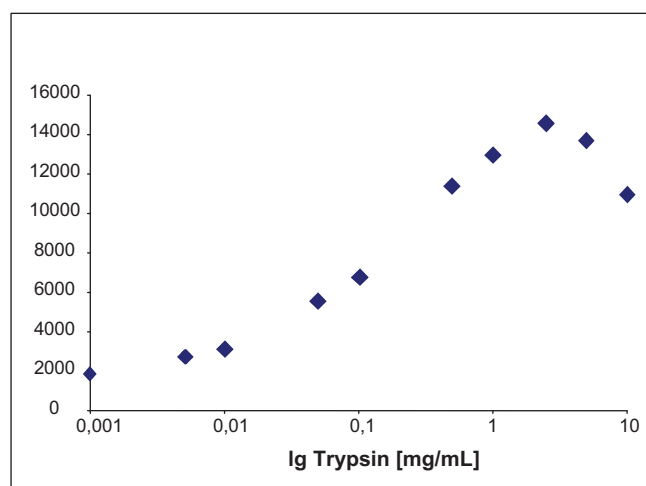


Fig.: Proteolytic activity of different concentrations of the serine protease Trypsin. Because of the curve shape we set the limits for our score at 1 μ g/mL for the minimal detectable and 2.5 mg/mL for the maximal detected activity

Proteolytic activity of latices in the genus *Euphorbia* could be expected as a chemotaxonomic characteristic because each tested sample was able to induce a significant change in fluorescence of the labeled substrate compared to the controls.

2.2. Inhibitory studies

To confirm that the proteolytic activity is due to a serine proteases mentioned in the literature, the latex samples were pre-incubated with the specific serine proteases inhibitors AEBSF and Aprotinin. The remaining proteolytic activity was determined and compared to the not inhibited samples.

Six plants show strong inhibition of proteolytic activity, in 43 samples the proteolytic activity was only partially inhibited, indicating that these belong to other types of endopeptidases which produce the remaining activity. Fifteen plants were not influenced by the pre-incubation with the serine specific inhibitors and the proteolytic activity remained completely (Table).

3. Discussion

The following species of the genus *Euphorbia* are known to have proteolytic activity in their latices; all of them which were characterized by type of endopeptidases are described to serine protease activity. Milin was purified from the latex of *Euphorbia milii* Des. Moul. (Euphorbiaceae). This plant is native to Madagascar and cultivated as an ornamental plant in India. The latex of the plant is used to control mollusks proliferation by its embryo-fetotoxicity. It is frequently used in traditional medicine against liver fluke, schistosomiasis in sheep, cattle, and even in humans. Milin is a glycoprotein with a detectable carbohydrate moiety (7–8%) which is essential for the activity. It is strongly inhibited by serine specific inhibitors (Yadav

Table: Euphorbiaceae and their proteolytic activity

Euphorbiaceae Euphorbia	Accession number of Berlin Botanical Garden	Origin	Leafy/succulent	Residual activity	Proteolytic activity
<i>E. amygdaloides</i> L.*	028-10-01-10	Europe	Leafy	0	+
<i>E. aphylla</i> Brouss. ex. Willd.	080-34-74-80	Canary Islands	Succulent	#	+
<i>E. avasmontana</i> Dinter	115-15-74-80	Namibia	Succulent	#	+
<i>E. balsamifera</i> Ait.	236-01-83-80	Canary Islands, Niger, Senegal, Mali, Burkina Faso, Mauretania	Succulent	#	+
<i>E. bubalina</i> Boiss.	305-01-97-80	South Africa	Succulent	#	+
<i>E. cereiformis</i> L.	027-89-74-80	South Africa	Succulent	#	+
<i>E. characias</i> L. ssp. <i>characias</i>	194-67-92-14	Mediterranean	Leafy	#	+
<i>E. coerulescens</i> Haw.*	042-59-74-80	South Africa	Succulent	#	+
<i>E. confinalis</i> R.A. Dyer	084-26-83-50	South Africa, Zimbabwe, Mozambique,	Succulent	##	+
<i>E. cooperi</i> N.E.Br.	124-98-74-80	South Africa, Zimbabwe	Succulent	#	+
<i>E. cyparissias</i> L.*	025-31-88-10	Europe	Leafy	#	+
<i>E. deightonii</i> Croizat	125-01-74-80	Sierra Leone	Succulent	#	+
<i>E. dendroides</i> L.	353-05-86-14	Canary Islands	Succulent	#	+
<i>E. dulcis</i> L.	007-32-84-10	Europe	Leafy	#	++
<i>E. epithymoides</i> L.	072-23-87-70	Europe	Leafy	#	+
<i>E. esula</i> L.*	069-63-74-80	Europe/Asia	Leafy	#	++
<i>E. evansii</i> Pax	042-62-74-80	South Africa	Succulent	#	++
<i>E. fortissima</i> L. C. Leach	084-28-83-50	Zambia, Zimbabwe	Succulent	#	+
<i>E. franckiana</i> Bgr.	012-20-74-80	South Africa	Succulent	##	+
<i>E. grandicornis</i> Goebel	027-72-74-80	South Africa, Swaziland, Mozambique	Succulent	#	+
<i>E. grandidens</i> Haw.	042-60-74-80	South Africa	Succulent	0	+
<i>E. gregaria</i> Marloth	125-02-74-80	South Africa, Namibia	Succulent	#	+
<i>E. hamata</i> Sweet	012-24-74-80	South Africa, Namibia	Succulent	#	++
<i>E. handiensis</i> Burchard	027-01-91-20	Fuerteventura	Succulent	##	+
<i>E. heterochroma</i> Pax	125-03-74-80	Tanzania	Succulent	0	++
<i>E. heterodoxa</i> Müll.Arg	078-14-96-40	Brazil	Succulent	#	+
<i>E. jansenvillensis</i> Nel	012-94-74-80	South Africa	Succulent	##	+

Table: (Continued)

Euphorbiaceae Euphorbia	Accession number of Berlin Botanical Garden	Origin	Leafy/succulent	Residual activity	Proteolytic activity
<i>E. lagunillarum</i> Croizat	231-05-90-84	Venezuela	Succulent	#	+
<i>E. lathyris</i> L.*	019-02-94-14	Europe/Asia	Leafy	#	+
<i>E. ledienii</i> A. Berger	014-29-74-80	South Africa	Succulent	#	++
<i>E. leucodendron</i> Drake	027-94-74-80	Madagascar	Succulent	0	+
<i>E. leuconeura</i> Boiss.	203-33-98-84	Madagascar	Succulent	##	+
<i>E. marginata</i> Pursh	094-53-74-84	North America	Leafy	##	+
<i>E. milii</i> Des Moul. var. <i>bevilanensis</i> (Croizat) Ursch & Leandri	043-62-74-80	Madagascar	Succulent	##	+
<i>E. milii</i> Des Moul. var. <i>longifolia</i> Rauh	027-92-74-80	Madagascar	Succulent	#	+
<i>E. milii</i> Des Moul. var. <i>milii</i> *	027-75-74-80	Madagascar	Succulent	#	+
<i>E. myrsinites</i> L.	186-54-02-80	Mediterranean	Leafy	#	+
<i>E. neohumbertii</i> Boit.	027-90-74-80	Madagascar	Succulent	#	+
<i>E. nicaeensis</i> All.	116-07-81-10	Mediterranean	Leafy	##	+
<i>E. nivulia</i> Buch.-Ham.	125-05-74-80	India	Succulent	#	+
<i>E. onoclada</i> Drake	001-11-80-10	Madagascar	Succulent	0	+
<i>E. officinarum</i> L.	001-82-74-70	Morocco	Succulent	#	+
<i>E. ornithopus</i> Jacq.	012-48-74-80	South Africa	Succulent	0	++
<i>E. pentagona</i> Haw.	014-33-74-80	South Africa	Succulent	#	+
<i>E. pilosa</i> L.	069-60-74-80	Europe	Leafy	#	++
<i>E. polyacantha</i> Boiss.	236-03-83-80	Sudan	Succulent	##	+
<i>E. pseudocactus</i> A. Berger	012-84-78-80	South Africa	Succulent	#	+
<i>E. pseudoglobosa</i> Marloth	012-38-74-80	South Africa	Succulent	##	+
<i>E. pteroneura</i> A. Berger	012-86-74-80	Mexico	Succulent	#	+
<i>E. resinifera</i> Berg	042-58-74-80	Morocco	Succulent	#	+
<i>E. royleana</i> Boiss.*	125-06-74-80	India	Succulent	##	+
<i>E. schimperi</i> C. Presl	125-07-74-80	Yemen, Saudi Arabia	Succulent	#	+
<i>E. seguieriana</i> Neck.	069-59-74-80	Europe, West Asia	Leafy	##	+
<i>E. sipolisii</i> N. E. Br.	231-04-90-30	Brazil	Succulent	#	+
<i>E. spinosa</i> L.	202-04-95-10	Canary Islands	Leafy	#	+
<i>E. submammillaris</i> A. Berger ex Pax	125-13-74-80	South Afrika	Succulent	##	+
<i>E. sudanica</i> A. Chev.	221-04-94-20	Mali	Succulent	##	+
<i>E. teixeirae</i> L. C. Leach	027-67-74-80	Angola	Succulent	#	+
<i>E. tirucalli</i> L.*	043-05-90-80	Africa, India	Succulent	#	+
<i>E. triangularis</i> Desf.	042-63-74-80	South Africa	Succulent	##	+
<i>E. trigona</i> Haw.*	125-16-74-80	Namibia	Succulent	#	+
<i>E. villosa</i> Waldst. & Kit. ex Willd.	033-03-04-70	Europe	Leafy	#	+
<i>E. woodii</i> N. E. Br.	151-31-74-80	South Africa	Succulent	#	+
<i>E. xylophylloides</i> Brongn. ex. Lem	129-03-04-84	Madagascar	Succulent	#	+

+ proteolytic activity (between 1 µg/mL and 2.5 mg/mL)

++ proteolytic activity (higher than 2.5 mg/mL)

0–10% residual activity

10–95% residual activity

not influenced by the inhibitors

* Known for proteolytic activity or serine protease activity

et al. 2006). In the latex of *Euphorbia supina* Rafin. are two proteolytic enzymes, the major protease B, which is characterised as a cucumisin-like serine protease, the N-terminal sequence of the first fifteen residues was determined and six of the residues match those of cucumisin [EC 3.4.21.25] and a minor, termed *E. supina* protease A which is still not characterized. Euphorbain L a serine protease from the latex of *Euphorbia lathyris* L. (Euphorbiaceae), commonly known as caper spurge, is a biennial plant which grows to a height of about 1 m. The amino acid composition of euphorbain L was expressed as percent residue weight; there is a notable similarity between euphorbain L and cocoonase (cocoonase has also been identified as a serine protease) (Lynn and Clevette-Radford 1983; Lennox

and Ellis 1945). Three serine (Y-1, Y-2, and Y-3) proteolytic enzymes were isolated from the latex of *Euphorbia cyparissias* L. (cypress spurge, Euphorbiaceae). The proteases which are glycoproteins are immunologically distinct from euphorbain L, but related to that enzyme in amino acid composition. The three euphorbains have different activities to both esterolytic and proteolytic substrates and react in individual ways in digesting of insulin B-chain (Lynn and Clevette-Radford 1985b). Euphorbain P purified from the latex of *Euphorbia pulcherrima* Willd., Poinsettia, Christmas star (Euphorbiaceae). This multi-chain enzyme is similar in composition to one in *Euphorbia lathyris* L., but is larger in size and has a more restricted activity. Euphorbain P is a glycoprotein containing glucosamine

(Lynn and Clevette-Radford 1984). The latices of two succulent Euphorbiaceae, *Euphorbia lactea* Haw. Candelabra plant and *Euphorbia lactea* Haw. “*cristata*” (brain cactus), a crested ‘*monstrosa*’ variety which bears little physical resemblance to *E. lactea*, were examined. The euphorbains from *E. lactea* (La1, La2, La3) and *E. lactea cristata* (Lc) are related to each other in amino acid composition even though they display different physical and biochemical properties (Lynn and Clevette-Radford 1986). The proteases from both are distinct from those isolated from other members of the genus *Euphorbia*. Euphorbain T1-T4 were isolated from the latex of the succulent *Euphorbia tirucalli* L. (known as milk bush, Euphorbiaceae), which is native to Uganda, Zaire and Tanzania. Each enzyme has several different charge forms. The four proteases examined are of similar amino acid composition but yield differing two-dimensional maps of tryptic digests. Euphorbain T1 is a glycoprotein containing glucosamine. There is no close relationship in the amino acid composition of the Euphorbains T1-T4 when comparison is made with the other euphorbains (Lynn and Clevette-Radford 1985a). Euphorbia protease B from the latex of *Euphorbia pseudochamaesyce* Fisch. has six out of ten amino-terminal residues which were identical to those of cucumisin. The enzyme reacted with anti-cucumisin antibody, demonstrating that *Euphorbia* protease B from *E. pseudochamaesyce* belongs to cucumisin-like proteases (Shimada et al. 2000). The plants which were tested for proteolytic activity but not classified in one of the endopeptidase family are the following: *Euphorbia amygdaloides* L. (Demir et al. 2005), *Euphorbia cerifera* Alc. (Castaneda et al. 1948), *Euphorbia coerulescens* Haw. (Lynn and Clevette-Radford 1987), *Euphorbia esula* L., *Euphorbia helioscopia* L. (Gonashvili and Gonashvili 1968), *Euphorbia hirta* L. (Chary and Reddy 1983), *Euphorbia royleana* Boiss. (Singh and Singh 2002), *Euphorbia splendens* Bojer ex Hook, *Euphorbia trigona* Haw. (Lynn and Clevette-Radford 1987). Proteolytic activity in the latex of the genus *Euphorbia* might be a chemotaxonomic marker because every tested sample had measurable proteolytic activity. Proteolytic activity in the latex is not limited to the genus *Euphorbia*, also in the latex of the Euphorbiaceae *Synadenium grantii* Hook ‘f’, *Hevea brasiliensis* Muell. Arg. and *Elaeophorbium drupifera* (Schum.) Stapf. (Domsalla and Melzig 2008) proteolytic activity was described. The assumption that the proteolytic enzymes in the genus *Euphorbia* might be serine endopeptidases could not be confirmed, even the known plant latices of *E. milii* var. *milii*, *E. cyparissias* and *E. tirucalli* which contain serine protease activity, demonstrated a remaining proteolytic activity. We conclude that not only serine proteases are responsible for the proteolytic activity in genus *Euphorbia* but also other types of endopeptidases might be involved. Although the determination of the type of latex proteases might be a scientific aid in chemotaxonomy, not for the genus *Euphorbia*, but in other plant families like Asteraceae and Convolvulaceae only serine protease and in Caricaceae only cysteine protease activity were found (Domsalla and Melzig 2008).

4. Experimental

4.1. Materials and chemicals

Aprotinin, trypsin, PBS (phosphate buffered saline) were purchased by Sigma, Enzchek protease assay kit green (BODIPY FL-casein) Molecular Probes Inc. (Eugene, OR) Invitrogen Karlsruhe, AEBSEF (4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride) AppliChem Darmstadt. All chemicals used were of analytical reagent grade.

4.2. Plant material collection of latex

The latices were collected in the Botanical Garden Berlin, Free University Berlin. All plants were labelled and the reliability of identification

was assured by professional workers. 50 plants were succulent members cultivated in a greenhouse, 14 grew outside in the garden.

The crude latex of healthy plants was collected in distilled water (50 µl) in plastic tubes that were shaken gently, closed and maintained at environmental temperature, until handled in the laboratory. The samples were initially submitted to centrifugation at 4 °C during 20 min at 14400 rpm and frozen and freeze dried, the dry latices were stored at –18 °C for further experiments.

4.3. Protease assay

Protease activity was assayed using BODIPY FL- casein as substrate. The freeze dried latices were suspended with 200 µl PBS and centrifuged 20 min, 14400 rpm, 4 °C, 50 µl of the supernatant was diluted with 100 µl PBS and 200 µl of BODIPY FL- casein were added (Molecular Probes, Inc. (Eugene, OR)) and incubated 2 h at 37 °C. Each sample (200 µl) was transferred into a 96 well microtiter plate and read in a microtiter plate fluorescence reader (Tecan Spectra Fluor) against a blank (PBS + BODIPY FL-casein) plus a blank of the sample (PBS + sample) to eliminate auto fluorescence.

4.4. Determination of inhibition by specific serine proteases inhibitors

The suspended and centrifuged latices of the protease activity assay were also used for the inhibitor experiments, 50 µl latex were gently swirled with 100 µl of ABESF (10 mg/ml) or Aprotinin (20 µg/ml) and pre-incubated for 30 min at 37 °C. The residual activity was determined using BODIPY FL- casein as previously described. The assay performed without any of the inhibitors served as control with the reference initial activity.

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