Acta Bot. Neerl. 32(1/2), February 1983, p. 39–47.

LINAMARASE AND β-GLUCOSIDASE ACTIVITY IN NATURAL POPULATIONS OF TRIFOLIUM REPENS

P. BOERSMA, P. KAKES and A. W. SCHRAM

Genetisch Instituut, Universiteit van Amsterdam Kruislaan 318, 1098 SM Amsterdam

SUMMARY

Evidence is presented that linamarase is a cell-wall bound enzyme. It can be extracted using high concentrations of sodium chloride. The activity is maximal at pH 5.5. Bound and solubilized enzyme exhibit a Km value of about 8 mM on linamarin. Linamarase also hydrolyses the artificial substrate 4-methyl umbelliferyl-β-D-glucoside. Besides linamarase, at least three other β-glucosidases are present, exhibiting activity towards the artificial substrate. These enzymes exhibit no or hardly any activity towards linamarin and differ in isoelectric point from linamarase. The presence of linamarase is regulated by the gene Li, the other β-glucosidases being independent of Li.

In natural populations, plants with and without linamarase activity are observed. The percentage of plants exhibiting linamarase activity, is dependent on the altitude of the locality of the population.

Keywords: Linamarase, cyanogenesis, β-glucosidase, Trifolium repens

1. INTRODUCTION

Cyanogenesis in white clover is controlled by two non linked genes (ATWOOD & SULLIVAN 1943). The presence of the cyanogenic glucosides linamarin and lotaustralin is controlled by the gene Ac, whereas the gene Li controls the presence of linamarase. This enzyme hydrolyses the β-glucosidic linkage in linamarin and eventually yields glucose, acetone and hydrogen cyanide. Linamarase also exhibits activity towards artificial substrates with a β-glucosidic linkage like p-nitrophenyl-β-D-glucoside (HUHGES 1968, HUHGES & MAHER 1972). Most European populations of white clover are polymorphic for Ac, Li or both. Mediterranean populations at sea level have high frequencies of the dominant alleles, Ac and Li. Northern and north-eastern populations, or populations at elevations higher than 1000 m have low frequencies of Ac and Li (DADAY 1954a, b).

We tested plants of wild populations growing at different altitudes using a standard picrate test. The populations were collected in the Cevennes (S. France). In the picrate test the release of hydrogen cyanide from bruised leaf material is qualitatively determined by the color change of paper strips, impregnated with an alkaline picrate solution (CORKILL 1940). The tests revealed a great variation in cyanide production within the populations and classification in "cyanogenic" and "acyanogenic" was sometimes difficult. We therefore developed a procedure to extract linamarase and other β-glucosidases from leaves of white clover and determined enzyme activities in plants from different wild

populations. The results were compared to the results obtained using the picrate test.

2. MATERIALS AND METHODS

2.1. Plant material

Randon samples of cuttings (100/population) were taken from natural populations of *Trifolium repens* at different altitudes near Montpellier, France. Care was taken that each cutting represented a different plant. The cuttings were grown to plants in the greenhouse and subsequently cuttings of these plants were cultured in climate chambers at 22°C, 65% relative humidity and 6000 Lx (3 a.m -6 p.m.). Young just unfolded leaves were used for enzyme extraction and picrate tests. The populations used and their localities are given in *table 1.*

2.2. Picrate test

From each plant equal amounts of frozen leaflets were tested as indicated in *table 2,* using linamarin (Calbiochem AG), linamarase (isolated from linseed) or water as additives. Released hydrogen cyanide was determined by colouring of Whatman 003 paperstrips, impregnated with a solution of picric acid in water (7.5 g. 1^{-1}, pH 9.0). Incubations were carried out in standard test tubes for 24 hr at 35°C.

The results were compared to similar incubations with known amounts of hydrogen cyanide *(table 3).*

2.3. Enzyme extraction

Acetone powders were made by grinding frozen leaves (fresh weight: 0.5–1.5 g) in acetone (50 ml, -15°C) in a Waring blendor. The suspension was filtered, washed in acetone (100 ml, -15°C) and airdried. Extracts were made by homogenizing 20–30 mg of dry acetone powder in 1 m NaCl (± 5 ml) at 0°C in a Braun homogenizer. After 15 min incubation (0°C) the mixture was centrifuged at 1600 g.

The supernatant was filtered and the pellet was taken up in 5 ml 1 M NaCl. Supernatant and the resuspended pellet were assayed separately.

2.4. Enzyme assays

The reaction mixtures consisted of 50 µl enzyme extract, 50 µl sodium acetate buffer 100 mM pH 5.5 and 50 µl substrate (10 mM) in water. Activity on linamarin (Calbiochem AG) was measured by determining the release of hydrogen cyanide. The assay temperature was 35°C. The reaction was stopped after 20 min by injecting 400 µl 0.2 M NaOH into the reaction tube (Eppendorf 3810, 2 ml). After centrifuging at 18.000 g for 5 min 500 µl of the reaction mixture were used for determination of hydrogen cyanide by the pyrazolone method (MAO et al. 1965). In each experiment a blank was run by adding 400 µl 0.2 M NaOH before the enzyme extract.

Table 1. Specifications of population localities.

No. of population	Locality	Altitude	Exposition
Tr 2800	Col du Pas	830 m.	Z.O.
Tr 3600	Source de Galadet	1170 m.	Z.W.
Tr 4500	Gaseiral	900 m.	Z.
Tr 2600	Pic St. Loup	120 m.	Z.O.

Table 2. Picrate test with white clover of different genotypes.
+ positive reaction
− no reaction

Genotypes of white clover plant	Bruised leaf material incubated in the presence of		
	linamarase	linamarin	H_2O
Ac – Li –	+	+	+
Ac – lili	+	−	−
acac Li –	−	+	−
acac lili	−	−	−

Table 3. Scoring system of the picrate test.

Rank nr	Amount of HCN liberated per tube (3 leaves)
0	$< 1\ \mu g$
1	$\geqslant 1\ \mu g$
2	$\geqslant 2\ \mu g$
3	$\geqslant 5\ \mu g$
4	$\geqslant 10\ \mu g$
5	$\geqslant 20\ \mu g$
6	$\geqslant 50\ \mu g$

Activity on 4-methylumbelliferyl-β-D-glucoside (Koch-Light Ltd.) was measured by determining the release of 4-methyl-umbelliferone. The reaction was stopped after 20 min by adding 800 μl 1 M glycine/NaOH pH 10.6. After centrifuging at 18.000 g for 5 min 500 μl of the reaction mixture were used for determination of 4-methyl-umbelliferone using a λex. at 360 nm and a λF at 450 nm. In each experiment a blank was run by adding 800 μl 1 M glycine/NaOH pH 10.6 before the enzyme extract.

Enzyme activity in plants is expressed as mU/mg powder. One mU of enzyme activity is defined as hydrolysis of 1 nmol substrate/min.

Table 4. Effect of NaCl on extraction of linamarase from aceton powders of leaves from white clover.

Extraction medium	Enzyme Activity (mU/mg powder)	
	supernatant	pellet
H_2O	0.01	16.3
0.25 M NaCl	4.2	14.9
0.5 M NaCl	17.6	6.7
1 M NaCl	18.5	6.7

2.5. Chromatography

Polybuffer exchanger PBE 94 (Pharmacia AG) used in the chromatofocussing experiment, was eluted with polybuffer 74/HCl, pH 4 as eluent and 0.025 M imidazole/HCl, pH 7.5 as starting buffer. Before chromatofocussing the protein was dialyzed overnight against the starting buffer.

3. RESUTS AND DISCUSSION

3.1. Enzyme extraction and characterization

In preliminary experiments it appeared that linamarase activity was difficult to solubilize after homogenizing. This has been reported earlier (WILKINSON & MILLAR 1979). This could be explained by assuming localization of linamarase activity in cell walls. To investigate this we studied the relation between concentration of NaCl in the extraction medium and solubilized linamarase activity. The results are shown in *table 4*. Upon extraction of acetone powder with water most of the activity remained in the pellet. However, linamarase activity could be extracted from the pellet using NaCl, the amount of solubilized activity being

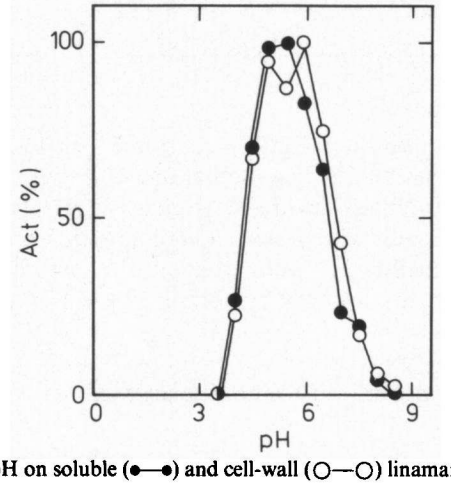

Fig. 1. The effect of pH on soluble (●—●) and cell-wall (○—○) linamarase activity.

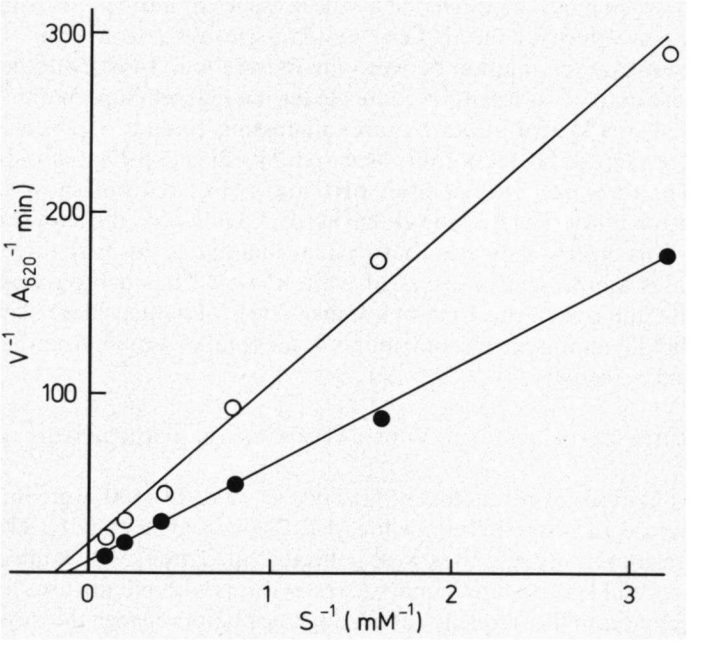

Fig. 2. Lineweaver-Burk plot of activity against linamarin in soluble (●—●) and cell-wall fraction (○—○) of plant Tr 2600-42.

dependent on the NaCl concentration. (It should be stressed that in all activity measurements the final concentration of NaCl in the reaction mixture was 125 mM.) Linamarase activity could not be extracted using Triton-X-100 (result not shown). These results indicate that linamarase is a cell-wall bound enzyme. We have difficulty to explain the difference in recovery of activity when extractions with water and NaCl are compared. It could be that the cell wall enzyme is less accessible for substrate than the solubilized enzyme.

The effects of pH on linamarase activity in supernatant and pellet are shown in *fig. 1.* They are quite similar: both activities were maximal at pH 5.5.

Km values using linamarin and 4-methylumbelliferyl-β-D-glucoside were determined using supernatant and pellet as enzyme sources. The Lineweaver-Burk plot obtained with linamarin as substrate showed linear relationships between 0.625 mM and 10 mM *(fig. 2)*. The Km values obtained with supernatant and pellet as enzyme sources were very similar. Using the supernatant a Km value of 7.4 ± 1.2 mM (mean ± SEM, n = 8) was obtained whereas a value of 8.2 ± 2.2 mM (mean ± SEM, n = 6) for using the pellet was obtained. This indicates that the solubilized activity is identical to the cell wall activity. The present values differ from the one (0.7 mM) reported by HUGHES & MAHER (1972). A possible explanation could be the different extraction procedures used to extract linamarase from leaves and acetone powders. The Lineweaver-Burk plot with 4-methyl-umbelliferyl-β-D-glucoside as substrate showed a clear deviation from

linearity, which might be explained by the presence of more β-glucosidases with different Km values for the artificial substrate (results not shown).

To investigate the relation between linamarase and 4-methylumbelliferyl-β-glucosidase activity in leaves of white clover, an extract (supernatant) of plant Tr 4500-24 was loaded into a chromatofocussing column. Upon elution with polybuffer *(fig. 3)* at least four 4-methylumbelliferyl-β-D-glucosidases were eluted. The three activities at pH7, pH6 and pH4.7 did not show significant linamarase activity. The fraction eluted at pH 4.3 however, did show linamarase activity. This clearly indicates that besides linamarase at least three other β-glucosidases are present in leaves of white clover. The cochromatography of linamarase and one of the 4-methyl-umbelliferyl-β-D-glucosidase acitivities indicates that linamarase itself contributes to the total of 4-methyl-umbelliferyl-β-D-glucosidase activity.

3.2. Picrate tests and Enzyme activities in populations of white clover

Three populations of white clover (Tr 2800, Tr 3600, Tr 4500) were investigated on linamarase and 4-methyl-umbelliferyl-β-D-glucosidase activity. The populations were chosen because they were collected on stations with contrasting microclimates and because preliminary investigations with picrate tests had shown clear differences in the frequency of cyanogenic plants between the three populations. Two cuttings were taken from a random sub-sample of the populations and grown in a climate chamber as described in material and methods. The cuttings are designated in *table 5* as clone 1 and clone 2. Of each clone leaves were collected for use in a picrate test and for preparation of acetone powders on the same day. The picrate tests were performed with the addition of linamarine (100 μg/3 leaves) in order to score for the presence/absence of linamarase *(table 2)*. The acetone powder was used to determine linamarase and 4-methyl-

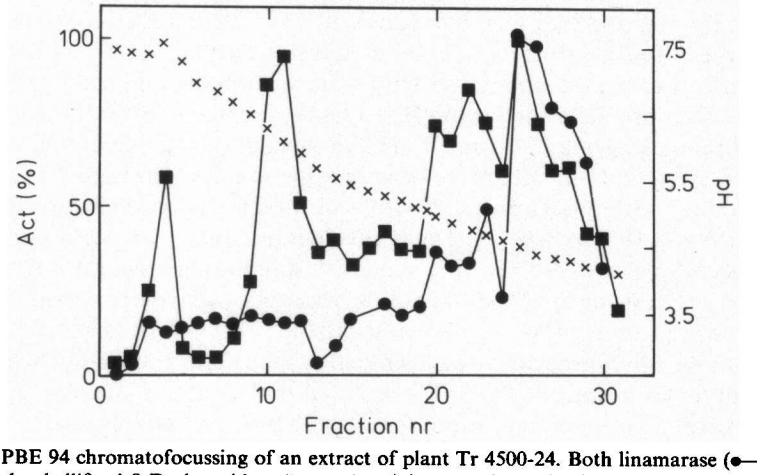

Fig. 3. PBE 94 chromatofocussing of an extract of plant Tr 4500-24. Both linamarase (●—●) and 4-methylumbelliferyl-β-D-glucosidase (■—■) activity were determined. ×—×: pH.

Table 5. β-glucosidase activity of leaves from plants of natural populations of *Trifolium repens*.

Population Tr 4500						Population Tr 2800						Population Tr 3600					
plant	activity[a]				picrate[b] test	plant	activity[a]				picrate[b] test	plant	activity[a]				picrate[b] test
	linamarin		4-mu-β-glu				linamarin		4-mu-β-glu				linamarin		4-mu-β-glu		
	clone 1	clone 2	clone 1	clone 2			clone 1	clone 2	clone 1	clone 2			clone 1	clone 2	clone 1	clone 2	
63	tr[c]	tr	–[d]	–	4	47	tr[c]	–[d]	–	–	3	23	tr[c]	–[d]	–	–	3
64	tr	tr	–	–	3	43	tr	tr	–	–	3	36	tr	tr	–	–	3
83	tr	tr	–	–	4	18	tr	tr	–	–	2/3	90	tr	tr	–	–	3
102	tr	tr	–	–	4	65	tr	tr	–	–	2/3	18	tr	tr	–	–	0
28	tr	tr	–	–	0/2	3	tr	tr	–	–	3	52	tr	tr	–	–	3
22	tr	tr	3.2	4.2	6	6	tr	tr	–	–	0/3	55	tr	tr	–	–	3
8	tr	tr	3.9	6.0	3	23	tr	tr	–	–	3	80	tr	tr	–	–	3/4
1	tr	tr	1.5	2.3	3/5	54	tr	tr	4.0	4.3	3	30	tr	–	3.8	–	2
97	tr	–	1.0	–	4	68	tr	tr	3.9	3.9	3	63	tr	–	2.9	–	2
95	tr	–	5.9	–	3	14	tr	–	1.8	–	3	69	tr	–	5.1	–	3
43	tr	tr	6.0	5.3	3/5	44	tr	tr	2.8	3.3	2/3	88	tr	–	4.1	–	3
65	tr	tr	3.0	4.1	5	62	tr	tr	3.6	1.3	2	22	tr	–	2.5	–	3
98	tr	–	7.1	–	5	11	tr	tr	4.1	5.0	2	90	tr	–	4.5	–	3
68	tr	tr	3.1	3.7	2/3	1	tr	tr	2.0	3.3	2	51	tr	tr	2.2	4.6	3
11	tr	–	2.8	–	3	37	tr	tr	1.7	3.9	2	68	tr	tr	2.5	2.3	3
69	5.0	4.2	–	–	6	29	tr	–	1.8	–	3	19	tr	tr	1.9	2.8	3
24	21.5	14.1	–	–	5/6	36	tr	tr	2.9	1.3	3/4	87	tr	tr	3.6	4.2	3
42	8.3	8.7	19.1	20.2	6	73	tr	tr	1.8	1.1	0/3	13	tr	tr	2.2	4.1	2
59	9.8	–	18.7	20.6	6	21	tr	–	0.5	–	4	81	tr	–	3.1	–	2
12	10.5	–	15.6	–	6	61	tr	tr	1.5	1.8	3	6	tr	–	5.0	–	3
34	6.0	6.5	12.8	14.3	6	56	tr	–	0.9	–	4	27	tr	–	6.2	–	3
20	6.9	–	16.6	–	6	31	tr	–	1.1	–	4	43	tr	tr	2.4	2.6	2/3
19	6.3	6.1	12.1	9.4	6	28	tr	–	1.2	–	4	59	tr	–	4.5	–	3
6	16.2	19.0	18.8	20.9	6	17	4.8	3.7	–	–	6	24	tr	tr	2.4	3.2	3
4	13.5	13.7	23.3	22.5	6	55	6.4	8.7	16.5	20.4	5	12	tr	–	4.4	–	3
41	3.1	–	10.0	–	3	38	7.0	–	16.8	–	3	32	tr	–	2.7	–	3
44	3.3	6.1	6.5	12.3	6	42	16.6	8.6	26.3	12.2	3	85	tr	–	5.5	–	3
46	4.1	–	9.0	–	6							83	tr	–	5.5	–	3
66	13.5	5.5	22.3	11.2	5												
72	6.9	8.6	–	–	5												
73	3.6	3.4	9.2	9.6	5/6												
87	2.2	2.8	7.5	3.4	5												
37	5.5	8.8	12.1	19.7	5												

a) activity expressed as mU/mg acetone powder
b) rank number of picrate test reaction (100 μg of linamarin were added)
c) trace of activity (0–0.1 mU/mg powder)
d) not determined

umbelliferyl-β-D-glucosidase activities. The results are given in *table 5*. The picrate tests were scored by comparison with a test series of known amounts of HCN (see *table 5*). If duplo tests gave different results, this is indicated in the table by two figures.

We first consider the results of the picrate tests. Tr 4500 can be divided into two groups, one with rank nr $\geqslant 5$ (producing $\geqslant 20$ μg HCN/3 leaves) and one with rank nr < 5. There is however, considerable overlap. In Tr 2800 no such distinction can be made and in Tr 3600 no plants ranking over 4 are present. There is apparently a difference in the mean amount of HCN produced between the populations, but there is no clear cut difference between plants, as would be expected if one gene segregated in the populations. If we now consider the 4-methylumbelliferyl-β-D-glucosidase activities, we are able to distinguish high activities ($\geqslant$ 10mU/mg) and low activities ($<$ 10 mU/mg) in populations Tr 4500 and Tr 2800, with some overlap. High activities are absent from Tr 3600. The linamarase activities can be clearly divided into "trace" and "high" ($>$ 2 mU/mg).

Our conclusions are as follows: Populations Tr 4500 and Tr 2800 are polymorphic for a gene that regulates linamarase activity (presumably Li). Plants dominant for Li are absent from our sample of Tr 3600. Because linamarase activity constitutes a major part of the total β-glucosidase pool in the leaves, the latter activity is reduced in lili plants. The low but positive results in the picrate test of for example population Tr 3600 are explained by a residual activity of the other β-gucosidases, exhibited under the conditions (long incubation) of the picrate test. The frequency of plants dominant for Li is, according to *table 5*: Tr 4500: 0.55, Tr 2800: 0.15, Tr 3600: 0. Preliminary experiments using the picrate test have shown that in the Cevennes (Southern France) the frequency of cyanogenic plants is high at sea level, shows a sharp drop between 800 and 1000 m and is virtually zero at altitudes over 1000 m. Consequently, the populations sampled between 800 and 1000 m show large differences in the frequency of cyanogenic plants. Populations Tr 2800 and Tr 4500 clearly show these local differences. Tr 2800 grows on an exposed ridge (Col du Pas) whereas Tr 4500 although somewhat higher, grows in a sheltered valley. In Tr 3600, at 1170 m, the frequency of Li-plants is virtually zero. This observation is in accordance with earlier findings (Daday 1954a, b) that the distribution of the Li- allele is temperature dependent.

The substantial differences between clones and the still larger differences between populations, both with regard to enzyme activities as to picrate tests, show that other factors, environmental and genetic, influence the hydrolysis of linamarin. Restricting ourselves to the genetic differences, it is possible that the differences between populations are caused by modifiers. It is our experience from picrate tests that plants from lowland populations generally produce more HCN than Li-dominant ones from high level populations. This could be due to modifiers enhancing the expression of the Li- and/or Ac allele in the former populations. Another reason for differences between populations could be a dose-effect. MAHER & HUGHES (1972) found a dose effect in backcrosses of plants heter-

ozygous for the Li gene. This effect could be present in the populations studied by us, expecially in Tr 4500 where the frequency of Lili plants, assuming Hardly Weinberg equilibrium would be 0.45. The number of plants studied is, however, too small for a definite conclusion. This effect of the Li gene is subject of further investigation.

ACKNOWLEDGEMENTS

The authors are grateful to Mrs. M. Mulder, Mr. P. Verwey and Mr. E. Anink for skilful technical assistance, to B. Dommée (C.N.R.S.-C.E.P.E. L. Emberger, Montpellier) who kindly assisted us in collecting the material and to prof. D. Stegwee for critically reviewing the manuscript.

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