The Presence of a Specialized-β-Glucosidase: Linamarase, in the Leaves of *Trifolium repens* is Controlled by the Gene *Li*

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Z. Naturforsch. **40c,** 509–513 (1985); received January 28, 1985

Cyanogenesis, β-Glucosidase, Linamarase, *Trifolium repens*

Linamarase was purified by a three-step procedure from leaves of a clone of *Trifolium repens*. The partially purified enzyme was used to produce antilinamarin. The antiserum contained antilinamarase as well as antibodies against one or more other β-glucosidases. By incubation with an extract of a plant without linamarase activity pure antilinamarase could be obtained. Comparison of the titration curves of crude and purified antilinamarase shows that linamarase is an enzyme antigenically different from other β-glucosidases, and that this enzyme is absent from plants recessive for the gene *Li*.

Introduction

Most European populations of white clover (*Trifolium repens* L.) are polymorphic for cyanogenesis: only plants which possess at least one functional allele of the genes *Ac* and *Li* are able to release hydrocyanic acid when the leaves of the plant are damaged [1]. This release is due to the hydrolysis of the cyanogenic glucosides linamarin and lotaustralin by a β-glucosidase called linamarase (E.C. 3.2.1.21) to produce a glucose molecule and an unstable cyanohydrin. The cyanohydrin in turn dissociates spontaneously or enzymatically to release HCN and either acetone or 2-butanone.

The *Ac*-allele, which has been shown to be incompletely dominant [2] determines the presence or absence of both cyanoglucosides.

The presence or absence of the enzyme linamarase is determined by the independently inherited gene *Li*, which is also incompletely dominant [3]. There is evidence for the existence of an *Li*-allele with reduced activity [4]. Linamarase also exhibits activity towards artificial substrates with a β-glucosidic linkage like *p*-nitrophenyl-β-D-glucoside (PNPG) [3] and 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) [5]. Leaf extracts of plants with the *lili*-genotype cannot hydrolyse the natural substrate linamarin, but they do possess low levels of activity against the synthetic substrates [4, 5]. The difference in response between *Li*- and *lili*-plants may be due either to different properties of one enzyme or to the presence of several enzymes, one of which is absent or inactive in *lili*-plants.

Boersma *et al.* [5] found by chromatofocussing of an extract of a plant homozygous dominant for *Li* at least four β-glucosidases exhibiting activity towards the artifical substrates, one of which, with an isoelectric point of 4.3–4.4, exhibited activity against linamarin. The other β-glucosidases exhibited hardly any activity against linamarin and differed from linamarase in isoelectric point. Extracts of plants with the homozygous recessive genotype *(lili)* also contained several β-glucosidases, but they lacked the enzyme with the isoelectric point of 4.3–4.4 and linamarase activity.

Immunological tests by Maher and Hughes [4] have shown that plants homozygous for the recessive *li*-allele do not contain large amounts of an enzymatically inactive protein antigenically related to the normal enzyme (CRM). However, since these authors used only the artificial substrate PNPG in their immunochemical tests, it is impossible to decide whether their results apply to total β-glucosidase activity or to linamarase activity only. To obtain information on this particular subject, we have tried to establish the separate identity of the enzymes in plants with and without linamarase-activity using antibodies raised against partially purified white clover linamarase. Experiments were undertaken to determine whether white clover plants of the *lili*-genotype synthesize a catalytically inactive protein that is immunologically similar to linamarase of *Lili* plants.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/85/0700–0509 $ 01.30/0

Materials and Methods

Plant material

Cuttings, taken from a natural population of *Trifolium repens* at Gaseiral (near Montpellier, France) were grown to plants in a greenhouse [5]. Two clones were selected on the basis of their genotype and their response to the picrate test; clone Tr 4506 being *Ac Ac LiLi* and clone Tr 4511 being *Ac - lili.* Young just unfolded leaves were collected and kept at −20 °C till used for enzyme-extraction.

Enzyme-extraction

Acetone powder of the collected leaves was prepared as previously published [5] and stored at 4 °C. Extracts were made by homogenizing 500 mg of dry acetone powder in 1 M NaCl, 1 mM EDTA, 1 mM cysteine at 0 °C in an Ultra-Turrax homogenizer for 3 min. After 15 min (0 °C) the mixture was centrifuged at 10,000 rpm. Only the supernatant was used in this study.

Enzyme assays

Linamarase-activity was measured by the amount of hydrogen cyanide released by hydrolysis of linamarin (Calbiochem) as described previously [5]. The reaction was stopped after 60 min by addition of 0.4 ml 0.2 M NaOH. An aliquot of 500 µl of the assay mixture was used to determine HCN by the method of MaO *et al.* [6].

β-Glucosidase activity against 4-methylumbelliferyl-B-D-glucopyranoside (Koch Light Ltd) was measured by determining the release of 4-methylumbelliferone as described previously [5].

Purification of linamarase

Acetone powder of clone Tr 4506 was extracted overnight at 4 °C in 1 M NaCl, 1 mM EDTA, 1 mM cysteine with continuous stirring. The proportion acetone powder/extractant was approximately 1:12 (w/v). The suspension was strained through cheesecloth and centrifuged at 38,000 g for 30 min. The supernatant was applied to a 150 ml S200 (Pharmacia) column and eluted with 0.2 M tris-maleate buffer, 1 mM EDTA, 1 mM cysteine, 0.05 M NaCl, pH 5.6. Fractions of 3 ml were assayed for linamarase activity. The active fractions of several runs were pooled, concentrated by pressure dialysis (Diaflo PM30) and dialysed against 0.025 M imidazol-HCl buffer pH 7.5 overnight at 4 °C.

The dialysed preparation was applied to a 30 ml PBE94 (Pharmacia) column that had been equilibrated with 0.025 M imidazol-HCl buffer pH 7.5. The column was eluted with polybuffer 74 (Pharmacia) pH 4.0. Fractions of 2 ml were collected and assayed for linamarase-activity. The active fractions (pH 4.9−4.3) were pooled, desalted over a Pharmacia PD-10 prepacked column (Sephadex G-25M) and freeze dried. The active fractions of several chromatofocussing runs were pooled again and purified by preparative isoelectric focussing and assayed for linamarase-activity. The active fractions were pooled, desalted and freeze dried. The yield from 50 g aceton powder was 600 µg protein as measured by the Biorad procedure [7]. An aliquot of the linamarase preparation after chromatofocussing was tested in 10% sodiumdodecylsulfate polyacrylamide gel electroforesis.

The result was one band at approximately 62,000 daltons and two faint bands at 47,000 daltons.

Preparation of antiserum to linamarase

Antibodies against the purified linamarase preparation of plant Tr 4506 were produced in a young male New Zealand white rabbit (Centraal Proefdierbedrijf TNO, Zeist, Holland). The antigen was given intravenous as 60 µg enzyme preparation in 1 ml with complete Freunds adjuvant. After ten days the rabbit received an injection with 60 µg enzyme preparation in 1 ml with incomplete Freunds adjuvant. This was repeated three times with intervals of ten days. Then 50 ml of blood was taken from the animal and clotted; 1 hour at 37 °C and overnight at 4 °C. The serum was removed and centrifuged 15 min 1000 × g (4 °C). The supernatant was incubated at 56 °C for 30 min in a shaking water bath to inactivate the complement, after which the serum was frozen in portions of 1 ml in reaction tubes (Eppendorf 3810, 2 ml) at −20 °C.

Antibody-assay

IgG-type antibodies were selectively absorbed from the antiserum by incubating varying amounts of antiserum with 100 µl Protein A-Sepharose CL4B (Pharmacia). The endvolume was adjusted to 350 µl with phosphate buffered saline (PBS). After 60 min incubation (at room temperature in a rotary shaker)

the mixture was centrifuged at 10,000 rpm for 2 min. The supernatant was removed and the pellet was washed three times with PBS to remove traces of the antiserum. The pellet was taken up in 200 µl of an enzyme extract of clone Tr 4506 or Tr 4511 of which the linamarase and total β-glucosidase activity had been previously determined and incubated for 90 min (constantly shaking at room temperature) to allow binding of antigen to immobilized antibodies. The mixture was centrifuged at 10,000 rpm for two min. The supernatant was assayed for enzyme-activity against 4 MUG and linamarin.

A modification of this assay was used to test for the presence of CRM in *lili*-plants. The immobilized IgG-type antibodies were incubated with an extract of a *lili*-plant (Tr 4511). After 90 min incubation the mixture was centrifuged at 10,000 rpm for 2 min. The supernatant was removed and assayed for enzyme-activity against 4-MUG. The pellet was washed three times with PBS to remove all traces of non-bound *lili*-extract. The pellet was taken up in 200 µl of an enzyme extract of a *LiLi*-plant (Tr 4506). The total β-glucosidase activity in the first incubation was six times that of the second incubation.

After 90 min continuous shaking at room temperature the mixture was centrifuged 2 minutes at 10,000 rpm. The supernatant was assayed for enzyme-activity against 4 MUG and linamarin. Results were compared with a parallel series of incubations using the same amounts of antiserum but without the preincubation with a Tr 4511 *lili*-extract.

Results and Discussion

Fig. 1a shows the decrease in β-glucosidase activity of an enzyme-extract of plant TR 4506 *(LiLi)* that has been treated with proteine A-sepharose to which varying amounts of antiserum were added. It appears that 5 µl of serum binds approximately 50% of the initial activity (2.2. µKat). Upon increasing amounts of antibody the activity in the supernatant further decreases to very low values, however, significantly over the background level. The binding must be specific as serum of an untreated rabbit does not give the same effect.

Fig. 1b shows results of the same experiment, but now linamarase-activity is measured. Apparently binding is complete with 50 µl antiserum.

Fig. 1c shows the decrease in β-glucosidase activity of an enzyme-extract of plant TR 4511 *(lili)*. The decrease is far less than that of the *LiLi* plant: even with 250 µl of antiserum, the binding is incomplete.

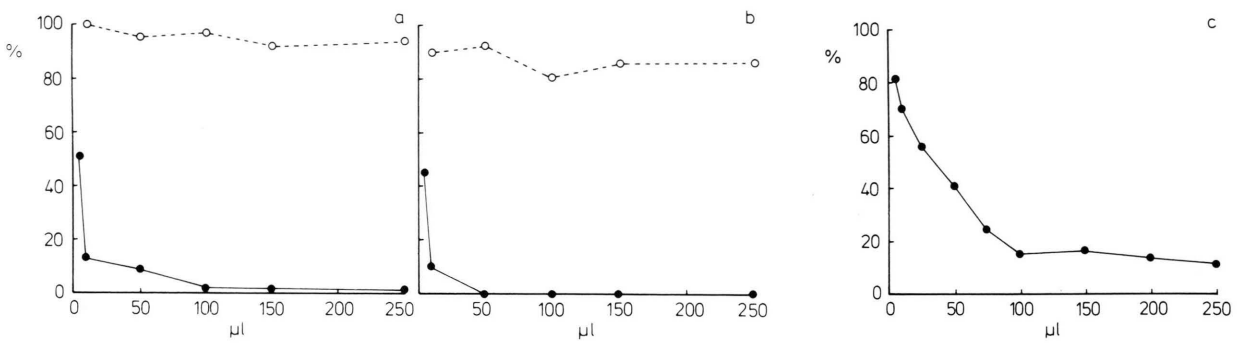

Fig. 1a, b. Antibody titration curves using extracts of Plant TR 4506 *(Li)*. Horizontal axis: amount of serum added to the protein A-Sepharose beads.
Vertical axis: activity in the supernatant, expressed as percentage of a blank to which no serum was added.
Full circles: antiserum added.
Open circles: normal rabbit serum added.
a. Shows the total β-glucosidase-activity (4-MUG as substrate).
b. Shows the linamarase activity of the same supernatant.
Fig. 1c. Antibody titration curves using extracts of Plant TR 4511 *(lili)*. Horizontal axis: amount of serum added to the protein A-Sepharose beads.
Vertical axis: activity in the supernatant, expressed as percentage of a blank to which no serum was added.
Full circles: antiserum added.
Open circles: normal rabbit serum added.
Only the total β-glucosidase-activity is shown (4-MUG as substrate).

In view of our earlier results [5] showing that about 50% of the total β-glucosidase activity is due to linamarase, these results can be explained in two ways:

Firstly the purified enzyme and consequently the antiserum, could be impure, containing besides antilinamarase also antibodies against other β-glucosidases.

Secondly the antilinamarase could react with other β-glucosidases as well.

To distinguish between the two possibilities a double incubation was used: Preincubation of the immobilized antibodies with a large amount (28 μKat of β-glucosidase activity) of an extract of an *lili* plant, followed by a second incubation with an extract of *LiLi* plant (5 μKat). After preincubation, the activity in the *LiLi* extract is reduced to appr. 20% (measured with the artificial substrate) by 10 μl of antiserum. No further reduction of activity is observed (Fig. 2a). Apparently, about 80% of the total β-glucosidase activity in the *LiLi* plant is due to linamarase, which is precipitated by the pretreated serum. This result is confirmed by the results shown in Fig. 2b, indicating that 90% of the linamarase activity (1 μKat) of Tr 4506 *(LiLi)* can be precipitated by 10 μl of pretreated antiserum. Obviously no significant reduction of antibody titer occurs upon preincubation (compare Figs. 1, 2a, 2b).

The results clearly show that the antiserum is impure, containing antibodies against at least two different β-glucosidases. As it is not possible to saturate the binding sites for linamarase with an *lili* extract, we conclude that linamarase is a protein antigenically different from all other β-glucosidases and that this protein is virtually absent from plant TR 4511.

These conclusions are also drawn by Hughes and Dunn [8]. They refer to an earlier paper [9] for the immunological work. However in this paper, only PNPG was used as a substrate. The results therefore apply to total β-glucosidase activity and show that the antiserum used contained antibodies against different β-glucosidases including linamarase. In the 1982 paper the authors state that "The low level of pnpgase in *lili* plants is not due to small amounts of linamarase". This implies the existence of different β-glucosidases, one of them being linamarase. The possibility of two different, but evolutionary related genes controlling the two enzymes is mentioned by them.

In our opinion the only firm conclusion that can be drawn from the observations published so far is that there is at least one gene, *Li*, controlling the level of linamarase protein in the leaves of *T. repens*. The *li* mutation is unusual, but not unique, in that plants recessive for *li* do not contain measurable amount of linamarase protein (for a discussion of this phenomenon, see Hughes and Dunn [8].

The hypothesis of Jones [10] that *Li* is the result of a mutation that causes overproduction of enzyme is difficult to reconcile with this property of the *li*-allele. One could speculate that somewhere in the lineage of *T. repens* changes in the structural gene coding for a β-glucosidase altered the catalytic and antigenic properties of this enzyme, giving rise to the present linamarase.

Other changes in the genome, included mutations in the primitive *Li*-gene would subsequently have changed the proportions between the β-glucosidases in the leaf cells, leading to the high contribution of linamarase to the β-glucosidase activity indicated in

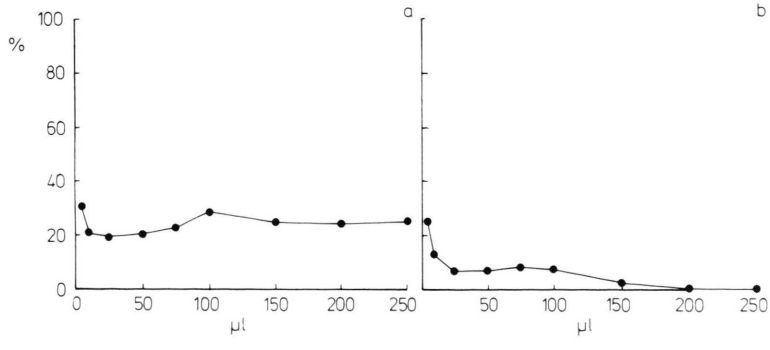

Fig. 2. Antibody titration curves using extracts of Plant TR 4506 *(LiLi)* and using immobilized antibodies pretreated with an extract of plant TR 4511 *(lili)*.
Horizontal axis: amount of serum added.
Vertical axis: activity in the supernatant expressed as percentage of a blank to which no serum was added.
Full circles: antiserum added.
Open circles: normal rabbit serum added.
a. Shows the total β-glucosidase-activity (4-MUG as substrate);
b. the linamarase activity of the same supernatant.

Fig. 2a, as well as causing the high relative amount of linamarase protein found by Hughes and Dunn [8]. Investigation into the quantitative and qualitative variation in linamarase in different populations, that are under way at this moment could shed light on details of the evolution of the cyanogenic system in *T. repens*.

Acknowledgements

The authors wish to thank Dr. A. W. Schram who suggested the experiments and gave technical advise throughout this study. E. Anink cultured the plants, R. Scholten gave excellent technical assistance, Karla ter Horst prepared the manuscript and H. v. d. Meyden the drawings.

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