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Aglycon specificity of the cyanogenic β-glucosidases of leaves of *Trifolium* species and Cassava (*Manihot esculenta*).

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ABSTRACT: The aglycon specificity of the cyanogenic β-glucosidases of *Trifolium repens*, *Trifolium nigrescens* and cassava (*Manihot esculenta*) towards their natural substrates linamarin and lotaustralin was studied. In cassava, a species in which linamarin predominates, both substrates are hydrolysed although the Km is slighty lower and the Vmax is higher for linamarin. The importance of this result for the determination of the cyanogenic potential in cassava is discussed. In the *Trifolium* species there is little difference in kinetic parameters, although the proportion of linamarin differs between the species. The *Trifolium* results are discussed in relation to the possible origin of the Li-gene in *T. repens*.

KEYWORDS: *Trifolium repens, Trifolium nigrescens*, Cassava, *Manihot esculenta*, linamarin, lotaustralin, cyanogenic β-glucosidases, cyanogenic potential.

Introduction

All plants produce minute amounts of HCN as a byproduct of ethylene production. Approximately 5 % of the higher plants produce HCN in much higher quantity after damage (SAUPE 1981). Such plants are called cyanogenic. HCN is produced in these plants by the action of one or more cyanogenic β-glucosidases on cyanogenic glycosides present in a different compartment in the

intact plant (GRUHNERT & al. 1994). Variation in the cyanogenic glycosides is caused by variation in the sugar moiety and in the aglycon. It has been known for a long time that β-glucosidases are specific for the sugar part of the cyanogenic glycoside (refs in CONN 1993). HÖSEL & CONN (1982) as well as CONN (1993) have shown that there is also a marked aglycon specificity. Generally speaking plants that contain cyanoglycoside(s) will also contain the corresponding β-glucosidase. Exceptions to this rule are the polymorphic species, like *Trifolium repens* L. and *Lotus corniculatus* L., where plants can be found that contain only cyanogenic glucosides or cyanogenic β-glucosidases or lack both (KAKES 1990). Studies on the aglycon specificity of β-glucosidases have compared artificial substrates e. g. p-nitrophenol-glycosides with natural substrates, or natural substrates with very different aglycons e. g. trichochinin versus taxiphyllin. Only one plant study is known to us comparing substrate specificity for linamarin and lotaustralin (SELMAR & al. 1987). These authors found a non-specific β-glycosidase in *Hevea braziliensis* (HUMB., BONPL. & KUNTH) MÜLL.-ARG.

We will in this study compare the substrate specificities and reaction velocities of the cyanogenic β-glucosidases of the closely related species *Trifolium repens* L. and *Trifolium nigrescens* VIV. against their natural substrates linamarin (2-hydroxyisobutyronitrile β-D-glucopyranoside) and lotaustralin (R-2-hydroxy-2-methylbutyronitrile β-D-glucopyranoside). The non related species *Manihot esculenta* CRANTZ (Cassava), in which primarely linamarin has been reported is included for comparison.

In an earlier study (KAKES & HAKVOORT 1994) we compared the cyanogenic β-glucosidases of *T. repens* and *T. nigrescens*. We showed that both species contain only one enzyme that hydrolyses linamarin. In view of the close resemblance between the enzymes (mol. weight, kinetic parameters, cross reacting antibodies) we postulated that *T. nigrescens* is the donor of the linamarase of *T. repens*. A later study showed that *T. nigrescens* differs from *T. repens* in the proportion of linamarin and lotaustralin, linamarin being more abundant in the latter species (this paper). We therefore decided to see if we could find a difference in the kinetics of the two enzymes with the two substrates.

Material and methods

Isolation and purification of lotaustralin: *T. nigrescens* was raised from seeds collected in the Cevennes (S. France). For the enzyme studies on *T. repens* a genotype originally collected in the Cevennes was used. This genotype is homozygous dominant for the genes Ac and Li, that govern the production of cyanogenic glucosides, resp. the cyanogenic β-glucosidase. For the determination of the proportion of linamarin and lotaustralin we used backcross progeny plants that were either homozygous or heterozygous for Ac as indicated in table 1. The plants were kept in a growth chamber, day temperature 20°C, night temperature 15° C, daylength 14 hours, RH 75 %. Illumination by incandescent lamps 200 $\mu E.sec^{-1}.m^{-1}$ at plant height. Young full grown leaves were harvested and kept at -20° C. Portions of 30 g were homogenized under liquid nitrogen in a mortar, transferred to boiling ethanol and boiled for 5 min. The

mixture was extracted in a Soxhlet with 400 ml ethanol untill a clear filtrate was obtained. The extract was reduced to approximately 100ml with a rotavapor. 25 ml of demineralized water was added and the concentrated extract was kept in a fume cupboard for 16 hrs to remove the remaining ethanol. The next day the extract was divided in 1 ml aliquots in Eppendorf vessels, frozen in liquid nitrogen and freezedried in a speedvac freezedryer. The dry sample was taken up in demineralized water, sonicated for 10 min and filtrated over a 0.2 µm filter. The end volume was 2.5 ml per batch. Purification over a preparative C18 column: A column was made from a 60 ml syringe fitted with a glass filter p3 at the bottom and filled with 35 ml C18. The C18 was loaded as a slurry with ethanol, washed with 5 volumes of ethanol and equilibrated with 5 volumes of demineralized water. 2.5 ml of the sample was loaded and the column eluted with 90 ml of each of the following series of ethanol in water: 0.5%, 2.5%, 5%, 10%, 20%, 40%, 100% (v/v). Elution was by gravity (head 25 cm). 15 ml fractions were collected. 100 µl aliquots of the fractions were dried, taken up in 50 µl of water and tested for the presence of cyanoglucosids with the microtiterplate assay (KAKES 1991). Aliquots of the active fractions were tested with HPLC (see below). It turned out that linamarin eluted with 2. 5% ethanol, and lotaustralin with 5% ethanol. The two cyanoglycosides were well separated. The lotaustralin containing fractions were combined and concentrated with a speedvac and taken up in 2 ml of water. The purity of the isolate was checked with HPLC. Only one symmetrical peak was found. The yield was 470 mg lotaustralin from 150 g of leaves.

Determination of the linamarin and lotaustralin content of cassava: 2.5 g of fresh leaves were pulverized in liquid nitrogen and transferred to 200 ml of boiling ethanol. After 15 min of boiling under reflux the volume was reduced to 100 ml, 15 ml of water was added and the mixture freezed dry in 1 ml aliquots. The residue of 1 ml was taken up in 0. 5 ml of water. 20 µl of the concentrate was injected in the HPLC column.

Enzyme isolation: Leaves of the cassava varieties Mprt-26 and Faroka were collected in the greenhouse of the University of Wageningen. The cassava linamarase was prepared by extracting 0.25 g aceton powder of fresh leaves overnight in 3 ml 0.1M Na phosphate buffer pH 7.4. The *T. repens* and *T. nigrescens* linamarase was obtained by extracting 1 g of acetonpowder with 10 ml extraction buffer (KAKES & EELTINK 1985).

Enzyme incubations: *Trifolium*: Linamarin (Sigma) and purified lotaustralin were incubated in the indicated concentrations with 65 mM acetate buffer pH 5.6 and 50 µl of enzyme in a total volume of 100 µl in Eppendorf vessels. The incubation time was 2 min and the temperature 37°C. The reaction was stopped by immersing the vessels in liquid nitrogen. *Cassava*: The buffer was a 0.1 M phosphate buffer pH 7.4 and the reaction temperature 55°C. The other conditions were as described for *Trifolium*.

HPLC separation and quantification of linamarin and lotaustralin: The apparatus used was equipped with a guard-pak Precolumn module P/N 099141 with a 10 µm retaining insert, a C18 column 15 cm x 3.9 mm, 4 µm particles.

Tab. 1: Proportion of linamarin in leaf extracts of the species and cultivars studied.

Plant	Genotype/cultivar	lin/lin+lot	95% conf.int.
Cassava	Mprt	0.96	-
	Faroka	0.96	-
T.repens	AcAc	0.40	0.01
T.repens	Acac	0.48	0.02
T.repens	AcAc	0.46	0.01
T.repens	Acac	0.42	0.01
T.repens	Acac	0.41	0.00
T.nigrescens	-	0.13	0.03

Elution was water (Superdemi). The elution speed was 1.6 ml/min, the silk software was on. The eluens was aspirated with 10 ml helium/min. The temperature of eluens and column was 30°C. For the detection a RI detector was used, at a temp of 37°C. The retention time of the linamarin peak was 4 min, that of the lotaustralin peak 13.5 min. The detection range is 0.125- 4 mM cyanoglucosid. The linamarin peak was calibrated with commercial linamarin. The lotaustralin peak was calibrated by determining the N-content of the pure fractions.

Results and discussion

The proportion of linamarin and lotaustralin in the plants used for the enzyme studies is shown in table 1. The values for Cassava and *T. repens* are in agreement with those published earlier (BUTLER 1965). The value found for *T. nigrescens* is higher than that found by VAN VALEN (1979). The kinetic parameters of the different species are given in table 2. (Both enzymes were tested by their published optimum temperature, which is higher for the cassava linamarase.) It is clear from this table that in both *Trifolium* species the Km for lotaustralin is much lower than that for linamarin. The cassava linamarase does not show a great difference in affinity for the two substrates. This is important

Table 2: Kinetic parameters of different linamarases.

Species	Substrate	Km (mM)	Vmax (kat/kg protein)
T. nigrescens	lin	3.1	4.1*10^-2
T. nigrescens	lot	0.28	2.8*10^-2
T. repens	lin	4.2	5.2*10^-2
T. repens	lot	0.22	1.1*10^-2
Cassava "Mprt 26"	lin	2.1	34*10^-2
Cassava "Mprt 26"	lot	3.1	14*10^-2
Cassava "Faroka"	lin	2.1	33*10^-2
Cassava "Faroka"	lot	3.9	17*10^-2

from a practical point of view: most methods currently in use to measure cyanogenic potential in cassava use cassava-linamarase to hydrolyse the cyanogenic glucosides and subsequently determine the amount of cyanide produced. As approximately 5% of the cyanoglucosides is lotaustralin a failure to hydrolyse lotaustralin would lead to an underestimate of the cyanogenic potential. There is in cassava a difference in Vmax: the Vmax for linamarin is about twice as high as that for lotaustralin. The tenfold difference in Vmax between the *Trifolium* species and cassava is partly due to the higher incubation temperature, but may also be caused by a higher linamarase activity on protein base in the cassava extracts. In view of the fact that in cassava there is much more linamarin than lotaustralin (table 1) the results of the cassava experiments are in accordance with earlier findings that the predominant cyanoglucoside is hydrolysed best by the endogenous β-glucosidase (KAKES 1990). The Km and Vmax for *T. repens* and *T. nigrescens* do not differ very much, in line with our earlier hypothesis (KAKES & HAKVOORT 1994), based on kinetic and immunological studies, that *T. nigrescens* is the donor of the linamarase gene in *T. repens*. The high affinity for lotaustralin in both species may reflect the preponderance of lotaustralin in *T. nigrescens*, a character retained in *T. repens* (table 1). The Km-values for linamarin are in good agreement with results published earlier in cassava: 2.08 mM (YEOH & WOO 1992) and 1.9 mM (MKPONG & al. 1990). For *T. repens* there is agreement with the most recent publication: 4.3 mM (POCSI & al. 1989). The value found by BOERSMA & al. (1983) is higher and that of HUGHES & MAHER (1973) is much lower. As the latter authors used a mixture of linamarin and lotaustralin, this outcome was to be expected. The Km and Vmax values for linamarin and lotaustralin have been determined for *Hevea braziliensis* (SELMAR & al. 1987) and for the butterfly *Zygaena trifolii* ESPER (FRANZL & al. 1989). In *Hevea*, that contains no specific linamarase a broad spectrum β-glucosidase has the same Km but a much higher Vmax for linamarin. In *Zygaena* the Km values are much higher than ours. Data for *T. nigrescens* have not been published earlier.

The difference in specificity between Cassava and *T. repens* can be illustrated by a series of experiments in wich the two substrates were used separately and in combination. In these experiments the reaction time was 20 min, and the enzyme activity adjusted so that both substrates showed an appreciable decrease. As shown in fig. 1 and 2 for cassava, the substrates act as competitors: each one lowers the rate of hydrolysis of the other, notably at higher initial concentrations. The effect of linamarin on lotaustralin is much more profound than vice versa. In *T. repens* (fig. 3 and 4) it is the other way around: lotaustralin is here the stronger inhibitor. At intermediate concentrations the effect of the inhibitor is less, because most of this substrate is converted early during the reaction. The cassava data clearly show that in the presence of an excess of linamarin lotaustralin will be mainly hydrolysed towards the end of the reaction time when the linamarin concentration is low. The practical meaning of this observation is that in the determination of the cyanogenic potential of cassava the reaction time is critical.

A general conclusion is that there is a clear relation between the cyanogenic glucoside(s) present in the species studied and the properties of the cyanogenic glucosidases, but that the evolutionary history of the species studied should be taken into account, especially in the young species *T. repens* and *T. nigrescens*.

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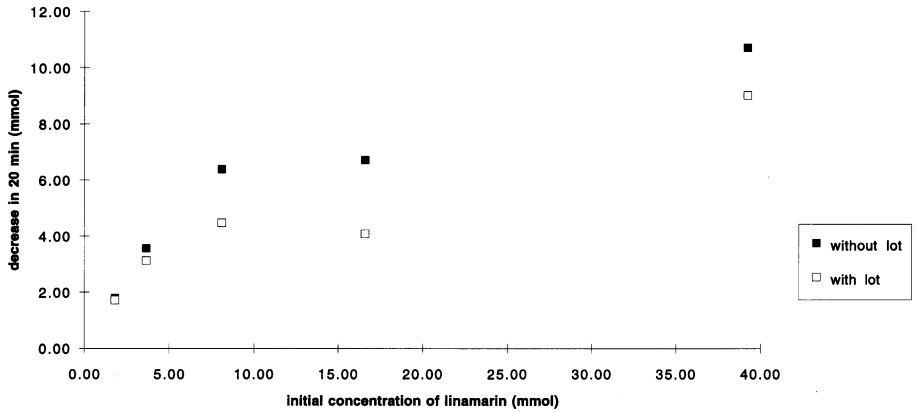

Fig 1. Hydrolysis of linamarin by Cassava linamarase.

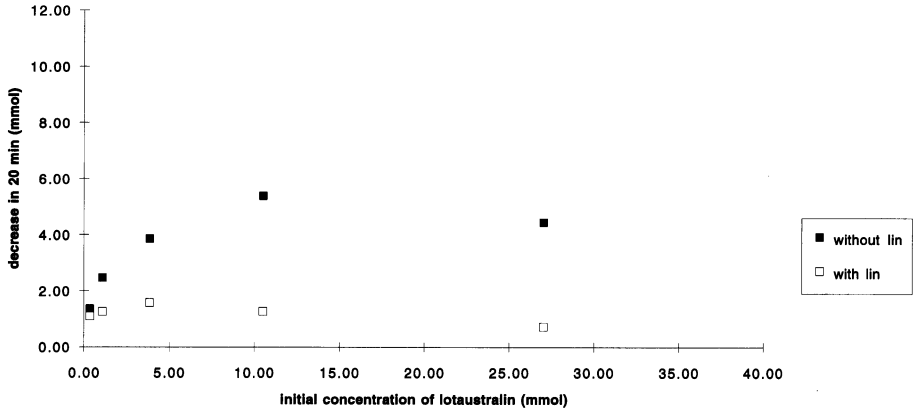

Fig 2. Hydrolysis of lotaustralin by Cassava linamarase.

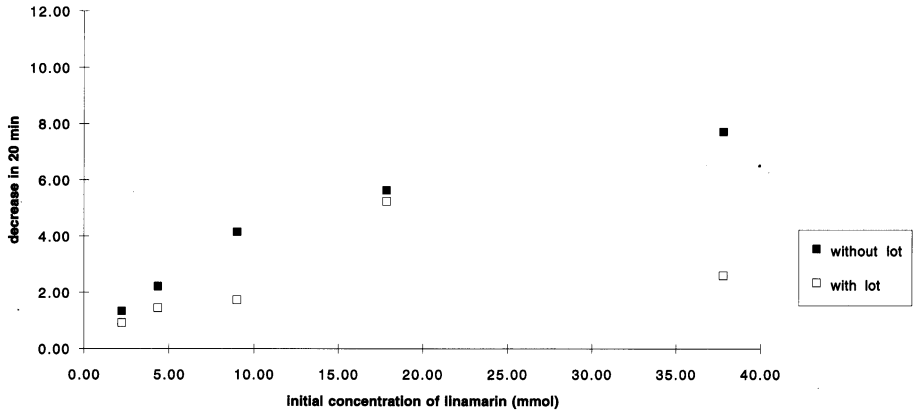

Fig 3. Hydrolysis of linamarin by *T. repens* linamarase.

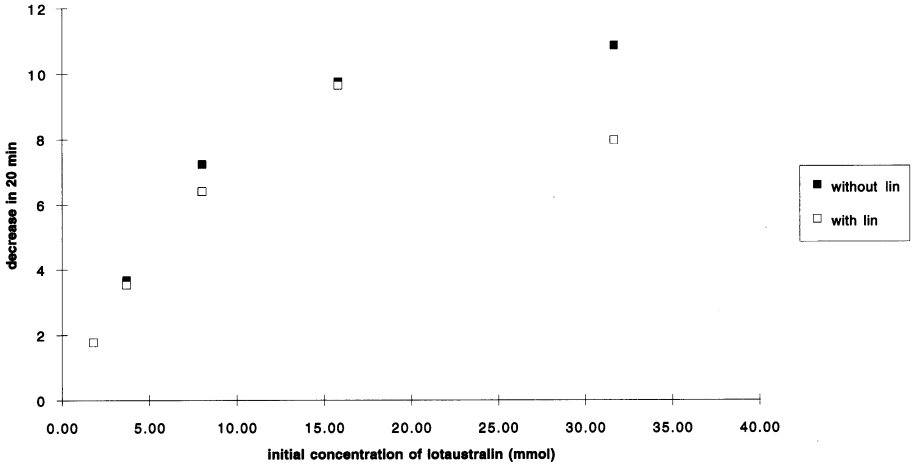

Fig 4. Hydrolysis of lotaustralin by *T. repens* linamarase.

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