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SHORT COMMUNICATION

ISOLATION OF LINAMARIN–LOTAUSTRALIN FROM *TRIFOLIUM REPENS*

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Abstract—Linamarin and lotaustralin were isolated as a mixture from *Trifolium repens* L. and used as substrate for the assay of linamarase.

IN A STUDY of the genetic control of linamarase activity in *Trifolium repens* L., it became necessary to isolate the native substrates, linamarin (α-hydroxyisobutyronitrile-β-D-glucoside) and lotaustralin (α-hydroxy-α-methylbutyronitrile-β-D-glucoside) for use in a quantitative assay of linamarase activity in crude leaf extracts. The first report of a preparative scale isolation of linamarin and lotaustralin from leaves of *T. repens* involved continuous extraction of an aqueous syrup with ethyl acetate.[1] Later attempts to repeat this method failed to give satisfactory crystallization and it was replaced by two modified techniques in which the aqueous syrup was either extracted with alcohol–acetone or exhaustively boiled with ethyl acetate.[2] Linamarin has also been extracted from tubers of *Manihot esculenta* by chromatography of an ethanolic extract with chloroform–methanol on silica gel and crystallization from ethyl acetate,[3] and by an adaptation of the dry column technique of Loev and Snader.[4,5] A method for the isolation of dhurrin from *Sorghum vulgare* involving chromatography on cellulose powder with butan-1-ol–water (9:1) has been described by Mao *et al.*[6] and has now been adapted for the isolation of a mixture of linamarin and lotaustralin from leaves of *T. repens*.

The rapid inactivation of glycosidases is an important preliminary to the extraction of glycosides from plant tissue.[7] This problem has been avoided in the present study by the collection of leaves from a single clone, genotype **AcAclili**,[8] which contained both linamarin and lotaustralin, but no detectable linamarase activity. Clarified, deionized extracts of these leaves were used in various unsuccessful attempts to isolate the cyanoglucosides by dry column chromatography and by extraction of dried residues with ethyl acetate. However, chromatography on columns of cellulose powder gave reproducible separations of the cyanoglucosides, which readily crystallized from butan-1-ol. Paper chromatography of the

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isolated mixture and densitometric scans of spots located with ammoniacal $AgNO_3$[9] suggested that the mixture was approximately 56% lotaustralin, 44% linamarin.

Linamarase activity of *T. repens* leaf extracts was assayed by hydrolysis of the cyanoglucosides and estimation of the evolved HCN. The 10-min reaction period, which was used, required a sensitive method for the determination of cyanide and this was provided by the modified pyrazolone method.[8] This short hydrolysis period provided a better indication of initial velocity of the reaction than the periods of one hour or more, which have been used in other studies. The reaction velocity was proportional to linamarase concentration and the optimum pH for maximum linamarase activity was 5·0. The linamarase assay has been used in a study of the properties of the enzyme in white clover and in comparisons of plants containing different alleles of the **Li** locus. The results of these experiments will be published elsewhere.

EXPERIMENTAL

Plant material. Material for the cyanoglucoside isolation was collected from a single clone of *T. repens*, variety S.100, genotype **AcAcLili**. Leaf, petiole and some stem tissue was collected over a number of weeks and stored at −20°. Linamarase was extracted from the folded leaves of a single clone, variety S.100, genotype **acacLiLi**, which were stored at −20° until required.

Paper chromatography. The solvent system was MeCOEt–Me_2CO–H_2O (30:10:6)[10] with Whatman No. 4 paper. The purified, crystalline material gave two spots with ammoniacal $AgNO_3$[9], R_fs 0·52 and 0·63 and no reaction with aniline-diphenylamine.[11] The two spots were the only regions to give positive reactions to a modified picrate test,[5] and also to the pyrazolone test,[6] when cut out, eluted and treated with linamarase. From this and published data[10,12] the two spots were identified as linamarin (R_f 0·52) and lotaustralin (R_f 0·63). Monitoring, by paper chromatography, of the fractions from cellulose columns showed that lotaustralin was the predominant cyanoglucoside in the early fractions, while linamarin, only, is present in the final fractions.

Estimation of the cyanoglucosides. Hydrolyses were performed in Thunberg tubes containing 1 ml test solution and 0·5 ml linamarase. The tube was stoppered, the side arm containing 1·5 ml 0·2 N NaOH. After incubation at 35° for 24 hr the solutions were mixed and aliquots removed for HCN determination by the pyrazolone method.[6] An aqueous solution of the purified crystals, containing 392 mμ moles of a 56/44 mixture of lotaustralin–linamarin gave 376 mμ moles HCN, 96% of expected yield.

Isolation. For the main procedure, 3·89 kg of frozen clover tissue was minced in a Hobart mincer and homogenized in a Waring blendor with 5 vol. 80% aq. EtOH. The slurry was boiled for 10 min, filtered, and the residue washed twice with 80% EtOH and re-filtered. Combined filtrates were reduced *in vacuo* at 40° to 4·14 l., containing 10·6 g of cyanoglucosides, and clarified with 700 ml 40% w/v basic lead acetate. The precipitate was spun down and washed twice with water. The supernatant and washings were combined, treated with H_2S, and PbS removed by filtration. The filtrate was reduced to 335 ml which was deionized on columns of Zeokarb 225 (H^+) and DeAcidite E (OH^-) to yield 1·4 l. containing 9·5 g of cyanoglucosides. This solution was reduced to 83 ml of thick, red syrup, which was chromatographed in three portions on columns of Whatman cellulose powder (cf 11) as described by Mao *et al.*[6] From each fraction 5 μl was spotted onto Whatman No. 4 paper, the spots were allowed to dry and each spot monitored for the presence of cyanoglucosides by the picrate test. In each case the cyanoglucosides first appear at an eluent volume of 220 ml and continued to approximately 475 ml. As in the dhurrin extraction,[6] a yellow pigment, eluting at 200 ml, acted as a marker for the cyanoglucosides. In each case the fractions containing the eluent volume from 220 to 420 ml were pooled and reduced *in vacuo* at 55° to a crystalline residue, which was dissolved in warm *n*-BuOH to give a yellow solution. On standing, this solution deposited colourless crystals which were dissolved in water and filtered, and the filtrate reduced *in vacuo* at 55° to a syrupy residue. This was dissolved in warm *n*-BuOH and left to crystallize at 8°. A further crystallization from *n*-BuOH provided colourless, needle-shaped crystals. The eluents from the 3 columns yielded respectively, 680, 670 and 630 mg of crystals in the final purified form. The combined solutions from which crystallization took place, yielded 840 mg of crude crystalline material, which has not been further purified.

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Linamarase preparations. Crude extracts were made by grinding 500 mg folded leaves in 15 ml citrate-phosphate buffer, pH 5·0. Insoluble plant material was removed by centrifugation. Partially purified extracts, used in the development of the linamarase assay, were prepared from acetone-dried powders of folded leaves by collecting the protein fraction precipitating between 45% and 75% saturated $(NH_4)_2SO_4$ and resuspending in 0·05 M Tris acid maleate–NaOH buffer pH 5·8, containing 1 mM cysteine.

Linamarase assay. The reaction mixture consisted of 0·9 ml citrate-phosphate buffer, pH 5·0, containing 3 μ moles linamarin–lotaustralin, preheated to 35°, plus 0·1 ml of enzyme solution. After 10 min at 35° the reaction was stopped by addition of 1·0 ml 0·2 N NaOH. An aliquot of the mixture was taken for determination of HCN by the pyrazolone method.[6] For the determination of pH optimum, the substrate was dissolved in a range of citrate-phosphate buffers, pH 3·6–8·0.

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