

CONDENSED TANNINS FROM WHITE CLOVER SEED DIFFUSATE

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(Received 14 May 1979)

Key Word Index—*Trifolium repens*; Leguminosae; *Rhizobium trifolii*; toxicity; condensed tannins; purification.

INTRODUCTION

It is a common agricultural practice to coat legume seeds before sowing with an inoculum of *Rhizobium* bacteria in order to ensure nodulation. With some cultivars of clover, however, there is a rapid decrease in the number of viable bacteria following such inoculation [1, 2]. Aqueous amyl alcohol extracts from such seeds have been studied by Fottrell *et al.* [3] and shown to contain two substances toxic to *Rhizobium* bacteria. One, a low molecular weight substance, was shown to be myricetin (3, 5, 7, 3', 4', 5'-hexahydroxyflavone). The other gave typical reactions for condensed tannins but Fottrell *et al.* [3] did not attempt to characterise it.

It is unlikely that alcoholic extracts are representative of the situation in practice, where the toxin is being leached out of the seed by water. Our results have indicated that in water extracts of clover (*Trifolium repens* L.) seeds, myricetin is only a minor factor in the observed toxicity and that condensed tannins, also found in the aqueous alcoholic extracts, seem to be the major factor. This paper reports on the purification and the chemical composition of these condensed tannins.

RESULTS AND DISCUSSION

Myricetin was clearly visible as a yellow band at R_f 0.5 on a paper chromatogram (BAW solvent) of a concentrated aqueous seed extract. However, division of the chromatogram into zones and recovery of these zones from the paper by elution with water showed that under the bioassay conditions used the only toxic zone was that at the origin. The yellow-brown eluate from this toxic zone gave a positive vanillin-HCl test and a deep purple colour on heating with HCl in *n*-BuOH. These reactions are consistent with the presence of condensed tannin. When a saturated aqueous solution of myricetin was tested, a toxic response was not obtained in the bioassay. A positive response could only be obtained when 100 μ l of a finely dispersed suspension of myricetin at 3.18 mg/ml was used.

Extraction of the tannin from white clover seeds was carried out at room temperature with water containing ascorbic acid. After concentration, the aqueous extract was washed with *n*-BuOH to remove organic soluble materials. A further crude separation was obtained by precipitation of the tannin material using a MeOH-

CHCl₃-H₂O mixture. This precipitation procedure afforded a very convenient 10-fold purification of the condensed tannin. Final purification was achieved by chromatography on Sephadex LH20 columns, which gave three fractions each of which was toxic to *Rhizobium* bacteria and gave a positive vanillin-HCl test. These fractions were labelled A-C in order of their elution; the major fraction, C, was rechromatographed on a second Sephadex LH20 column.

All three fractions remained at the origin on paper chromatograms developed with BAW. Cellulose TLC of a mixture of A, B and C in various solvents (MeOH-H₂O, 50%; Py-H₂O, 10, 25 and 50%; *n*-PrOH-H₂O, 50 and 70%) failed to separate the three fractions. The most successful was *n*-PrOH-H₂O (1:1) in which activity was found in a broad zone between R_f 0.1 and 0.5.

Hydrolysis of the tannin fractions with concentrated HCl in *n*-BuOH gave deep purple solutions which were analysed by PC in Forestal solvent. Each fraction was shown to produce delphinidin, identified by co-chromatography with authentic delphinidin and by a comparison of the visible spectrum. A very faint trace of a higher R_f product could be detected but there was insufficient for an identification to be made. Furthermore, it appeared to be unstable. Similar condensed tannins which produced delphinidin only on acid hydrolysis were found in the flowers of *T. repens* [4].

The MW distribution of the condensed tannins from the seeds is 6000–18 000 for B and C, and 6000–12 000 for A. This is in contrast to the results reported by Jones *et al.* [4] for the condensed tannins from leaves and flowers of various *Trifolium* species. For those tannins the MW ranged between 7000 and 9000. An attempt was made to clarify the wide MW range for the condensed tannins from the seeds by HPLC on a size exclusion column. Unfortunately there were large variations in peak shape and peak elution volume from run to run. Results appeared to be affected by temperature and, more importantly, by sample size. This suggests that the behaviour of condensed tannins in solution, at least in the solvents used (aqueous buffers and MeOH), is complex.

The toxicity of clover seed tannin towards *Rhizobium trifolii* is probably due, at least in part, to its high prodelphinidin content. Lucerne seeds extracted in a similar fashion produced a tannin which gave only cyanidin on acid hydrolysis. This condensed tannin was not toxic towards *R. trifolii* in the bioassay. This finding should assist in the selection of clover genotypes which do not cause problems with *Rhizobium* inoculation.

EXPERIMENTAL

Extraction and partial purification. Clover seed (120 g) was stirred in 1.2 l. of dist. H_2O containing ascorbic acid (1.2 g) for 3 hr at room temp. The seed was allowed to settle, the aq. layer filtered through gauze and centrifuged for 20 min at 10 000 rpm. The light yellow supernatant was evapd to 200 ml on the rotary evaporator at 35–40°, the concentrate washed with *n*-BuOH (100 ml \times 3) and the aq. layer evapd. to dryness at 35–40°. After drying at 20°/0.1 mm Hg the residue (3.91 g) was taken up in water (15 ml); MeOH (45 ml) followed by $CHCl_3$ (15 ml) were added with constant stirring. The ppt., designated MCW insolubles, was collected by centrifugation and dried at 20°/0.1 mm Hg. It weighed 0.43 g. After weighing it was immediately taken up in 0.01% ascorbic acid soln (30 ml).

Sephadex chromatography. The soln containing the MCW insolubles was chromatographed on a column (3.3 cm i.d. \times 37 cm) of Sephadex LH20 prepared in H_2O . The eluate was collected in flasks to which 100 μ l of 0.1% ascorbic acid soln was added. The LH20 column was eluted with H_2O (620 ml), 25% Me_2CO (320 ml), 30% Me_2CO (310 ml), 40% Me_2CO (100 ml), 50% Me_2CO (700 ml), 70% Me_2CO (440 ml). In a typical chromatogram of the MCW insolubles (430 mg), three fractions were shown to give a positive bioassay response. A (12.2 mg) started to elute off after 195 ml of H_2O and was contained in 169 ml. B (21.4 mg) started to elute off after 146 ml of 25% Me_2CO and was contained in 272 ml. C (43 mg) started to elute off after 102 ml of 40% Me_2CO and was contained in 948 ml. In several experiments the total mass recovered from the column ranged from 80 to 105%. This variation is probably due mainly to the retention of solvent in the residue which formed a hard film around the flask. Fraction C was further purified on a second LH20 column (0.9 cm i.d. \times 32 cm) prepared in 30% MeOH. Elution with 30% MeOH (76 ml) gave 6.7 mg of B. Further elution with 50% MeOH (20 ml) gave 2 mg of inactive material. Continued elution with 5% aq. Me_2CO (25 ml) and 50% aq. Me_2CO (50 ml) gave 32 mg of pure fraction C. Each fraction was freeze-dried for storage.

Chromatography. PC was carried out on Whatman 3 MM paper. Solvents used were *t*-BuOH-HOAc- H_2O (3:1:1)—BAW, and HOAc- H_2O -HCl (30:10:3)—Forestal. TLC was carried out on plates coated with Macherey, Nagel MN 300 cellulose powder. HPLC was carried out on Waters Associate equipment fitted with a 254 nm UV detector using Waters Associate E-linear μ Bondagel analytical columns (two

30 cm \times 4 mm). The solvents used were either MeOH or 0.1 M phosphate buffer (K salt) at pH 4.5.

Hydrolysis of purified tannin fractions. Tannin from each fraction (4 mg) was hydrolysed in *n*-BuOH containing 5% conc HCl (0.3 ml) at 90–95°. After 1.75 hr the mixture was applied to a paper chromatogram and developed in Forestal solvent. In each case an intense purple band was detected at R_f 0.30 and a very faint purple band at R_f 0.64. An authentic delphinidin sample co-chromatographed with the band at R_f 0.30. The coloured bands were eluted from the paper using 0.01% conc HCl in 90% aq. MeOH and the spectra measured in the same solvent. The R_f 0.3 band had λ_{max} 547 nm similar to that for authentic delphinidin. The R_f 0.64 band also had λ_{max} 547 nm. However, this absorption maximum rapidly disappeared on standing in the presence of air.

Molecular weight determination. The MWs were determined by the method of Jones *et al.* [4].

Bioassay. Aq. solns were made up to contain the test substances at concns of 5 μ g/ μ l. If there was less than 1 mg of material available, the soln was made up to 200 μ l regardless of concn. The solns under test were applied to sterile filter paper discs (1.3 cm dia) at the rate of 20 and 50 μ l per disc. Treated discs were placed on yeast mannitol agar plates [5] seeded with *Rhizobium trifolii* (PDDCC 2153) and incubated at 25° for 24–36 hr. In the presence of a toxic component an inhibition zone formed around the disc.

Acknowledgements—We are grateful to Dr. C. N. Hale, Plant Diseases Division, D. S. I. R., for many helpful suggestions and to Dr. W. T. Jones, Applied Biochemistry Division, D. S. I. R., for measuring the MWs.

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