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Assay of cis and trans-o-Hydroxycinnamic Acids in Sweetclover Extracts

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ASSAY OF cis- AND trans-o-HYDROXYCINNAMIC ACIDS IN SWEETCLOVER EXTRACTS[1]

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Various investigations related to the biosynthesis of o-hydroxycinnamic acid in sweetclover require that simple, rapid methods of assay be available for both the cis isomer (coumarinic acid) and the trans isomer (o-coumaric acid). This report deals with two such methods, both of which represent refinements of a technique for the fluorometric assay of coumarin described in an earlier publication from this laboratory (Haskins and Gorz, 1957). In one procedure, designated method I, o-coumaric acid and total o-hydroxycinnamic acid are determined, and coumarinic acid is estimated by difference; while in the other procedure, method II, coumarinic acid and total o-hydroxycinnamic acid are determined, and o-coumaric acid levels are calculated by difference. Both methods depend upon the ultraviolet-mediated interconversion of the non-fluorescent cis isomer and the fluorescent trans form illustrated in Fig. 1. In addition, method II depends upon the fact that o-hydroxycinnamic acid occurs in sweetclover almost exclusively in the form of glucosides (Rudorf and Schwarze, 1958; Kosuge, 1961[2]; Haskins and Gorz, 1961), and sweetclover β-glucosidase readily hydrolyzes coumarinyl glucoside but is virtually inert toward o-coumaryl glucoside (Kosuge and Conn, 1961).

The derivation of the "high-coumarin" lines (CuCuBB and CuCubb genotypes) of biennial white sweetclover (Melilotus alba Desr.) used in this work has been

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2 The authors are grateful to Dr. T. Kosuge for permitting examination of his manuscript before its publication.

described elsewhere, as has the procedure for obtaining aqueous extracts of leaf tissue (Haskins and Gorz, 1961). The extraction procedure provides for the rapid inactivation of β-glucosidase and thereby minimizes the hydrolysis of o-hydroxycinnamic acid glucosides during extraction. In many previous investigations such hydrolysis, followed by the spontaneous lactonization of liberated cis-o-hydroxycinnamic acid, has resulted in the presence of coumarin as an artifact in sweetclover extracts, and has led to the erroneous conclusion that this compound is a normal constituent of the intact plant (Haskins and Gorz, 1961).

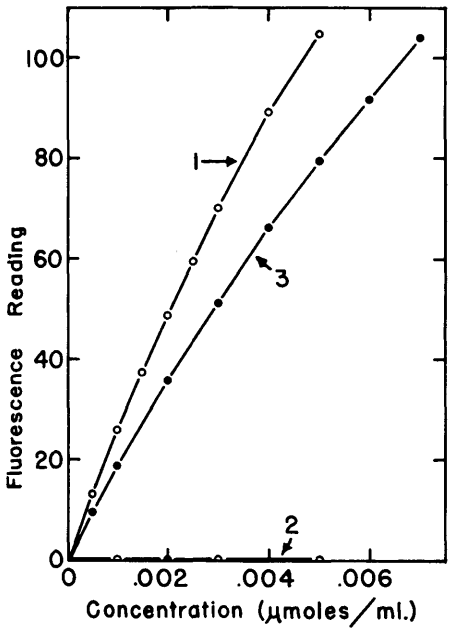

Figure 1. Relationship of fluorescence reading to concentration for non-irradiated solutions of o-coumaric acid (curve 1) and coumarinic acid (curve 2) in 0.2 N NaOH, and for solutions of either isomer irradiated with ultraviolet light (curve 3). The ultraviolet treatment consisted of a 15-min. exposure to the TF8 tube (energy peak at 360 mμ) of a Gates MR4 lamp. Quinine sulfate, 0.77γ/ml. in 0.1 N H_2SO_4, was used as the fluorescence standard (reading = 100).

Method I. In the darkroom (Kodak Safelight with series OA Wratten filter as a source of illumination) a 5-ml. portion of diluted leaf extract containing up to 0.7 micromoles of o-hydroxycinnamic acid was mixed with an equal volume of 5 N NaOH, and the resulting solution was autoclaved at 15 psi. for 45 min. to hydrolyze the glucosides. Two 1-ml. portions of the autoclaved solution were diluted to 10 ml. with 0.5 N NaOH. One of the diluted solutions was exposed to ultraviolet light (360 mμ) for 15 min., and the other was held in the dark. Fluorescence readings of both solutions were determined with the Beckman model DU spectrophotometer equipped with the fluorescence attachment. The fluorescence of the ultraviolet-treated solution was used as a measure of total o-hydroxycinnamic acid, and the fluorescence of the solution held in the dark was used as a measure of o-coumaric acid.

Method II. A 0.5-ml. portion of leaf extract containing up to 0.7 micromoles of o-hydroxycinnamic acid was mixed with 3.5 ml. of 0.07 M sodium acetate buffer, pH 4.9, and at zero time, 1.0 ml. of sweetclover glucosidase preparation was added. (This preparation consisted of a water suspension of the precipitate which formed between acetone concentrations of 25% and 40% during the fractional acetone precipitation of a homogenate of leaves representing the CuCuBB genotype. A concentration of 0.2 mg. of precipitate in 1 ml. of water was used.) Following an incubation period of 20 min. at 30° C., 5 ml. of 5 N NaOH was added to the reaction mixture, and the solution was carried immediately to the darkroom where a 1-ml. sample was withdrawn. The remaining solution was autoclaved for 45 min. at 15 psi. after which a second 1-ml. sample was taken. Both samples were diluted to 10 ml. with 0.5 N NaOH and exposed to ultraviolet light for 15 min. Fluorescence readings were then made. The sample taken after enzymatic hydrolysis provided a measure of coumarinic acid level, and that taken after alkaline hydrolysis of the glucosides served as the basis for estimating total o-hydroxycinnamic acid.

Results of applying the two assay methods to extracts of young leaves from first-year, field-grown plants representing two genotypes of sweetclover are shown in Table 1. Plants of the two genotypes were obviously similar with respect to contents of o-coumaric and coumarinic acids. In the leaves sampled, the cis isomer accounted for approximately 80% of the o-hydroxycinnamic acid present. Values for the ratio of the sum of the two isomers to the total o-hydroxycinnamic acid content were uniformly near 1.00, as shown by the mean ratios and standard errors in the table. Good agreement between the two methods is thus indicated.

Results of applying method I to leaves of various ages from greenhouse-grown plants of the CuCuBB genotype are shown in Table 2. The youngest leaf was removed from each of 10 stems, and the resulting 10 leaves were bulked to form sample 1. Samples 3, 5, and 7 were composed of successively older leaves from these same 10 stems. Similarly, sample 2 consisted of the bulked youngest leaves from a second set of 10 stems on which the leaves were slightly more ad-

Table 1.--Content of o-coumaric and coumarinic acids in young leaves from CuCuBB and CuCubb plants, as indicated by assay methods I and II. Ten plants of each genotype were used in these assays.

Genotype	Method	Acid measured	Content (% of dry wt.)	o-coumaric + coumarinic / o-hydroxycinnamic*
			mean ± SE	mean ± SE
CuCuBB	I	o-coumaric	0.94 ± 0.130	1.005 ± 0.011
		o-hydroxycinnamic	4.86 ± 0.285	
	II	coumarinic	3.92 ± 0.210	
		o-hydroxycinnamic	4.91 ± 0.310	
CuCubb	I	o-coumaric	0.80 ± 0.094	0.996 ± 0.007
		o-hydroxycinnamic	4.75 ± 0.146	
	II	coumarinic	3.94 ± 0.127	
		o-hydroxycinnamic	4.78 ± 0.140	

* Means of the o-hydroxycinnamic acid values obtained by the two methods were used in this computation.

Table 2.--Content of o-coumaric and coumarinic acids in leaves of various ages from CuCuBB plants.

Leaf sample	Dry matter per leaflet (mg.)	Content of o-coumaric acid: γ/leaflet	Content of o-coumaric acid: %	Content of coumarinic acid: γ/leaflet	Content of coumarinic acid: %	o-coumaric acid / coumarinic acid
1	0.19	7.4	3.89	4.3	2.26	1.72
2	0.62	20.6	3.32	16.4	2.65	1.26
3	1.35	24.2	1.79	39.8	2.95	0.61
4*	2.58	31.1	1.21	62.2	2.41	0.50
5	2.91	21.2	0.73	63.1	2.17	0.34
6	3.01	15.5	0.51	57.2	1.90	0.27
7	3.56	11.1	0.31	64.2	1.80	0.17
8	3.26	10.3	0.32	57.2	1.75	0.18

* Sample 4 consisted of the youngest leaves that were fully expanded. Leaves at this stage have been used in previous assays for coumarin (Haskins and Gorz, 1957).

vanced in age than those on the first set of stems. Samples 4, 6, and 8 were composed of successively older leaves from the second set of stems. The apparent association between gain in dry matter per leaflet and gain in quantity of o-hydroxycinnamic acid per leaflet suggests that o-hydroxycinnamic acid was synthesized most rapidly during the period of leaf expansion. The ratio of o-coumaric acid to coumarinic acid decreased with increasing age of the leaves,

lending support to the suggestion of Kosuge and Conn (1961) that o-coumaryl glucoside acts as a precursor to coumarinyl glucoside in the sweetclover plant. A similar relationship between these two glucosides may be inferred from studies of Brown, Towers, and Wright (1960) on sweet grass (Hierochloë odorata Beauv.)

A few of the wide variety of fluorescent compounds known to occur in plants (Goodwin, 1953) were examined for possible interference with the assays for o-coumaric and coumarinic acids. The compounds tested were umbelliferone, daphnetin, and aesculin, and it was found that none of these compounds contributed significantly to the fluorescence ascribed to o-hydroxycinnamic acid. More convincing evidence on the absence of interfering fluorescence comes from the work of Kosuge (1961) demonstrating that, following treatment with ultraviolet light, alkaline solutions of hydrolyzed sweetclover extract and o-coumaric acid have identical fluorescence spectra.

Extracts to be assayed for o-coumaric and coumarinic acids must be protected from bright light. Exposure of an aqueous extract of sweetclover leaves to standard cool white fluorescent light at an intensity of 650 foot-candles for a period of 4 hours resulted in the apparent conversion of approximately 50% of the o-coumaryl glucoside to coumarinyl glucoside. Ultraviolet effects this conversion more rapidly than visible light, and this fact has been applied in the laboratory synthesis of coumarinyl glucoside (Lutzmann, 1940; Kosuge and Conn, 1961). In preliminary work with excised but intact leaflets from plants of the CuCuBB genotype, it was found that exposure to sunlight or ultraviolet irradiation caused a material reduction in o-coumaryl glucoside while coumarinyl glucoside was correspondingly increased. This observation suggests that the trans to cis conversion postulated by Kosuge and Conn (1961) may be a non-enzymatic, photochemical reaction. Further studies of this effect of light are in progress.

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