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Genetic and Biochemical Aspects of o-Hydroxycinnamic Acid Synthesis in *Melilotus alba*

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SECTION 3

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GENETIC AND BIOCHEMICAL ASPECTS OF *o*-HYDROXYCINNAMIC ACID SYNTHESIS IN *MELILOTUS ALBA* [1]

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Summary

In sweetclover *(Melilotus alba* Desr.) the *cis* and *trans* isomers of *o*-hydroxycinnamic acid occur primarily as the respective β-D-glucosides. Available evidence indicates that these glucosides are formed via the following pathway: phenylalanine (formed from shikimic acid) → *trans*-cinnamic acid → *o*-coumaric acid *(trans-o*-hydroxycinnamic acid) → *o*-coumaryl glucoside *(trans*-β-D-glucosyl-*o*-hydroxycinnamic acid) → coumarinyl glucoside *(cis*-β-D-glucosyl-*o*-hydroxycinnamic acid). In tissues that are disrupted, coumarinyl glucoside is rapidly hydrolyzed by the action of endogenous β-glucosidase to yield coumarinic acid, which lactonizes spontaneously to form coumarin. The *cu* gene influences the content of the glucosides, apparently by controlling the *o*-hydroxylation of cinnamic acid, while the *b* gene is associated with loss of β-glucosidase activity.

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Introduction

The pioneering studies of Schofield (21) and Roderick and Schalk (18) established the fact that the mysterious bleeding disease of cattle was associated with ingestion of spoiled sweetclover hay. This observation provided the initial impetus for investigations that have subsequently embraced many scientific disciplines. It was soon discovered that the development of the toxic principle in poorly cured sweetclover hay depended upon the presence of coumarin (23). This was substantiated when the hemorrhagic agent (named Dicumarol) was isolated and identified as 3,3'-methylenebis-(4-hydroxycoumarin) (25).

The recent review of Smith and Gorz (24) summarizes work that has been done on various aspects of the problem of coumarin in sweetclover. Although coumarin was reported to be a constituent of sweetclover more than 100 years ago (28), only within the past 10 years has it been recognized that intact tissues of sweetclover contain little if any coumarin in the free, molecular form. Thus, the term "coumarin" is a misnomer when applied to the compounds present in normal plants of sweetclover, but it will be retained in some parts of this paper for convenience.

The development of a low-coumarin variety of sweetclover was the major objective in breeding programs initiated at several experiment stations approximately 30 years ago. Many methods of assaying for coumarin were developed in conjunction with the breeding programs but results were extremely variable. Thus, attempts were made to explain and reduce this variation by studying coumarin biosynthesis and the chemical reactions associated with the extraction and assay of coumarin. These studies have led to a much better understanding of the problems inherent in analyzing for coumarin and in breeding for sweetclover varieties that are low in coumarin content.

The work reported here is particularly concerned with associating the effects of the *Cu/cu* and *B/b* genes with specific steps in the biosynthetic pathway for coumarin in *Melilotus alba* Desr. The application of these studies to breeding for low-coumarin varieties of sweetclover will be discussed.

General considerations

Coumarin is the lactone form of coumarinic acid (*cis-o*-hydroxycinnamic acid). The lactone ring of coumarin remains intact in acidic solutions, but the ring is broken in basic solutions to yield coumarinic acid, or more correctly, the coumarinate ion. Irradiation of an alkaline solution of coumarinic acid with light of wavelengths less than 450 mμ results in the partial conversion of the nonfluorescent *cis-o*-hydroxycinnamic acid to the fluorescent *trans* isomer (*o*-coumaric acid). Various equilibria are attained between the *cis* and *trans* isomers, depending upon the wavelength of light used. Within the range of effective wavelengths, decreases in wavelength are associated with increases in percentage of *trans* isomer in the equilibrium mixture (7).

Sweetclover contains both *cis-* and *trans-o*-hydroxycinnamic acids, predominantly as β-D-glucosides (8, 13). The β-D-glucosides of coumarinic and *o*-coumaric acid are known as coumarinyl glucoside and *o*-coumaryl glucoside, respectively. Both isomers are readily extracted from plant tissue with hot water and are nonfluorescent in alkaline solution (9). Hydrolysis of both glucosides is achieved by heating in strong acid or base or by treatment with emulsin, but only coumarinyl glucoside is readily hydrolyzed by the β-glucosidase prepared from sweetclover (13).

The independent genes, *Cu/cu* and *B/b*, influence the amount of *o*-hydroxycinnamic acid glucosides and the activity of β-glucosidase, respectively. Plants of the *CuCu* genotype are high in content of *o*-hydroxycinnamic acid glucosides and preparations of the *BB* genotype possess β-glucosidase activity. The *Cu* gene displays partial dominance (3, 16), and the *B* gene exhibits no dominance (10).

Biosynthesis

Available evidence indicates that *o*-hydroxycinnamic acid in sweetclover is synthesized from phenylalanine which, in turn, is formed from carbohydrate precursors via the shikimic acid pathway for the biosynthesis of aromatic compounds. Phenylalanine is converted to *trans*-cinnamic acid by the action of the enzyme phenylalanine deaminase (15), as shown in Figure 1. Apparently, *trans*-cinnamic acid undergoes hydroxylation *ortho* to the side chain

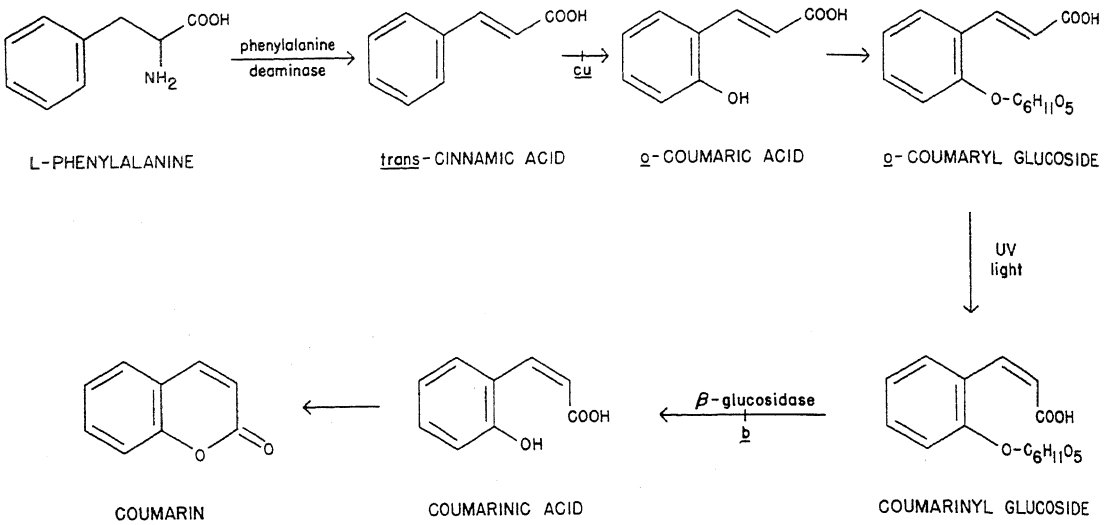

Figure 1. Probable biosynthetic pathway from L-phenylalanine to coumarin in sweetclover with the action of two enzymes and the *Cu/cu* and *B/b* genes indicated at specific steps in the pathway.

yielding *o*-coumaric acid, which is rapidly converted to *o*-coumaryl glucoside. Recent results by Haskins and Kosuge (11) support the hypothesis proposed by Brown (2) that the *o*-hydroxylation of *trans*-cinnamic acid is the step that is influenced by the *Cu/cu* alleles. Coumarinyl glucoside is formed from *o*-coumaryl glucoside by means of a nonenzymatic *trans* to *cis* isomerization, induced by exposure of leaves to light of wavelengths below 360 mμ (12). Stoker (26), on the other hand, has suggested that an isomerase is involved in this conversion. In tissues that are disrupted, coumarinyl glucoside is rapidly hydrolyzed by the action of endogenous β-glucosidase to yield coumarinic acid (8). This step is under the influence of the *B/b* alleles which control the activity of the β-glucosidase specific for the *cis*-glucoside (20), but most evidence indicates that this hydrolysis probably does not occur to any great extent in normally growing sweetclover plants. When coumarinic acid is formed, it lactonizes spontaneously to form coumarin.

Although studies of the site of synthesis of *o*-hydroxycinnamic acid within the sweetclover plant have not given completely uniform results, it is generally agreed that the primary site of synthesis is in the young, actively growing leaves. This conclusion is supported by evidence that young leaves are high, stems are intermediate, and roots are low in *o*-hydroxycinnamic acid glucosides (1), and that only limited translocation of the glucosides occurs in grafting experiments (5).

Discussion

The point of view concerning the form in which coumarin exists in normal, coumarin-containing sweetclover plants has changed. Thus, early workers thought that the free form predominated in the plant, while later studies demonstrated that both free and bound coumarin were present in substantial quantities. Subsequently, extracts virtually devoid of free coumarin were obtained by using procedures (e.g. submerging the plant tissue in boiling water) which prevented β-glucosidase activity during extraction (8, 19). Thus, normally growing sweetclover plants are now known to contain little if any free coumarin, although free coumarin is rapidly formed in plants containing β-glucosidase when the cells are disrupted by such treatments as maceration, freezing, or drying.

The presence of a bound form of coumarin in sweetclover was demonstrated as early as 1920 (27), and Roberts and Link (17), in describing their colorimetric method of analysis which measured only the content of free coumarin, provided for the enzymatic release of bound coumarin by including an incubation period. Later, it was demonstrated that the

compound known as "bound coumarin" was identical with coumarinyl glucoside (13). Since sweetclover β-glucosidase hydrolyzes coumarinyl glucoside but not o-coumaryl glucoside (14), any method of testing that is dependent upon endogenous enzymatic activity to release coumarin from its combined form (4, 17) measures only coumarinyl glucoside as bound coumarin. Also, such methods are influenced greatly by the quality and intensity of light received by the leaves. Actually, most fluormetric methods of analysis employ hydrolysis in strong acid or alkali, and therefore measure the sum of both isomeric aglycons as bound coumarin. The separate assay of the 2 isomers is readily accomplished by 2 recently described fluorometric procedures (9).

Several qualitative methods of analyzing for *o*-hydroxycinnamic acid have been developed as an aid in breeding for low-coumarin varieties of sweetclover. A rapid fluorometric method (4), suitable for screening large populations, involves maceration of a small piece of leaf tissue on white filter paper, followed by treatment with base and examination under an ultraviolet light. This method, as well as other modifications of the paper test (Gorz and Haskins, unpublished data), combine unusual simplicity with acceptable reliability. Since both *o*-hydroxycinnamic acid and β-glucosidase activity can be detected simultaneously, the paper test is of value in genetic studies and could serve as an efficient tool for inspectors checking for contamination in foundation and certified seed fields of low-coumarin sweetclover varieties.

Conclusions

Studies of *o*-hydroxycinnamic acid biosynthesis in sweetclover have provided a much better understanding of the problems associated with the breeding of a low-coumarin variety of sweetclover. This knowledge has permitted the development of improved techniques for *o*-hydroxycinnamic acid determination in plant tissues. These include new methods of sampling and extraction, as well as assay procedures that permit the separate detection of coumarinic acid and *o*-coumaric acid. Evidence has been obtained which indicates that the *cu* gene prevents efficient *ortho* hydroxylation of *trans*-cinnamic acid, and that the *b* gene is associated with loss of β-glucosidase activity. Additional studies are needed to clarify the enzymology of steps concerned with *o*-hydroxylation, glucosylation, and isomerization.

Practical benefits from coumarin research have been realized in the form of the anticoagulants (such as Dicumarol), the rodenticides (such as Warfarin), and 3 low-coumarin varieties of *M. alba* that have recently been released for use by farmers. These are the Canadian variety Cumino (6), the German variety Acumar (19), and the U.S.A. variety Denta (22). Low-coumarin strains of *M. officinalis* are under development at the Nebraska Agricultural Experiment Station.

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