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GENETIC CONTROL OF COUMARIN BIOSYNTHESIS AND β-GLUCOSIDASE ACTIVITY IN *MELILOTUS ALBA*[1]

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Two pairs of alleles, *Cu/cu* and *B/b*, are known to influence coumarin biosynthesis in *Melilotus alba* Desr. (Goplen *et al.*, 1957; Haskins and Gorz, 1957). Young leaves from *CuCuBB* plants characteristically contain substantial amounts of both free and bound coumarin, *CuCubb* leaves are high in "coumarin" but virtually all the substance is in the bound form, and *cucuBB* and *cucubb* leaves are low in both forms. It has been suggested that the *cu* gene is involved in the formation of bound coumarin from coumarin precursors and that the *b* gene is concerned with the conversion of the bound form to the free form (Gorz and Haskins, 1960). Evidence indicates that bound coumarin, so called because it responds in the usual coumarin assays only after a hydrolytic treatment, is the glucoside of *cis*-o-hydroxycinnamic acid (Rudorf and Schwarze, 1958), the lactone form of which is coumarin. It seemed reasonable, therefore, to investigate the possibility that tissues of the *BB* genotype might differ from *bb* tissues with respect to β-glucosidase activity.

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Materials and Methods

Plant material: The *M. alba* plants used were homozygous with respect to *Cu/cu* and *B/b*. All plants were derived from a cross of an individual of the *cucuBB* genotype and a *CuCubb* plant. The original cross was followed by five generations of self-pollination of doubly heterozygous individuals to produce a high degree of isogenicity in the resulting lines. Plants were grown in the greenhouse.

Enzyme preparations: Three types of enzyme preparation were used--(a) crude preparations, made by grinding the tissue in sand and cold water in a mortar, diluting to 10 ml. per gm. of tissue with cold water, and centrifuging briefly in a clinical centrifuge to remove sand and debris; (b) supernatants obtained by centrifuging crude preparations at 25,000 x *g* for 25 min. at room temperature; and (c) acetone precipitates, made by fractional precipitation of the foregoing supernatants with cold acetone. The fraction used in this work was that which precipitated over the range of acetone concentration from 40 to 57% of the total volume. Nitrogen contents of the enzyme preparations were determined by a modification of the method of Koch and McMeekin (1924) applied to trichloroacetic acid precipitates of the preparations.

Substrates: Two substrates were used--p-nitrophenyl-β-D-glucoside (California Corporation for Biochemical Research) and bound coumarin. The bound coumarin was partially purified by ascending paper chromatography of hot-water extracts of leaves from plants of the *Cub* phenotype. The solvent was a mixture of 3 volumes of n-propyl alcohol and 1 volume of 1% aqueous ammonia. Bound coumarin was detected as an ultraviolet-absorbing spot at Rf 0.36 and was eluted from the paper with water.

Reaction conditions: After considerable preliminary work (Schaeffer, 1960), the following conditions were selected for assaying β-glucosidase activity in preparations representing various genotypes and various portions of the *M. alba* plant: total volume of reaction mixture--6 ml.; buffer--0.025 *M* sodium acetate, pH 4.8; p-nitrophenyl-β-D-glucoside concentration--7.0 x 10^{-3} *M*; enzyme preparation--0.10 ml. of crude homogenate or supernatant from

high-speed centrifugation. For estimation of Km values for both substrates, acetone-precipitated material from CuCuBB plants was used as the enzyme preparation. At specified times after addition of enzyme preparation to the reaction mixture, samples of the mixture were transferred to measured portions of 0.2 N NaOH. Aglycon concentrations were then determined colorimetrically at 400 mμ in those instances where p-nitrophenyl-β-D-glucoside was the substrate and fluorometrically (Haskins and Gorz, 1957) where bound coumarin was used. A reaction temperature of 30° C. was used throughout the determinations.

Results and Discussion

Supernatants from high-speed centrifugation of leaf homogenates representing various genotypes displayed the following specific activities (micromoles p-nitrophenol liberated/min./mg. protein N): CuCuBB--2.20; CuCubb--no activity detected; cucuBB--1.53; cucubb--no activity detected. With the methods used in these determinations, specific activities as high as 0.08 might have escaped detection. Despite this fact, the bb genotype was clearly associated with great reduction in β-glucosidase activity. This decrease was particularly apparent in other experiments in which, with bound coumarin as the substrate, the activity of a BB leaf preparation was at least 500 times as great as that of a preparation of bb leaves. The reduction in activity was probably not due to soluble glucosidase inhibitors in the bb material, since no inhibition was shown when CuCuBB and CuCubb preparations were mixed.

Assay of crude preparations from various portions of CuCuBB plants indicated the following specific activities: young leaves--1.09; old leaves--0.30; stems--0.23; roots--0.07. This pattern of distribution is similar to that observed for free coumarin (Schaeffer, 1960).

Km values (Lineweaver and Burk, 1934) were 5.9×10^{-3} M for p-nitrophenyl-β-D-glucoside and 4.0×10^{-4} M for bound coumarin. It is apparent that the enzyme had a lower affinity for the synthetic substrate than for bound coumarin.

On the basis of the foregoing evidence, the hypothesis is proposed that in the M. alba plant free coumarin is formed through the action of β-glucosidase on bound coumarin and that the b gene influences coumarin metabolism

through its influence on the enzyme, β-glucosidase.

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