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ABSENCE OF DOMINANCE OF THE *B* GENE IN INFLUENCING β-GLUCOSIDASE ACTIVITY IN *MELILOTUS ALBA*[1]

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IN sweetclover (*Melilotus alba* Desr.) the allelic pair designated *B/b* was originally thought to be concerned with coumarin biosynthesis. Extracts of *BB* plants were reported to contain substantial amounts of both free and bound coumarin, while extracts of *bb* plants contained only bound coumarin (GOPLEN, GREENSHIELDS and BAENZIGER 1957; HASKINS and GORZ 1957), more recently identified as coumarinic acid glucoside (β-D-glucosyl-*cis*-*o*-hydroxycinnamic acid) (KOSUGE 1961; STOKER and BELLIS 1962). The bound-coumarin character behaved as a simple recessive in crosses between *bb* and *BB* plants (GOPLEN, GREENSHIELDS and BAENZIGER 1957).

SCHAEFFER, HASKINS and GORZ (1960) reported enzyme preparations of leaves of the *BB* genotype to be high in β-glucosidase activity but detected no activity in preparations of *bb* leaves. Inasmuch as the activity of *BB* leaf extracts was not reduced by the addition of *bb* preparations, the absence of activity in the latter preparations probably was not caused by soluble β-glucosidase inhibitors. Plants of the *Bb* genotype were not included in this comparison. These authors suggested that the effect of the *B* gene on β-glucosidase activity was responsible for the influence of this gene on coumarin formation. Thus, in *BB* plants a portion of the coumarinic acid glucoside was presumed to be hydrolyzed by β-glucosidase, yielding coumarinic acid (*cis*-*o*-hydroxycinnamic acid) which lactonized spontaneously, producing coumarin.

Other studies (RUDORF and SCHWARZE 1958; HASKINS and GORZ 1961a) have shown that the existence of free coumarin in sweetclover extracts is largely an artifact of the extraction procedure, and have therefore raised doubt as to whether β-glucosidase actually has a role in coumarin formation *in vivo*. Regardless of whether β-glucosidase has such a role, there is no apparent reason to doubt the influence of the *B/b* alleles on the activity of this enzyme in sweetclover leaf preparations. Accordingly, the present experiments were designed to furnish information on the degree of dominance of the *B* gene with respect to β-glucosidase activity.

MATERIALS AND METHODS

Plant material: Plants of biennial white-blossomed sweetclover (*Melilotus alba*) of the

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following genotypes were used in this study: *cu cu b b*, *cu cu B b*, and *cu cu B B*. The *Cu/cu* alleles influence level of glucosidically bound *o*-hydroxycinnamic acid. In comparison with *CuCu* plants, *cu cu* individuals are low in content of this constituent (HASKINS and GORZ 1961a). The *cu cu b b* and *cu cu B B* plants were grown from F_{12} seed obtained by self-pollination of two homozygous F_{11} plants that were derived from a single, doubly heterozygous F_9 plant. The original cross from which this F_9 plant was derived (by selfing a single doubly heterozygous plant in each generation) involved a *cu cu B B* plant as the female parent and a *Cu Cu b b* plant as the male parent. Seed for these parent plants was obtained from DR. W. K. SMITH, Crops Research Division Agricultural Research Service, U. S. Department of Agriculture and the University of Wisconsin.

Seed of the *cu cu B b* genotype was obtained by crossing the two homozygous F_{11} plants described in the preceding paragraph, with the *cu cu b b* plant serving as the female parent. Plants resulting from this cross were tested qualitatively for β-glucosidase activity. Any plant lacking such activity was assumed to have resulted from selfing of the *cu cu b b* parent and was therefore discarded.

Ten plants of each of the three genotypes, *cu cu b b*, *cu cu B b*, and *cu cu B B*, were established from seed in the greenhouse in the fall of 1963. During the subsequent winter and early spring months, cuttings were made from each of these 30 plants until a ten-plant clone was available from each. All plants were grown in soil in greenhouse pots.

Three weeks before sampling, all plants were cut to a uniform height of approximately 8 cm so that plants would be as uniform as possible when sampled. At the time the plants were pruned they were also arranged in a randomized complete block design, with each of ten blocks containing one plant from each of the 30 clones.

Plants were sampled in five groups of 60 plants each over a period of 3 days. Each group included two clones of each of the three genotypes. After plant heights were determined, the terminal portion of the main branch was cut from each plant. These branch portions, pooled within clones, were placed in water and carried to the laboratory for immediate processing.

Enzyme preparations: The youngest fully expanded leaf was removed from each of the ten sampled branches representing a clone. These ten leaves were weighed and then ground in sand and a small volume of cold water in a cold mortar. After dilution with cold water (total water used—10 ml/g of tissue) the ground material was centrifuged briefly in a clinical centrifuge to remove sand and debris. The supernatant was then centrifuged at $19{,}000 \times g$ for 20 min at approximately 0°C. For nitrogen determination duplicate aliquots of the supernatant from this centrifugation were mixed with trichloroacetic acid; the resulting precipitates were digested with H_2SO_4 and H_2O_2 and assayed colorimetrically by a modification of the method of KOCH and and MCMEEKIN (1924). The remaining supernatant was frozen immediately for later enzyme assay. Tests indicated that such frozen supernatants could be stored for several months without appreciable loss of β-glucosidase activity.

Substrate: Young leaves of the sweetclover varieties Spanish and Evergreen (both varieties are of the *CuB* phenotype) were extracted by autoclaving in water. The resulting extract was lyophilized to yield a powder containing 0.06 μmoles of *o*-coumaric acid glucoside (β-D-glucosyl-*trans*-*o*-hydroxycinnamic acid) and 0.52 μmoles of coumarinic acid glucoside per mg, and no more than trace amounts of free *o*-coumaric acid and coumarin. An aqueous solution containing 1 mg of this powder per ml was used as the substrate. As shown by KOSUGE and CONN (1961) sweetclover β-glucosidase is highly active in hydrolyzing coumarinic acid glucoside but only slightly active in hydrolyzing *o*-coumaric acid glucoside.

β-Glucosidase assays: Reaction mixtures consisted of 3.0 ml of 0.1M acetate buffer, pH 5.0; 0.8 ml of substrate; 2.1 ml of distilled water; and 0.1 ml of enzyme preparation. A water bath was used to maintain the reaction temperature at 30°C. Supernatants representing the *bb* clones were used as enzyme preparations without further dilution, and those from *Bb* and *BB* clones were diluted tenfold with cold water immediately before use. One-ml aliquots of the reaction mixture were mixed with 9 ml portions of 2.5 N NaOH at 0.5 and 5.5 min after addition of the enzyme preparation. In preliminary tests, extending the reaction time to 10.5 min resulted in only a slight departure from linearity in the plot of extent of hydrolysis against time. Assays

for free coumarin and *o*-coumaric acid in reaction mixture samples were made by the fluorometric procedure of HASKINS and GORZ (1961b), using a Turner model 110 fluorometer. In no case was appreciable free *o*-coumaric acid detected.

Each enzyme preparation was assayed in duplicate. Each preparation was also subjected to a control (no added substrate) assay. No activity was observed in these control determinations; thus, no corrections for endogenous substrate were required. The essential absence of endogenous substrate resulted from the exclusive use of plants of the *cu cu* genotype. As previously indicated, such plants contain only small amounts of coumarinic acid glucoside.

The substrate concentration used in the routine assays was too low to permit maximum activity of enzyme preparations from *Bb* and *BB* clones. However, as shown in Figure 1, sufficient substrate was present to afford linearity between extent of hydrolysis and quantity of enzyme preparation over a considerable range. The linear portion of this curve includes enzyme concentrations at least twice as high as those encountered in the routine assay of the most active *BB* preparations. Therefore, the assay conditions were satisfactory for comparative purposes.

RESULTS AND DISCUSSION

The average β-glucosidase activity of preparations representing the *BB* genotype was approximately 2.5 times as great as that of *Bb* preparations, and no activity was detected in *bb* preparations (Table 1). Thus, despite the dominant nature of the *B* allele indicated by measurements of free and bound coumarin in certain sweetclover extracts (GOPLEN, GREENSHIELDS and BAENZIGER 1957), this allele appears to be without dominance in its influence on β-glucosidase activity. Absence of dominance of certain alleles governing β-glucosidase activity in yeast also has been reported (HERMAN and HALVORSON 1963). An apparently similar case was observed in the effect of the ry^+ ($rosy^+$) allele on xanthine dehydrogenase activity in Drosophila (GLASSMAN and MITCHELL 1959; GRELL 1962; GLASSMAN, KARAM and KELLER 1962). Other examples in which heterozygous individuals display intermediate levels of enzyme activity have been found in the tryptophan oxidase system of *Ephestia kühniella* (EGELHAAF and CASPARI

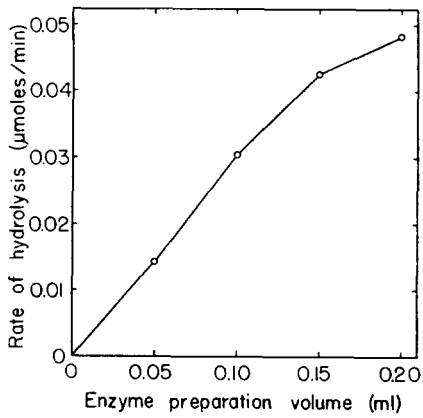

FIGURE 1.—Relationship between rate of hydrolysis and quantity of enzyme preparation. See text for reaction conditions. The enzyme preparation consisted of a five-fold dilution of supernatant from clone D-10. Water volume was varied to keep the total volume of the reaction mixture at 6.0 ml.

TABLE 1

β-Glucosidase activity of sweetclover leaf preparations from plants of the cu cu b b, cu cu B b, *and* cu cu B B, *genotypes*

Genotype	Clone	Average plant height (mm)	Average fresh weight (mg/leaf)	Protein N (mg/ml of supernatant)	Specific activity (μmoles hydrolyzed/min/mg N)
cu cu bb	R-1	266	49.7	2.56	No activity detected
	R-2	290	58.3	2.06	No activity detected
	R-3	249	51.5	2.44	No activity detected
	R-4	286	53.7	2.15	No activity detected
	R-5	305	62.1	1.83	No activity detected
	R-6	338	50.2	2.00	No activity detected
	R-7	284	57.2	2.02	No activity detected
	R-8	329	51.6	1.90	No activity detected
	R-9	344	53.7	1.92	No activity detected
	R-10	344	49.2	1.77	No activity detected
	Mean ± SE	304 ± 11	53.7 ± 1.3	2.07 ± 0.08	No activity detected
cu cu Bb	H-1	367	54.1	2.41	1.05
	H-2	350	49.9	2.06	0.97
	H-3	355	56.5	1.87	1.17
	H-4	296	54.5	1.96	1.35
	H-5	340	57.6	1.82	0.87
	H-6	359	56.0	1.96	0.86
	H-7	357	50.6	1.82	0.75
	H-8	381	50.8	1.89	1.24
	H-9	306	48.9	1.80	1.16
	H-10	359	47.5	1.69	0.98
	Mean ± SE	347 ± 8	52.6 ± 1.1	1.93 ± 0.06	1.04 ± 0.05
cu cu BB	D-1	245	58.8	2.33	1.45
	D-2	273	47.3	2.25	3.13
	D-3	198	50.5	2.33	2.40
	D-4	315	49.3	1.94	2.78
	D-5	316	58.1	1.83	2.24
	D-6	278	52.0	1.87	2.97
	D-7	326	56.8	1.80	2.31
	D-8	353	62.7	1.96	3.19
	D-9	364	51.6	1.89	2.18
	D-10	312	53.9	1.80	3.06
	Mean ± SE	298 ± 16	54.1 ± 1.5	2.00 ± 0.07	2.57 ± 0.18

1960) and the phenylalanine oxidizing (HSIA, DRISCOLL, TROLL and KNOX 1956) and galactose-1-phosphate uridyl transferase (BRETTHAUER, HANSEN, DONNELL and BERGEN 1959) systems in man.

The heterozygote is not always intermediate in activity as is clearly shown in Drosophila by the work of GRELL (1962) and GLASSMAN, KARAM and KELLER (1962) on *ma-l* (maroon-like) strains and of KELLER and GLASSMAN (1964) on *lxd* (low xanthine dehydrogenase) strains. Like *ry*, *ma-l* and *lxd* affect xanthine dehydrogenase activity. However, xanthine dehydrogenase preparations from

individuals heterozygous with respect to *ma-l* or *lxd* were as active as preparations from homozygous normal flies.

In the present study, sensitivity of the assay method was such that specific activities as low as 0.003 could have been detected in the preparations from *bb* plants with reasonable confidence. Thus, on the average, *Bb* and *BB* preparations were at least 300 and 800 times as active, respectively, as *bb* preparations. On this basis it is reasonable to classify the *b* allele as essentially amorphic with respect to β-glucosidase activity. Similarly, the experiments of CORKILL (1942) and ATWOOD and SULLIVAN (1943) indicate that white clover strains homozygous for the *li* allele display no activity of the glucosidase, linamarase. This amorphic characteristic is not shared by mutations influencing β-glucosidase in all organisms. For example, EBERHART, CROSS and CHASE (1964) found that β-glucosidase preparations from conidia of the *gluc-1* mutant of Neurospora ranged from about 1/10 to 1/3 as active as preparations from *gluc-1*$^+$ conidia. The β-glucosidase activity observed by HERMAN and HALVORSON (1963) in preparations of B^l yeast cells was reduced to a similar extent in comparison with B^h cell preparations. EBERHART, CROSS and CHASE (1964) suggested that the *gluc-1* gene in Neurospora has a regulatory function, whereas HERMAN and HALVORSON (1963) concluded that the *B* alleles in yeast are structural alleles. Unfortunately, available information does not permit any such conclusions relative to the *B/b* locus in sweetclover.

The specific activities listed in Table 1 indicate considerable variation among very closely related clones. This variation does not seem to be strongly associated with variation in the average values for plant height or leaf weight shown in the table. As indicated by the data in Table 2, however, β-glucosidase activity is highly dependent upon stage of leaf development. A considerable part of the apparent interclonal variation within a genotype is probably due to small differences in the developmental stage of the leaves sampled.

SUMMARY

Preparations of young leaves from *Melilotus alba* plants representing the *BB*, *Bb*, and *bb* genotypes were assayed for β-glucosidase activity. On the average,

TABLE 2

β-Glucosidase activity of preparations of sweetclover leaves representing various stages of development. The youngest fully expanded leaves are classified as stage 2, and stages 1 and 3 represent the next younger and next older leaves, respectively, on the same plants. Seven plants of clone D-1 were used in this experiment

Stage of development	Average fresh weight (mg/leaf)	Protein N (mg/ml of supernatant)	Specific activity (μmoles hydrolyzed/min/mg N)
1	18.0	4.08	3.52
2	46.6	3.92	1.49
3	70.9	2.22	0.53

preparations of *BB* leaves were about 2.5 times as active as those of *Bb* leaves. No activity was detected in *bb* leaves. Some of the variation encountered between closely related clones was ascribed to small differences in stage of development of the leaves sampled.

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