COMPARISON OF PROTEIN CONSTITUENTS RELATING TO β-GLUCOSIDASE ACTIVITY IN BB AND bb INBRED LINES OF MELILOTUS ALBA

by

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INTRODUCTION AND REVIEW OF LITERATURE

The variation in phenotypic behavior attributable to mutant genes has provided geneticists with both research material and challenge since the turn of the century. The efforts of Beadle and Tatum (2), demonstrating that the action of a specific gene is related directly to a particular chemical reaction in the living cell, have stimulated dramatically the combined research efforts of geneticists and biochemists in the past two decades. This fusion of interests has enabled a molecular analysis of genetic lesions, leading to an understanding of the chemical basis of genetic control as well as providing extensive insight into the sequence of biosynthetic pathways.

On the basis of extensive evidence, primarily from microorganisms, gene mutations appear, in general, to result from small changes in the DNA code carried at the mutated locus (63). Such changes in the DNA sequence are transcribed subsequently as complementary alterations in the messenger-RNA sequence and ultimately are translated as alterations in the amino acid sequence of the polypeptide specified by the locus in question (66). The amino acid sequence (primary structure) of a given polypeptide is involved directly in determining the unique configuration of the higher order structures (secondary, tertiary, and quaternary) of proteins required for the biological specificity characteristic of enzymes (42). Single amino acid alterations in either the α-or β-chains of hemo-

globin or in the A-protein of tryptophan synthetase result in molecules which are both structurally and functionally distinct from the normal (31,6). Thus, though a given gene may be the ultimate agent of control of a particular biochemical reaction, immediate control is exerted at the phenotypic level usually, if not always, by an appropriate enzyme.

With respect to microbial systems, gene-protein relationships are well documented (63). Phage T_4 lysozyme, alkaline phosphatase and β-galactosidase from Escherichia coli, and tryptophan synthetase from E. coli and Neurospora have all been studied in great depth, both from genetic and biochemical approaches. The tryptophan synthetase A-protein mutants of E. coli provide illustrations of effects that a mutational event may have upon the protein for which the mutated gene codes.

Various mutant strains of E. coli, all having in common a specific requirement for tryptophan, have been reported which show a deficiency in the enzyme tryptophan synthetase (28). Nevertheless, many of the mutants produce a protein which, though catalytically different, is similar enough to the normal tryptophan synthetase to be recognized by antibodies which have been produced in experimental animals in response to injection of the wild type enzyme (antigen) (66). Mutant proteins of this kind are termed CRM (cross reacting material) due to their serological relationship to the protein used as the antigen. Certain serological techniques are sufficiently sensitive to distinguish mutant proteins whose differences are no greater than single amino acid substitutions (10). The wild type enzyme can

catalyze three different but related reactions, only one of which appears to be physiologically essential. The CRMs may be able to effect one or two of the reactions but never all three and, most importantly for screening purposes, only the wild type tryptophan synthetase can catalyze the physiologically important reaction (65). At the molecular level, the CRM proteins can be distinguished from each other by amino acid sequence analysis of their altered polypeptide (9).

Yanofsky and co-workers, using genetic, serological, and biochemical data, were able to deduce what the nucleotide changes must have been in the DNA code to give particular CRM proteins (22). The tryptophan synthetase system provides convincing evidence that the genetic map is colinear with the amino acid sequence of the corresponding polypeptide (64). For microorganisms, it now appears that the gene structure-protein structure picture is quite clear, that the nature of mutations and their varied effects on protein structure is related directly to the phenotypic effect through a modification in the catalytic capability of the enzyme involved (63). For those research workers interested in genetic control and improvement of higher organisms, ". . .the definitive information derived from studies of lower organisms should indicate new approaches to and intensify investigations of the fundamental bases of phenotype in higher organisms" (59).

Biochemical genetic investigations with higher plants have been pursued much less eagerly than such studies in fungi and bacteria due to several attendant difficulties as discussed by Nelson (46).

First, in relatively few cases is there a clue in the phenotype as to the metabolic lesion that may conceivably be produced by the mutation. Secondly, many mutants are lethals and in only limited cases are techniques available for their culture through supplementation of media as is common with fungi and bacteria. Thirdly, isolation of multiple mutants within a cistron or biosynthetic pathway appears to be formidable as is true for any diploid organism. In spite of these difficulties, significant contributions have been made to the understanding of control of organelle development and biosynthetic pathways in plants, and interesting indications have been provided as to the nature of heritable charge differences between isozymes (46).

Organelle development in higher plants has been investigated by studying chloroplast development in normal plants. These studies have shown a strong interdependence between structural elements of the chloroplasts and the photosynthetic pigments in leading to normal organelle development (62). In addition, a number of reports of unique species of DNA in chloroplasts indicate still other interesting possibilities for biochemical genetic investigations of these structures (35).

Multiple molecular forms of an enzyme found within the same organism (isozymes) have been recognized since 1952 (47) and several reviews of recent work involving isozymes are available (56,61).

Isozymes have been detected in several higher plants and their presence has been shown to be under genetic control. Schwartz and co-workers have studied several of these isozyme systems (51).

The most intensively studied is the E_1 gene in maize which controls the production of pH 7.5 esterases (52,53,54). At least seven alleles of the E_1 gene are known and individual allelic products appear to interact in a predictable fashion to form the electrophoretically distinct active enzymes. Plants heterozygous for any two alleles produce the esterase characteristic of each allele and another esterase with a mobility intermediate between the two parental forms. Information concerning the physiological function and evolutionary preservation of multiple alleles on such a wide scale is lacking, however (56).

As examples of the limited number of instances in higher plants where genetic systems have provided a tool for biochemical study of the genetic block and for examination of the biosynthetic pathway involved, cyanogenesis and coumarin synthesis in Trifolium repens L. and Melilotus alba Desr., respectively, will be discussed.

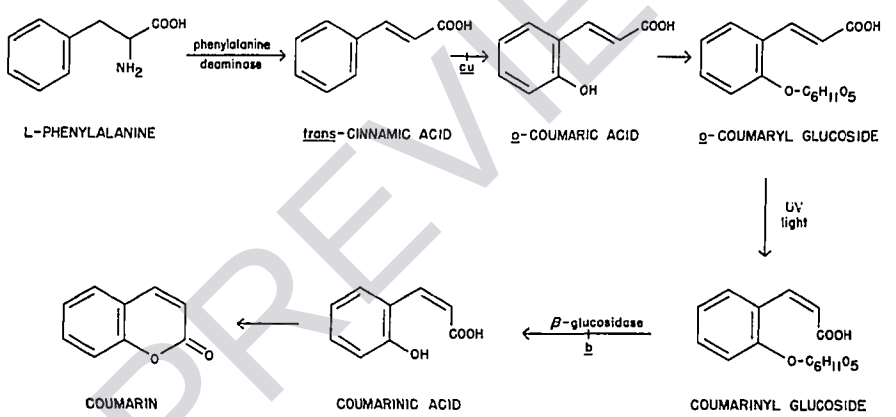
The cyanogenetic character in Trifolium repens (white clover) is dependent on the presence in a single plant of a functional allele at each of two loci. Alleles at the Ac locus control the production of two related glucosides and alleles at the Li locus control the production of β-glucosidase (29). Hydrolysis of the glucoside by β-glucosidase in damaged leaves and the subsequent breakdown of the resulting unstable aglycones give rise to hydrocyanic acid (HCN). The function of the Li locus in controlling the presence or absence of β-glucosidase was the first demonstrated effect of genetic control of enzyme function in higher plants (13). Using extracts from commercial varieties of white clover, Hughes (29)

was able to identify two distinct β-glucosidase enzymes following DEAE-cellulose chromatography. One of the enzyme forms was found to be associated primarily with seed extracts, the other with young leaves. Two isozymes of β-glucosidase also have been reported in sorghum (43), flax (8), and yeast (16). No specific physiological function for the alternate forms has been described, nor has any definitive study of the mode of action of the li allele been reported.

Coumarin metabolism in white-flowered sweetclover (Melilotus alba Desr.) is in many respects analagous from a genetic standpoint to the cyanogenesis situation in white clover. Several recent reviews on the subject of coumarin metabolism are available and should be consulted for a more comprehensive insight (Brown (7), Smith and Gorz (58), Gorz, et. al. (20)). Two gene pairs designated as Cu/cu and B/b are known to influence the amount of free coumarin released when the sweetclover plant is macerated (48). The biosynthetic pathway from phenylalanine to coumarin in sweetclover, with the sites of action of the two loci designated, is outlined in Figure 1 (20). The sequence as outlined terminates with coumarin, but points of clarification should be made. Most evidence now indicates that the hydrolysis of cis-o-hydroxycinnamic acid (cis-o-HCA) glucoside (coumarinyl glucoside) does not occur to any great extent in normally growing, intact sweetclover plants (24). Only when the tissues are disrupted is appreciable free coumarin detected, so that, in vivo, the term "coumarin metabolism" is a misnomer (20). However, intact sweetclover does contain both cis

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.



and trans isomers of o-hydroxycinnamic acid, predominately as their respective β-D-glucosides (36).

As shown in Figure 1, phenylalanine is converted to trans-cinnamic acid by the action of the enzyme phenylalanine deaminase (phenylalanine ammonia-lyase) (36). Although in vitro enzymatic o-hydroxylation of trans-cinnamic acid has not been demonstrated, the reaction apparently does take place in vivo yielding trans-o-HCA which is rapidly converted enzymatically to trans-o-HCA glucoside (12). Haskins and Kosuge (26) presented evidence supporting Brown's (7) earlier proposal that o-hydroxylation of trans-cinnamic acid is the step most directly influenced by the Cu/cu alleles. However, since the enzymatic o-hydroxylation of trans-cinnamic acid has not been demonstrated, assignment of the primary effect of the Cu locus to a specific enzyme is not possible. Kleinhofs, et. al. (36) have shown that the Cu/cu alleles appear to have no direct effect on either the phenylalanine ammonia-lyase step or the glucosylation step. The glucosylation of o-coumaric acid proceeds by a uridine disphosphate glucose (UDPG)-requiring glucosylating enzyme reported and partially characterized by Kleinhofs, et. al. (37). In leaves, cis-o-HCA glucoside is formed from trans-o-HCA glucoside by means of a non-enzymatic trans to cis isomerization, induced by exposure to light of wavelengths below 360 nm (27). In tissues that are disrupted, cis-o-HCA glucoside is rapidly hydrolyzed by the stereospecific endogenous β-glucosidase to yield cis-o-HCA, which spontaneously lactonizes to form coumarin (24). The hydrolytic step, which is directly influenced by

the B/b allelic pair (48), is the subject of this thesis.

Schaeffer, Haskins, and Gorz (48) reported enzyme preparations of leaves of the BB genotype to be high in β-glucosidase activity but detected no activity in preparations from bb leaves. Furthermore, they concluded that since no influence on β-glucosidase activity was observed when BB and bb preparations were mixed, the action of the b allele did not involve soluble β-glucosidase inhibitors. Subsequently, Haskins and Gorz (25) analyzed cucubb, cucuBb, and cucuBB genotypes for β-glucosidase activity and reported that preparations of BB leaves were about 2.5 times as active as those of Bb leaves. No activity was detected in the bb preparations with the sensitivity limit of the assay restricting this statement to indicate that the BB preparations were at least 800 times as active as the bb preparations.

Sweetclover β-glucosidase was first purified and partially characterized by Kosuge and Conn (40). A procedure was established which gave a 40-fold purification of β-glucosidase with an overall yield of 64%. In tests of substrate specificity utilizing a number of phenolic glucosides, activity was exhibited toward cis-o-HCA glucoside and the β-glucosides of salicylic acid, melilotic acid, and salicylaldehyde, but essentially no activity toward trans-o-HCA glucoside was observed. The enzyme exhibited maximal activity toward melilotyl glucoside at pH 5.25 and appeared to be most stable at pH 7.0. The apparent Michaelis constant (Km) for the β-glucoside of cis-o-HCA was reported to be 2.2×10^{-3} M. Schaeffer, et. al.(48), using cis-o-HCA glucoside, reported a Km for sweetclover β-gluco-

sidase of 4.4×10^{-4} M. Schön (50) reported conflicting data for substrate specificity in that his enzyme preparations were active against cis-o-HCA glucoside, but not against the β-glucoside of melilotic acid. Schön also found β-glucosidase activity with the β-glucosides salicin, aesculin, helicin, and cellobiose.

The research efforts to be described in the succeeding sections of this thesis involved a comparison of protein constituents in extracts of plants representing BB and bb genotypes with specific reference to β-glucosidase activity.

MATERIALS AND METHODS

Plant Material

Sweetclover plants of the cucuBB and cucubb genotypes were used in this study. Alleles at the Cu locus have been shown to influence the content of glucosidically bound o-hydroxycinnamic acid. Homozygous cucu individuals are low in content of this constituent compared with CuCu individuals (24). The cucu genotype was chosen to reduce the possible interference of high levels of endogenous substrate in assays for β-glucosidase activity. As previously stated, the B/b alleles affect the level of β-glucosidase activity; preparations of bb plants apparently lack this activity, and BB extracts are extremely active (25,48).

The homozygous genotypes used in this study were derived from an initial cucuBB x CuCubb cross. A single plant, heterozygous for both Cu/cu and B/b alleles, was selfed in each generation from F_1 to F_{17}. The cucuBB and cucubb genotypes were isolated in the F_{18} generation and were progeny-tested for homozygosity in the F_{19} generation. The F_{21} seed of each genotype used in this study was obtained by selfing in the F_{19} and F_{20} generations. Thus, the two homozygous lines used are closely related and highly inbred. The seeds were hand scarified and planted in the greenhouse in flats containing a mixture of soil, sand, and vermiculite. For sampling, the plants were cut to an 8-cm height at the early bud stage. The young healthy leaves were removed from the branches, ground in

liquid nitrogen with a mortar and pestle, freeze-dried, and stored at -10 C pending extraction.

β-Glucosidase and Protein Assay

β-Glucosidase assays were conducted routinely by the fluorometric procedure of Haskins and Gorz (25). The substrate was a lyophilized extract of young leaves of the sweetclover varieties Spanish and Evergreen (CuB phenotype) containing 0.075 u moles of trans-o-HCA glucoside and 0.469 u moles of cis-o-HCA 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 for all assays except those involving Km determinations.

A partially purified preparation of cis-o-HCA glucoside was obtained chromatographically for use in determination of Km values.[1] Young leaves of the variety Goldtop (CuB phenotype) were extracted by autoclaving in water. The lyophilization of the extract to dryness yielded 465 mg of dry powder per gm of dry leaf tissue. For chromatography, a solution containing 93 mg of this dry powder per ml of water was used. A 0.4-ml aliquot of the concentrated extract was applied per sheet, in a band 15-cm in length, and at right angles to the long dimension of $18\frac{1}{2}$ x $28\frac{1}{2}$ cm sheets of distilled water-washed Whatman #3 MM paper. Ascending chromatography was conducted with a solvent consisting of 8 parts 2-propanol, 1 part concentrated NH_4OH, and 2 parts distilled water. The solvent front was allowed to migrate 15 cm from the origin after which the cis-o-HCA glucoside

[1]The technical assistance of Ralph Kulm and Richard Ronnenkamp in the preparation of the purified cis-o-HCA glucoside is gratefully acknowledged.

was located as an ultraviolet-absorbing band (254 nm) with an Rf of 0.41. The ultraviolet-absorbing band was cut from the chromatogram and eluted with water. The eluate was rechromatographed in a second solvent consisting of 8 volumes 2-propanol, 1 volume glacial acetic acid, and 3 volumes water. The ultraviolet-absorbing cis-o-HCA glucoside band was located at Rf 0.73, cut from the chromatogram, eluted with water, and held at -10 C pending assay. Fluorometric assay (42) indicated that the final eluate contained 40.55 u moles of cis-o-HCA glucoside and 6.43 u moles of trans-o-HCA glucoside per ml with negligible amounts of free trans and cis-o-hydroxycinnamic acids.

Reaction mixtures for routine β-glucosidase assays consisted of 3.0 ml of 0.1 M acetate buffer, pH 5.0; 2.1 ml of deionized distilled water; 0.8 ml of substrate; and 0.1 ml of enzyme preparation. Prior to assay, all enzyme preparations were diluted to a concentration such that the enzymatically released o-hydroxycinnamic acid concentration in a reaction mixture incubated for five minutes would be no more than 3×10^{-2} u moles per ml. The assays were conducted at 30 C. The reaction was started by addition of the enzyme, and 0.5-ml aliquots of the reaction mixture were mixed with 4.5-ml portions of 2.5 N NaOH at 0.5 and 5.5 minutes after addition of the enzyme. These conditions resulted in no departure from linearity, over a period of 10 minutes, in the plot of extent of hydrolysis against time. A unit of enzyme was defined as the amount that would liberate 1 u mole of cis-o-hydroxycinnamic acid per minute at 30 C. The specific activity was expressed as enzyme units per mg of protein.