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NOTES AND COMMENTS

ALLELIC GLUTAMIC DEHYDROGENASE ISOZYMES IN MAIZE—A SINGLE HYBRID ISOZYME IN HETEROZYGOTES?

A. J. PRYOR

Division of Plant Industry, CSIRO, Canberra, A.C.T., Australia

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SUMMARY

Unexpectedly, plants heterozygous for any two of three *Gdh* alleles contain only a single hybrid isozyme, which is broad and diffuse and has an electrophoretic migration rate intermediate to that of the parental forms. Different electrophoretic techniques did not resolve this broad hybrid band but elution from a DEAE column showed that it is composed of a population of isozymes of different migration rates.

1. INTRODUCTION

THE use of gel electrophoresis to distinguish allelic products and to elucidate the interactions that occur between these products in the formation of active enzyme molecules has proved valuable in genetic analyses of diploid organisms. In maize many examples of allelic isozymes variants have been described and these have provided information about the subunit structure of the active enzyme.

For example in plants heterozygous for E_1 esterase alleles, Schwartz (1960) observed three bands, two of which corresponded to the parental isozyme forms, while the third was a new form with an intermediate migration rate. Schwartz argued that these data fitted the hypothesis that the E_1 esterase was a dimer, and that the new or hybrid isozyme form represented a hetero-dimer composed of dissimilar subunits produced by the two alleles. Similarly from the occurrence of five isozyme bands (two parental and three hybrid forms) in heterozygotes for maize catalase alleles this enzyme has been inferred to be a tetramer (Beckman *et al.*, 1964). Putting this argument in general terms we can say, given an enzyme composed of one or more subunits determined by a single gene, then the heterozygotes of alleles producing isozyme variants are expected to contain $n+1$ isozyme bands, where n is the number of subunits in the active enzyme. There are a number of assumptions implicit in this argument and a general consideration of these has been given in a number of review articles (see Shaw, 1969). In the simplest case where the enzyme is functionally a monomer only the two parental forms are expected in heterozygotes. In maize, catechol oxidase appears to be an example of this type (Pryor and Schwartz, 1973).

Further evidence for the validity of the argument that the number of isozyme bands observed in heterozygotes reflects the subunit nature of the enzyme can be provided by the demonstration that the new or novel bands that appear are in fact hybrid molecules composed of dissimilar subunits. By subjecting an *in vitro* mixture of two catalase isozymes to conditions known to promote the dissociation and reassociation of protein subunits,

Scandalios (1965) was able to generate *in vitro* the five banded pattern (two parental and three hybrid isozymes) that appears in heterozygotes.

Even if some of these assumptions do not apply there is no simple reason why the two parental bands should not be observed in heterozygotes. If, in homozygotes, like subunits polymerise to form functional enzyme, then this should also occur in heterozygotes. In this paper we describe an exceptional observation in which maize plants heterozygous for alleles determining isozyme variants appear to contain a single hybrid isozyme and lack both parental forms.

2. Methods

Standard horizontal starch gel techniques were used with a number of buffer systems (see Smith, 1968 for review). At high *p*H (>8·3) there was little resolution of the isozyme variants, while at low *p*H (<7·0) activity was lost from the zymograms. Gels in the *p*H range 7·0-8·5 were generated using a Tris-citrate buffer system (Poulik, 1957). The most satisfactory resolution was obtained using a gel buffer at *p*H 7·6 and 0·3M Borate-NaOH (*p*H 8·6) for the electrodes. The gels contained 12 per cent (w/v) hydrolysed starch (Connaught) and electrophoresis was carried out in the cold with a constant voltage (200 v) for 4-5 hours. Significantly longer time produced diffuse zymograms without increasing the resolution.

Disk acrylamide gel electrophoresis was also carried out using several buffer systems. Enzyme samples of 10 μl containing 90-100 μg of protein (Biuret) and 10 per cent sucrose were applied to each gel. Spacer gels gave no increased resolution. As with starch gels, extremes of *p*H were not satisfactory for GDH isozymes (see Smith, 1968 for methods). Rapid and differential migration of isozyme variants was achieved using the following Tris-citrate buffer system: A. *Gel buffer*: 50 ml of 0·2M Tris containing 0·23 ml of TEMED were adjusted to *p*H 7·6 with 0·005M tri-Sodium citrate. B. *Acrylamide solution*: 30 g of acrylamide and 0·8 g of bis acrylamide in 100 ml of water. C. *Persulphate solution*: 0·14 g ammonium persulphate in 100 ml of water. Gels were prepared by adding 1 part A+2 part B+1 part water to 4 parts of C. The reservoir buffer was 0·03 M Borate-NaOH at *p*H 8·65 and electrophoresis was carried out at room temperature for 1 hour with about 2 mA/gel. Different acrylamide gel concentrations were prepared using appropriate dilutions of B.

Individual 5-day old dark grown seedlings were assayed in starch gels by absorbing the juice from squashed root and mesocotyl tissue into filter paper pieces which were inserted directly in the gels.

Extracts containing GDH isozymes were prepared by grinding root and mesocotyl tissue in liquid nitrogen and extracting in buffer (0·1M Tris-HCl *p*H 8·0, containing 0·001 β-mercaptoethanol). The 40-70 per cent ammonium sulphate pellet was taken up in and dialysed against diluted buffer (0·01M). Protein content was determined by the Biuret method using bovine serum albumin as standard.

After electrophoresis gels were stained in a standard dehydrogenase diazo stain in 0·1M Tris-HCl (*p*H 8·0) with L-sodium glutamate (0·02M) as substrate. Under these conditions activity was specific for NAD.

Fractionation of enzyme extracts (1 ml containing approximately 10 mg of protein) on 1×10 cm DEAE cellulose columns (Whatman DE52) was achieved by eluting with a 100-ml linear gradient of 0·05M to 0·3M NaCl

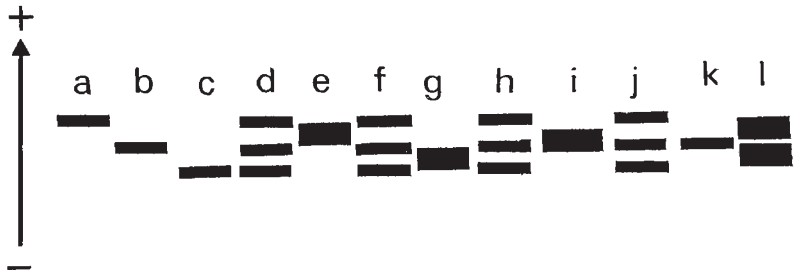

FIG. 1.—Starch gel zymograms of GDH isozymes. The samples are: a, Fast variant; b, k, Normal variant; c, Slow variant; d, f, h, j, *in vitro* mixtures of the three variants. e, Fast/Normal heterozygote; g, Normal/Slow heterozygote; i, Fast/Slow heterozygote; l, *in vitro* mixture of the Fast/Normal and Normal/Slow heterozygotes.

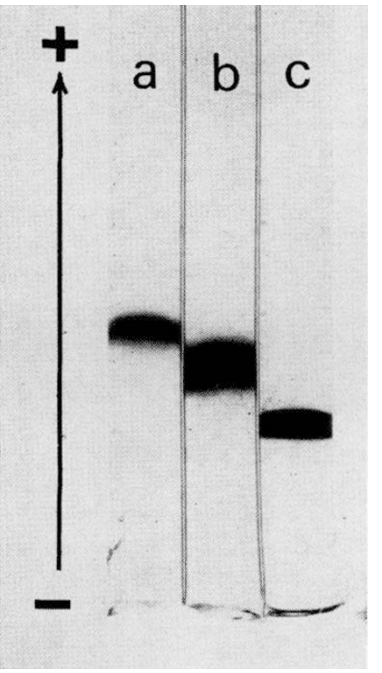

FIG. 2.—Disk acrylamide gel zymograms of GDH isozymes. a, Fast variant. b, Fast/Slow heterozygote. c, Slow variant.

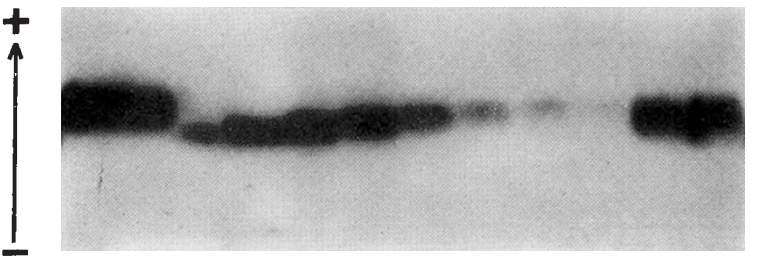

FIG. 3.—Starch gel zymograms of GDH isozyme fractions eluted from a DEAE cellulose column.

The first and last samples are the F/S GDH isozyme extract which was applied to the column. Between these are eight fractions (alternate) which were eluted off the column and had GDH activity.

in 0·01M Tris-HCl *p*H 8·0 containing 0·001M β-mercaptoethanol. GDH activity was eluted at about 0·15M NaCl and was located in the 1·0-ml fractions by using the standard dehyrogenase diazo stain as a spot test.

3. Results and discussion

Three electrophoretic variants of glutamic dehydrogenase (GDH) with different anodal migration rates at *p*H 7·6 have been found. In addition to the common or Normal form (N) there are Faster (F) and Slower (S) forms. The migration rate of the N isozyme is not mid-way between F and S forms but is slightly displaced towards S (fig. 1).

Table 1

Inheritance of GDH isozyme variants

Cross	F	N	S	F/N	F/S	N/S	Total	χ^2	p
N × F/N	—	29	—	24	—	—	53	0·47	> 0·3
N × F/S	—	—	—	19	—	15	34	0·47	> 0·3
N × N/S	—	25	—	—	—	19	44	0·82	> 0·3
F × F/S	25	—	—	—	29	—	54	0·29	> 0·5
N/S × S	—	—	24	—	—	26	50	0·08	> 0·7
F/N selfed	32	25	—	53	—	—	110	1·036	> 0·5

F, N and S stand for true breeding lines that carried the Fast (F) Normal (N) and Slow (S) variants and χ^2 was calculated on the hypothesis that F, N and S are inherited as alleles of a single gene. Source of material: F was derived from inbred line C103A, N the common or wild type was for most of this work from inbred line B48 and S was from line P32.

In heterozygotes between any two variants only a single band is observed. The migration rate of this band is intermediate to that of the two parental forms and is displaced from the mid-point towards the faster migrating parent. This is most clear in the case of heterozygotes between the F and S forms (figs. 1, i and 2, b), and these single bands are characteristically broader and more diffuse than any of the three parental forms.

The best resolution of GDH isozymes was achieved at *p*H 7·6 in both starch and acrylamide gel electrophoresis. Increasing the time of electrophoresis or alternation of the gel concentration or *p*H did not resolve the hybrid bands. They still appeared as single broad diffuse bands except at high *p*H (> 8·3) where none of the GDH isozymes is resolved and all migrate as a compact band.

The single band in heterozygotes appears to be the result of allelic interaction, for in backcrosses to either parent the parental isozyme appears in 50 per cent of the progeny (table 1). Similarly, F_2 data (table 1) are consistent with the hypothesis that the three electrophoretic variants are specified by alleles of the same gene. On this basis the three alleles are defined as Gdh^F, Gdh^N and Gdh^S.

The genetic data are clear and indicate that the single novel band that appears in heterozygotes is a result of the interaction of alleles of the *Gdh* gene. Three models can be considered to explain the observation.

Model 1. The gene controlling the GDH variant is not the structural gene for the enzyme but is instead involved in the addition of some side group which alters the electrophoretic migration,

perhaps a system analogous to the addition of sialic acid residues to alkaline phosphatase in chickens (Law, 1967). For argument's sake we could say that Gdh^F adds twice the number of side groups as does Gdh^N while Gdh^S adds none. If these genes were additive in effect this would explain the occurrence of the single band of intermediate migration in heterozygotes.

Model 2. The single hybrid band results from the preferential association of dissimilar subunits produced by the two alleles. This model requires that the genetic alterations which produce the three electrophoretic variants are also responsible for preferential association to the extent of exclusion of the parental forms.

Model 3. The single broad band could be an unresolved mixture of isozymes. In several animals GDH has been shown to be a complex enzyme in which the smallest active unit consists of six or more identical subunits (see Goldin and Frieden, 1971 for review). In plants the subunit nature of GDH is not clear, but let us suppose that maize GDH is a hexamer. Random association of dissimilar subunits in a heterozygote will produce seven forms with different subunit constitution of which five forms are hetero-hexamers and comprise $\frac{62}{64}$ of the total enzyme. The parental forms would each contribute only $\frac{1}{64}$. (This contribution will decrease as $(\frac{1}{2})^n$ as the number of subunits (n) increases.) Thus the single broad GDH isozyme band seen in heterozygotes could be an unresolved mixture of isozymes in which the contribution of the parental homo-hexamers is so small that it is not visible on the starch gel zymograms.

The difference in the widths of the parental and heterozygous isozyme bands is not expected on Model 1. This model predicts that the N and F bands should be as broad as heterozygous bands and this is not observed. This same observation argues against Model 2. The broad band could reflect some inherent instability of the hybrid molecule that is maintained during electrophoresis, but this is unlikely since there is no suggestion of interaction between the constituent subunits of an *in vitro* mixture of two heterozygous isozymes (fig. 1, l). However, the broad band in the heterozygote is expected on Model 3.

In addition it can be shown that this broad hybrid band is composed of a population of isozymes of different migrations. When GDH from hybrid seedlings is eluted off a DEAE cellulose column with a linear salt gradient, it is separated into isozymes which have a range of migration rates within the limits of the broad hybrid band (fig. 3). This result was obtained twice for the Fast/Slow heterozygote and once with each of the remaining heterozygotes.

Thus although Model 3 seems the most probable and not inconsistent with the data, our inability to resolve the putative mixture of isozymes forms still leaves a degree of uncertainty. The model may be tested if conditions for dissociating and reassociation subunits can be found (attempts using the method of Hart (1971) were unsuccessful). If the F isozyme form is reassociated in the presence of increasing amounts of the S form, then the

distribution of the enzyme activity within the broad band should initially be skewed towards the F form and tend to the S form as the proportion of S isozyme is increased. The same test can be made genetically using tetraploid stocks carrying different doses of the *Gdh* alleles and thus avoid having to dissociate and reassociate the enzyme *in vitro*.

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SELECTION BEHAVIOUR OF WILD BLACKBIRDS AT HIGH PREY DENSITIES

PAUL H. HARVEY

School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex

CATHERINE A. JORDAN

Department of Zoology, University College, Swansea

and

JOHN A. ALLEN

Department of Zoology, University of Dar es Salaam, Tanzania

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Summary

In experiments with wild blackbirds eating artificial pastry baits of two colours presented at high density. it has been argued elsewhere that selection was directional. The experiments have been repeated with a slightly different experimental design and the results indicate apostatic selection. Some indications of changes in selection behaviour by the blackbirds, both within and between visits, are also mentioned.

1. Introduction

As a result of the development of a " specific searching image " (Tinbergen, 1960) predators may preferentially take the more common varieties of a