SUBSTRATE-PREFERENCE POLYMORPHISM AT AN ESTERASE LOCUS OF DROSOPHILA MOJAVENSIS

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ABSTRACT

In a larval esterase of Drosophila mojavensis there are alleles whose products preferentially hydrolyze α-naphthyl esters, whereas the majority of the alleles hydrolyze preferentially β-naphthyl esters. In a collection of laboratory stocks α alleles have a frequency of 15%. Three different mobilities of α alleles were discovered, suggesting a polymorphism rather than a single mutation event. If substrate-preference polymorphisms are common among “multiple-substrate” enzymes (category II of Gillespie and Langley 1974), allozyme variation at these enzyme loci may well be maintained by balancing selection.

It is a well-established phenomenon that natural populations contain large amounts of genetic variability at a large fraction of their enzyme loci. A large amount of this variability can be detected electrophoretically (Lewontin 1974); but recent studies have shown that the allelic variants may differ in many other aspects, such as specific catalytic activity or functional stability under a variety of conditions (see, for example, Day, Hillier and Clarke 1974a; Miller, Pearcy and Berger 1975; Loukas, Vergini and Krimbas 1981), regulation of expression (Gibson 1972; Day, Hillier and Clarke 1974b; Chovnick et al. 1976), ability to form multimers (Cobbs 1976), post-transcriptional modification, or coenzyme-binding ability (Finnerty and Johnson 1979). Some of these differences are sensitive enough to be used to uncover variation which cannot be detected by routine electrophoresis (Singh, Lewontin and Felton 1976).

One type of variation that has not been shown among allozymes is specificity for different substrates of alleles at the same locus. Yet this is a type of variation usually envisaged in hypotheses purporting to explain the maintenance of enzyme polymorphisms in populations. The best-known of these hypotheses is the one by Gillespie and Langley (1974), who have drawn a distinction between “single-substrate” and “multiple-substrate” enzyme loci. Enzymes of the first category (Group I) recognize only one substrate, which is usually the intracellular product of another enzymatic reaction. Selection is expected to establish a single best allele for this substrate, whose quantity may also be under

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the organism’s genetic control. Enzymes of the second category (Group II) recognize a variety of chemically-related substances as substrates; these substrates may be of external origin to the organism; and their availability may not be under the organism’s control. Polymorphism can easily be established at group II loci, especially if it could be shown that the various allelic forms take preferentially one substrate over another, or have maximal catalytic activities for different substrates.

Singh (1976) has attempted to discover just such a type of variation at the alcohol dehydrogenase loci of Drosophila pseudoobscura. He examined eight such loci, of which three were polymorphic. Although there were marked differences in the substrate specificities of enzymes coded by different loci, Singh could find no differences in the specificities of enzymes coded by different alleles at the same locus. On the basis of these findings he suggested that substrate specific differences exist only between isozyme loci, and that a substantial fraction of allelic variation in group II loci may indeed be selectively neutral. Here we report a clear case of differential substrate preference among enzymes coded by various alleles of an esterase locus of Drosophila mojavensis.

MATERIALS AND METHODS

The Drosophila mojavensis stocks used in this study were established as multifemale lines from collections made between 1971 and 1977. Each line was maintained in the laboratory as a mass culture. There is no way of knowing how many “wild” chromosomes were in each line at the time of its establishment, nor is there any way of knowing how many of the original chromosomes are lost during culture in the laboratory. We will conservatively assume that there is only one original chromosome left in each line, except if the line is segregating for electrophoretically-detectable variants, in which case the original chromosomes are assumed to be as many as are the variants. Eighteen stocks of D. mojavensis race B and one stock of D. mojavensis race A were surveyed (see Zouros 1973 for a description of the races of D. mojavensis). In addition, seven stocks of D. arizonensis, a close relative of D. mojavensis, were also surveyed. Nine stocks of D. mojavensis B and two stocks of D. arizonensis were found to segregate each for two Est-4 electromorphs. The other stocks contained one electromorph. Thus, a conservative estimate of chromosomes sampled is 37: 27 for D. mojavensis B, 1 for D. mojavensis A, and 9 for D. arizonensis.

Esterase allozymes were separated on a polyacrylamide gel as described in Prakash, Lewontin and Hubby (1969). Third instar larvae were used for these assays. After electrophoresis, the gel was incubated in 0.5 boric acid for half an hour, then rinsed and developed in the following solution: 100 ml of phosphate buffer pH 6.4 (1.75 × 10⁻²M Na₂HPO₄ and 4.39 × 10⁻⁴M KH₂PO₄), 50 mg Fast Blue BB salt, 1 ml of 0.1 M MgCl₂, 1 ml of 0.1 M MnCl₂, 1 ml of 0.1 M NaCl, and 2 ml of 50% acetone into which there were resolved 30 mg of substrate α and 30 mg of substrate β. The substrates used were α-naphthyl acetate, α-naphthyl propionate, α-naphthyl butyrate, and their β analogs. An α substrate was used in combination with any one of the β substrates; all nine combinations were tested. After 1 ½ hours of development in room temperature the gel was rinsed and fixed in 1:5:5 mixture of acetic acid, methanol and water.

RESULTS

Extracts from third instar larvae of Drosophila mojavensis or Drosophila arizonensis show multiple bands of esterase activity after electrophoresis on a polyacrylamide gel. Formal Mendelian genetics and surveys of variation of larval esterases are published in Zouros (1973) and Zouros and Johnson (1976).
Three polymorphisms are reported in these papers: esterase 2 (Est-2), esterase 4 (Est-4), and esterase 5 (Est-5). All bands stain brown when α-naphthyl acetate is used as substrate, and purple when β-naphthyl acetate is used as substrate, and Fast Blue BB is the coupling agent. When both substrates are used simultaneously, Est-2 bands stain brown, and Est-4 and Est-5 bands stain purple. On this basis, Est-2 is classified as an α esterase, whereas Est-4 and Est-5 as β esterases. This classification has long been in use (i.e., Narise, 1973). Brown coloration also appears when α-naphthyl propionate or α-naphthyl butyrate are used as substrates, and purple coloration appears when the β analogs of these esters are used.

Est-4 and Est-5 probably represent a gene duplication (Zouros, E., W. van Delden, R. Odense and H. van Dijk, unpublished results). In the process of studying this duplication, it was observed that in gels stained with both α- and β-naphthyl acetates, certain electromorphs of Est-4 produce a brown band instead of the purple band characteristic of β esterases. This prompted a detailed survey of all D. mojavensis and D. arizonensis stocks at our disposal, with the results shown in Table 1. In these tests an α and a β substrates were used, and since we tested three different α and three different β substrates, there were nine substrate combinations. We observed that in all cases the substrate preferences depended on the position of the acid residue on the naphthyl ring (position α or β), and not on the residue itself.

### TABLE 1

Substrate preferences of electrophoretic alleles of Est-4 in laboratory stocks of Drosophila mojavensis and Drosophila arizonensis

<table>
<thead>
<tr>
<th>Electrophoretic mobility</th>
<th>Substrate specificity*</th>
<th>Number of stocks possessing the allele</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>D. mojavensis B</td>
</tr>
<tr>
<td>77</td>
<td>β</td>
<td>2</td>
</tr>
<tr>
<td>82</td>
<td>α</td>
<td>1</td>
</tr>
<tr>
<td>86</td>
<td>β</td>
<td>3</td>
</tr>
<tr>
<td>88</td>
<td>β</td>
<td>2</td>
</tr>
<tr>
<td>94</td>
<td>β</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>α</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>β</td>
<td>1</td>
</tr>
<tr>
<td>102</td>
<td>β</td>
<td>2</td>
</tr>
<tr>
<td>102</td>
<td>α</td>
<td>2</td>
</tr>
<tr>
<td>104</td>
<td>β</td>
<td>4</td>
</tr>
<tr>
<td>86</td>
<td>β</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. mojavensis A</td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>β</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>β</td>
<td>1</td>
</tr>
<tr>
<td>104</td>
<td>β</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>D. arizonensis</td>
<td></td>
</tr>
</tbody>
</table>

* α: preferential hydrolysis of α-naphthyl acetate, α-naphthyl propionate or α-naphthyl butyrate;
* β: preferential hydrolysis of β-naphthyl acetate, β-naphthyl propionate or β-naphthyl butyrate.
Among the minimum number of 37 original chromosomes sampled, four carried an \( \alpha \) allele at the \( \text{Est-4} \) locus. All four were found in \( Drosophila \) \textit{mojavensis} \( B \): the \( 82\alpha \) allele was discovered in the Los Planes (LP) stock originated from the southern part of Baja California; the \( 100\alpha \) was found in stock A-426 from near Mulegé, Baja California; one \( 102\alpha \) was found in stock A-713 from the island of San Esteban, and another in stock A-506 from the island of Tiburon, both in the Gulf of California (see Figure 1 in ZOUROS and d'ENTREMONT 1980 for the geographical origins of these stocks). Through pair matings homozygous lines for the \( \alpha \) alleles were established from each stock. Following are some observations obtained so far from these lines.

1. Formal genetics showed that the \( \alpha \) bands are allelic to \( \text{Est-4} \) \( \beta \) bands, as well as to each other. Table 2 gives the results from one cross involving two alpha alleles (\( 82\alpha \) and \( 100\alpha \)) and two beta alleles (\( 86\beta \) and \( 104\beta \)).

2. \( \alpha \) monomers dimerize normally with other \( \beta \) or \( \alpha \) monomers to form active dimers; when the dimerization is with a \( \beta \) monomer, the color of the hybrid band is an intermediate between purple and brown.

3. The age-specific and tissue-specific expression profiles of \( \alpha \) alleles are the same as those of \( \beta \) alleles. \( \text{Est-4} \) activity can be first detected when the larva enters the third instar, and reaches its peak two to four hours before pupation; it diminishes rapidly thereafter and it does not reappear in the adult stage. The highest concentration of esterase 4 occurs in the larval carcass; low levels of activity can be detected in the hemolymph and the fat body.

Figure 1 demonstrates the polymorphism. Three alleles of \( \text{Est-4} \)---102\( \beta \), 82\( \alpha \) and 100\( \alpha \)---can be seen under varied conditions of staining. The OPNM \( \times \) LP \( F_1 \) hybrid illustrates the allelic state of the \( \alpha \) and \( \beta \) bands. The weaker bands between \( \text{Est-4} \) and \( \text{Est-5} \) are dimers between \( \text{Est-4} \) and \( \text{Est-5} \) products. The intensities of these bands are much weaker when compared to bands produced by intra-allelic dimerization.

These observations establish that, at the \( \text{Est-4} \) locus, there is variation for substrate preference. The fact that the four alleles with altered preference were discovered in stocks that had originated in different localities, together with the fact that the four alleles fall in three distinct electrophoretic classes exclude the

<table>
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<th>TABLE 2</th>
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<td><strong>Crosses showing the allelism of alpha and beta bands</strong></td>
</tr>
</tbody>
</table>

| 1. \( \text{Est-4} \) (\( 82\alpha/82\alpha \)) \( \times \) \( \text{Est-4} \) (\( 100\alpha/100\alpha \)) |
| \( F_1 \): \( \text{Est-4} \) (\( 82\alpha/100\alpha \)) |
| 2. \( \text{Est-4} \) (\( 86\beta/86\beta \)) \( \times \) \( \text{Est-4} \) (\( 104\beta/104\beta \)) |
| \( F_1 \): \( \text{Est-4} \) (\( 86\beta/104\beta \)) |
| 3. \( \text{Est-4} \) (\( 82\alpha/100\alpha \)) \( \times \) \( \text{Est-4} \) (\( 86\beta/104\beta \)) |
| \( F_1 \): \( \text{Est-4} \) (\( 82\alpha/86\beta \)) 28  
\( \text{Est-4} \) (\( 82\alpha/104\beta \)) 23  
\( \text{Est-4} \) (\( 100\alpha/86\beta \)) 14  
\( \text{Est-4} \) (\( 100\alpha/104\beta \)) 21 |
FIGURE 1.—Esterase profiles of *D. mojavensis* larvae from four different stocks and from one cross between two of these stocks. Eight 3rd instar larvae were ground together and spun, and three wells of the gel were loaded with equal amounts of supernatant. The gel was then divided in three parts. Part A (wells 1 to 5) was stained with α-naphthyl acetate, part B (wells 6 to 10) was stained with β-naphthyl acetate, and part C (wells 11 to 15) was stained with α- and β-naphthyl acetate. Wells 1, 6 and 11: stock OPNM; wells 2, 7 and 12: F, from cross OPNM × LP; wells 3, 8, and 13: stock LP; wells 4, 9, and 14: stock A426; wells 5, 10 and 15: stock A506.4. Stock OPNM is *Est*-4102β, stock LP is *Est*-4k2α, stock A426 is *Est*-4100α, and stock A506.4 is *Est*-4102β. Bands between *Est*-4 and *Est*-5 are products of interlocus dimerization.

The possibility of a single recent mutation of a transient nature. Rather, it appears that this is a true polymorphism with a crude estimate of the frequency of α bands of 4/27 ≈ 0.15. Previous studies of *Est*-4 variation (Zouros, 1973; Zouros and Johnson 1976) failed to detect this polymorphism because in these studies one type of substrate was used for the staining of gels. The alternative hypothesis that α alleles represent mutations that arose independently in the laboratory and were subsequently selected under the laboratory conditions appears less likely, but it cannot be dismissed.

DISCUSSION

Several authors have demonstrated differences in the catalytic properties of allozymic products (Vigue and Johnson 1973; Harper and Armstrong 1973; Day, Hillier and Clarke 1974a; Miller, Pearcy and Berger 1975; Narise, 1979; Fucci et al. 1979). An important observation emerging from these studies is that when a single substrate is used in the tests, one and the same allozyme performs better than any other under most circumstances. When more substrates
are tested, the allozyme that gives the highest score for one substrate also does so for almost all other substrates (Singh 1976; Dranford and Beardmore 1979). A serious limitation of these studies (and of this one) is that compensatory and homeostatic mechanisms may exist in organisms which smooth out the physiological impact of interallelic differences seen in *in vitro* experiments (Bargiello and Grossfield 1979). For at least one enzyme, the ADH of *Drosophila melanogaster*, there is convincing evidence that *in vitro* catalytic activities are good predictors of outcomes of competition experiments (Van Delden, Kamping and van Dijk 1975; Van Delden, Boerema and Kamping 1978.)

If one allozyme is always more efficient than any other in the use of any given substrate, it is difficult to see how the variation of multiple substrate loci can be selectively maintained. This has led Singh (1976) to speculate that the most efficient solution to the multiple substrate problem is gene duplication and subsequent diversification of the substrate specificities of the resulting isozyme loci. Myers (1978) has theorized that this will be the expected strategy if the substrate supplies are constant and predictable, whereas allozymic variation (with substrate specificities) will be expected if the supplies are variable or unpredictable.

One way that allelic variation can be maintained in the face of a single most efficient allele is when high rates of turnover of a certain substrate are occasionally disadvantageous to the organism, or when the catalytically most efficient allozyme is also more thermoliable. Both situations have been shown to occur in the ADH of *Drosophila melanogaster* (Morgan 1975; Day, Hillier and Clarke 1974). Another, more obvious, way occurs when different allozymes have different substrate preferences. Here we report a clear case of the latter type.

Because of its nature, one would expect this type of polymorphism to be an obvious target of balancing selection. Also, this polymorphism is different from other types (such as electrophoretic or heat-sensitivity polymorphisms) in that it probably resulted from mutations at the active center of the enzyme molecule. As such, it is expected to be under strong selective constraints; its mere presence in natural populations may signify the operation of selection at the molecular level.

However, the interpretation of balancing selection, although appealing, appears premature for several reasons, of which the most obvious are listed below.

1. We do not know how widespread this type of polymorphism is. In our search for it we exploited the fact that when the hydrolyzed products of α- and β-naphthyl esters are coupled with certain dyes they produce precipitates of different colors. This simple assay cannot be applied to most other multiple-substrate enzymes. Therefore, substrate-preference may exist in other enzyme systems but has not yet been detected because of lack of an appropriate technique. Our survey of the same stocks of *Drosophila mojavensis* failed to reveal any substrate-specific variants at Est-5 or Est-2, both of which are electrophoretically polymorphic.

2. Since we have not a clear idea of what the physiological function of Est-4 is (or, for that matter, of any other esterase), we can only speculate as to whether
the substrate specificity we have observed is relevant to the organism. We have already mentioned that Est-4 occurs in the third instar larva and early pupa, and then it is mostly concentrated in the carcass; it does not occur in the digestive system. Hence, it is less likely that it is exposed to external substrates; but this cannot be excluded, nor can one overlook the possibility that esterases are exposed to a variety of internally-produced substrates.

3. It may be argued that the organism has more than one esterase locus whose products may equally well serve the same metabolic needs. Under such conditions, mutations that reduce or alter the metabolic efficiency of one locus may well be tolerated for long periods of time. However, the larval esterases of D. mojavensis have distinctly different tissue expressions, so it is difficult to see how one of them might substitute for the physiological function of the other.

In conclusion, the demonstration of a substrate-preference polymorphism provides empirical justification for one of the assumptions of the hypothesis that multiple substrate enzyme polymorphisms are maintained by balancing selection. The manifestation of the selection itself is not established.

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LITERATURE CITED


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