Activity differences between acid phosphatase allozyme variants of *Drosophila virilis*: Differences in intracellular localization of allozymes

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SUMMARY

Three acid phosphatase allozyme strains (Acph-1, Acph-2 and Acph-4) of *Drosophila virilis* show large differences of enzyme activity when examined by means of starch gel electrophoretic technique, Acph-4 strain showing approximately four times the activity of Acph-1 and twice that of Acph-2, as reported previously (Narise, 1976). Crude extract difference between Acph-4 and Acph-1 strains is less than twofold and this compared with larger differences in supernatants. Cell fractionation and density gradient centrifugation demonstrated that the acid phosphatase resides mainly in lysosomes and becomes soluble in part during preparation without structural damage to lysosomes. The solubility of the allozymes from lysosomes was variable among the three strains. ACPH⁴ allozyme was released in the highest degree. However, the release-rate of other lyso-somal enzymes, such as α-glucosidase, β-galactosidase and β-glucuronidase was similar among these strains. These results suggest that the strain variation in ability of the allozymes to be incorporated into lysosomes is due to the allozymes themselves, not due to alteration in the lysosomes.

1. INTRODUCTION

Many enzymes occur in electrophoretic forms in a large number of species. These electromorphs in *Drosophila* species have been found to be specified by alleles at a single locus and are called allozymes. Different allozyme variants often show differences in enzyme activity (Rasmuson *et al.* 1966; Doane, 1969; Gibson & Miklovich, 1971; Hicky, 1977). Recent studies on alcohol dehydrogenase and amylase have demonstrated that such variation in activity can be controlled by regulatory genes (Pipkin & Hewitt, 1972; Ward, 1975; McDonald & Ayala, 1978 for alcohol dehydrogenase; Abraham & Doane, 1978; Powell & Lichtenfels, 1979; Tejima & Ohba, 1981 for amylase). Although genetic and biochemical studies have documented that the regulatory genes affect the number of the enzyme molecules (Gibson, 1972; McDonald *et al.* 1977; Hicky, 1981), the molecular basis of the variation is still unclear.

Acid phosphatase (EC 3.1.3.2) (ACPH) exists as electromorphs in adult flies of *Drosophila virilis* occuring in natural populations of Japan (Ohba, 1977). These

forms are coded by alleles at the *Acph* locus. The homozygous flies (Acph-1, Acph-2 and Acph-4) for each allele ($Acph^1$, $Acph^2$ and $Acph^4$) show variation in enzyme activity which can be detected by measurement of the relative intensity of the allozyme bands; Acph-4 activity was approximately four times that of Acph-1 and twice that of Acph-2 (Narise, 1976). In addition, surveys of the allele frequency in wild populations by Ohba (1977) have demonstrated that most populations have one allele ($Acph^2$) which comprises more than 95 % of the genes at *Acph* locus. $Acph^4$ is one of the rare alleles (mean frequency of 0·56 %) in spite of its high enzyme activity.

Recently, preliminary experiments have demonstrated similar activity of whole fly homogenates from these allozyme strains (Narise, 1983). Furthermore, electro-phoretic study has shown that in Acph-1 and Acph-2 strains, besides the allozymes, ACPH activity was observed near origin after electrophoresis, but not in flies of Acph-4 strain. These activities were diminished after treatment of flies with a detergent, Triton X-100, while the intensity of the allozyme bands increased (Narise, 1983).

These results suggest that the activity variation for these electrophoretically detectable allozymes may correlate with the differences in ability of the allozymes to be incorporated into particles and such alterations in cellular organization may affect efficiency of the function of this enzyme *in vivo*. Therefore, it seems important to inquire further into the nature of the activity variation in order to discover what genetic factors control intracellular localization of the enzyme and what the physiological role of the enzyme is. The purpose of this report is to confirm the subcellular localization of the enzyme and to test whether or not there are differences among strains in release of the allozymes from the particles.

2. MATERIALS AND METHODS

Drosophila virilis *stocks*

Three acid phosphatase (ACPH) variants were used in this study; Acph-1, Acph-2 and Acph-4, each of which is homozygous for each allele ($Acph^1$, $Acph^2$ and $Acph^4$) at *Acph* locus. They were isolated from isofemale lines from natural populations surveyed by Ohba (1977) and have been maintained in a mass culture. Flies were reared in vials on ordinary yeast-sugar-agar media. Adult flies were collected within 24 h after eclosion and used immediately.

Electrophoresis

Horizontal electrophoresis was carried out according to the method described by Shaw & Prasad (1970). After electrophoresis enzyme activity was localized by a diazo-coupling method (Narise, 1984).

Preparation of fly homogenates

Adult flies were homogenized with a Teflon pestle in 30 vols w/v of 20 mM-Tris-HCl pH 7·0 containing 1 mM-EDTA in a Potter–Elvehjem homogenizer, unless otherwise stated.

Preparation of cell fractions

Fresh flies were homogenized in cold 0·25 M sucrose and 1 mM-EDTA buffered at pH 7·0 with 20 mM-Tris-HCl, as described above. The homogenate was centrifuged for 10 min at 1000 g and the precipitate was resuspended with the same solution and centrifuged again. The pellet combined was referred to as cell debris and nuclear fraction. The combined supernatant was then centrifuged for 20 min at 10000 g to precipitate both mitochondria and lysosomes. After the pellet was washed with sucrose solution, the supernatant was combined and centrifuged for 2 h at 100000 g. The pellet was washed in the same manner. The pellet obtained was referred to as microsomal fraction and the supernatant as the soluble fraction.

For some experiments a combined cytoplasmic particle fraction was prepared by centrifuging at 100000 g for 30 min after removal of the nuclear fraction.

Sucrose density gradient centrifugation

A non-linear gradient of sucrose, ranging in concentration from 0·4 to 2·1 M, buffered at pH 7·0 with 20 mM-Tris-HCl was prepared according to Bock & Ling (1954). Centrifugation was carried out at 80000 g for 1 h using a swinging rotor in a Hitachi centrifuge 70P-72.

Enzyme assays

ACPH activity was measured by the method previously reported (Narise, 1984). Succinate oxidase activity was determined at 25 °C by spectrophotometric method following the reduction of 2,6-dichlorophenol indophenol (King, 1967). α-Glucosidase, β-galactosidase and β-glucuronidase activities were assayed in 0·1 M acetate buffer pH 5·0. p-Nitrophenyl glycosides (final concentration, 2·5 mM) of sugars on which these enzymes act were used as substrate. Assay methods were the same as the method for ACPH, except for differences in substrate concentration.

Release of ACPH allozymes from lysosomal fraction

Adult fly homogenates prepared in 0·25 M sucrose containing 20 mM-Tris-HCl pH 7·2 were centrifuged at 1000 g for 10 min. The supernatant was centrifuged at 18000 g for 30 min to precipitate lysosomes. The pellet was resuspended in the sucrose solution and the suspension was divided into two fractions with the same volume and centrifuged again separately. The pellet obtained from one fraction was suspended in the sucrose solution and that from the other was suspended in the same volume of Tris-HCl pH 7·2 as the sucrose solution used. After being kept in ice-water for 1 h both suspensions were centrifuged at 35000 g for 30 min. Enzyme activities of the final suspension, supernatant and sediment were determined.

3. RESULTS

Activity differences among acid phosphatase allozymes

Previous reports (Narise, 1976, 1983) have demonstrated the considerably different activities of three allozymes (ACPH[1], ACPH[2] and ACPH[4]) produced by

Table 1. *Acid phosphatase activity in crude extracts and supernatants from ACPH allozyme homozygous strains*

Strains	Activity (μmol p-nitrophenol/min/g flies)		Ratio B/A (%)
	(A) Crude extract	(B) Supernatant	
Acph-1	$6{\cdot}57 \pm 1{\cdot}08$	$1{\cdot}60 \pm 0{\cdot}34$	24·4
Acph-2	$8{\cdot}15 \pm 0{\cdot}97$*	$2{\cdot}83 \pm 0{\cdot}39$**	34·7
Acph-4	$12{\cdot}21 \pm 1{\cdot}66$***	$7{\cdot}04 \pm 0{\cdot}77$****	57·6

Crude extracts were obtained by squeezing homogenates through two layers of gause and supernatants were by centrifuging crude extracts at 18000 **g** for 30 min.

Each value for activity is mean $\pm$ S.E. ($N = 8$).

*, ** Differ from the corresponding value of Acph-1 at the 0·05 and 0·01 probability levels.

, * Differ from the corresponding value of Acph-2 at the 0·05 and 0·01 probability levels.

Table 2. *The effects of isotonic and detergent on extraction of ACPH allozymes*

Strains		Activity (μmol p-nitrophenol/min/g flies)		
		Addition of 0·25 M sucrose	Control	Addition of 0·5% Triton X-100
Acph-1	Cell-free extract	$2{\cdot}92 \pm 0{\cdot}56$	$3{\cdot}25 \pm 0{\cdot}32$	$5{\cdot}90 \pm 0{\cdot}74$***
	Supernatant	$1{\cdot}79 \pm 0{\cdot}36$	$1{\cdot}97 \pm 0{\cdot}25$	$4{\cdot}10 \pm 0{\cdot}26$***
	%	61·3	60·6	69·5
Acph-2	Cell-free extract	$4{\cdot}37 \pm 0{\cdot}85$	$4{\cdot}89 \pm 0{\cdot}47$	$7{\cdot}58 \pm 0{\cdot}43$**
	Supernatant	$2{\cdot}95 \pm 0{\cdot}66$	$3{\cdot}29 \pm 0{\cdot}61$	$6{\cdot}18 \pm 0{\cdot}65$***
	%	67·7	67·3	81·5
Acph-4	Cell-free extract	$6{\cdot}22 \pm 0{\cdot}46$***	$8{\cdot}98 \pm 0{\cdot}40$	$12{\cdot}27 \pm 0{\cdot}13$***
	Supernatant	$4{\cdot}49 \pm 0{\cdot}27$***	$7{\cdot}79 \pm 0{\cdot}78$	$10{\cdot}73 \pm 0{\cdot}25$***
	%	72·2	86·7	87·4

Activity values are means $\pm$ S.E. ($N = 5$).

% indicates ratio of activity of supernatant to the corresponding activity of cell free extract.

Cell-free extracts were obtained by centrifuging homogenates for 10 min at 1000 **g**. Supernatant was by centrifuging cell free extracts for 30 min at 35000 **g**. Preparation of homogenate for control is described in Materials and Methods.

, * Differ from the corresponding values of control at the 0·05 and 0·01 probability levels.

Acph locus in *D. virilis* by means of starch gel electrophoresis. Since these homozygous flies have exhibited one major band of acid phosphatase, staining intensity of the allozyme bands should correspond to the enzyme activity in whole flies. In this experiment, the ACPH activity of flies from these allozyme strains was examined. Table 1 shows ACPH activity in crude extracts and supernatants of adult flies. From the results of *t*-tests, the enzyme activities of crude extracts were statistically different among the three strains. The enzyme activity in the supernatant of Acph-4 flies, however, was more than four times that of Acph-1 and twice that of Acph-2. This activity ratio corresponds to that of the staining intensity of the allozyme bands (Narise, 1976). As shown in Table 1, this can be attributed to the fact that 58% of the activity in the crude extract from the Acph-4 strain was found in the supernatant, compared with only 24% from Acph-1 and

35 % from Acph-2 strain, respectively. It may be suggested from these results that strain variation in ACPH activity detected by electrophoretic technique is dependent on differences in amount of the soluble form of the enzyme.

The strain difference in the soluble activity of the allozymes was also observed when these allozymes were extracted under conditions which affect the structure of cellular organelles. As shown in Table 2, in the Acph-1 and Acph-2 strains, the addition of 0·25 M sucrose to the homogenizing buffer for protecting the organelles from osmotic shock had no effect on enzyme activity in both cell free extracts and supernatants, while in Acph-4 strain enzyme activities in both fractions were considerably reduced. On the other hand, when 0·5 % Triton X-100 (which had no effect on the enzyme activity *in vitro*) was added in order to lyse the organelles, the enzyme activity in cell free extracts as well as in supernatants increased significantly in the three strains. The activity ratios of supernatant to cell free extract increased in the Acph-1 and Acph-2 strains, but did not change in the Acph-4 strain. This indicates that almost all the ACPH[4] allozyme has been released to the cytoplasm even without structural damage of cell organelles by the detergent. ACPH[2] allozyme was almost all released by the addition of Triton X-100, but the effect of Triton on ACPH[1] allozyme was rather small. These results suggest that ACPH allozymes are differentially released from the organelles.

Intracellular distribution of acid phosphatase allozymes

In order to discover the intracellular location of the allozymes, the intracellular distribution of the enzyme was investigated. Homogenates from fresh flies of each strain were fractionated by differential centrifugation and the ACPH activity of each fraction was determined (Table 3). Distribution of the enzyme activity in the Acph-1 strain among four fractions (cell debris and nuclei, mitochondria and lysosomes, microsomes and soluble) is similar to that in the Acph-2 strain. In contrast, in the Acph-4 strain, higher activity was observed in the soluble fractions and lower activity in the particulate fractions.

Furthermore, the enzyme localization was examined by means of density gradient centrifugation. A combined cytoplasmic fraction was centrifuged through a 0·4–2·0 M non-linear sucrose gradient and fractions after centrifugation were assayed for ACPH and succinate oxidase, a marker enzyme for mitochondria. The typical pattern of enzyme distribution in the Acph-2 strain is shown in Fig. 1. ACPH activity was found in a single peak. The peak corresponds to lysosomes on the basis of the succinate oxidase distribution and sucrose concentration. The activity at the top of the gradient is probably soluble and released from lysosomes during centrifugation. Fig. 2 shows results of a similar experiment using the Acph-4 strain. The ACPH activity was greatly reduced in lysosomal fractions and increased in soluble fractions as compared with the Acph-2 strain. However, the activity of the mitochondrial enzyme was similar.

Solubility differences of acid phosphatase allozymes

The data described above suggest that ACPH allozymes reside mainly in lysosomes and ACPH[4] enzyme is most easily solubilized. To obtain direct evidence

Table 3. *Intracellular distribution of acid phosphatase activities in ACPH allozyme strains*

Strains		Homogenates	Cell debris and nuclei	Mitochondria and lysosomes	Microsomes	Soluble	Recovery (%)
Acph-1	Activity	6·43 ± 0·38	1·92 ± 0·12	2·14 ± 0·25	0·59 ± 0·04	1·14 ± 0·11	
	%	100	29·9	33·3	9·2	17·7	90·1
	RSA	1	1·06 ± 0·02	1·77 ± 0·25	2·02 ± 0·30	0·53 ± 0·08	
Acph-2	Activity	8·16 ± 1·02	2·16 ± 0·18	2·54 ± 0·36	0·71 ± 0·03	1·89 ± 0·30	
	%	100	26·5	31·1	8·7	23·1	89·4
	RSA	1	1·06 ± 0·14	1·52 ± 0·22	1·67 ± 0·06	0·65 ± 0·05	
Acph-4	Activity	10·38 ± 1·48	1·66 ± 0·49	1·87 ± 0·61	0·42 ± 0·03	5·23 ± 0·37	
	%	100	16·0	18·0	4·0	50·4	88·4
	RSA	1	0·55 ± 0·08	1·04 ± 0·19	1·27 ± 0·02	1·16 ± 0·08	

Activity is expressed as μmol p-nitrophenol/min/g flies.

RSA (relative specific activity) means specific activity of each fraction divided by that of homogenate in respective strains. Each value is mean ± S.E. ($N = 5$).

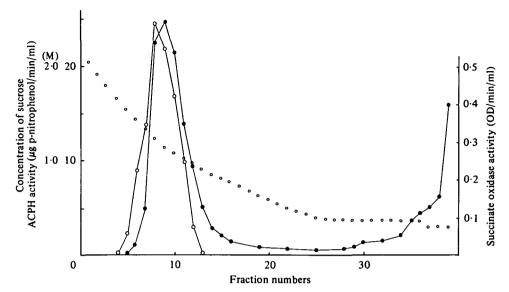Preparation procedures are described in Materials and Methods.

Fig. 1. Distribution of ACPH after sucrose-density gradient centrifugation of cytoplasmic particles from Acph-2 strain. The cytoplasmic particles (1·8 ml) were layered over a sucrose-density gradient and centrifuged at 80000 *g* for 1 h. Each 1 ml fraction was collected and assayed for acid phosphatase and succinate oxidase activities. Total acid phosphatase activity in cytoplasmic particles used was 1·42 units and recovery was 88%. ●, Acid phosphatase activity; ○, succinate oxidase activity; °, sucrose molarity.
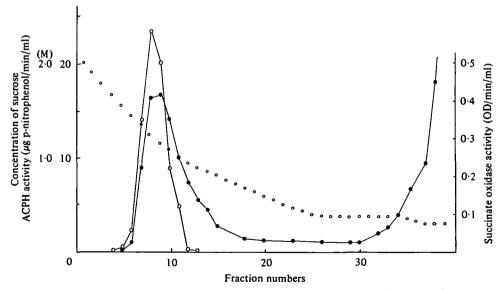

Fig. 2. Distribution of ACPH after sucrose-density gradient centrifugation of cytoplasmic particles from Acph-4 strain. Procedures and symbols are identical to those of Fig. 1. Total enzyme activity used was 1·90 units and recovery was 80%.

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for this, solubility of the allozymes from lysosomal fractions to supernatant was compared among three strains according to the method described in Materials and Methods. The percentages of released activity to the total activity of lysosomal suspension were illustrated in Table 4. In the presence of sucrose, the activity solubilized from lysosomes was low and similar among the strains, but, the activity

Table 4. *Release of ACPH allozymes from lysosomes*

Strains	Number of experiment	Treatment	
		With sucrose	Without sucrose
Acph-1	7	$10\cdot2 \pm 1\cdot46$	$20\cdot1 \pm 1\cdot83$
Acph-2	10	$9\cdot7 \pm 2\cdot10$	$22\cdot0 \pm 3\cdot40$
Acph-4	10	$10\cdot0 \pm 1\cdot10$	$27\cdot2 \pm 1\cdot49$***

Numerals show percentages of supernatant activity to total activity in lysosomal suspension after treatment with and without sucrose for 1 h at 0 °C. Each value is mean ± s.e.
*** Differ from the corresponding values of Acph-1 and Acph-2 at the 0·01 probability level (For statistical analysis, Arc sin transformation was made and *t*-test was conducted).

Table 5. *The ratio(%) of soluble enzyme to total enzyme activity in cell free extracts from ACPH allozyme strains*

Enzymes	Number of experiment	Acph-1	Acph-2	Acph-4
Acid phosphatase	8	$63\cdot8 \pm 5\cdot0$	$69\cdot4 \pm 5\cdot7$	$81\cdot0 \pm 5\cdot4$***
α-Glucosidase	8	$71\cdot5 \pm 9\cdot4$	$76\cdot7 \pm 4\cdot7$	$78\cdot8 \pm 5\cdot0$
β-Galactosidase	5	$86\cdot1 \pm 3\cdot7$	$91\cdot7 \pm 3\cdot7$	$87\cdot6 \pm 8\cdot6$
β-Glucuronidase	5	$89\cdot4 \pm 4\cdot7$	$87\cdot0 \pm 7\cdot7$	$84\cdot3 \pm 4\cdot0$

Each value is mean ± s.e.
*** Differ from Acph-1 and Acph-2 at the 0·01 probability level (for statistical analysis, *t*-test was conducted after Arc sin transformation was made).

increased in the absence of sucrose and the activity of Acph-4 was highest among the strains. This means that the allozymes are differentially released from lysosomes and also raised the question whether this phenomenon is caused by the enzyme itself or by lysosomes. If the release of the allozymes is due to breakage of lysosomes, other enzymes contained in lysosomes should also be released in the same manner as observed in ACPH allozymes. Therefore, the release of α-glucosidase, β-galactosidase and β-glucuronidase was examined. Homogenates prepared in 0·25 M sucrose, after removal of cell debris and nuclei, were centrifuged at 35000 *g* for 30 min and ratios of the enzyme activity in the supernatant to the total activity were compared with that of ACPH. (Table 5). Though a large percentage of these enzymes was found in supernatant, there were no differences between allozymes strains as seen in the case of ACPH.

4. DISCUSSION

The results of this study showed that ACPH allozymes in *D. virilis* are mainly located in the lysosomes and solubilized from lysosomes in varying degrees

depending on allelic differences at the *Acph* locus, and consequently display different cytosolic activities.

It was not possible to demonstrate whether all ACPH is located in the lysosomes. Table 3 indicates considerably broad distribution of the enzyme into subcellular fractions, although a high relative specific activity was found in mitochondrial and lysosomal fraction. However, it is clear from Figs. 1 and 2 that ACPH localized in the lysosomes is partly released to cytoplasm. In fact, histochemical study has already shown that acid phosphatase-1 activity in *D. melanogaster* is localized to organelles having morphological characteristics of lysosomes (Sawicki & MacIntyre, 1978). DeAraujo, Mies & Miranda (1976) separated two acid phosphatases from rat, of high molecular weight and of low molecular weight. The former possessing molecular weight of 100000 is of lysosomal origin and the latter possessing that of 30000 is restricted to the cell sap. Narise (1984) has reported that ACPH^2 allozyme purified from *D. virilis* has molecular weight of 100000 which is composed of two subunits and corresponds to the high molecular weight acid phosphatase in mammals on the basis of inhibition study. Although it is still possible that there would be two forms, one is lysosomal and another cytosolic, what is certain from the present study is that the solubility of the allozymes from lysosomes varies among the allozyme strains. This difference is likely due to the enzyme, not to the lysosomes (Tables 4 and 5).

The mechanism of the organization of acid hydrolases into lysosomes has been studied. Cultured fibroblasts of patients homozygous for a single gene mutation, the I-cell disease, exhibit deficiency of lysosomal hydrolases in contrast to excess of the same enzyme activities in culture media. (Hickman & Neufeld, 1972; Neufeld, Lim & Shapiro, 1975). Neufeld and colleagues (1975) suggested on the basis of these and other results that the enzymes produced by these patient's fibroblasts lack a recognition marker for entry into lysosomes and carbohydrate residues of glycoproteins function as recognition markers. Later, phosphohexose has been identified as a recognition site, and treatment of the hydrolase with alkaline phosphatase has resulted in change of both electrophoretic mobility and uptake by fibroblasts (Kaplan, Achord & Sly, 1977; Natowicz *et al.* 1979). In addition, for lysosomal enzymes, many of which are glycoproteins, it has been reported that treatment with neuraminidase alters electrophoretic mobility (Robinson & Stirling, 1968; Ostrowski *et al.* 1970; Dizik & Elliott, 1977) by removal of terminal neuraminic acids. Dizik & Elliott (1977) showed that such a post-translational modification is inherited in a dominant fashion. ACPH^2 allozyme is a glycoprotein containing 20 % neutral sugars and a low content of hexosamines (Narise, 1984). However, ACPH allozymes show codominant inheritance (Ohba, 1977), suggesting mutation of a structural gene.

Meanwhile, Neuberger *et al.* (1972) have reported that carbohydrate chains in mammalian glycoproteins is linked to the polypeptide chains through asparagine residues. If this is applicable to ACPH, conversion of asparagine to the other amino acid caused by mutation of structural gene would disturb the linkage of carbohydrate chains. Consequently, integration of the enzyme into lysosomes would also be hindered. One observation which may be relevant in this regard is that ACPH^4 allozyme has less amount of neutral sugars than does ACPH^2 (Narise,

Unpublished data). Alternatively, it is probable that alteration of enzyme(s) which catalyse the formation of the carbohydrate-polypeptide linkage would result in the same phenomenon, suggesting mutation of gene(s), not identical to the structural gene. Detailed study of the carbohydrate composition and amino acid sequence of ACPH[4] as well as a more detailed genetic study will be required.

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