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Received 18 June 2001; accepted 17 December 2001.

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Quantitative Analysis of Phenol Oxidase Activity in Insect Hemolymph

BioTechniques 32:815-823 (April 2002)

ABSTRACT

*We describe a simple, inexpensive, and robust protocol for the quantification of phenol oxidase activity in insect hemolymph. Discrete volumes of hemolymph from *Drosophila melanogaster* larvae are applied to pieces of filter paper soaked in an L-3, 4-dihydroxyphenylalanine (L-DOPA) solution. Phenol oxidase present in*

*the samples catalyzes melanin synthesis from the L-DOPA precursor, resulting in the appearance of a roughly circular melanized spot on the filter paper. The filter paper is then scanned and analyzed with image-processing software. Each pixel in an image is assigned a grayscale value. The mean of the grayscale values for a circular region of pixels at the center of the image of each spot is used to compute a melanization index (MI) value; the computation is based on a comparison to an external standard (India ink). Numerical MI values for control and experimental larvae can then be pooled and subjected to statistical analysis. This protocol was used to evaluate phenol oxidase activity in larvae of different backgrounds: wild-type, lozenge, hopscotch (tumorous-lethal (which induces the formation of large melanotic tumors), and body-color mutations ebony and yellow. Our results demonstrate that this assay is sensitive enough for use in genetic screens with *D. melanogaster* and could conceivably be used for evaluation of MI from hemolymph of other insects.*

INTRODUCTION

The phenol oxidase enzyme catalyzes the synthesis of melanin from any of several precursor molecules, including L-3, 4-dihydroxyphenylalanine (L-DOPA) (1,17). It has been demonstrated that the blood (hemolymph) of many insects, including the model system insect *Drosophila melanogaster*, contains phenol oxidase, either carried within specialized blood cells (hemocytes) and/or dissolved in solution (8). In *D. melanogaster* larvae, these specialized hemocytes are called crystal cells. In insects, melanin synthesis has been linked to several key biological processes, including sclerotization and darkening of cuticle and wings (15,16). Melanization has also been implicated in humoral recognition of foreign pathogens, wound healing (1,3,17), and melanotic encapsulation of, and cytotoxic effects on, self and nonself tissue (9). However, the exact role of melanin in these processes and the genetic and regulatory mechanisms that govern its synthesis are only partially understood. It should be possible to identify the genes involved in the regulation of melanin synthesis, transport, and deposition by screening large numbers of novel genetic back-

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grounds with a rapid biochemical assay. One previously described assay makes use of a spectrophotometer to detect melanin synthesis in solution (7,14). This protocol has the advantage of being quantifiable but is unwieldy when used to screen large numbers of samples. A simpler, qualitative alternative is the "spot test", in which insect hemolymph is applied to filter paper in the presence of L-DOPA, and the quality of the melanized spot that appears is noted (2, 12,14). However, because the spot test is qualitative in nature, it only allows comparison of the most obviously different samples. Here we use a commercially available image scanner to quantify the darkness of melanized hemolymph samples for comparison to an external standard. We present a simple, low-cost, and quantifiable method capable of screening large numbers of insect hemolymph samples with sufficient acuity as to detect 2-fold differences in phenol oxidase activity. With appropriate modifications, this protocol should also prove to be useful in systems other than *D. melanogaster* and possibly for any chromatographic assay.

MATERIALS AND METHODS

Fly Stocks

Canton S, *y v hop^{Tum-l/Basc}* (C. Dearolf), *e*, *y cv v f*, *amx¹ lz⁸ v¹/Binsinscy* (generated from *amx¹ lz⁸ v¹/C(1) DX y¹ f¹*), and *lz^K* were all from Bloomington Stock Center (Bloomington, IN, USA). For explanations of genotypes, see Flybase (<http://flybase.bio.indiana.edu/>).

Egg Laying

Adult flies were permitted to lay eggs at 25°C for 2 or 6 h (24 h for *lz* stocks) on a standard yeast/agar/cornmeal/sucrose medium sprinkled with dry yeast. Temperature shifts were performed at 48 h after the midpoint of the egg-laying period.

Hemolymph Samples

Larvae were examined at a time at which most were in the feeding L3 stage. *Canton S*: 18°C at 10 days; 25°C

at 5 days; and 29°C at 5 days. *y v hop^{Tum-l/Basc}*: 18°C at 11 days; 25°C at 6 days; and 29°C at 4 days. *lz* stocks: 25°C at 6 days; all other stocks: 25°C at 5 days. Live larvae were washed twice in PBS, then once in 95% ethanol, and transferred to an ethanol-sterilized glass slide. Using two pairs of fine forceps (Style no. 5; Sigma, St. Louis, MO, USA), the larval cuticle was torn, allowing hemolymph to pool around the larval carcass. For each sample, enough larvae were simultaneously bled so as to produce a 1-μL aliquot of hemolymph (the number of larvae needed to produce 1 μL hemolymph varied: *Canton S*: 18 and 25°C, 2–3 larvae; 29°C, 3–5 larvae; *Basc/Y*: 18°C, 4 larvae; 25°C, 4–10 larvae; 29°C, 3–5 larvae; *hop^{Tum-l/Y}*: 18°C, 3–5 larvae; 25°C, 3–7 larvae; 29°C, 2–6 larvae; *e*: 3–7 larvae; *y cv v f*: 3–4 larvae; and *lz*: 5 larvae).

Melanization Assay

Single (1-μL) aliquots of hemolymph were immediately transferred to small pieces of white filter paper (Whatman No. 52) soaked in 2 mg/mL L-3,4-dihydroxyphenylalanine (L-DOPA; Sigma) in a 10 mM sodium phosphate buffer (pH 6.6). Application of the samples to filter paper was performed with the pipettor held perpendicular to the filter paper; samples were gently and slowly applied to the filter paper to avoid bubbling, splashing, and other uneven distributions of hemolymph. Samples were kept for 30 min at room temperature. To minimize error associated with the formation of layers of melanin product, which would result in an underestimation of the strength of a melanization reaction, we kept filter paper moist by rewetting with more L-DOPA solution (not water) as necessary, so as (i) to allow phenol oxidase molecules to continuously diffuse away from the center of the spot and (ii) to provide an excess of L-DOPA substrate. After 30 min, samples were transferred to a clean paper towel and allowed to air-dry at room temperature.

Quantification of Melanization

Multiple pieces of filter paper were placed facedown on an AGFA Duoscan™ T1200 scanner (Agfa-

Gevaert, Mortsel, Belgium). Individual pieces of filter paper, after being soaked in L-DOPA solution and then air-dried, do not remain flat but become irregularly warped. This warping results in unpredictable alterations in the detection of the darkness of individual melanized hemolymph spots, as well as an overall darkening of the image. To accentuate any color contrast between the melanized hemolymph spot and the rest of the filter paper, several sheets of clean white paper were placed over the samples. We then ensured that samples were completely flattened against the scanner glass by placing a heavy ream of paper on top of the clean white paper layer. Samples were scanned using AGFA Fotolook™ 32 V3.00.07 (settings: original, reflective; mode, grayscale; bits per color, 8 bits; input, 200 ppi; scale to, 100%; range, automatic; tone curve, none; sharpness, high (60%); descreen, none; and flavor, none). Images of each sample were then examined using Corel® Photopaint® 8.0. For each sample, we obtained the mean grayscale pixel value of a circular region (diameter, 20 pixels; area, 348 square pixels) at the visually estimated center of the melanized spot (see Figure 2C). Corel Photopaint 8.0 grayscale pixel values are a scale from 0 (completely black) to 255 (completely white). Because we wished to quantify darkness so that darker samples would have higher "darkness values", we reversed the gradient of the scale by subtracting the mean (of 348 pixels) grayscale pixel value from 255, resulting in a raw mean pixel value (RMPV) ranging from 0 (completely white) to 255 (completely black). Filter paper soaked in L-DOPA for 30 min, but to which hemolymph was not added (i.e., negative controls), had an RMPV of 0.00 ($n = 30$). RMPVs were then converted to melanization index (MI) values: $MI = MI = \{e^{[(RMPV - 277.62) / 43.627]}\} * 100\%$. This formula represents the natural logarithmic function (as calculated by Microsoft® Excel® 2002) that best represents (by least-squares method) the RMPVs obtained for the smallest series of India ink samples that completely contained the range of RMPVs obtained for all hemolymph values (see Figure 1B). The correlation coefficient r was calculated

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as: $r = n(\sum xy) - \sum x \sum y / \{[n \sum x^2 - (\sum x)^2][n \sum y^2 - (\sum y)^2]\}^{1/2}$. It is important to note that this algorithm allows detection of 2-fold differences in darkness. Assay sensitivity can be increased by modifying spot images through user-defined parameters associated with a given image-processing program.

The MI value thus represents the average darkness of a sample as a percentage of the average darkness of undiluted India ink (Faber-Castell Waterproof drawing ink no. 4415). Mean MI (MMI) values for each class of larvae were compared by modified Student's *t* test:

$$t = (X_1 - X_2) / ((s_1^2/n_1) + (s_2^2/n_2))^{1/2}$$
$$v = \{[(s_1^2/n_1) + (s_2^2/n_2)]^2 / [(s_1^2/n_1)^2 / (n_1 + 1)] + [(s_2^2/n_2)^2 / (n_2 + 1)]\} - 2.$$

where *X* is the mean, *s* is the standard deviation, and *n* is the sample size.

Differences between MMI values were considered significant if *P* < 0.01.

RESULTS AND DISCUSSION

Standardization of a Darkness Scale

Comparison of the melanization capacities of different hemolymph sam-

ples requires an external scale against which samples can be measured. The Corel Photopaint 8.0 program assigns grayscale values to pixels on a scale that ranges from 0 (completely black) to 255 (completely white). Corel Photopaint 8.0 is also able to compute the mean of all individual pixel values within a user-defined region of a sample image. As our melanized hemolymph samples are roughly circular in shape, we made use of a circular field of 348 square pixels (200 ppi; diameter of circle is 20 pixels) at the approximate center (estimated visually) of each melanized hemolymph sample. The mean grayscale value for the 348 pixels was computed by Corel Photopaint 8.0 and then subtracted from 255 to provide an RMPV. This had the effect of reversing the scale so that the darker the sample, the higher the RMPV (0, completely white; 255, completely black).

However, the relationship between the perceived darkness of any point in a sample and its assigned grayscale value is not necessarily linear; that is, a pixel with an original grayscale value of 200 is not necessarily half as dark as a pixel with an original grayscale value of 100. To determine the manner in which our scanner (AGFA Duoscan T1200; see

Materials and Methods section) assigned grayscale pixel values, we scanned samples of filter paper onto which had been placed 1-μL aliquots of a series of dilutions of India ink. The use of an external standard such as India ink allowed us to produce spots with little variation in the magnitude of their darkness. Mean RMPVs for three aliquots at each dilution level (dilution levels range from undiluted to a dilution factor of 2⁻¹², in decrements of powers of 2) are presented in Figure 1A. When plotted on a semilogarithmic (base 10) set of axes, a clear S-curve pattern emerges. Thus, the function of the relationship between the detected darkness of a sample and its assigned grayscale pixel value is indeed not linear.

The function of the relationship depicted in Figure 1A also displays local variation in slope that could decrease the accuracy of predictions made with our scanner. We reasoned that if our hemolymph samples produced spots with RMPVs in a region of the function that is relatively constant in slope, then a relationship between the RMPV of a sample and a darkness scale as defined by an India ink standard could be inferred. The lowest mean RMPV for a larval class was 65.90, and the highest

Figure 1. Derivation of MI function using India ink standard. (A) Standard curve for mean RMPV of India ink: spots on filter paper produced by 1-μL aliquots of a series of dilutions of India ink were assayed for RMPVs (see Materials and Methods). Horizontal (x) axis: dilution factor of India ink on a base-10 logarithmic scale; we tested dilution factors ranging (in increments of powers of 2; see also Figure 2A) from 2⁻¹² to 2⁰ (undiluted). Vertical (y) axis: mean RMPV on a linear scale; each point represents the mean RMPV of three spots produced by a given dilution of India ink; standard deviation is indicated by error bars. In all cases, the mean RMPV for each dilution factor was significantly different from the value above and below it (*P* < 0.01; Student's *t* test). (B) Standardization of the MI within the hemolymph range of values: a closer view of the smallest portion of the function described in Figure 1A that includes the entire range of all RMPVs from individual hemolymph samples, regardless of class. The best-fit function and *r* value are included. Dotted lines represent the boundary of a 99% confidence interval for the best-fit function, within which lie all data points. The MI of a single sample is the *x* value in the equation multiplied by 100 and represents the average darkness of the center region of a melanized spot as a percentage of the average darkness of undiluted India ink.

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was 247.54. These two extreme hemolymph values are contained within the region of the function bounded by India ink diluted by a factor of 2^{-7} (RMPV = 44.78), and that of undiluted India ink (RMPV = 254.66). This region of the function, as well as a best-fit equation, is depicted in Figure 1B. The best-fit equation and all data points are within the region defined by a 99% confidence interval, and the correlation coefficient has a value of $r = 0.9805$ (see Materials and Methods), indicating that the derived function is a very close fit of the data. Thus, for the range of RMPVs encountered in our assay, the best-fit function is an excellent approximation of the relationship between a sample's RMPV and its darkness relative to the India ink standard. MI values for each sample can then be obtained by rewriting the best-fit equation as: $MI = \{e^{[(RMPV - 277.62) / 43.627]}\} * 100$.

Melanization Capacity of Hemolymph Samples

Using our melanization assay, we tested the ability of larval hemolymph samples obtained from wild-type and mutant strains to catalyze melanin synthesis, as indicated by the average darkness of a spot produced by introducing hemolymph to an excess of L-DOPA (see Materials and Methods). Because of the variability in the amount of hemolymph that can be obtained from any single larva and because the actual amount of hemolymph from a single larva is often too small for efficient manipulation by pipettor, we decided to dissect as many larvae as were necessary (*Canton S* at 25°C: 2–3 larvae; for other larval classes, see Materials and Methods) to obtain a standard volume of 1 μ L. Upon visual inspection, wild-type melanized spot samples are relatively uniform in appearance, and examples from wild-type (*Canton S* and *Basc/Y*) larvae raised at 25°C are presented in the middle column of Figure 2. The MMI value for *Canton S* 25°C samples (quantified as described above) is 46.23 ± 5.11 (Figure 3); therefore, hemolymph from *Canton S* larvae raised at 25°C produces melanized spots whose darkest regions are, on average, 46.23% as dark as undiluted India ink. Sample quality is highly

reproducible: 30 *Canton S* samples, each obtained from the hemolymph of 2–3 larvae, exhibited a relatively narrow range of MMI values (34.17–55.33) and a standard deviation value that is 11.06% of the MMI value (Figure 3). This ratio of variation is very low for a biological system. *Basc/Y* 25°C samples exhibited an MMI value (45.19 ± 6.05) that was statistically indistinguishable from that of *Canton S* at 25°C. Finally, our protocol allowed us to quickly produce the large numbers of samples (in the majority of cases, at least 30 samples/larval class) necessary for sound statistical analysis.

To determine whether this assay

would be able to detect alterations in melanization capacity that result from widely varying pathologies, we examined the melanization capacities of *lozenge*, *yellow*, *ebony*, and *hopscotch-Tumorous-lethal* mutant larvae.

Rizki and Rizki (13,14) observed that *lz^{ffg}* mutants, which do not have crystal cells, are also deficient in phenol oxidase activity. An additional allele, *lz^g*, also has a drastically reduced population of crystal cells (Govind and Leung, unpublished observations). Interestingly, extracts from *lz^g* pupae are also deficient in the ability to catalyze melanin synthesis (10). Therefore, we determined the melanization capacity

Figure 2. Melanized spots. (A) Examples of spots produced by 1- μ L aliquots of a series of dilutions of India ink. Dilution factor is noted above or below each spot. (B) Examples of melanized spots produced by 1- μ L aliquots of hemolymph taken from *D. melanogaster* larvae of different classes. Rows: top, *Canton S*; middle, *Basc/Y*; bottom, *hop/Tum-I/Y*. Columns: left, 18°C; middle, 25°C; right, 29°C. Images in parts A and B are to the same scale (hemolymph samples spread farther). Note the significantly lighter spots produced by hemolymph from *hop/Tum-I/Y* larvae raised at 25°C and 29°C. (C) The portion of the image that is subjected to analysis (a circular region 20 pixels in diameter) has been removed from the image of the *Canton S* 25°C spot from part B.

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of hemolymph from *lz^g/Y* larvae and found that they exhibited an extremely low MMI of 2.63 ± 4.82 , a value just over 6% that of *Binsinscy/Y* sibling controls (41.93 ± 8.80 ; Figure 3). It is worth noting that the *Binsinscy/Y* internal control value is indistinguishable from that of *Canton S*. In contrast, the MMI of *lz^K/Y* larvae, which exhibit no major defects in melanin synthesis (11) and no reduction in crystal cell population (Govind and Leung, unpublished observations), is in the wild-type range of values, at 44.71 ± 5.74 (Figure 3).

Having validated our protocol, we tested the possibility that mutations that alter the melanization patterns of the adult cuticle could also have an effect on the melanization capacity of larvae carrying the same mutation. We obtained MMI values for hemolymph from larvae carrying the mutations *yellow* (*y*; adult cuticle is yellow instead of the wild-type brown) and *ebony* (*e*;

adults have black cuticle). The *y cv v f* samples produced an MMI value (46.68 ± 7.27 ; Figure 3) indistinguishable from that of *Canton S*, whereas *e* samples exhibited an MMI of 27.64 ± 12.68 , a highly significant ($P < 0.001$) 40% reduction in hemolymph melanization capacity (Figure 3). This novel result suggests that the same processes that regulate melanin synthesis, transport, and deposition in the cuticle may also be at work in larval hemolymph. Interestingly, the *e* gene encodes a putative β -alanine-dopamine synthetase (4,5), and its roles in recognition, wound healing, or encapsulation bear further investigation.

To investigate a possible link between the formation of melanotic encapsulation of self tissue and the melanization capacity of larval hemolymph, we tested *hopscotch^{Tumorous-lethal}/Y* (*hop^{Tum-l}/Y*) mutants. The *hop^{Tum-l}* mutation is temperature-sensitive, dominant, and

Figure 3. Comparison of MMIs. Numerical MMI values for hemolymph samples from larvae of different classes are indicated to the right of each bar; sample sizes (number of spots, not number of larvae) are in parentheses. Standard deviations are represented by error bars. Shaded bars represent temperature at which larvae were raised: 18°C, white; 25°C, light gray; 29°C, dark gray. Solid bars represent wild-type and control larvae; spotted bars represent mutant larvae. Significant differences from controls, as determined by modified Student's *t* test (see Materials and Methods) are indicated: *, $P < 0.001$. *Canton S* (wild-type) MMI values are similar at 18°C, 25°C, and 29°C; MMIs for control classes (*Base/Y* and *Binsinscy/Y*) are indistinguishable from *Canton S* values. *ebony* larvae exhibit a significant 40% reduction in MMI (compared to *Canton S* value), whereas another body-color mutation (*yellow*) has no effect on MMI. *hop^{Tum-l}* reduces MMI values by over 50%; this effect is temperature-linked and parallels the perceived qualitative difference in Figure 2B. *lz^g* shows the strongest effect, reducing MMI to nearly zero. Sample quality in our assay is highly reproducible. Standard deviations, indices of sample variation within a class of larvae, are generally in the range of 10%–15% of the values of their respective means, which is very low for a biological system. Mutant organisms often exhibit greater magnitudes of phenotypic variation; thus, it is not surprising that standard deviations are higher for mutant classes for which we observed significant reductions in MI (*hop^{Tum-l}/Y* at 25°C and 29°C, *lz^g/Y*, *e*).

semilethal; it causes overproliferation of larval hemocytes and the encapsulation by hemocytes of self tissue, which then melanizes (6). However, its effect on crystal cells is unknown. As the *hop^{Tum-l}* mutation is temperature-sensitive (effects increase in magnitude with increasing temperature), we carried out tests on hemolymph samples from *Basc/Y* and *hop^{Tum-l}/Y* siblings raised at 18°C, 25°C, and 29°C. *Canton S* samples are similar at all three temperatures (Figure 2B, top row), and *Basc/Y* samples, as expected, follow the same pattern (Figure 2B, middle row). The MMI values for *Canton S* and *Basc/Y* larvae are statistically indistinguishable (Figure 3), consistent with visual appraisal (Figure 2B). However, while samples from *hop^{Tum-l}/Y* raised at 18°C are indistinguishable from controls, those from larvae raised at 25°C and 29°C are noticeably lighter in appearance (Figure 2B, bottom row). Consistently, MMI values for these larvae show a highly significant reduction ($P < 0.001$) in average darkness to about half that of *Canton S* and *Basc/Y* samples (Figure 3). Thus, as indicated by both *Canton S* and *Basc/Y* samples, temperature appears to have no effect on MMI. Furthermore, there is a temperature-linked effect on MMI by the temperature-sensitive *hop^{Tum-l}* mutation. However, it is worth noting that when raised at 18°C our *hop^{Tum-l}/Y* larvae displayed nearly as great a number and size of melanized encapsulations as those exhibited by mutant larvae raised at 29°C (results not shown). Therefore, the presence of melanized encapsulations in larvae does not necessarily correlate with a reduction in MMI.

There are at least four genes encoding prophenol oxidases present in the *D. melanogaster* genome: *Black cells* (*Bc*, monophenol monooxygenase), *Dox-A2* [*l(2)37Bf*; diphenol oxidase-A2], *Dox-A3* [*freckled*; diphenol oxidase-A3, and *CG8193* (these genes are described on Flybase at <http://flybase.bio.indiana.edu/>)). However, the spatial expression of these genes and precise biological functions of the enzymes they encode are unknown. The use of different mutants, substrates, and enzyme-specific inhibitors can be incorporated in this protocol to understand the biological role of melanization in *D. melanogaster*.

In conclusion, this assay is useful in

several ways. Screening large numbers of samples for alterations in phenol oxidase activity (as a result of genetic background or experimental manipulation) is feasible and takes less time to quantify than standard spectrographic techniques. Furthermore, it is reasonable to assume that this assay could be used on any fluid containing phenol oxidase, such as hemolymph from insects other than *D. melanogaster*, and even homogenized tissue samples. Additionally, while we chose India ink as our standard, it is certainly possible to standardize a scale of darkness generated by other reagents and enzymes. Finally, it is possible that any chromatographic assay that produces a colored precipitate can be standardized and quantified in a similar manner.

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This work was supported by funds from the American Heart Association, Heritage Affiliate, Inc., and American Cancer Society grant no. RPG 98-228-01-DDC. Support from the NIH-RCMI RR03060-16, CRS, and PSC-CUNY is also gratefully acknowledged. Address correspondence to Dr. Shubha Govind, Graduate School and University Center of the City, University of New York, 138th Street and Convent Avenue, New York, NY 10031, USA. e-mail: sgovind@scisun.sci.ccny.cuny.edu

Received 26 September 2001; accepted 2 January 2002.

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