A Commentary on Experiments with Tyrosinase

Rob L. Dean

The enzyme tyrosinase (polyphenol oxidase) occurs in a wide variety of organisms. In vertebrates, it is involved in the synthesis of melanin, a pigment responsible for the color of skin, hair and eyes. In insects, tyrosinase catalyses the synthesis of phenols involved in tanning the cuticle and also participates in wound repair. In plants and fungi, this enzyme converts phenols, which are liberated at wound sites, to quinones. Since quinones are toxic to microorganisms, this response probably functions to prevent wound infection.

Tyrosinase catalyzes two types of reactions: **(1)** the aerobic hydroxylation of a phenol in the ortho position and **(2)** the oxidation of a catechol to its corresponding ortho-quinone (Ingraham 1966). The latter activity, using L-dihydroxyphenylalanine (L-DOPA) or catechol as substrate, has been exploited in a variety of undergraduate laboratory exercises (e.g. Bowen & Baxter [1980], Boyer [1993] and Stewart [1991]). However, assays with tyrosinase present a few challenges not encountered in simpler systems, such as the hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase. For example, the reaction with L-DOPA is linear for only a brief time while that with catechol only becomes linear after a lag phase. A second problem is quantitation because the colored oxidation products of both L-DOPA and catechol are unstable.

For the past year I have been using a potato tyrosinase preparation in my section of a mandatory second year laboratory course, Scientific Methods in Biology (Biology 290). In this paper I will present the methods we have used to isolate the enzyme and to assay its activity. Throughout the paper I refer to methods given by others and give reasons why I have adopted or modified them. Finally, I suggest a new laboratory exercise using tyrosinase for those instructors who have a taste for adventure in class because the outcome is variable but frequently dramatic.

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Equipment

- Spectrophotometers and cuvettes
- Domestic blender
- Volumetric pipets
- Variable volume micropipets (10-100 µl) ideally (or disposable glass microcapillary pipets)
- Parafilm™
- Clinical centrifuge (ideally in a cold room. I keep mine in a refrigerator.)
- Beakers
- Ice bucket

Stock Solutions

1. Reaction buffer, 20 mM sodium phosphate buffer (pH 6.5). Made by titrating 20 mM Na_2HPO_4 into 20 mM $NaH_2PO_4 \cdot H_2O$ until the required pH is obtained.
2. Substrate, 7.3 mM L-dihydroxyphenylalanine (L-DOPA) (Sigma) in distilled water.
3. Sodium fluoride, 25 mM in distilled water.
4. Saturated and half-saturated ammonium sulphate in distilled water.

Safety Note: Sodium fluoride is toxic if ingested. Phenolic compounds like L-DOPA and catechol are toxic if ingested and can also cause eczematous dermatitis on skin contact or severe eye inflammation. They should therefore be handled with care and disposed of in an appropriate manner.

Tyrosinase Preparation

If you plan to purchase the enzyme, Boyer (1993) gives a good account of how to use it. If not, the following protocol has been reliable.

Step 1. Weigh a peeled potato (for a class with 10 groups of students, I usually use 60 g). Cut into small cubes and add to an ice-cold 25 mM solution of sodium fluoride (1 ml NaF solution/g of potato) in a blender cup that has been stored in the freezer. Blend for 1 minute at full speed.

Step 2. Filter the blendate through one layer of Miracloth™ (Calbiochem) into a beaker on ice.

Step 3. Transfer a volume of filtrate to another beaker on ice and add an equal volume of saturated ammonium sulphate solution while stirring with a glass rod (Bowen & Baxter 1980).

Step 4. Transfer 12 ml aliquots of the mixture to 15-ml graduated conical centrifuge tubes. Centrifuge at 1300 × g (full speed, setting #7 on my clinical centrifuge) for 10 minutes.

Step 5. Remove and discard the supernatant with a pasteur pipet.

Step 6. Resuspend each pellet to 12 ml with half-saturated ammonium sulphate. Centrifuge at 1300 × g for 10 minutes.

Step 7. Discard the supernatant with a pasteur pipet. Resuspend the pellet to 3 ml with 20 mM sodium phosphate buffer (pH 6.5) by repeated aspiration (15 cycles) with a pasteur pipet.

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Step 8. Centrifuge at 410 × g (setting #5) for 5 minutes to pellet the starch. Decant the supernatant, which is an opalescent solution containing tyrosinase-activity. Store on ice.

Commentary on the Steps in the Preparation Protocol

Step 1. Sodium fluoride is included in the protocol of Bowen and Baxter (1980) but not that of Stewart (1991). Since discussions with enzymologists have not provided a specific reason for its inclusion, and since sodium fluoride is toxic, I have prepared preparations of tyrosinase in its presence and absence with the hope that it could be eliminated. However, preparations from the same potato made with 25 mM NaF show ~ 40% greater activity (0.075 ± 0.006 [2 × S.E.M] μmoles product/minute, n = 4) than those made with distilled water (0.053 ± 0.003 [2 × S.E.M] μmoles product/minute, n = 4).

Step 3. Again, the ammonium sulphate precipitation is included in the protocol of Bowen and Baxter (1980) but not that of Stewart (1991). Ammonium sulphate precipitates some of the potato proteins, including tyrosinase. The majority of other types of compounds can therefore be discarded with the supernatant. I have tried three concentrations of ammonium sulphate to determine the optimal concentration; 75% saturated, 50% saturated (as described in Step 3), and 25% saturated. At the lowest concentration used, tyrosinase activity is barely detectable. The activities of preparations made with 75% and 50% saturated ammonium sulphate were 0.077 ± 0.009 and 0.073 ± 0.005 μmoles product produced/minute (± 2 × S.E.M, n = 4) respectively. The lack of significant difference indicates that the use of 50% saturated ammonium sulphate is appropriate.

Step 5. Do not pour off the supernatant as suggested in Bowen and Baxter (1980). The pellet is not sufficiently compacted to remain in the tube. The pellet produced has two components, a fine white powder at the bottom (probably starch as it produces an intense black color in the presence of Lugol's iodine) covered by a fine, whitish, flocculent material (protein). Preparations made in the presence of different ammonium sulphate concentrations produced different pellet volumes. At the lowest concentration (25% saturated) the pellet was ~ 0.1 ml and appeared to be exclusively starch. Pellets from 75% and 50% saturated $(NH_4)_2\ SO_4$ preparations were about 2 ml and 1 ml respectively, including the starch. Therefore, although the highest $(NH_4)_2\ SO_4$ concentration precipitates twice as much protein, the tyrosinase activity is associated with the protein fraction precipitated by 50% saturated $(NH_4)_2\ SO_4$.

Step 6. This step is to wash endogenous substrate out of the precipitated protein. It is not essential but allows the preparation to be stored for long periods on ice without discoloration. Preparations treated in this way are relatively stable. Activity declines to about 80% to 95% after 24 hours and to about 72% after three days of storage.

Step 7. Avoid introducing air into the preparation when dissolving the protein pellet in buffer. Foaming tends to denature the protein. At this stage, the protein containing tyrosinase activity is concentrated into a relatively small volume. This has two advantages: proteins are usually more stable in higher concentration and very small aliquots can be used in enzyme assays so that temperature changes caused by adding ice-cold enzyme preparations to room temperature buffered substrate mixtures are minimized.

The Basic Assay

Tyrosinase oxidizes L-DOPA, which is colorless, to dopaquinone, which is chemically rearranged to produce the reddish-orange product, dopachrome. The initial velocity (v_o) of the reaction is calculated in μmoles of product produced per minute from the increase in absorbance at 475 nm at timed intervals during the reaction. Since the reaction between tyrosinase and L-DOPA is linear for only a brief period of time (Behbahani et al. 1993), we read values from the spectrophotometer at 15 second intervals. We use analog spectrophotometers so it is easier to read percent transmittance (% T) values from the linear scale, to one estimated decimal place, than absorbance (A_{475}) values from the exponential scale. Percent transmittance values are then converted to A_{475} values from Equation 1:

$$A = \log_{10}\frac{1}{T}$$

(e.g. 92.1% T = 0.921 T ∴

$$A = \log_{10}\frac{1}{0.921} \quad (1)$$

$$= \log_{10} 1.086$$

$$= 0.0357 \text{ or } 0.036\ A_{475})$$

Reaction mixtures to which the tyrosinase preparation is added contain 1 ml of 20 mM sodium phosphate buffer (pH 6.5), a volume of 7.3 mM L-DOPA to give a final concentration of 2.4, 1.9, 1.7, 1.46, 1.2 or 0.97 mM (a satisfactory concentration range for a Lineweaver-Burk plot), and distilled water to bring the volume to 3 ml. The volume of enzyme to be added is determined empirically for each preparation (see below).

1. Use a reaction mixture without enzyme to adjust the needle on the spectrophotometer to 100% T.
2. Add a volume of enzyme preparation (typically ~30 μl).
3. Cover the tube with Parafilm™ and invert three times (do not shake) to mix the contents and return the tube to the spectrophotometer.
4. The % T value will be declining. When the % T value reaches a convenient whole number (e.g. 93% or 89% T), start timing. Record this % T value at 0 seconds and % T values at 15-second intervals for 2 minutes.

The amount of enzyme preparation to be added to reaction mixtures is determined by trial and error. We choose a volume that causes a decline in percent transmittance from 100% to about 50% T in 2 minutes in the presence of 2.4 mM L-DOPA. In this way

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the reaction rate is such that the rates at lower substrate concentrations can still be measured.

Commentary on the Assay

Tyrosinase exhibits an interesting phenomenon called "reaction inactivation" (Ingraham 1966) which means that it loses activity while functioning as a catalyst but does not lose activity when not functioning under similar conditions. Consequently it is not desirable to run the reaction for 16 minutes (Bowen & Baxter 1980) or 10 minutes (Stewart 1991). In fact, using sophisticated apparatus, Behbahani et al. (1993) showed that the reaction between tyrosinase and L-DOPA is linear for only 60 to 90 seconds. Consequently, any data relevant to the determination of initial velocity can be obtained in 2 minutes or less. A second reason for using short reaction times is the instability of the product (see below). Short assays provide less time for the dopachrome to be converted to other compounds with different absorbance properties. Also, if you run assays for 90 or 120 seconds, you not only collect superior data, you can also collect much more of it in a 2 or 3 hour laboratory class.

Because we never know for how long the reaction will remain linear, it seems appropriate to record absorbance readings as frequently as possible. An experienced person can easily take readings at 15-second intervals and my students manage this well if one watches the clock while another watches the spectrophotometer.

Analysis of Results

Step 1. Make a graph of the change in absorbance (ΔA_{475}) against time to determine how long the reaction is linear.

Step 2. Determine the ΔA_{475}/minute for the initial linear part of the reaction, e.g. if the reaction is linear for 45 seconds with a cumulative ΔA_{475} of 0.015, divide this value by 3 and multiply by 4 to give a ΔA_{475}/minute value of 0.020.

Step 3. This cumulative change in absorbance/minute corresponds to an increase in the concentration of the product, dopachrome (and an equal decrease in the concentration of the substrate, L-DOPA). This concentration change (Δc) can be determined by interpolation of standard curve of dopachrome concentration against A_{475} (see below) or by using the absorption coefficient (E) determined from the slope of the standard curve where:

$$A = \epsilon.c.L$$

where A = absorbance
ϵ = absorption coefficient
ϵ = concentration in moles/liter
L = 1 cm ($\therefore$ omit)

$$\therefore c = \frac{A}{\epsilon} \text{ and}$$

$$\Delta c = \frac{\Delta A}{\epsilon}$$

Step 4. The derived value is the increase in concentration of dopachrome in moles/liter/minute. To find the number of moles of dopachrome produced/minute, correct for the total volume of the reaction mixture (e.g. if 30 µl of tyrosinase preparation is added to 3 ml of buffered substrate, the total volume is 3.03 ml.).

$$\therefore Vo = \Delta c/\text{minute} \times \frac{3.03 \text{ ml}}{1000 \text{ ml}} = \text{moles of dopachrome produced/minute.}$$

Standard Curve for Dopachrome

For many quantitative colorimetric assays, it is easy to generate a standard curve of product concentration against absorbance at its wavelength of maximum absorbance. Where the product is known and chemically stable, one can purchase a sample of the product, make a dilution series, and read the absorbances. However, dopachrome is not chemically stable. Therefore when we produce a solution of dopachrome by the oxidation of L-DOPA, we cannot be sure what is in the solution that we are measuring as it becomes increasingly heterogeneous over time. Consequently the method suggested by Bowen and Baxter (1980) is problematic. They suggest diluting a completely oxidized solution of L-DOPA with reaction buffer and reading the A_{475} values to generate a standard curve. This assumes that a completely oxidized solution of 5 mM L-DOPA will contain 5 mM dopachrome, which is probably true for only a brief time. Their method involves producing a "100% oxidized" solution of 5 mM L-DOPA by adding several drops of concentrated tyrosinase preparation. The reaction is stopped when the solution is "very dark orange" by poisoning the enzyme with a few crystals of phenylthiourea. (Although phenylthiourea inhibits the enzyme, it will not inhibit the further modification of any dopaquinone that has already been produced as these reactions are not enzyme dependent [Behbahani et al. 1993]). I have tried a similar approach in class by adding a volume of saturated sodium periodate (Sigma) to a known volume of 7.3 mM L-DOPA and distributing aliquots to the students for further dilution. Since it takes 10 to 20 minutes from the time that the oxidizing agent is added to the L-DOPA to the time that the students take their A_{475} readings, the results generate absorption coefficients that consistently underestimate published values. We typically obatined ϵ values between 1400 to 1800 liters/mole/cm compared to values of 3600-3700 reported by Mason (1948).

In an attempt to resolve this discrepancy, I made reaction mixtures containing 1 ml of 20 mM sodium phosphate buffer (pH 6.5), 100, 80, 60, 40, 20 or 10 µl of 7.3 mM L-DOPA and distilled water to bring the volume to 2.9 ml. I then read A_{475} at 30-second intervals after the addition of 100 µl of saturated sodium periodate. The relationship between the assumed dopachrome concentration (= L-DOPA concentration) and absorbance at 475 nm is linear at 30, 60, 90 and 120 seconds after the addition of oxidant and deteriorates thereafter. Since the reaction between tyrosinase and L-DOPA is usually only linear for 2 minutes or less and since we only run the assays for 2 minutes, I recommend this method for producing the standard curve. This method gives an absorption coefficient of about 4300 liters/mole/cm. The difference between the value reported by Mason (1948) and that given here is probably attributable to differences in the buffers used. Mason's buffer was 0.5 M with respect to KH_2PO_4 adjusted to pH 5.6 with 0.1 N NaOH.

A Diversion on Catechol as Substrate

To determine if there are any practical advantages to using catechol instead of L-DOPA as substrate, I followed the protocol of Stewart (1991). In this method, 2 ml of a dilute aqueous potato extract is added to 2 ml of buffered catechol and the increase

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in absorbance at 540 nm is monitored at 30-second intervals. When the ΔA_{540} values were plotted against elapsed time, a consistent pattern emerged. In the first 2 minutes the reaction proceeds relatively slowly. This is followed by a faster, linear increase in rate which persists for 4 to 5 minutes after which the reaction rate declines. Since the initial lag phase might result from the addition of a large volume of ice-cold enzyme preparation to the buffered substrate at room temperature, I modified the assay so that a very small volume of enzyme, prepared as outlined in this paper, could be used. I found the same response, namely an initial lag phase followed by a period in which the reaction rate is faster and linear. There are several possible explanations for this type of response, none of which I have the experience to investigate. However, the reaction pattern appears to be consistent and raises two points. First, although a line can be fitted through the data points without significantly changing the calculated initial velocity, the true V_O for the reaction is probably best calculated from the middle linear part of the reaction. Second, if we are training scientists, we should encourage them to evaluate the data critically and not make *a priori* assumptions about whether or not they are linear.

I attempted to make a standard curve of the product of catechol oxidation against absorbance at 540 nm by adding saturated sodium periodate to buffered catechol at final concentrations of 6.33, 5.0, 3.33, 2.67, 2.0, 1.33 or 0.67 mM. Again absorbance values were read at 30-second intervals for 5 minutes. The absorbance values increased rapidly over time. At the highest and lowest catechol concentrations, the absorbance at 5 minutes into the reaction was 4.3-fold and 2.6-fold higher, respectively, than the absorbance at 30 seconds after addition of the oxidant. Given this magnitude of change, it is impossible to know what is being measured. Presumably the situation would be even more complex in assays run for periods greater than 5 minutes. Consequently, I have no confidence in our ability to quantify the reaction between tyrosinase and catechol.

A Class Experiment

We have performed a variety of kinetic assays in class with tyrosinase and L-DOPA. These include the demonstration of a linear relationship between initial velocity and enzyme concentration with a fixed substrate concentration, determination of K_m and V_{max} from Lineweaver-Burk plots (K_m values typically range from ~5 to 10 mM), determination of K_m and V_{max} in the presence of competitive and noncompetitive inhibitors, and the effect of pH on enzyme activity in both phosphate and tricine buffers (pH has only a small influence in the range 6.0 to 8.0; pH 6.5 produces the highest initial velocities).

The experiment described here arose from a simple observation. I cut a potato in half to make two preparations, one earlier and one later in the morning. When I peeled and blended the second half of the potato, the blendate rapidly became orange in color compared to the white blendate obtained from the first potato half. This suggested that endogenous substrate was being more rapidly oxidized by tyrosinase in the preparation made at the later time, and led to the hypothesis that tyrosinase activity might be enhanced by injury. To address this hypothesis, I cut a large potato into quarters and placed three of the quarters in a polythene bag. The first quarter was peeled at once, trimmed to 30.0 ± 0.1 g and blended in 30 ml of 25 mM sodium fluoride. I then followed the tyrosinase preparation protocol as previously outlined. Tyrosinase activity was assayed in quadruplicate using 1.9 mM buffered L-DOPA as substrate. Initial velocities were determined as outlined previously and mean initial velocities and standard errors were calculated using a one-way Anova and Scheffe's Multiple Contrast Test in the StatView II™ program (Abacus Concepts Inc., Berkeley, CA). The experiment was repeated using the same volume of tyrosinase preparation from the remaining three quarters of the potato at one, two and three hours after the initial cut. In each case the original cut surfaces were removed from the potato when preparing the 30 g sample. Results from experiments using six different potatoes suggest that the precise pattern of the response to injury varies greatly between individual samples (Figure 1a-f). However, with one exception (Figure 1b), all show an increase in initial velocity ranging from ~23% (1f) to more than double (1e) at one or two hours after the initial injury. These data suggest that potato cells at some distance from the cut site are responding to the injury which, in turn, implies cell-to-cell communication.

Experiments of this type, in which the outcome is always uncertain, are very useful in science education where a frequenly asked question is "What is the right answer?" In this case, the "right answer" can only be the one that you obtain by working as carefully and consistently as is possible.

I welcome comments from any instructor who has experience with tyrosinase or who tries any of the suggestions given in this paper.

Acknowledgment

I thank my colleague Irene Krajnyk for the statistical analysis and for generating the figure.

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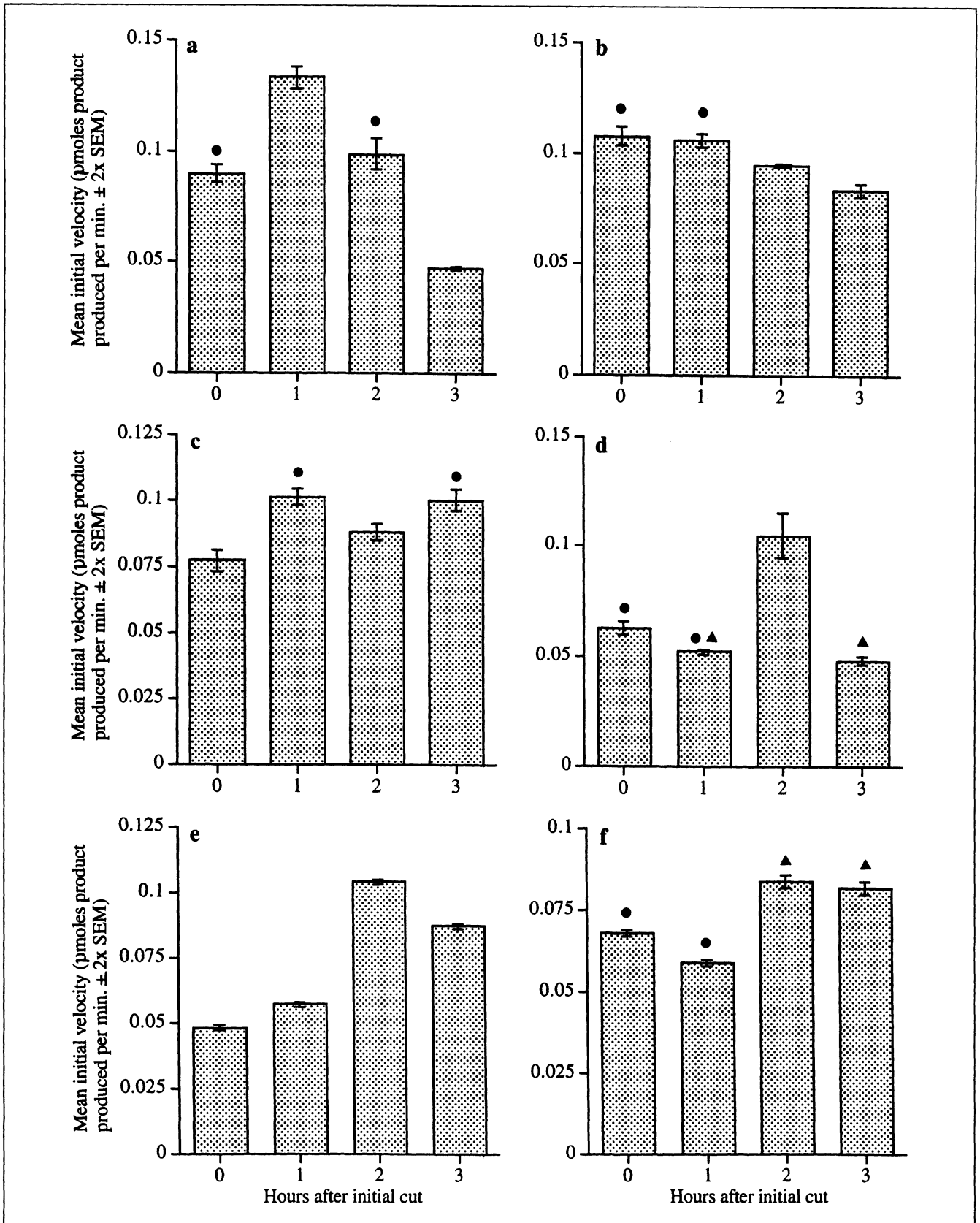

Figure 1. Mean initial velocities (n = 4) for the oxidation of L-DOPA by equal volumes of tyrosinase preparations made from potato quarters at 0, 1, 2 or 3 hours after the potato was first cut. Data from six experiments (a-f) with different potatoes are shown. In each case, bars marked with a circle and those marked with a triangle are not significantly different at the 95% confidence level. All other differences are significant.

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