An electrometric method for the determination of tyrosinase activity

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1. The pathway of dopachrome formation from L-dopa involves the net release of one proton for each molecule of dopachrome formed. 2. The protons produced as a consequence of the enzymic step catalysed by tyrosinase can be measured by an electrometric device able to monitor changes in H^+ concentration below $1\ \mu M$. 3. This electrometric recording can be used as a simple, sensitive and continuous method for determining tyrosinase activity. 4. The electrometric method can also be used in the presence of ascorbate by the spontaneous coupling of ascorbate oxidation to dopaquinone reduction, but measuring proton uptake instead of proton release.

Tyrosinase (EC 1.14.18.1) is the main enzyme participating in the process of melanin biosynthesis, catalysing the oxidation of tyrosine to dopachrome in two steps by using molecular O_2 as the oxidant. The first step is the *o*-hydroxylation of L-tyrosine to L-dopa, and the second one is the oxidation of L-dopa to dopachrome.

The pathway of melanin biosynthesis, initially proposed by Raper (1928) and Mason (1948), was later slightly modified by Lerner & Fitzpatrick (1950), by including the possible oxidation of leucodopachrome by dopaquinone, yielding dopachrome and L-dopa, therefore recycling one product of the enzymic reaction catalysed by tyrosinase (Scheme 1).

To date, spectrophotometric methods have been the most widely used for determining tyrosinase activity, measuring either the increase in the dopachrome absorbance at 475 nm (Mason, 1948), or dopaquinone production by coupling it to ascorbate oxidation at 265 nm (El-Bayouimi & Frieden, 1957). Radiometric methods have also been employed (Pomerantz, 1976) based on the $^3H^+$ release from [2,5,6- 3H]dopa, when leucodopachrome is formed.

A detailed study on the melanin biosynthesis has been carried out (Cánovas *et al.*, 1982), showing that the Raper–Mason–Lerner pathway is the main one, and only at pH values lower than 4 should two pathways compete for the protonated form of *o*-dopaquinone (*o*-dopaquinone- H^+), one of them involving topa and topaquinone forma-

tion. Besides, the participation of *o*-dopaquinone- H^+ and its amino-group deprotonation to undergo a ring-closure process to yield leucodopachrome has been postulated as the limiting step in the process of dopachrome formation (Cánovas *et al.*, 1982; Carmona *et al.*, 1982). If so, at pH values below the pK_a of the amino group of dopaquinone the dopaquinone consumed by the cyclation step must be replaced from dopaquinone- H^+ , and one proton would be released for each dopachrome formed.

In the present paper we show that the enzymic process of dopachrome formation from L-dopa at neutral and slightly acidic pH involves the release of one proton for each molecule of dopachrome formed. These results can be used as a new and direct confirmation of the melanin biosynthesis pathway (Lerner & Fitzpatrick, 1950; Cánovas *et al.*, 1982). On the other hand, they can also be used as the basis for a new electrometric method in assaying tyrosinase activity.

Experimental

Chemicals

Mushroom tyrosinase was purchased from Sigma Chemical Co. All the reagents used were analytical grade.

Measurements

Spectrophotometric measurements were carried out with a Varian spectrophotometer. Dopachrome accumulation was monitored at 475 nm (Mason, 1948). Dopaquinone production was determined by coupling it to ascorbate oxidation, with measurement of the decrease in absorbance at

Abbreviations used: dopa, 3,4-dihydroxyphenylalanine; topa, 2,4,5-trihydroxyphenylalanine.

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Scheme 1. Raper-Mason-Lerner pathway of melanogenesis

Only L-dopa oxidation is catalysed by tyrosinase. In the presence of ascorbate, dopaquinone is converted back into L-dopa, and no other intermediates are formed.

265 nm (El-Bayouimi & Frieden, 1957). Ascorbate concentration was $30\mu\text{M}$. The reaction medium always contained 1 mM-sodium phosphate and 0.150 M- KNO_3 in order to compare these measurements with the electrometric assays.

Electrometric measurements were carried out in a potentiometric set-up composed of a combined Radiometer electrode (model GK2322C), a Radiometer pH-meter, a recorder and an inserted bucking voltage device as described by Muñoz-Delgado *et al.* (1983). Basically this device may subtract most of the signal coming from the pH-meter, by simply varying the resistance of the circuit and hence allowing the observation of variations of only small percentages of the total signal. In this way smaller variations in pH that could not be detected by a conventional pH-stat can now be detected. The reaction mixture contained 1 mM-sodium phosphate buffer to stabilize pH, and 0.150 M- KNO_3 to ensure constant ionic strength and for stabilizing the electrode response. The pH, L-dopa and enzyme concentration were changed in different experiments. The reaction was started by adding the enzyme and the change in pH was followed. The variation of pH

was never greater than 0.1 unit in the course of the assay. Therefore it could be assumed that the enzyme activity was not affected by the pH change through the assay, and that the measurements provided meaningful rate data. The system was calibrated by addition of $50\mu\text{l}$ of 0.1 mM-HCl during the course of each experiment, because the variation of the pH as result of a fixed amount of H^+ released shows a dependence on the initial conditions of the assay media and the possible contamination of the enzymic preparation by other chemicals. This HCl solution was prepared from stock 100 mM-HCl titrated against freshly prepared NaOH, previously titrated against potassium hydrogen phthalate. The non-enzymic H^+ release was negligible, so that a stabilized pH recording was obtained before addition of the enzyme.

The total volume of the reaction mixture was 3 ml for both spectrophotometric and electrometric measurements.

Specific enzyme activity was expressed either as μmol of dopachrome produced (spectrophotometric method) or μmol of H^+ released (electrometric method) per min per mg of protein. In the assays when ascorbate was present the activity

was expressed as μmol of ascorbate consumed or μmol of H^+ taken per min per mg of protein.

Results and discussion

Fig. 1(a) shows a typical electrometric recording of L-dopa oxidation into dopachrome by tyrosinase. A linear release of H^+ can be seen when enzyme is added. Since pH variation is not a linear function of proton release and depends on the buffering power of the reaction mixture, an internal calibration must be carried out during the assay by adding a known amount of H^+ and determining the variation of the electrometric signal. Fig. 1(b) shows a parallel assay in the presence of $10\mu\text{l}$ of 10mM -ascorbate. There is an initial uptake of H^+ (and subsequently a release of H^+) that is approximately twice the rate of H^+ release in the 'second phase' of the reaction (see slope values in Fig. 1b).

These experimental observations confirm the postulated pathway shown in Scheme 1 (Lerner & Fitzpatrick, 1950). In the absence of ascorbate, L-dopa oxidation leads to dopachrome, and one proton is released for each dopachrome molecule formed due to the dopaquinone deprotonation step previous to the ring closure, which yields leuco-

dopachrome. In the oxidation of leucodopachrome by dopaquinone- H^+ to dopachrome and L-dopa, only half of the tyrosinase-catalysed L-dopa oxidation will release protons, and therefore the rate recorded for measuring H^+ release is half the enzymic rate expressed as O_2 consumed in the tyrosinase reaction. In the presence of ascorbate, dopaquinone- H^+ is converted back into L-dopa by coupling it to the ascorbate oxidation into dehydroascorbate. One proton will be consumed per ascorbate molecule oxidized to dehydroascorbate, since pK_a values for ascorbic acid are $\text{pK}_1 = 4.19$ and $\text{pK}_2 = 11.57$ (Morris & Redfearn, 1972), and at pH 7 only one acidic hydroxy group should be dissociated. Therefore the rate of H^+ uptake will be the same as the enzymic rate of L-dopa oxidation, and twice that obtained in the absence of ascorbate, according to our experimentally obtained data. These results show good correlation to those obtained when tyrosinase activity is determined spectrophotometrically either at 265nm, measuring ascorbate consumption, or at 475nm, after dopachrome production (Pomerantz, 1963; Fling *et al.*, 1963).

The electrometric determination of tyrosinase activity by measuring H^+ release was compared with the spectrophotometric method based on

Fig. 1. Electrometric recording of the time course of L-dopa oxidation catalysed by mushroom tyrosinase (a) The initial mixture contained 3 ml of 3.8mM -L-dopa in 1mM -sodium phosphate buffer, pH 7, containing 150mM - KNO_3 . The assay was started by the addition of $25\mu\text{l}$ of tyrosinase (0.5mg/ml). During the assay, $50\mu\text{l}$ of $88.8\mu\text{M}$ -HCl was added for internal calibration. (b) The initial mixture was as for (a) but with the addition of $10\mu\text{l}$ of 10mM -ascorbic acid previously neutralized. When the enzyme is added, there is a pH increase that is reversed when the ascorbate is exhausted. Slopes are expressed in nmol of H^+/min .

dopachrome production at different pH values. All assays were carried out at saturating L-dopa concentration. In accordance with Scheme 1, Fig. 2 shows a very good correlation between the reaction rates, expressed either as μmol of H^+ released or μmol of dopachrome formed per min per mg of protein, in a 1:1 ratio up to pH 8. At pH above 8 there is an abrupt decrease in H^+ release, which can be explained by taking into account the dissociation constant for the amino group of L-dopa, $\text{p}K_a = 8.72$ (Gray & Weitzman, 1972). According to this value, the main form of L-dopa at pH below 8 will have its amino group protonated. When pH rises to reach values close to $\text{p}K_a$, the proportion of non-protonated molecules of L-dopa will increase in the solution, and the tyrosinase-catalysed oxidation will directly produce non-protonated dopaquinone, which with subsequent ring closure to yield leucodopachrome will not produce a net release of H^+ . Therefore electrometric measurements are not suitable for measurement of the enzyme activity at pH above 8, but can be used as a reliable method for determining tyrosinase activity below that pH.

As mentioned above, this method can be used either in the absence or in the presence of ascorbate. In the first case, there is a release of H^+ , whereas in the second one there is an uptake of H^+ . Fig. 3 shows the dependence of the reaction rate with the enzyme concentration. In both cases there is a linear relationship between the recorded rate of

Fig. 2. Plots of specific activity of mushroom tyrosinase versus pH

●, Electrometric method, units expressed as μmol of H^+ released/min per mg of protein ▲, Spectrophotometric method, units expressed as μmol of dopachrome formed/min per mg of protein. The initial mixture always contained 3 ml of 3.8 mM-L-dopa in 1 mM-sodium phosphate and 150 mM- KNO_3 . The assays were started by the addition of 50 μl of tyrosinase (0.5 mg/ml), and during each one 50 μl of 88.8 μM -HCl was added for internal calibration.

pH change and enzyme concentration, and the slope in the presence of ascorbate is twice the one in the absence of it. Furthermore, the reciprocal of the time elapsed for the whole consumption of ascorbate is also a linear function of enzyme amount, as shown in Fig. 4. This relationship has already been used in 'chronometric' methods for measuring plant catechol oxidase activities (Mayer *et al.*, 1966).

To check the validity of the electrometric assay, the V_{max} and K_m values for L-dopa obtained by using the electrometric and spectrophotometric methods, in the presence and in the absence of ascorbate, were compared. These values were

Fig. 3. Plots of reaction rate versus amount of mushroom tyrosinase in the absence (○) and in the presence (●) of ascorbate

The slopes were 4.6 and 10 μmol of H^+ /min per mg of protein respectively. The assays were carried out as described for Fig. 1, except for the amount of enzyme used.

Fig. 4. Plot of the reciprocal of the time elapsed to total consumption of ascorbate versus amount of mushroom tyrosinase

Assays were as described for Fig. 1(b), except for the amount of enzyme used.

Table 1. K_m for L-dopa and V_{max} values of mushroom tyrosinase, obtained by linear regression from Hanes–Woolf plots, for spectrophotometric and electrometric methods in the presence and in the absence of ascorbate

Initial pH was 7 in all cases, and the reaction was always started by adding 50 μ l of mushroom tyrosinase (0.5 mg/ml). For full experimental details see the text. Values are the means of three determinations.

Method	K_m (mM)	V_{max}
Spectrophotometric, dopachrome formation	0.178 ± 0.025	$1.43 \pm 0.12 \mu\text{mol of dopachrome formed} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$
Spectrophotometric, ascorbate disappearance	0.174 ± 0.013	$2.94 \pm 0.22 \mu\text{mol of ascorbate consumed} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$
Electrometric, H^+ release in the absence of ascorbate	0.192 ± 0.020	$1.48 \pm 0.15 \mu\text{mol of } \text{H}^+ \text{ released} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$
Electrometric, H^+ uptake in the presence of ascorbate	0.186 ± 0.019	$2.99 \pm 0.37 \mu\text{mol of } \text{H}^+ \text{ taken} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$

determined in triplicate by linear regression from Hanes–Woolf plots, the substrate concentration being varied in the range 0.0475–3.8 mM. All the experiments were carried out in a reaction volume of 3 ml, at pH 7, and the reaction was started by adding 50 μ l of tyrosinase (0.5 mg/ml). Fig. 5 shows a Hanes–Woolf plot for the electrometric and spectrophotometric methods in the absence of ascorbate, which demonstrates the adherence of the tyrosinase experimentally obtained data to the Michaelis–Menten equation. Table 1 shows the good correlation among the values obtained from the different methods, taking into account Scheme 1, and this proves that the electrometric method is a good alternative option for measuring tyrosinase activity, mainly in coloured or turbid enzymic crude extracts, where spectrophotometric determinations cannot be accurately performed. However, in such cases, the reaction should be started by adding the substrate last, after the reaction mixture had been previously incubated with the enzymic extract until there was no fluctuation in the electrometric signal. In that way, possible interference due to contamination by other enzymes of the extract can be avoided.

The electrometric method in the absence of ascorbate, with the use of the ultrasensitive bucking voltage device built in our laboratory (Muñoz-Delgado *et al.*, 1983), allows changes in H^+ amount below 1 nmol (concentration change below 1 μM for reaction volume around 1 ml) to be measured, its sensitivity being similar to that of the spectrophotometric method assuming $\epsilon_{475} = 3600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for dopachrome (Mason, 1948).

The methods with ascorbate have a higher sensitivity, owing to the $\epsilon_{265} = 7000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for ascorbic acid (Morris & Redfearn, 1972) and the double pH change in the electrometric method compared with the pH change in the absence of ascorbate. Also, they have the advantage of not accumulating quinone species; therefore no side reactions can occur, such as enzyme inactivation due to the reactivity of quinones, giving a better

Fig. 5. Hanes–Woolf plot for mushroom tyrosinase ●, Spectrophotometric method, dopachrome formation. ○, Electrometric method, proton release in the absence of ascorbate. Reaction rates are expressed as μmol of either dopachrome or protons $\cdot \text{l}^{-1} \cdot \text{s}^{-1}$. The reaction was always started by adding 50 μ l of tyrosinase (0.5 mg/ml).

linearity between enzyme activity and time course of reaction. On the other hand, these methods also have some additional disadvantages, and they have to be used with caution for measuring tyrosinase activity in crude extracts, when substances capable of oxidizing ascorbate may be present. Moreover, ascorbate can produce deviations from the Raper–Mason–Lerner pathway of melanogenesis, such as the formation of 5-hydroxydopa reported by Hansson *et al.* (1981).

In conclusion, we think that the assays in the absence of ascorbate are more accurate than in the presence of it, in spite of the lower sensitivity, and they must be used mainly for measuring activity in non-purified enzyme extracts. The electrometric method may be necessary in some circumstances, such as highly absorbent media. Thus we have used that method successfully for studying the inhibitory effect of melanin suspensions on melanoma tyrosinase activity, when spectrophotometric methods cannot be used.

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