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LIPOXYGENASE-CATALYZED OXIDATION OF CATECHOLAMINES

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SUMMARY: Dopa and structurally related catecholamines in presence of hydrogen peroxide are oxidized in vitro by soybean lipoxygenase producing the corresponding melanin pigments. The kinetic parameters of the catecholasic reaction, measured as aminochrome formation, have been calculated. The rate of peroxidation depends on catecholamine and hydrogen peroxide concentration. The optimum pH for the peroxidative activity of the enzyme is around 8.5. The enzyme, at higher pH values (pH 9 - 9.5), is also able to perform an oxidative reaction of the substrates. Implications of the possible biochemical relevance of the reactions are discussed.

Two enzymes are generally considered responsible for melanin synthesis: tyrosinase and peroxidase (1). Tyrosinase (catechol-oxidase, catecholase: EC 1.14.18.1) is able to produce melanin through the hydroxylation of tyrosine to dopa and the oxidation of dopa to dopachrome (2). The Mason-Raper pathway further involves the synthesis of 5,6-dihydroxyindole or 5,6-dihydroxyindole-2-carboxylic acid that are oxidatively polymerized to melanin. Peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) as well was demonstrated to catalyze the oxidation of catechols (4) and dihydroxyindoles (5) to pigmented derivatives.

Recently, we reported that tyrosinase from mushroom and sepia is also capable of utilizing opioid peptides as substrates (6) and to convert the latter compounds into soluble melanin-like pigments retaining the peptide moiety (7,8).

We have now focused our attention on the possibility that lipoxygenase, another enzyme widely distributed in tissues, can contribute to melanin generation from catechol precursors. Lipoxygenase (linoleate:oxygen oxidoreductase EC 1.13.11.12) is able to transform unsaturated fatty acids to hydroperoxy-

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derivatives giving ultimately rise to a wide list of biological compounds such as leukotrienes, hepoxylins and hydroxy-fatty acids (9).

Few reports have also been recently presented on the peroxidative action carried out by the enzyme in presence of H_2O_2 (10,11).

In this paper we report that the lipoxygenase/H_2O_2 system can accomplish the in vitro oxidation of dopa and other catecholamines to aminochromes with the consequent production of melanin.

MATERIALS AND METHODS

Materials: Dopa, dopamine, adrenaline, noradrenaline, α-methyldopa, N-acetyl dopamine, isoproterenol and soybean lipoxygenase type V (646,600 Units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were analytical grade products from Merck (D-6100 Darmstadt, Germany).

H_2O_2 concentration was determined using a molar extinction coefficient of 72.4 at 230 nm (4).

Catecholamine oxidation: Dopachrome and dopaminechrome were assayed at 475 nm using a millimolar extinction coefficient of 3.7 (2). Adrenochrome and noradrenochrome were followed at 487 nm using a millimolar extinction coefficient of 3.0 (4). Spectrophotometric measurements were performed with a Varian spectrophotometer DMS200 in thermostated cuvettes at 25°C.

The reaction mixture, unless otherwise specified, contained 1 mM catecholamine, 0.05 mM hydrogen peroxide, 27 µg enzyme, 0.1 M phosphate buffer (pH 8) in a volume of 1 ml; the reaction was started by the addition of the enzyme.

For measuring oxidizing reaction, H_2O_2 was omitted; in some cases samples were run with O_2 bubbling.

Suitable blanks were contemporaneously performed to subtract the oxidation of the substrates by H_2O_2 or O_2. The boiled enzyme did not carry out the reactions.

Melanin production: Incubation mixtures contained 25 mM dopa, 2.5 mM H_2O_2, 500 µg of enzyme, 0.2 M phosphate buffer pH 8.0 or pH 7.0 in a final volume of 40 ml. After 2 hour incubation at 37° C, 4 ml of 1M HCl were added to block the reaction. Incubation mixtures were centrifuged for 30 min at 12,000 g and the supernatant was removed. The precipitated pigment, washed three times, centrifuged and recovered, was lyophilized and weighed.

RESULTS

In figure 1 the spectra at various times of an incubation mixture of the lipoxygenase/H_2O_2 system with dopa as substrate are reported. The reaction reached the completeness after about 20 minutes. The spectrum with absorption maxima at 305 and 475 nm is indicative of the formation of dopachrome (2,4). The initial rate, calculated as dopachrome production/min, was found to be dependent upon the enzyme concentration.

The Lineweaver-Burk plot of oxidation of dopa and N-acetyl dopamine by the lipoxygenase/H_2O_2 system is showed in the inset of figure 1. The kinetic

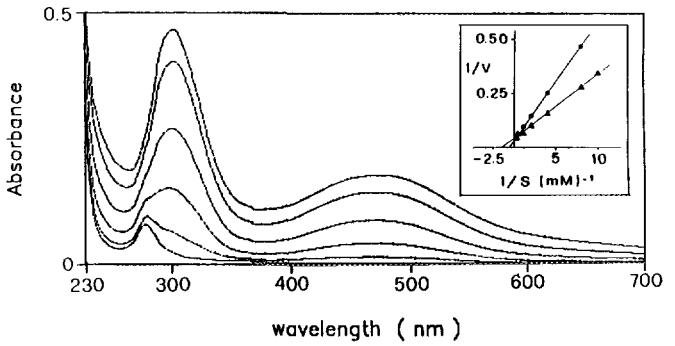
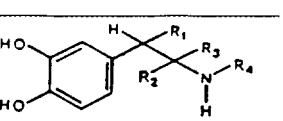


Fig. 1. Spectral modification of dopa oxidized by the lipoxygenase/H_2O_2 system. Spectra were automatically recorded every three minutes.
Inset: Lineweaver-Burk plot for the oxidation of dopa (circles) or N-acetyldopamine (triangles) by the lipoxygenase/H_2O_2 system.
Reaction rate (V) is expressed as nmoles of aminochrome produced/min.

parameters of the reaction involving all the catecholamines tested as substrates are illustrated in Table I. The best substrate appears to be N-acetyldopamine (Km 0.62 mM).

Reaction obeyed Michaelis and Menten kinetic also for H_2O_2, having a Km value of 71.4 μM at pH 8.0 at saturating concentration of the reductant, as determined by Lineweaver-Burk plot (fig. 2). Dopachrome formation rate plotted as a function of hydrogen peroxide concentration (see inset fig. 2) indicates that the optimum value is 0.1-0.15 mM but the amount of the peroxide is critical because levels of H_2O_2 higher than 0.2 mM were found to inhibit lipoxygenase. This result is in agreement with previous studies demonstrating lipoxygenase sensitivity to H_2O_2 (11). A very similar behaviour is on the other hand displayed by plant and animal peroxidases (12).

TABLE I. Kinetic parameters for the oxidation of various catecholamines by the lipoxygenase/H_2O_2 system

Substrate	Km (mM)	Kcat (Vmax/mg E)	Kcat/Km				
				R_1	R_2	R_3	R_4
N-acetyldopamine	0.62	2.85	4.60	H	H	H	$COCH_3$
dopamine	1.15	2.44	2.12	H	H	H	H
α-methyl dopa	1.25	4.63	3.70	H	CH_3	COOH	H
noradrenaline	1.30	4.63	3.56	OH	H	H	H
dopa	1.66	3.88	2.42	H	H	COOH	H
adrenaline	2.77	3.96	1.43	OH	H	H	CH_3
isoproterenol	8.33	5.92	0.71	OH	H	H	$CH(CH_3)_2$

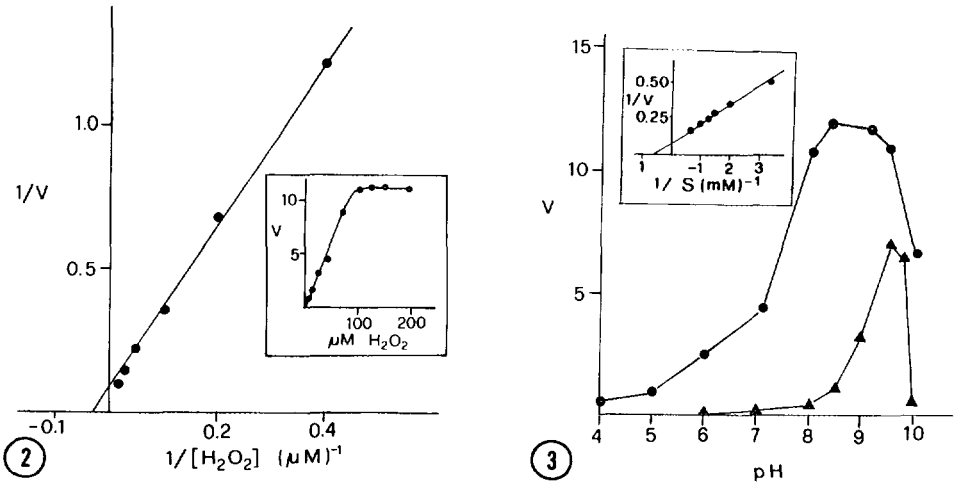


Fig. 2. Lineweaver-Burk plot for lipoxygenase-catalyzed oxidation of dopa in function of H_2O_2 concentration. For details see materials and methods.
Inset: reaction rate as a function of H_2O_2 concentration (5-200 μM).
Reaction rate (V) is expressed as nmoles of dopachrome produced/min.

Fig. 3. Effect of pH on oxidation rate of dopa by the lipoxygenase/H_2O_2 system (circles) or lipoxygenase/O_2 system (triangles).
Inset: Lineweaver-Burk plot for the oxidation of dopa by the lipoxygenase/O_2 system. The incubation was performed at pH 9.
Reaction rate (V) is expressed as nmoles of dopachrome produced/min.

The effect of pH on the oxidation of catecholamines by the lipoxygenase/H_2O_2 system was also investigated. In figure 3 dopachrome formation as a function of pH values is shown, the optimum being about 8.5.

In absence of H_2O_2 lipoxygenase can also catalyze an oxidative reaction of catecholamines; in figure 3 the reaction rate in presence of oxygen is plotted as a function of pH. The best activity is, in this case, at higher pH values (pH 9.5) with respect to the peroxidative reaction. The Km value of lipoxygenase toward dopa in the oxidative reaction was 1.8 mM (see inset fig. 3).

When the latter reaction was performed with O_2 bubbling (see Table II, experiment B), the rate of substrate oxidation was enhanced. In this case lipoxygenase can act as an oxidase even at pH 7. When dopa was oxidized in absence of H_2O_2 (Table II, experiment B) a more consistent amount of dopa-melanin was recovered with respect to that obtained during the peroxidation (Table II, experiment A). In this latter case however the initial rate of dopachrome formation was more pronounced and represents the highest value obtained among the various experimental conditions.

TABLE II. Dopachrome and melanin production from lipoxygenase catalyzed oxidation of dopa

oxidation conditions	dopachrome production (nmoles/min)	melanin yield[a] (%)
A) dopa + E + H_2O_2	6.40	4.8
B) dopa + E + O_2 (bubbled)[b]	0.55	6.4
C) dopa + E + O_2[c]	0.35	1.7
D) dopa + boiled E + H_2O_2	0.02	0.1
E) dopa + H_2O_2	0.02	0.1

a) amount of melanin formed after 2 hours of incubation, expressed as percent (w/w) of dopa incubated.
b) incubation was performed at pH 7, with O_2 bubbling.
c) incubation was performed in presence of atmospheric oxygen.
For details see materials and methods.

DISCUSSION

Lipoxygenases are a family of almost ubiquitous enzymes containing iron. Several mammalian organs, including brain, have been found to possess lipoxygenase activity (10).

Native soybean lipoxygenase contains a nonheme ferrous ion that must be oxidized to yield the catalytically active ferric form (Fe III) (13). It is ascertained that compounds able to mantain lipoxygenase in its ferrous state (Fe II) act as inhibitors in the dioxygenasic reaction of unsaturated fatty acids (14). This function is notably exerted by catechols (14) or dihydroxyindoles (15) that are considered as lipoxygenase inhibitors for their capacity to efficiently reduce the enzyme keeping it in the inactive form (14,16).

In the presence of H_2O_2, lipoxygenase functions as a peroxidase rather than a dioxygenase (11). In this case, and in analogy with the behaviour of horseradish peroxidase (16), H_2O_2 appears to have the ability of maintaining the enzyme into the active form that directly reacts with the hydrogen donor.

Our results indicate that lipoxygenase is able to catalyze the peroxidation of the catecholamines. Data obtained are in keeping with a Michaelis-Menten kinetic for both hydrogen donor and H_2O_2. The Km of 71.4 μM toward H_2O_2 reflects a high affinity, probably due to the formation of a covalent bond between enzyme and H_2O_2, as suggested by Radi et al. (17).

Lipoxygenase can perform catechol oxidation also in absence of hydrogen peroxide but only at higher pH values (pH 9), that can permit the maintenance of iron in

the ferric status at the catalytic site. The presence of H_2O_2 allows the oxidation of substrates at lower pH because in this case is the peroxide that acts as iron oxidant, thereby increasing the turnover rate of the enzyme.

With regard to the melanin formation, pigments from the various catechols dissolved in alkali showed the typical spectrum of dopa-melanin, i.e. a continous regular increasing absorption over the range 600-250 nm, but they were different for colour and solubility behaviour (1).

The high value obtained for dopachrome formation in the course of lipoxygenase oxidation of dopa by H_2O_2 (Table II, experiment A) induced to expect a more substantial melanin yield with respect to that recovered; the low melanin recovery can be explained by the well known cleavage exerted by hydrogen peroxide on the melanin polymer (1) that consistently reduces the final amount of the pigment.

As a whole it may be advanced the hypothesis that lipoxygenase, able to perform an easy peroxidation of catechols in vitro, could be an enzyme producing melanin in vivo.

Several brain enzymes liberate H_2O_2 during their action (18) and hydrogen peroxide may be also actively generated during non-enzymatic reactions, the major source being the dismutation of the superoxide anion radical (19). The peroxidasic activity of lipoxygenase could play some role in melanin formation when the level of H_2O_2 is not decreased by catalase, as actually occurs in some pathological situations (for example, in Parkinson's disease) (20), where basal lipid peroxidation also extensively occurs (20,21).

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