Action of imidazole derivatives on oxidation of unsaturated fatty acids by soybean lipoxygenase

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The effects of imidazole derivatives on the oxidation of linoleic (or arachidonic) acid by soybean lipoxygenase was studied. Carbimazole (at the concentrations employed) was found to have an effect on the reaction. The initial reaction velocity, the maximum velocity and the affinity constant were all modified. These results suggest that carbimazole exerts actions both on the affinity of the enzyme for the substrate and on the final product of the reaction. Carbimazole has the highest electron donating potential and, at the doses used, was the most active of the three compounds tested. The other derivatives were found to have similar but much slower actions on enzyme activity. Such compounds, which are often used in thyroid disorders, could also act on polyunsaturated fatty acid metabolism and so could provoke secondary effects.

Unsaturated fatty acid (Soybean) Lipoxygenase Imidazole derivative

1. INTRODUCTION

Lipoxygenases catalyse the oxidation of unsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene structure such as linoleic and arachidonic acid [1] to form hydroperoxides with a conjugated *cis-trans* double bond structure [2,3]. Smith and Lands [4] have shown that the enzymatic activity of soybean lipoxygenase drops during the reaction according to a first order rate law. This rate of inactivation has a characteristic value for each fatty acid. Moreover, the hydroperoxides suppressed the lag period of these oxidative enzymes [5,6]. In biological tissues, these hydroperoxides are metabolized by various routes which may be enzymatic [7–9] or non-enzymatic. Heat, metal ions and metallo-proteins all play a role in these complex reactions [10]. Various substances such as cysteine, GSH, iodide and lipoic acid can reduce the hydroperoxides to hydroxylated derivatives. Here we report the action of imidazole derivatives on the enzymatic oxidation of unsaturated fatty acids.

2. MATERIALS AND METHODS

2.1. *Materials*

Soybean lipoxygenase type I, linoleic and arachidonic acids were purchased from Sigma. Carbethoxythio-1-methylglyoxaline (carbimazole) is a product of Aspro-Nicholas Pharmaceuticals. *N*-Methylimidazole was obtained from Fluka (product no. 67560) and 2-(1,2,3,4-tetrahydro-1-naphthyl)-Δ2-imidazoline (tetrahydrozoline) from Sigma (product no. 4264).

Absorption spectra were measured in a double beam Perkin-Elmer Lambda 5 UV-VIS spectrophotometer fitted with a Peltier effect thermostatted sample holder. Absorbance was analysed with a Hewlett Packard 85 microcomputer.

The thin layer silica gel plates (60F 254) were purchased from Merck.

2.2. *Activity of soybean lipoxygenase*

Soybean lipoxygenase activity was measured spectrophotometrically from the increase in absorption at 234 nm due to the formation of the conjugated diene hydroperoxide from either

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linoleic or arachidonic acid at pH 7.4 [E (molar) = 23000 $M^{-1} \cdot cm^{-1}$].

The reaction medium contained various concentrations of dispersed substrate (50–300 μM) in 3 ml sodium phosphate buffer (pH 7.4; 0.05 M). After incubating at 20°C for 5 min, 10 μg (1235 units) of soybean lipoxygenase was added to initiate the reaction.

2.3. *Effects of the different molecules on the reaction kinetics*

The compounds were dissolved in distilled water and used at concentrations from 2.7 to 2700 nM in the reaction medium. They were incubated for 5 min with the substrate, before adding the enzyme. For controls an identical volume of distilled water was used under the same conditions.

The HP 85 microcomputer calculated the values for initial reaction velocity. The amount of product formed [P = (absorbance × 2.303)/23000] is given by the kinetic equation (saturation function) $P = A(1 - e^{-\alpha t})$. A curve-fitting program calculated the parameters A and α.

The rate of formation of P at any time is the differential of the above equation and is given by $A\alpha e^{-\alpha t}$. For $t = 0$, the initial velocity is $A\alpha$. For the same concentration of enzyme, α is constant and the initial velocity (v_i) is a function of A only, which depends on the substrate concentration (fig.1). For a given substrate concentration, A remains constant and v_i is a function of α, which depends on enzyme concentration (fig.2).

2.4. *Chromatography*

The Merck thin layer silica gel plates were treated by an 'in situ' chemical reaction with alkyl chlorosilanes as described [11,12]. These treated plates were able to separate the hydroperoxides from the hydroxylated derivatives, and could also separate out the hydroperoxide or hydroxylated isomers depending on the position of the hydroxyl group on the carbon chain (5,8,9,11,12,15-hydroxyeicosatetraenoic acids).

3. RESULTS

3.1 *Effects of imidazole derivatives on soybean lipoxygenase activity*

Absorbance was recorded every 5 s after the first 15 s for 1 min, then every 15 s up to 2 min and

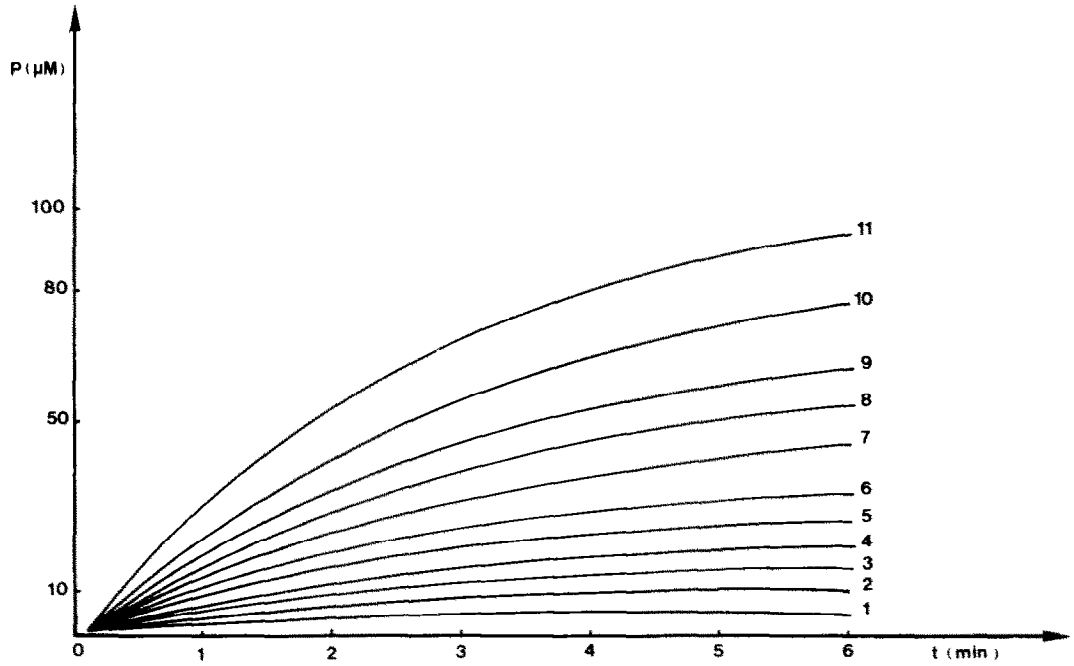

Fig.1. Change in initial reaction velocity as a function of substrate concentration (37 nM soybean lipoxygenase). Linoleic acid (curves): 38 (1); 75 (2); 107 (3); 138 (4); 167 (5); 231 (6); 286 (7); 343 (8); 400 (9); 457 (10); 572 μM (11).

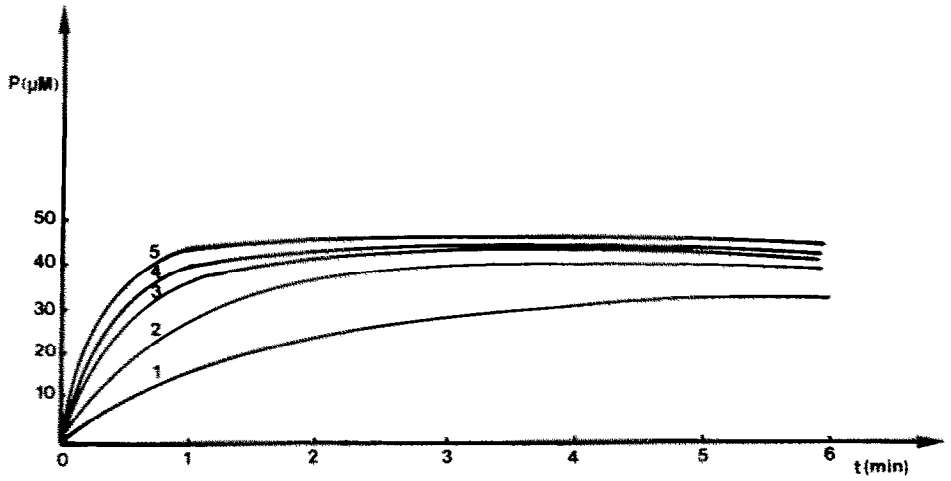

Fig.2. Change in initial reaction velocity as a function of enzyme concentration (120 μM linoleic acid). Soybean lipoxygenase (curves): 37 (1); 74 (2); 111 (3); 148 (4); 185 nM (5).

every 30 s up to 5 min. From these values, the computer using a specially developed program produced a curve of the reaction course, and calculated the initial reaction velocity.

Table 1 gives the values of initial velocity for different concentrations of linoleic acid and various concentrations of carbimazole. The enzyme concentration was fixed at 0.037 μM. To determine the kinetic parameters V_{max}, K_m, Hill's coefficient) the molar concentrations of enzyme and carbimazole were fixed at 0.037 μM and 27 nM, respectively. The substrate (linoleic acid) concentration in phosphate buffer varied between 50 and 300 μM. At these concentrations the lag period was less than 5 s.

Table 2 gives the initial reaction velocities for each concentration of substrate, the Michaelis constant and Hill's coefficient for reactions both with and without carbimazole.

Table 1

Action of carbimazole on the initial reaction velocity of the oxidation of linoleic acid by soybean lipoxygenase

Linoleic acid (μM)	Control	Carbimazole (nM)			
		2.7	27	270	2700
90	0.49	0.58	0.62	0.66	–
100	0.61	0.76	0.79	0.86	–
150	0.94	0.96	1.03	1.13	1.24
200	1.05	1.13	1.26	1.32	1.43
300	1.29	1.31	1.33	1.43	1.54
400	1.45	1.47	1.50	1.75	1.76

Different concentrations of linoleic acid were incubated with a fixed amount of soybean lipoxygenase (10 μg) in phosphate buffer (pH 7.4) alone or with various concentrations of carbimazole. The HP 85 computer produced a plot of the reaction kinetics, and calculated the initial reaction velocity in μmol/min per 10 μg enzyme

Table 2

Action of carbimazole on the Michaelis constant K_m, the maximum reaction velocity V_m and Hill's coefficient of the oxidation reaction of linoleic acid by soybean lipoxygenase

Linoleic acid (μM)	Initial reaction velocity	
	Control	+ 27 nM carbimazole
50	0.17	0.25
60	0.25	0.48
70	0.32	0.53
80	0.34	0.59
100	0.60	0.72
150	0.89	0.95
200	1.01	1.22
300	1.16	1.33
K_m (μM)	1.21	0.79
V_m (μmol/min per 10 μg enzyme)	9	9
Hill's coefficient	1.165	1.172

These results suggest that carbimazole has an activating action on the enzyme. K_m was reduced and Hill's coefficient was unchanged. It would appear that carbimazole has an effect on the affinity of the enzyme for the substrate. The percent activation, measured by the change of initial velocity both with and without a given amount of carbimazole, varied inversely with substrate concentration (fig.3).

For the same concentration of substrate, the degree of activation of the reaction was directly dependent on the amount of carbimazole added (fig.4). The amount of carbimazole which led to a 50% activation at a given enzyme concentration varied with substrate concentration (fig.4).

The other compounds tested (*N*-methylimidazole and tetrahydrazoline) had smaller and non-significant effects on the reaction kinetics.

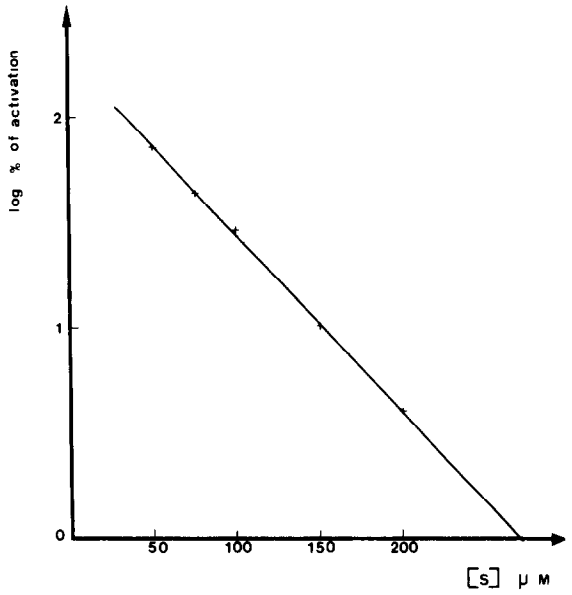

Fig.3. Percent activation of initial reaction velocity by carbimazole (27 nM) as a function of substrate concentration (50–200 μM linoleic acid).

3.2. *Spectroscopic data on imidazole derivatives*

The spectrum of carbimazole changed during the reaction, with a progressive reduction in the

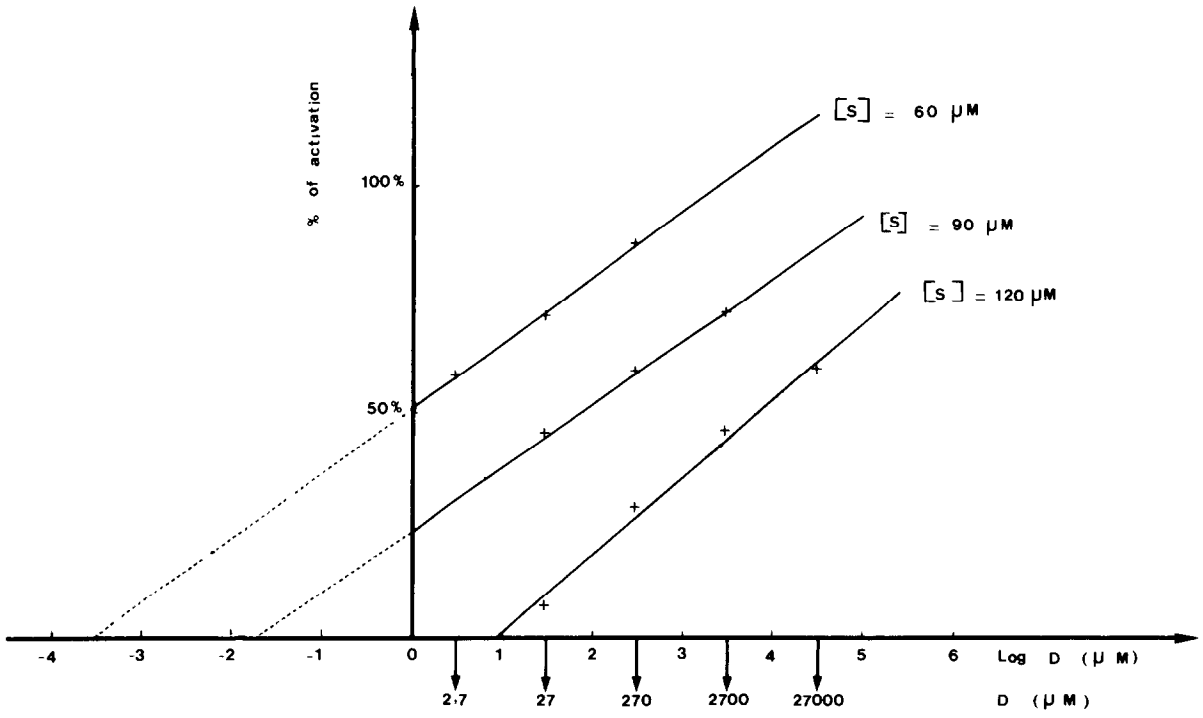

Fig.4. Percent activation of initial reaction velocity with increasing concentrations of carbimazole (2.7–27000 μM) at different concentrations of linoleic acid (60, 90 and 120 μM).

290, 226 and 198 nm peaks and the appearance of a new peak at 210 nm.

This change was observed immediately when carbimazole was added towards the end of the enzymatic reaction, i.e. when the hydroperoxide concentration was high. To test whether the compound was in fact being oxidized, the spectrum was measured in the presence of hypochlorite (carbimazole 29 μM; and oxidising agent 8×10^{-6} g equiv.). The same changes were seen as during the enzyme reaction. It was concluded that carbimazole was being oxidised in the course of the enzymatic oxygenation. Similar changes were observed with the other compounds at analogous concentrations, although, since they are weaker electron donors than carbimazole, the reactions were slower.

3.3. *Chromatographic analysis of the reaction products*

Reaction of 200 μM linoleic or arachidonic acid and 37 nM soybean lipoxygenase in phosphate buffer (pH 7.4) was studied alone or in the presence of 116 μM carbimazole.

After 3 min incubation the reaction was stopped by the addition of 3 N HCl. The reaction products were extracted into diethyl ether. The ether layer was separated and evaporated to dryness under nitrogen. The residue was taken up in 100 μl ethanol. 2 μl aliquots were placed on the siliconized plates and run in heptane-methylformate-diethyl ether-acetic acid (65:20:10:2, v/v) over 8 cm.

In the control experiment (no carbimazole), the hydroperoxides (15-HPETE; 12-HPETE, 11-HPETE for arachidonic acid) were detected. In the presence of carbimazole all the hydroperoxides were reduced to the hydroxylated derivatives (15-HETE, 12-HETE, 11-HETE; fig.5), and the amount of fatty acid remaining at the end of the reaction was less. Carbimazole was also found to migrate differently, which could be a result of oxidation.

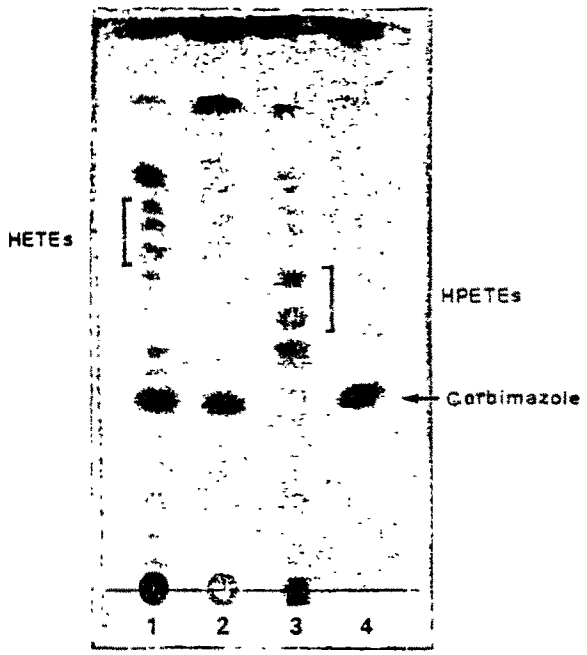

Fig.5. Thin layer chromatography on siliconized plates run in heptane methylformate-diethyl ether-acetic acid (65:20:10:2, v/v). Lanes: 1, arachidonic acid + soybean lipoxygenase + carbimazole; 2, arachidonic acid + carbimazole; 3, arachidonic acid + soybean lipoxygenase; 4, soybean lipoxygenase + phosphate buffer (pH 7.4) + carbimazole.

4. DISCUSSION

Carbimazole is the most active of the antithyroid agents and is the strongest electron donor with respect to iodine [13]. It was the compound we tested that had the greatest effect on the kinetics of the oxidation reaction of unsaturated fatty acids by soybean lipoxygenase. It acted both on the affinity of the enzyme for the substrate (reduced K_m) and on the reaction products (the hydroperoxides).

It is suggested that the enzyme-oxidized substrate complex described by Smith and Lands [4] is altered, and that the enzyme is thus protected by carbimazole at the start of the reaction, hence explaining the increase in initial reaction velocity. As the reaction proceeds, carbimazole is progressively oxidized and loses it protective capacity.

Thin layer chromatography confirmed that the hydroperoxides formed by the enzymatic reaction were in fact converted to the corresponding hydroxylated derivatives of the unsaturated fatty acids.

Since most molecules having an action on soybean lipoxygenase would also have effects on mammalian lipoxygenases [14], it is suggested that these imidazole derivatives, which are used in the treatment of thyroid disorders, could act on poly-

unsaturated fatty acid metabolism, via the lipoxygenase pathway. Moreover, these results could suggest an action on the other enzymes, such as the cycloxygenase.

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