Enzymatic determination of - and -naphthols using peanut peroxidase

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Novel cationic peanut peroxidase has been used for developing a technique for the determination of - and -naphthols based on their differing effect towards enzyme activity in the *o*-dianisidine oxidation reaction.

Cationic peanut peroxidase (CPP) belongs to those plant peroxidases (alfalfa, tobacco) whose isolation and investigation has only been started relatively recently.[1-4] We began a systematic study of the inhibitors and substrates of this enzyme and a development of procedures for their determination only 2 years ago;[5,6] until that time CPP had not been used in chemical analysis. The data obtained from our recent research has shown that the catalytic activity of CPP in the reactions of aryldiamine (and *o*-dianisidine, in particular) oxidation with H_2O_2 is inhibited by phenols (phenol and resorcinol, for example) with redox potentials higher than that of *o*-dianisidine (1.08, 1.04 and 0.85 V, respectively),[7] and phenols with potentials less than that of *o*-dianisidine (such as pyrocatechol, hydroquinone and pyrogallol with potentials 0.74, 0.71 and 0.61 V, respectively) are the second substrates of the enzyme.

The aim of this work is to study the effect of condensed phenols (- and -naphthols, in particular) on the catalytic activity of CPP, and to show the possibility of applying this enzyme in chemical analysis for the determination of these phenols.

Studying the influence of - and -naphthols on CPP catalytic activity. The reaction of *o*-dianisidine (3,3′-dimethoxybenzidine) oxidation with H_2O_2 catalysed by CPP was selected as an indicator for studying the possibility of determining - and -naphthols.† The rate of the enzymatic reaction was monitored spectrophotometrically by an increase in solution absorbance because of the formation of coloured products.

Studying the effect of naphthols on the CPP catalytic activity has shown that the introduction of -naphthol causes a decrease in the indicator reaction rate which is inversely proportional to -naphthol concentration. Kinetic curves of the reaction in the presence of -naphthol are characterised by the occurrence of an induction period, whose duration is directly proportional to the concentration of -naphthol, and the slope of the second part of the kinetic curves does not change at different concentrations of -naphthol (Table 1).

So, it has been shown that in spite of the similar structure of the condensed phenols studied, the nature of their effect was different, and might be explained in terms of their redox properties as well as in the case of polyphenols. Thus, -naphthol having a redox potential higher than that of *o*-dianisidine (1.09 and 0.85 V, respectively)[7] belongs to the CPP inhibitors, and -naphthol, which is more readily oxidised than *o*-dianisidine (E = 0.80 V), acts as the second substrate of CPP in the indicator reaction.

Table 1 Dependence of the indicator reaction rate on - and -naphthol concentration (n = 3).

Naphthol	C^a/ M	V_0^b/ M min^{-1}	τ_{ind}^c/s
In the absence of naphthol	—	4.27±0.03	—
-Naphthol	0.5	4.30±0.03	36±2
	2.5	4.20±0.07	92±5
	5.0	4.17±0.07	155±3
-Naphthol	1.0	3.87±0.07	—
	5.0	3.43±0.03	—
	10.0	2.83±0.03	—

[a]Naphthol concentration. [b]The initial indicator reaction rate. [c]The duration of the induction period.

Using the dilution method[9] we have shown that -naphthol is a reversible inhibitor of CPP. The results of studying the interaction of the inhibitor and enzyme over time have also confirmed the reversible character of the inhibition: the preliminary incubation[10] of a -naphthol solution (3 M) with CPP for 1 h does not change the extent of its inhibitory effect. The presentation of the experimental data obtained in the coordinates of Lineweaver–Buerck and Dixon[11,12] confirmed the mixed nature of the CPP inhibition with -naphthol. The values of the Michaelis constant are (10.4±0.5)×10^{-5}, (11.3±0.3)×10^{-5}, (13.0±0.3)×10^{-5} and (14.2±0.3)×10^{-5} M (at -naphthol concentrations 2, 4, 6 and 8 M, respectively), and the inhibition constant is (1.2±0.3) M.

Optimisation of the determination conditions of naphthols. To obtain the optimum conditions for the naphthol determination the dependences of the rate of the indicator reaction on pH and concentrations of *o*-dianisidine and H_2O_2 in the presence of - and -naphthols were studied. The conditions were considered to be optimum if the inhibitory effect of -naphthol was a maximum and the duration of the induction period in the presence of -naphthol was sufficiently long, but did not exceed 3 min. At the same time, the rate of *o*-dianisidine oxidation starting after the induction period needed to be high enough in the case of -naphthol. The choice of optimum peroxidase concentration was conditioned by the necessity to maintain a reaction rate convenient for spectrophotometric monitoring. These conditions are as follows: Tris-HCl buffer

Table 2 Analytical characteristics of the enzymatic determination of naphthols using oxidation of *o*-dianisidine catalysed by peanut peroxidase.

Compound	Applicable concentration range/ M	Equation of calibration graph	C_l^a/ M	RSD^b (%) (n = 3, P = 95%)
-Naphthol	0.5-5. 0	τ^c = 25.52x^d + 23.41	0.5	20
-Naphthol	1.0-10 .0	V_0^e = -0. 08x + 3.81	1.2	15

[a]The lower limit of analytical concentration. [b]The relative standard deviation for naphthol determination at a concentration equal to C_l. [c]The duration of the induction period/s. [d]The naphthol concentration. [e]The initial indicator reaction rate/ M min^{-1}.

† *Experimental details*. Cationic peanut peroxidase (1.11.1.7) was isolated from a medium of cultured cells.[8] Aqueous enzyme solutions were obtained by dissolving the enzyme preparation in Tris buffer (pH 7.5). Solutions of *o*-dianisidine and naphthols (analytical grade reagents from 'Soyuz Reactive', Moscow, Russia) were prepared daily by dissolution of accurately weighed amounts in ethanol (working naphthol solutions of lower concentration were prepared by diluting an ethanol solution with water), solutions of H_2O_2 (Merck) in water. Double distilled and demineralized water was used. Optimum conditions of the indicator reaction:[5] Tris-HCl buffer pH 5.0, concentrations of CPP 0.15 nM, *o*-dianisidine 0.12 mM, H_2O_2 0.15 mM.

The absolute value of the initial rate of the indicator reaction (V_0, M min^{-1}) was calculated according to the formula: $V_0 = c/\tau = A \cdot l/t \cdot l \cdot \varepsilon = \mathrm{tg}\,\alpha/\varepsilon \cdot l$, where c is the concentration of the reaction product; τ is the time of the reaction; ε is the molar absorbance coefficient; l is the cuvette length, tg α is the slope of a kinetic curve plotted as absorbance *vs*. time.

Table 3 Combined effect of - and -naphthol (ratio 1:5) on the indicator reaction rate ($n = 3$, $P = 95\%$).

Naphthol	C^a/ M	V_0^b/ M min^{-1}	τ_{ind}^c/s
In the absence of naphthol	—	4.20±0.07	—
-Naphthol	0.7	4.07±0.09	40±3
-Naphthol	3.5	3.67±0.07	—
-Naphthol + -Naphthol	0.7 3.5	3.70±0.08	38±1

[a]The naphthol concentration. [b]The initial indicator reaction rate. [c]The duration of the induction period.

pH 4.5–5.0 for -naphthol and pH 5.0–5.5 for -naphthol, concentrations: CPP 0.15 nM, *o*-dianisidine 0.25 mM, H_2O_2 75 M.

Analytical characteristics of the proposed procedures for the determination of naphthol are presented in Table 2.

As the effect of the nature of the naphthol on the catalytic activity of CPP was different, their combined action (at various ratios due to the concentration ranges applicable) on the indicator process was studied. It has been found that the reaction rate in the presence of both - and -naphthols (in 1:1–1:10 ratio) was the same as that in the presence of -naphthol alone, while the induction period was the same as that with -naphthol alone (Table 3). If the ratio of the naphthol concentrations is more than 1:10 (1:15, for instance), the reaction rate decreases significantly and it is difficult to measure the duration of the induction period. It has therefore been shown that - and -naphthols might be determined together in ratios 1:1–1:10.

Determination of -naphthol and -naphthol using the o-*dianisidine oxidation reaction*. 7 ml of a 0.05 M Tris-HCl buffer (pH 5.0), 0.05 ml of 30 nM CPP solution and the required volume of standard -naphthol (or -naphthol) solution over the concentration range 0.1–1 mM (or 0.05–0.5 mM) were placed sequentially in a glass test-tube fitted with a ground-glass stopper. 0.1 ml of a 25 mM *o*-dianisidine solution and H_2O up to10 ml volume of the reaction mixture in all cases were then added into the same test-tube. Finally, 0.1 ml of a 7.5 mM H_2O_2 solution was introduced. At the moment when the H_2O_2 was added and the reaction solution was mixed a stop-watch was started and the absorbance at 440 nm was measured at 15 s intervals for 2 min (or 5 min). The initial rate of the reaction (V_0) in the presence of -naphthol was characterised by the slope of a kinetic curve plotted as absorbance *vs*. time. The calibration graph for the determination of -naphthol was plotted as V_0 (M min^{-1}) *vs*. concentration. In the case of -naphthol determination the duration of the induction period (τ_{ind}/s) was determined. The calibration graph for the determination of -naphthol was plotted as τ_{ind} *vs*. concentration.

Thus, it can be stated that the nature of the effect of the condensed phenols (as well as polyphenols studied earlier) on the catalytic activity of peanut peroxidase is mainly governed by their redox properties. The fact that - and -naphthols belong to different groups of phenols — substrates or inhibitors of CPP — made it possible to elaborate a sensitive procedure not only for their individual determination, but also for the determination of one of them in the presence of the other in different ratios (1:1–1:10).

The data obtained show the potential for the application of CPP in chemical analysis for the determination of phenols and isomers, and in particular, inhibitors and substrates (such as - and -naphthols) in mixtures of them, without preliminary separation.

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