5 Kinetics of KCN binding to membrane bound and soluble cytochrome *d* in *Salmonella typhimurium*

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Introduction

Cytochrome *bd* type ubiquinol oxidase is one of the terminal oxidases in a number of gram-negative bacteria [1–3]. It catalyzes the oxidation of ubiquinol and the reduction of molecular oxygen to water [4]. Most of the studies on cytochrome *bd* have been done in *Escherichia coli*. In that organism it consists of two subunits I and II [5,6]. Extensive spectrophotometric studies, MCD, ESR and potentiometric titrations showed that the *bd* complex exhibited three redox centers, namely the low spin heme *b558* and the two high spin hemes *b595* and *d* [7–9]. Cytochrome *d* shows an α absorption band at 630 nm but little if any contribution in the Soret region. In contrast, cytochromes *b558* and *b595* show both α and Soret absorption bands. Interaction of various ligands with cytochromes *bd* type have been investigated [9 and references thereafter]. KCN was found to bind to the oxidized form of cytochrome *d* [1] but not to its reduced form [10]. Our previous studies in *Salmonella typhimurium* also showed that, for intact cells and spheroplasts, KCN bound to cytochrome *d* in the oxidized state [11]. In this research, we show that for membrane fragments and for solubilized cytochrome *d* from *S. typhimurium*, KCN binds to both the oxidized and the reduced form of cytochrome *d*. However, the absorption maximum of reduced-cytochrome *d*-KCN complex is at 635 nm instead of 648 nm, absorption maximum of the oxidized-cytochrome *d*-KCN complex.

Materials and methods

S. typhimurium was grown in medium containing 7 g peptone, 5 g yeast extract and 3 g/L NaCl, pH 7.0. After culture at 37°C for 48 h, cells were washed twice and resuspended in phosphate buffer 0.1 M, pH 7.0. Spheroplasts were prepared according to reference [12]. To prepare membrane fragments, spheroplasts were suspended in 0.05 M Tris-HCl (pH 9.0). The suspension was homogenized in a Waring blender, then centrifuged at 10,000 g for 40 min. The yellow cloudy supernatant consisting of membrane fragments and soluble proteins was centrifuged at 40,000 g for 40 min. The pellet consisting of membrane fragments was washed once with phosphate buffer 0.1 M, pH 7.0 and recentrifuged at 40,000 g for 40 min. To prepare soluble cytochrome *d*, membrane fragments were treated with 1% Triton X-100 at ~ 25°C for 45 min, then centrifuged at 50,000 g for 40 min. The yellow, pale supernatant containing cytochrome *d* was separated from the insoluble material (pellet) and used for further studies. Formation of a cytochrome *d*-KCN complex was observed spectrophotometrically using Aminco DW2 spectrophotometer. Samples were either air oxidized or dithionite reduced before addition of KCN; references were identical to the samples, except for KCN. All assays were carried at room temperature and, except for pH dependency studies, all assays were done at pH 7.0.

Results and discussion

Fig. 5.1 shows the effect of KCN on solubilized cytochrome *d* obtained from Triton-treated membranes. Addition of 20 mM KCN to the oxidized form of solubilized cytochrome *d* resulted in the formation of a trough at 648 nm which progressively increased, as shown in Fig. 5.1A. In contrast, addition of 20 mM KCN to dithionite-reduced solubilized cytochrome *d* resulted in the progressive formation of a trough with a maximum at 635 nm and 2 minor absorption bands at 599 nm and 678 nm (Fig. 5.1B). Thus a shift of 13 nm (from 648 nm to 635 nm) was observed between the absorption band maximum due to the oxidized-cytochrome *d*-KCN complex and the absorption maximum due to the reduced-cytochrome *d*-KCN complex. Fig. 5.2 shows the effect of increasing amounts of KCN on the formation of the oxidized-cytochrome *d*-KCN complex and dithionite-reduced-cytochrome *d*-KCN complex for both membrane bound and solubilized cytochrome *d*. When cytochrome *d*, either membrane bound or solubilized, was in the oxidized form, the amount of cytochrome *d*-KCN complex formed increased with the amount of KCN added up to roughly 15 mM KCN when a saturation effect was observed (Fig. 5.2A). The amount of complex formed per mg protein was about 3 times higher for solubilized cytochrome *d* when compared to membrane-bound cytochrome *d*. In contrast, when cytochrome *d*, either membrane bound or solubilized, was reduced, no saturation effect was observed even at 40 mM KCN. The amount of complex

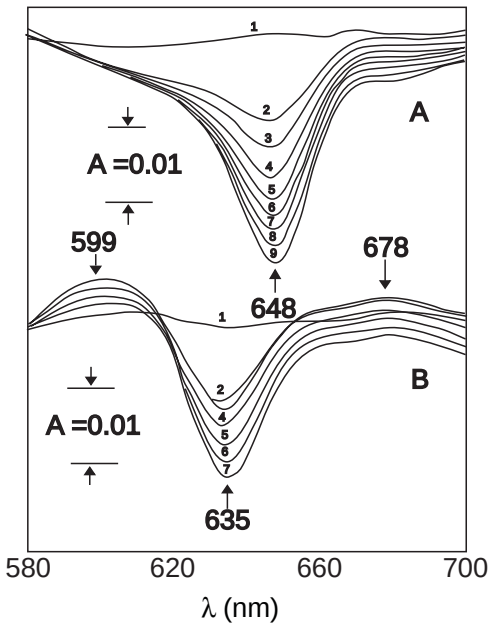

Fig. 5.1 Formation of cytochrome *d*-KCN complex upon addition of KCN (20 mM) to solubilized Cytochrome *d*. A: Air oxidized sample-KCN-minus-air oxidized sample (11.5 mg prot/ml); B: dithionite-reduced sample-KCN-minus-dithionite reduced sample (5.75 mg prot./ml). Difference spectra were recorded at 1 min intervals. The figure shows a progressively deepening trough at 648 (A) and 635 (B).

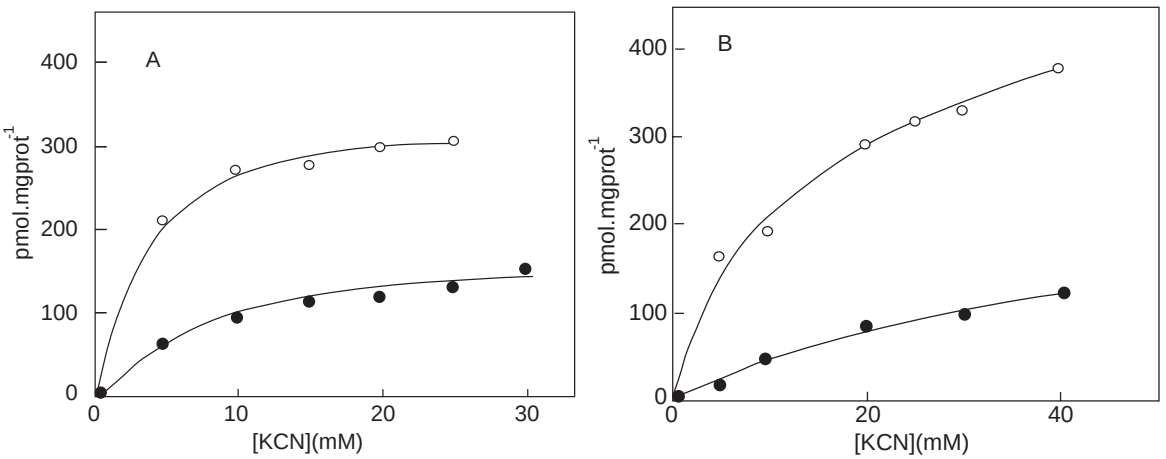

Fig. 5.2 Effect of increasing concentrations of KCN on the formation of cytochrome *d*-KCN complex in the soluble fraction (○) and in membrane fragments (•). A: oxidized samples; B: reduced samples. Each point was the average of 3 separate experiments.

formed per mg protein was still about 3 times higher for solubilized cytochrome *d* when compared to membrane-bound cytochrome *d*. Thus, unlike the observations made in intact cells and spheroplasts in which KCN bound only to the oxidized

Table 5.1 pH dependency of K_s and V_{max} for the formation of cytochrome *d*-KCN complex.

pH	K_s (mM)		V_{max} (pmol/min/mg prot)	
	Membrane	Soluble	Membrane	Soluble
5.0	4.0 ± 0.35	11.8 ± 0.90	27.5 ± 7.7	138 ± 7
6.0	4.5 ± 0.70	12.0 ± 3.00	54.0 ± 0.5	340 ± 24
7.0	4.0 ± 0.50	10.4 ± 0.35	101.0 ± 17.0	435 ± 7
8.0	4.2 ± 0.20	10.5 ± 0.70	114.0 ± 5.0	408 ± 40
9.0	4.4 ± 0.14	Denatured	65.0 ± 2.5	Denatured
10.0	4.1 ± 0.40	Denatured	48.0 ± 3.5	Denatured

Table 5.2 Effect of NaCl on K_s and V_{max} for the formation of cytochrome *d*-KCN complex.

NaCl (M)	K_s (mM)		V_{max} (pmol/min/mg prot)	
	Membrane	Soluble	Membrane	Soluble
0	4.0 ± 0.50	10.4 ± 0.35	101 ± 17	435 ± 7
0.1	3.9 ± 0.14	12.0 ± 1.40	118 ± 10	365 ± 49
0.2	4.0 ± 0.14	12.1 ± 1.60	132 ± 11	382 ± 74
0.5	4.2 ± 0.21	12.5 ± 1.10	107 ± 3.5	350 ± 28
1.0	4.2 ± 0.28	12.9 ± 0.50	102 ± 3.5	330 ± 0

Table 5.3 Effect of sucrose on K_s and V_{max} for the formation of cytochrome *d*-KCN complex.

Sucrose (M)	K_s (mM)		V_{max} (pmol/min/mg prot)	
	Membrane	Soluble	Membrane	Soluble
0	4.0 ± 0.50	10.4 ± 0.35	101 ± 17	435 ± 7
0.1	4.1 ± 0.14	12.2 ± 1.5	98 ± 3.5	360 ± 14
0.2	4.3 ± 0.17	13.0 ± 1.2	84 ± 9	350 ± 28
0.5	4.5 ± 0.14	12.0 ± 3.0	81 ± 5.6	350 ± 28
1.0	4.3 ± 0.42	11.8 ± 0.9	76 ± 1.4	350 ± 42

form of cytochrome *d* [11], KCN bound to both the oxidized and the reduced form of cytochrome *d* in membrane fragments and in the soluble fraction.

The effects of pH, ionic strength and sucrose molarity on the values of K_s and V_{max} for the formation of cytochrome *d*-KCN complex with either membrane bound or solubilized cytochrome *d* were shown in Tables 5.1, 5.2 and 5.3. For membrane bound cytochrome *d*, K_s was around 4 mM at all pH, NaCl concentrations and sucrose concentrations studied. The value of V_{max} varied as follows: it was minimal at pH 5.0 and 10.0 and maximal at pH 7.0 and 8.0. The value at pH

5.0 was 27% that at pH 7.0 and the value at pH 10.0 was 48% of the pH 7.0 value. In the presence of NaCl, V_{max} was the highest in 0.2 M NaCl. Nevertheless, with various NaCl concentrations, the value of V_{max} was close to the control value at pH 7.0.

Sucrose had a depressing effect on the V_{max} of KCN binding to membrane fragments. For example, in 1 M sucrose, V_{max} was 75% of the value of V_{max} in the absence of sucrose. For the solubilized cytochrome *d* K_s was around 12 mM for pH 5 to 8 and all NaCl and sucrose concentrations studied, but at pH 9 and 10, because of denaturation of cytochrome *d*, K_s and V_{max} were unmeasurable. In contrast, V_{max} for the formation of KCN-cytochrome *d* increased as a function of pH from pH 5.0 to pH 7.0; the value at pH 8.0 was just 7% less than that at pH 7.0 while that at pH 6.0 was 25% less than that at pH 7.0. In the presence of NaCl, V_{max} decreased progressively with increasing NaCl concentration. However, the lowest V_{max} value (in 1 M NaCl) was still 76% of the control value. In the presence of sucrose, V_{max} decreased to about 80% of the control value for all sucrose concentrations studied.

Our results showed that in KCN-inhibited cytochrome *d*, no change in K_s value was observed under our various experimental conditions. However, the V_{max} for the formation of the complex between cytochrome *d* (oxidized or reduced) and KCN was sensitive to changes in the pH, indicating that the conformation of both ferri and ferro-cytochrome *d* in the KCN inhibited enzyme was sensitive to proton binding, presumably in the active site of the enzyme. Differences in the K_s and V_{max} of the membrane bound KCN-inhibited cytochrome *d* and solubilized KCN-inhibited cytochrome *d* were consistent with the observation of Merle and Kadenbach [13] who showed that for cytochrome *c* oxidase the K_m and V_{max} values differed in the membrane bound and the deoxycholate solubilized enzyme. Thus, like cytochrome *c* oxidase reported in reference [13], the cytochrome *d* oxidase reported here behaved kinetically differently in the membrane bound state and in the solubilized state. Finally, both membrane bound and solubilized cytochrome *d* differed from the cytochrome *d* of intact cells in that even in the reduced state the membrane bound or solubilized enzyme would bind KCN.

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6 Kinetics of isoperoxidases and their differential sensitivity to inhibitors in saffron (*Crocus sativus* L.) bulb

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Introduction

Peroxidases (EC 1.11.1.7) are widely distributed throughout the animal and plant kingdoms. They have been isolated and characterized from several organisms [1,2]. Peroxidases fulfill a wide range of physiological functions. In human their involvement goes from asthma and respiratory diseases [3] to arteriosclerosis [4]. In plants, they play various roles in leaf and flower abscission, aging and senescence, cold tolerance, fruit development and ripening, etc [1,2]. Moreover, peroxidases have been used in analytical chemistry (blood sugar and cholesterol), immunochemistry, biosensor construction [5], food processing and food storage [2,5]. Welinder and coworkers [6] divided isoperoxidases into three distinct classes. In class I are peroxidases of prokaryotic origin including mitochondrial cytochrome c peroxidase and ascorbate peroxidase; class II peroxidases comprise extracellular fungal peroxidases such as lignin and manganese peroxidases; class III peroxidases include secretory plant peroxidases such as horseradish peroxidase.

For thousands of years, saffron has been valued and used because it flavors food and because of its medicinal values. However, besides a few recent studies on the anti-cancerous effect of saffron [7,8], research on the basic aspect of the physiology and biochemistry of this plant is unavailable. Because of the importance of peroxidases and their functional differentiation, we decided to study the kinetic properties of isoperoxidases in saffron bulb by using multiple substrates and inhibitors. Our results showed the presence of several kinetically distinct peroxidases in saffron bulb extract. These peroxidases have distinct kinetic properties, substrate oxidation and have different sensitivities to inhibitors such as azide and cyanide.

Materials and methods

Dormant saffron (*Crocus sativus* L.) bulbs were used throughout these studies. They showed neither roots nor shoots. Extracts were prepared from bulbs weighing each between 3 and 6 g, by homogenization in phosphate buffer 0.1 M, pH 7.0. After centrifugation at 4,000 g for 3 min, then at 10,000 g for 5 min, a clear, transparent supernatant termed 'crude extract' was obtained and used for our studies. Protein concentrations were determined by the Lowry method.

Peroxidase activity was determined by following the oxidation of o-dianisidine at 460 nm, guaiacol at 470 nm, KI at 350 nm, pyrogallol at 430 nm and ascorbate at 290 nm with extinction coefficients 11.3 $mM^{-1}cm^{-1}$, 26.6 $mM^{-1}cm^{-1}$, 26 $mM^{-1}cm^{-1}$, 2.47 $mM^{-1}cm^{-1}$ and 2.8 $mM^{-1}cm^{-1}$, respectively. Assays were carried out at room temperature (≈22–25°C), in the presence of 0.3 mM H_2O_2, using Aminco DW2 and Milton Roy spectrophotometers. Results were average of 3 different experiments conducted on crude extracts from 3 different batches of bulbs.

The pH activity curve was determined for each substrate using a citrate-phosphate-borate buffer system (range 3–10) at a concentration of 0.1 M.

Results and discussion

Kinetic studies for identification of various types of isoperoxidases in saffron (*Crocus sativus* L.) bulbs were based on the following criteria as suggested in references [9] and [10]: (i) variation in activity as a function of pH; (ii) variation in activity as a function of substrate concentration (K_m and V_{max}); (iii) variation in activity as a function of substrate structure, e.g., KI, o-dianisidine, ascorbate, etc.; (iv) effect of inhibitors on activity (differential effects of azide and cyanide).

Fig. 6.1 shows the rate of oxidation of o-dianisidine and guaiacol by saffron bulb extract at different pH, ranging from 3 to 10. Two optima peaks were found, at pH 4.0 and 6.5, respectively, for both o-dianisidine and guaiacol, indicating the presence of two distinct enzymes for each substrate [10]. The K_m values found for o-dianisidine at pH 4.0 (0.05 mM) and at pH 6.5 (0.25 mM) differed (Table 6.1), suggesting possibly distinct metabolic roles for the corresponding isoperoxidases [11]. Indeed, the variation in velocity of the reactions plotted as a function of the concentration of o-dianisidine gave two distinct curves (Fig. 6.2).

In contrast, the K_m values found for guaiacol at pH 4.0 and at pH 6.5 were similar (around 3 mM) (Table 6.1) and the variation in velocity of the reactions plotted as a function of the concentration of guaiacol gave two curves superimposing on each other (Fig. 6.3). However, the sensitivities to the inhibitory effects of azide and cyanide (KCN) were different (Table 6.2), confirming the presence of two distinct isoperoxidases for guaiacol as well, as suggested by the two pH optima. The guaiacol isoperoxidase active at pH 4.0 showed a high sensitivity to KCN but was

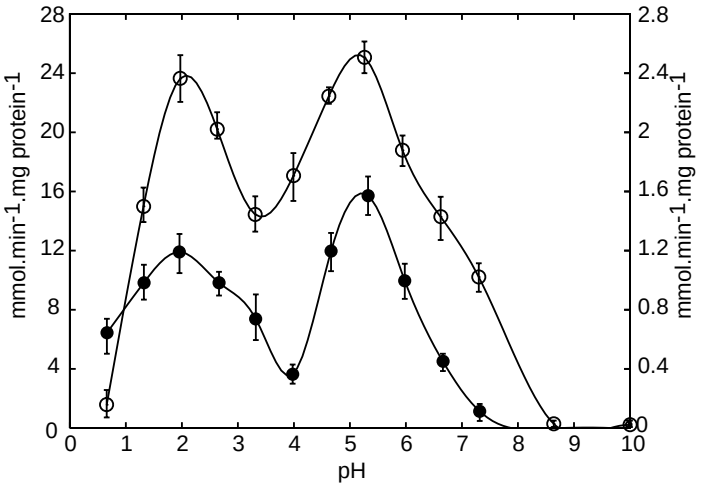

Fig. 6.1 pH dependency of o-dianisidine (○) and guaiacol (•) present in dormant saffron bulb.

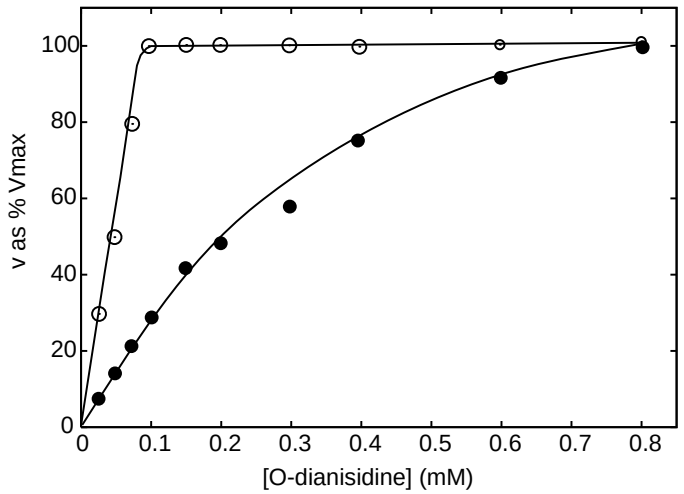

Fig. 6.2 Variation in velocity of the reactions catalysed by o-dianisidine isoperoxidases peroxidases with concentration of o-dianisidine, at pH 4.0 (○) and pH 6.5 (•).

much less sensitive to azide. In 1 μM azide the enzyme retained 100% activity but exhibited only 10% activity in 1 μM KCN. On the other hand, the guaiacol isoperoxidase active at pH 6.5 showed similar sensitivities to both azide and cyanide. In 1 μM azide or cyanide, the enzyme showed 65 to 70% activity. Furthermore, guaiacol oxidation at pH 4.0 was enhanced by 20% upon addition of 0.1 mM and 0.2 mM Mn^{2+} to the assay system; such sensitivity was used as evidence of the presence of Mn peroxidase in some organisms [12].

For KI, pyrogallol and ascorbate as substrates a single optimum peak at pH 5.0 for KI (Fig. 6.4), pH 7.5 for pyrogallol (Fig. 6.4) and pH 8.0 for ascorbate (re-

Table 6.1

Substrate	K_m (mM)	V_{max} (nmol.min^{-1}.mg prot^{-1})	k_4[a] ($\times 10^5$)
o-dianisidine (pH 4.0)	0.05	12.5	25
o-dianisidine (pH 6.5)	0.25	30.0	12
guaiacol (pH 4.0)	2.8	3.0	0.11
guaiacol (pH 6.5)	3.3	4.5	0.14
KI (pH 5.0)	20.5	56.0	0.27
Pyrogallol (pH 7.0)	0.8	47.0	5.9
Ascorbate (pH 8.0)	0.12	17.0	14

[a] calculated according to [13] and expressed in l.min^{-1}.mg prot^{-1}

Table 6.2

Inhibitor	Relative activity (% control)						
	o-dianisidine		guaiacol		KI	Pyrogallol	Ascorbate
	pH 4.0	pH 6.5	pH 4.0	pH 6.5	pH 5.0	pH 7.0	pH 8.0
None	100	100	100	100	100	100	100
Azide							
1 μM	90	100	100	65	100	85	100
10 μM	70	100	50	50	80	85	98
100 μM	20	70	–	10	75	75	95
Cyanide							
1 μM	70	90	10	70	90	95	100
10 μM	25	55	5	40	80	65	95
100 μM	–	15	0	5	0	15	90

sults not shown) was obtained. The ascorbate peroxidase detected was assumed to be cytosolic since there are no chloroplasts in saffron bulbs. Table 6.1 shows the K_m, V_{max} and k_4 values for each substrate at optima pH. Results suggested 6 different K_m, V_{max} and k_4 for the 5 substrates studied. Variable values for kinetic parameters such as K_m and k_4 have been used as criteria for the detection of isoperoxidases in bone marrow under immunostimulation [13]. The values found in this study, along with the pH-activity curves, suggested the presence of several isoenzymes, perhaps up to seven. These results were further substantiated with the effect of inhibitors (Table 6.2) as shown above for guaiacol isoperoxidases. For the other peroxidases, different sensitivities were found as well. For example, in 100 μM azide, o-dianisidine isoperoxidase pH 4.0 showed 20% activity while o-dianisidine isoperoxidase pH 6.5, as well as KI and pyrogallol isoperoxidases still conserved around 70% activity. In 100 μM KCN, no activity was detectable for KI isoperoxidase while 15% activity was still detected for o-dianisidine isoperoxidase pH 6.5 and pyrogallol isoperoxidase; in contrast, in 10 μM KCN, the activity

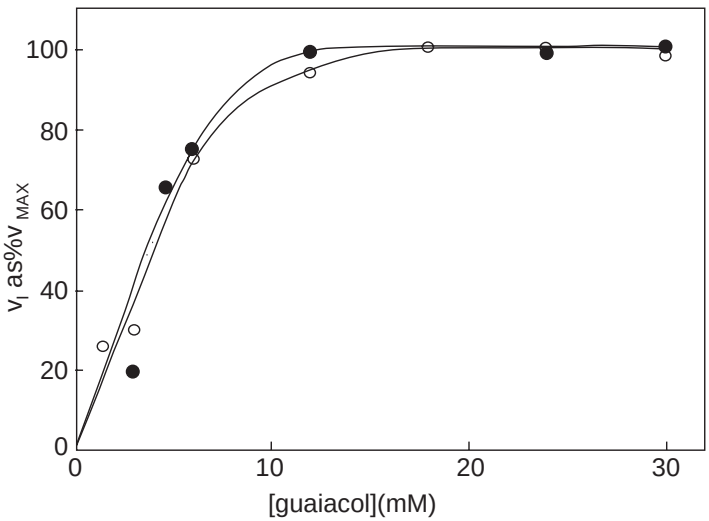

Fig. 6.3 Variation in velocity of the reactions catalysed by guaiacol isoperoxidases with concentration of guaiacol, at pH 4.0 (◦) and pH 6.5 (•).

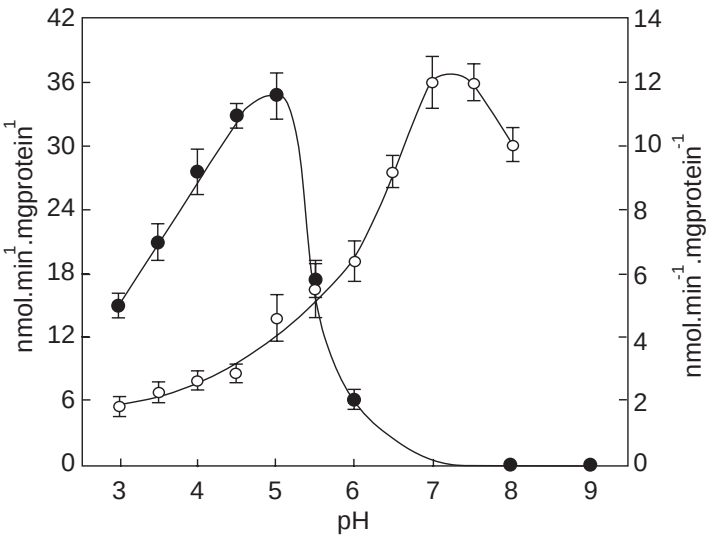

Fig. 6.4 pH dependency of KI (•) and pyrogallol (◦) peroxidases present in dormant saffron bulb.

dropped at 55% for o-dianisidine isoperoxidase pH 6.5 and 65% for pyrogallol isoperoxidase, but was still 80% for KI isoperoxidase. Ascorbate peroxidase was remarkably insensitive to both azide and cyanide for concentrations up to 100 μM.

Our studies suggested that in dormant bulb of saffron several isoperoxidases could be kinetically distinguished. Some of them, such as guaiacol peroxidase, Mn peroxidase and ascorbate peroxidase, were probably similar to those already described in fungi [12] and plants [14] while others such as KI peroxidase with

distinctive pH optima and kinetics values, needed to be characterized. Their distinctive kinetic properties suggested possible different metabolic roles for each isoenzyme in the future development of the plant.

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