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THE EFFECT OF SODIUM DODECYL SULPHATE ON POLYPHENOL OXIDASE

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Key Word Index—*Vicia faba*; Leguminosae; polyphenol oxidase; SDS activation.

Abstract—Polyphenol oxidase (PPO) from *Vicia faba* is enzymically inactive in aqueous buffers at neutral pH, but is active at acidic pHs (pH 3–4). However, the presence of sodium dodecyl sulphate (SDS) in the reaction medium eliminates the activity at such acid pH and greatly increases the activity at neutral pH. This activation by SDS at various pHs is not dependent on the substrate. Cresolase and catecholase activities of the enzyme were activated by SDS at neutral pH. This activation is rapid and dependent on detergent concentration. The stability of the enzyme during relatively long treatments with SDS is also pH dependent. This relationship between proton and SDS concentrations is interpreted as a displacement of the sensitive p*K*s of the enzyme caused by the interaction with SDS molecules. This displacement depends on the binding of SDS to a specific centre, with a dissociation constant of 0.52 mM.

INTRODUCTION

Polyphenol oxidase (PPO) (EC 1.14.18.1) catalyses the *o*-hydroxylation of monophenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity) at the expense of molecular oxygen. In healthy leaves, PPO is localized in the chloroplast, where it is bound to be thylakoid membranes. However, it is not an intrinsic membrane protein and can be released from the thylakoids by sonication, mild detergent treatment or protease treatment [1]. One unusual and intriguing characteristic of the enzyme is its ability to exist in an inactive or latent state. Catecholase activity has been activated by a variety of treatments or agents such as proteases [2, 3], urea [4], fatty acids [5], polyamines [6], divalent cations [7], polyglucan elicitors [8], acid or basic shock [9] and anionic detergents such as SDS [10, 11]. First experiments carried out by Kenten [9] showed that crude extracts of broad bean leaves contained much latent phenolase and its activity could be released by brief exposure of the extracts to acid (pH 3–3.5) or alkaline (pOH 2.5–3) conditions. Kenten [10] was also the first to report the activation of crude bean PPO by denaturants. The enzyme was activated with anionic wetting agents, such as aerosol OT (sodium dioctylsulphosuccinate) and SDS. Cationic and non-ionic wetting agents had no effect. Robb *et al.* [12] and Swain *et al.* [4] took Kenten's studies [10] further. Using a partially purified enzyme, they found that prolonged incubation with SDS resulted in a decrease in activity. One of the more recent and thorough studies examining SDS activation of the enzyme was carried out by Moore and Flurkey [11]. They determined the effect of SDS on plant PPO, and suggested that a limited conformational change due to binding of small amounts of SDS, may induce or initiate the activation of the latent enzyme. They also reported that the low pH optimum (below pH 4.0) obtained for the latent enzyme was abolished in the presence of SDS and stated that it remained to be seen whether the acid-shocking and the SDS activation processes were related through a common mechanism. In connection with this observation and in order to study the possible relation between these two activating factors (SDS and pH) we have carried out PPO activation at several SDS concentrations and different pH values. Although recent studies carried out with apple PPO [13] have pointed out a relation between SDS and pH effects on this fruit enzyme, the possible influence of pH on the effect of SDS, and vice versa, has not been thoroughly investigated in the case of leaf PPO. Marquès *et al.* [13] have also suggested the existence of a regulatory domain of PPO, shifted by the addition of SDS, which might control the enzymic activity. We have therefore analysed in more detail in this paper the complex effect of SDS and proton concentration on latent broad bean PPO.

Besides being activated by anionic detergents, PPO can also be activated by a variety of treatments, e.g. by proteases such as trypsin [2, 14]. In this work we have also activated the enzyme with trypsin and the results

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obtained for proteolytic activation are compared with those obtained when SDS was used.

The cresolase activity of PPO is characterized by a lag period which depends on the substrate and enzyme concentrations, as has been reported for other PPOs when cresolase activity was measured [15–17]. We have also obtained this latter activity in latent state and, like catecholase activity, it can be activated when different concentrations of SDS are added to the reaction medium. SDS was able to activate broad bean PPO, increasing both activities of the enzyme and shortening the characteristic lag of the cresolase activity.

RESULTS AND DISCUSSION

The activation process of broad bean PPO by SDS was studied with three substrates: 4-methylcatechol, *t*-butylcatechol and chlorogenic acid. Figure 1 shows the results obtained when enzyme activity was measured, at different pH values, in the absence or presence of 0.2 mM SDS. At this detergent concentration the enzyme was fully active. The increase of activity was higher when 4-methylcatechol was used as substrate (Fig. 1A) than when *t*-butylcatechol (Fig. 1B) or chlorogenic acid (Fig. 1C) was used. In the absence of SDS the maximal activity was obtained at acid pH values (at *ca* pH 3.4 for all substrates). This low pH optimum is a result of the activity being induced by acid shocking [4, 9]. Moore and Flurkey [11] observed a pH optimum in a similar range of values.

Assays in the presence of SDS eliminated the pH optimum found below pH 4.0 and a new maximum appeared at or about pH 5.0 for 4-methylcatechol and *t*-butylcatechol (Figs 1A and 1B, respectively) and at pH 4.8 for chlorogenic acid. Activation with SDS depends on the surfactant concentration and, as Fig. 1 shows, it also depends on pH. In order to study whether this action also depended on the substrate used, we determined the degree of activation for each substrate at different pH values (Fig. 2). There was no significant difference in the activation values for any substrate. Activity in the presence of SDS, increased *ca* 65±5 fold. Maximal activation was obtained at slightly different pH values: at pH 5.1 for *t*-butylcatechol and at pH 4.8 for chlorogenic acid. As regards 4-methylcatechol, maximal activation appeared at pH 5.4; however, we routinely determined the activity with 4-methylcatechol at pH 5.0 since at this pH the activity was higher than at pH 5.4 (as seen in Fig. 1A) and, in addition, the enzyme is practically latent at this pH value. As can be seen in Fig. 2, activation at pH 4–4.5 was higher in the case of 4-methylcatechol than when *t*-butylcatechol or chlorogenic acid were used as substrates. It was not possible to determine the degree of activation at pH values higher than the ones shown in Fig. 2 since at those pHs the activity of the latent enzyme was practically undetectable for all three substrates. The results shown in this figure clearly demonstrate that SDS activation of broad bean PPO

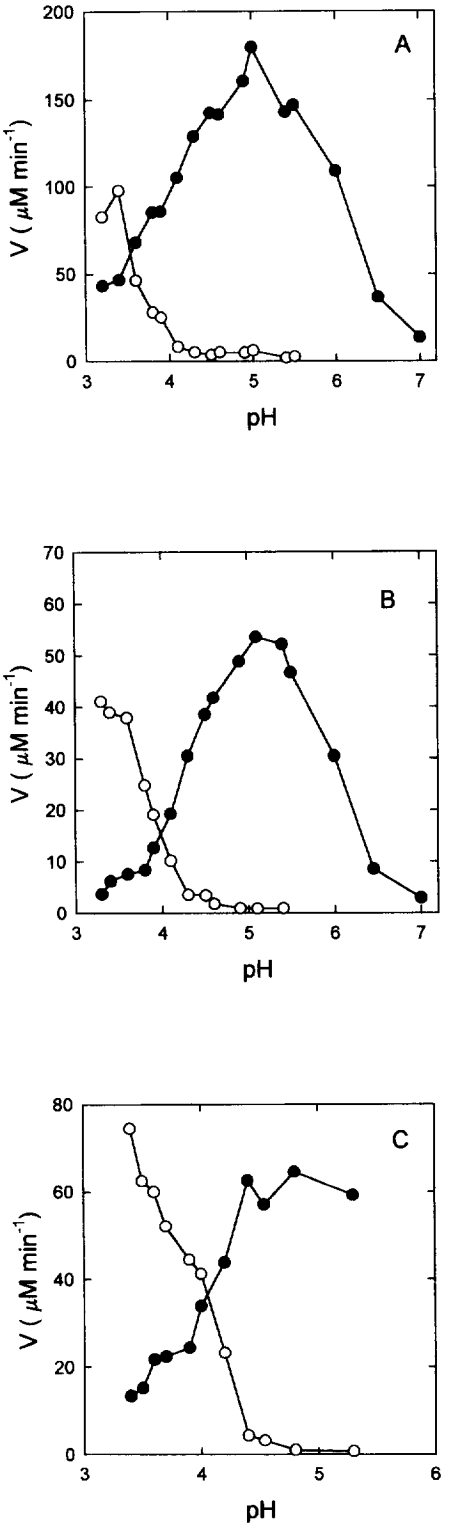


Fig. 1. Catecholase activity of broad bean leaf PPO at different pH values, with (●) and without (○) SDS, towards (A) 4-methylcatechol, (B) *t*-butylcatechol and (C) chlorogenic acid. The reaction medium, at 25°, contained the substrate, at the concentration indicated in the Experimental, in 0.1 M acetate (pH 3.2–5.5) and phosphate (pH 5.5–7.0) buffers. The SDS concentration used was 0.2 mM. Enzyme concentration was 0.5 μg ml^{-1} (A and B) and 3.75 μg ml^{-1} (C).

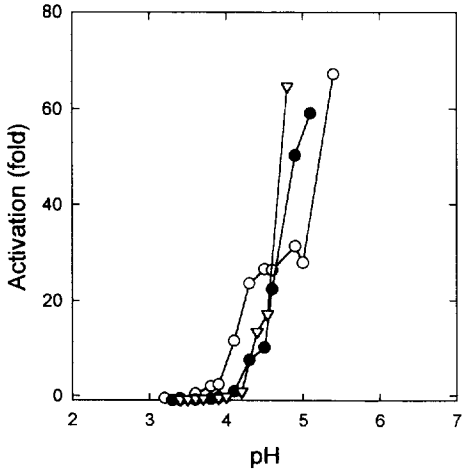


Fig. 2. Degree of activation at different pH values when (○) 4-methylcatechol, (●) *t*-butylcatechol or (▽) chlorogenic acid was used as substrate. Activation fold is expressed as the ratio between the activity of the enzyme activated with 0.2 mM SDS and the activity of the latent PPO.

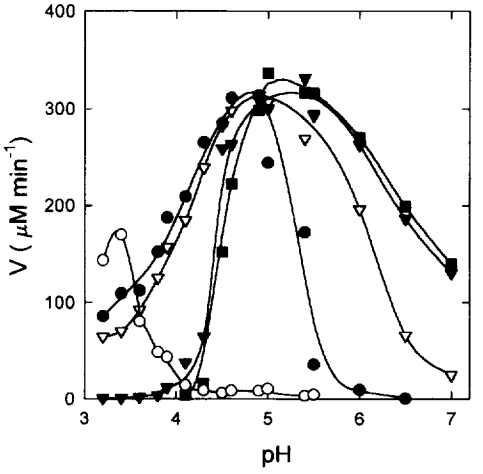


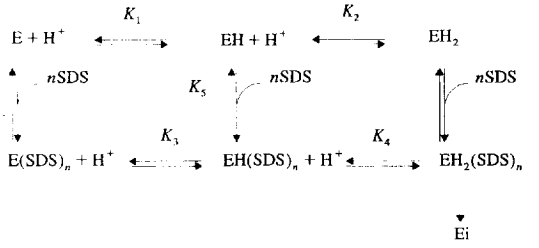
Fig. 3. Effect of pH on the catecholase activity of broad bean PPO at different SDS concentrations. The reaction medium contained 5 mM 4-methylcatechol in 0.1 M sodium acetate (pH 3.2–5.5) or phosphate buffers (pH 5.5–7.0) and different concentrations of SDS: (●) 0.08 mM, (▽) 0.2 mM, (▼) 1 mM, (■) 3.5 mM and (○) without SDS. Enzyme concentration was 0.9 μg ml^{-1}.

does not depend on the substrate used. Sánchez-Ferrer *et al.* [17], However, obtained different degrees of SDS activation, depending on the substrate, in the case of potato leaf PPO.

For the purpose of characterizing in more detail the behaviour of PPO towards each substrate, kinetic parameters of the activated enzyme were determined at pH 5.0, obtaining the following values for 4-methylcatechol (V_m = 241.0 μM min^{-1}, K_m = 1.0 mM, $V_m/K_m = 24.1 \times 10^{-2}$ min^{-1}), for *t*-butylcatechol (V_m = 73.0 μM min^{-1}, K_m = 2.8 mM, $V_m/K_m = 2.6 \times 10^{-2}$ min^{-1}) and for chlorogenic acid (V_m = 14.0 μM min^{-1}, K_m = 4.4 mM, $V_m/K_m = 0.32 \times 10^{-2}$ min^{-1}). At this pH, it was impossible to determine the corresponding kinetic parameters for the latent enzyme due to the very low activity in the absence of SDS. 4-Methylcatechol showed the lowest K_m and highest V_m values, and so the catalytic efficiency was higher than with the other two substrates. For this reason and despite the fact that the degree of activation obtained was similar for the three substrates, standard enzymic assays were made with 4-methylcatechol.

The results obtained (Fig. 1) clearly show that the low pH optimum was abolished in the presence of activating concentrations of SDS. This effect suggested the possible existence of a relationship between the activation by proton concentration and the activation by anionic detergents such as SDS. In order to investigate this possibility, the enzyme was activated at different pH values with increasing SDS concentrations. The results of these experiments are shown in Fig. 3. As can be seen, there is a displacement in the pH optimum curves towards higher pH values as SDS concentration increased. The results shown in this figure suggest that at very acidic pH values the term 'activation' is not the most appropriate to explain the effect of SDS since, at these pH values, the addition of detergent caused a decrease in enzyme activity. However, at more basic pHs, SDS increased enzymic activity and, therefore, we think that the word 'activation' used in general terms to explain the effect of SDS on PPO activity might be misleading and should be restricted only to pH values greater than 4.0.

To explain these results the following mechanism is proposed:



where the existence of two sensitive p*K*s which determine the presence of a maximum in the activity/pH profile has been considered. EH and EH(SDS)$_n$ are the active forms of the enzyme, the form present at high SDS and proton concentration [EH$_2$(SDS)$_n$] being susceptible to inactivation (Ei). The equilibrium constants are defined by the following equations:

$$K_1 = \frac{[E][H^+]}{[EH]} \quad (1)$$

$$K_2 = \frac{[EH][H^+]}{[EH_2]} \quad (2)$$

$$K_3 = \frac{[E(SDS)_n][H^+]}{[EH(SDS)_n]} \quad (3)$$

$$K_4 = \frac{[EH(SDS)_n][H^+]}{[EH_2(SDS)_n]} \quad (4)$$

$$K_5 = \frac{[EH][SDS]^n}{[EH(SDS)_n]} \quad (5)$$

and the rate equation is

$$v = k_1[EH] + k_2[EH(SDS)_n] \quad (6)$$

k_1 and k_2 being the specific rate constants.

By simply resolving the corresponding mathematical calculations the following rate equation can be established:

$$v = \frac{(k_1K_5K_2K_4 + k_2K_4K_2[SDS]^n[H^+]}{K_1K_2K_4K_5 + K_4K_5[H^+]^2 + K_4K_5K_2[H^+] + K_3K_4K_2 \times [SDS]^n + K_2[SDS]^n[H^+]^2 + K_4K_2[H^+][SDS]^n} \quad (7)$$

If the SDS concentration is kept constant, equation (7) can be simplified as follows:

$$v = \frac{a[H^+]}{b + c[H^+] + [H^+]^2} \quad (8)$$

where

$$a = \frac{k_1K_5K_2K_4 + k_2K_4K_2[SDS]^n}{K_4K_5 + K_2[SDS]^n} \quad (9)$$

$$b = \frac{K_1K_2K_4K_5 + K_3K_4K_2[SDS]^n}{K_4K_5 + K_2[SDS]^n} \quad (10)$$

$$c = \frac{K_4K_5K_2 + K_4K_2[SDS]^n}{K_4K_5 + K_2[SDS]^n} \quad (11)$$

The experimental data shown in Fig. 3 can be fitted to equation (8) and for each SDS concentration the values of the corresponding parameters, a, b and c were evaluated, as seen in Table 1. Since these parameters are dependent on SDS concentration, we can plot a, b and c against the different detergent concentrations. Figure 4 shows the plots corresponding to the kinetic

Table 1. Kinetic parameters, a, b and c, at different SDS concentrations*

[SDS](mM)	a	b	c
0.00	3.28×10^{-1}	6.28×10^{-8}	7.55×10^{-4}
0.08	3.47×10^{-2}	7.59×10^{-10}	5.87×10^{-5}
0.20	2.36×10^{-2}	1.15×10^{-10}	5.13×10^{-5}
1.00	7.50×10^{-3}	1.00×10^{-11}	1.65×10^{-5}
3.50	8.00×10^{-3}	1.00×10^{-11}	1.60×10^{-5}

*These values were obtained from the curve fit, according to equation (8), to the experimental data shown in Fig. 3.

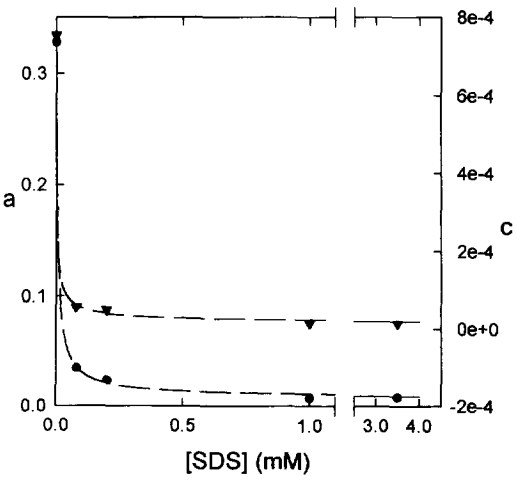


Fig. 4. Variation of the kinetic parameters, (●) a and (▼) c, with SDS concentration. Curves (dashed lines) fitted according to equations (9) and (11).

parameters a and c. We can proceed in a similar way with b (figure not shown). From the limits of equations (9), (10) and (11), when $[SDS] \rightarrow \infty$ and $[SDS] \rightarrow 0$, it can be calculated that $a_\infty = k_2K_4$ and $a_0 = k_1K_2$; $b_\infty = K_3K_4$ and $b_0 = K_1K_2$ and finally $c_\infty = K_4$ and $c_0 = K_2$, respectively. From these relations, the values for the kinetic constants can be evaluated ($K_1 = 8.32 \times 10^{-5}$ M, $K_2 = 7.55 \times 10^{-4}$ M, $K_3 = 0.63 \times 10^{-6}$ M, $K_4 = 1.6 \times 10^{-5}$ M, $k_1 = 4 \times 10^2$ sec^{-1} and $k_2 = 5 \times 10^2$ sec^{-1}).

The experimental data in Fig. 4 can be fitted (dotted lines) according to equations (9) and (11), respectively, by using the values previously obtained for the constants k_1, k_2, K_1, K_2, K_3 and K_4 in order to evaluate the values of K_5 and n. Similarly, the values for the kinetic parameter b can be fitted to equation (10) (data not shown). From these curve fits, an average value for $K_5 = 0.52 \pm 0.05$ mM and $n = 0.9 \pm 0.1$ were obtained. These results indicate that, among the binding centers for SDS, there is one which is responsible for the pH dependence of PPO activity, the dissociation constant being 0.52 mM. To ratify these results further an activation profile by SDS at pH 5.5, which favours the species $EH(SDS)_n$, was established (figure not shown), and from the data fitting, a constant value of 0.4 mM was obtained, which was very close to the previously obtained value for the dissociation constant (K_5).

An excess of SDS in the reaction medium results in loss of activity (data not shown), which is in accordance with previous studies [4, 11]. To investigate whether this activity loss was also pH dependent, the enzyme was incubated with increasing detergent concentrations at different pH values. Aliquots were removed from the incubation media at different times and the activity was measured as described in Experimental. Figure 5 shows only the data obtained after 10 min of incubation. As can be seen, the enzyme was inactivated rapidly by

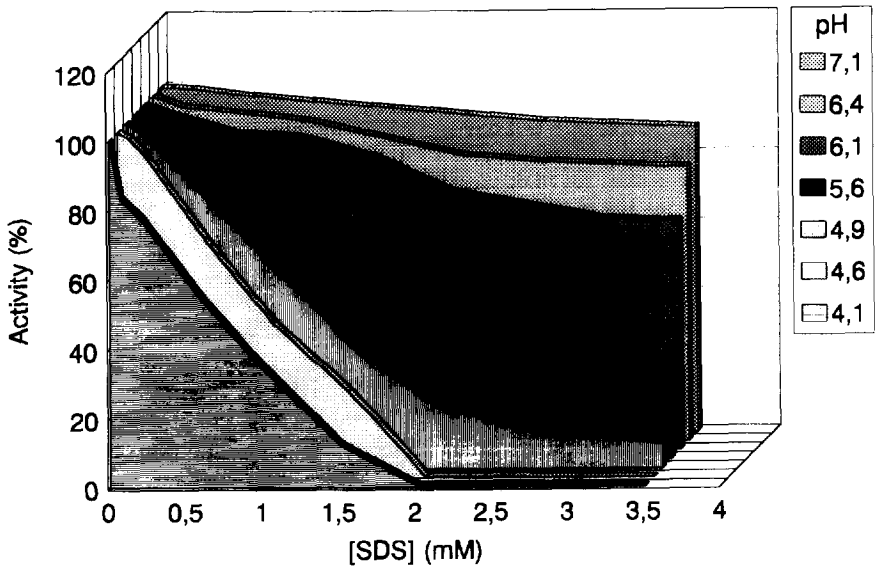


Fig. 5. Enzyme inactivation by SDS at different pH values. Assays were carried out as described in the Experimental.

SDS concentrations above 2 mM when the pH was acid (pH 4.1–4.6). However, these concentrations did not inactivate the enzyme at pH values close to 7.0. Therefore, at relatively high SDS concentrations, when the pH is maintained close to neutrality, the enzyme is not inactivated. However, relatively low SDS concentrations cause a rapid inactivation of the enzyme at acid pH.

In order to compare SDS activated enzyme with enzyme activated by other procedures we used trypsin as proteolytic agent. Broad bean leaf PPO is proteolytically cleaved by trypsin, yielding a 45-kDa peptide that had PPO activity and an inactive peptide of 15 kDa [1]. Trypsin was less effective than SDS in activating the enzyme [2, 14]. In our experiments, activation by trypsin was half that obtained in the presence of detergent (data not shown). To investigate whether the protease- and detergent-activated enzymes behaved similarly towards pH, PPO was activated with trypsin (as described in Experimental) and the activity was measured at different pH values, as shown in Fig. 6. The results plotted for *t*-butylcatechol are the result of multiplying the experimental data by three. The optimum pH for proteolytic activated enzyme was *ca* 4.0 with 4-methylcatechol or *t*-butylcatechol. As Fig. 6 shows, the pH profile for trypsin-activated enzyme is different from the pH profile for the detergent-activated enzyme and even different from the pH profile obtained for the latent enzyme (see Fig. 1). As can be seen, trypsin did not eliminate enzymic activity at very acidic pHs, as in the case of SDS. It seems, therefore, that the behaviour of PPO at different pHs depends on the agent used to provoke activation.

Although PPO also catalyses the hydroxylation of monophenols to *o*-diphenols, data referring to plant cresolase activity are scarce, probably due to the very low activity values usually obtained [10, 14]. This activity was also increase by SDS (figure not shown), the enzyme being completely activated at detergent concentrations close to 2 mM. The characteristic lag for cresolase activity was drastically shortened by the presence of SDS in the reaction medium. At the lowest SDS concentration tested, the lag lasted 47 min. This high lag value obtained in the absence of SDS could explain the difficulty found by other authors in measuring cresolase activity.

This paper, therefore, demonstrates the antagonistic effect between SDS and proton concentrations in the expression of polyphenol oxidase. We propose that 'activation' is not the most appropriate term used to explain the effect of SDS at acidic pH, and it should be restricted to pH values higher than 4. A kinetic model

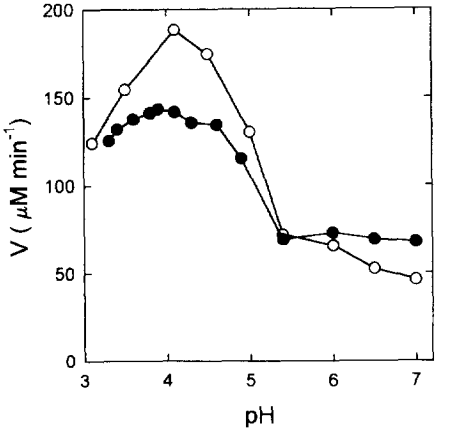


Fig. 6. Catecholase activity of broad bean PPO activated with trypsin at different pH values. The reaction medium included (○) 5 mM 4-methylcatechol or (●) 5.25 mM *t*-butylcatechol in 100 mM acetate (pH 3.1–5.4) and phosphate (pH 6.0–7.0) buffers. Enzyme concentration was 1 μg ml^{-1}.

has been presented that explains the experimental results as a specific effect of SDS on the p*K*s, which determine the enzymic activity. The equilibrium constants as well as the specific rate constants involved in the activation scheme for PPO by SDS and proton concentration have been determined and our results indicate that, among the binding centres for SDS, there is one which is responsible for the pH dependence of PPO. The existence of a regulatory domain where pH controls the enzymic activity has been suggested for apple PPO [13]. This region or domain could be cut out by proteolysis or shifted by the addition of SDS.

EXPERIMENTAL

Reagents. SDS was purchased from Sigma and contained *ca* 99% C_{12}, as determined by GC. Trypsin was obtained from Boehringer. *t*-Butylcatechol was obtained from Aldrich. Triton X-114 (TX-114) was purchased from Fluka and condensed ×3 as described [18], but using 100 mM Na-Pi buffer, pH 7.3. The detergent phase of the third condensation had a concn of 25% (w/v) TX-114 and was used as stock soln for all experiments. All other chemicals were of reagent grade and obtained from Sigma.

Plant material. Broad bean (*Vicia faba* L. cv. Muchamiel) plants were grown in vermiculite for 3 weeks at 23° and 70% humidity. They were watered twice a week with Cron medium [19].

Enzyme extraction. All procedures were carried out at 4°. Broad bean leaves (50 g) of assorted sizes [20] were homogenized in 500 ml 100 mM Na-Pi buffer, pH 7.3, containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethyl sulphonylfluoride and 1 mM benzamidine. The slurry was filtered through 8 layers of gauze and centrifuged at 600 *g* for 2 min. The pellet was discarded, and the supernatant was centrifuged at 6000 *g* for 10 min to ppt. the thylakoid membranes. The resulting pellet was washed ×2 by resuspending in 40 ml grinding buffer without sorbitol and centrifuging for 10 min at 6000 *g*. The final pellet was resuspended in 15 ml homogenization buffer without sorbitol and sonicated for 5 min. The sonicated thylakoid suspension was centrifuged at 50 000 *g* for 1 hr. This supernatant was subjected to temp. phase partitioning by adding TX-114 at 4°, so that the final detergent concn was 8% (w/v). The mixt. was kept at 4° for 15 min and then warmed to 37°. After 15 min. the soln became spontaneously turbid due to formation, aggregation and pptn of large mixed micelles composed of detergent, hydrophobic proteins and the remaining chlorophylls [21]. This soln was centrifuged at 5000 *g* for 10 min at 23°. The green detergent-rich phase with no PPO activity was discarded and the clear supernatant was used as enzyme source and had a specific activity 4 times lower than the enzyme described in ref. [1].

Enzyme assay. Catecholase and cresolase activities were determined spectrophotometrically at 400 nm [22]. Unless otherwise stated, the reaction medium (1 ml), at 25°, contained 100 mM NaOAc buffer, pH 5, 5 mM 4-methylcatechol ($\varepsilon_{o\text{-quinone}} = 1433\ M^{-1}\ cm^{-1}$). When other *o*-diphenols, such as *t*-butylcatechol ($\varepsilon_{o\text{-quinone}} = 1682\ M^{-1}\ cm^{-1}$) and chlorogenic acid ($\varepsilon_{o\text{-quinone}} = 1018\ M^{-1}\ cm^{-1}$), were used as substrates, the concns were 5.25 and 6 mM, respectively. One unit of enzyme was defined as the amount of enzyme that produces 1 μmol *o*-benzoquinone min^{-1}. In activation assays with SDS, detergent was added, unless otherwise specified, at a routine concn of 2 mM. In the proteolytic activation assays, the sample was preincubated with trypsin (1000 units ml^{-1}) for 5 min at 37°. As regards cresolase activity, the reaction medium (1 ml) contained 100 mM Na-Pi buffer, pH 7, 0.5 mM *p*-cresol and SDS at different concns.

pH studies were carried out using 0.1 M NaOAc and Na-Pi buffers. Enzyme assays were performed in the presence and absence of activating agent at each pH, and at different concns of SDS. After catalysis, the pH of the assay soln was again measured at room temp.

Inactivation with SDS. These experiments were carried out by preincubating the enzyme at 25° with different SDS concns in 0.1 M Na-Pi buffer or 0.1 M NaOAc buffers at different pH values. Aliquots were removed at various times and assayed for PPO activity, as described in enzyme assays, in 0.1 M NaOAc buffer, pH 5 and 5 mM 5-methylcatechol. Although the incubation media contained different amounts of SDS, the final detergent concn in the reaction medium was kept constant at 0.175 mM.

Other methods. Protein was determined according to the method of ref. [23] using BSA as a standard.

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