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pH-INDUCED HYSTERESIS OF LATENT BROAD BEAN POLYPHENOL OXIDASE

MERCEDES JIMÉNEZ and FRANCISCO GARCÍA-CARMONA*

Departamento de Bioquímica y Biología Molecular A, Unidad Docente de Biología, Facultad de Veterinaria, Universidad de Murcia, Espinardo, E-30001 Murcia, Spain

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Key Word Index—*Vicia faba*; Leguminosae; broad bean; polyphenol oxidase; latent enzyme; hysteresis; co-operativity; lag period.

Abstract—A study of the catecholase activity of a latent plant polyphenol oxidase, extracted and purified from the chloroplast membranes of broad bean leaves (*Vicia faba* L. cv Muchamiel) showed the existence of a lag period which was dependent on pH. Steady-state rate was reached immediately when pH values fell below 3.3, suggesting that the enzyme was of hysteretic nature. Steady-state rates were linear with enzyme concentration but the lag period remained constant, indicating that the 'slow' conformational changes in the protein during the assay were pH-induced. Results obtained by varying substrate concentration showed that the system presents hyperbolic or co-operative kinetics, depending on the pH of the assay.

INTRODUCTION

It has been reported that some enzymes on cell envelopes exhibit a different kinetic behaviour depending on the pH of the medium [1–3]. It is believed that this unusual pH response represents a regulatory device that is operative *in vivo* [4].

In addition to the classical enzyme co-operativity generated by site–site interactions, many enzymes display a special type of kinetic co-operativity which results from 'slow' conformational transitions occurring under non-equilibrium conditions. These enzymes, which may be polymeric or monomeric, were given the name of 'hysteretic enzymes' [5, 6]. An important example of this type of kinetic co-operativity occurs when the hysteresis is induced by pH changes in the medium. Ricard *et al.* [7] developed a kinetic model that explained the pH-induced cooperative effects in hysteretic enzymes. This model was applicable to the kinetic study of a plant cell-wall β-glucosyltransferase [8] and of a fructose-1,6-bisphosphatase obtained from spinach (*Spinacia oleracea*) chloroplasts [9]. The basis of this model is that the protein undergoes a 'slow' conformational transition, upon ionization or protonation of a strategic ionizable group.

Polyphenol oxidase (PPO; EC 1.14.18.1) is a widely distributed enzyme and is mainly involved in the biosynthesis of melanins in animals and in the browning phenomenom in plants. The enzyme catalyses two distinct reactions involving molecular oxygen: the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity). PPO is localized in the chloroplast, where it is bound to the thylakoid membranes [10]. However, it is not an intrinsic membrane protein and can be released from the thylakoids by sonication, mild detergent or protease treatments [11]. In some species it exists in an inactive or latent state. Catecholase activity can be activated by different treatments such as proteases [12], fatty acids [13], ageing [14], divalent cations [15], polyglucan-type elicitors [16], anionic detergents [17] and acid or basic shock [18]. As regards activation of the enzyme by pH changes in the medium, this phenomenom was first reported by Kenten [18], who ascribed the process to the removal of an inhibitor. Lerner *et al.* [19] found that prolonged exposure to acid pH provoked irreversible activation of the enzyme. This activation was apparently due to conformational changes in the enzyme; Lerner and Mayer [20] went further and showed that the process was accompanied by a change in the Stokes' radius of the protein.

In the present paper we have studied the catecholase activity of a latent plant polyphenol oxidase extracted from broad bean leaves. We report the appearance of a pH-dependent lag period in the expression of catecholase activity, which suggests the hysteretic nature of the enzyme. These results agree with previous studies [21] on PPO extracted from grape berries. The enzyme also shows negative co-operativity depending on the pH of the assay. This kinetic behaviour of polyphenol oxidase is consistent with the model described by Ricard *et al.* [7] to explain pH-induced co-operative effects of hysteretic enzymes.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

When catecholase activity of latent broad bean leaves PPO was measured at pH 3.1, the enzyme became active due to acid shock, which agrees with previous studies [18]. The reaction rate immediately reached its steady-state (Fig. 1, curve a). However, when the activity was measured at pH 3.9 (Fig. 1, curve b), the steady state was reached after a discernible lag period. The appearance of this lag phase in the expression of catecholase activity of PPO is a phenomenom that has been previously reported only for the case of latent grape PPO [21]. It is not an artefact of the enzyme assay since similar results were obtained when other *o*-diphenols were used to measure enzymatic activity.

This change of kinetic behaviour in response to different pH values in the medium has been described as a characteristic of 'hysteretic' enzymes. These enzymes, in response to a ligand binding, undergo a slow transition to another kinetically different form during catalysis [6]. The lag shown by a hysteretic enzyme can be abolished by preincubation with the ligand responsible for the slow transition (protons in our case). Accordingly, the latent enzyme was dialysed against 1 mM phosphate buffer, pH 7.3, and then incubated in 100 mM acetate buffer, pH 3.1, for 10 min at 25°. Catecholase activity was determined at pH 3.9 with this preincubated enzyme. Curve c in Fig. 1, clearly shows that the lag observed in the expression of the catecholase activity of latent polyphenol oxidase was abolished when the enzyme was preincubated at very acidic pH values. This result indicates that the lag period can be caused by slow pH-induced conformational changes in the enzyme to a catalytically more active form, as in previous studies [21]. Besides, if the enzyme is preincubated in the same experimental conditions as before, i.e. with the ligand responsible for the slow transition, but the activity is now measured at pH 6.6, the enzyme is returned to its previous condition, and therefore a progress curve in the opposite direction (a burst) is obtained (Fig. 1, curve d). This indicates that, in this case, pH induces a slow transition to a less catalytically active form. Moreover, this process is reversible since the enzyme recovers its activity after the addition of more acetic acid to the reaction medium (curve d) once the steady state has been reached (Fig. 1, curve e).

In order to study in more detail the kinetic behaviour of the enzyme in response to pH changes, the catecholase activity of latent broad bean polyphenol oxidase and the length of the lag phase were determined at different pH values. As Fig. 2 shows, catecholase activity decreased as pH increased, and at very acidic pH values the reaction rate reached its steady-state value and the lag was negligible. However, when this lag period was determined over a wider pH range, the result was a bell-shaped curve, with a maximum at pH 4.2.

To confirm that hysteretic behaviour of the enzyme was due to pH-induced isomerization of the enzyme to another form with different catalytic activity and was not due to an oligomerization phenomenon, the catecholase activity and the lag were determined as functions of enzyme concentration. As Fig. 3 shows, the lag period remained constant and steady-state rates were linear at different enzyme concentrations.

Another factor that influences the length of the lag period is substrate concentration. Figure 4 shows the effect of substrate concentration on the steady-state rates and lag period at two different pH values. As can be seen from this figure, the lag decreased when 4-methylcatechol

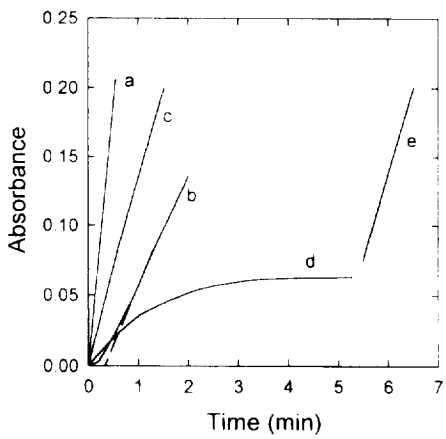

Fig. 1. Progress curves for catecholase activity of latent broad bean polyphenol oxidase. Assays were carried out with 5 mM 4-methylcatechol in 100 mM sodium acetate buffer at pH (a) 3.1, and (b) 3.9. Enzyme concentration was 5 $\mu g\,ml^{-1}$. (c) Latent PPO (8.5 μg) was preincubated at pH 3.1 with 100 mM acetic acid for 10 min at 25°, and then catecholase activity was measured in 100 mM sodium acetate buffer, pH 3.9. (d) Enzyme was preincubated as in (c) but activity was measured in 100 mM sodium phosphate buffer pH 6.6. (e) Once the steady state was reached in curve d, 100 μl of acetic acid 2 M was added to the reaction medium.

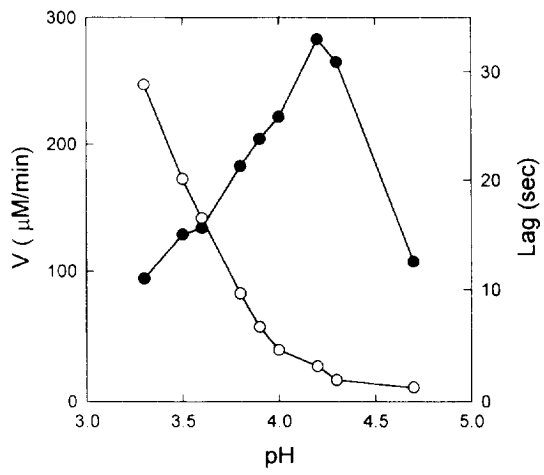

Fig. 2. Effect of pH on catecholase activity and lag period of latent broad bean polyphenol oxidase. (○) Steady-state rates, (●) length of the lag period. Assays were carried out with 5 mM 4-methylcatechol in 100 mM sodium acetate buffer. Enzyme concentration was 5 $\mu g\,ml^{-1}$.

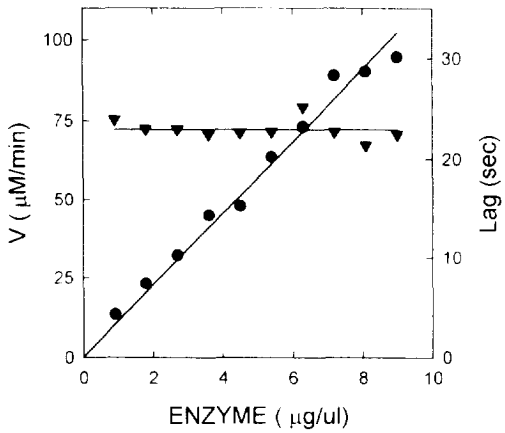

Fig. 3. Effect of enzyme concentration on (●) steady-state rate, and (▼) lag period at pH 3.9.

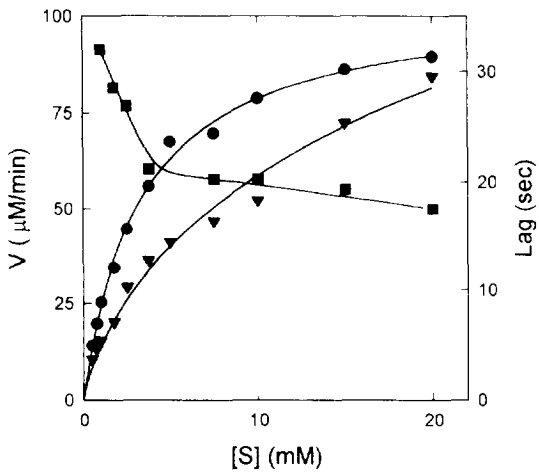

Fig. 4. Effect of substrate concentration on steady-state rates at pH (●) 4.5, and (▼) 6.1, and on the lag period at (■) pH 4.5. Enzyme concentration was 22 $\mu g\,ml^{-1}$. The results plotted at pH 6.1 are the result of multiplying experimental data by 10.

concentration was increased. As regards catalytic activity, at pH 4.5, the enzyme displayed classical Michaelis–Menten kinetics. From the data fit, the kinetic parameters, K_m and V_{max}, were evaluated (K_m = 3.25 mM, V_{max} = 104 $\mu M\,min^{-1}$). However, at pH 6.1, the enzyme exhibited kinetic co-operativity. The experimental data obtained at this pH can be fitted to the Hill equation ($v = V_{max}[S]^h/(K_H^h + [S]^h)$), giving the following values for the kinetic parameters: V_{max} = 186.9 $\mu M\,min^{-1}$, K_H = 28.25 mM and a Hill coefficient of 0.75. This kinetic behaviour of latent broad bean polyphenol oxidase is consistent with the kinetic model of pH-induced co-operativity for hysteretic enzymes reported by Ricard *et al.* [7]. The basis of the model is that co-operativity is not due to the existence of two conformations of the active site; but rather arises from the ionization or the protonation, of a group located outside the active centre, in a different region of the protein, which undergoes spontaneous 'slow' conformational transition.

This kinetic behaviour of latent broad bean polyphenol oxidase has only been observed in the grape enzyme [21] probably due to the fact that most kinetic studies have been carried out with enzymes extracted by disruptive methods which partially activate the enzyme. Our Triton X-114 extraction method [22] has allowed us to obtain the enzyme in latent state. The pH response of latent broad PPO shows that the activation of the enzyme by pH changes of the medium can be considered as a hysteretic phenomenom.

EXPERIMENTAL

Reagents. Biochemicals were purchased from Sigma and used without further purification. Triton X-114 was obtained from Fluka and condensed × 3 as described in ref. [23] but using 100 mM Na-Pi buffer, pH 7.3. The detergent phase of the third condensation had a concentration of 25% Triton X-114 and was used as the stock soln for all the experiments.

Plant material. Broad bean (*Vicia faba* L. cv Muchamiel) plants were grown in vermiculite for 3 weeks, at 23° and 70% RH. They were watered twice a week with Cron medium [24].

Enzyme extraction. All procedures were carried out at 4°. Chloroplasts were prepared by homogenizing 50 g of broad bean leaves of assorted sizes [25] in 500 ml of 100 mM Na-Pi buffer, pH 7.3, containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride and 1 mM benzamidine. The slurry was filtered through 8 layers of gauze and centrifuged at 600 *g*, 2 min. The pellet was discarded, and the supernatant was centrifuged at 6000 *g* for 10 min to ppt the thylakoid membranes. The resultant pellet was washed × 2 by resuspending in 40 ml of grinding buffer without sorbitol and centrifuging for 10 min at 6000 *g*. The final pellet was resuspended in 15 ml of 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 Triton X-114 at 4°, so that the final detergent concn was 8% (w/v). The mixture was kept at 4° for 15 min and then warmed to 37°. After 15 min the soln became spontaneously turbid due to the formation, aggregation and precipitation of large mixed micelles composed of detergent, hydrophobic proteins and the remaining chlorophylls [22]. The soln was centrifuged at 5000 *g* for 10 min at 23°. The clear supernatant was used as enzyme source and the green detergent-rich phase with no PPO activity was discarded.

Enzyme assay. Catecholase activity was determined spectrophotometrically at 400 nm [26]. One unit of enzyme activity is the amount of enzyme that produces 1 μmol of *o*-methylbenzoquinone/min. Steady-state rate was defined as the slope of the linear zone of the product accumulation curve. The lag period was estimated by extrapolation of the linear portion of the product accumulation curve to the abscissa axis. Unless otherwise

stated, the reaction media contained 5 mM 4-methylcatechol ($\varepsilon_{o\text{-quinone}} = 1433\ M^{-1}\ cm^{-1}$) at the indicated pH in 100 mM NaOAc (pH 3.1–4.7) or Na-Pi (pH 6.1) buffers, as required.

Other methods. Protein content was determined by the dye binding method of ref. [27] using BSA as a standard.

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