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Characterization of polyphenol oxidase from Napoleon grape

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Abstract

Polyphenol oxidase (PPO) from Napoleon grape was isolated using a two-phase partitioning approach with Triton X-114. The enzyme was purified in a latent form and could be optimally activated by the presence of 0.2% of sodium dodecyl sulphate (SDS) at pH 6.0. In the absence of SDS, the enzyme showed maximum activity at acid pH (3.0). The enzyme was kinetically characterized at pH 3.0 and pH 6.0 in the presence of 0.2% of SDS, using 4-*tert*-butylcatechol (TBC) as a substrate. The V_m/K_M ratio showed that Napoleon grape PPO presents greater affinity for TBC at acid pH (0.1 min^{-1}) that at pH 6.0 in the presence of SDS (0.02 min^{-1}). The enzyme was highly heat stable, 80% of activity remaining at 70 °C. Selected inhibitors were also studied, tropolone being the most active with a K_i value of 27 μM at acid pH and pH 6.0 in the presence of 0.2% SDS.

Keywords: Polyphenol oxidase; Browning; Triton X-114; Latent enzyme; Table grape

1. Introduction

Napoleon is a large, dark purple, thick-skinned table grape. As with all grapes, it is one of the major sources of phenolic compounds (Maxcheix, Fleuriet, & Billot, 1990), with multiple biological effects, acting as antioxidant (Bors & Saran, 1987; Chang, Ostric-Matijasevic, Hsieh, & Huang, 1977), antiinflammatory (Moroney, Alcaraz, Forder, Carey, & Hoult, 1988), inhibitor of platelet aggregation (Wauwe & Gossenc, 1983), antimicrobial (Capasso et al., 1995) and antiageing agent (Rice-Evans, 2001). These phenolics are potentially oxidized by polyphenol oxidase (PPO) to their corresponding quinones. The quinones thus formed are highly reactive substances, which normally react further with other quinones, amino acids or proteins to produce coloured compounds that cause food quality deterioration (Martínez & Whitaker, 1995; Vamos-Vigyazo, 1981).

In the case of black grapes, the enzymatic oxidation of phenolics leads not only to the browning of the flesh and loss of nutritional and organoleptic properties, but also to anthocyanin degradation (Yokotsuka & Singleton, 1997). These anthocyanins are directly degraded by PPO (Raynal & Moutounet, 1989) and by the quinones formed from the phenol substrates by PPO (Pifferi & Cultrera, 1974; Wesche-Ebeling & Montgomery, 1990; Yokotsuka & Singleton, 1997). Thus, the enzymatic effects on the colour of this type of grape juice are believed to be largely influenced by the composition of the anthocyanins and oxidizable phenols, as well as the degree of PPO activity (Gunata, Sapis, & Moutounet, 1987; Wissemann & Lee, 1981; Yokotsuka & Singleton, 1997).

Polyphenol oxidase (PPO) (monophenol, dihydroxy-L-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is a widely distributed copper-containing enzyme, which is associated with undesirable browning reactions in fruits and vegetables. This enzyme catalyses two distinct reactions, each using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the

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oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) (Sánchez-Ferrer, Rodríguez-López, García-Cánovas, & García-Carmona, 1995). This enzyme is present in all plants (Whitaker, 1972), but its activity is particularly high in those fruits and vegetables containing significant levels of phenolic compounds, such as grapes (Cantos, Espín, & Tomás-Barberán, 2002; Maxcheix et al., 1990).

Grape PPO activity is considerably influenced by several factors such as variety, stage of development and environmental conditions (Mayer & Harel, 1979; Sapis, Macheix, & Cordonnier, 1983). A common characteristic of this enzyme is its ability to exist in an inactive or latent state (Mayer & Harel, 1979). PPO can be activated by a variety of treatments or agents, including acid and base shock (Kenten, 1957), urea (Swain, Mapson, & Rob, 1966), polyamines (Jiménez-Atiénzar, Pedreño, & García-Carmona, 1991), anionic detergents such as SDS (Flurkey, 1986; Moore & Flurkey, 1990; Jiménez-Atiénzar & García-Carmona, 1996), proteases (Laveda, Núñez-Delicado, García-Carmona, & Sánchez-Ferrer, 2001) and fatty acids (Goldbeck & Cammarata, 1981). SDS is particularly interesting as an activating agent because few enzymes are known to be activated by it, although many enzymes are inactivated. Moore and Flurkey in 1990 demonstrated that SDS activation of PPO is related with a conformational change in the latent enzyme (Moore & Flurkey, 1990). Later, in 2000, our research group demonstrated the total reversibility of this activatory process mediated by SDS, using cyclodextrins as stripping agent to remove SDS from the reaction medium (Laveda, Núñez-Delicado, García-Carmona, & Sánchez-Ferrer, 2000).

In this paper, latent PPO from Napoleon table grapes was isolated and its diphenolase activity was kinetically characterized. This resulting information will be useful for devising effective methods for enhancing the quality of Napoleon grape juice.

2. Materials and methods

2.1. Sampling and raw material

Napoleon table grapes grown under an integrated production system were kindly supplied by COATO (Totana, Murcia, Spain). Grape berries, in the optimal commercial stage of maturity, as measured by the soluble solids content (SSC = 13°Brix), were picked from three different plantations. From each plantation, 10 samples of 500 g were picked from 10 differently oriented vines. All samples were mixed, transported to the laboratory and frozen at −80 °C until they were used as PPO source.

2.2. Reagents

Biochemicals were purchased from Fluka (Madrid, Spain) and used without any further purification. Inhibitors (cinnamic acid, L-minmosine, tropolone, ascorbic acid, L-cysteine, metabisulfite and diethyldithiocarbamate) were from Sigma (Madrid, Spain). Triton X-114 was obtained from Fluka (Sigma–Aldrich Quimica S.A., 28760, Madrid, Spain) and was condensed (concentrated by buffer removal in temperature-induced phase partitioning) three times as described by Bordier (1981), using 100 mM sodium phosphate buffer (pH 7.3). The detergent-rich phase of the third condensation had a concentration of 25% Triton X-114 (w/v).

2.3. Enzyme purification

Napoleon grape PPO was extracted using the method described by Sánchez-Ferrer, Bru, and García-Carmona (1989). All extractions were made in triplicate as explained below.

Grape berries (250 g) were washed and homogenized for 1 min with 100 ml of 100 mM sodium phosphate buffer (pH 7.3) containing 10 mM ascorbic acid. The homogenate was filtered through eight layers of gauze and centrifuged at 4000*g* for 15 min. The supernatant was discarded and the precipitate was extracted with 20 ml of 4% (w/v) Triton X-114 in 100 mM sodium phosphate buffer (pH 7.3). The mixture was subjected to temperature-induced phase partitioning and kept at 4 °C for 15 min and then warmed to 37 °C for 15 min. At this time, the solution became spontaneously turbid due to the formation, aggregation and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins, anthocyanins and phenolic compounds. This turbid solution was centrifuged at 10,000*g* for 15 min at 25 °C. After centrifugation, the detergent-rich phase was discarded and the clear detergent-poor supernatant, which was used as enzyme source, was stored at −20 °C.

2.4. Enzyme activity

Diphenolase activity was determined spectrophotometrically at 400 nm (Sánchez-Ferrer, Laveda, & García-Carmona, 1993a) with 4-*tert*-butylcatechol (TBC) ($\varepsilon_{400} = 1150$ M^{-1} cm^{-1}). One unit of enzyme was defined as the amount of the enzyme that produced 1 μmol of *tert*-butyl-*o*-quinone per minute. The standard reaction medium at 25 °C contained 6.5 ng/ml of partially purified PPO, 10 mM sodium acetate buffer (pH 3.0) and 16 mM TBC in a final volume of 1 ml.

In the SDS standard assay, samples contained the above mixture and 0.2% SDS detergent in a cuvette. To determine the effect of the inhibitors, we measured PPO activity in the steady state in standard reaction media in the presence or absence of the stated concentration of inhibitors.

Each sample was assayed in triplicate and the mean and standard deviation were plotted.

2.5. Electrophoresis

SDS–PAGE was carried out as described by Angleton and Flurkey (1984). Samples were mixed with glycerol,

SDS and bromophenol blue before being applied to 12.6% polyacrylamide gels. Electrophoresis was carried out for 1 h at 25 °C in a Mini protein cell (Bio-Rad). The gels were stained for PPO activity in 100 ml of 10 mM sodium acetate buffer (pH 3.0) containing 5 mM 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and 3 mM 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH).

2.6. Determination of proteins

The protein content was determined according to Bradford's dye binding method, using bovine serum albumin (BSA) as a standard (Bradford, 1976).

2.7. Thermal stability

The enzyme was incubated at various temperatures between 30 and 100 °C for 5 min. and the residual activity was determined at pH 3.0 and pH 6.0 and 0.2% SDS at 25 °C.

3. Results and discussion

The purple skin colour of Napoleon c.v. table grape hinders the extraction of PPO in a clear solution free of phenols and anthocyanins, since it interferes with the spectrophotometric determination of PPO activity. Therefore, PPO was extracted using the Triton X-114 method (Sánchez-Ferrer et al., 1989), which permitted phenolics and anthocyanins to be removed, yielding a PPO in a clear solution. Indeed, the removal of phenols by TX-114 was sufficient to avoid browning of the enzyme solution, even after many cycles of freezing and thawing or after months of storage at −20 °C. The enzyme thus isolated was in a latent state, as has previously been described for other fruits and vegetables (Fraignier, Marques, Fleuriet, & Macheix, 1995; Yoruk & Marshall, 2003).

In the case of latent PPO, pH is a determining factor to express enzymatic activity and its activation by acid or basic shock has been widely described (Chazarra, Cabanes, Escribano, & García-Carmona, 1996; Kenten, 1957; Núñez-Delicado, García-Carmona, & Sánchez-Ferrer, 2003). On the other hand, latent PPOs can also be activated by the anionic detergent, SDS (Moore & Flurkey, 1990). For these reasons, the kinetics and optimal pH of the enzyme were determined in the presence and absence of SDS.

In the absence of SDS the activity increased at acidic pH (Fig. 1, filled circles) as a result of acid shocking (Núñez-Delicado et al., 2003). However, this optimum acidic pH for Napoleon PPO activity was subject to change when assayed in the presence of anionic detergents, such as SDS. The presence of SDS eliminated the acidic pH optimum and two new maxima appeared at pH 4.0 and pH 6.0 (Fig. 1, filled squares). This effect of SDS has previously been described for other latent PPOs extracted from vegetable sources using the TX-114 method and measured using

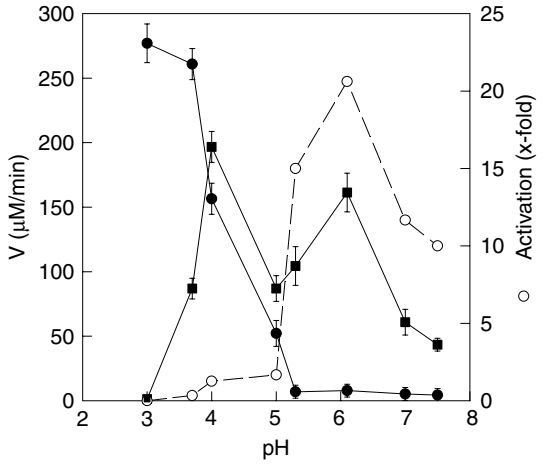

Fig. 1. Effect of pH on Napoleon grape PPO activity in 10 mM sodium acetate buffer (pH 3.0–5.5) and 10 mM sodium phosphate buffer (pH 6.0–7.5) in the presence (■) or absence (●) of 0.2% SDS. The reaction medium at 25 °C contained 16 mM TBC and 6.5 ng/ml PPO. Activation degree at different pHs (○).

TBC as substrate (Chazarra et al., 1996; Laveda et al., 2000; Núñez-Delicado et al., 2003; Sánchez-Ferrer et al., 1993a).

The pH scan in the presence or absence of SDS (Fig. 1) revealed that the highest value in the activation process was obtained at pH 6.0 (22-fold) (Fig. 1, open circles). It was therefore decided to study the activation by the detergent at pH 6.0, although assays in the absence of SDS were carried out at pH 3.0. The degree of SDS activation obtained for Napoleon grape PPO (22-fold) was quite similar to that obtained for Monastrell grape (19.2-fold) (Sánchez-Ferrer et al., 1989) and peach PPO (25-fold) (Laveda et al., 2000), and higher than that obtained for lettuce (5-fold) (Chazarra et al., 1996), mushroom (6-fold) (Núñez-Delicado, Bru, Sánchez-Ferrer, & García-Carmona, 1996) and persimmon PPO (15-fold) (Núñez-Delicado et al., 2003).

The degree of SDS activation with SDS depended on surfactant concentration, as shown in Fig. 2. The optimum SDS concentration for activating the enzyme (0.2%) was higher than that described for banana (0.06%) (Sojo, Núñez-Delicado, García-Carmona, & Sánchez-Ferrer, 1998) or peach PPO (0.05%) (Laveda et al., 2000). Such activation of the latent enzyme by SDS is a common feature of other latent PPOs, and has been attributed to a reversible conformational change in the protein (Laveda et al., 2000). In addition, the latent enzyme was also activated by 12.5% SDS–PAGE, appearing as multiple activity bands when L-DOPA was used as substrate (Fig. 2, inset). This result indicated the presence of different isoenzymatic PPO forms in the Napoleon grape extract.

Activation by SDS depended not only on the surfactant concentration and pH, but also on the substrate used (Sánchez-Ferrer, Laveda, & García-Carmona, 1993b). For example, when TBC was used as substrate, the activation obtained for Napoleon grape PPO was 20.6-fold (at pH

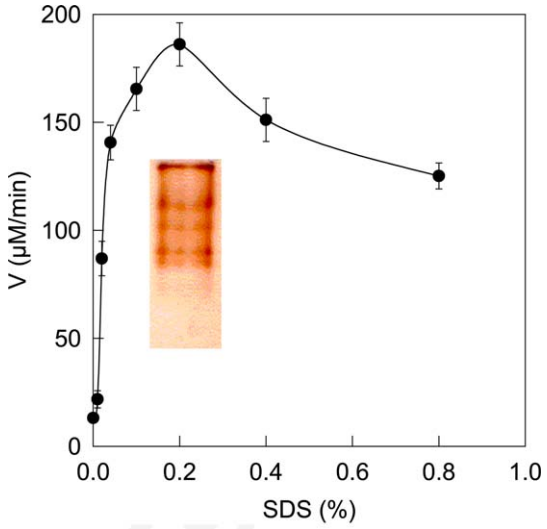

Fig. 2. Effect of SDS concentration on Napoleon grape PPO activity. The reaction medium at 25 °C contained 10 mM sodium phosphate buffer, pH 6.0, 16 mM TBC, 6.5 ng/ml PPO and increasing concentrations of SDS (0–0.8%). (Inset) SDS–PAGE (12.6% gel) of Napoleon grape PPO stained with 5 mM L-DOPA and 3 mM MBTH in 10 mM sodium acetate buffer (pH 3.0).

6.0 in the presence of 0.2% of SDS) (Fig. 2). However, when 4-methylcatechol (4MC) was used as substrate the activation obtained was 15.7-fold (data not shown). These results seem to be related to the degree of hydrophobicity of the substrate used (TBC > 4MC), the conformational change produced by the surfactant favouring access of the hydrophobic substrates to the active centre. This effect has previously been described for potato leaf PPO (Sánchez-Ferrer et al., 1993b).

With regard to enzyme stability and temperature, the results obtained at pH 3.0 and pH 6.0 in the presence of SDS 0.2% are shown in Fig. 3. The enzyme was stable between 30 and 60 °C. From 60 to 70 °C, the residual activity decreased slightly (20%), but rapid inactivation occurred from 70 to 80 °C, when only 20% of activity remained. At 100 °C, the residual activity was 0%. The temperature stability of this enzyme was relatively high in both conditions compared with other grape PPOs (Nakamura, Amano, & Kagami, 1983; Yokotsuka, Makino, & Singleton, 1988).

The study of the kinetic parameters (V_m and K_M) of latent Napoleon grape PPO was carried out in the absence of SDS at pH 3.0 and in the presence of 0.2% SDS at pH 6.0, using TBC as substrate (Fig. 4). The apparent kinetic parameters (V_m and K_M) were calculated by nonlinear regression to the Michaelis-Menten equation using the data obtained at pH 3.0 and pH 6.0 in the presence of 0.2% SDS. Fig. 4 shows the variation in initial velocity vs. substrate concentration in these conditions. The value obtained for each maximum velocity ($V_m = 310$ μM/min at pH 3.0 and $V_m = 180$ μM/min at pH 6.0 with 0.2% SDS) showed that the PPO was strongly activated by acid shocking. The K_M value obtained at pH 3.0 (3.1 mM) was lower than that obtained at pH 6.0 in the presence of SDS (8.2 mM). The V_m/K_M ratio was 0.1 min^{-1} at pH 3.0 and 0.02 min^{-1} at pH 6.0 with 0.2% SDS, indicating that latent Napoleon grape PPO presents more affinity for TBC at acid pH that at pH 6.0 in the presence of SDS.

To further characterize latent Napoleon grape PPO, a detailed study of its inhibition was carried out. All inhibitors used in this study inhibited PPO activity, the extent of the inhibition being dependent on the concentration of the compound used. Among reducing agents (Table 1), metabisulphite, L-cysteine and diethyldithiocarbamate (DEDTC) appeared to be the most effective inhibitors at pH 3.0 and pH 6.0 in the presence of 0.2% SDS. However, the action mechanism differs according to the reducing

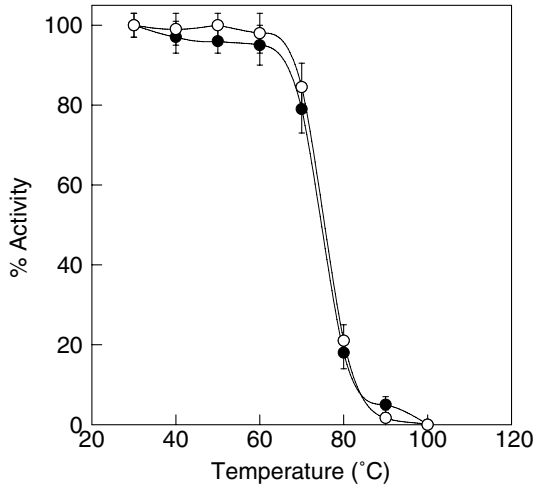

Fig. 3. Thermal stability of the enzyme after heating for 5 min. at temperatures between 30 and 100 °C, as determined by activity assay at 25 °C. The reaction medium contained 16 mM TBC and 6.5 ng/ml PPO in 10 mM sodium acetate buffer pH 3.0 (•) or 10 mM sodium phosphate buffer, pH 6.0 with 0.2% SDS (○).

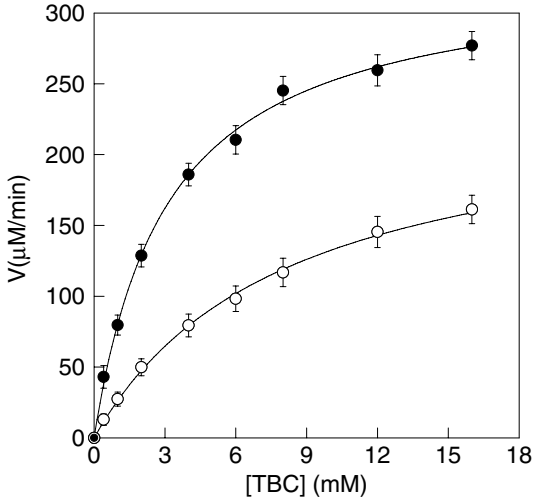

Fig. 4. Effect of TBC concentration on Napoleon grape PPO activity. The reaction medium at 25 °C contained 6.5 ng/ml PPO and TBC concentrations ranging from 0 to 16 mM in (•) 10 mM sodium acetate buffer (pH 3.0) or (○) 10 mM sodium phosphate buffer pH 6.0 with 0.2% SDS.

Table 1
Inhibition percentage of partially purified Napoleon grape PPO by reducing agents[a]

	pH 3.0				pH 6.0 + SDS 0.2%			
	10 μM	0.1 mM	0.4 mM	1 mM	10 μM	0.1 mM	0.4 mM	1 mM
Ascorbic acid	0	0	49	100	0	0	20	100
DEDTC	7	49	100	100	0	0	47	100
L-cysteine	0	12	71	100	0	5	100	100
Metabisulfite	17	27	100	100	0	4	81	100

[a] Assayed under the standard reaction conditions with the suitable concentration of inhibitor.

Table 2
Inhibition percentage of partially purified Napoleon grape PPO by substrate analogues[a]

	pH 3.0				pH 6.0 + SDS 0.2%			
	10 μM	0.1 mM	0.4 mM	1 mM	10 μM	0.1 mM	0.4 mM	1 mM
Tropolone	0	49.5	71.1	85.6	18.6	66.14	83.86	89.42
L-mimosine	0	15	20	28	0	4	9	30
Kojic acid	10	13	17	18	3	12	17	23

[a] Assayed under the standard reaction conditions with the suitable concentration of inhibitor.

agent used. The inhibition by thiol compounds may be due to an addition reaction with the quinones to form stable colourless products (Ikediobi & Obasuyi, 1982) and/or a binding to the active centre of the enzyme, as in the case of metabisulphite (Valero, Varón, & García-Carmona, 1992). Ascorbate acts as an antioxidant rather than as an enzyme inhibitor because it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes the secondary reaction that leads to browning (Whitaker, 1972). Ascorbic acid has also been reported to cause irreversible inhibition (Golan-Goldhirsh & Whitaker, 1984). Finally, DEDTC may act by complexing the copper prosthetic group of the enzyme, as has been seen for other plant PPOs (Anosike & Ayaebene, 1981).

Of the substrate analogues (Table 2), tropolone was the most effective inhibitor, inhibiting the enzyme about 50% at 0.1 mM and almost completely at 1 mM at pH 3.0 and pH 6.0 in the presence of 0.2% SDS. This result is in accordance with other studied PPOs, such as persimmon (Núñez-Delicado et al., 2003), banana (Sojo et al., 1998) or potato (Sánchez-Ferrer et al., 1993a). Surprisingly, neither L-mimosine nor kojic acid (Table 2) had much of an inhibiting effect, in any of the conditions used.

The inhibition of tropolone was determined by Lineweaver-Burk plots of $1/v$ vs. $1/S$ at three inhibitor concentrations (data not shown) and confirmed by a Dixon plot of $1/v$ vs. I (Fig. 5). Straight lines were obtained both at pH 3.0 (Fig. 5(a)) and pH 6.0 in the presence of SDS 0.2% (Fig. 5(b)). The inhibition constant, K_i, was deduced from the points of interception of the plots. Tropolone showed a competitive inhibition, and a K_i value of 27 μM in both conditions studied, an inhibition type in accordance with other data in the literature for PPO from other sources such as Airen grape (Valero, García-Moreno, Varón, & García-Carmona, 1991), banana (Sojo et al., 1998) or persimmon (Núñez-Delicado et al., 2003).

In conclusion, a detailed kinetic study of Napoleon grape PPO, which appeared as a multiple band profile in

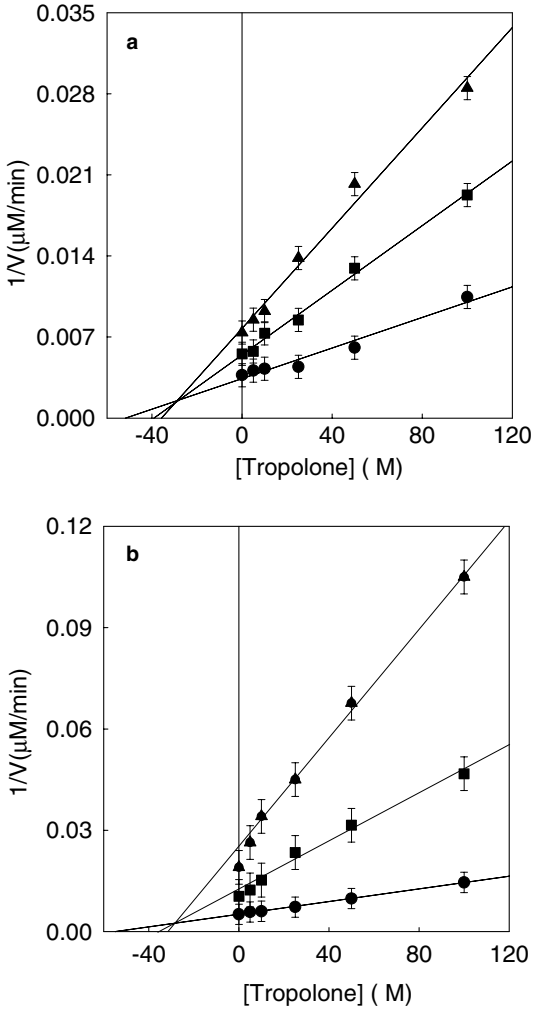

Fig. 5. Dixon plot ($1/v$ vs. I) for the competitive type of inhibition presented by tropolone: (a) the reaction medium at 25 °C contained 10 mM sodium acetate buffer (pH 3.0), 6.5 ng/ml PPO, tropolone (0–120 μM), and three different concentrations of TBC [2 mM (●), 4 mM (■) and 16 mM (▲)]; (b) the reaction medium at 25 °C contained 10 mM sodium phosphate buffer (pH 6.0) with SDS 0.2%, 6.5 ng/ml PPO, tropolone (0–120 μM), and three different concentrations of TBC [2 mM (●), 4 mM (■) and 16 mM (▲)].

electrophoresis when isolated using TX-114 was presented for the first time. This enzyme was present in a latent stage, but could be activated by the anionic detergent SDS and was strongly inhibited by tropolone.

Acknowledgements

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