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Some biochemical properties of polyphenol oxidase from two varieties of avocado

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Abstract

A study of crude polyphenol oxidases from two avocado (*Persea americana* Mill.) varieties, Booth 1 (BIPPO) and Julio Millán (JMPPO), were carried out to provide information useful for guiding food processing operations. Results are reported as ''apparent'' data because crude extracts were used, activities of which can be derived from a combination of isoenzymes. The pH-activity optimum was pH 7.5–7.6 for both extracts. Heat inactivation, between 67 and 84 °C was biphasic with activation energies ranging from 21.4 to 64.1 kcal/mol. The apparent substrate specificity was established from values V_{max}/K_m as: 4-methyl catechol > chlorogenic acid >pyrogallol >catechol >caffeic acid >DL-DOPA for BIPPO. The substrate specificity for JMPPO was: 4-methyl catechol >chlorogenic acid >pyrogallol >caffeic acid >catechol >DL-DOPA >protocatechuic acid. The order of inhibitor effectiveness was: L-cysteine >ascorbic acid >resorcinol >glycine >NaCl. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Polyphenol oxidase; Avocado; Enzymatic browning; *Persea americsna*; Tropical fruit

1. Introduction

Avocado is a tropical and subtropical fruit, very rich in oil. One of the main problems associated with preserving the fruit is the enzymatic browning, catalyzed by polyphenol oxidase (PPO). PPO catalyses the oxidation of phenols into quinones that subsequently polymerize into brown pigments. Several reviews, devoted to PPO characteristics have been published (Mayer & Harel, 1979; Vámos-Vigyázó, 1981; Zawistowski, Biliaderis, & Michael, 1991).

The inactivation of avocado PPO is very difficult to achieve because of its high activity and resistance to treatments. In a study designed to compare PPO from five fruits, following the same methods of extraction and characterization, it was found that PPO avocado activity was 30 times higher than the second most active fruit enzyme (Weemaes, Ludikhuyze, Van den Broeck, Hendrickx, & Tobback, 1998). Avocado PPO in vitro activity was the most difficult to suppress among those from eight vegetable sources and mushroom tested by Almeida and Nogueira (1995), using 10 inactivation treatments.

Several attempts to obtain avocado products have failed because of the browning (Gerdes & Parrino-Lowe, 1995); none of them were based on PPO characterization and a designed approach to inhibition. A stable avocado puree made by high pressure treatment together with NaCl and acidification was successfully obtained (López-Malo, Palou, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1999); however, that technology is very expensive to use for extensive marketing in undeveloped countries.

Varietal differences in enzyme activity and susceptibility to browning are well-known phenomena, reported for the first time in avocados by Kahn (1975). Some PPO characterizations have been performed in avocado varieties such as Spinx (Samish, 1937), Lula (Knapp, 1965), and Fuerte and Lerman (Kahn, 1977). Avocado PPO has been studied mainly in Fuerte (Kahn, 1976a,b, 1977; Sharon & Kahn, 1979; Van Lelyveld, Gerrish, & Dixon, 1984), an unknown variety in the Venezuelan market. In this work, some characteristics of the PPO from avocados harvested in Venezuela, intended for processing were studied. This aims to prevent browning during their processing and shelf-life.

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2. Materials and methods

2.1. Plant materials and crude enzyme preparation

Two avocado varieties, Booth 1 (BIPPO) and Julio Millán (JMPPO), harvested at the CENIAP orchard, Maracay, Venezuela, were used.

The flesh of five ripe fruits was pooled and homogenized by a pestle. The acetone powder was prepared blending the fresh avocado homogenate with cold acetone (1:3, w:v) at −20 °C by an Omni-Mixer for up to 2 min, and filtered through a Whatman No. 1 filter paper. The precipitate was extracted twice more with cold acetone and the resultant white powder was dried at ambient temperature and stored at −20 °C (Sharon & Kahn, 1979). The same procedure was followed for the preparation of both acetone powders.

2.2. Protein determination

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin fraction V as standard.

2.3. Enzyme assay

Avocado acetone powder (100 mg) was suspended in 20 ml of 0.1M phosphate buffer, pH 6.5 (called work buffer). After stirring continuously for 24 h, the suspension was centrifuged at 15 000×***g*** for 20 min. The supernatant (referred to as the crude enzyme extract) was used as the enzyme source (Kahn, 1975), so that two enzyme extracts were obtained, called BIPPO (from Booth 1 variety) and JMPPO (from Julio Millán variety). The enzyme was routinely kept at 4 °C for up to 3 days (Kahn, 1977).

PPO activity was determined by measuring the increase in absorbance at 410 nm (475 nm for DL-DOPA) with a spectrophotometer (Beckman model DU 7500). Reaction velocity was computed from the linear slopes of the absorbance–time curve. The standard reaction mixture contained 1.25 ml of freshly prepared 20 mM catechol in work buffer, 1.15 ml of work buffer, and 0.1 ml of crude enzyme extract. One unit of PPO activity was defined as the change in one unit of absorbance/min/ml crude extract, and the specific activity as activity/mg protein.

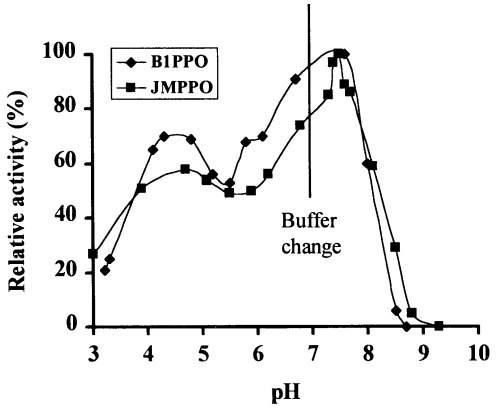

Fig. 1. pH activity profiles for BIPPO and JMPPO, using catechol as substrate.

2.4. pH optima of crude PPO

The PPO activity from both sources was determined in a pH range of 3–7 in 0.1M citrate-phosphate buffer and in a pH range of 7–10 in bicarbonate-phosphate buffer. PPO activity was assayed using the standard reaction mixture but changing the buffer.

2.5. Temperature stability of crude PPO

The temperature stability of crude PPO was studied at 67, 73, 80 and 84±0.5 °C. For the study, 1.5 ml of crude enzyme extract was poured into a pre-heated test tube immersed in a water bath at the required temperature. At intervals, a 0.15 ml aliquot of the extract was taken out and quickly cooled in another test tube immersed in an ice and water bath. Finally, PPO assays were performed in the standard reaction mixture. The residual PPO activity was calculated by comparison with unheated enzyme and the energy of activation (E_a) was calculated from the Arrhenius equation.

2.6. Substrate specificity

PPO activity was measured with 14 potential substrates, used without further purification, diluted in work buffer to a final concentration of 10 mM for all cases, except for caffeic (3 mM) acid, due to solubility difficulties.

Activity assays were performed in triplicate measurements employing the standard reaction mixture but with different substrates. K_M's and V_{max}'s were calculated by the Lineweaver–Burk plot, with substrate concentrations ranging from 0.3 to 3 K_M in the standard reaction mixture (Whitaker, 1972).

2.7. Effect of inhibitors on crude PPO

Inhibitors examined included L-cysteine, ascorbic acid, resorcinol, glycine and NaCl. The reaction mixture contained 20 mM catechol (final concentration), inhibitor solution at the concentrations indicated in Fig. 3 (see later), work buffer, and 0.1 ml crude enzyme extract; the final volume was 2.5 ml. The percentage relative inhibition for each compound was compared with that of the control (100% activity). The rate of the

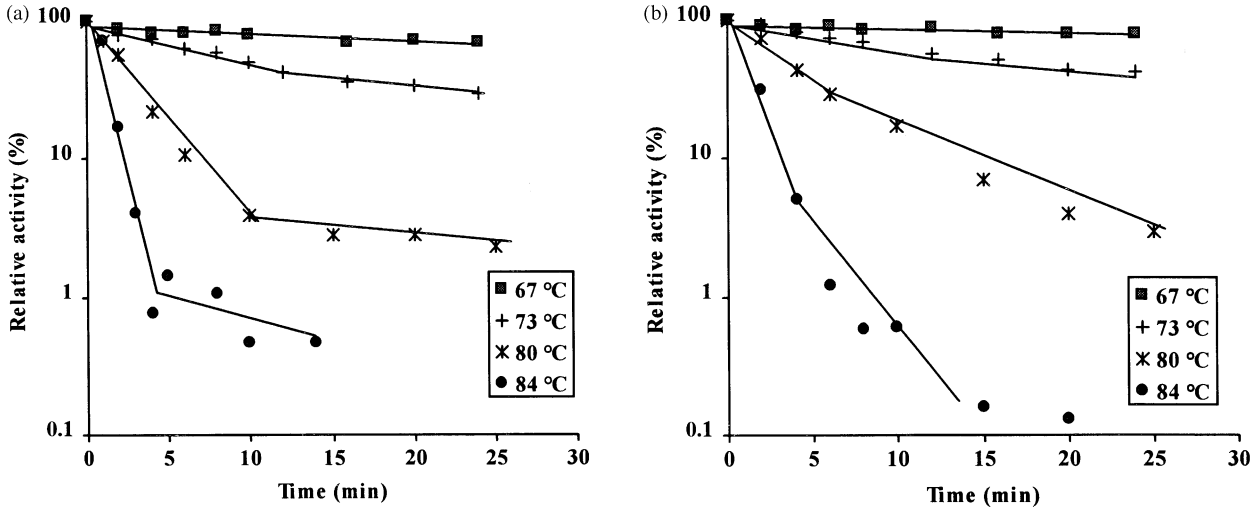

Fig. 2. Thermal inactivation of (A) B1PPO and (B) JMPPO.

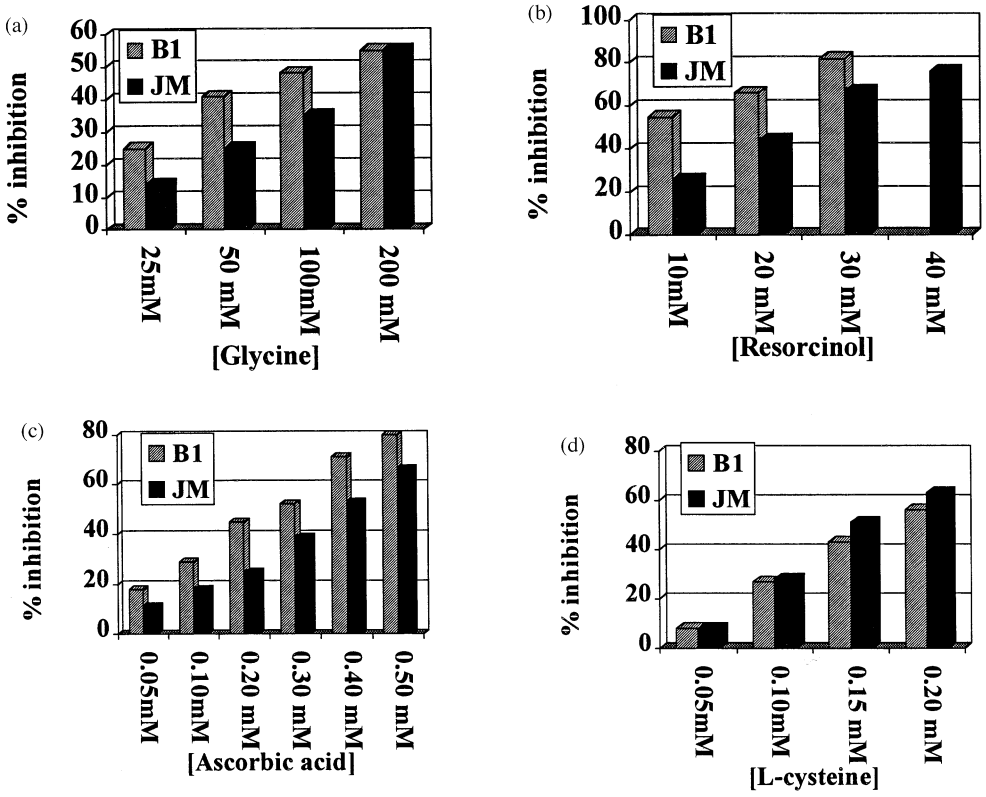

Fig. 3. Effect of inhibitors on B1PPO and JMPPO. Inhibitors were: (A) glycine, (B) resorcinol, (C) ascorbic acid, (D) L-cysteine.

reaction was computed from the linear portion of the curve absorbance-time excluding lag-phases when they occurred.

3. Results and discussion

In this discussion it must be pointed out that results were derived from a study of crude enzyme preparations extracted from acetone powders, so the properties reported hereby may be derived from a combination of isoenzymes and interactions with nonenzymatic proteins. The properties described below are therefore apparent quantities, even where the description (apparent) has not been used. However, the properties of a crude preparation can be as relevant to the Food Industry as those of the purified or isolated enzyme (Duangmal & Owusu-Apenten, 1999).

3.1. pH optima of crude PPO activity

Both extracts assayed in the pH range of 3–10, using catechol as substrate had two activity peaks, with higher activity at neutral than at acidic pH (Fig. 1). This result may be explained as an effect caused by the presence of isoenzymes, since some workers have isolated several isoenzymes of avocado PPO (Dizik & Knapp, 1970; Kahn, 1976b, 1977; Van Lelyveld et al., 1984). The peak at acidic pH is in agreement with that reported by Knapp (1965), while the other one is in agreement with that reported by Weemaes et al. (1998) but one unit higher than the value reported by Kahn (1977). All of the cited works reported only one peak, although a narrow plateau between pH 4 and 5 seems to have been obtained by Weemaes et al. (1998) from an extract from an unidentified variety, subjected to ammonium sulphate precipitation and further dialysis. The optimum pH depends on genetic properties (variety), nature of phenolic substrates and extraction methods (Duangmal & Owusu-Apenten, 1999). As can be seen from the graph, the PPO from both sources was still 50% active at pH 4.0.

3.2. Temperature stability of crude PPO

Temperature-stability profiles for crude BIPPO and JMPPO, presented in the form of the residual percentage activity, are shown in Fig. 2. The thermal inactivation could be described by a biphasic first-order decay process, the first phase was called thermolabile and the last one thermoresistant. Kahn (1977) also found first-order decay of Fuerte PPO at 70 °C, but noticed deviation from log-linear decay at 74 and 79 °C. Biphasic thermal inactivation profiles have been reported in plum PPO (Weemaes et al., 1998) and banana PPO (Padrón, Lozano, & González, 1995). The biphasic behavior might reflect the existence of isoenzymes with different thermal properties. Wong, Luh and Whitaker (1971) found different temperature-stability profiles for purified PPO isoenzymes from peaches. Heating at 84 °C for 10 min almost inactivated BIPPO and JMPPO completely.

The E_a's of the thermal inactivation for the thermolabile fraction were 64.1 ($r^2 = 1.00$) and 61.4 ($r^2 = 0.98$) kcal/mol for BIPPO and JMPPO; and for the thermoresistant fraction were 21.4 ($r^2 = 0.70$) and 44.0 ($r^2 = 0.89$) kcal/mol for BIPPO and JMPPO respectively. The computed E_a's are lower for the thermoresistant fractions and close to that reported for an avocado PPO from an unidentified variety (87 kcal/mol) (Weemaes et al., 1998). They are also in agreement with results from other vegetable sources (18.4–84.8 kcal/mol) (Padrón et al., 1975) and (25.2 kcal/mol; Robert, Cadet, Rouch, Pabion & Richard-Forget, 1995).

3.3. Substrate specificity

It was found that the PPO of either source was active toward the *o*-diphenols: catechol, 4-methyl catechol, DL-DOPA, caffeic acid and chlorogenic acid. Ten and 50 mM protocatechuic acid were oxidized by JMPPO, but not BIPPO. This difference might be assigned to an activity lower than the method sensitivity.

There was activity toward pyrogallol, but no activity was detected toward its carboxylated derivate, gallic acid (10 mM), which might due to a non-productive binding of this phenol to the active site as a consequence of the carboxyl group (Zawistowski et al., 1991).

No m-phenolase activity was detected toward 2–10 mM orcinol and 2–10 mM resorcinol, or toward 2–18 mM phloroglucinol. m-Phenols are generally considered not oxidizable by PPO, but phloroglucinol oxidase activity in cabbage has been reported (Fujita, Nazamid, Maegawa, Tetsuka, Hayashi, & Tono, 1995). No laccase (10 mM hydroquinone) or cresolase (0.8 mM DL-Tyrosine and 10 mM *p*-coumaric acid) activity was detected.

Table 1 shows the total and specific enzymatic activities. The substrate with highest activity was 4-methyl catechol, followed by catechol, and then by pyrogallol and DL-DOPA.

Apparent affinities toward phenolic substrates showed in Table 2 for avocado PPO (0.11–29.12 mM) are of the same order of magnitude as those reported for PPO from other avocado varieties (Dizik & Knapp, 1970; Kahn, 1977).

In terms of physiological efficiency (V_{max}/K_M) 4-methyl catechol appeared to be the best substrate (Table 2).

3.4. Effect of inhibitors on crude PPO

The effects of five inhibitors, namely NaCl, glycine, resorcinol, ascorbic acid and L-cysteine on crude BIPPO and JMPPO activity were studied. The results are shown in Fig. 3. L-cysteine was the most effective inhibitor followed closely by ascorbic acid. The effect of each inhibitor, on both crude extracts is discussed below.

3.4.1. Effect of NaCl on crude PPO

The activity of PPO decreased, 37% for BIPPO and 39% for JMPPO, when using 0.8M sodium chloride. No additional tests were done at other NaCl concentrations because of the relatively low inhibition achieved at high concentration. From the data, it may be concluded that the inhibitory effect of NaCl was not satisfactory.

It is believed that the action of NaCl is due to the formation of a complex between the halide ion and copper in the enzyme (Zawistowski et al., 1991). Several

Table 1
Activities (△abs/min/ml extract) and specific activities (activity/mg protein) of crude PPOs from two avocado varieties

Substrate and parameter	Crude BIPPO	Crude JMPPO
4-methyl catechol		
Activity	1.89±0.045	8.11±0.184
Specific activity	9.72±0.23	52±1.18
Catechol		
Activity	1.78±0.038	6.28±0.117
Specific activity	9.15±0.20	40.3±0.75
Pyrogallol		
Activity	0.935±0.189	2.98±0.188
Specific activity	6.87±1.39	22.4±1.39
DL-DOPA		
Activity	1.13±0.020	4.46±0.017
Specific activity	5.84±0.10	28.6±0.11
Chlorogenic acid		
Activity	0.181±0.055	1.49±0.055
Specific activity	0.92±0.28	10.73±1.67
Caffeic acid (3 mM)		
Activity	0.049±0.006	0.330±0.042
Specific activity	0.25±0.03	2.12±0.27
Protocatechuic acid		
Activity	No detected	0.074±0.014
Specific activity		0.56±0.10

Table 2
Apparent K_M and V_{max} of PPO from two avocado varieties for some phenolic substrates

Substrate and parameter	Crude BIPPO	Crude JMPPO
4-Methyl catechol		
K_M (mM)	0.73	0.58
V_{max} (D.O./min)	0.147	0.694
$(V_{max}/K_M)\times 100$	20.2	120
Chlorogenic acid		
K_M (mM)	0.37	0.34
V_{max} (D.O./min)	0.020	0.144
$(V_{max}/K_M)\times 100$	5.41	42.4
Pyrogallol		
K_M (mM)	1.98	2.10
V_{max} (D.O./min)	0.0883	0.335
$(V_{max}/K_M)\times 100$	4.46	16.0
Caffeic acid		
K_M (mM)	0.25	0.11
V_{max} (D.O./min)	0.0038	0.0153
(V_{max}/K_M) x 100	1.52	13.9
Catechol		
K_M (mM)	10.4	9.24
V_{max} (D.O./min)	0.283	1.01
$(V_{max}/K_M)\times 100$	2.71	10.9
DL-DOPA		
K_M (mM)	11.3	9.88
V_{max} (D.O./min)	0.16	0.987
$(V_{max}/K_M)\times 100$	1.43	9.99
Protocatechuic acid		
K_M (mM)		29.1
V_{max} (D.O./min)	No detected	0.0281
$(V_{max}/K_M)\times 100$		0.10

works have been reported on the effect of NaCl on PPO, in which a high inhibitor concentration was necessary to achieve inhibition. It was shown that the inhibition of PPO from eggplant by 5.8% sodium chloride (0.99M) was 90% (Knapp, 1965), and the inhibition of four isoenzymes from clingstone peach by 4.5% sodium chloride (0.77M), ranged between 47 and 93% (Wong et al., 1971).

3.4.2. Effect of glycine on crude PPO

As seen from Fig. 3 A, the PPO activity decreased as the concentration of glycine increased. However, glycine was not a good inhibitor. Glycine added to concentrations as high as 200 mM did not inhibit PPO from either source much more than 50%.

The inhibitory effect of glycine could be explained in at least two ways: by reacting with the *o*-quinones, and by chelating the copper at the active site of PPO (Kahn, 1985).

Glycine and some other amino acids, added to a reaction mixture of catechol and avocado PPO, caused pronounced changes in the absorbance of the product formed. For example, in the presence of glycine the pigment formed upon the addition of avocado PPO (added last) was optically red (maximum absorbance 481–491 nm). This pigment faded during the first 90 s of the reaction. Thereafter, an optically green color (absorption band around 653 nm) appeared in the reaction mixture after 10 min. Similar results were also reported for PPO from tobacco leaves (Pierpoint, 1966), mushroom and avocado (Kahn, 1985), and sweet potato (Lourenço, Neves, & Da Silva, 1992). These results might indicate that the use of some kind of amino acids, or their sources, as browning inhibitors is more complex in comparison with other substances and, despite amino acids inhibting PPO and browning, they could be less useful because of the formation of side colours.

3.4.3. Effect of resorcinol on crude PPO

As shown in Fig. 3B, the inhibitory effect of resorcinol on PPO activity is stronger than those discussed above, and increased as its concentration increased.

Resorcinol resembles the structure of the substrates for PPO; these types of inhibitors are typically competitive. Knapp (1965) has shown that resorcinol competitively inhibits chlorogenic acid oxidation by Lula

avocado PPO. However, Kahn (1976a) found that resorcinol markedly increased the formation of dark-coloured *o*-quinones by PPO extracted from three avocado varieties, using 4-methyl catechol as substrate.

3.4.4. Effect of ascorbic acid on crude PPO

The activity of PPO decreased as the concentration of ascorbic acid increased (Fig. 3C). The concentration of this substance necessary to achieve 50% inhibition is at least two orders of magnitude lower than those necessary for the compounds reported above, being an effective inhibitor of the PPO from both sources.

In PPO assays, where ascorbic acid was used as an inhibitor, a lag period was observed before any changes in absorbance were measured. A similar result was also reported in Fuerte and Lerman PPO extracts by Kahn (1977). The mechanism of inhibition by ascorbic acid involves the reduction of quinones, generated by PPO, back to phenolic compounds, which arrests brown pigment formation until nearly all ascorbic acid is depleted (Tate, Luh, & York, 1964). Two other mechanisms of inhibition involving direct interaction with the enzyme have been reported: chelation of the copper at the active site (Zawistowski et al., 1991) and reduction of Cu^{++} to Cu^{+} (Hsu, Shieh, Bills, & White, 1988).

Ascorbic acid was used by Makower and Schwimmer (1957) to retard browning of avocado slices. Dorantes (1971) showed that the inhibitory effectiveness of ascorbic acid on avocado browning depends on the variety. Kahn (1977) inhibited Fuerte PPO using concentrations between 0.66 and 1.0 mM, getting a lag-phase proportional to inhibitor concentration.

3.4.5. Effect of L-cysteine on crude PPO

From the data in Fig. 3D, L-cysteine was the most effective PPO inhibitor, although at a level very similar to that of ascorbic acid. Also, the higher the L-cysteine concentration, the greater the inhibition.

Colour development in the reaction mixture employing cysteine as inhibitor showed a lag period. This result might be explained by the effect of cysteine trapping *o*-quinones as a colourless cysteine-quinone addition compound (Richard-Forget, Goupy, & Nicolas, 1992). Using this substance, Kahn (1977) also found a lag period with Fuerte and Lerman PPO extracts.

Using the results reported herein, a combined method approach could be tested to preserve avocado pulp from browning.

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