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Reactivity and Stability of Lignostilbene-α, β-Dioxygenase-I in Various pHs, Temperatures, and in Aqueous Organic Solvents

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Abstract The reactivity and stability of purified Lignostilbene-α, β-dioxygenase (LSD)-I were examined. Its optimum temperature was 50°C at pH 8.5, but it was stable only up to 30°C. The activity of LSD-I increased 12-fold by 30% methanol, with increased V_{max} and lowered K_m. LSD-I was stable in 10% methanol.

Key words: Lignostilbene-α, β-dioxygenase, enzyme reactivity, enzyme stability, dissolved oxygen, enzyme reaction in organic solvents

Lignostilbene-α, β-dioxygenase (EC.1.13.11.43, LSD), purified and cloned by Kamoda *et al.* [4, 5], is a dioxygenase which cleaves the double bond of 1,2-bis(3-hydroxy-4-methoxyphenyl)-ethylene (compound 1) and produces 2 equivalent moles of vanillin (Fig. 1). While studying the possibility of industrial production of vanillin from stilbene by LSD, we investigated its reactivity and stability in various conditions to find out its optimum reaction conditions. Reactivity in aqueous organic solvents was tested, because the poor solubility of stilbene in water might limit the productivity and reaction, therefore, an aqueous organic solvent might be preferable.

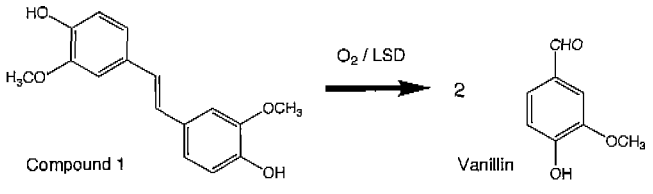

Fig. 1. Reaction scheme of LSD. Compound 1; 1,2-bis(3-hydroxy-4-methoxyphenyl)-ethylene.

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Enzyme activity was monitored by the UV photometric method. To 0.95 ml of 50 mM Tris-HCl buffer, 50 nmol of the compound 1 in 1 μl dimethylformamide was added. The reaction was started by adding 0.05 ml of enzyme solution, and UV absorption at 360 nm, which is specific to the vanillin carbonyl group, was monitored. Enzyme activity was calculated on the basis of the molar extinction coefficient of vanillin and the compound 1.

LSD-I, the major isozyme of LSD that is responsible for the metabolism of the compound 1 [4, 6], was purified from the cell free extract of *Pseudomonas paucimobilis* TMY1009, which was previously isolated from pulping waste sludge [1, 9]. Cells were cultured aerobically at 26.5°C in a low salt LB media (Bacto Tryptone 10 g/l, Bacto Yeast Extract 5 g/l, sodium chloride 5 g/l, pH 7.4). After 22 h of culture, cells were harvested by centrifugation (3,500 ×g, 20 min), suspended in 20 mM sodium phosphate buffer (pH 7.4, buffer A), and disrupted by sonification in an ice-water bath with a Branson Sonifer B-15 (25% pulse, 20 min). The supernatant after centrifugation (20,000 ×g, 60 min) was applied to ammonium sulfate (1 M) precipitation, Butyl-Toyopearl hydrophobic chromatography (5.5 cm i.d. ×9 cm length) with stepwise reduction of ammonium sulfate (1, 0.8 and 0.5 M) concentration in buffer A, QAE-toyopearl anion-exchange chromatography (3 cm i.d. ×28 cm length) with linear gradient of NaCl (0 to 0.5 M in 1-l) in buffer A, and finally to a hydroxyapatite column (3×3 cm) with 10 mM potassium phosphate buffer (pH 6.8). Purified LSD-I showed a single band in native PAGE.

Purified LSD-I was subjected to evaluation of its reactivity and stability in various conditions. First, effects of pH and temperature were investigated. The optimum pH of LSD has already been reported to be 8.5 [4]. At pH 8.5, the reaction was stimulated with increasing temperature up to 50°C (Table 1). At 60°C, the activity was completely lost. As a result, the optimum temperature of LSD-I was considered to be 50°C.

Table 1. Effect of temperature on the activity of LSD-I.

Temperature (°C)	Activity (nmol/sec/mg protein)
8	2.39
20	1.93
30	3.80
37	3.98
50	10.07
60	not detected

Incubation at 30°C proved that LSD-I was stable over 20 h at pHs 7.0 and 8.5 with no loss of activity, but unstable at pH 10.5, showing 67% of deactivation within 1 h. LSD-I was unstable in all pH ranges at 50°C, having complete deactivation within 1 h. The best reaction pH and temperature for the continuous production of vanillin was considered to be pH 8.5 and 30°C, because LSD-I was both active and stable under this condition.

Next, we investigated the reactivity and stability of LSD-I in aqueous organic solvents at pH 8.5 and 30°C. Methanol and 1,4-dioxane, two solvents which are water miscible and transparent at 360 nm, were selected as test organic solvents.

In aqueous methanol, the reaction was stimulated, with increasing concentration of methanol up to 30%, to 12-fold (Table 2). Above 30% of methanol concentration, the activity was decreased, possibly by enzyme denaturation. At 30%, the activity was 10 times as high as the control. In aqueous 1,4-dioxane, the reaction was also stimulated up to 2-fold (Table 3), but its effect was weaker than methanol.

No loss of activity was observed in 10% methanol solution over 3 h, and moderate stability was observed in 30% methanol with 33% deactivation in 3 h, but a complete deactivation was observed in 40% methanol within 2 h. In 1,4-dioxane, LSD-I was found to be unstable and 78% of activity was lost in 10% 1,4-dioxane solution within 1 h. Therefore, continuous production of vanillin was possible in 10% methanol.

To investigate the kinetic properties of stimulated activities in 30% methanol, K_m and V_{max} were determined. In 30% methanol, the K_m was 22.6 μM and Vmax was 4.16 nmol/sec/mg protein, whereas their values in the control were 4.5 μM and 14.20 nmol/sec/mg protein, respectively.

Table 2. Effect of methanol on the activity of LSD-I.

Methanol (%)	Activity (nmol/sec/mg protein)	% of control
0	3.98	100
10	6.76	170
20	14.52	364
30	49.70	1,247
40	46.95	1,178
50	19.02	477
60	0.38	9

Table 3. Effect of 1,4-dioxane on the activity of LSD-I.

1,4-Dioxane (%)	Activity (nmol/sec/mg protein)	% of control
0	3.98	100
10	6.20	156
20	7.96	200
30	1.20	30
40	not detected	-

In conclusion, LSD-I was stable in a pH range of 7.0 and 8.5 at 30°C. Its optimum temperature was 50°C, but continuous reaction was possible only at 30°C because LSD-I was unstable at 50°C. LSD-I was active and stable in 10% methanol, and reaction in 10% methanol was possible. The reaction was stimulated in aqueous methanol, but its mechanism has not yet been clarified. For other unstable dioxygenases, stabilization by organic solvent is commonly observed [2, 3, 8] and stabilization may affect the apparent reactivity, whereas LSD-I was proved to be a very stable enzyme. Although dioxygenases are two-substrate enzymes and the addition of methanol will increase the concentration of dissolved oxygen, the K_m of LSD-I for oxygen was 110 μM [4] which is lower than the maximum dissolved oxygen in water (265 μM at 25°C, 1 atm [7]). This indicates that, although the oxygen concentration increased, the increase in activity would be no more than 2-folds (by Michaelis-Menten kinetics) and more than a 10-fold increase, which in fact can not be explained. K_m and V_{max} in both conditions indicate that the reaction was accelerated both by the increase in V_{max} and the decrease in K_m.

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