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Kinetics of Inhibition of Polyphenol Oxidase Mediated Browning in Apple Juice by β-Cyclodextrin and L-Ascorbate-2-triphosphate

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Polyphenol Oxidase (PPO) mediated browning in raw fruits and vegetables is a major cause of quality deterioration in fruits and vegetables and derived food products. Here the rate of browning reaction in apple juice treated individually and in combination(1:1) of β-Cyclodextrin (β-CD) and L-Ascorbate-2-triphosphate (L-AATP) is described. It was observed that the rate of quinone formation can be minimized using a combination of β-CD and L-AATP as compared to individual treatment with these agents. Kinetic experiments revealed that both compounds are non-competitive inhibitors of PPO.

Keywords: Polyphenol oxidase; L-Ascorbate; β-Cyclodextrin; Browning; Apple juice

INTRODUCTION

Enzyme catalyzed browning is a commonly observed phenomenon when fruits and vegetables are bruised or wounded. Enzymatic browning is catalyzed by two major groups of enzymes: PPO (EC 1.14.18.1) and peroxidase (EC 1.11.1.7). The relative contribution of these two groups is still not clearly resolved and is likely to differ with plant source.[1] A generalized reaction scheme for PPO from plants is shown in Figure 1, where mono- and dihydroxy phenol conversion is mediated by the cresolase and catecholase activity of PPO.[2] The PPO mediated quinones are highly reactive and will polymerize, either with themselves, or with amino acids or proteins to yield complex heterogenous high molecular weight structures generally called "melanin" or melanoidin. This is usually brown but can also be red, black, blue, or various combinations of these colours.[3]

Because of the detrimental effects of the browning reaction in most commercially important food products, its control is a high priority for the food processing industries.[4] A variety of adsorbing agents such as gelatin, activated carbon and polyvinylpolypyrrolidone have been used to remove soluble phenolic substrates from wines and beers.[5] The use of sulphiting agents (SO_2, sodium sulfite, sodium metabisulfite, sodium bisulfite) is the most wide spread chemical method for controlling browning.[6] However the use of sulphites is restricted due to their allergic and mild corrosive nature.[7] Another commonly used method of controlling enzymatic browning is by the addition of ascorbic acid (vitamin C). This vitamin has a lower redox potential than the quinones formed by PPO action so it is preferentially oxidized and the quinones are reduced back to their parent dihydroxy phenols, thus preventing browning.[8] Sulfur amino acids such as Lcysteine and the tripeptide glutathione (reduced form) have been reported as effective inhibitors of browning in fruit juices.[9] The use of natural substances such as honey and certain fractions from green pea have also been encountered as potential natural inhibitors of PPO.[10,11] Recently various ascorbyl derivatives, which release ascorbic acid when hydrolyzed by acid phosphatases,[6,12] have been reported to be useful antioxidants for the food industry. Cyclodextrins (CD's), cyclic oligosaccarides containing 6 or more glucose units which

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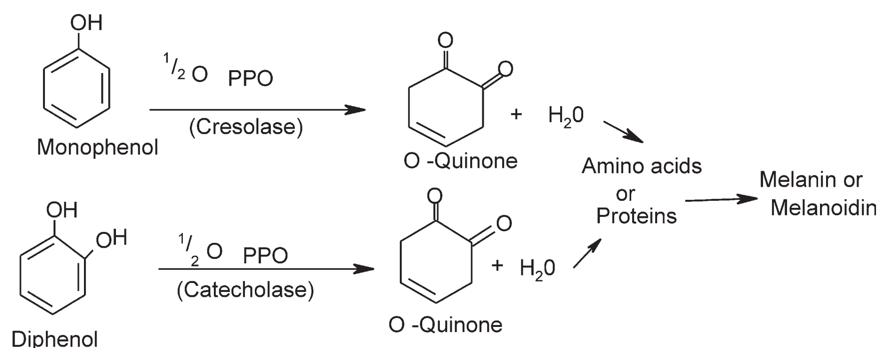

FIGURE 1 A generalized reaction mechanism for plant PPO.

form inclusion complexes with various organic compounds, have been reported to be retardants of browning in some fruits.[13]

In the present work attempts have been made to inhibit PPO mediated browning in apple juice treated individually and with a combination of β-CD and L-AATP (L-ascorbate-2-triphosphate). Kinetic studies were carried out to understand the nature of the inhibition process.

MATERIALS AND METHODS

Materials

Apples were purchased from the local market at Nanded city, β-CD was obtained from Himedia Laboratories Pvt. Ltd. Mumbai, L-AATP and L-DOPA (3,4-dihydroxy phenyl L-alanine) a substrate for PPO[14] were obtained from s.d. fine chemicals Ltd. Mumbai. Other chemicals were of AR grade.

A. Determination of Rate of Browning (Quinone Formation) Using β-CD and L-AATP

Apples (100 g) were chilled, peeled and blended. The preparation was diluted with 200 ml distilled water, filtered through 8 layers of muslin cloth and re-filtered through filter paper to obtain a clear juice. Individual concentrations of β-CD and L-AATP were prepared from freshly prepared 50 mM stock solutions. Three sets of six beakers (for β-CD, L-AATP and combination of both) each containing 9 ml apple juice were used for studies. To these beakers, 1 ml individual concentration of β-CD and L-AATP were added serially and the 6th beaker was kept as a control. In the third set, 0.5 ml individual concentrations of β-CD and L-AATP were mixed together and added serially, keeping the 6th beaker as a control. At 5 min intervals the contents of the beakers were observed spectrophotometrically at 420 nm (Figures 2–4).

B. Extraction, Protein Determination, Assay and Activity Measure of PPO

To understand the nature of PPO inhibition by β-CD and L-AATP a semi-pure preparation of PPO was used. Extraction of PPO was carried out using 50% saturated ammonium sulfate. In brief, 3 × 100 g freshly peeled apple pieces were blended and extracted in chilled phosphate buffer (pH 7.0, 20 mM) to a final volume of 150 ml, which was filtered through 8 layers of muslin cloth and centrifuged. To the clear supernatant (150 ml) 75 g ammonium sulfate was added with continuous stirring to achieve 50% saturation. The mixture was kept at 0°C for 3 h for enzyme precipitation to occur. The residue was then collected by centrifugation and re-dissolved in phosphate buffer (pH 7.0, 20 mM). This preparation was stored at 4°C and used for protein determination, assay, activity measurements and kinetic studies. Irrespective of the speed of the whole procedure the preparation of PPO always ended up with slightly brown colouration.

The protein determination and PPO assay was carried out by the methods described.[15,16] The activity measure of PPO with individual concentrations (1–5 mM) of β-CD/L-AATP/combination(1:1) of both was carried out separately. The reaction mixture contained L-DOPA (0.2 ml, 0.2 mM)

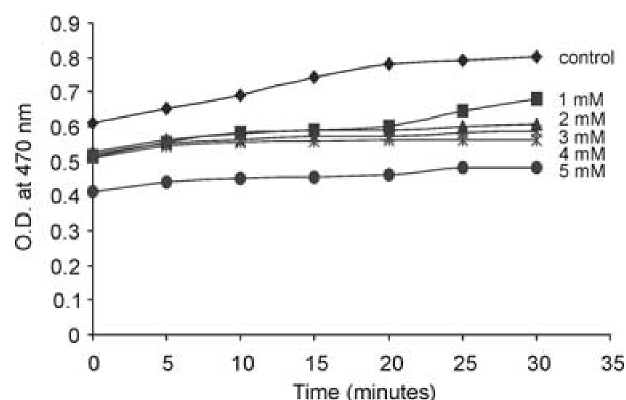

FIGURE 2 Rate of browining (quinone formation) of apple juice treated with different concentrations of β-CD.

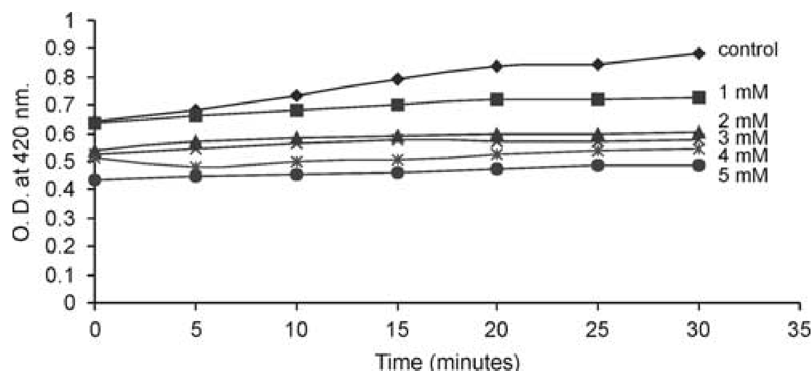

FIGURE 3 Rate of browining (quinone formation) in apple juice in control and L-AATP-treated samples.

enzyme solution (0.1 ml, 0.26 mg protein), 0.2 ml individual concentration of either β-CD/L-AATP/ or a combination of both (0.1 ml + 0.1 ml), and phosphate buffer (2.5 ml, pH 7.0, 20 mM). One unit of enzyme activity was defined as the amount of enzyme which caused an increase in absorbance of 0.001 min^{-1} at 470 nm at 25°C and pH 7.0 which corresponds to the formation of 0.01 μM of product (Dopachrome). The profile of PPO activity in different concentrations of β-CD, L-AATP and combination(1:1) is shown in Table I.

C. Kinetics of Inhibition of PPO by β-CD, L-AATP and Combination(1:1) of Both

To study the nature of the inhibition, a set of five concentrations of β-CD and L-AATP (1–5 mM) were used and compared with the kinetic behavior of a control set (without addition of β-CD or L-AATP). In these reaction mixtures the total volume of reaction mixture was kept constant but the substrate concentration (L-DOPA) varied (0.1–0.5 mM). The results were plotted (Figures 5–7) as double reciprocal plots.

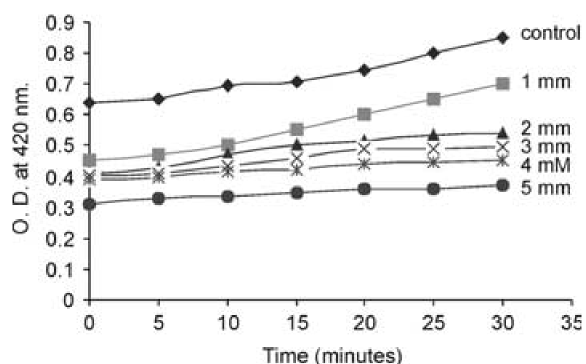

FIGURE 4 Rate of browining (quinone formation) in apple juice in a control and treated with different concentrations of a combination(1:1) of β-CD and L-AATP.

RESULTS

Inhibition of PPO from apple juice was observed for 30 min after preparation of the juice. Figures 2–4 depicts the absorbance at 420 nm *vs.* time for 1–5 mM concentrations of β-CD, L-AATP and the combination(1:1) respectively. It is evident from the results summarized in Figures 2–4 that the individual and combined treatment of β-CD and L-AATP shows reduction in the rate of browning over time as compared to controls. The amount of protein from 100 g apples was estimated to be 2.6 mg/ml. It is also observed that with an increase in time the nature of the inhibition is only attributed to higher concentrations of β-CD, L-AATP and combination of both. This also holds true from the results summarized in Table I. Comparatively it is clear that β-CD is a more potent inhibitor of PPO than L-AATP, while the combination(1:1) of both proved to be an efficient strategy over individual treatment with β-CD and L-AATP.

The double reciprocal Lineweaver-Burk plots (Figures 5–7) for β-CD, L-AATP and the combination of both shows that the nature of the inhibition is non-competitive. The Km values 0.253, 0.263, and 0.390 mM calculated for L-AATP, β-CD and the combination(1:1) respectively remain relatively constant, while the value of V_{max} reduces ($1/V_o$ increases).

TABLE I Profile of PPO activity with different concentrations of β-CD or L-AATP or a combination(1:1) of both

	Activity (U/mg)		
Inhibitor (mM)	β-CD	L-AATP	Combination
0	392	392	392
1	238	276	231
2	207	246	200
3	184	215	176
4	146	176	116
5	101	103	91

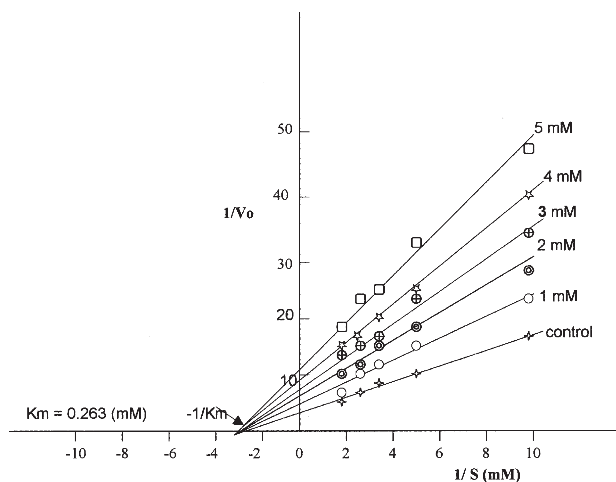

FIGURE 5 Double reciprocal Lineweaver-Burk plot of inhibition of PPO by β-CD (1–5 mM) with L-Dopa (0.2 ml, 0.1–0.5 mM), enzyme (0.1 ml, 0.26 mg protein) in phosphate buffer (20 mM, pH 7.0) to a total volue of 3 ml.

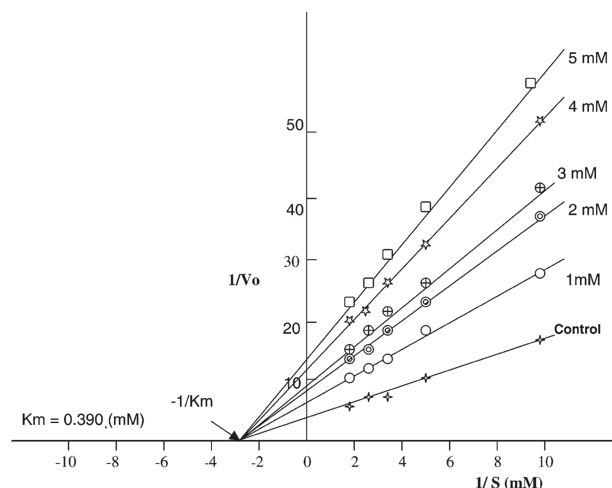

FIGURE 7 Double reciprocal Lineweaver-Burk plot of inhibition of PPO by a combination(1:1) of β-CD and L-AATP (1–5 mM) with L-Dopa (0.2 ml, 0.1–0.5 mM), enzyme (0.1 ml, 0.26 mg protein) in phosphate buffer (20 mM, pH 7.0) to a total volue of 3 ml.

DISCUSSION

There are two main classes of PPO inhibitors. The first class interacts with the copper site in the enzyme while the second class interferes with the site for the phenolic substrates. The first and second class show a competitive and non-competitive type of inhibition respectively.[2] The latter type of inhibition can not be overcome by increasing substrate concentration.[17] This type of inhibition is reversible and the inhibitor binds to the enzyme or enzyme-substrate complex.[18] Km values are the indicators of the affinity of an enzyme towards its substrates; the greater the value of Km, the less is the afinity.[19]

β-CD, a group of complexing agents, inhibit browning by the formation of inclusion complexes with, or entrapment of PPO substrates or products, thus removing the source of substrate. Their effectiveness is dependent on the equilibrium between free and complexed PPO-substrate or products and the rate of complex formation.[20] The potential of L-AATP as a antioxidant agent lies in the release of ascorbic acid when hydrolyzed by acid phosphatases.[6]

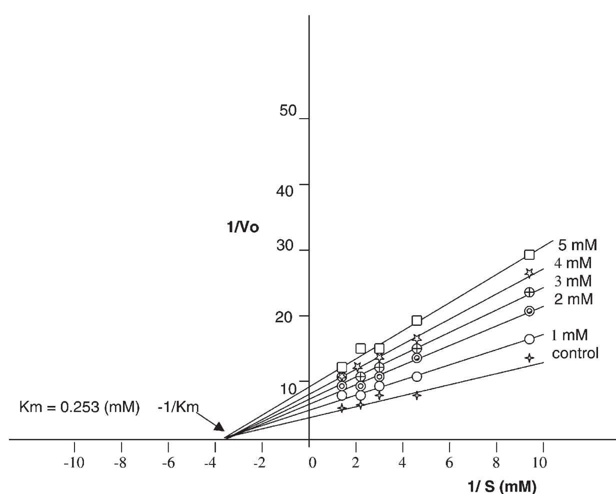

FIGURE 6 Double reciprocal Lineweaver-Burk plot of inhibition of PPO using L-AATP (1–5 mM) with L-Dopa (0.2 ml, 0.1–0.5 mM), enzyme (0.1 ml, 0.26 mg protein) in phosphate buffer (20 mM, pH 7.0) to a total volue of 3 ml.

In the present investigations, irrespective of whether β-CD and L-AATP bind to the PPO or PPO-substrate complex, the order of affinity of PPO towards substrate decreases in the presence of a combination(1:1) of β-CD and L-AATP as compared to the individual agents, while β-CD seems to be a potent inhibitor of PPO as compared to L-AATP, as shown by the calculated Km values for the combination(1:1)/β-CD/L-AATP (0.390, 0.263 and 0.253 mM respectively). The results summarized in Figures 5–7 clearly shows that V_{max} is greater ($1/V_o$ decreases) in the case of L-AATP indicating faster turnover of products as compared to β-CD and the combination.

In conclusion it is clear from the present studies that combination of β-CD and L-AATP can be effectively used for the prevention of PPO mediated browning in apple juice. As both compounds are non-toxic, their addition in the apple juice could be advantageous on health grounds.

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