# Comparison of β-Cyclodextrin Effect on Polyphenol Oxidation Catalyzed by Purified Polyphenol Oxidase from Different Sources

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The effects of β-cyclodextrin (β-CD) on polyphenol oxidation catalyzed by apple polyphenol oxidase (PPO), endive PPO, or mushroom tyrosinase have been compared. β-CD forms a complex with phenolic substrates of PPO by inclusion. Assuming a 1:1 β-CD/phenol stoichiometry, and assuming that PPO is inactive on the complex β-CD/phenol, $K_D$ values were similar when determined kinetically by inhibition of apple PPO or endive PPO. However, the experimental velocities found during inhibition of mushroom tyrosinase by β-CD were higher than the values predicted by this model. In this latter case, it was assumed that mushroom tyrosinase is able to act on the complex β-CD/phenol. A new model based on this assumption allows experimental and calculated velocities to be fit in presence of β-CD.

**Keywords:** *Enzymatic browning; polyphenol oxidase; apple; endive; mushroom; inhibition; cyclodextrin*

## INTRODUCTION

Sulfites are highly effective in controlling enzymatic browning in fruits and vegetables. However, because of adverse health effects, the Food and Drug Administration banned their use in the fruit and vegetable industry (Taylor, 1986). To meet the needs of the food industry for alternatives to sulfites, several methods for controlling enzymatic browning are proposed (Sapers and Hicks, 1989; Nicolas *et al*., 1994). Several chemical inhibitors have been examined, including ascorbic acid and its derivatives either separately or in combination with citric acid (Sapers *et al*., 1989, 1994; Sapers and Miller, 1995; Langdon, 1987; Santerre *et al*., 1988a,b; Lattanzio and Linsalata, 1989) and cysteine (Richard-Forget *et al*., 1991, 1992), other sulfur amino acids like *N*-acetyl-L-cysteine or reduced glutathione (Molnar-Perl and Friedman, 1990), halide ions (Rouet-Mayer and Philippon, 1986), aromatic carboxylic acids (Janovitz-Klapp *et al*., 1990), sulfated polysaccharides (Tong and Hicks, 1991), polyphosphates (Sapers *et al*., 1989), kojic acid (Chen, 1991; Kahn *et al*., 1995), resorcinol derivatives (McEvily *et al*., 1992; Monsalve-Gonzalez *et al*., 1995), and extracts from honey (Oszmianski and Lee, 1990).

These compounds mainly affect the enzyme, polyphenolic substrate, or reaction products, although, in some cases, two or three of these targets can be affected at the same time. Recently, cyclodextrin (CD), especially β-CD, has been proposed to control the enzymatic browning of apple products (Sapers *et al*., 1989; Crouzet *et al*., 1991; Billaud *et al*., 1995), and the use of CD has been patented (Hicks *et al*., 1990).

β-CD consists of seven glucopyranose units linked by α (1−4) glycosidic bonds. It is characterized by a cylindric-shaped cavity of hydrophobic internal surface and hydrophilic outer surface (Szejtli, 1991). The internal cavity of β-CD is slightly apolar and, therefore, can induce inclusion complex formation with many guest molecules. Thus, β-CD has been shown to inhibit enzymatic browning in apple juice (Sapers *et al*., 1989; Crouzet *et al*., 1991). In these studies, the authors proposed that β-CD forms inclusion complexes with the substrates of polyphenol oxidase (PPO), thereby preventing their oxidation to quinones and subsequent polymerization to brown pigments. Interaction between β-CD and main phenolic substrates, *i.e.*, chlorogenic acid (Irwin *et al*., 1994, 1995) and flavan-3-ol (Cai *et al*., 1990), have been studied. These studies, mainly using NMR spectroscopy and UV spectrophotometry, showed that the dissociation constants of the phenol/β-CD complexes varied according to the nature of the phenol.

The purpose of this work was to study the effect of β-CD on the enzymatic phenolic oxidation catalyzed by three PPOs (endive, apple, and mushroom) which exhibited different kinetic properties (Goupy *et al*., 1994; Nicolas *et al*., 1994; Whitaker, 1985). The results of these studies are also compared to data obtained from apple PPO by Billaud *et al*. (1995).

## MATERIALS AND METHODS

**Materials.** Endive core (*Cichorium endivia* L.) was supplied by INRA-Avignon (France). It was lyophilized and stored at −18 °C until use. According to the method of Goupy *et al*. (1994) with some modifications, endive PPO was purified in three steps: extraction, fractional precipitation by ammonium sulfate (20−80%), and hydrophobic chromatography with Phenyl Sepharose CL4B (Pharmacia). All these steps were carried out at 4 °C. Apple PPO was partially purified according to the method of Janovitz-Klapp *et al*. (1989). Mushroom tyrosinase was provided by Fluka and was utilized at 40% in 50 mM phosphate buffer at pH 6.5. The different phenolics substrates were reagent grade from Sigma (St. Louis) and used without further purification. β-CD was provided by Roquette (Lestrem, France) and used as received.

**Measurement of PPO Activity.** PPO was assayed by polarography at 30 °C according to the method of Janovitz-Klapp *et al*. (1990), using 4-methylcatechol (20 mM) as substrate in a McIlvaine buffer solution at pH 4.5. Activity was expressed in nanomoles of oxygen consumed per second (nkat).

**Inhibition of PPO Activity by β-CD.** The phenolic substrate concentrations ranged from 0.1 to 20 mM (the limits were dependent on the $K_m$ values) in the control and with two

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**Table 1. Inhibition of Phenolic Oxidation by β-CD (Given in % of the Value Obtained without β-CD)**

| | | phenolic compounds | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (+)-catechin | | | (−)-epicatechin | | | chlorogenic acid | | | 4 methylcatechol | | | hydrocaffeic acid | | |
| | | 1 mM | 5 mM | 10 mM | 0.5 mM | 1 mM | 2.5 mM | 0.5 mM | 1 mM | 2.5 mM | 0.5 mM | 1 mM | 2 mM | 0.5 mM | 1 mM | 5 mM |
| 2 mM [β-CD] | apple | 80 | 30 | 14 | | | | | | | | | | | | |
| | endive | 75 | 22 | 5 | | | | | | | | | | | | |
| | mushroom | 73 | 20 | 1 | | | | | | | | | | | | |
| 4 mM [β-CD] | apple | 93 | 61 | 25 | | | | | | | | | | | | |
| | endive | 95 | 53 | 13 | | | | | | | | | | | | |
| | mushroom | 80 | 50 | 5 | | | | | | | | | | | | |
| 5 mM [β-CD] | apple | | | | 64 | 64 | 60 | 62 | 58 | 51 | | | | 39 | 34 | 17 |
| | endive | | | | 61 | 54 | 43 | 55 | 50 | 33 | | | | 27 | 19 | nd[a] |
| | mushroom | | | | 48 | 48 | 27 | 35 | 31 | 25 | | | | 25 | 18 | 13 |
| 10 mM [β-CD] | apple | | | | 81 | 78 | 77 | 78 | 75 | 71 | 32 | 32 | 26 | 56 | 51 | 30 |
| | endive | | | | 78 | 73 | 62 | 69 | 67 | 55 | 32 | 25 | 24 | 49 | 41 | 9 |
| | mushroom | | | | 60 | 59 | 35 | 41 | 40 | 33 | 13 | 5 | 1 | 34 | 23 | nd[a] |
| 20 mM [β-CD] | apple | | | | | | | | | | 50 | 49 | 44 | | | |
| | endive | | | | | | | | | | 50 | 42 | 42 | | | |
| | mushroom | | | | | | | | | | 22 | 15 | 8 | | | |

[a] nd = not determined.

concentrations of β-CD. Enzyme extract was added to 1.5 mL of the air-saturated phenolic solution at 30 °C.

**Calculation of $K_d$ (Dissociation Constant) of the β-CD/Phenol Complex from Kinetic Experiments.** Assuming a 1:1 stoichiometry,

$$K_D = \frac{[CD][S_f]}{[CDS]}$$

where $[S_f]$, [CD], and [CDS] are the concentrations of free substrate and free and complexed cyclodextrin, respectively.

The mass balance equations for S and β-CD are

$$[S_t] = [CDS] + [S_f]$$

$$[CD_t] = [CDS] + [CD]$$

where $[S_t]$ and $[CD_t]$ are the total amounts of phenol and β-CD in the solution, respectively.

Hence,

$$K_D = \frac{([CD_t] - [S_t] + [S_f])[S_f]}{[S_t] - [S_f]}$$

After rearranging,

$$[S_f]^2 + ([CD_t] - [S_t] + K_D)[S_f] - K_D[S_t] = 0$$

The negative solution can be ignored because $[S_f]$ must be positive.

$$[S_f] = \frac{-([CD_t] - [S_t] + K_D) + \sqrt{([CD_t] - [S_t] + K_D)^2 + 4K_D[S_t]}}{2} \quad (1)$$

This expression, representing the amount of free phenol available for PPO, may be substituted in the velocity equation, assuming PPO is inactive on the β-CD/phenol complex.

$$v = V_m \frac{[S_f]}{K_m + [S_f]} \quad (2)$$

$K_m$ and $V_m$ were determined from the control experiments by using a nonlinear regression program developed by Leatherbarrow (1987). $K_D$ was evaluated by fitting the experimental and calculated velocities (using eqs 1 and 2) in the presence of β-CD.

## RESULTS AND DISCUSSION

**Effect of β-CD on Oxidation of Different Phenolic Compounds Catalyzed by Three PPOs.** The inhibition effect of β-CD on the oxidation of five phenolic compounds has been studied with three different partially purified PPOs, the apple and endive PPOs and the tyrosinase from mushroom. The three enzymes were chosen because they exhibited large differences in their kinetic properties (Nicolas *et al.*, 1994; Goupy *et al.*, 1994; Whitaker, 1985). The inhibition study was carried out at pH 4.5. Although the optimum pH was higher for endive or lettuce PPOs (Fujita *et al.*, 1991; Heimdal *et al.*, 1994; Goupy *et al.*, 1994) and mushroom tyrosinase (Mayer and Harel, 1979), the value of pH 4.5 was selected because it ensures that spontaneous oxidation of all the phenols tested is almost nil. At pH 6 (or higher), corresponding to the optimum pH for endive PPO and mushroom tyrosinase, the autoxidation of some of the phenols tested (stereoisomers of catechin) cannot be neglected. It can be seen that, whatever the enzyme and the phenolic substrate considered, increasing the amount of β-CD resulted in an increasing inhibition of oxygen uptake (Table 1). In the same way, for the same concentration of β-CD, increasing substrate concentration always resulted in a decreasing inhibition. Among the different phenolic substrates, the greatest inhibition was always observed with (+)-catechin. Thus, with 4 mM β-CD and for a (+)-catechin concentration of 1 mM, the inhibition was at least 80%, whereas for the other phenolics at the same concentration, it was less than this value, even for β-CD concentration as high as 10 mM. For the three enzymes, the decreasing inhibition rank was always the same: (+)-catechin < (−)-epicatechin < chlorogenic acid < hydrocaffeic acid < 4-methylcatechol. Last, under the same conditions, β-CD exhibited the highest inhibition values in almost all cases with apple PPO, followed by endive PPO and mushroom tyrosinase.

**Model for Apple and Endive PPO.** Since β-CD is able to form a complex with phenolic compounds, the inhibition pattern observed can be first explained by the formation of inactive complex. Using eqs 1 and 2 (see Materials and Methods), a dissociation constant $K_D$ of

**Table 2. Dissociation Constants ($K_D$) of the Complexes $\beta$-CD/Diphenol and $K_m$ Values Determined from Kinetic Studies with the Three Enzymes**

| | apple PPO | | endive PPO | | mushroom tyrosinase | |
|---|---|---|---|---|---|---|
| phenolic compd | $K_D$[a] (mM) | $K_m$ (mM) | $K_D$[a] (mM) | $K_m$ (mM) | $K_D$[a] (mM) | $K_m$ (mM) |
| 4-methylcatechol | 18 | 5.2 | 20 | 5.6 | 45 | 0.55 |
| (−)-epicatechin | 4.1 | 6.2 | 4.0 | 3.8 | 7.0 | 5.4 |
| (+)-catechin | 0.19 | 6.1 | 0.19 | 4.9 | 0.55 | 2.5 |
| chlorogenic acid | 2.5 | 5.6 | 3.0 | 1.8 | 7.5 | 1.8 |
| hydrocaffeic acid | 6.0 | 2.2 | 7.0 | 2.1 | 14 | 0.50 |

[a] $K_D$ values are calculated by fitting experimental data with eqs 1 and 2.

the complex $\beta$-CD/phenol can be calculated by fitting experimental and theoretical velocities in the presence of $\beta$-CD. The values obtained for the five phenolic compounds tested are given in Table 2, together with the $K_m$ values for each enzyme.

The $K_D$ constants found from experiments with apple and endive PPOs were very close for each phenol. These values calculated from kinetic experiments are in agreement with those obtained by NMR experiments for chorogenic acid and (−)-epicatechin by Irwin *et al.* (1994) and for the two isomers of catechin by Cai *et al.* (1990). In this last case, we confirm the stereoselectivity of $\beta$-CD, which exhibited a much higher affinity for (+)-catechin than for (−)-epicatechin.

Cai *et al.* (1990) have proposed that a hydrogen bond between $C_3$ of (+)-catechin and the peripheral hydroxy group at $C_2$ and $C_3$ on the top rim of the $\beta$-CD cavities was responsible for this high affinity, whereas the position of this hydroxy group in (−)-epicatechin makes this hydrogen bond impossible. Moreover, the $K_D$ values found in this study with apple and endive PPOs were comparable to those obtained by inhibition studies with apple PPO and NMR spectroscopy by Billaud *et al.* (1995). The highest inhibition level found with (+)-catechin as substrate is obviously due to the high affinity of $\beta$-CD for this phenolic compound. Similarly, endive PPO exhibited in most cases a higher affinity for phenolic substrates than apple PPO, which explained why the latter enzyme was more inhibited by $\beta$-CD than the former one under the same conditions.

Taking into account the $K_m$ and the $V_m$ of the apple and endive PPOs toward each phenol, a standardized model of inhibition was established by representing $v_{exptl}/V_m$ versus $[S_t]/K_m$. An excellent fit was obtained between theoretical and experimental values for the inhibition by $\beta$-CD of the two enzymes, as shown by the uniform representation applied to (+)-catechin (Figure 1A) and chlorogenic acid (Figure 1B). Similar results were obtained for the three other phenolic compounds (results not shown).

**Model for Mushroom Tyrosinase.** The previous pattern (eqs 1 and 2) did not apply to model solutions containing phenolic substrate, $\beta$-CD, and mushroom tyrosinase. The $K_D$ constants obtained after fitting experimental and theoretical values were almost always twice those determined with solutions containing endive or apple PPO. Since the dissociation constant of the complex $\beta$-CD/phenol cannot have different values, we have supposed first the presence of phenolic compounds in mushroom tyrosinase extract. These phenolic compounds could displace the equilibrium, leading to a modified $K_D$ value. This hypothesis was rejected because, after extensive dialysis of the extract at 4 °C against a solution of phosphate buffer (50 mM at pH 6.5), a similar pattern of inhibition was obtained.

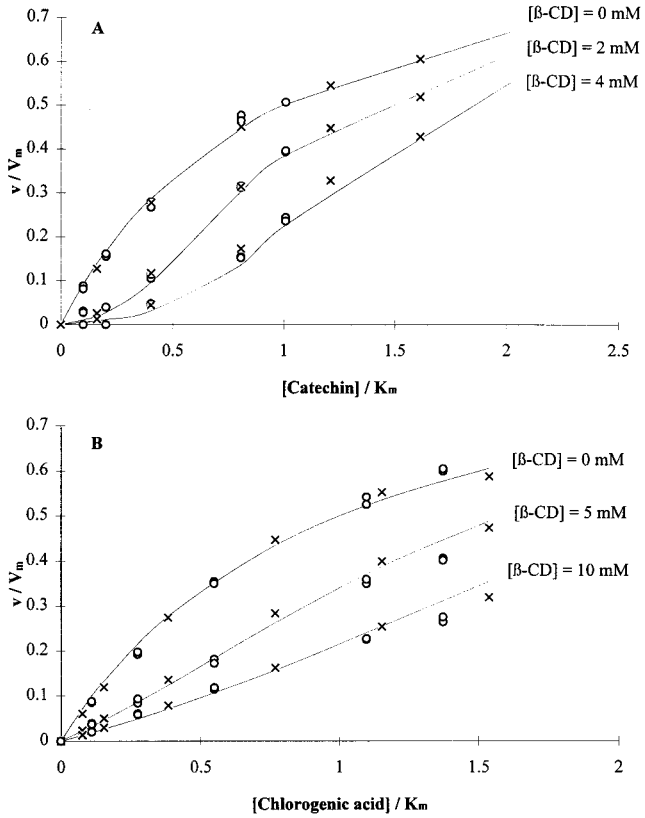


**Figure 1.** (A) Inhibition by $\beta$-CD of catechin oxidation catalyzed by endive or apple PPO. Lines are for values calculated using eqs 1 and 2. (○) Catechin oxidation catalyzed by endive PPO ($K_m$ = 4.9 mM; $V_m$ = 31.2 nkat mL$^{-1}$). (×) Catechin oxidation catalyzed by apple PPO ($K_m$ = 6.1 mM; $V_m$ = 130 nkat mL$^{-1}$). (B) Inhibition by $\beta$-CD of chlorogenic acid oxidation catalyzed by endive or apple PPO. Lines are for values calculated using eqs 1 and 2. (○) Chlorogenic acid oxidation catalyzed by endive PPO ($K_m$ = 1.8 mM; $V_m$ = 250 nkat mL$^{-1}$). (×) Chlorogenic acid oxidation catalyzed by apple PPO ($K_m$ = 5.6 mM; $V_m$ = 275 nkat mL$^{-1}$).

Second, we have supposed that the $\beta$-CD/phenol complex could be used as a substrate by the enzyme. This complex could then be oxidized by mushroom tyrosinase, whereas the apple and endive PPOs were not able to act on this complex due to steric hindrance leading to differences in accessibility of the catalytic centers of these enzymes. This new model can be considered as an enzymatic reaction with two phenolic substrates, the free *o*-diphenol and the complex $\beta$-CD/phenol. Tyrosinase oxidized the $\beta$-CD/*o*-diphenol complex with kinetic parameters ($V_m$ and $K_m$) which are different from those of the free *o*-diphenolic compound. Using the same equation (eq 1) for the determination of $[S_f]$ as in the previous model, the new velocity equation is given by (Janovitz-Klapp *et al.*, 1990)

$$v_t = \frac{V_{m1}([S_f]/K_{m1}) + V_{m2}([CDS]/K_{m2})}{1 + ([S_f]/K_{m1}) + ([CDS]/K_{m2})}$$

$$\text{thus,} \quad v_t = \frac{V_{m1}([S_f]/K_{m1}) + V_{m2}([S_t - S_f]/K_{m2})}{1 + ([S_f]/K_{m1}) + ([S_t - S_f]/K_{m2})} \qquad (3)$$

where $V_{m1}$, $K_{m1}$, $V_{m2}$, and $K_{m2}$ are the kinetic parameters of mushroom tyrosinase toward free and complexed *o*-diphenolic compound, respectively.

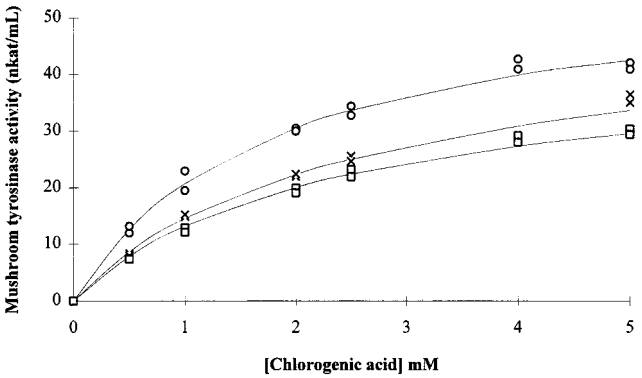


**Figure 2.** Effect of $\beta$-CD on chlorogenic acid oxidation catalyzed by mushroom tyrosinase. (○) Control without $\beta$-CD; (×) $\beta$-CD = 5 mM; (□) $\beta$-CD = 10 mM. Lines are for values calculated using eqs 1 and 3 with $K_D$ = 2.5 mM, $K_{m1}$ = 1.8 mM, $V_{m1}$ = 58 nkat mL$^{-1}$, $K_{m2}$ = 2.1 mM, and $V_{m2}$ = 35 nkat mL$^{-1}$.

**Table 3. Kinetic Parameters of Mushroom Tyrosinase toward Free and Complexed *o*-Diphenolic Substrates**

| | $K_m$ (mM) | | $V_m$ (%)[a] | |
|---|---|---|---|---|
| phenolic compd | free ($K_{m1}$) | complexed ($K_{m2}$) | free ($V_{m1}$) | complexed ($V_{m2}$) |
| 4-methylcatechol | 0.55 | 3.5 | 100 | 93 |
| (−)-epicatechin | 5.4 | 10 | 34 | 20 |
| (+)-catechin | 2.5 | 10 | 10 | 5 |
| chlorogenic acid | 1.8 | 2.1 | 12 | 7 |
| hydrocaffeic acid | 0.50 | 1.0 | 22 | 19 |

[a] $V_m$ values are expressed as % of $V_m$ obtained at pH 4.5 for 4-methylcatechol.

Using eqs 1 and 3 with the $K_D$ values determined with apple or endive PPOs, the values of $V_{m2}$ and $K_{m2}$ can be calculated by fitting experimental and theoretical velocities in the presence of $\beta$-CD with mushroom tyrosinase. An excellent fit was obtained, as shown in Figure 2 for the $\beta$-CD inhibition of the chlorogenic acid oxidation catalyzed by mushroom tyrosinase. Similar results were obtained with the other phenolic compounds using the calculated $V_{m2}$ and $K_{m2}$ kinetic parameters given in Table 3.

The kinetic parameters of complexed *o*-diphenol showed that the mushroom tyrosinase efficiency was largely decreased since, compared to the free *o*-diphenolic substrate, the values of $K_m$ increased, whereas those of $V_m$ decreased. Thus, the decrease in efficiency ($V_m/K_m$) was between 50% for chlorogenic acid and 87% for (+)-catechin and 4-methylcatechol.

In conclusion, the inhibition effect of $\beta$-CD on enzymatic browning varied greatly from one phenol to another and from one enzyme to another. For some PPOs, such as the apple and endive enzymes, the inhibition level can be predicted, provided the dissociation constant $K_D$ of the complex phenol/$\beta$-CD and the $K_m$ values are known. Obviously, the greatest inhibition is observed with the phenolic compound which exhibited the highest affinity for $\beta$-CD and the highest value of $K_m$. In comparison with the two previous enzymes, mushroom tyrosinase was less inhibited by $\beta$-CD. Assuming that this enzyme was able to act on the phenol/$\beta$-CD complex, we have developed a model which is in agreement with the experimental results. According to the calculated kinetic constants, the enzyme was much less efficient on the complex than on the free phenol. Nevertheless, further experiments are needed to elucidate why mushroom tyrosinase seems able to act upon the phenol/$\beta$-CD complexes, whereas the two other PPOs (apple and endive) are not.

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